

BACTERIAL POPULATION DYNAMICS
UNDER MULTIDRUG TREATMENTS

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Summary

Since the discovery of penicillin, antibiotics have been a cornerstone of modern medicine, saving countless lives. This achievement is now under threat, as bacteria have evolved resistance mechanisms against most available drugs. Resistance arises through the acquisition of resistance genes—either via mutations or through horizontal transfer on mobile genetic elements such as plasmids—and is selected for according to the administered drugs, their concentrations, and the pharmacodynamics that link treatment to its effect on the bacterial population.

To curb the spread of resistance, antibiotics should ideally be chosen on the basis of phenotypic information. In emergency situations, however, this is not always feasible. Therapy then relies on predefined treatment protocols rather than tailored data. Chapter 2 examines how these default protocols (treatment strategies) influence the persistence of plasmid-mediated resistance and the emergence of new double resistance. We performed large-scale automated in-vitro experiments that mimic hospital-like transmission dynamics, using two antibiotics in combination with two resistance plasmids derived from clinical isolates.

We found that administering both drugs to every patient (combination therapy) was the most effective strategy in most scenarios. Although theoretical models often predict the success of combination therapy, the clinical literature is less conclusive. This gap has several causes: some drug pairs act synergistically, whereby the combined effect exceeds the sum of single-drug effects, whereas others act antagonistically, reducing overall efficacy.

Drug interactions have so far been studied mainly at concentrations below the minimal inhibitory concentration (MIC), where growth can be tracked conveniently by optical density (OD). OD cannot measure bacterial decline, and probing the clinically relevant super-MIC range—where bacteria are actively killed—requires colony-forming-unit (CFU) assays, which are labor-intensive and unsuited to high throughput.

Chapter 3 therefore evaluates whether a luminescence-based high-throughput method, previously validated for sub-MIC conditions, can also track population dynamics across the entire concentration range. Using bacteria engineered to emit bioluminescent light, we show that this method accurately estimates net growth rates for drugs that do not induce significant filamentation.

Building on these insights, Chapter 4 explores drug interactions for six antibiotics that performed well in the preceding validation. We analysed 15 drug pairs across 144 concentration combinations each, introducing a mathematical framework to describe the pharmacodynamics of combinations. By comparing observed effects to null models of non-interaction, we classified pairs as neutral, synergistic or antagonistic, and demonstrated that interaction types can shift markedly between sub- and super-MIC conditions.

Together, these studies combine experimental and theoretical approaches to elucidate antibiotic effects on bacterial populations and resistance evolution. They deliver robust tools for quantifying population dynamics and bolster the case for combination therapy, while refining our understanding of drug–drug interactions across clinically relevant concentration ranges.

Zusammenfassung

Seit der Entdeckung des Penicillins bilden Antibiotika einen Grundpfeiler der modernen Medizin und haben unzählige Leben gerettet. Dieser Erfolg ist jedoch bedroht, da Bakterien gegen die meisten verfügbaren Wirkstoffe Resistenzmechanismen entwickelt haben. Resistenzen entstehen durch den Erwerb von Resistenzgenen – entweder über Mutationen oder durch horizontalen Gentransfer via mobile genetische Elemente wie Plasmide – und werden abhängig von verabreichten Wirkstoffen, deren Konzentrationen und den Pharmakodynamiken selektiert, die Behandlung und Wirkung miteinander verknüpfen.

Um die Ausbreitung von Resistenzen einzudämmen, sollten Antibiotika idealerweise auf Basis phänotypischer Informationen ausgewählt werden. In Notfallsituationen ist dies jedoch oft nicht möglich, sodass auf vordefinierte Behandlungsprotokolle zurückgegriffen wird. Kapitel 2 untersucht, wie diese Standardprotokolle (Behandlungsstrategien) die Persistenz plasmidvermittelter Resistenzen und das Auftreten neuer Doppelresistenzen beeinflussen. Hierfür führten wir groß angelegte, automatisierte In-vitro-Experimente durch, die krankenhausähnliche Übertragungsdynamiken simulieren; zum Einsatz kamen zwei Antibiotika in Kombination mit zwei aus klinischen Isolaten stammenden Resistenzplasmiden.

Die Gabe beider Wirkstoffe an jeden Patienten (Kombinationstherapie) erwies sich in den meisten Szenarien als wirksamste Strategie. Obwohl theoretische Modelle diesen Erfolg häufig vorhersagen, ist die klinische Datenlage weniger eindeutig. Ursachen hierfür sind unter anderem synergistische Wirkstoffpaarungen, bei denen der kombinierte Effekt größer ist als die Summe der Einzeleffekte, sowie antagonistische Paarungen, bei denen er kleiner ausfällt.

Bisher wurden Arzneimittelinteraktionen überwiegend bei Konzentrationen unterhalb der minimalen Hemmkonzentration (MIC) untersucht, da Wachstumsraten dort bequem mittels optischer Dichte (OD) erfassbar sind. OD misst jedoch keinen Bakterienrückgang. Der klinisch relevante Super-MIC-Bereich, in dem Bakterien aktiv abgetötet werden, erfordert koloniebildende Einheiten (CFU), ein arbeitsintensives und wenig durchsatzstarkes Verfahren.

Kapitel 3 prüft daher, ob eine bereits für Sub-MIC-Bedingungen validierte, lumineszenzbasierte Hochdurchsatzmethode auch über den gesamten Konzentrationsbereich einsetzbar ist. Durch gentechnisch veränderte, biolumineszente Bakterien konnten wir zeigen, dass diese Methode die Nettowachstumsrate für Wirkstoffe ohne ausgeprägte Filamentierung präzise erfasst.

Aufbauend darauf untersucht Kapitel 4 Wirkstoffinteraktionen für sechs im Vorversuch bewährte Antibiotika. Wir analysierten 15 Wirkstoffpaare mit jeweils 144 Konzentrationskombinationen und entwickelten einen mathematischen Rahmen zur Beschreibung der Pharmakodynamik von Kombinationen. Durch Vergleich der beobachteten Effekte mit Nullmodellen ohne Interaktion klassifizierten wir Paare als neutral, synergistisch oder antagonistisch und zeigten, dass sich diese Klassifikationen zwischen Sub- und Super-MIC-Bereichen deutlich ändern können.

Insgesamt verbinden diese Arbeiten experimentelle und theoretische Ansätze, um die Wirkung von Antibiotika auf bakterielle Populationen und die Resistenzentwicklung zu beleuchten. Sie liefern robuste Methoden zur Quantifizierung von Populationsdynamiken, stärken die Evidenz

für Kombinationstherapien und verfeinern unser Verständnis von Arzneimittelinteraktionen über klinisch relevante Konzentrationsbereiche hinweg.

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Introduction

1.1 Antibiotic resistance evolution and treatment strategies

Antibiotics have nearly eliminated premature death from bacterial infections in most parts of the developed world, to the extent that effective treatment is now often taken for granted. The threat to this achievement posed by antibiotic resistance was recognized early, for instance by [1], and explicitly highlighted by Alexander Fleming in his 1945 Nobel Prize speech. Today, this threat has materialized: antimicrobial resistance (AMR) contributed to an estimated 4.71 million deaths in 2021, with 1.14 million deaths directly attributable to resistant infections [2]. Chevereau2015 For some of the most worrisome infectious diseases, including tuberculosis, drug resistance evolves mainly via spontaneous mutations that render antibiotics ineffective [4,5]. A possible way of averting the looming resistance crisis is developing novel treatment strategies that use established drugs in ways that minimize resistance evolution [6–9]. To rationally design such strategies, it is crucial to understand the genetic source for MDR tuberculosis: <https://doi.org/10.1016/j.mcna.2013.03.012> The possibility of returning to a world where antibiotics are largely ineffective underscores the importance of understanding the ecological and evolutionary forces shaping AMR.

Evolution of antibiotic resistance is driven by two key processes: mutation and selection. Resistance genes arise de novo through mutations, occurring either on bacterial chromosomes or on extrachromosomal mobile genetic elements that replicate independently, such as plasmids (small circular DNA molecules) or bacteriophages (viruses targeting bacteria). Once established, these genes can spread.

Resistance genes spread in two fundamentally different ways: vertically, when a mother cell passes them to its progeny during division, and horizontally, when previously susceptible bacteria acquire foreign DNA. Horizontal transfer is often mediated by plasmids, many of which can actively initiate their own conjugation by assembling a pilus — a physical tunnel connecting

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donor and recipient cells — through which plasmid DNA is delivered. However, not all plasmids initiate conjugation: some rely exclusively on vertical inheritance, while others lack conjugation machinery but can opportunistically transfer by exploiting the conjugation apparatus of co-resident plasmids. Plasmid conjugation can facilitate gene exchange across bacterial lineages and even kingdom boundaries [3].

Whether these emerged resistance genes can persist depends on selection. Selection is shaped by the interplay between (i) pharmacokinetics (PK), which describes how drug concentrations change over time within the host, and (ii) pharmacodynamics (PD), which describes how these concentrations influence bacterial population dynamics [4].

Each use of antibiotics imposes selective pressure on microbial populations, which can favor the emergence or spread of resistant variants. This is consistent with the widely accepted view that antibiotic use is the main driver of resistance evolution [5, 6]. However, because infections can be life-threatening, antibiotic use is often unavoidable, creating a trade-off between effective treatment and the risk of selecting for resistant bacteria.

This raises the fundamental question of how to best use antibiotics to treat infections; with and without knowledge about the phenotypic properties of the infecting pathogen. Furthermore, this question arises at multiple biological scales: within individual hosts, where antibiotic concentrations and bacterial populations interact dynamically; and between hosts, where treatment strategies influence the transmission and spread of resistance at the population level. Both scales are relevant for evaluating the effectiveness and evolutionary consequences of antibiotic use.

Treatment Strategies

In an ideal scenario, the treatment of infections is tailored to the resistance phenotype of the infecting pathogen. However, in clinical practice this is often not feasible: in emergency settings, immediate treatment is required and microbiological characterisation often takes 24 to 72 hours [7]; resistance may exist below detection thresholds, rendering diagnostics inconclusive; and in prophylactic contexts such as surgery or treatment of immunocompromised patients, antibiotics are often prescribed preemptively.

In such cases of limited information, clinicians rely on empirical treatment strategies that aim to minimize the risk of selecting for resistance. Commonly discussed approaches include com-

bination therapy (simultaneous use of multiple antibiotics), mixing (random patient allocation to different antibiotics), and cycling (periodic rotation of antibiotics over time). Among these, combination therapy, which was first introduced in agriculture to prevent resistance in plant pathogens [8–10] is often considered the most promising strategy in theoretical models [11–13]. Yuan2000, While many modern drugs originated from plants, unpuri- fied herbal preparations are used extensively in both Europe and Asia. Western herbal medicines are often standard- ized ex- tracts of single herbs used for particular conditions. For exam- ple, valerian and St. John’s wort are used for sleep aid and mild depression, respectively. In comparison, traditional Chi- nese medicines (TCMs) are decoctions of mixtures of up to 20 herbs that are customized for each individual patient. TCMs represent one aspect of Chinese medical philosophy that is characterized by its emphasis on maintaining and restoring balance. This medical approach is much more appropriate to disease prevention and the treatment of chronic diseases with- out an unacceptably high level of collateral damage. Most Chinese formulations contain a mixture of herbs. There are different methods of classifying the ways in which these can be combined. When combined, two biolog- ically active substances can be observed to have the follow- ing effects: mutual accentuation, mutual enhancement, mu- tual counteraction, mutual suppression, mutual antagonism, and mutual incompatibility. The principal ingredient is a substance that provides the main therapeutic thrust, the sec- ond principal ingredient enhances or assists the therapeutic actions of the first. The rest serve one of the following func- tions: treat accompa- nying symptoms, moderate the harsh- ness or toxicity of the primary ones, guide the medicine to the proper organs, or exert a harmonizing effect.

However, concerns persist. One major concern is that combination therapy might accelerate the selection for resistance, given the observed correlation between overall antibiotic consumption and resistance [5, 6], not only regarding the targeted pathogen but also for the microbiome [14]. Furthermore, combining antibiotics may impose a higher burden on patients, for example through an increased risk of toxicity [15].

In the clinical context combination therapy plays a central role in clinical protocols for rapidly evolving pathogens such as HIV, *Mycobacterium tuberculosis*, and *Plasmodium falciparum* [16]. Nonetheless, the clinical evidence remains inconclusive. A recent meta-analysis found no consis- tent advantage of combination therapy in preventing resistance across bacterial infections [17].

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The discrepancy between theoretical predictions and inconclusive clinical outcomes can stem both from limitations in modelling or clinical study design. On the theoretical side, models often rely on simplifying assumptions and may overlook important biological complexities, such as heterogeneity in patients, pathogens, and treatment responses. On the clinical side, most trials are not designed to detect resistance outcomes and are therefore underpowered for this purpose [17]. Additional variability arises from differences in study design, pathogens, treatment regimens, and clinical endpoints. Moreover, the choice of antibiotic combination itself can increase or decrease treatment success, further contributing to outcome variability.

To bridge the gap between theoretical models and clinical studies, Angst et al. [18] developed an *in vitro* experimental setup mimicking within- and between-host dynamics under different treatment strategies, and showing that combination therapy typically best prevents resistance emergence best.

In Chapter 2, we refine this framework to study plasmid-mediated resistance. Our results confirmed that combination therapy remains the most effective strategy in most scenarios.

Population Dynamics under Combination Therapy

To better understand the potential of combination therapy, a deeper insight into drug interactions is needed. Drug interaction studies evaluate how the combined effect of two antibiotics compares to a predicted neutral effect derived from their individual actions. **Gjini2021: - Drug interactions can shape resistance evolution. - Difficult to draw general conclusion (e.g. synergistic combinations always accelerate resistance”)**

Two classical null models are typically used for this purpose: the Loewe additivity model [19] and the Bliss independence model [20]. Numerous studies have investigated drug interactions below the minimum inhibitory concentration (MIC) [21–23], providing valuable insights into sublethal interactions. However, it remains unclear whether these findings extend to the super-MIC range, where antibiotics are expected to kill bacteria. This uncertainty arises from the scarcity of studies exploring interactions above the MIC.

The reason for this is methodological: to quantify treatment effects, we estimate the exponential change in time-series population curves. Below the MIC, bacterial growth is typically quantified using optical density (OD), which allows for high-throughput measurement. However, OD does

not distinguish between live and dead cells, making it unreliable for estimating net growth rates above the MIC. At super-MIC concentrations, colony-forming unit (CFU) assays remain the gold standard, but they are labor-intensive, costly, and can become unreliable at high drug levels.

To address this gap, Chapter 3 evaluates luminescence as a high-throughput alternative to CFU counting and tests how reliably each method captures bacterial dynamics at inhibitory drug levels. We find that luminescence assays tend to track biomass more closely than viable cell numbers; which can cause divergence for estimated rates when cells filament.

Using this validated approach, Chapter 4 systematically characterizes pharmacodynamic interactions for multiple antibiotic pairs, examining whether interaction classifications are consistent across sub- and super-MIC concentration ranges.

1.2 Thesis outline

In this thesis we develop experimental and analytical methods to quantify how antibiotic-induced selection pressure shapes the evolution and transmission of resistance at both within-host and between-host scales.

In Chapter 2, we use an in-vitro hospital-ward model, to compare three multidrug strategies (combination, mixing, cycling), two monotherapies (Mono A, Mono B) and an untreated control. We show that combination therapy suppresses plasmid-borne double resistance most efficiently under the majority of conditions, but also find that in certain scenarios, mixing, cycling, or even monotherapy can perform equally well. Surprisingly, we find that omitting treatment entirely can accelerate the emergence of multi-resistance—a phenomenon specific to plasmid-mediated resistance dynamics.

In Chapter 3, we assess the applicability of luminescence assays as a high-throughput method for estimating net growth above the MIC, benchmark them against CFU counts, and develop a mathematical framework that explains when each read-out reflects biomass, viable cell number, or neither. We find that light intensity correlates more closely with biomass than with viable cell number, which leads to discrepancies in growth-rate estimates under conditions that induce filamentation. However, we encountered two well-documented yet commonly ignored limitations of CFU counting — antibiotic carry-over and loss of culturability — which make rate estimates unreliable at high drug concentrations.

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In Chapter 4, we build on the validated luminescence workflow, and use it to map net-growth surfaces for 2160 drug–drug–concentration combinations, using six antibiotics. From these data, we derive interaction parameters for each pair and compare them to the expectations under Loewe additivity and Bliss independence. We find that the classification of drug interactions can shift between sub- and super-MIC ranges, implying that interaction types at sub-MIC levels do not necessarily generalise to inhibitory concentrations. Furthermore, we extend the pharmacodynamic formalism to be applicable for drug combinations, allowing us to analyse how pharmacodynamic parameters of individual drugs combine under co-treatment.

We conclude the thesis in Chapter 5 with a synthesis of our findings and a discussion of their implications for optimising antibiotic use.

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2

The Impact of Treatment Strategies on the Epidemiological Dynamics of Plasmid-Conferred Antibiotic Resistance

2.1 abstract

The issue of antibiotic resistance is a critical concern for public health, prompting numerous investigations into the impact of treatment strategies on preventing or slowing down the emergence of resistance. While existing studies have predominantly focused on chromosomal resistance mutations, the consequences of often clinically more relevant plasmid-conferred resistance remain insufficiently explored.

To address this gap, we conducted three extensive in vitro experiments utilising a liquid-handling platform. These experiments evaluated the efficacy of five distinct treatment strategies using two antibiotics (tetracycline and ceftazidime) along with two horizontally transmissible clinical resistance plasmids conferring the respective resistances.

Among the experimentally investigated treatment strategies, combination therapy proved to be the most effective in preventing the emergence of double resistance while minimising the number of infections.

To verify the reliability of these findings, we constructed a computational model of our experiments that we parameterised using the experimental data. We employed this model to augment the experimental data by conducting an *in silico* parameter sensitivity analysis. The sensitivity analysis corroborated our experimental results, demonstrating that combination therapy consistently outperformed other treatment strategies across a range of parameter values.

2.2 Introduction

In light of the growing threat of antimicrobial resistance (AMR) to human health, various multidrug strategies are being considered to improve the sustainability of antibiotic use. These approaches include combination therapy (simultaneous use of multiple antibiotics), mixing therapy

2. THE IMPACT OF TREATMENT STRATEGIES ON THE EPIDEMIOLOGICAL DYNAMICS OF PLASMID-CONFERRED ANTIBIOTIC RESISTANCE

(randomly assigning patients to receive different antibiotics), and cycling therapy (alternating between multiple antibiotics over time).

Combination, originally proposed alongside cycling therapy to prevent biocide resistance in plant pathogens [1–3], was later adopted in human medicine. Combination therapy proved its effectiveness in preventing resistance evolution in highly adaptable pathogens such as HIV, *Mycobacterium tuberculosis*, and *Plasmodium falciparum* [4]. However, a recent meta-analysis investigating the effect of combination therapy on resistance across various bacterial infections and antibiotic combinations found no evidence for a difference in the risk of resistance acquisition [5]. Also, a comprehensive cluster-randomised crossover study comparing mixing and cycling by van Duijn et al. [6], spanning nearly two years across eight ICUs, found no significant differences in outcomes.

A review of the available model literature by Uecker et al. [7] reveals the complexity and context-dependent efficacy of treatment strategies such as combination, cycling or mixing strategies. Yet, theoretical models often identify combination therapy as the best strategy to prevent new resistance [8, 9]. It remains unclear whether the inconclusive results regarding the effectiveness of multidrug treatment strategies in the literature are due to the theoretical models failing to account for key processes, or if clinical studies lack statistical power, as suggested by Siedentop et al. [5]. This lack of power may be caused by patient and bacterial strain heterogeneity, stochasticity in infection dynamics, and other unknown factors that make it difficult to isolate single effects.

We recently started experiments to make a foray into the large gap between theoretical models and clinical trials. In an in vitro experiment mimicking the epidemiological scenario of transmission in a hospital ward, Angst et al. [10] investigated the effect of cycling, mixing, and combination therapy on resistance evolution and showed that for chromosomal resistance mutations combination therapy outperformed the other strategies. One potential reason why combination therapy succeeded in that study and tends to be superior in mathematical models is that it increases the genetic barrier to resistance by requiring the acquisition of multiple mutations in the same background.

Here, we explore the effect of horizontal gene transfer (HGT) on resistance evolution under treatment by conducting three large-scale in vitro experiments. The experiments mimic epi-

demiological transmission dynamics of symptomatic infections by a focal strain in an intensive care unit (ICU) and include patient discharge and admission, infection between patients, and treatment. We use two antibiotics, ceftazidime (A) and tetracycline (B), along with two clinical resistance plasmids [11] we call p_A and p_B , conferring resistance to the corresponding antibiotics. The plasmids are compatible, can conjugate, and were isolated from clinical samples collected and characterised in a study at University Hospital Basel [12]. We model patients as wells in a 384-well microtiter plate filled with LB medium. These “patients” can be infected with a mixture of bacteria, which may carry up to two resistance plasmids. Depending on the presence of bacteria and resistance, we assign each “patient” a resistance profile: uninfected (U), sensitive infected (S), single-resistant (A_r , B_r , or $(A_r \& B_r)$), or double-resistant (AB_r).

In each experiment, we model six hospital wards to assess the ability of five treatment strategies (mixing, cycling, combination therapy with two antibiotics and two monotherapies with each antibiotic alone) and one control (no antibiotics) to contain the spread of plasmid-borne resistance and prevent the emergence of double resistance. All patients in each ward are treated daily according to the assigned strategy. A schematic of the experimental setup is shown in [Figure S2.1](#). Each of the three experiments addresses a different scenario (Table 2.1), varying in patient turnover probability (admission/discharge), infection probability, and the distribution of resistance profiles for incoming patients (sampling proportions). The prevention scenario addresses a situation with low levels of pre-existing single and no double resistance brought into the hospital ward from the community. The containment scenario focuses on the ability of treatment strategies to contain pre-existing double resistance and in the maximum-emergence scenario, we maximised the opportunities for emerging double resistance through HGT by admitting single-resistant patients only.

Alongside our experiments, we created a computational model that mimics the experiment and is parameterised but not fitted using the experimental data. We used the model to assess the robustness of our findings to the randomisation of the experimental decisions and conducted an in silico sensitivity analysis to augment the experimental data.

Results

In each of our three experiments, we simulated the transition dynamics across six hospital wards on six 384-well plates. Each 384-well plate simulates four replicate hospital wards, with each replicate comprising 96 wells representing 94 patients and two negative controls. We replace each assay plate daily to renew the treatment and medium ([Figure S2.1](#)). Based on the turnover probability τ , we randomly decide if a patient stays. If this is the case, we inoculate the well on the new plate from the same well on the old plate. Else we replace this patient with a new incoming patient by inoculating the well on the new plate from a strain plate containing all resistance profiles. The resistance profile of the incoming patient is randomly selected based on predefined probabilities (sampling proportions c_ϕ). Based on the infection probability β , we randomly decide if a patient will infect another randomly chosen patient. These infections are then simulated in vitro by passing a drop to the infected well on the new plate. All inoculations are carried out using the same pintoool.

Multidrug strategies keep the overall number of infections lowest and best suppress single resistance. The prevention scenario is characterised by a moderate proportion of single-resistant admissions to the hospital ward, the absence of pre-existing double resistance, and a moderately spreading infection dynamic ($R_0 = 1.5$, [Equation S2.1, SI Methods](#)). In this scenario, there are no differences between combination, mixing, and cycling on the frequency of uninfected, single-resistant-infected and double-resistant-infected wells ([Fig. 2.1A](#), time series in [Figure S2.2](#)).

However, all multidrug strategies are significantly better at suppressing single resistance and increasing the number of cleared wells than the single-drug strategies and the control without treatment ([Fig. 2.1A](#)). In all scenarios, combination therapy was one of the most successful treatment strategies in minimising single-resistant and overall infections. At the same time, we

Table 2.1 Parameter sets and R_0 used in the three experiments: c_ϕ is the proportion of admitted patients with resistance profile ϕ , τ denotes the probability that a patient is replaced with a new patient sampled from the community and β denotes the infection probability.

| scenario | c_S | c_{Ar} | c_{Br} | c_{ABr} | c_U | τ | β | R_0 |
|-------------------|-------|----------|----------|-----------|-------|--------|---------|-------|
| prevention | 0.75 | 0.05 | 0.05 | 0 | 0.15 | 0.20 | 0.30 | 1.5 |
| containment | 0.58 | 0.11 | 0.11 | 0.05 | 0.15 | 0.20 | 0.35 | 1.75 |
| maximum-emergence | 0 | 0.50 | 0.50 | 0 | 0 | 0.50 | 0.25 | 0.5 |

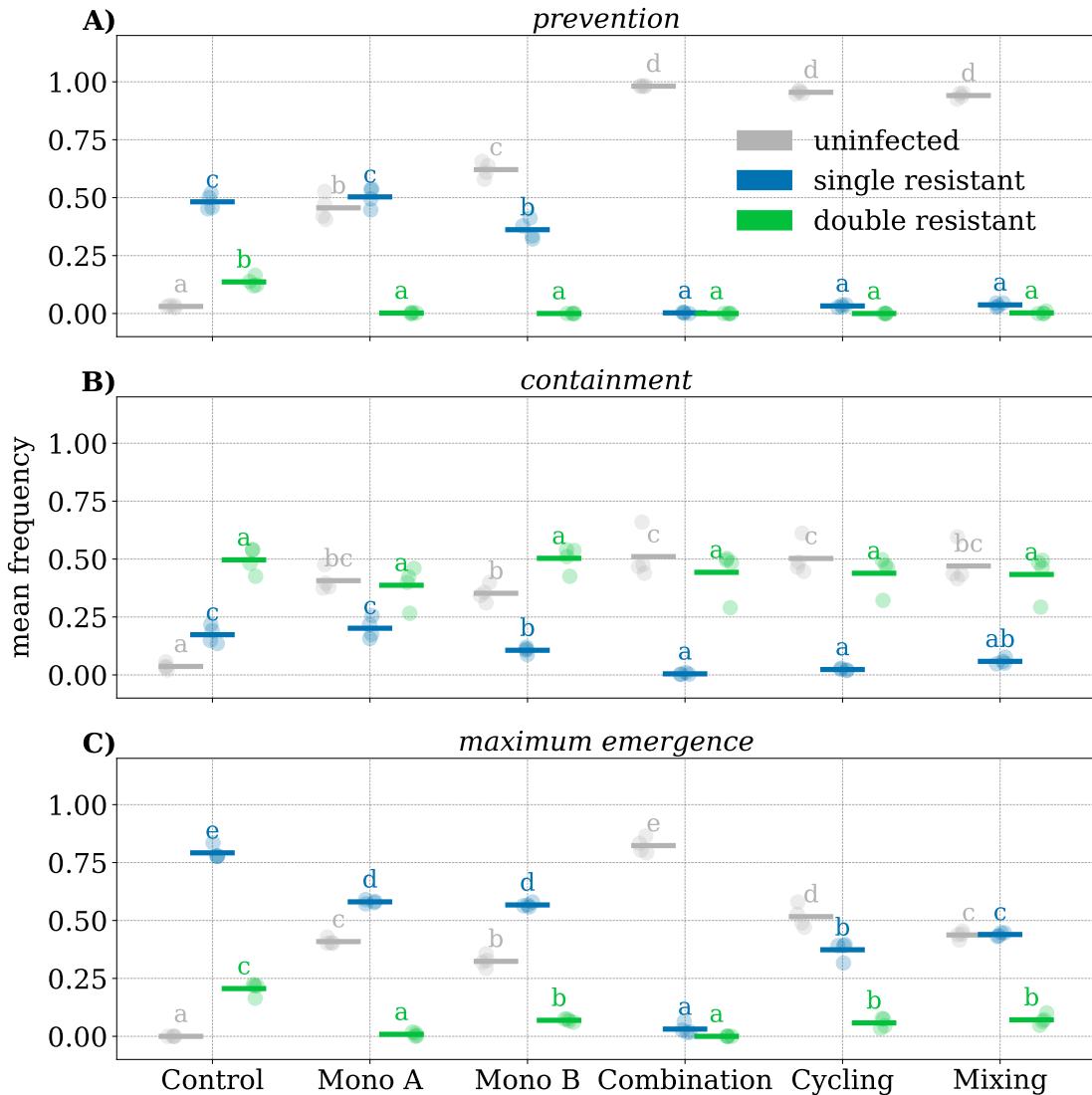


Fig. 2.1 Panels A–C show the mean frequency of uninfected (grey), single-resistant infected (blue), and double-resistant infected wells (green) during the last four transfers of the three scenarios. Circles represent replicates ($n = 4$), and bars represent means. Within resistance categories, bars not sharing a letter are significantly different (pairwise Tukey post hoc test, $p < 0.05$; ANOVA tables and all p-values can be found in Table S2.34 – S50).

observed most single and double resistance in the untreated control. All strategies (but not the control) were able to clear sensitive infections effectively with clearance probabilities of 97% for drug A, 73% for drug B, and 86% for AB (Table S2.8).

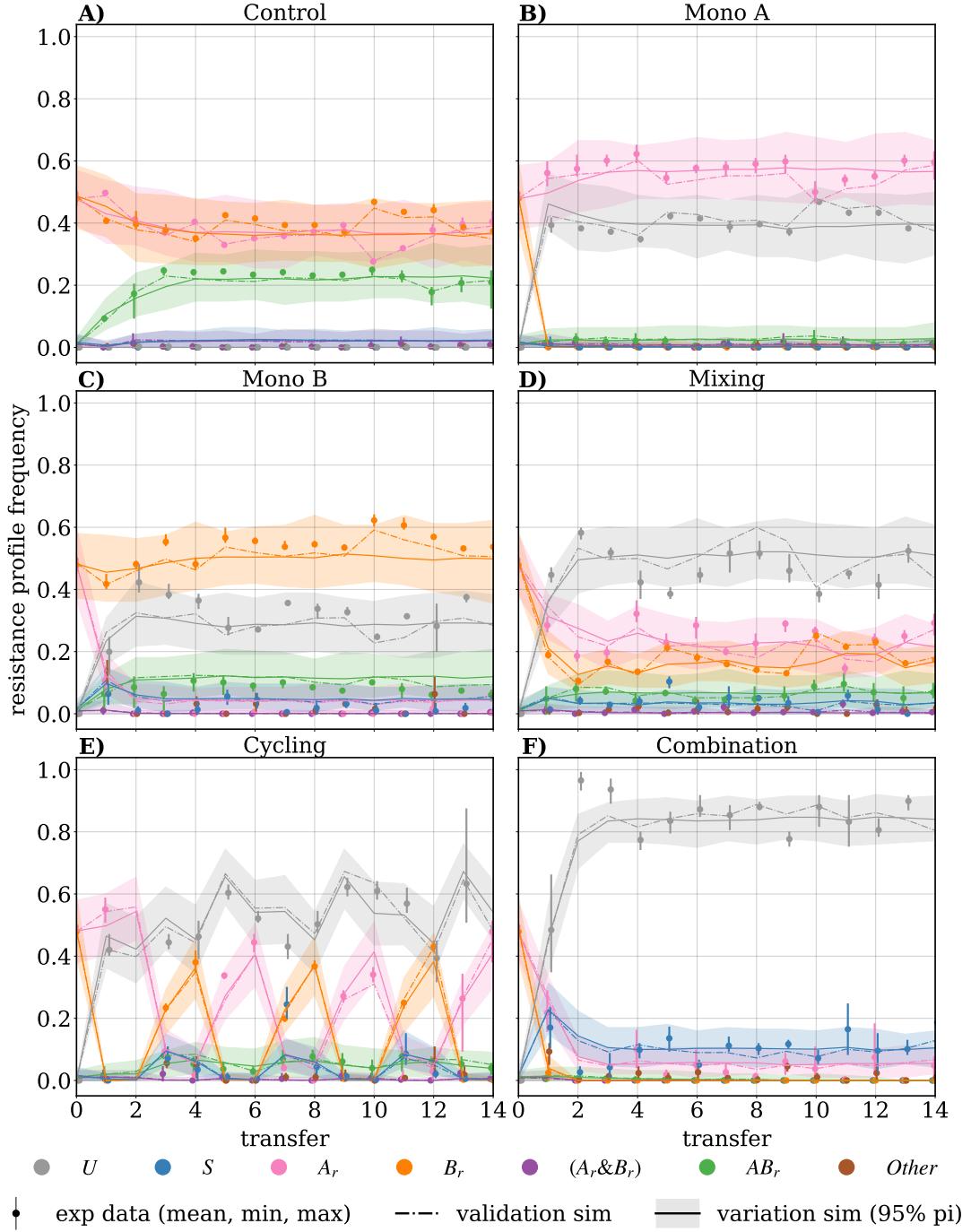


Fig. 2.2 Frequencies of resistance profiles over time during the maximum-emergence scenario. Panels (A–F) show the six tested strategies. Dots and bars show the mean and min/max interval of the four replicates. The dash-dotted line shows the mean value of 100 stochastic simulations based on the instruction set used in the in vitro experiment (validation simulation). The solid line shows the mean value of 100 simulations with randomly created instruction sets based on the parameter set used in the experiment (variation simulations). The shaded error band indicates the 95-percentile interval between the variation simulations.

All treatment strategies fail to contain pre-existing double resistance. The containment scenario explores a situation in which patients infected with double-resistant bacteria are continuously admitted to the hospital. No strategy was able to contain the spread of double resistance, resulting in increased frequencies of double resistance ($> 40\%$) in all treatment arms at the conclusion of the experiment (Fig. 2.1B).

Treatment strategies affect the emergence of double resistance. In our experiments, double resistance primarily emerges in wells inoculated with both single-resistance plasmids via HGT, as the evolution of de novo resistance (e.g. by point mutations) to high drug concentrations ($> 50\times\text{MIC}$) is unlikely. As the inoculum volumes for turnover, infection, and passage are identical in our experiments, we do not distinguish between wells containing A-resistance (A_r) infecting wells containing B-resistance (B_r) or vice versa and simply refer to these events as superinfections.

During the prevention and containment scenario, we could not identify differences in the strategies' abilities to suppress the emergence of double resistance. We attribute this to a lack of statistical power because we observed only a few instances of double resistance emerging, mostly in the untreated control. To address this, we selected parameters for the maximum-emergence scenario designed to maximise superinfection opportunities between wells carrying complementary resistance. To this end, all admitted patients carried bacteria with only one of the two resistance plasmids (at equal proportions). In addition, we set the probability of admission and discharge to $\tau = 0.5$ and the infection probability to $\beta = 0.25$, resulting in a basic reproduction number $R_0 = 0.5$ (Equation S2.1). An $R_0 < 1$ makes double resistance more likely to be replaced by newly admitted patients than to spread, thus maintaining a high potential for emergence. We implement this scenario to explore emergence under a magnifying glass, being aware that it does not reflect a likely clinical situation. In this scenario, combination therapy and monotherapy with drug A lead to the lowest frequency of double resistance during the last four transfers (Fig. 2.1C, Fig. 2.2).

For the maximum-emergence scenario, we observed that combination therapy, cycling, and monotherapy with drug A were most effective in preventing newly emerging double resistance. Combination was the only strategy in which we did not observe a single case of emerging double resistance after the first transfer (Fig. 2.3A). Furthermore, combination therapy is the most successful treatment strategy in minimising the number of both single-resistant and overall infec-

tions, while the control leads to the highest number of double- and single-resistant, and overall infections.

Combination therapy suppresses the emergence of double resistance by preventing superinfections. Treatment strategies can impact the emergence of double resistance by suppressing superinfections. The number of superinfections $n_{\mathcal{S}}$ is dependent on the abundance of the single resistance carrying wells A_r and B_r . Hence, we expect the highest number of superinfections and most opportunities for emerging double resistance when both single resistances are unaffected by the treatment and the fewest if the treatment successfully suppresses both single resistances. Our measurements confirmed these expectations during the maximum-emergence scenario. Here $n_{\mathcal{S}}$ is highest in the control group (no treatment) and lowest under combination therapy (Fig. 2.3B).

Treatment strategies influence the emergence of double resistance within superinfected wells. We observed the highest average frequency of superinfections developing double resistance ($\frac{n_{\mathcal{S}}}{n_{\mathcal{S}}}$) in antibiotic-free medium and in medium treated with antibiotic B (tetracycline). In contrast, superinfections resulting in double resistance rarely occur in medium treated with antibiotic A (ceftazidime) or both drugs (Fig. 2.3C). We think the impact of treatment on cell densities within superinfected wells (both in infected and infecting wells) can best explain these findings.

Firstly, applying a drug affects the in-well population dynamics of superinfected wells. Reducing the cell density for one or both single-resistant strains within a superinfected well reduces the probability of bacteria with complementary resistance to encounter and conjugate (see [SI Results](#)). As drug A (bactericidal) decreases the cell density faster than drug B (bacteriostatic), more conjugation opportunities occur in wells treated with drug B.

Secondly, the treatment strategies influence the number of transferred single-resistant bacteria that inoculate superinfections by curbing the bacterial density within the infecting wells (see [SI Results](#)).

Due to the differences in the abilities of drugs A and B to prevent conjugation, there are times (cycles) and places (beds) during cycling and mixing where using drug B offers increased opportunities for the emergence of double resistance, which is never the case with combination therapy.

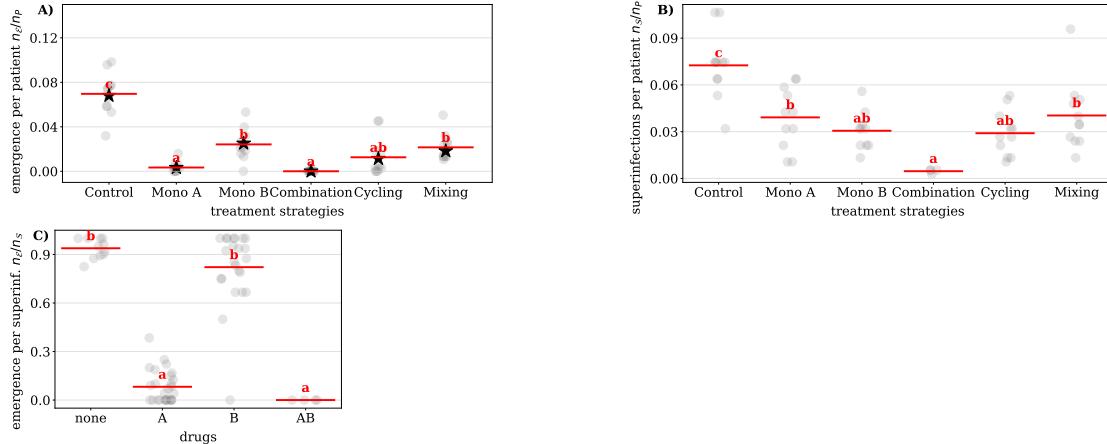


Fig. 2.3 Analysis of the emergence of double resistance in vitro and superinfection between single resistant A_r and B_r wells during the maximum-emergence scenario, from transfer four onwards. Each dot corresponds to data from a single plate, with each plate representing a distinct treatment arm, encompassing 376 patients for one transfer. Mean values are represented by red bars. Bars not sharing a letter are significantly different ($p < 0.05$, ANOVA tables and pairwise Tukey post hoc results can be found in Table S2.51 – Table S2.56). A) number of newly emerged cases of double resistance per plate (n_g), normalised to the total number of patients (wells) per plate ($n_p = 376$). Here the stars indicate predictions based on the ratio of superinfections per patient and the emergence per superinfection (SI Computational Model). B) Number of superinfections per plate (n_s), normalised to the total number of patients (wells) per plate ($n_p = 376$). C) Number of superinfected wells treated with (A, B, AB and none) that develop double resistance (n_g) divided by the number of all superinfected wells (n_s) receiving the respective treatment.

Computational model corroborates the robustness of experimental outcomes. The experiments are conducted by a liquid handling platform that carries out predefined instructions, specifying which infections occur and who is admitted or discharged. The instructions are randomized based on parameter sets we defined for each scenario, including the overall infection and turnover probability as well as the distribution of the resistance profiles of admitted patients. We call the entirety of all instructions that come up during one experiment an ‘instruction set’. Due to the scale and technical complexity of the experiments, it was not feasible to carry out individual instruction sets for each replicate, so we opted to apply the same instruction sets for all replicates. This raises the question of whether the experimental results are a consequence of a specific instance of this random process and whether they are robust to the randomisation in the instruction set. To address this, we developed a discrete-time stochastic model comprising 94 individual in silico patients mimicking the epidemiological dynamics of the experiment (SI Computational Model). The model was parametrised, but not fitted, with transition probabilities (Table S2.18–Table S2.25) that we estimated based on the transition frequencies measured

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in vitro. We used the same transition probabilities in the simulations for all scenarios.

First, we validated the model by averaging 100 validation simulations, each employing the identical instruction sets used in vitro. The aim of the validation simulations is to recreate the experiments in silico ([Figure S2.3B](#)). We found that the simulation results are in good agreement with the experimental data, indicating that the model reflects the dynamics observed in the in vitro experiments well (see Fig. 2.2, [Figure S2.2](#), and [Figure S2.4](#)). One exception is the spread of A-resistance during the prevention scenario in control and Mono A. This could indicate an increased number of contaminations at the beginning of the prevention scenario. We also observe some discrepancies for the spread of double resistance during the prevention scenario, which we attribute to contamination artefacts in the transition probabilities (see [SI Computational Model](#)).

Second, we averaged 100 variation simulations to assess the robustness of the experimental outcomes against variations in the instruction sets. In these variation simulations, each of the 100 instruction sets was randomized based on the same three parameter sets used in vitro ([Figure S2.3C](#)). Differences between the validation and variation simulations indicate differences in outcome due to the randomization of the instruction sets. For instance, with a turnover probability $\tau = 0.2$ and an admission probability $c_A = 0.05$, we expect $0.94 A_r$ admissions per transfer. However, random fluctuations can result in either more (or fewer) A_r admissions, leading to a temporarily higher (or lower) frequency of A_r in the validation simulations, creating a temporary spread between the validation and variation simulations. We observed that the validation simulations fluctuate around the variation simulations and never diverge far (see Fig. 2.2, [Figure S2.2](#), and [Figure S2.4](#)), indicating robustness of the experimental results to the randomisation of the instruction sets.

In silico sensitivity analysis indicates that the superiority of combination therapy is robust. Given that the validation simulations agreed well with the experiments, we used the model to perform an in silico parameter sensitivity analysis of the experimental results ([Figure S2.3D](#)). To this end, we ran ten simulations for each of 20,000 randomly generated parameter sets by varying the turnover and infection probability and the five sampling proportions for incoming patients: $(\tau, \beta, c_S, c_{A_r}, c_{B_r}, c_{AB_r}, c_U)$. For half of the parameter sets, we forced the frequency of incoming patients with double resistance (c_{AB_r}) to zero.

We used the frequency of uninfected *in silico* patients to measure treatment success. Using this criterion, the control strategy (no treatment) always performed worst, and accordingly, we excluded this treatment arm from the following analysis. Strategies were then classified as (i) 'single winners' if they are significantly better than all other strategies; (ii) 'winners' if they are not outperformed by any other strategy; (iii) 'losers' if they do not outperform at least one other strategy; or (iv) 'single losers' if all other strategies outperform them.

In parameter sets with and without pre-existing double resistance, combination therapy ranks most often as one of the best strategies (87% and 98%, respectively). It is the single best strategy in 55% of the tested parameter sets with pre-existing double resistance and in 93% of cases without pre-existing double resistance (Fig. 2.4, [Table S2.14](#), [Table S2.15](#)).

In some situations (for example, when one strategy is much worse than all others), it is more important to avoid the worst strategy than selecting the very best strategy among the good ones. Our analysis finds that combination therapy is almost never among the worst strategies, while usually one of the two monotherapies performs worst. As expected, single-drug strategies perform particularly poorly when there is a high frequency of pre-existing single-resistance to the applied drug ([Table S2.11](#), [Table S2.13](#)).

Cycling and mixing lose substantially less than the monotherapies but are rarely the single best strategy.

2.3 Discussion

In our study, multi-drug strategies, particularly combination therapy, outperformed monotherapies in reducing overall infections and the emergence of double resistance across most scenarios, while we observed most emergence of double resistance in the untreated control. Interestingly, the effectiveness of combination therapy does not stem from an increased efficacy associated with higher doses. This is because an asymmetrical antagonism exists between the bactericidal antibiotic ceftazidime (drug A) and the bacteriostatic antibiotic tetracycline (drug B), resulting in a lower clearance rate for the combination A+B compared to drug A alone ([SI Results](#)). This observation implies that combination therapy may be even more advantageous when drugs are neutral or synergistic towards each other.

Why does the absence of treatment lead to worse outcomes, and why is combination therapy

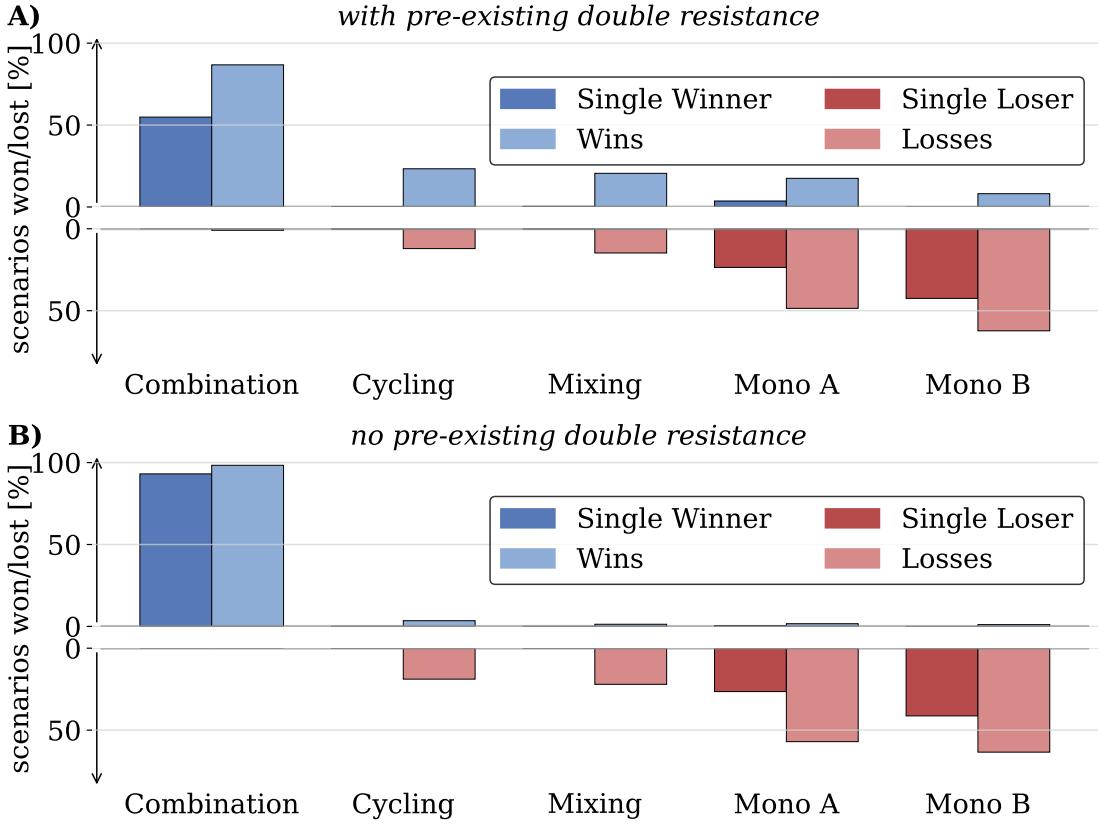


Fig. 2.4 Effectiveness of the five treatment strategies in maximising the frequency of uninfected individuals across randomly generated parameter sets. Strategies not significantly better than any other are marked as losers (pastel red), and those significantly worse than all others as single losers (dark red). Strategies not significantly worse than any other are classified as winners (pastel blue), and those significantly better than all others as single winners (dark blue). Strategies without significant differences were excluded. (A) 10,000 parameter sets with pre-existing double resistance. 606/10,000 sets yielded no significant difference between the strategies. (B) 10,000 parameter sets without pre-existing double resistance. 100/10,000 sets yielded no significant difference between the strategies.

preventing the emergence of double resistance so effectively?

First, we measured the presence, not the density, of resistant bacteria in wells by assessing if small aliquots of the liquid culture could grow on treated agar plates. This approach quantifies the number of wells hosting a specific resistance but can not quantify the frequency of resistance in the in-well population. The information about presence/absence alone yields important information about potential treatment success and is used in analogous clinical diagnostic methods, such as disk diffusion tests [13].

We would only recognize a loss of resistance (in the experiments and clinical samples) if the

resistant strain were fully outcompeted. This was not observed during the containment scenario in the untreated control. Such an outcome was expected due to the short average patient stay of 2–5 days in our experiments and 5–6 days in clinical situations [14]. For the same reason, we would not expect an eradication of resistance but only a shift in resistance density, even if there were more substantial costs of resistance or higher segregational loss. In our experiments, we found no evidence of a cost of resistance (see [SI Methods](#), [Figure S2.6](#), and [Table S2.3](#)) or segregational loss (see [SI Methods](#) and [Table S2.4](#)).

Second, in our experiments, the emergence of double resistance requires conjugation, which relies on superinfection between hosts with complementary resistance profiles. As demonstrated in Fig. 2.3B, the lowest number of superinfections occur in combination therapy, where both single-resistant strains can be cleared. Conversely, without treatment, the abundance of single resistance is highest resulting in the highest number of superinfections.

Third, the applied antibiotics affect the frequency of superinfections leading to double resistance, likely by influencing the growth dynamics within the superinfected well and potentially the conjugation rate [15]. However, our experimental data are unsuitable for supporting or rejecting the impact on conjugation rates. We observed the least emergence of double resistance in superinfected wells treated with both drugs and most in untreated wells, contributing to the superiority of combination therapy and the high rates of double resistance in the absence of treatment (Fig. 2.3C). This effect on the in-well dynamics may be a property of the chosen drugs and concentrations, and we expect better results for cycling and mixing if both drugs were equally effective in suppressing double resistance or worse results for combination therapy if the combination of both drugs was less effective.

Fourth, we observed that the number of single-resistant bacteria inoculating superinfections impacts the frequency of emerging double resistance (see [SI Results](#), [Table S2.1](#)). In our setup, superinfected wells receive two inocula, with at least one inoculum transferred from the previous plate (by infection) that has already undergone treatment for one day. When prior treatment led to a low bacterial density in the source wells, we did not observe any cases of double resistance emerging. This could magnify the effectiveness of combination therapy, where all potential single-resistant inocula transferred from the previous plate contain low bacterial densities due to effective treatment. On the one hand, this may be more a characteristic of our experimental

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setup due to the fixed length of the treatment interval and high clearance probabilities. On the other hand, we indeed expect fewer cases of emergence in superinfected patients if the infecting inocula are small.

In our experiments and simulations, combination therapy showed superior results in minimizing infections and preventing double resistance. This advantage may partly result from assumptions and simplifications, including the chosen strain, drugs, plasmids, and inoculum size, the discrete setup with fixed treatment durations, colonization-independent infection and turnover probabilities, and the absence of an immune system and microbiome. Also, treating all patients irrespective of colonization diverges from clinical reality in two ways: i) in a clinical setting, some untreated patients may serve as a sanctuary for resistance and a potential source of double resistance and ii) treating all patients, regardless of infection status, contrasts with clinical efforts to promote targeted antibiotic use. However, since patients as we model them in our in vitro experiments lack a microbiome, treating uninfecteds should have no impact on the resistance dynamics.

Despite the numerous differences between our experiments and a real clinical situation, we argue that the relative effectiveness of combination therapy in suppressing double resistance would likely translate to real patients. The reason is that the emergence of double resistance hinges on two critical processes: 1) preventing superinfections between patients carrying bacteria with complementary resistance plasmids and 2) the probability that superinfected hosts develop double resistance. We think that combination therapy offers a strategic advantage in addressing both processes.

Our results complement the findings by Angst et al. [10], who observed similar outcomes in the context of chromosomal resistance. We believe that such in vitro experimental models, which explore admittedly idealised and simplified epidemiological scenarios, can help to bridge the divide between mathematical models and randomised clinical trials. However, ultimately the evidence for or against the benefits of combination therapy must be confirmed by rigorous clinical trials with sufficient statistical power to support or challenge the effectiveness of combination therapy.

2.4 Methods

Drugs and Media. In all experiments, we used LB (Sigma L3022) with $25 \mu\text{g}/\text{ml}$ (prevention scenario) or $5 \mu\text{g}/\text{ml}$ (containment and max-emergence scenario) chloramphenicol as a liquid medium and the same LB and drugs with 1.5% agar as a solid medium. Chloramphenicol was added to prevent external contaminations. We could not measure any significant growth effects of the chloramphenicol concentrations on the chloramphenicol-resistant strains (see [Table S2.5](#)). We used $80 \mu\text{g}/\text{ml}$ ceftazidime as drug A and $40 \mu\text{g}/\text{ml}$ tetracycline as drug B, with identical concentrations for liquid and solid media.

Strains and Plasmids. We used two compatible plasmids p_A and p_B derived from samples ESBL9 and ESBL25 from a clinical transmission study [12]. Samples were kindly provided by Adrian Egli and sequenced and analysed by Huisman et al. [11]. Plasmids p_A and p_B provide (among other resistances) resistance against drug A and drug B, respectively. We used these plasmids and the chloramphenicol-resistant host MDS42-YFP [16] (sensitive to drugs A and B) to create three additional strains by conjugation ([Table S2.2](#)) (i) A-resistant, containing p_A ; (ii) B-resistant, containing p_B ; and (iii) AB-resistant, containing both plasmids (see [SI Methods](#)).

Treatment arms. We simulated the epidemiological dynamics of six hospital wards in vitro, with each ward exploring a different treatment arm: (i) control with no treatment, (ii) monotherapy with ceftazidime (mono A), (iii) monotherapy with tetracycline (mono B), (iv) cycling therapy (A, A, B, B, ...), (v) mixing therapy (treatment A and B are randomly assigned daily, without knowledge of prior treatment), and (vi) combination therapy (treating all patients with both drugs, each at full concentration).

Assay plates. Each hospital ward was simulated in vitro on a 384-well microtiter plate (Greiner 781186). Wells are interpreted as beds in four replicate hospital wards with 94 beds each. The remaining wells contained only growth medium and remained untouched, acting as sentinels for contamination. Across all experiments and treatment arms, 2752 control wells were used, 67 of which became contaminated. Wells with growth medium but no bacteria represent uninfected patients, whereas wells with growth medium and (resistant or sensitive) bacteria represent infected patients.

Experimental procedure. Experiments were performed using a Tecan Evo 200 automated liquid handling system (Tecan) with an integrated, automated incubator (Liconic STX100, Liconic), a Tecan Infinite F200 spectrophotometer (Tecan), and a camera (Pickolo, SciRobotics).

Every day new assay plates were filled with 45 μ l fresh medium and 5 μ l antibiotic stock, according to its designated treatment strategy (see [Figure S2.1](#)). At each of these transfers, we simulate patients staying overnight in the hospital (passage), the admission and discharge of patients (turnover), and infections between patients (infection). Passage, turnover and infections were all done by inoculating the new plate using a pintoool with retractable pins, as detailed below, carrying $\approx 0.3 \mu$ l drops between wells ($\approx 1 : 150$ dilution) leading to an approximately 6-8 hours exponential phase. The assay plates were then incubated at 37°C and 95% relative humidity. The incubation duration varied due to variations in the time it takes to set up a new transfer and occasional transfer repetitions made necessary because of machine errors or user mistakes. The mean incubation duration was 27 hours.

We initiated the experiment by inoculating one 384-well plate from fresh overnight cultures representing patients from an outside community. We assume that this community is sufficiently large to be unaffected by interactions with the hospital ward. Incoming patients are either uninfected or carry one of the four strains (sensitive, each single resistant or double resistant) and are sampled according to predefined sampling proportions, defining the probability of a resistance profile being admitted to the hospital. (Table 2.1). This initial plate remained untreated and was used as the initial population for all six treatment arms.

Turnover. Every transfer, each patient has a turnover probability τ to be discharged from the hospital and replaced by a newly admitted patient. Wells representing staying patients were passed from the previous to the new assay plate using the pintoool. Here, the pins for discharged patients are retracted. Vacant beds on the assay plate were then reoccupied by patients from the community analogous to the initial setup.

Infections. To simulate infections, each well has an infection probability β to infect another randomly chosen well on the next assay plate during the transfer. Therefore, each patient can infect at most one other patient per transfer, but several patients could potentially infect the same patient.

Resistance Profiles. To assess the resistance profile of each well, we spotted the previous assay plate onto four agar plates, using the pintool. Three plates were treated with antibiotics (A, B, or AB), while one was untreated (none). After incubation at 37 °C and 95% relative humidity, images of the agar plates are taken and analysed using the Pickolo package (SciRobotics, Kfar Saba, Israel). The software automatically detects the presence of colonies at each well position, which we also manually verified. The growth pattern on the four agar plates allowed us to determine the resistance profile of a well, which reflects how the well would behave if treated.

By default, we distinguish six resistance profiles ([Table S2.6](#)). The wells may either be 1) uninfected (U), 2) exclusively infected with sensitive bacteria (S), 3) infected with A-resistant bacteria (A_r), 4) infected with B-resistant bacteria (B_r), 5) infected with AB-resistant bacteria (AB_r), 6) or be infected with a mixed population containing A-resistant and B-resistant bacteria, but no AB-resistant bacteria ($(A\&B)_r$). The way we classify the resistance profiles of the bacterial population in a well leads to the dominance of resistance, in the sense that a predominantly sensitive population harbouring a resistant minority would be classified as resistant (see [Table S2.7](#)). Any observed growth pattern not corresponding to the six resistance profiles mentioned above is classified as 'other'. The resistance profile 'other' primarily occurs when bacterial densities are low (see also [SI Methods](#)).

Scenarios. We conducted experiments for three scenarios (prevention, containment, and maximum-emergence) with 14 to 27 transfers each. Each experiment was defined by a different parameter set consisting of (i) the infection probability β within the hospital, (ii) the turnover probability τ and (iii) the sampling proportions c_ϕ of patients with resistance profile $\phi \in \{U, S, A_r, B_r, AB_r\}$ (see Table 2.1).

The prevention scenario ([Figure S2.2](#)) addresses how the treatment strategies perform with a moderately resistant community and a moderate infection regime in the hospital ward and how well they are able to prevent the upcoming double resistance.

The containment scenario ([Figure S2.4](#)) corresponds to a scenario in which some patients entering the hospital are infected with double-resistant bacteria to compare the ability of treatment strategies to contain the spread of pre-existing double resistance.

During the maximum-emergence scenario (Fig. 2.2) 50 % of the incoming patients are infected

with A-resistant bacteria, and the other 50 % are infected with B-resistant bacteria. These conditions maximally favour opportunities for horizontal gene transfer. The basic reproduction number was set to $R_0 = 0.5$ ([Equation S2.1](#)) to ensure that double-resistant strains are flushed out, reducing the stochastic dependency on earlier emergence events while maintaining a high potential for new emergence.

Instruction Sets. Based on the parameter defined for each experiment (see Table 2.1), we generated instructions that were passed to the liquid handling platform. These instructions specify which patients are passaged or discharged and admitted, who infects whom, and the treatment for mixing therapy. Instructions are randomly generated prior to each transfer. We call the entirety of all instructions that come up during an experimental run an instruction set. Instruction sets are identical across all treatment arms and replicates.

Computational Model. We created a stochastic model ([SI Computational Model](#)) incorporating 94 in silico patients, each capable of adopting one of six resistance profiles $\phi \in \{U, S, A_r, B_r, AB_r, (A_r \& B_r)\}$. The model is structured analogue to the in vitro experiments ([Figure S2.1](#)) and alternates between modelling the transactions between wells and the effect of treatment during incubation. Admission and discharge (turnover) were simulated by replacing the resistance profile of the current patient with that of the incoming patient, as defined by the instruction set. Infections are simulated by combining the resistance profiles of the receiving well i and the infecting well j . The resulting resistance profile $\phi_i + \phi_j$ is determined using the rules based on the dominance of resistance specified in [Table S2.9](#). Calculations involving more than two resistance profiles apply the associative law and are determined pairwise, e.g. $(U + S) + A_r = S + A_r = A_r$.

To model treatment effects, we use transition probabilities to assign the post-incubation resistance profile $\phi(\hat{T})$ stochastically based on the treatment and the pre-incubation resistance profile $\phi(T)$. The transition probabilities ([Table S2.18 – Table S2.25](#)) were estimated based on experimental data across all experiments.

In Silico Sensitivity Analysis. To augment the experimental data, we conducted an in silico sensitivity analysis. We randomly generated 10,000 parameter sets with and 10,000 without pre-existing double resistance. Turnover and infection probabilities were uniformly sampled

[0.05, 0.95], allowing for $R_0 \in [0.0526, 19]$. The sampling proportions c_ϕ for all incoming resistance profiles ($\phi \in \{U, S, A_r, B_r, AB_r\}$) were randomised by sampling a number $n_\phi \in [0, 1]$ from a uniform distribution and subsequently normalising by the sum: $c_\phi = n_\phi / \sum_j n_j$. We created ten randomised instruction sets for each parameter set and conducted one simulation per instruction set (Figure S2.3D) for 28 transfers.

For this analysis, the frequency of non-infected individuals during the last four transfers was used as a performance metric for treatment strategies, as it also indirectly reflects the frequency of both double- and single-resistant patients. We conducted an ANOVA test to assess if the effect of the treatment strategies significantly ($p < 0.05$) influences the frequency of uninfecteds. For significant tests, we proceeded with Tukey's post hoc analysis ($p < 0.05$), identifying significantly distinct pairs of strategies. Strategies not significantly inferior to others were classified as 'winners', while strategies not significantly superior to any were classified as 'losers'. Strategies that win or lose a parameter set alone are 'single winners' or 'single losers'.

Data Availability Experimental data and analysis scripts, as well as code for the computational model, have been deposited in Zenodo (<https://doi.org/10.5281/zenodo.14137410>).

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2.1 Supplementary Information Chapter 2

2A SI Methods

Strains. We chose two plasmid-carrying donors, ESBL9 and ESBL25, and two drugs, ceftazidime and tetracycline, based on the resistance conferred by the plasmids contained in the strains and the compatibility of the plasmids. ESBL9 and ESBL25 were collected as part of a clinical transmission study at the University Hospital Basel, Switzerland [1] and fully sequenced, including identification of the carried plasmids [2]. The strains were a generous gift from Adrian Egli, University Hospital Basel.

ESBL9 contains an IncI1 plasmid, referred to here as p_A , conferring, among others, resistance to ampicillin and ceftazidime but not tetracycline or chloramphenicol. ESBL25 contains an IncF1 plasmid, referred to here as p_B , conferring, among others, resistance to ampicillin and tetracycline but not ceftazidime or chloramphenicol.

The two plasmids were transferred by conjugation from the original clinical isolates to the chloramphenicol-resistant and ampicillin-sensitive Escherichia coli MDS42-YFP (recipient) [3], followed by selection for ampicillin and chloramphenicol resistance. This results in the ceftazidime-resistant (A-resistant) strain and the tetracycline-resistant (B-resistant) strain. The double-resistant (AB-resistant) strain was created by a further round of conjugation to receive both plasmids and subsequent selection for ceftazidime and tetracycline resistance. Strains are listed in Table S2.2. All transconjugants were verified by PCR replicon typing using primers specific for the respective replicon [4].

Drugs. We used ceftazidime, referred to as drug A, at a concentration of $80 \frac{\mu\text{g}}{\text{mL}}$. $80 \frac{\mu\text{g}}{\text{mL}}$ is substantially lower than the MIC for A-resistant bacteria and more than 50 times the MIC for sensitive or B-resistant bacteria. Using the same reasoning, we used tetracycline, referred to as drug B, at a concentration $40 \frac{\mu\text{g}}{\text{mL}}$. The antibiotic concentrations in the liquid and the solid media were identical. To avoid contamination, we used $25 \frac{\mu\text{g}}{\text{mL}}$ chloramphenicol for prevention scenario and $5 \frac{\mu\text{g}}{\text{mL}}$ chloramphenicol for containment and maximum-emergence scenarios. We could not measure any significant growth effects of the chloramphenicol concentrations on the chloramphenicol-resistant strains (see Table S2.5).

Conjugation Protocol. We used ampicillin-resistant and chloramphenicol-sensitive original donors [1, 5] and the chloramphenicol-resistant, ampicillin-sensitive recipient [3]. Fresh overnight cultures of both donors and recipients were diluted 1:1000 and grown to mid-exponential phase. Following this, the donor and recipient cultures were combined in a culture tube and incubated for four hours at 37 °C with constant shaking at 180 rpm. We then spotted a 100 μl drop of this mixture on an agar plate treated with 25 $\frac{\mu\text{g}}{\text{ml}}$ chloramphenicol and 100 $\frac{\mu\text{g}}{\text{ml}}$ ampicillin, allowing only the transconjugants to grow. Conjugation was verified by PCR replicon typing [4].

Plasmid costs To measure plasmid costs, we grew three replicates of overnight cultures of all strains in selective medium. The cultures were then diluted approximately 1:150 into LB with 5 $\mu\text{g}/\text{ml}$ chloramphenicol using the pintoool, following the same procedure as in the main experiments. Subsequently, we recorded OD growth curves using the same plate reader. The maximum growth rate was estimated by applying linear regression to a sliding window on the log-transformed data (window size: 1 hour, step size: approximately 5 minutes). Pairwise comparisons were performed between the maximum growth rates of the sensitive strain and the plasmid-carrying strains using the Mann-Whitney U test (scipy.stats [6]), followed by a Bonferroni correction to account for multiple testing. We observed no significant difference in the maximum growth rate between any of the pairs (Table S2.3).

Segregational Loss We estimated plasmid segregation loss over 24 hours ($t_0 - t_1$) without treatment and with treatment as a control. For this, we grew overnight cultures in selective medium for three replicates $k \in \{1, 2, 3\}$ of each plasmid-carrying strain. We diluted the cultures and plated each on drug-free agar plates, followed by replica plating onto selective plates to identify the presence or absence of resistance plasmids in each colony. This initial step represents time point t_0 . The overnight cultures were then transferred to i) drug-free medium and ii) selective medium (control), using the same pintoool as in the main experiments. The cultures were incubated for 24 hours, after which we diluted and plated them again on drug-free plates and used replica plating on selective plates to assess plasmid presence for time point t_1 . We compared the frequencies $f_k(t)$ of plasmid presence between time points t_0 and t_1 using the Mann-Whitney U test (scipy.stats [6]). No significant loss of plasmids was observed in either the control or the experimental conditions (Table S2.4). We estimated the mean frequency of plasmid pres-

ence $f(t)$ for each strain and time point and the confidence intervals $CI(t)$ for the frequency by bootstrapping the pooled colony presence-absence data.

Growth rates and bacterial density. We assessed the final bacterial density of overnight cultures following an 18-hour incubation period (Table S2.5) for each bacterial strain in its respective selective medium for two different chloramphenicol concentrations: 5 $\frac{\mu\text{g}}{\text{mL}}$ and 25 $\frac{\mu\text{g}}{\text{mL}}$. To estimate the bacterial density, we plated 200 μL of various dilutions of the cultures on agar plates using glass beads. The 95 % confidence intervals for the colony counts were calculated using the Poisson distribution.

In addition, we monitored the optical density (OD) in 384-well plates over an 18-hour period in the respective selective medium containing either 5 $\frac{\mu\text{g}}{\text{mL}}$ or 25 $\frac{\mu\text{g}}{\text{mL}}$ chloramphenicol. To determine the maximum growth rates, we employed a sliding window approach with a one-hour width, linearly fitting the growth rate to the log transformed values within this window for each replicate. The 95 % confidence intervals for the maximal growth rates were calculated using the Student's t-distribution.

Basic Reproductive Number. R_0 represents the number of secondary infections generated by one patient in a fully susceptible population. Let τ denote the probability that a patient leaves the hospital ward after one timestep, $\tau' = 1 - \tau$ the probability that the patient stays and β the probability that a patient infects another during one timestep. Then we can write the total number of infections caused by one patient introduced into a susceptible population as a geometric series:

$$R_0 = (\tau')^0 \cdot \beta + (\tau')^1 \cdot \beta + (\tau')^2 \cdot \beta + \dots = \beta \sum_{n=0}^{\infty} (\tau')^n$$

This geometric series can then be rewritten as:

$$R_0 = \beta \cdot \frac{1}{1 - \tau'} = \frac{\beta}{\tau} \quad (\text{S1})$$

Phenotyping -- Limitations. The phenotyping procedure enables high throughput identification of resistance profiles. Although this method is generally reliable and effective for most wells, it is difficult to accurately determine the resistance profile for wells with very low bacterial densities

2. THE IMPACT OF TREATMENT STRATEGIES ON THE EPIDEMIOLOGICAL DYNAMICS OF
PLASMID-CONFERRED ANTIBIOTIC RESISTANCE

due to the potential for stochastic effects. We analysed 1784 A_r turnover wells treated with antibiotic B during the maximum-emergence scenario. Here, we observed, alongside the expected A_r and U wells, 9% 'other' and 17% S wells, as detailed in Fig. S2.5a. It might be tempting to interpret the S wells as those in which all bacteria lost their plasmids and the 'other' wells as artefacts of measurement errors. Although these interpretations are not incorrect, a more critical factor influencing the measured resistance profile distribution is the inherent stochasticity of the method if applied to low-density wells.

As an example, we will analyse wells identified as A_r -wells during the previous transfer and subsequently treated with antibiotic B. To simplify the following analysis, we only consider agar plates treated with drug $\vartheta \in \{N, A\}$ (no drug, drug A) and disregard plates treated with drug B (B-plates) and AB (AB-plates). Furthermore, we will use a prime ('') to indicate a counter probability ($w' = 1 - w$). Drawing a drop with volume V_{drop} from a well with volume V_{well} leads to a probability $p = \frac{V_{\text{drop}}}{V_{\text{well}}}$ of drawing a specific bacterium. The probability that the drawn drop contains no bacteria of phenotype $\psi \in \{\emptyset, a\}$ (without resistance and A-resistance) is $d'_\psi = (1 - p)^{Z_\psi}$, with Z_ψ representing the number of bacteria with phenotype ψ inside the well. We denote the probability that a drop can grow on a plate treated with drug ϑ as g_ϑ . The probability g'_N of drawing a drop that will not form a colony on an N-plate can then be defined as $g'_N = d'_\emptyset d'_a$, and the probability g'_A of drawing a drop that will not grow on an A-plate is $g'_A = d'_a$.

Assuming that the drawn drop does not significantly alter the well volume and composition, we obtain the following probabilities for the possible resistance profiles (see also Fig. S2.5b):

$$\begin{aligned}\mathbb{P}(U) &= g'_N \cdot g'_A \\ \mathbb{P}(S) &= g_N \cdot g'_A \\ \mathbb{P}(A_r) &= g_N \cdot g_A \\ \mathbb{P}(\text{other}) &= g'_N \cdot g_A\end{aligned}$$

Assuming $p = 0.006$ (value for our experiment), a well containing 25 A-resistant and 15 sensitive bacteria will result in 70% U , 26% S , 3% A_r , and 11% other. This result is similar to the experimentally measured distribution (compare Fig. S2.5a).

Advanced Resistance Profiles We introduced advanced phenotypes into our analysis to determine the approximate bacterial density inside the wells. Wells that retain their phenotype after one transfer, despite being sensitive to a particular antibiotic, are expected to exhibit a low bacterial density post-treatment and are labelled ϕ^l . Conversely, wells that were either untreated or treated with an ineffective antibiotic are expected to contain a high bacterial density and are labelled ϕ^h . In addition, wells that underwent a change in resistance profile or were mixed with other wells are denoted as $\phi^?$ and are excluded from further analysis. We summarised the influence of the bacterial densities within the inoculating wells on the frequency of superinfections developing double resistance in Table S2.1.

Statistical Analysis. To compare the performance of different treatment strategies in vitro, we summarized the resistance profiles into groups and focused our analysis on three groups: uninfected, single-resistant and double-resistant. These labels stem from the properties within the wells. That means single resistant wells (A_r , B_r or $(A_r \& B_r)$) would be wells that contain only single resistant (or sensitive) bacteria, but no double resistant bacteria and therefore can be cured by combining drugs. In contrast, double-resistant wells cannot be cured using both antibiotics simultaneously since they contain AB-resistant bacteria. We then averaged the frequency of each group over the last four transfers for every replicate. Four transfers correspond to a complete cycle in the cycling strategy with a period of 2 (e.g., A-A-B-B). The effect of the treatment strategy on the average frequencies of uninfected, single-resistant, and double-resistant wells was then tested using a one-way ANOVA. In the case of a significant test ($p < 0.05$), we conducted a pairwise Tukey post hoc analysis to relate the mean frequencies.

In addition, we analysed superinfections between patients and the emergence of double resistance across different strategies in the maximum-emergence scenario. We considered all measurement points from the fourth transfer onwards as at near-stationary level for the non-cycling strategies. Consequently, the initial conditions for each new transfer remain approximately the same or are repeated every fourth transfer in the case of cycling.

For this analysis, we counted the number of newly emerged double-resistant wells n_{ϕ} and the number of superinfections $n_{\mathcal{S}}$ across all replicates for each plate, with each plate representing one treatment arm for a single transfer. Newly emerged double-resistant wells are defined as those exhibiting double resistance but not having previously been passaged or infected by a

double-resistant well. Additionally, for each plate, we analysed all wells treated with treatment ϑ , counting the number of superinfected wells $n_{\mathcal{S}}^{\vartheta}$ and those among them that developed double resistance $n_{\mathcal{E}}^{\vartheta}$.

We then tested whether the treatment strategy significantly affects the emergence frequency $f_{\mathcal{E}} = n_{\mathcal{E}}/n_p$ and the frequency of superinfection $f_{\mathcal{S}} = n_{\mathcal{S}}/n_p$, with n_p being the number of patients in a hospital ward across all replicates (376) using an ANOVA. Additionally, we used an ANOVA to assess if treatment ϑ significantly influences the frequency of superinfected wells that develop double resistance $n_{\mathcal{E}}^{\vartheta}/n_{\mathcal{S}}^{\vartheta}$.

Subsequent to a significant ANOVA test, we conducted pairwise Tukey post hoc comparisons between the treatment arms ($p < 0.05$).

All statistical analyses were performed in Python 3.8.5 using SciPy's `f_oneway()` [6] for ANOVA tests and Statsmodels' `pairwise_tukeyhsd()` [7] for conducting Tukey's honest significant difference post hoc analyses.

Maximum-emergence scenario: Predicting the Emergence Probability. We counted for each plate i the number of superinfected wells $n_{\mathcal{S}}^i$ and normalized them by the number of patients per plate n_p to calculate the frequency of superinfection ($f_{\mathcal{S}}^i = \frac{n_{\mathcal{S}}^i}{n_p}$). Then, we estimated the probability of superinfection $\mathbb{P}(\mathcal{S})$ for each treatment arm by averaging $f_{\mathcal{S}}^i$. In addition, we approximated the probability of emergence for superinfected wells $\mathbb{P}_{\vartheta}(\mathcal{E}|\mathcal{S})$ under treatment ϑ , by normalizing the total number of newly emerged resistances across all plates $N_{\mathcal{E}}^{\vartheta}$ by the total number of superinfected wells under treatment ϑ ($N_{\mathcal{S}}^{\vartheta}$): $\frac{N_{\mathcal{E}}^{\vartheta}}{N_{\mathcal{S}}^{\vartheta}}$. The estimates for $\mathbb{P}_{\vartheta}(\mathcal{E}|\mathcal{S})$ were then utilized to approximate the average probability of superinfected wells developing double resistance for each treatment arm. The weighted average of all $\mathbb{P}_{\vartheta}(\mathcal{E}|\mathcal{S})$ were computed using the proportion of patients receiving treatment ϑ as weights. For example, in mixing, the average probability $\bar{\mathbb{P}}(\mathcal{E}|\mathcal{S})$ is given by $0.5\mathbb{P}_A(\mathcal{E}|\mathcal{S}) + 0.5\mathbb{P}_B(\mathcal{E}|\mathcal{S})$. We then used Equation S2 to predict the average probability of emerging double resistance $\mathbb{P}(\mathcal{E})$ for each strategy, as indicated in Figure 3A by black stars.

$$\mathbb{P}(\mathcal{E}) = \mathbb{P}(\mathcal{S})\bar{\mathbb{P}}(\mathcal{E}|\mathcal{S}) \quad (\text{S2})$$

2B SI Results

Impact of Treatment on the Emergence of Double Resistance. As demonstrated in Figure 3B, our findings indicate that treatment substantially influences the frequency of emerging double resistance in superinfected wells. Population dynamics within wells can potentially explain these results. Here we approximate E_θ , the expected number of conjugations during one treatment phase under treatment ϑ , as $E_\theta \propto \gamma_\vartheta \int_{t1}^{t2} {}^A X_\theta(t) {}^B X_\theta(t) dt$. ${}^i X$ represents the density of bacteria with resistance i , and γ_ϑ is the treatment dependent conjugation rate. The experimentally generated data are insufficient for adequately estimating γ_ϑ . However, if we assume identical initial bacterial populations, we can qualitatively rank the cumulative product of bacterial densities $\int_{t1}^{t2} {}^A X_\theta(t) {}^B X_\theta(t) dt$. The highest cumulative product is achieved when ${}^A X$ and ${}^B X$ grow without or with ineffective treatment. Additionally, we know that the clearance rate of ceftazidime (drug A) is substantially higher than that of tetracycline (drug B), as shown in Table S2.8, resulting in a larger cumulative product over time when treated with antibiotic B. Lastly, the lowest cumulative product is associated with treatment AB, where neither of the two strains can grow. Therefore, if we disregard γ_ϑ , the above reasoning predicts the following ranking for the number of emergences per superinfection: None, B, A, AB. This predicted ranking aligns with the ranking observed in [Figure 3C](#).

Another potential explanation are potential differences in the plasmid-specific conjugation rates. If, for example, p_B had a higher conjugate rate than p_A , then drug A would have a stronger impact on the emergence of double resistance, even if we assumed identical clearance rates.

Treatment strategies influence the number of bacteria inoculating superinfections. We observed that the number of single-resistant bacteria that inoculate superinfections affects the emergence of double resistance (Table S2.1). At least one of the two superinfection-initiating inocula originates from infections between patients and is sourced from the previous assay plate. The cell densities and compositions within these source wells, which have already undergone treatment for one day, vary considerably depending on the resistance profile ϕ and the treatment history. We used 'advanced phenotypes' (see SI Methods) to distinguish between high-density (ϕ^h) and low-density (ϕ^l) wells, assigning these based on the wells resistance profile (ϕ) and treatment history.

During the maximum-emergence scenario, which contained the highest number of superinfections, we made two noteworthy observations. First, superinfections between ϕ^h and ϕ^l ($A_r^l + B_r^h$ (43 superinfections) and $A_r^h + B_r^l$ (one superinfection)) occur significantly less frequently than superinfections between $A_r^h + B_r^h$ (a total of 1176 superinfections, as outlined in Table S2.1). This discrepancy can be attributed to the high clearance rates of both drugs, resulting in a higher prevalence of ϕ^h compared to ϕ^l .

Second, none of the 44 superinfections involving $A_r^l + B_r^h$ and $A_r^h + B_r^l$ resulted in double resistance.

Antagonism. Adding the bacteriostatic antibiotic tetracycline (drug B) reduces the probability of clearing sensitive bacteria with the bactericidal antibiotic ceftazidime (drug A). The clearance probability drops from 0.97 to 0.86, as shown in Table S2.8. Antagonism between bactericidal and bacteriostatic antibiotics has been documented by various researchers since the 1950s, as exemplified by the works of Cates [8] and Jawetz [9], and also more recently by Ocampo [10]. The antagonistic effect may arise because the bacteriostatic drug (tetracycline) lowers the growth rate, resulting in a decreased kill rate of the bactericidal drug (ceftazidime) [11]. Accordingly, this antagonistic effect is anticipated to be less pronounced for a tetracycline-resistant strain, where the impact on the growth rate is diminished. This hypothesis is supported by the measured clearance rates for B_r wells, where the clearance probability remains at 0.98 for treatment with drugs A and AB Table S2.8.

2C SI Computational Model

Stochasticity We conducted three experiments, each defined by one parameter set consisting of a turnover probability τ , an infection probability β and the probability distribution for sampling patients c_ϕ with different resistance profiles $\phi \in \{U, S, A_r, B_r, AB_r\}$. For each experiment, we randomly generated one instruction set based on the given parameter set. Due to the scale and complexity of the experiment, it was infeasible to conduct a unique instruction set for each replicate. Therefore, we opted to employ identical instruction sets for all replicates, which reduces the number of robot arm movements (and time) for infection and turnover by a factor of four.

As a consequence, our replicates may be interpreted as patients with identical histories regarding original infection, treatment and exchange with other patients. However, due to the accumulated biological stochasticity along the patient histories, their phenotypic properties may vary, as

reflected by the variance of the replicates.

Since we only tested one instruction set per replicate in vitro, we wondered whether the measured results depend on the randomisation of the instruction set and if we would expect a qualitatively different result if we reran the experiment 100 times. To answer this question, we created a computational model that, for a given parameter set, rerandomises the instruction set and conducts a stochastic simulation to mimic the biological variability. In Fig. S2.3, we visualised the different sources of experimental and computational variability.

Transition Probabilities. We used the experimental data to calculate the transition frequencies for all pre-treatment $\phi_\vartheta^i(T)$ to post-treatment $\phi_\vartheta^i(\hat{T})$ resistance profile combinations for each plate i and treatment ϑ . $\phi(\hat{T})$ is measured during the phenotyping procedure, while $\phi(T)$ is estimated by employing one plate-handling simulation step to $\phi(\hat{T} - 1)$ as described in the methods (e.g. $A_r + S \rightarrow A_r$). Then, we estimated the transition probabilities as the weighted average of transition frequencies across all plates, with the count of $\phi_\vartheta(T)$ on each plate as a weight.

For each treatment ϑ , we created one transition matrix M^ϑ , with the pre-treatment resistance profile $\phi(T)$ as columns and the post-treatment profile $\phi(\hat{T})$ in the rows (Table S2.18–S2.21). To simulate the incubation phase, we use M^ϑ to stochastically assign the post-treatment resistance profile $\phi_\vartheta(\hat{T})$, using the respective column of the transition matrix as a probability distribution.

Transition Probabilities for Transfer 1. All patients are untreated during transfer 0, leading to exceptionally high rates of superinfections and high emergence rates per superinfection during transfer 1. To account for this, we created four additional transition matrices for simulating the first transfer (see Table S2.22–S2.25).

Choice of Model. We also considered using a continuous model. However, a typical population-based model would not match the experimental measurements for effectively treated patients. This is due to the discrete nature of our experimental setup. Here, the frequency of infected patients has a local maximum before treatment and a local minimum after treatment, creating sawtooth-shaped frequencies over time. We conducted the phenotyping at the end of the incubation period, at the local low point, diverging from the average frequencies predicted by a continuous model. Therefore, a continuous model would either use realistic clearance rates and

not fit through the experimental data points or use exaggerated clearance rates and fit through the data points. For example, treating an S well with a hypothetical drug C leads to a steady decline in bacterial density over time, resulting in a bacterial density below the detection limit after 24 hours (our first experimental measurement point). If initially, all wells are infected, and at the first measurement point, the infection drops to 0%, fitting a continuous compartmental model to these data would result in infinite clearance rates.

Contamination of the Transition Matrix. The computational simulations employ four transition matrices ($M^{\text{none}}, M^A, M^B, M^{AB}$) derived from the observed transitions during the three experiments. During the experiments, we observed a low rate of contamination affecting neighbouring wells, likely due to pipette and plate movements by the liquid handling platform. Quantifying the exact contamination rate is challenging, though the observed mean transition probability from U to U is 0.99%.

These contaminations can be inconsequential; for example, an S well contaminating an A_r well will not cause a shift of the resistance profile in the contaminated well. However, they can also lead to artefactual transitions that are reflected in the transition matrices, such as $U \rightarrow S$ (Table S2.18), $U \rightarrow A_r$ (Table S2.19), or $U \rightarrow B_r$ (Table S2.20). The impact of the recorded artefactual transitions in the transition matrices depends on the frequency and the transferred resistance profile. For instance, in Mono A, a high frequency of A_r contaminations is observed due to the predominant presence of A_r wells, creating the impossible transition $U \rightarrow A_r$ described above, which now occurs in all simulations independent of the presence or frequency of A_r during the simulation. Similarly, in the containment experiment, the abundance of AB_r wells in all treatment arms led to a higher rate of double-resistant contamination reflected in the transitions: $A_r \rightarrow AB_r$ (Table S2.18) and $A_r \rightarrow AB_r \& B_r \rightarrow AB_r$ (Table S2.20). Resistance mutations could also explain these transitions; however, because they occurred mainly during the containment scenario and the fact that they exclusively came up in wells neighbouring double-resistant wells, we believe that they are an artefact of unintended infections.

Artefactual transitions such as $U \rightarrow S$, or $U \rightarrow A_r$ have a neglectable effect on the simulation of all scenarios, as their occurrence in the regular infection and admission processes outweighs the contribution through the artefactual transitions. Similarly, double-resistant contaminations minimally impact the simulations of both the containment scenario (where double resistance

is regularly admitted) and the maximum-emergence scenario (due to a low R_0 and frequent emergence of double resistance). However, they pose a problem to the simulation of prevention scenario, where a low (untruthful) influx of AB_r can spread ($R_0 > 1$).

Filtered Transition Probabilities. To mitigate the effect of contaminated transition matrices, we introduced filtered transition matrices. For this, we assumed no resistance mutations and forbidding impossible transitions (by setting the transition probability $U \rightarrow U$ to 1; see Table S2.26 – S2.33).

Using these filtered probabilities to simulate the prevention scenario leads to better-matching results, almost removing the spread of double resistance in Mono A and the multidrug strategies and thereby matching the experimental data better (compare the green error bands between Fig. S2.2 & Fig. S2.7). In addition, we conducted a secondary sensitivity analysis with these filtered probabilities (see Fig. S2.8). Because the overall conclusions are consistent between the simulations using filtered and unfiltered transition probabilities, we opted to use the unfiltered transition probabilities in the main paper for a more direct representation of the experimental data.

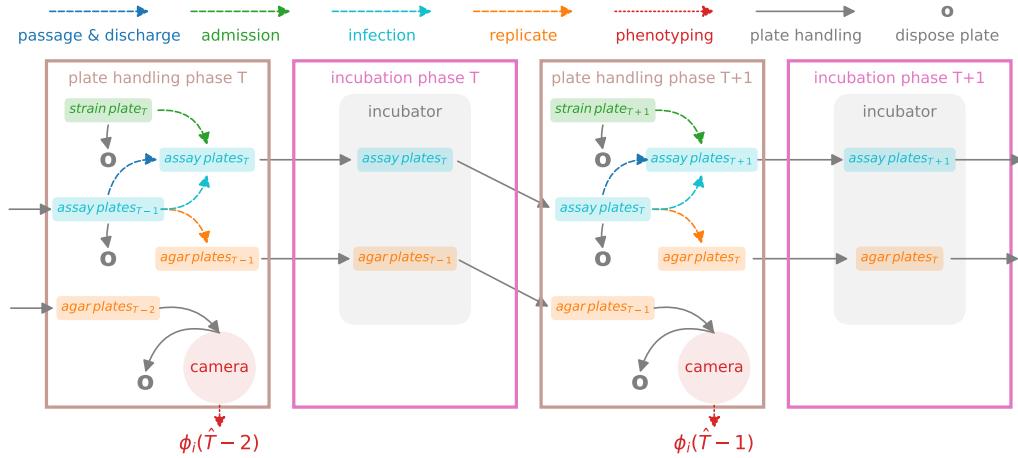


Fig. S2.1 Schematic illustrating the procedure used in the experiment for transfers T and T+1 in the liquid handling platform after adding medium and drugs to the assay plates. Every transfer (day), we provide new assay and agar plates. Plates from the previous transfers are removed. To inoculate the new assay plates with newly admitted patients from the strain plate, along with staying patients and infection between patients from the previous assay plate, we use a pintoil with retractable pins (dashed lines). Discharged patients are not transferred (pins retracted) to the new assay plates. Plates are then automatically transferred (solid lines) to the incubator for overnight incubation. Subsequently, we replicate each assay plate onto four agar plates using the pintoil. These plates are treated with antibiotics A, B, and AB, and one remains untreated. Once the agar plates have been incubated overnight, we capture images (dotted lines) to determine the resistance profile ϕ_i for each well i .

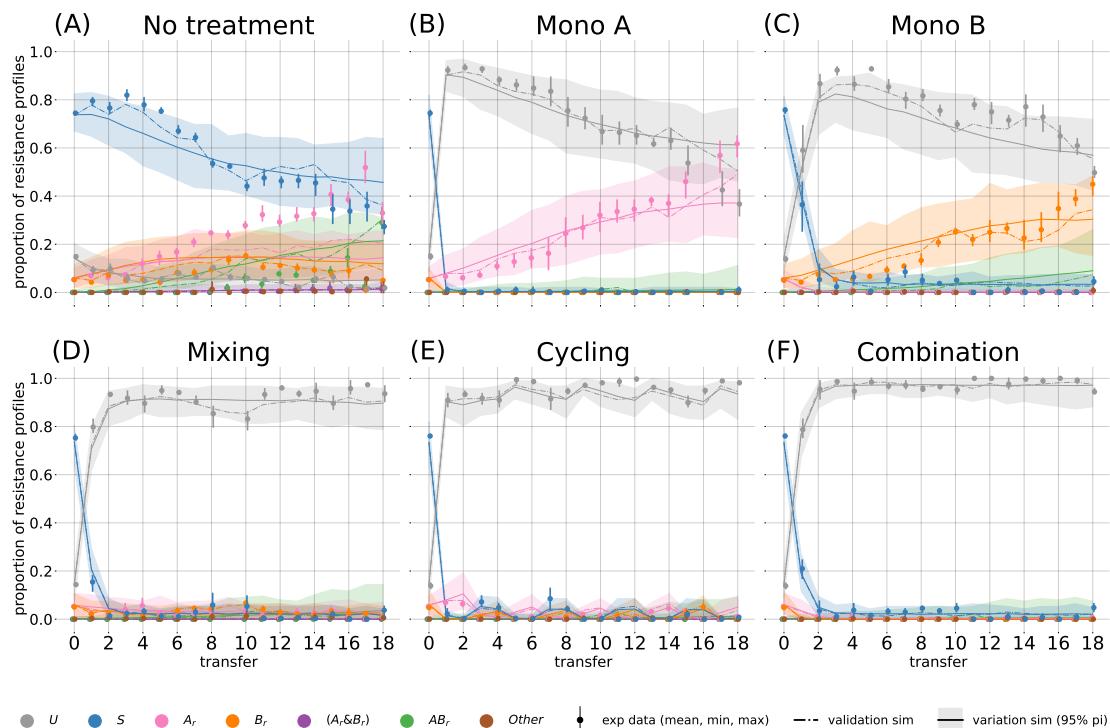


Fig. S2.2 Prevention scenario: Frequencies of resistance profiles (colours) over time during the prevention scenario. The dots show the experimental measurements, and the error bar indicates the min/max interval between the replicates. The dash-dotted line shows the mean value of 100 stochastic simulations based on the instruction set used in the in vitro experiment. The solid line represents the mean value of 100 simulations with randomly created instruction sets based on the parameter set used in the experiment. The shaded error band indicates the 95-percentile interval between the simulations.

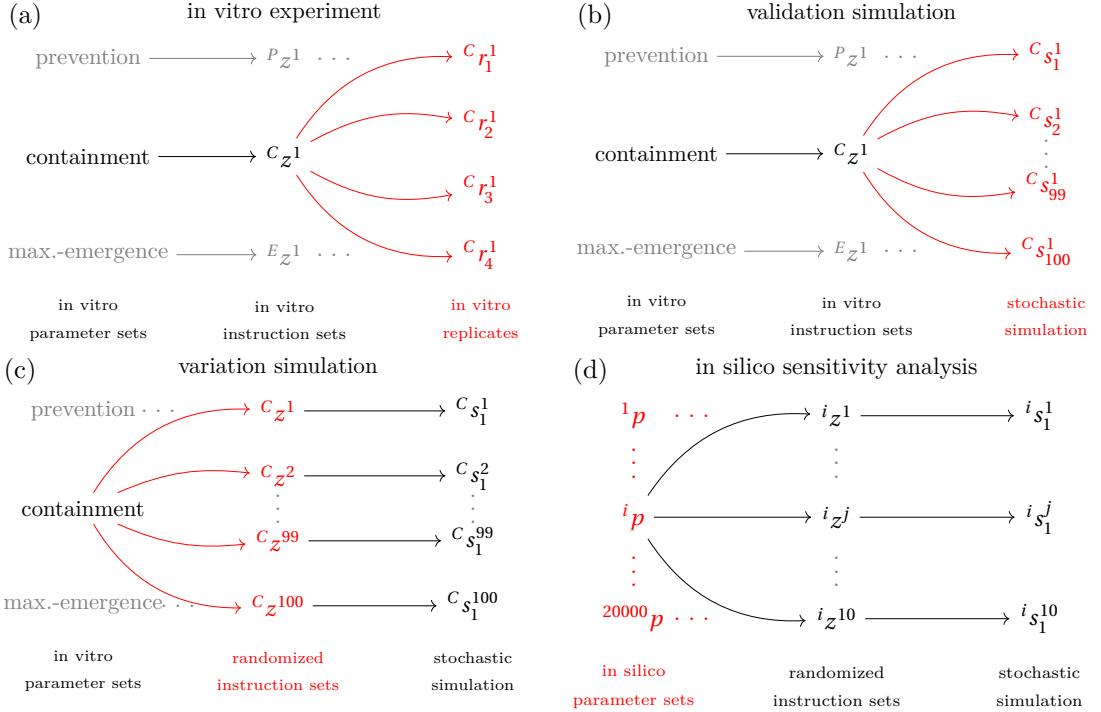


Fig. S2.3 Illustration depicting the various sources of stochasticity and variation in experiments and simulations. Our experiments and simulations investigate various sources of stochasticity and their contributions to the variability of the outcome. In panels A-D, we sketch the different sources of stochasticity for each experiment and simulation, highlighting our primary focus in red. (A) In vitro Experiment. Each experiment explores a scenario and is defined by a distinct parameter set (prevention (P), containment (C), maximum emergence (E)). We randomly generated one instruction set for each parameter set i : i_z^1 . For each instruction set i_z^1 , we replicated the cumulative in-well dynamics four times $i_r_j^1$. (B) Validation Simulation. To assess our computational model, we employed identical parameter sets and instruction sets i_z^1 , as employed in the in vitro experiments. For each instruction set i_z^1 , we conducted 100 stochastic simulations $i_s_j^1$. (C) Variation Simulation. For every in vitro parameter set, we randomly generated 100 alternative instruction sets i_z^k to quantify the influence of experimental decisions on the experiment's outcomes. For each instruction set, we performed one simulation $i_s_j^k$. (D) In Silico Sensitivity Analysis. We examined the sensitivity of our experimental findings to the input parameters by examining the effects of varying the input parameters on the resulting frequency of uninfected cases for different treatment strategies. To achieve this, we generated 20,000 alternative parameter sets i_p . We created ten randomised instruction sets i_z^k for each parameter set i_p and simulated each instruction set one time ($i_s_j^k$).

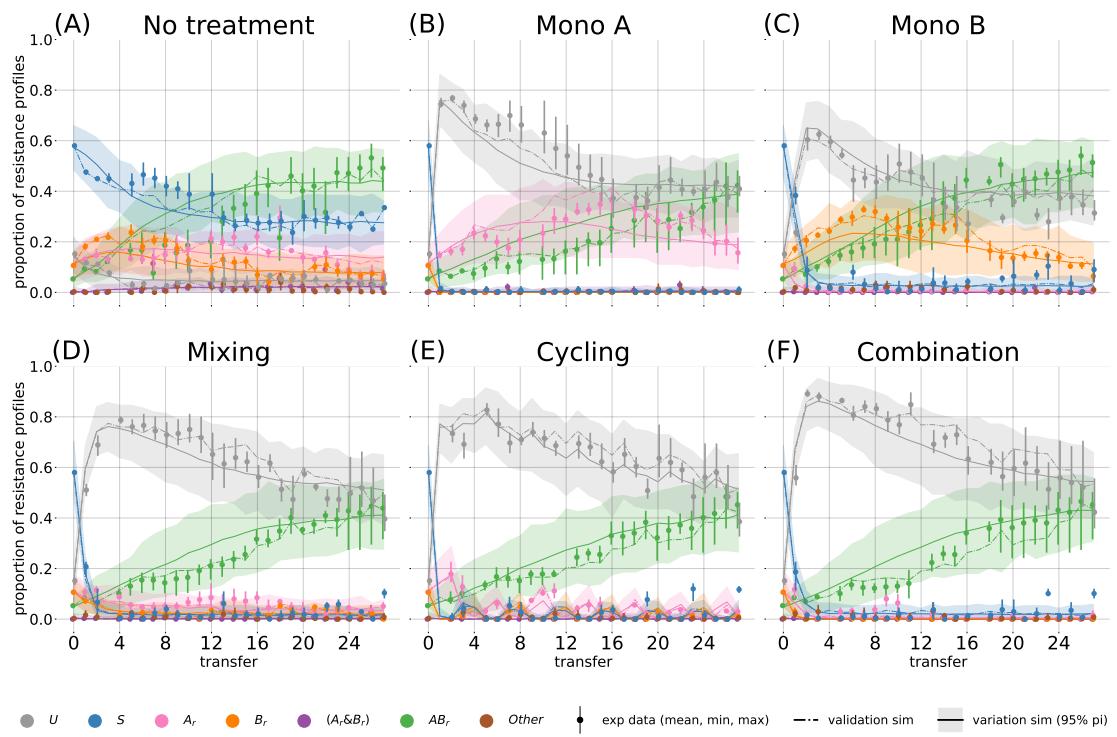


Fig. S2.4 Containment scenario: Frequencies of resistance profiles (colours) over time during the containment scenario. The dots show the experimental measurements, and the error bar indicates the min/max interval between the replicates. The dash-dotted line shows the mean value of 100 stochastic simulations based on the instruction set used in the in vitro experiment. The solid line represents the mean value of 100 simulations with randomly created instruction sets based on the parameter set used in the experiment. The shaded error band indicates the 95-percentile interval between the simulations.

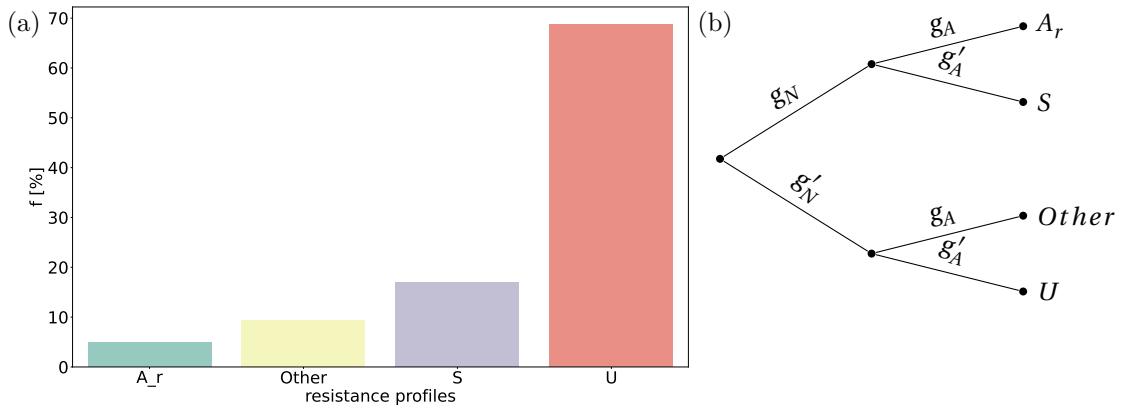


Fig. S2.5 (A) Experimentally measured resistance profiles for 1784 wells with the pre-treatment profile A_r and treatment with drug B during the maximum-emergence scenario. (B) Decision tree to calculate the distribution of measured phenotypes for a well that contains Z_\varnothing sensitive and Z_A A-resistant bacteria. g_ϑ is the probability of drawing a drop that forms a colony on a plate treated with drug ϑ , while g'_ϑ is the probability that it does not form a colony.

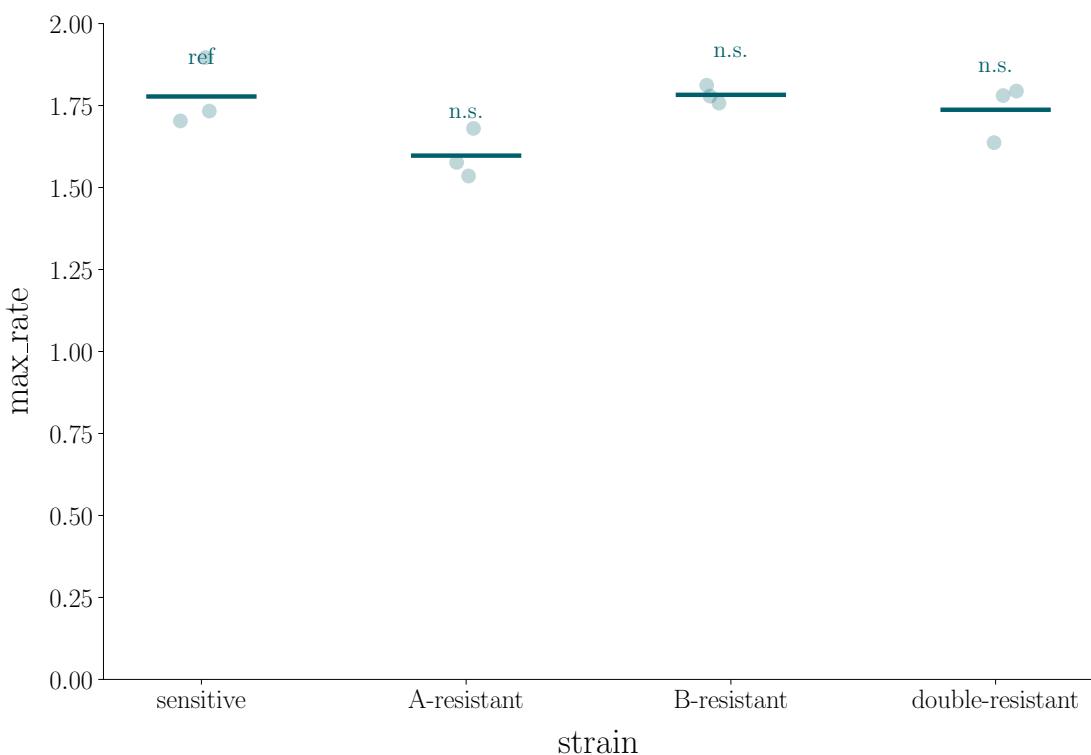


Fig. S2.6 Maximum growth rates of sensitive and plasmid-carrying strains, measured using OD-growth curves. Each dot represents an individual well, and vertical bars indicate the mean. The sensitive strain was used as the reference ("ref") for pairwise comparisons to the plasmid-carrying strains to identify potential plasmid costs. We used the Mann-Whitney U tests with the Bonferroni correction to identify significant differences in growth rates. All pairwise comparisons were not significant (n.s.).

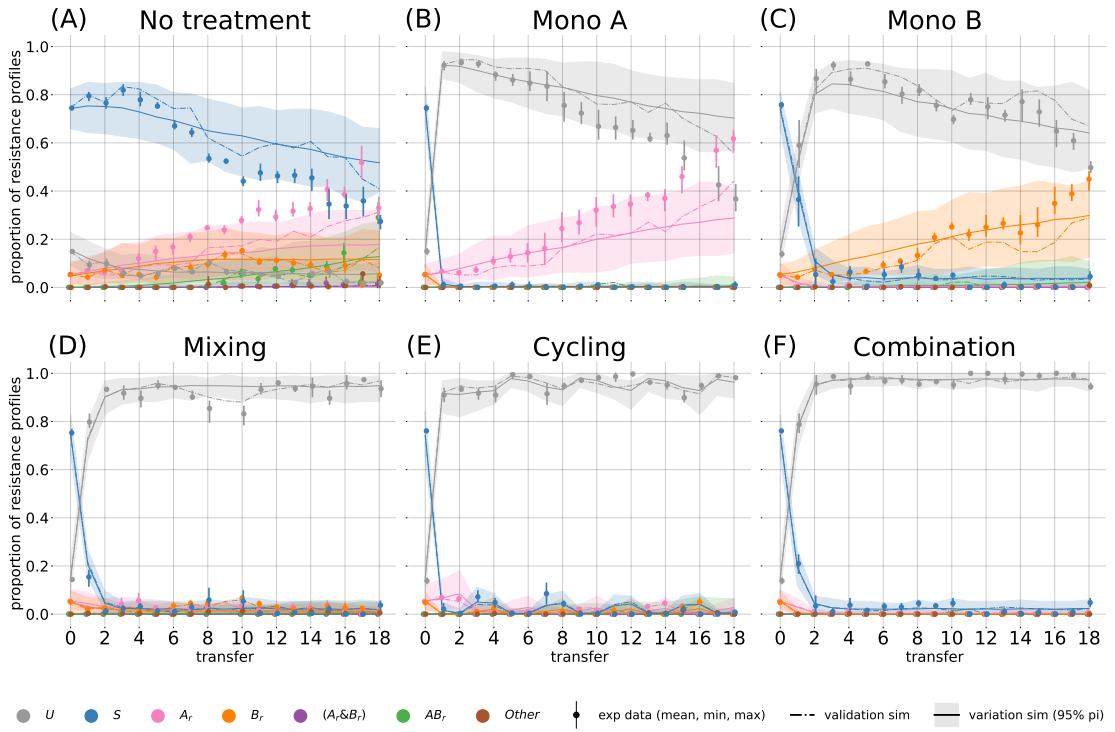


Fig. S2.7 Prevention scenario with filtered transition probabilities. Frequencies of resistance profiles (colours) over time during the prevention scenario. The dots show the experimental measurements, and the error bar indicates the min/max interval between the replicates. The dash-dotted line shows the mean value of 100 stochastic simulations based on the instruction set used in the in vitro experiment. The solid line represents the mean value of 100 simulations with randomly created instruction sets based on the parameter set used in the experiment. The shaded error band indicates the 95-percentile interval between the simulations.

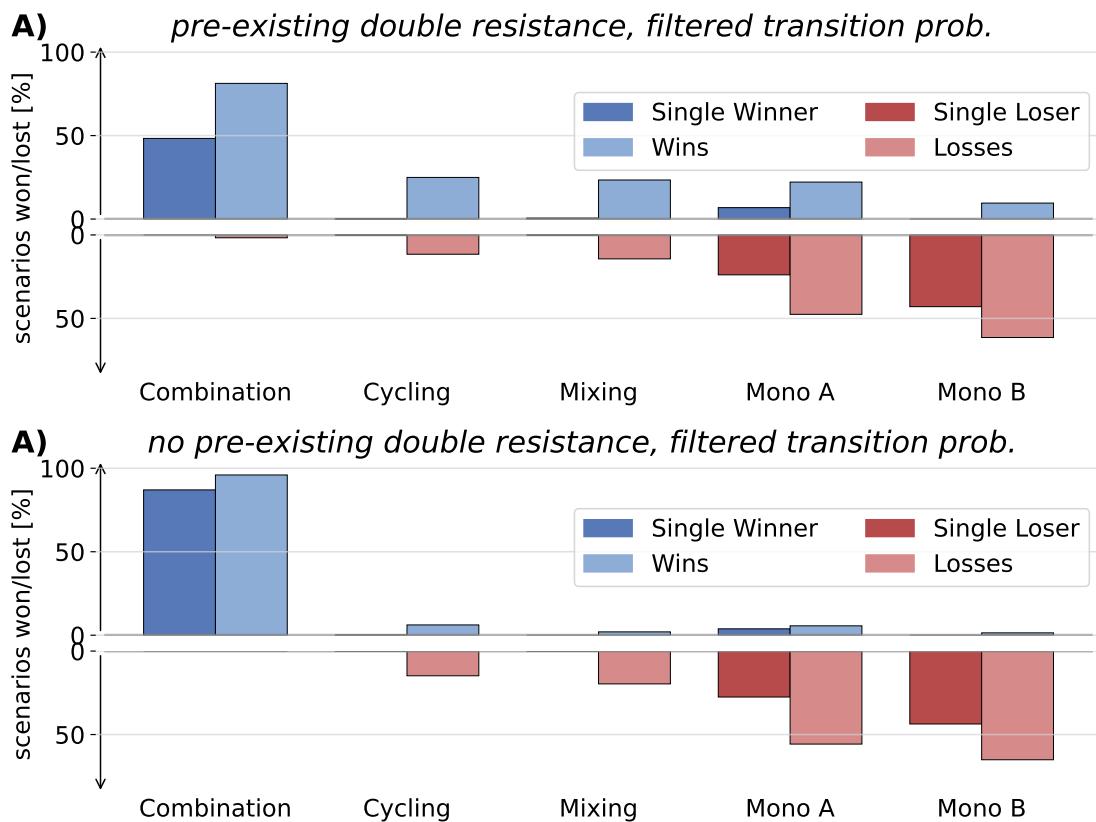


Fig. S2.8 Sensitivity analysis using filtered transition probabilities. We evaluated the effectiveness of the five treatment strategies in maximising the frequency of uninfected *in silico* patients across randomly generated parameter sets. Strategies not significantly better than any other are marked as losers (pastel red), with those significantly worse than all others being labelled as single losers (dark red). Conversely, strategies that are not significantly worse than any other are classified as winners (pastel blue), and those significantly better than all others as single winners (dark blue). (A) Evaluation of 10,000 parameter sets with preexisting double resistance. 659 out of 10,000 parameter sets yielded no significant difference between the strategies. (B) Evaluation of 10,000 parameter sets without preexisting double resistance. 8 out of 10,000 parameter sets yielded no significant difference between the strategies.

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Table S2.1 Number of superinfections ($N_{\mathcal{S}}$) between high- and low-concentrated A_r and B_r wells and the number of double resistances that emerged ($N_{\mathcal{E}}$) under treatment ϑ across all three experiments.

| Treatment ϑ | A_r^x | B_r^x | $N_{\mathcal{E}}^\vartheta$ | $N_{\mathcal{S}}^\vartheta$ | $\frac{N_{\mathcal{E}}^\vartheta}{N_{\mathcal{S}}^\vartheta}$ |
|-----------------------|---------|---------|-----------------------------|-----------------------------|---|
| A | A_r^h | B_r^h | 55 | 399 | 0.14 |
| AB | A_r^h | B_r^h | 2 | 35 | 0.06 |
| B | A_r^h | B_r^h | 257 | 322 | 0.8 |
| none | A_r^h | B_r^h | 390 | 420 | 0.93 |
| A | A_r^h | B_r^l | 0 | 1 | 0.0 |
| A | A_r^l | B_r^h | 0 | 13 | 0.0 |
| AB | A_r^l | B_r^h | 0 | 15 | 0.0 |
| B | A_r^l | B_r^h | 0 | 15 | 0.0 |

Table S2.2 Strains used in this study and their relevant phenotypes. The phenotype in brackets is conferred by the respective plasmid. Cm^R: Chloramphenicol resistance, Amp^R: Ampicillin resistance, Caz^R: Ceftazidime resistance, Tet^R: Tetracycline resistance.

| Name | Relevant phenotype | Reference |
|---|---|-----------------|
| Escherichia coli MDS42-YFP;Cm ^R ;[3] A-resistant | Cm ^R pA (Amp ^R , Caz ^R) | this study, [2] |
| B-resistant | Cm ^R pB (Amp ^R , Tet ^R) | this study, [2] |
| AB-resistant | Cm ^R pA (Amp ^R , Caz ^R) pB (Amp ^R , Tet ^R) | this study, [2] |

Table S2.3 Statistical comparison of maximum growth rates between the sensitive and plasmid-carrying strains. We used a Mann-Whitney U test for pairwise comparisons, and the p-values were adjusted using the Bonferroni correction.

| Comparison | U-statistic | P-value | Corrected P-value | Significant after Bonferroni |
|-------------------------------|-------------|----------|-------------------|------------------------------|
| sensitive vs A-resistant | 9.000000 | 0.100000 | 0.300000 | False |
| sensitive vs B-resistant | 3.000000 | 0.700000 | 1.000000 | False |
| sensitive vs double-resistant | 5.000000 | 1.000000 | 1.000000 | False |

Table S2.4 Plasmid segregation loss was estimated over 24 hours without treatment and with selective treatment as a control. Frequencies of plasmid retention were compared between $t_0 - t_1$ using the Mann-Whitney U test. Confidence intervals and the mean frequencies were estimated by bootstrapping the binary data (plasmid retained or lost) pooled across replicates. No significant plasmid loss was observed in either the main data or the control.

| plasmids | $f(t_0)$ | $CI(t_0)$ | $f(t_1)$ | $CI(t_1)$ | p -value | $f(t_1)$ control | $CI(t_1)$ control | p -value control |
|--------------|----------|-------------|----------|-------------|------------|------------------|-------------------|--------------------|
| p_A | 1.00 | (1.0, 1.0) | 1.00 | (1.0, 1.0) | 1.00 | 1.00 | (1.0, 1.0) | 1.00 |
| p_B | 1.00 | (1.0, 1.0) | 0.97 | (0.93, 1.0) | 0.20 | 1.00 | (1.0, 1.0) | 1.00 |
| $p_A \& p_B$ | 0.99 | (0.97, 1.0) | 1.00 | (1.0, 1.0) | 0.50 | 1.00 | (1.0, 1.0) | 0.50 |

Table S2.5 95 % confidence intervals for the final bacterial density measured by colony plating and the maximal growth rates measured by evaluating OD-growth curves.

| strain | antibiotic | cmp [$\mu\text{g}/\text{ml}$] | cfu [$1/\mu\text{l}$] | growthrate [$1/\text{h}$] |
|------------------|------------|---------------------------------|-----------------------------|-----------------------------|
| double-resistant | AB | 5 | $(2.14 - 3.33) \times 10^5$ | (0.43 – 0.86) |
| double-resistant | AB | 25 | $(2.68 - 3.99) \times 10^5$ | (0.4 – 0.85) |
| A-resistant | A | 5 | $(1.58 - 2.62) \times 10^5$ | (0.51 – 0.7) |
| A-resistant | A | 25 | $(2.05 - 3.21) \times 10^5$ | (0.46 – 0.71) |
| sensitive | None | 5 | $(1.03 - 1.27) \times 10^6$ | (0.68 – 0.74) |
| sensitive | None | 25 | $(1.15 - 1.4) \times 10^6$ | (0.55 – 0.96) |
| B-resistant | B | 5 | $(4.05 - 5.62) \times 10^5$ | (0.45 – 0.74) |
| B-resistant | B | 25 | $(3.53 - 5.01) \times 10^5$ | (0.4 – 0.87) |

Table S2.6 Definition of resistance profiles (rows) by growth patterns on differently treated agar plates (columns). X indicates colony formation, whereas o indicates no growth.

| | None | A | B | AB |
|----------------|------|---|---|----|
| U | o | o | o | o |
| S | X | o | o | o |
| A_r | X | X | o | o |
| B_r | X | o | X | o |
| $(A_r \& B_r)$ | X | X | X | o |
| AB_r | X | X | X | X |

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Table S2.7 Association between bacterial phenotypes (rows) and resistance profiles ϕ (columns). An 'X' denotes that a phenotype is obligatory for a given profile, while a ' \checkmark ' indicates that it is optional.

| | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
|------------------|-----|--------------|--------------|--------------|----------------|--------------|
| sensitive | X | \checkmark | \checkmark | \checkmark | \checkmark | \checkmark |
| A-resistant | | X | | X | X | \checkmark |
| B-resistant | | | X | X | X | \checkmark |
| double-resistant | | | | | | X |

Table S2.8 Clearance probability of well phenotypes across the three experiments.

| | S | A_r | B_r |
|----|------|-------|-------|
| A | 0.97 | 0.02 | 0.98 |
| B | 0.73 | 0.62 | 0.01 |
| AB | 0.86 | 0.4 | 0.98 |

Table S2.9 Mixing rules. During the plate-handling phase, we mix wells due to infections. The resulting phenotype of the two mixed wells can be calculated using this table. More than two phenotypes can be combined by applying associative logic.

| $\phi_1(\hat{T}) \backslash \phi_2(\hat{T})$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
|--|----------------|----------------|----------------|----------------|----------------|--------|
| U | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
| S | S | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
| A_r | A_r | A_r | A_r | $(A_r \& B_r)$ | $(A_r \& B_r)$ | AB_r |
| B_r | B_r | B_r | $(A_r \& B_r)$ | B_r | $(A_r \& B_r)$ | AB_r |
| $(A_r \& B_r)$ | $(A_r \& B_r)$ | $(A_r \& B_r)$ | $(A_r \& B_r)$ | $(A_r \& B_r)$ | $(A_r \& B_r)$ | AB_r |
| AB_r | AB_r | AB_r | AB_r | AB_r | AB_r | AB_r |

Table S2.10 Mean parameter leading to n single wins during the sensitivity analysis without preexisting double resistance. Strategies that did not yield at least one single win were excluded.

| | turnover | infection | U | S | A_r | B_r | AB_r | n |
|-------------|----------|-----------|------|------|------|------|------|---------|
| Combination | 0.50 | 0.50 | 0.26 | 0.24 | 0.24 | 0.25 | 0.00 | 9311.00 |
| Mono A | 0.80 | 0.27 | 0.26 | 0.29 | 0.37 | 0.08 | 0.00 | 56.00 |
| Cycling | 0.10 | 0.39 | 0.33 | 0.42 | 0.14 | 0.10 | 0.00 | 4.00 |
| Mixing | 0.11 | 0.33 | 0.12 | 0.49 | 0.38 | 0.01 | 0.00 | 1.00 |

Table S2.11 Mean parameter leading to n single losses during the sensitivity analysis without preexisting double resistance. Strategies that did not yield at least one single loss were excluded.

| | turnover | infection | U | S | A_r | B_r | AB_r | n |
|---------|----------|-----------|------|------|------|------|------|---------|
| Mono B | 0.60 | 0.43 | 0.26 | 0.25 | 0.16 | 0.33 | 0.00 | 4132.00 |
| Mono A | 0.55 | 0.43 | 0.27 | 0.25 | 0.36 | 0.12 | 0.00 | 2648.00 |
| Cycling | 0.07 | 0.87 | 0.28 | 0.34 | 0.27 | 0.11 | 0.00 | 8.00 |
| Mixing | 0.07 | 0.90 | 0.69 | 0.25 | 0.01 | 0.05 | 0.00 | 1.00 |

Table S2.12 Mean parameter leading to n single wins during the sensitivity analysis with preexisting double resistance. Strategies that did not yield at least one single win were excluded.

| | turnover | infection | U | S | A_r | B_r | AB_r | n |
|-------------|----------|-----------|------|------|------|------|------|---------|
| Combination | 0.61 | 0.47 | 0.21 | 0.18 | 0.22 | 0.22 | 0.17 | 5487.00 |
| Mono A | 0.52 | 0.56 | 0.23 | 0.20 | 0.16 | 0.15 | 0.26 | 365.00 |
| Mixing | 0.13 | 0.30 | 0.23 | 0.21 | 0.18 | 0.15 | 0.22 | 40.00 |
| Cycling | 0.14 | 0.20 | 0.23 | 0.20 | 0.21 | 0.20 | 0.16 | 9.00 |

Table S2.13 Mean parameter leading to n single losses during the sensitivity analysis with preexisting double resistance. Strategies that did not yield at least one single loss were excluded.

| | turnover | infection | U | S | A_r | B_r | AB_r | n |
|---------|----------|-----------|------|------|------|------|------|---------|
| Mono B | 0.57 | 0.46 | 0.21 | 0.19 | 0.13 | 0.28 | 0.18 | 4250.00 |
| Mono A | 0.51 | 0.43 | 0.23 | 0.19 | 0.30 | 0.10 | 0.18 | 2359.00 |
| Cycling | 0.42 | 0.72 | 0.23 | 0.21 | 0.14 | 0.14 | 0.28 | 6.00 |
| Mixing | 0.34 | 0.71 | 0.23 | 0.20 | 0.18 | 0.09 | 0.30 | 2.00 |

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Table S2.14 Wins and losses during the sensitivity analysis. With preexisting double resistance. 606 parameter sets yielded an insignificant result.

| strategy | single winner [%] | single loser [%] | loser [%] | winner [%] | single winner | single loser | loser | winner |
|-------------|-------------------|------------------|-----------|------------|---------------|--------------|-------|--------|
| Combination | 54.87 | 0.00 | 0.95 | 86.76 | 5487 | 0 | 95 | 8676 |
| Cycling | 0.09 | 0.06 | 12.10 | 23.32 | 9 | 6 | 1210 | 2332 |
| Mixing | 0.40 | 0.02 | 14.78 | 20.54 | 40 | 2 | 1478 | 2054 |
| Mono A | 3.65 | 23.59 | 48.57 | 17.49 | 365 | 2359 | 4857 | 1749 |
| Mono B | 0.00 | 42.50 | 62.29 | 8.11 | 0 | 4250 | 6229 | 811 |

Table S2.15 Wins and losses during the sensitivity analysis without preexisting double resistance. 100 parameter sets yielded an insignificant result.

| strategy | single winner [%] | single loser [%] | loser [%] | winner [%] | single winner | single loser | loser | winner |
|-------------|-------------------|------------------|-----------|------------|---------------|--------------|-------|--------|
| Combination | 93.11 | 0.00 | 0.00 | 98.35 | 9311 | 0 | 0 | 9835 |
| Cycling | 0.04 | 0.08 | 18.89 | 3.49 | 4 | 8 | 1889 | 349 |
| Mixing | 0.01 | 0.01 | 22.05 | 1.32 | 1 | 1 | 2205 | 132 |
| Mono A | 0.56 | 26.48 | 57.03 | 1.65 | 56 | 2648 | 5703 | 165 |
| Mono B | 0.00 | 41.32 | 63.44 | 1.11 | 0 | 4132 | 6344 | 111 |

Table S2.16 Wins and losses during the sensitivity analysis, using filtered transition probabilities and preexisting double resistance. 659 parameter sets yielded an insignificant result.

| strategy | single winner [%] | single loser [%] | loser [%] | winner [%] | single winner | single loser | loser | winner |
|-------------|-------------------|------------------|-----------|------------|---------------|--------------|-------|--------|
| Combination | 48.31 | 0.00 | 1.72 | 81.27 | 4831 | 0 | 172 | 8127 |
| Cycling | 0.07 | 0.10 | 11.57 | 24.94 | 7 | 10 | 1157 | 2494 |
| Mixing | 0.59 | 0.03 | 14.34 | 23.40 | 59 | 3 | 1434 | 2340 |
| Mono A | 6.85 | 23.91 | 47.57 | 22.15 | 685 | 2391 | 4757 | 2215 |
| Mono B | 0.00 | 42.97 | 61.45 | 9.57 | 0 | 4297 | 6145 | 957 |

Table S2.17 Wins and losses during the sensitivity analysis with filtered transition matrices and no preexisting double resistance. 8 parameter sets yielded an insignificant result.

| strategy | single winner [%] | single loser [%] | loser [%] | winner [%] | single winner | single loser | loser | winner |
|-------------|-------------------|------------------|-----------|------------|---------------|--------------|-------|--------|
| Combination | 87.04 | 0.00 | 0.00 | 95.98 | 8704 | 0 | 0 | 9598 |
| Cycling | 0.08 | 0.06 | 14.80 | 6.16 | 8 | 6 | 1480 | 616 |
| Mixing | 0.00 | 0.04 | 19.66 | 2.00 | 0 | 4 | 1966 | 200 |
| Mono A | 3.82 | 27.53 | 55.71 | 5.62 | 382 | 2753 | 5571 | 562 |
| Mono B | 0.00 | 43.67 | 65.10 | 1.41 | 0 | 4367 | 6510 | 141 |

Table S2.18 M^{none} . Unfiltered transition matrix for untreated wells.

| $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
|-----------------|----------------|------|-------|-------|----------------|--------|
| $\phi(\hat{T})$ | U | 0.97 | 0.0 | 0.0 | 0.0 | 0.0 |
| | S | 0.03 | 0.98 | 0.01 | 0.02 | 0.01 |
| | A_r | 0.0 | 0.01 | 0.96 | 0.0 | 0.07 |
| | B_r | 0.0 | 0.01 | 0.0 | 0.97 | 0.02 |
| | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.01 | 0.01 | 0.08 |
| | AB_r | 0.0 | 0.0 | 0.02 | 0.0 | 0.82 |

Table S2.19 M^A . Unfiltered transition matrix for wells treated with antibiotic A.

| $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r | |
|-----------------|----------------|------|-------|-------|----------------|--------|------|
| $\phi(\hat{T})$ | U | 0.99 | 0.96 | 0.02 | 0.97 | 0.03 | 0.02 |
| | S | 0.0 | 0.02 | 0.0 | 0.01 | 0.0 | 0.0 |
| | A_r | 0.01 | 0.02 | 0.98 | 0.01 | 0.59 | 0.01 |
| | B_r | 0.0 | 0.0 | 0.0 | 0.01 | 0.0 | 0.0 |
| | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.18 | 0.01 |
| | AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.96 |

Table S2.20 M^B . Unfiltered transition matrix for wells treated with antibiotic B.

| $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r | |
|-----------------|----------------|------|-------|-------|----------------|--------|------|
| $\phi(\hat{T})$ | U | 0.99 | 0.8 | 0.63 | 0.01 | 0.03 | 0.02 |
| | S | 0.0 | 0.19 | 0.18 | 0.0 | 0.0 | 0.0 |
| | A_r | 0.0 | 0.0 | 0.18 | 0.0 | 0.0 | 0.0 |
| | B_r | 0.01 | 0.01 | 0.0 | 0.98 | 0.21 | 0.0 |
| | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.03 | 0.0 |
| | AB_r | 0.0 | 0.0 | 0.01 | 0.01 | 0.73 | 0.98 |

Table S2.21 M^{AB} . Unfiltered transition matrix for wells treated with antibiotic AB.

| $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r | |
|-----------------|----------------|-----|-------|-------|----------------|--------|------|
| $\phi(\hat{T})$ | U | 1.0 | 0.9 | 0.49 | 0.98 | 1.0 | 0.02 |
| | S | 0.0 | 0.1 | 0.32 | 0.02 | 0.0 | 0.0 |
| | A_r | 0.0 | 0.0 | 0.19 | 0.0 | 0.0 | 0.0 |
| | B_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.98 |

Table S2.22 M_1^{none} . Unfiltered transition matrix for the first time point in untreated wells.

| $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
|-----------------|----------------|------|-------|-------|----------------|--------|
| $\phi(\hat{T})$ | U | 0.96 | 0.0 | 0.0 | 0.0 | 0.0 |
| | S | 0.03 | 0.99 | 0.0 | 0.0 | 0.0 |
| | A_r | 0.01 | 0.01 | 1.0 | 0.0 | 0.0 |
| | B_r | 0.0 | 0.0 | 0.0 | 1.0 | 0.17 |
| | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.02 |
| | AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.81 |

Table S2.23 M_1^A . Unfiltered transition matrix for the first time point in wells treated with antibiotic A.

| $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r | |
|-----------------|----------------|------|-------|-------|----------------|--------|-----|
| $\phi(\hat{T})$ | U | 0.99 | 0.97 | 0.0 | 0.99 | 0.23 | 0.0 |
| | S | 0.0 | 0.02 | 0.0 | 0.01 | 0.0 | 0.0 |
| | A_r | 0.01 | 0.01 | 1.0 | 0.0 | 0.54 | 0.0 |
| | B_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.11 | 0.0 |
| | AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.12 | 1.0 |

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Table S2.24 M_1^B . Unfiltered transition matrix for the first time point in wells treated with antibiotic B.

| $\phi(T)$ | $\phi(\hat{T})$ | | | | | |
|----------------|-----------------|------|-------|-------|----------------|--------|
| | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
| U | 1.0 | 0.48 | 0.51 | 0.02 | 0.01 | 0.0 |
| S | 0.0 | 0.52 | 0.21 | 0.01 | 0.0 | 0.0 |
| A_r | 0.0 | 0.0 | 0.28 | 0.0 | 0.0 | 0.0 |
| B_r | 0.0 | 0.0 | 0.0 | 0.97 | 0.31 | 0.0 |
| $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 |
| AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.58 | 1.0 |

Table S2.25 M_1^{AB} . Unfiltered transition matrix for the first time point in wells treated with antibiotic AB.

| $\phi(T)$ | $\phi(\hat{T})$ | | | | | |
|----------------|-----------------|------|-------|-------|----------------|--------|
| | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
| U | 0.98 | 0.79 | 0.32 | 0.67 | 0.24 | 0.0 |
| S | 0.02 | 0.21 | 0.21 | 0.24 | 0.21 | 0.0 |
| A_r | 0.0 | 0.0 | 0.47 | 0.0 | 0.41 | 0.0 |
| B_r | 0.0 | 0.0 | 0.0 | 0.09 | 0.02 | 0.0 |
| $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.07 | 0.0 |
| AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.05 | 1.0 |

Table S2.26 M^{none} . Filtered transition matrix for untreated wells.

| $\phi(T)$ | $\phi(\hat{T})$ | | | | | |
|----------------|-----------------|-----|-------|-------|----------------|--------|
| | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
| U | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S | 0.0 | 1.0 | 0.01 | 0.02 | 0.01 | 0.01 |
| A_r | 0.0 | 0.0 | 0.99 | 0.0 | 0.07 | 0.03 |
| B_r | 0.0 | 0.0 | 0.0 | 0.98 | 0.02 | 0.0 |
| $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.08 | 0.04 |
| AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.82 | 0.92 |

Table S2.27 M^A . Filtered transition matrix for wells treated with antibiotic A.

| $\phi(T)$ | $\phi(\hat{T})$ | | | | | |
|----------------|-----------------|------|-------|-------|----------------|--------|
| | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
| U | 1.0 | 0.98 | 0.02 | 0.98 | 0.03 | 0.02 |
| S | 0.0 | 0.02 | 0.0 | 0.01 | 0.0 | 0.0 |
| A_r | 0.0 | 0.0 | 0.98 | 0.0 | 0.59 | 0.01 |
| B_r | 0.0 | 0.0 | 0.0 | 0.01 | 0.0 | 0.0 |
| $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.18 | 0.01 |
| AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.96 |

Table S2.28 M^B . Filtered transition matrix for wells treated with antibiotic B.

| $\phi(T)$ | $\phi(\hat{T})$ | | | | | |
|----------------|-----------------|-----|-------|-------|----------------|--------|
| | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
| U | 1.0 | 0.8 | 0.64 | 0.01 | 0.03 | 0.02 |
| S | 0.0 | 0.2 | 0.18 | 0.0 | 0.0 | 0.0 |
| A_r | 0.0 | 0.0 | 0.18 | 0.0 | 0.0 | 0.0 |
| B_r | 0.0 | 0.0 | 0.0 | 0.99 | 0.21 | 0.0 |
| $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.03 | 0.0 |
| AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.73 | 0.98 |

Table S2.29 M^{AB} . Filtered transition matrix for wells treated with antibiotic AB.

| $\phi(T)$ | $\phi(\hat{T})$ | | | | | |
|----------------|-----------------|-----|-------|-------|----------------|--------|
| | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
| U | 1.0 | 0.9 | 0.49 | 0.98 | 1.0 | 0.02 |
| S | 0.0 | 0.1 | 0.32 | 0.02 | 0.0 | 0.0 |
| A_r | 0.0 | 0.0 | 0.19 | 0.0 | 0.0 | 0.0 |
| B_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.98 |

Table S2.30 M_1^{none} . Filtered transition matrix for the first time point in untreated wells.

| $\phi(\hat{T})$ | $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
|-----------------|----------------|-----|-----|-------|-------|----------------|--------|
| U | U | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S | S | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| A_r | A_r | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 |
| B_r | B_r | 0.0 | 0.0 | 0.0 | 1.0 | 0.17 | 0.0 |
| $(A_r \& B_r)$ | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.02 | 0.0 |
| AB_r | AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.81 | 1.0 |

Table S2.31 M_1^A . Filtered transition matrix for the first time point in wells treated with antibiotic A.

| $\phi(\hat{T})$ | $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
|-----------------|----------------|-----|------|-------|-------|----------------|--------|
| U | U | 1.0 | 0.98 | 0.0 | 0.99 | 0.23 | 0.0 |
| S | S | 0.0 | 0.02 | 0.0 | 0.01 | 0.0 | 0.0 |
| A_r | A_r | 0.0 | 0.0 | 1.0 | 0.0 | 0.54 | 0.0 |
| B_r | B_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| $(A_r \& B_r)$ | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.11 | 0.0 |
| AB_r | AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.12 | 1.0 |

Table S2.32 M_1^B . Filtered transition matrix for the first time point in wells treated with antibiotic B.

| $\phi(\hat{T})$ | $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
|-----------------|----------------|-----|------|-------|-------|----------------|--------|
| U | U | 1.0 | 0.48 | 0.51 | 0.02 | 0.01 | 0.0 |
| S | S | 0.0 | 0.52 | 0.21 | 0.01 | 0.0 | 0.0 |
| A_r | A_r | 0.0 | 0.0 | 0.28 | 0.0 | 0.0 | 0.0 |
| B_r | B_r | 0.0 | 0.0 | 0.0 | 0.97 | 0.31 | 0.0 |
| $(A_r \& B_r)$ | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 |
| AB_r | AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.58 | 1.0 |

Table S2.33 M_1^{AB} . Filtered transition matrix for the first time point in wells treated with antibiotic AB.

| $\phi(\hat{T})$ | $\phi(T)$ | U | S | A_r | B_r | $(A_r \& B_r)$ | AB_r |
|-----------------|----------------|-----|------|-------|-------|----------------|--------|
| U | U | 1.0 | 0.79 | 0.32 | 0.67 | 0.24 | 0.0 |
| S | S | 0.0 | 0.21 | 0.21 | 0.24 | 0.21 | 0.0 |
| A_r | A_r | 0.0 | 0.0 | 0.47 | 0.0 | 0.41 | 0.0 |
| B_r | B_r | 0.0 | 0.0 | 0.0 | 0.09 | 0.02 | 0.0 |
| $(A_r \& B_r)$ | $(A_r \& B_r)$ | 0.0 | 0.0 | 0.0 | 0.0 | 0.07 | 0.0 |
| AB_r | AB_r | 0.0 | 0.0 | 0.0 | 0.0 | 0.05 | 1.0 |

Table S2.34 Prevention scenario: Effect of treatment strategies on the frequency of uninfecteds (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|---------|---------|
| Between Groups | 2.833 | 5 | 0.567 | 779.436 | < 0.001 |
| Within Groups | 0.013 | 18 | < 0.001 | | |
| Total | 2.846 | 23 | | | |

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Table S2.35 Prevention scenario: Multiple comparison between the effects of treatment strategies on the frequencies of uninfecteds using Tukey's post-hoc analysis.

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|--------|--------|
| Combination | Cycling | -0.027 | 0.729 | -0.087 | 0.034 | False |
| Combination | Mixing | -0.041 | 0.317 | -0.101 | 0.020 | False |
| Combination | Mono A | -0.525 | 0.000 | -0.586 | -0.465 | True |
| Combination | Mono B | -0.360 | 0.000 | -0.421 | -0.300 | True |
| Combination | No treatment | -0.951 | 0.000 | -1.011 | -0.890 | True |
| Cycling | Mixing | -0.014 | 0.975 | -0.074 | 0.047 | False |
| Cycling | Mono A | -0.499 | 0.000 | -0.559 | -0.438 | True |
| Cycling | Mono B | -0.334 | 0.000 | -0.394 | -0.273 | True |
| Cycling | No treatment | -0.924 | 0.000 | -0.985 | -0.864 | True |
| Mixing | Mono A | -0.485 | 0.000 | -0.545 | -0.424 | True |
| Mixing | Mono B | -0.320 | 0.000 | -0.380 | -0.259 | True |
| Mixing | No treatment | -0.910 | 0.000 | -0.971 | -0.850 | True |
| Mono A | Mono B | 0.165 | 0.000 | 0.104 | 0.226 | True |
| Mono A | No treatment | -0.425 | 0.000 | -0.486 | -0.365 | True |
| Mono B | No treatment | -0.590 | 0.000 | -0.651 | -0.530 | True |

Table S2.36 Prevention scenario: Effect of treatment strategies on the frequency of single resistance (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|---------|---------|
| Between Groups | 1.133 | 5 | 0.227 | 290.494 | < 0.001 |
| Within Groups | 0.014 | 18 | < 0.001 | | |
| Total | 1.147 | 23 | | | |

 2.1. SUPPLEMENTARY INFORMATION CHAPTER 2

Table S2.37 Prevention scenario: Multiple comparison between the effects of treatment strategies on the frequencies of single resistance using Tukey's post-hoc analysis.

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|--------|--------|
| Combination | Cycling | 0.030 | 0.659 | -0.033 | 0.093 | False |
| Combination | Mixing | 0.035 | 0.518 | -0.028 | 0.097 | False |
| Combination | Mono A | 0.501 | 0.000 | 0.438 | 0.563 | True |
| Combination | Mono B | 0.359 | 0.000 | 0.296 | 0.422 | True |
| Combination | No treatment | 0.479 | 0.000 | 0.417 | 0.542 | True |
| Cycling | Mixing | 0.005 | 1.000 | -0.058 | 0.067 | False |
| Cycling | Mono A | 0.471 | 0.000 | 0.408 | 0.533 | True |
| Cycling | Mono B | 0.329 | 0.000 | 0.266 | 0.392 | True |
| Cycling | No treatment | 0.450 | 0.000 | 0.387 | 0.512 | True |
| Mixing | Mono A | 0.466 | 0.000 | 0.403 | 0.529 | True |
| Mixing | Mono B | 0.325 | 0.000 | 0.262 | 0.387 | True |
| Mixing | No treatment | 0.445 | 0.000 | 0.382 | 0.508 | True |
| Mono A | Mono B | -0.142 | 0.000 | -0.204 | -0.079 | True |
| Mono A | No treatment | -0.021 | 0.884 | -0.084 | 0.042 | False |
| Mono B | No treatment | 0.120 | 0.000 | 0.058 | 0.183 | True |

Table S2.38 Prevention scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|---------|---------|
| Between Groups | 0.061 | 5 | 0.012 | 157.486 | < 0.001 |
| Within Groups | 0.001 | 18 | < 0.001 | | |
| Total | 0.063 | 23 | | | |

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Table S2.39 Prevention scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|-------|--------|
| Combination | Cycling | 0.000 | 1.000 | -0.020 | 0.020 | False |
| Combination | Mixing | 0.003 | 0.998 | -0.017 | 0.022 | False |
| Combination | Mono A | 0.002 | 1.000 | -0.018 | 0.022 | False |
| Combination | Mono B | 0.000 | 1.000 | -0.020 | 0.020 | False |
| Combination | No treatment | 0.136 | 0.000 | 0.117 | 0.156 | True |
| Cycling | Mixing | 0.003 | 0.998 | -0.017 | 0.022 | False |
| Cycling | Mono A | 0.002 | 1.000 | -0.018 | 0.022 | False |
| Cycling | Mono B | 0.000 | 1.000 | -0.020 | 0.020 | False |
| Cycling | No treatment | 0.136 | 0.000 | 0.117 | 0.156 | True |
| Mixing | Mono A | -0.001 | 1.000 | -0.021 | 0.019 | False |
| Mixing | Mono B | -0.003 | 0.998 | -0.022 | 0.017 | False |
| Mixing | No treatment | 0.134 | 0.000 | 0.114 | 0.153 | True |
| Mono A | Mono B | -0.002 | 1.000 | -0.022 | 0.018 | False |
| Mono A | No treatment | 0.134 | 0.000 | 0.115 | 0.154 | True |
| Mono B | No treatment | 0.136 | 0.000 | 0.117 | 0.156 | True |

Table S2.40 Containment scenario: Effect of treatment strategies on the frequency of uninfecteds (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|--------|---------|
| Between Groups | 0.639 | 5 | 0.128 | 28.906 | < 0.001 |
| Within Groups | 0.080 | 18 | 0.004 | | |
| Total | 0.718 | 23 | | | |

Table S2.41 Containment scenario: Multiple comparison between the effects of treatment strategies on the frequencies of uninfecteds using Tukey's post-hoc analysis.

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|--------|--------|
| Combination | Cycling | -0.008 | 1.000 | -0.157 | 0.141 | False |
| Combination | Mixing | -0.041 | 0.951 | -0.190 | 0.109 | False |
| Combination | Mono A | -0.104 | 0.282 | -0.253 | 0.046 | False |
| Combination | Mono B | -0.158 | 0.034 | -0.308 | -0.009 | True |
| Combination | No treatment | -0.474 | 0.000 | -0.623 | -0.325 | True |
| Cycling | Mixing | -0.033 | 0.980 | -0.182 | 0.117 | False |
| Cycling | Mono A | -0.096 | 0.361 | -0.245 | 0.054 | False |
| Cycling | Mono B | -0.150 | 0.048 | -0.300 | -0.001 | True |
| Cycling | No treatment | -0.466 | 0.000 | -0.616 | -0.317 | True |
| Mixing | Mono A | -0.063 | 0.758 | -0.212 | 0.086 | False |
| Mixing | Mono B | -0.118 | 0.175 | -0.267 | 0.032 | False |
| Mixing | No treatment | -0.433 | 0.000 | -0.583 | -0.284 | True |
| Mono A | Mono B | -0.054 | 0.850 | -0.204 | 0.095 | False |
| Mono A | No treatment | -0.370 | 0.000 | -0.520 | -0.221 | True |
| Mono B | No treatment | -0.316 | 0.000 | -0.465 | -0.166 | True |

Table S2.42 Containment scenario: Effect of treatment strategies on the frequency of single resistance (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|--------|---------|
| Between Groups | 0.129 | 5 | 0.026 | 40.881 | < 0.001 |
| Within Groups | 0.011 | 18 | < 0.001 | | |
| Total | 0.140 | 23 | | | |

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Table S2.43 Containment scenario: Multiple comparison between the effects of treatment strategies on the frequencies of single resistance using Tukey's post-hoc analysis.

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|--------|--------|
| Combination | Cycling | 0.019 | 0.895 | -0.038 | 0.075 | False |
| Combination | Mixing | 0.054 | 0.067 | -0.003 | 0.110 | False |
| Combination | Mono A | 0.197 | 0.000 | 0.140 | 0.253 | True |
| Combination | Mono B | 0.102 | 0.000 | 0.045 | 0.158 | True |
| Combination | No treatment | 0.169 | 0.000 | 0.112 | 0.225 | True |
| Cycling | Mixing | 0.035 | 0.389 | -0.021 | 0.092 | False |
| Cycling | Mono A | 0.178 | 0.000 | 0.122 | 0.235 | True |
| Cycling | Mono B | 0.083 | 0.002 | 0.027 | 0.140 | True |
| Cycling | No treatment | 0.150 | 0.000 | 0.094 | 0.207 | True |
| Mixing | Mono A | 0.143 | 0.000 | 0.086 | 0.199 | True |
| Mixing | Mono B | 0.048 | 0.125 | -0.009 | 0.104 | False |
| Mixing | No treatment | 0.115 | 0.000 | 0.059 | 0.172 | True |
| Mono A | Mono B | -0.095 | 0.001 | -0.152 | -0.039 | True |
| Mono A | No treatment | -0.028 | 0.626 | -0.084 | 0.029 | False |
| Mono B | No treatment | 0.067 | 0.015 | 0.011 | 0.124 | True |

Table S2.44 Containment scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|-------|-------|
| Between Groups | 0.038 | 5 | 0.008 | 1.169 | 0.362 |
| Within Groups | 0.116 | 18 | 0.006 | | |
| Total | 0.154 | 23 | | | |

Table S2.45 Maximum-emergence scenario: Effect of treatment strategies on the frequency of uninfecteds (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|---------|---------|
| Between Groups | 1.432 | 5 | 0.286 | 383.054 | < 0.001 |
| Within Groups | 0.013 | 18 | < 0.001 | | |
| Total | 1.445 | 23 | | | |

Table S2.46 Maximum-emergence scenario: Multiple comparison between the effects of treatment strategies on the frequencies of uninfecteds using Tukey's post-hoc analysis.

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|--------|--------|
| Combination | Cycling | -0.306 | 0.000 | -0.368 | -0.245 | True |
| Combination | Mixing | -0.386 | 0.000 | -0.447 | -0.324 | True |
| Combination | Mono A | -0.414 | 0.000 | -0.476 | -0.353 | True |
| Combination | Mono B | -0.499 | 0.000 | -0.561 | -0.438 | True |
| Combination | No treatment | -0.823 | 0.000 | -0.885 | -0.762 | True |
| Cycling | Mixing | -0.079 | 0.008 | -0.141 | -0.018 | True |
| Cycling | Mono A | -0.108 | 0.000 | -0.169 | -0.046 | True |
| Cycling | Mono B | -0.193 | 0.000 | -0.254 | -0.131 | True |
| Cycling | No treatment | -0.517 | 0.000 | -0.578 | -0.455 | True |
| Mixing | Mono A | -0.029 | 0.681 | -0.090 | 0.033 | False |
| Mixing | Mono B | -0.114 | 0.000 | -0.175 | -0.052 | True |
| Mixing | No treatment | -0.438 | 0.000 | -0.499 | -0.376 | True |
| Mono A | Mono B | -0.085 | 0.004 | -0.146 | -0.024 | True |
| Mono A | No treatment | -0.409 | 0.000 | -0.470 | -0.347 | True |
| Mono B | No treatment | -0.324 | 0.000 | -0.385 | -0.262 | True |

Table S2.47 Maximum-emergence scenario: Effect of treatment strategies on the frequency of single resistance (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|---------|---------|
| Between Groups | 1.311 | 5 | 0.262 | 524.241 | < 0.001 |
| Within Groups | 0.009 | 18 | < 0.001 | | |
| Total | 1.320 | 23 | | | |

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Table S2.48 Maximum-emergence scenario: Multiple comparison between the effects of treatment strategies on the frequencies of single resistance using Tukey's post-hoc analysis.

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|-------|--------|
| Combination | Cycling | 0.342 | 0.000 | 0.292 | 0.393 | True |
| Combination | Mixing | 0.408 | 0.000 | 0.358 | 0.459 | True |
| Combination | Mono A | 0.549 | 0.000 | 0.499 | 0.600 | True |
| Combination | Mono B | 0.536 | 0.000 | 0.486 | 0.586 | True |
| Combination | No treatment | 0.761 | 0.000 | 0.710 | 0.811 | True |
| Cycling | Mixing | 0.066 | 0.006 | 0.016 | 0.116 | True |
| Cycling | Mono A | 0.207 | 0.000 | 0.157 | 0.257 | True |
| Cycling | Mono B | 0.194 | 0.000 | 0.143 | 0.244 | True |
| Cycling | No treatment | 0.418 | 0.000 | 0.368 | 0.469 | True |
| Mixing | Mono A | 0.141 | 0.000 | 0.091 | 0.191 | True |
| Mixing | Mono B | 0.128 | 0.000 | 0.077 | 0.178 | True |
| Mixing | No treatment | 0.352 | 0.000 | 0.302 | 0.403 | True |
| Mono A | Mono B | -0.013 | 0.956 | -0.064 | 0.037 | False |
| Mono A | No treatment | 0.211 | 0.000 | 0.161 | 0.262 | True |
| Mono B | No treatment | 0.225 | 0.000 | 0.174 | 0.275 | True |

Table S2.49 Maximum-emergence scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|--------|---------|
| Between Groups | 0.109 | 5 | 0.022 | 71.779 | < 0.001 |
| Within Groups | 0.005 | 18 | < 0.001 | | |
| Total | 0.115 | 23 | | | |

Table S2.50 Maximum-emergence scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|--------|--------|
| Combination | Cycling | 0.058 | 0.002 | 0.019 | 0.097 | True |
| Combination | Mixing | 0.071 | 0.000 | 0.032 | 0.110 | True |
| Combination | Mono A | 0.009 | 0.980 | -0.031 | 0.048 | False |
| Combination | Mono B | 0.069 | 0.000 | 0.030 | 0.108 | True |
| Combination | No treatment | 0.206 | 0.000 | 0.167 | 0.245 | True |
| Cycling | Mixing | 0.013 | 0.884 | -0.026 | 0.052 | False |
| Cycling | Mono A | -0.049 | 0.009 | -0.088 | -0.010 | True |
| Cycling | Mono B | 0.011 | 0.937 | -0.028 | 0.051 | False |
| Cycling | No treatment | 0.148 | 0.000 | 0.109 | 0.188 | True |
| Mixing | Mono A | -0.062 | 0.001 | -0.102 | -0.023 | True |
| Mixing | Mono B | -0.002 | 1.000 | -0.041 | 0.037 | False |
| Mixing | No treatment | 0.135 | 0.000 | 0.096 | 0.174 | True |
| Mono A | Mono B | 0.060 | 0.001 | 0.021 | 0.100 | True |
| Mono A | No treatment | 0.198 | 0.000 | 0.158 | 0.237 | True |
| Mono B | No treatment | 0.137 | 0.000 | 0.098 | 0.176 | True |

Table S2.51 Maximum-emergence scenario: Effect of treatment strategies on the frequency of newly emerging double resistance (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|--------|---------|
| Between Groups | 0.035 | 5 | 0.007 | 41.272 | < 0.001 |
| Within Groups | 0.010 | 60 | < 0.001 | | |
| Total | 0.045 | 65 | | | |

2. THE IMPACT OF TREATMENT STRATEGIES ON THE EPIDEMIOLOGICAL DYNAMICS OF
PLASMID-CONFERRED ANTIBIOTIC RESISTANCE

Table S2.52 Maximum-emergence scenario: Multiple comparison between the effects of treatment strategies on the frequencies of newly emerging double resistance using Tukey's post-hoc analysis.

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|--------|--------|
| Combination | Cycling | 0.013 | 0.227 | -0.004 | 0.029 | False |
| Combination | Mixing | 0.021 | 0.004 | 0.005 | 0.038 | True |
| Combination | Mono A | 0.003 | 0.990 | -0.013 | 0.020 | False |
| Combination | Mono B | 0.024 | 0.001 | 0.008 | 0.041 | True |
| Combination | No treatment | 0.070 | 0.000 | 0.053 | 0.086 | True |
| Cycling | Mixing | 0.009 | 0.597 | -0.007 | 0.025 | False |
| Cycling | Mono A | -0.009 | 0.569 | -0.026 | 0.007 | False |
| Cycling | Mono B | 0.012 | 0.308 | -0.005 | 0.028 | False |
| Cycling | No treatment | 0.057 | 0.000 | 0.041 | 0.073 | True |
| Mixing | Mono A | -0.018 | 0.022 | -0.035 | -0.002 | True |
| Mixing | Mono B | 0.003 | 0.997 | -0.014 | 0.019 | False |
| Mixing | No treatment | 0.048 | 0.000 | 0.032 | 0.065 | True |
| Mono A | Mono B | 0.021 | 0.005 | 0.004 | 0.037 | True |
| Mono A | No treatment | 0.066 | 0.000 | 0.050 | 0.083 | True |
| Mono B | No treatment | 0.045 | 0.000 | 0.029 | 0.062 | True |

Table S2.53 Maximum-emergence scenario: Effect of treatment strategies on the frequency of super-infections (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|--------|---------|
| Between Groups | 0.019 | 5 | 0.004 | 11.731 | < 0.001 |
| Within Groups | 0.017 | 52 | < 0.001 | | |
| Total | 0.036 | 57 | | | |

Table S2.54 Maximum-emergence scenario: Multiple comparison between the effects of treatment strategies on the frequencies of superinfections using Tukey's post-hoc analysis.

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|-------------|--------------|----------|-------|--------|-------|--------|
| Combination | Cycling | 0.024 | 0.202 | -0.007 | 0.055 | False |
| Combination | Mixing | 0.036 | 0.015 | 0.005 | 0.067 | True |
| Combination | Mono A | 0.035 | 0.021 | 0.004 | 0.066 | True |
| Combination | Mono B | 0.026 | 0.160 | -0.005 | 0.057 | False |
| Combination | No treatment | 0.068 | 0.000 | 0.037 | 0.099 | True |
| Cycling | Mixing | 0.011 | 0.674 | -0.011 | 0.034 | False |
| Cycling | Mono A | 0.010 | 0.768 | -0.013 | 0.033 | False |
| Cycling | Mono B | 0.002 | 1.000 | -0.022 | 0.025 | False |
| Cycling | No treatment | 0.043 | 0.000 | 0.021 | 0.066 | True |
| Mixing | Mono A | -0.001 | 1.000 | -0.024 | 0.021 | False |
| Mixing | Mono B | -0.010 | 0.810 | -0.033 | 0.013 | False |
| Mixing | No treatment | 0.032 | 0.001 | 0.009 | 0.055 | True |
| Mono A | Mono B | -0.009 | 0.881 | -0.032 | 0.015 | False |
| Mono A | No treatment | 0.033 | 0.001 | 0.011 | 0.056 | True |
| Mono B | No treatment | 0.042 | 0.000 | 0.019 | 0.065 | True |

Table S2.55 Maximum-emergence scenario: Effect of drug ϑ on the frequency of emergence per superinfection (ANOVA).

| | Sum of Squares | df | Mean Square | F | Sig. |
|----------------|----------------|----|-------------|---------|---------|
| Between Groups | 10.194 | 3 | 3.398 | 143.661 | < 0.001 |
| Within Groups | 1.443 | 61 | 0.024 | | |
| Total | 11.637 | 64 | | | |

Table S2.56 Maximum-emergence scenario: Multiple comparison between the effects of drug ϑ on the frequencies of emergence per superinfection using Tukey's post-hoc analysis.

| group1 | group2 | meandiff | p-adj | lower | upper | reject |
|--------|--------|----------|-------|--------|-------|--------|
| A | AB | -0.082 | 0.752 | -0.300 | 0.136 | False |
| A | B | 0.740 | 0.000 | 0.624 | 0.855 | True |
| A | none | 0.857 | 0.000 | 0.711 | 1.002 | True |
| AB | B | 0.822 | 0.000 | 0.602 | 1.042 | True |
| AB | none | 0.939 | 0.000 | 0.701 | 1.176 | True |
| B | none | 0.117 | 0.174 | -0.032 | 0.266 | False |

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3

Antimicrobial Combination Effects at Sub-inhibitory Doses do not Reliably Predict Effects at Inhibitory Concentrations

Abstract

Assessing whether drug combinations synergise or antagonise is difficult for several reasons:

(i) measuring bacterial death rates at clinically relevant drug concentrations is methodologically challenging, (ii) there is no unifying definition of what constitutes synergistic or antagonistic interactions, and (iii) both synergism and antagonism may be concentration and mixing ratio dependent. To assess how well sub-inhibitory measurements predict inhibitory behaviour, we quantified drug interactions for 15 pairwise drug combinations on a concentration checkerboard covering a wide range of inhibitory and sub-inhibitory concentrations. To this end, we recorded 8640 time-resolved luminescence trajectories that have been shown to track bacterial population declines. To handle time-varying treatment effects and allow fair comparisons between drugs with distinct killing dynamics, we introduced a growth-integrated rate-like metric ψ to summarise each trajectory, and assigned interaction types (synergistic/independent/antagonistic) based on Bliss independence and Loewe additivity. We found that the interaction type frequently changes as the concentration increases from sub-inhibitory to inhibitory concentrations. Moreover, interaction type depended on the mixing ratio, implying that single-point sub-inhibitory measurements are not sufficient to predict interactions at clinically relevant concentrations.

3.1 Introduction

Antimicrobial resistance (AMR) poses a major global health burden, with an estimated 1.14 million deaths directly attributable to resistant infections in 2021 alone [1]. Moreover, the lag between the introduction of a new antibiotic class and the emergence of clinical resistance appears to be shrinking [2]. To develop a more sustainable approach to antibiotic use, a deeper understanding of the influence of treatment on the evolution of resistance is necessary.

3. ANTIMICROBIAL COMBINATION EFFECTS AT SUB-INHIBITORY DOSES DO NOT RELIABLY PREDICT EFFECTS AT INHIBITORY CONCENTRATIONS

One strategy that has been shown to slow the evolution of resistance in notoriously fast-evolving pathogens, such as HIV, malaria, and tuberculosis, is combination therapy [3, 4]. In theoretical models, combination therapy seems to outperform other strategies (such as mixing or cycling) [5–7], and it also seems to perform best in in vitro simulations of epidemics [8, 9]. However, clinical evidence for the efficacy of combination therapy is inconclusive, and a comprehensive meta-analysis did not identify a significant overall benefit [10].

Several factors contribute to the inconclusiveness of clinical studies, ranging from ethical constraints that limit focal pathogens to comparatively benign ones (i.e. excluding HIV and M. tuberculosis) to the lack of statistical power to track resistance evolution [10]. Another potential reason is drug interactions. Drug combinations can exhibit synergistic or antagonistic interactions, depending on whether the combined effect is stronger or weaker than the expectation based on the effects of the single drugs. However, defining this expectation is non-trivial, as null models are numerous and the best choice to describe drug independence remains debated [11, 12]. Here, we use two classical and widely applied reference frameworks to define independence: the response-based Bliss independence [13] and dose-based Loewe additivity [14].

Bliss independence is based on the assumption that the probability of being affected by one drug is statistically independent of being affected by the other [13]:

$$p_{AB}^{\text{Bliss}} = p_A + p_B - p_A p_B. \quad (3.1)$$

Under this assumption, Bliss independence is expected to be more appropriate for drug pairs with distinct and non-overlapping mechanisms of action [15].

In contrast, Loewe additivity – first discussed as iso-additivity by Frei in 1913 [16] and later formalised by Loewe and Muischnek [14] – uses dose equivalence as a null model. A combination is considered additive if pairs of concentrations that produce the same effect ψ (i.e. a measure of net growth) lie on a linear isobole connecting the equivalent single-drug doses [14]:

$$\frac{c_A}{f_A^{-1}(\psi(c_A, c_B))} + \frac{c_B}{f_B^{-1}(\psi(c_A, c_B))} = 1. \quad (3.2)$$

Here f_i^{-1} represents the inverse of a pharmacodynamic function $f_i(c)$ that maps drug concentrations c_i to an effect measure ψ .

There are many studies on drug interactions below the minimum inhibitory concentration (MIC) (e.g. [17–25]) and at the MIC level (e.g. [26–28]). However, studies covering the arguably clinically

more relevant inhibitory regime are scarce and often cover only a few points in the inhibitory range (e.g. [29–33]). The primary reason for this is methodological: under sub-inhibitory conditions, growth rates can be easily assessed at high throughput using optical density measurements, whereas inhibitory conditions typically require labour-intensive, low-throughput colony-forming unit assays [34]. This lack of data on effects at high concentrations is a problem, not only because inhibitory conditions are more relevant therapeutically, but also because the right, inhibitory, tail of the pharmacodynamic curve has a strong influence on resistance evolution [35].

It has already been shown that drug interactions can depend on dose and mixing ratio [18, 23, 36–38]. However, the existing literature provides limited quantitative insight into how frequently interaction classifications remain stable versus how often they change with concentration and mixing ratio. Here, we provide an extensive map of interactions for 15 antibiotic pairs, each measured on a 12×12 concentration checkerboard that includes inhibitory concentrations using time-resolved bioluminescence trajectories. We quantify how often sub-inhibitory interaction classifications based on Bliss independence and Loewe additivity generalise to inhibitory conditions and how often they fail, including cases of qualitative reversals across regimes.

3.2 Results

We quantified drug interactions for 15 pairwise combinations of six antibiotics falling into three mechanistic classes: polymyxins (colistin, COL; polymyxin B, POL), β -lactams (amoxicillin, AMO; penicillin, PEN), and ribosome-targeting drugs (chloramphenicol, CHL; tetracycline, TET). For each drug pair, we assessed 144 conditions (c_A, c_B) in a 12×12 checkerboard. We measured light intensity trajectories $I(t)$ for 8,640 bioluminescent cultures to infer changes in population size over time. Single-drug trajectories are shown in Fig. S3.1.

Time-variant growth rates and treatment effects. Most drug-interaction metrics are based on estimates of treatment effects, which collapse the trajectories of the bacterial population size into a scalar. These estimates are typically either rate-based, obtained by fitting an exponential rate of change to a growth trajectory [17, 31, 39–41], or area under the curve (AUC)-based, obtained by integrating a (transformed) growth trajectory over time [24, 30, 32, 33, 42, 43]. Rate-based approaches are easy to interpret and can be used directly in epidemiological models. However, slopes are sensitive to the choice of the fitting window when treatment effects are time-

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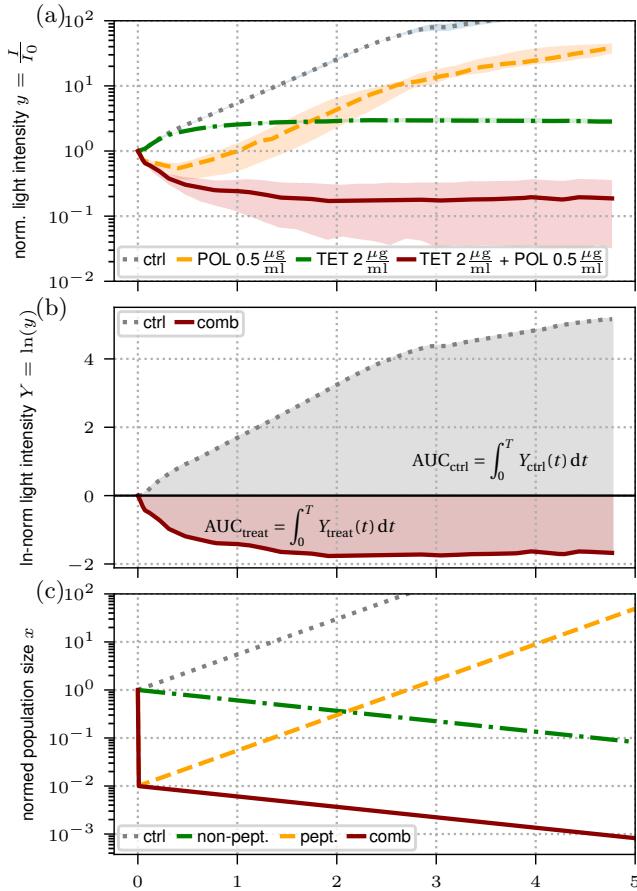


Fig. 3.1 Panel (a) shows the median of normalized light-intensity trajectories $y(t) = I(t)/I(0)$ for the combination of polymyxin B (POL, 0.5 $\mu\text{g mL}^{-1}$) and tetracycline (TET, 2 $\mu\text{g mL}^{-1}$), together with the corresponding single-drug and untreated control trajectories, with shaded bands indicating the interquartile range across biological replicates. Panel (b) shows the log-transformed signal $Y(t) = \ln(y(t))$ for the untreated control and the combination treatment from panel (a), together with their associated areas under the curve. Panel (c) shows a simple peptide–antibiotic interaction model illustrating qualitatively distinct dynamics under: no drugs, antibiotic (slow constant decline), peptide (rapid initial decline), and peptide plus antibiotic (rapid initial decline followed by a constant slow decline).

variant. This sensitivity is especially problematic when assessing interactions among drugs with very different killing dynamics (e.g., in the example shown in Fig. 3.1a), where treatment effects start at different time points and persist for different durations.

Given that many trajectories exhibited time-varying treatment effects, we summarised each trajectory by ψ (Methods), a time-weighted average of the instantaneous net growth rate $\hat{\psi}(t)$ (Equation S3.1). This weighting emphasises early treatment effects, reflecting that early changes in net growth shape the population for a longer duration than equally sized changes occurring later. ψ is proportional to the area under the log-normalised luminescence trajectory $Y(t) = \ln(I(t)/I(0))$ (Equation S3.3). Treatment effects, τ , are then defined as the difference between the time-weighted net growth rates of an untreated control and the treated condition, and correspond to the scaled area between curves (Fig. 3.1b).

Time-weighted net growth rates for one example (CHL+TET) are shown in Fig. 3.2, with markers indicating conditions as inhibitory (stars), sub-inhibitory (diamonds), or intermediate (no marker). Rates for all conditions are shown in Fig. S3.3. For further details see Methods. We then fitted pharmacodynamic curves to the single-drug estimates (Fig. S3.2).

For each condition (c_A, c_B) we estimate the bootstrap distribution $\boldsymbol{\tau}(c_A, c_B)$ by resampling pairs of control and treatment wells with replacement (Methods, Appendix 3G). Throughout this manuscript, we denote distributions with bold symbols. For each bootstrap draw b , we compute $\tau_b(c_A, c_B) = \psi_b(\emptyset) - \psi_b(c_A, c_B)$.

Bliss independence implies additive treatment effects. Because the original Bliss formulation is probabilistic, we have to translate it into our growth-based treatment-effect framework (see Appendix 3D). Bliss independence implies multiplicative survival $S_{AB}(T) = S_A(T)S_B(T)$ (Equation S3.12). After substituting $S_i(T) = \exp(-\int_0^T \hat{\tau}_i(u) du)$ with $\hat{\tau}_i(t)$, denoting the instantaneous treatment effect, we can derive that Bliss implies additive time-weighted treatment effects:

$$\tau^{\text{Bliss}}(c_A, c_B) = \tau(c_A) + \tau(c_B). \quad (3.3)$$

Peptide -- non-peptide interaction model. We observed that a combination of non-peptide drugs with relatively constant inhibition dynamics and peptide-like drugs, which induce a sharp early decline followed by (almost) unimpaired growth, is dominated by the peptide drug in the early phase and by the non-peptide drug in the later phase. A well-behaved example of such a com-

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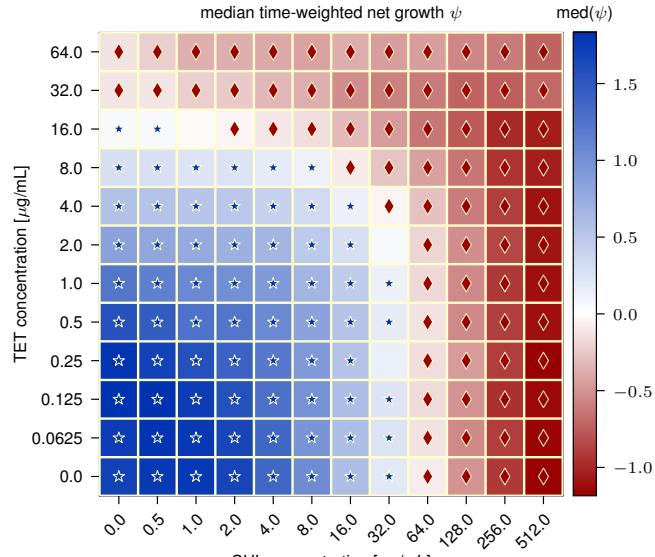


Fig. 3.2 Time-weighted growth rate ψ for combinations of CHL and TET. Each cell corresponds to one concentration pair, with colour indicating the median of ψ across $n = 4$ biological replicates. Markers denote whether the distribution of ψ is significantly different from zero (see Methods): stars indicate significantly positive values (net growth), and diamonds indicate significantly negative values (net killing).

bination is shown in Fig. 3.1a for polymyxin B (POL) and tetracycline (TET). Because most interaction metrics do not explicitly account for time-varying effects, it is unclear what combined treatment effect to expect. To answer this, we constructed a mathematical model for an idealised scenario. Drug A immediately reduces the population by a factor α but does not affect the subsequent dynamics, while drug B acts invariantly over time by constantly reducing the net growth rate from $\hat{\psi}_{\text{ctrl}}$ to $\hat{\psi}_B$. In combination, the population is first reduced by α and then grows with $\hat{\psi}_B$ (Fig. 3.1c). Substituting our rate definition (Equation 3.4) into this model yields additive treatment effects, $\tau_{AB} = \tau_A + \tau_B$ (see Appendix 3F), equivalent to the Bliss prediction derived above.

Interaction scores (μ, ν). Based on the Bliss prediction of combined treatment effects derived above, we define the Bliss interaction score μ as the normalised deviation from the prediction (Equation S3.16). The distribution of μ can be inferred based on the distributions of treatment effects $\boldsymbol{\tau}$ described above. For Loewe additivity, we define the interaction score ν as the deviation from dose equivalence (Equation S3.18). We obtain the distribution of ν from the inverted single-drug pharmacodynamic curves $f_i^{-1}(\boldsymbol{\psi})$ (Equation S3.10). We then assign interaction types

(synergistic, independent, antagonistic) depending on whether the 95% interval of $\boldsymbol{\mu}$ or $\boldsymbol{\nu}$ lies below, contains, or lies above zero. The resulting interaction types based on $\boldsymbol{\mu}$ and $\boldsymbol{\nu}$ for each combination are shown in Figures S3.4 and S3.5.

Disagreement between sub-inhibitory and inhibitory interaction types within reference models.

Using the per-condition distributions of interaction scores described above, we next assessed whether interaction classifications remain consistent across concentration regimes. We normalised concentrations c_i by the corresponding zMIC_i (defined by $f_i(\text{zMIC}_i) = 0$; Table S3.1), setting $z_i = c_i/\text{zMIC}_i$. We then separately summarised inhibitory and sub-inhibitory conditions using a second, higher-level bootstrap. Specifically, we sampled conditions $(c_A, c_B)_r$ for $r = (1, \dots, 200)$ with replacement, weighted by their mixing ratio to bias the sampling towards more equal ratios (Equation S3.22). For each sampled condition, we drew one estimate from the condition's distributions $\boldsymbol{\mu}(c_A, c_B)$ and $\boldsymbol{\nu}(c_A, c_B)$. To compare regimes and reference models, we define three alignment classes: agreement (same classification), soft disagreement (independent in one but synergistic or antagonistic in the other), and strong disagreement (opposite classifications). The resulting comparisons between sub-inhibitory and inhibitory regimes for $\boldsymbol{\nu}_{\text{sub}}$, $\boldsymbol{\nu}_{\text{inh}}$ and $\boldsymbol{\mu}_{\text{sub}}$, $\boldsymbol{\mu}_{\text{inh}}$ are shown in Fig. 3.3a,b and the resulting interaction types in Table S3.2.

Assuming Bliss independence, we observed six combinations with agreement and 9 cases of disagreement, of which one resulted in strong disagreement (AMO+PEN) and eight in soft disagreement. Assuming Loewe additivity, we observed agreement for six combinations (three of which also agreed under Bliss) and disagreement for nine combinations. Here, we observed two cases of strong disagreement (COL+TET and POL+TET) and seven with soft disagreement.

Disagreement between reference models, within concentration regimes. Fig. 3.3c replots the same interaction summaries described above, but now compares the Bliss interaction score $\boldsymbol{\mu}$ to the Loewe interaction score $\boldsymbol{\nu}$ across both sub-inhibitory and inhibitory regimes in a single panel. In Fig. S3.6a,b we show the same comparison but separated by regime. Across all 30 (2×15) comparisons, the Bliss and Loewe-based classifications agree in 14 cases, show soft disagreement in 15 cases, and show strong disagreement in one case (CHL+TET) (Fig. 3.3c).

Interaction types can change with dose even at fixed mixing ratio. Above, we compared interaction summaries between sub-inhibitory and inhibitory regimes by aggregating condition-wise

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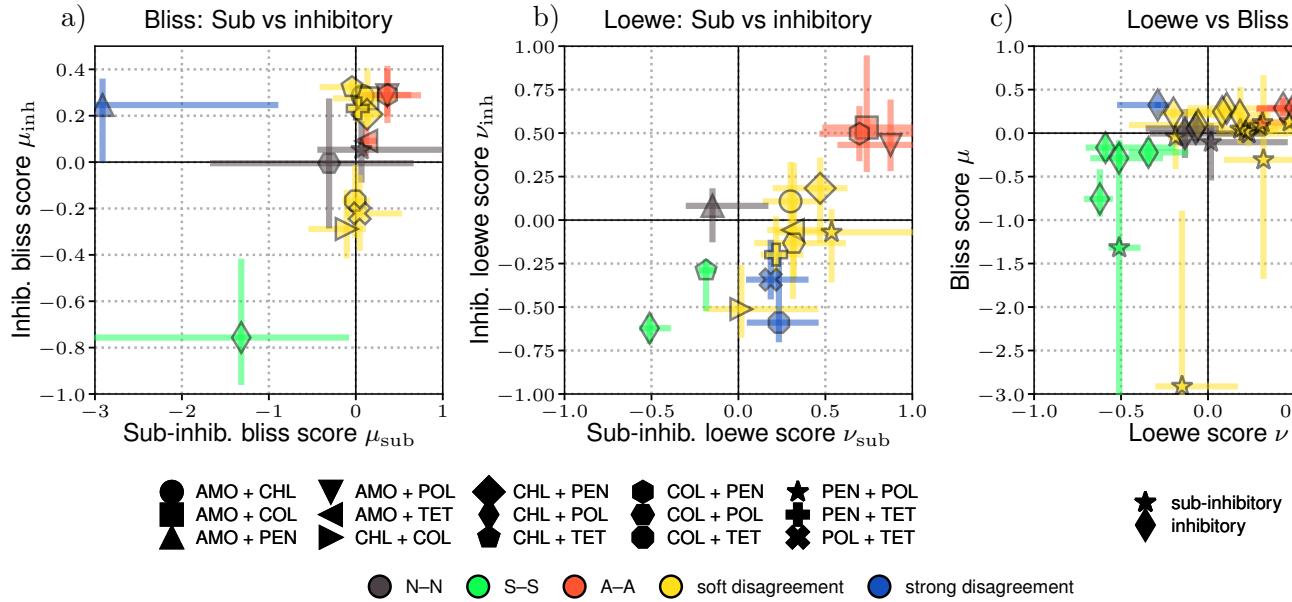


Fig. 3.3 Interaction summaries across regimes and models. Panels (a) and (b) compare sub-inhibitory vs. inhibitory interaction scores, using (a) the Bliss interaction score μ and (b) the Loewe interaction score ν . Each point represents one drug combination, plotted as the median estimate in the sub-inhibitory regime (x-axis) against the median estimate in the inhibitory regime (y-axis), with 95% bootstrap intervals shown as horizontal and vertical error bars. (c) Loewe vs. Bliss comparison across regimes: each point corresponds to one combination and regime, plotted as the median Loewe interaction score ν (x-axis) against the corresponding Bliss interaction score μ (y-axis), with 95% bootstrap intervals. Markers encode the drug combination (shared legend for panels (a) and (b)), while marker shape encodes the regime in panel (c). We classify interactions as synergistic (S), antagonistic (A), or independent (N; not significantly different from zero). Colors indicate interaction type agreement between the two compared axes in each panel: agreement if the two interaction types coincide (N–N, S–S, A–A), soft disagreement if one interaction type is independent and the other is non-independent (N–S, N–A), and strong disagreement if the classifications are opposite (S–A).

estimates across a range of doses and mixing ratios. We next ask whether interaction types also change (i) as the dose increases at a fixed mixing ratio, and (ii) as the mixing ratio varies at a fixed effect level. To facilitate both analyses, we reparameterize concentration pairs (c_A, c_B) in polar coordinates (z, ϕ) , where z is the combined dose and ϕ is the mixing angle (Equation 3.8) that encodes the mixing ratio.

For each drug combination, we fitted 25 continuous, monotonically decreasing surface splines on bootstrap datasets of $\psi(c_A, c_B)$ (Fig. S3.7). Based on these splines, we estimate polar pharmacodynamic curves, which show ψ as a function of the combined dose z at a fixed mixing angle $\phi = 45^\circ$ (equal mixing in units of zMIC). At a given dose, predicted values above the observed ψ indicate synergy, whereas predicted values below the observed ψ indicate antagonism. For

both reference models, there are examples where the interaction changes direction as the combined dose increases. For the combination AMO+COL (Fig. 3.4a), the Loewe-based interaction shifts from antagonism at lower doses to synergy at higher doses. For AMO+PEN (Fig. 3.4b) Bliss-based interaction flips, with synergy at lower doses and strong antagonism at higher doses. Polar pharmacodynamic curves at 45° for all combinations are shown in Fig. S3.8.

Interaction types can depend on the mixing ratio. To assess whether interaction types depend on the mixing ratio at a fixed effect level, we extracted isoboles from the median surface spline, i.e., the path $(z, \phi)_i$ along which the time-weighted net growth rate is constant ($\psi_i = 0 \frac{1}{h}$; Fig. S3.9). Along each isobole, we evaluate Bliss- and Loewe-based predictions and plot these predictions as a function of ϕ . For many combinations, the inferred interaction type is stable across ϕ , as exemplified by CHL+TET (Fig. 3.4c). However, combinations showing mixing-ratio dependence are also common, as exemplified for COL+PEN (Fig. 3.4d). Plots for all combinations are shown in Fig. S3.10.

3.3 Discussion

We quantified interaction patterns across a wide concentration range for several drug combinations with different modes of action. Quantifying the size of the bacterial population is notoriously difficult because bacterial death has many facets, and no single method captures all of them [44, 45]. In this work, we used bioluminescence as a proxy for population size. This choice enabled us to record 8640 finely time-resolved (every 10 minutes for five hours) growth trajectories at high throughput. Bioluminescence has been shown to be a better proxy for biomass than for cell number [45], which makes interpreting our readout as biomass the more robust interpretation. However, since we restricted our analysis to drugs for which changes in biomass align with changes in cell number (i.e. limited filamentation) [45], we do not expect qualitatively different conclusions when using an alternative readout of population size.

For many of the observed trajectories treated with higher concentrations of polymyxins (COL and POL), the light intensity dropped below the detection limit within the first few minutes. In principle, slope-based estimates can still be inferred for such curves. However, comparing these estimates across drugs with different onsets and durations of treatment effects is conceptually difficult to justify. To enable fair comparisons across drugs, we therefore used a weighted growth

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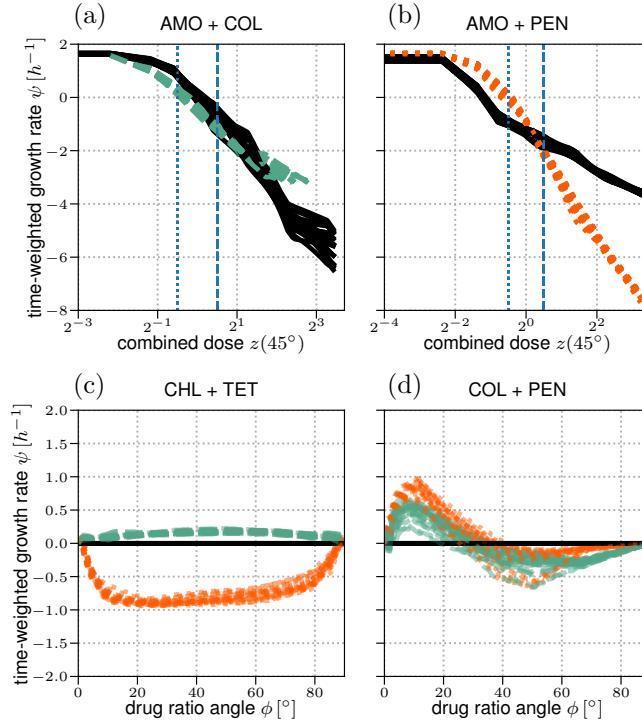


Fig. 3.4 Panels (a,b) show polar pharmacodynamic curves at $\phi = 45^\circ$ (corresponding to a 1:1 ratio in units of zMIC) for (a) AMO+COL and (b) AMO+PEN. The x-axis shows the combined dose z , where $z = 1/\sqrt{2}$ (blue dotted line) corresponds to both single-drug doses equaling 0.5zMIC , and $z = \sqrt{2}$ (blue dashed line) corresponds to both equaling 1zMIC . Panels (c,d) show the Bliss- and Loewe-based predictions for ψ over the mixing angle ϕ along the observed isobole at $\psi = 0\frac{1}{h}$ for (c) COL+PEN and (d) CHL+TET.

measure integrated over a shared timeframe that is equal for all trajectories ($T \approx 2\text{ h}$). However, this choice comes at the cost of having to discard all trajectories that fall below the detection limit too early.

To investigate how different treatment dynamics combine, we developed a simplified antibiotic-peptide interaction model. This model predicts that combinations of a short-acting, peptide-like drug with a drug with time-invariant treatment effects should follow Bliss independence. The observed isoboles for peptide–non-peptide pairs in Fig. S3.9 confirm this behaviour, as they were closer to the Bliss-based prediction than to the Loewe-based prediction for all pairs except COL+PEN.

To address our core question — how predictive sub-MIC interaction patterns are for inhibitory interactions — we aggregated interaction estimates for sub-inhibitory and inhibitory conditions

and compared them. For both reference models (Bliss independence and Loewe additivity), more than half of the combinations showed soft disagreement between inhibitory and sub-inhibitory regimes, i.e., a significant synergy or antagonism in one regime but not in the other. This does not necessarily imply that sub-inhibitory measures are uninformative, because non-significance of synergistic/antagonistic interactions is not evidence of independence and can arise from variance. Such variance can result from aggregating across diverse conditions and mixing ratios, which we showed can influence the interaction type (Fig. S3.10). Importantly, for both reference models, we observed more cases of synergistic or antagonistic agreement than strong disagreement, indicating that sub-inhibitory interaction measures retain some qualitative predictive value.

Our results also confirm the practical limitations of Loewe-based interaction measures at high concentrations that have been reported previously ([46]). Since Loewe relies on the inverse of the single-drug pharmacodynamic functions, it is only defined when the combined effect lies within the effect ranges spanned by both single-drug treatments. For drug pairs with very different maximal killing rates, this condition fails in large parts of the checkerboard (see undefined regions in Fig. S3.5). This is a severe limitation that prohibits the quantification of drug interactions for a large number of therapeutically relevant conditions. Consistent with previous work (e.g. [47]), we found that the Bliss and Loewe frameworks can produce opposite classifications.

Our findings show that conclusions about synergy or antagonism depend on the concentration range, mixing ratio, and the reference model on the basis of which synergy or antagonism is determined. Accordingly, single-point measurements at a single sub-inhibitory concentration are insufficient to reliably characterise drug interactions at clinically relevant inhibitory concentrations.

3.4 Methods

Strains and Media. We used the bioluminescent strain *Escherichia coli* MG1655 Δ galK::(kan^R-luxCDABE) constructed previously [45]. Cultures were grown in LB medium (Sigma L3022).

Drug preparation. We explored drug interactions among six drugs (Table 3.1), resulting in 15 drug–drug pairs. For each pair, one compound was designated drug A and the other drug B. Each compound was prepared as a $\times 20$ stock of its highest working concentration. We then performed a twofold dilution series in a 12-column deepwell plate, resulting in 11 diluted concentrations

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Table 3.1 Drugs used in the experiments, the solvent, the estimated zMIC (by fitting the single-drug pharmacodynamic curves), and the supplier with catalogue number.

| drug | solvent | zMIC [$\frac{\mu\text{g}}{\text{mL}}$] | supplier |
|-----------------------|---------|--|-----------------------------|
| amoxicillin (AMO) | water | 8.42 | ThermoFischer, J61290.14 |
| chloramphenicol (CHL) | DMSO | 54.11 | Sigma, C0378 |
| colistin (COL) | water | 0.58 | Sigma, C4461 |
| penicillin (PEN) | water | 153.86 | Roth, HP48.2 |
| polymyxin B (POL) | water | 0.51 | Roth, 0235.1 |
| tetracycline (TET) | DMSO | 15.15 | Sigma, T3383 |

and one drug-free column. For drug A, we transferred 125 μL per well into a 96-well plate (subreservoir A) with a horizontal concentration gradient. For drug B, we transferred 50 μL per well into two 96-well plates (antibiotic reservoir plates I and II) with vertical concentration gradients (each 6×12 layout). All plates were stored at -80°C to minimise degradation over time, accepting a one-time freeze–thaw-related degradation.

Dose response assays. For each assay, we prepared four overnight cultures grown for 14 h. Cultures were distributed to fill an entire 384-well source plate (Greiner 781073), so that the replicates formed a 2×2 block format (e.g. C1–rep1, C2–rep2, D1–rep3, D2–rep4). We then prefilled two white 384-well assay plates (Greiner 781073) with 54 μL LB per well and transferred inocula from the source plate to both assay plates (I, II) using an Evo 200 liquid handling platform (Tecan) with a pintool. The dilution is approximately 1:150, although we observed substantial well-to-well variation in the effective inoculum size (see below). Plates were incubated for 2 h to reach exponential phase. Simultaneously, subreservoir A and both antibiotic-reservoir plates were thawed. 50 μL from each well of the subreservoir A were transferred to the corresponding wells of both antibiotic-reservoir plates, generating a 10x mixture of drugs A and B. For both assay plates, the assay started as follows: a baseline luminescence reading was taken, 6 μL of the 10x drug mixture was added (defining $t = 0$ at dosing for the respective wells), and a second luminescence reading was taken. Subsequently, we alternated between reading assay plates I and II for a total duration of 5 h. Luminescence was recorded with an Infinite F200 plate reader (Tecan) using a 250 ms integration time.

Data preprocessing. To estimate stray-light contamination, we conducted a calibration experiment in which six source wells (E5, E12, E20, L5, L12, L20) contained stationary-phase cultures while all other wells remained empty. From a single luminescence read of the full plate, we constructed a distance-dependent stray-light kernel (Fig. S3.11) and corrected each well by subtracting the summed contributions from neighbouring wells (restricted to distances $d \leq 3$, Equation S3.8). We defined the lower limit of detection as $I_{\text{LoD}} = 10 \text{ RLU}$ and the upper limit of detection as 10^6 RLU . We defined the common analysis horizon T as the earliest time point at which any untreated control well exceeded the upper detection limit, yielding $T \approx 2 \text{ h}$. For each well, we computed the fraction of observations up to T below I_{LoD} . Wells with more than 20% of observations below I_{LoD} were excluded from all subsequent analyses. For retained wells, values below I_{LoD} were censored by replacing them with I_{LoD} . For numerical integration, we interpolated the log-normalised signal to obtain values on the shared horizon $t = T$ (see also Appendix 3B).

Time-weighted net growth rate. We define ψ as the linearly time-weighted net growth rate (SI: Equation S3.1) over the common time horizon T . We infer ψ by calculating the scaled area under the log-normalised light intensity $Y(t) = \ln(I(t)/I(0))$ (SI: Equation S3.2, Equation S3.3),

$$\psi = \frac{2}{T^2} \int_0^T Y(t) dt. \quad (3.4)$$

Classification of inhibitory and sub-inhibitory conditions. We classify each checkerboard condition as inhibitory, sub-inhibitory, or not significantly different from zero by testing whether replicate time-weighted growth-rate estimates $\psi(c_A, c_B)$ are consistently positive or negative. With four replicates, the binomial probability of observing all positive (or all negative) signs with equal probability is $0.5^4 = 0.0625$. This classification is used to annotate the ψ heatmaps (Fig. 3.2, Fig. S3.3) and to define inhibitory and sub-inhibitory regime pools.

Single-drug pharmacodynamic curve fitting. For each drug, we aggregated all single-drug data across experiments and fitted pharmacodynamic curves $f(c)$ to the corresponding time-weighted growth-rate estimates ψ :

$$f(c) = \psi_0 - \frac{(\psi_0 - \psi_{\min})(c/z\text{MIC})^\kappa}{(c/z\text{MIC})^\kappa - (\psi_{\min}/\psi_0)}, \quad [0, \infty) \rightarrow [\psi_{\min}, \psi_0] \quad (3.5)$$

Here, ψ_0 denotes the maximum and ψ_{\min} the minimum time-weighted growth rate, corresponding to $c = 0$ and $c \rightarrow \infty$, respectively. κ denotes the Hill coefficient and $z\text{MIC}$ the concentration at

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which $f(z\text{MIC}) = 0$. The model parameters ψ_{i_0} , ψ_{\min} , κ , $z\text{MIC}$ were jointly estimated by least-squares fitting of Equation 3.5.

Distribution of treatment effects. We define the treatment effect τ as the linearly time-weighted average difference between the untreated and treated instantaneous net growth rates over the common time horizon T (SI: Equation S3.6). Equivalently, τ is the scaled area between the control and treatment trajectories on the log-normalised scale (illustrated in Fig. 3.1b). Because τ is a comparative measure between one untreated and one treated trajectory, we bootstrap treated-control well pairs with replacement ($B = 200$; details in Appendix 3G). For each condition (c_A, c_B) and draw b , we compute $\tau_b(c_A, c_B) = \psi_b(\emptyset) - \psi_b(c_A, c_B)$, yielding the distribution of treatment effects $\boldsymbol{\tau}(c_A, c_B)$. Throughout this manuscript, distributions are denoted by bold symbols. We classify such bootstrap distributions as significant if the central 95% bootstrap interval excludes zero, and as not significant otherwise.

Bliss-based interaction score μ . We define the Bliss based interaction score as the normalised deviation from the Bliss-prediction for combined treatment effects (see Appendix 3D and Equation 3.3):

$$\mu_b(c_A, c_B) = \frac{\tau_b(c_A) + \tau_b(c_B) - \tau_b(c_A, c_B)}{\text{median}(\boldsymbol{\tau}(c_A)) + \text{median}(\boldsymbol{\tau}(c_B))}. \quad (3.6)$$

To avoid misinterpreting noise as interaction, we only evaluate $\nu(c_A, c_B)$ if $\boldsymbol{\tau}(c_A, c_B)$ is significantly greater than zero and at least one of $\boldsymbol{\tau}(c_A)$ or $\boldsymbol{\tau}(c_B)$ is significantly greater than zero.

Loewe-based interaction score ν . We define a Loewe interaction score (details in Appendix 3E) that quantifies deviations from dose equivalence as

$$\nu_b(c_A, c_B) = \frac{c_A}{f_A^{-1}(\psi_b(c_A, c_B))} + \frac{c_B}{f_B^{-1}(\psi_b(c_A, c_B))} - 1. \quad (3.7)$$

Here f_i denotes a pharmacodynamic function mapping concentration to net growth, $f_i(c) = \psi$. The inverse $f_i^{-1}(\psi)$ is the corresponding single-drug equivalent concentration of drug i . Our definition of ν is analogous to the combination index (CI – 1) [36], but uses a different pharmacodynamic model f_i (either Equation S3.10 or inferred numerically from the single-drug edges of the surface splines). To avoid misinterpreting noise as interaction, we only evaluate $\nu(c_A, c_B)$ if $\boldsymbol{\tau}(c_A, c_B)$ is significantly greater than zero and at least one of $\boldsymbol{\tau}(c_A)$ or $\boldsymbol{\tau}(c_B)$ is significantly greater than zero.

Polar reparametrization. We normalise concentrations of drug i using the corresponding zMIC_i estimates obtained from pharmacodynamic curve fits (Table 3.1): $z_i = c_i / \text{zMIC}_i$. We then define polar coordinates based on the normalised concentrations:

$$z = \sqrt{z_A^2 + z_B^2}, \quad \phi = \arctan 2(z_B, z_A), \quad (3.8)$$

with ϕ corresponding to the mixing angle and z to the combined dose. These coordinates were used to plot polar pharmacodynamic curves, which show the treatment effect over the combined dose z for a fixed mixing angle ϕ . These curves represent cross-sections through the surface splines at fixed ϕ .

Inoculum effects. We noticed a much larger-than-expected variation in pre-treatment light intensity I_0 in our data, which we use as a proxy for inoculum size. To assess the impact of this variation on the results, we regressed ψ for each single-drug and concentration on the pre-treatment signal I_0 (Appendix S3I). We found negligible inoculum effects for AMO, CHL, PEN, and TET, but substantial effects for COL and POL at intermediate concentrations (see Fig. S3.12). Because the size of the inoculum did not show a significant directional trend along the concentration index ($p = 0.092$), the variance of the inoculum mainly adds noise at intermediate concentrations of COL and POL, contributing to an increased scatter in Fig. S3.2c,e.

3.5 Data, Materials, and Software Availability

Experimental datasets and code are available at Zenodo (DOI: 10.5281/zenodo.18374151).

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3.1 Supplementary Information Chapter 4

3.2 SI text

3A Time weighted net growth rates

We define ψ the linearly time weighted average of the temporal net growth rate $\hat{\psi}$:

$$\psi = \int_0^T \hat{\psi}(t) w(t) dt, \quad w(t) = \frac{2}{T^2}(T-t), \quad (\text{S3.1})$$

where the kernel $w(t)$ decreases linearly from T to 0 and is normalised such $\int_0^T w(t) dt = 1$. To derive ψ from the luminescence trajectories $I(t) = I_0 e^{\int_0^t \hat{\psi}(u) du}$, we first normalise

$$y(t) = \frac{I(t)}{I(0)}. \quad (\text{S3.2})$$

We then define the log-normalised trajectory

$$Y(t) = \ln y(t) = \ln I(t) - \ln I(0) = \int_0^t \hat{\psi}(u) du, \quad (\text{S3.3})$$

and obtain ψ directly from $Y(t)$ by integrating over time. Applying Fubini's theorem yields

$$\int_0^T Y(t) dt = \int_0^T \left(\int_0^t \hat{\psi}(u) du \right) dt = \int_0^T (T-u) \hat{\psi}(u) du, \quad (\text{S3.4})$$

so that

$$\psi = \frac{2}{T^2} \int_0^T Y(t) dt. \quad (\text{S3.5})$$

The area between two curves (control and treatment) is

$$\int_0^T (Y_{\text{ctrl}}(t) - Y_{\text{treat}}(t)) dt = \int_0^T (T-t) (\hat{\psi}_{\text{ctrl}}(t) - \hat{\psi}_{\text{treat}}(t)) dt, \quad (\text{S3.6})$$

and we define the treatment effect as the difference between the corresponding time-weighted net growth rates,

$$\tau = \psi_{\text{ctrl}} - \psi_{\text{treat}} = \frac{2}{T^2} \int_0^T (Y_{\text{ctrl}}(t) - Y_{\text{treat}}(t)) dt. \quad (\text{S3.7})$$

Intuition for ψ and τ .

Non-monophasic case. If rates vary over time, Equation S3.6 shows that τ represents a time-weighted average of instantaneous rate differences,

$$\tau = 2/T^2 \int_0^T (T-t) (\hat{\psi}_{\text{ctrl}}(t) - \hat{\psi}_{\text{treat}}(t)) dt,$$

where the weighting factor $(T-t)$ decreases linearly from T to 0. Early rate differences thus contribute more strongly than later ones. This weighting is desirable because an early

population reduction has a bigger therapeutical impact than a delayed effect, even if both reach the same endpoint.

Constant-rate case. If the temporal net growth rate is constant $\psi(t) = \hat{\psi}$, Equation S3.5 simplifies to:

$$\psi = \hat{\psi}.$$

Accordingly; if the temporal net growth rate of control and treatment condition are constant; the treatment effect (Equation S3.6) simplifies to:

$$\tau = \psi_{\text{ctrl}} - \psi_{\text{treat}}.$$

Hence, when trajectories are log-linear, τ directly measures the difference in net rates.

3B Quantifying Light Noise.

We quantified luminescence leakage by a calibration plate in which six source wells (E5, E12, E20, L5, L12, L20) contained 50 μL of overnight culture and all other wells were empty. A single luminescence read yielded raw intensities \hat{I} . For each empty well i we calculated the ratio $I_{\text{empty}}/I_{\text{culture}}$ relative to the nearest source well j at the distance of the grid d_{ij} (with diagonal neighbours at distance $\sqrt{2}$). For each discrete distance, we summarized ratios by their median to obtain a kernel $r(d)$ (Fig. S3.11). We estimate the light contribution from well j into well i as $\hat{I}_{ij} = r(d_{ij}) \hat{I}_j$. For each experimental plate and time point, we corrected each well by subtracting received light noise,

$$I_i = \hat{I}_i - \sum_j r(d_{ij}) \hat{I}_j, \quad (\text{S3.8})$$

where the sum is restricted to wells within $d \leq 3$.

3C Drug conditions and response functions

Single-drug pharmacodynamic curves. For each drug i we describe the relationship between concentration c and net growth rate by the fitted pharmacodynamic curve

$$f_i(c) = \psi_0 - \frac{(\psi_0 - \psi_{\min,i})(c/z_{\text{MIC},i})^{\kappa_i}}{(c/z_{\text{MIC},i})^{\kappa_i} - (\psi_{\min,i}/\psi_0)}, \quad [0, \infty) \rightarrow [\psi_{\min,i}, \psi_0]. \quad (\text{S3.9})$$

On this domain, f_i is monotone and therefore invertible, with inverse $f_i^{-1} : [\psi_{\min,i}, \psi_0] \rightarrow [0, \infty)$.

Closed-form inversion. Using Equation S3.9, the inverse f_i^{-1} can be written in closed form as

$$f_i^{-1}(\psi) = z_{\text{MIC},i} \left(\frac{(\psi_0 - \psi) \psi_{\min,i} / \psi_0}{(\psi_0 - \psi) - (\psi_0 - \psi_{\min,i})} \right)^{1/\kappa_i}, \quad \psi \in [\psi_{\min,i}, \psi_0]. \quad (\text{S3.10})$$

Cartesian conditions. A condition is defined by a set of drug concentrations $\{c_i\}$, with drug $i \in \{\text{AMO, CHL, COL, PEN, POL, TET}\}$. The control condition corresponds to $\{c_i = 0 \ \forall i\}$ and is denoted by \emptyset .

Throughout this work, quantities characterizing a treatment are written as functions of the underlying condition $\{c_i\}$. For example, $\psi(\{c_i\})$ denotes the effective growth rate associated with a condition. For brevity, we write $\psi(c_A)$ when only one drug is present, $\tau(c_A, c_B)$ when two drugs are present, and ψ_0 for the untreated control.

Polar pharmacodynamic curves. For two-drug combinations, conditions are parameterized in polar coordinates (z, ϕ) , and the corresponding effective growth rate is written as

$$\psi(z, \phi) = \psi_0 - \frac{(\psi_0 - \psi_{\min}(\phi))(z/z_{\text{MIC}}(\phi))^{\kappa(\phi)}}{(z/z_{\text{MIC}}(\phi))^{\kappa(\phi)} - \psi_{\min}(\phi)/\psi_0}. \quad (\text{S3.11})$$

3D Bliss independence

Bliss independence is defined by assuming that the probabilities of a cell being killed by drugs A and B within a fixed observation window $T > 0$ are statistically independent [1]. Under this assumption,

$$p_{AB}^{\text{Bliss}} = p_A + p_B - p_A p_B, \quad (\text{S3.12})$$

where p_A and p_B denote the single-drug kill probabilities within T .

Time-varying hazards. Let $\hat{\tau}_i(t) \geq 0$ denote a time-dependent kill hazard under treatment i on $t \in [0, T]$. The survival fraction at time T is $S_i(T) = \exp(-\int_0^T \hat{\tau}_i(u) du)$. Bliss independence implies multiplicative survival $S_{AB}(T) = S_A(T) S_B(T)$, hence additive cumulative hazards:

$$\int_0^T \hat{\tau}_{AB}^{\text{Bliss}}(u) du = \int_0^T \hat{\tau}_A(u) du + \int_0^T \hat{\tau}_B(u) du. \quad (\text{S3.13})$$

To connect this survival formulation to the growth-based log-trajectories, we interpret the instantaneous treatment effect as the reduction in temporal net growth relative to the untreated control, i.e. $\hat{\tau}_i(t) = \hat{\psi}_{\text{ctrl}}(t) - \hat{\psi}_i(t)$. With $Y_i(t) = \int_0^t \hat{\psi}_i(u) du$ from Equation S3.3, it follows that

$$Y_i(t) = Y_{\text{ctrl}}(t) - \int_0^t \hat{\tau}_i(u) du.$$

Using additive cumulative hazards under Bliss then yields

$$Y_{AB}^{\text{Bliss}}(t) = Y_A(t) + Y_B(t) - Y_{\text{ctrl}}(t). \quad (\text{S3.14})$$

Integrating Equation S3.14 over time yields the Bliss prediction for the effective treatment effect:

$$\tau^{\text{Bliss}}(c_A, c_B) = \tau(c_A) + \tau(c_B). \quad (\text{S3.15})$$

Bliss-based interaction index μ . Based on the Bliss expectation for a combination at concentrations (c_A, c_B) , we define the interaction index

$$\mu(c_A, c_B) = \frac{\tau(c_A) + \tau(c_B) - \tau(c_A, c_B)}{\text{median}(\tau(c_A)) + \text{median}(\tau(c_B))}. \quad (\text{S3.16})$$

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To avoid division by zero, normalization is performed using the medians of the monotherapy effects. The index is only evaluated where a condition (c_A, c_B) is classified as eligible if the combined effect $\tau(c_A, c_B)$ is significant and at least one of the two monotherapy treatment effects, $\tau(c_A)$ or $\tau(c_B)$, is significant.

Interpreting μ .

Positive values of μ indicate synergy ($\tau(c_A, c_B) > \tau(c_A) + \tau(c_B)$), negative values indicate antagonism, and $\mu = 0$ corresponds to neutral interactions.

3E Loewe additivity

Loewe additivity is a form of dose additivity and formalizes self-additivity, i.e. that a drug combined with itself should behave like a higher dose of the same drug. This concept is independent of the particular choice of effect measure, provided the effect can be represented on a shared, monotone dose-response scale. In our case, this effect scale is the effective net growth rate ψ (see Equation S3.5).

For a combination with observed effect $\psi(c_A, c_B)$, Loewe additivity requires that the fractions of the corresponding monotherapy-equivalent concentrations sum to one,

$$\frac{c_A}{f_A^{-1}(\psi(c_A, c_B))} + \frac{c_B}{f_B^{-1}(\psi(c_A, c_B))} = 1, \quad (\text{S3.17})$$

where f_A and f_B are the single-drug pharmacodynamic curves mapping concentrations to ψ , and f_A^{-1} and f_B^{-1} map an effect level ψ to the corresponding monotherapy-equivalent concentration. As pharmacodynamic function f_i , we use the fitted single-drug pharmacodynamic curve (Equation S3.9) for the well-based interaction analysis, whereas for the surface-based analysis we infer f_i from the monotherapy edge profiles of the chequerboard surface splines. The Loewe prediction $\psi_{\text{Loewe}}(c_A, c_B)$ is obtained by numerically solving Equation S3.17 for ψ .

Loewe interaction index (ν). We quantify deviations from Loewe additivity by the centered dose-equivalence index

$$\nu(c_A, c_B) = \frac{c_A}{f_A^{-1}(\psi(c_A, c_B))} + \frac{c_B}{f_B^{-1}(\psi(c_A, c_B))} - 1. \quad (\text{S3.18})$$

The index is only evaluated where a condition (c_A, c_B) is classified as eligible if the combined effect $\tau(c_A, c_B)$ is significant and at least one of the two monotherapy treatment effects, $\tau(c_A)$ or $\tau(c_B)$, is significant.

Because f_A^{-1} and f_B^{-1} are only defined on $\text{Im}(f_A) = [\psi_{\min,A}, \psi_0]$ and $\text{Im}(f_B) = [\psi_{\min,B}, \psi_0]$, ν is only defined if

$$\psi(c_A, c_B) \in \text{Im}(f_A) \cap \text{Im}(f_B) = [\max(\psi_{\min,A}, \psi_{\min,B}), \psi_0]. \quad (\text{S3.19})$$

Interpreting ν .

By construction $\nu = 0$ corresponds to Loewe additivity, $\nu < 0$ indicates synergy, and $\nu > 0$ indicates antagonism.

Because Loewe additivity requires monotherapy-equivalent concentrations for both drugs at the observed effect level, ν can only be evaluated when $\psi(c_A, c_B)$ lies in the overlap of the two monotherapy effect ranges, i.e. $\psi \geq \max(\psi_{\min,A}, \psi_{\min,B})$.

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3F Peptide--antibiotic interaction model

We consider two drugs, A and B , acting on a population with size trajectory $x(t)$. Drug A represents a peptide-like effect that causes an instantaneous multiplicative reduction in population size by a factor $\alpha \in (0, 1]$, whereas drug B represents a conventional antibiotic that changes the subsequent net growth rate but does not cause an initial drop. We assume that the combination of two drugs induces a sharp drop by the factor α followed by net growth, determined by drug B :

$$\begin{aligned}x_{\text{ctrl}}(t) &= x_0 \exp(\hat{\psi}_{\text{ctrl}} t), \\x_B(t) &= x_0 \exp(\hat{\psi}_B t), \\x_A(t) &= \alpha x_{\text{ctrl}}(t), \\x_{AB}(t) &= \alpha x_B(t),\end{aligned}$$

and:

$$\begin{aligned}Y_{\text{ctrl}}(t) &= \ln x_0 + \hat{\psi}_{\text{ctrl}} t, \\Y_B(t) &= \ln x_0 + \hat{\psi}_B t, \\Y_A(t) &= Y_{\text{ctrl}}(t) + \ln \alpha, \\Y_{AB}(t) &= Y_B(t) + \ln \alpha.\end{aligned}$$

Using the definition of effective-rate $\tau_i = \frac{2}{T^2} \int_0^T (Y_i(t) - Y_{\text{ctrl}}(t)) dt$, we obtain the following.

$$\tau_A = \frac{2}{T^2} \int_0^T (-\ln \alpha) dt, \quad \tau_B = \frac{2}{T^2} \int_0^T (\hat{\psi}_{\text{ctrl}} - \hat{\psi}_B) t dt,$$

and

$$\tau_{AB} = \frac{2}{T^2} \int_0^T ((\hat{\psi}_{\text{ctrl}} - \hat{\psi}_B) t - \ln \alpha) dt. \quad (\text{S3.20})$$

Separating the two terms in Equation S3.20 yields

$$\tau_{AB} = \tau_A + \tau_B, \quad (\text{S3.21})$$

which matches the Bliss expectation for additive treatment effects.

3G Condition-wise interaction inference on the checkerboard

Each condition $\{c_i\}$ is realized by a pool of replicate wells $\mathcal{W}(c_A, c_B)$, from which effective growth rates are inferred as described above. Treatment effects are estimated by bootstrap resampling

from the pools of replicate wells. We draw $B = 200$ pairs consisting of one control well and one condition well, sampled with replacement from $\mathcal{W}(\emptyset)$ and $\mathcal{W}(c_A, c_B)$, respectively. For each draw b , the treatment effect is defined as

$$\tau_b(c_A, c_B) = \psi_b(\emptyset) - \psi_b(c_A, c_B),$$

yielding a bootstrap distribution of treatment effects.

For combination conditions, the corresponding mono-treatment growth rates $\psi_b(c_A, 0)$ and $\psi_b(0, c_B)$ are sampled analogously and included in each bootstrap draw, as they are required for interaction analysis.

Regime-wise aggregation across checkerboard conditions. To summarise interactions for a given drug pair within a regime (sub-inhibitory or inhibitory), we combine the condition-wise bootstrap interaction estimates from all eligible checkerboard cells. In each aggregation draw, we sample 200 eligible conditions with replacement, using sampling probabilities proportional to

$$w(\phi) = \sin(2\phi). \quad (\text{S3.22})$$

This excludes monotherapy edges and gives the highest weight to near-equal mixing. For every sampled condition, we draw one interaction score from its condition-wise bootstrap distribution. Repeating this procedure yields an aggregated distribution, from which we report the median and central 95% interval for the regime-level interaction estimate.

3H Continuous interaction surfaces and geometric exploration

Density-based restriction of the surface domain. For some drugs, the empirical data density decreases at high concentrations as trajectories drop below the detection limit and are excluded. We therefore restrict the chequerboard to the high-density subdomain. For each drug pair, we compute, for every grid cell (i,j) , the valid fraction $f(i,j) = n_{\text{valid}}(i,j)/4$ (with n_{valid} counting non-NaN values of ψ). We then fit a monotone-decreasing helper surface $f_{\text{dens}}(i,j)$ by alternating one-dimensional isotonic regression sweeps (`sklearn.isotonic.IsotonicRegression`) along both index axes, followed by bivariate spline interpolation on the (i,j) grid (`scipy.interpolate.RectBivariateSpline`). Cells with $f_{\text{dens}}(i,j) < f_{\text{min}}$ are excluded, and all subsequent bootstrap resampling and surface-spline fitting are performed only on the remaining cells.

Bootstrap datasets and coordinate transform. For each pair of drugs (A,B) we first collect all wells belonging to that pair together with their estimated effective net growth rates ψ . Each well is indexed by a normalised concentration pair (z_A, z_B) . To stabilise interpolation near zero, we transform the axes to $Z_A = \log_2(z_A + 1)$ and $Z_B = \log_2(z_B + 1)$. For each bootstrap draw b we generate a resampled surface dataset by sampling, with replacement, one well for every occupied grid point, and retaining its triplet (Z_A, Z_B, ψ) .

Monotone surface construction. For each bootstrap dataset we enforce a monotone-decreasing ψ -surface by alternating one-dimensional isotonic regressions along rows and columns. This is important to ensure that the backtransformation $z_\phi(\psi)$ is unique. Specifically, for each column and then for each row we apply an isotonic regression with a nonincreasing constraint using `sklearn.isotonic.IsotonicRegression` [2]. On each monotone grid we fit a bivariate spline $s_{AB}^{(b)}(Z_A, Z_B)$ using `scipy.interpolate.RectBivariateSpline` with a linear basis functions in both directions. For each drug combination we calculate a consensus surface ψ^{cons} based on the median of the single splines.

Bliss and Loewe interaction indices on the surface. Given the consensus surface $\psi^{\text{cons}}(z_A, z_B)$, we evaluate Bliss and Loewe interaction measures on the same effect scales defined above. In particular, we use the surface-derived treatment effects $\tau(\cdot)$ (see Equation S3.7) to compute the

Bliss expectation

$$\tau_{\text{Bliss}}(z_A, z_B) = \tau(z_A) + \tau(z_B),$$

where $\tau(z_A)$ and $\tau(z_B)$ are obtained from the consensus edge profiles $\psi^{\text{cons}}(z_A, 0)$ and $\psi^{\text{cons}}(0, z_B)$, with $\psi^{\text{cons}}(\emptyset) = \psi^{\text{cons}}(0, 0)$. We then define a normalized Bliss interaction index on the surface by

$$\mu_{\text{surf}}(z_A, z_B) = \frac{\tau_{\text{Bliss}}(z_A, z_B) - \tau(z_A, z_B)}{\tau_{\text{Bliss}}(z_A, z_B)},$$

using the same sign convention as Equation S3.16.

For Loewe additivity, we evaluate $v(z_A, z_B)$ in the same way as Equation S3.18, using the consensus monotherapy edges to obtain the monotherapy-equivalent doses z_A^* and z_B^* at the observed effect $\psi^{\text{cons}}(z_A, z_B)$:

$$v(z_A, z_B) = \frac{z_A}{z_A^*} + \frac{z_B}{z_B^*} - 1, \quad \psi^{\text{cons}}(z_A^*, 0) = \psi^{\text{cons}}(0, z_B^*) = \psi^{\text{cons}}(z_A, z_B).$$

3I Single-drug inoculum effect analysis

During data analysis, we noted that inocula varied more than expected, which we traced to variability induced by the pintoil. Across all wells, the pre-treatment luminescence standard deviation was ($\sigma(I_0) = 6.89 \times 10^4$ RLU), between experiments ($\sigma_{\text{between}} = 4.33 \times 10^4$ RLU) and within experiments ($\sigma_{\text{within}} = 5.47 \times 10^4$ RLU). To investigate whether the observed net growth rates ψ systematically depended on inoculum size, we used the pre-treatment luminescence signal (I_0) as a proxy for initial cell density.

For all monotherapy drug concentration conditions with at least $n \geq 5$ replicates, we fitted an ordinary least-squares regression of ψ on I_0 to obtain a concentration-specific slope β_{inoculum} together with a confidence interval, the value p and the determination coefficient R^2 . These slopes and their uncertainty summaries were visualised as concentration–slope profiles with 95% intervals in Fig. S3.12. Across AMO, CHL, PEN, and TET, the estimated slopes were negligible across concentrations, whereas COL and POL showed the strongest dependence on I_0 at intermediate concentrations (around $1 \frac{\text{pg}}{\text{mL}}$).

To assess whether inoculum size varies systematically along the one-dimensional concentration index, we pooled all single-drug wells and mapped each well to its concentration index. We then performed a permutation test on the ordinary least-squares slope of $\log_{10} I_0$ versus index by permuting $\log_{10} I_0$ across indices to obtain a null distribution. We found no evidence for a systematic

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trend of inoculum size with index in our data ($p = 0.092$), suggesting that inoculum variation contributes noise but does not introduce a strong directional bias along the concentration series. Consistent with this, the inoculum effect primarily adds scatter at intermediate concentrations of COL and POL, contributing to the increased spread in the corresponding single-drug PD curves.

3J SI tables

Table S3.1 Fitted single-drug pharmacodynamic (PD) curve parameters for Equation S3.9. For each antibiotic, we report the point estimates of ψ_0 , ψ_{\min} , κ , and z_{MIC} , together with the corresponding lower (L) and upper (U) uncertainty bounds (95% intervals).

| drug | ψ_0 | ψ_0^{L} | ψ_0^{U} | ψ_{\min} | ψ_{\min}^{L} | ψ_{\min}^{U} | κ | κ^{L} | κ^{U} | z_{MIC} | $z_{\text{MIC}}^{\text{L}}$ | $z_{\text{MIC}}^{\text{U}}$ |
|-----------------|----------|---------------------|---------------------|---------------|--------------------------|--------------------------|----------|---------------------|---------------------|------------------|-----------------------------|-----------------------------|
| polymyxinB | 1.70 | 1.47 | 1.94 | -4.43 | -5.37 | -3.48 | 1.48 | 1.01 | 1.95 | 0.51 | 0.42 | 0.59 |
| tetracycline | 1.77 | 1.75 | 1.79 | -0.24 | -0.28 | -0.20 | 1.15 | 1.07 | 1.22 | 15.15 | 13.80 | 16.49 |
| amoxicillin | 1.73 | 1.70 | 1.77 | -3.93 | -4.02 | -3.84 | 1.69 | 1.60 | 1.77 | 8.42 | 8.18 | 8.66 |
| colistin | 1.78 | 1.60 | 1.96 | -6.08 | -6.97 | -5.18 | 1.48 | 1.18 | 1.78 | 0.58 | 0.52 | 0.65 |
| chloramphenicol | 1.78 | 1.75 | 1.81 | -0.93 | -1.02 | -0.84 | 0.96 | 0.88 | 1.04 | 54.11 | 50.45 | 57.77 |
| penicillin | 1.78 | 1.73 | 1.83 | -4.54 | -4.84 | -4.24 | 1.07 | 0.99 | 1.16 | 153.86 | 146.77 | 160.94 |

Table S3.2 This table summarises interaction classifications (N: neutral, S: synergistic, A: antagonistic) for each drug combination based on Loewe additivity (μ) and Bliss independence (ν), evaluated separately for sub-inhibitory and inhibitory concentration regimes based on bootstrap confidence intervals.

| combination | loewe | | bliss | |
|------------------------------|----------------|------------|----------------|------------|
| | sub-inhibitory | inhibitory | sub-inhibitory | inhibitory |
| amoxicillin_chloramphenicol | A | N | N | A |
| amoxicillin_colistin | A | A | N | A |
| amoxicillin_penicillin | N | N | S | A |
| amoxicillin_polymyxinB | A | A | A | A |
| amoxicillin_tetracycline | A | N | A | A |
| chloramphenicol_colistin | N | S | N | S |
| chloramphenicol_penicillin | A | N | N | A |
| chloramphenicol_polymyxinB | S | S | S | S |
| chloramphenicol_tetracycline | S | S | N | A |
| colistin_penicillin | A | A | A | A |
| colistin_polymyxinB | A | N | N | N |
| colistin_tetracycline | A | S | N | S |
| penicillin_polymyxinB | A | N | N | N |
| penicillin_tetracycline | A | N | N | A |
| polymyxinB_tetracycline | A | S | N | S |

3K SI figures

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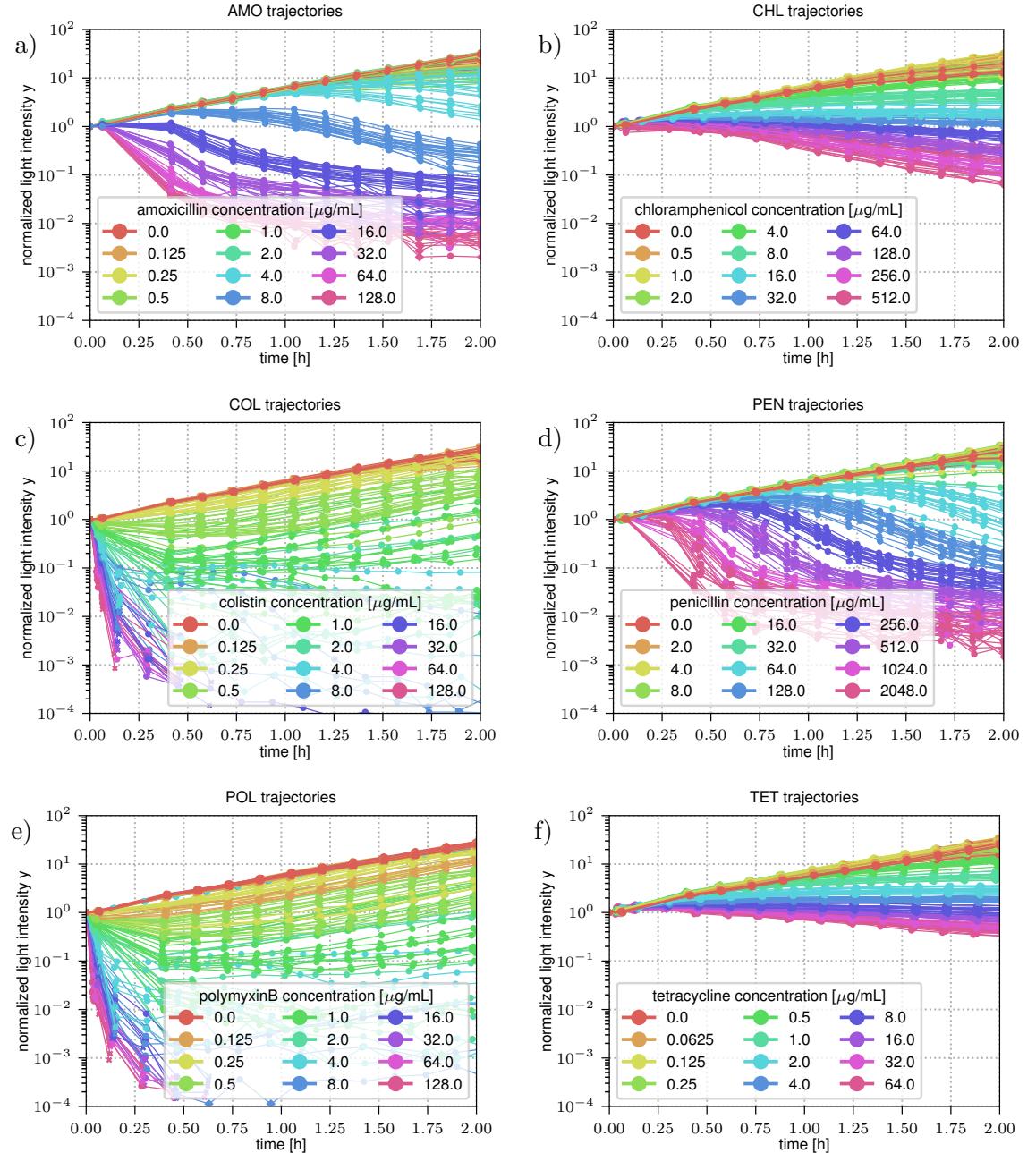


Fig. S3.1 Single-drug timecourse data for the six antibiotics used in this study. Panels (a–f) show the single-drug trajectories across 12 concentrations for amoxicillin, chloramphenicol, colistin, penicillin, polymyxin B, and tetracycline, respectively. For each concentration we aggregate 20 replicate curves, obtained from five independent combination experiments with four technical replicates each. Lines represent the concentration-wise median over replicates at each measurement cycle, and shaded bands indicate the central 50% percentile interval (25th–75th percentiles). Time on the horizontal axis corresponds to the per-cycle mean acquisition time across wells to account for 200 ms per-well readout staggering.

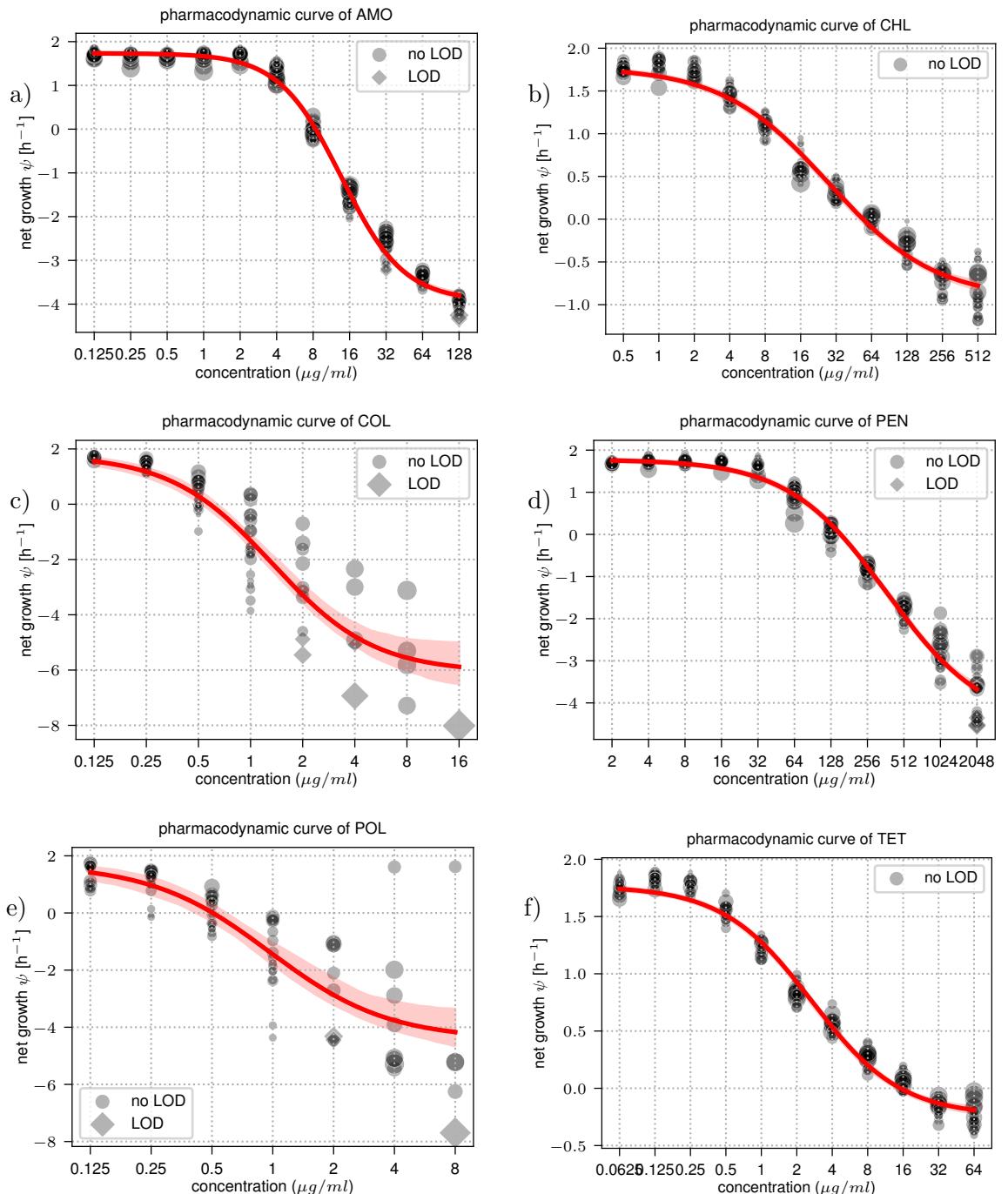
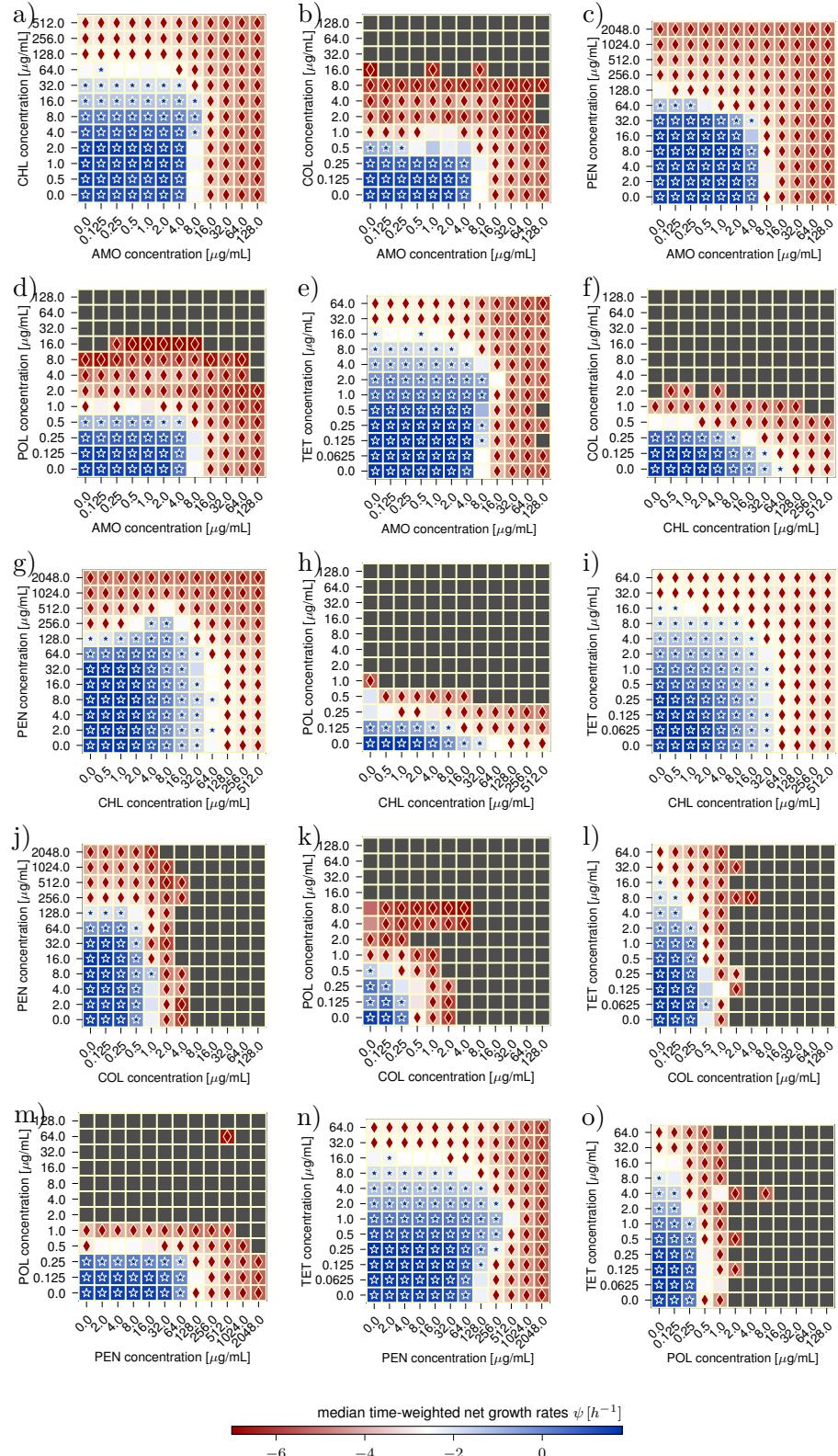


Fig. S3.2 Single-drug pharmacodynamic (PD) curves corresponding to the timecourse data in Fig. S3.1. Panels (a–f) show the net rate of population change as a function of drug concentration for amoxicillin, chloramphenicol, colistin, penicillin, polymyxin B, and tetracycline, respectively. Curves are fitted to the same replicate-aggregated datasets as in Fig. S3.1, and intervals indicate uncertainty in the fitted PD relationship.

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Fig. S3.3 Distribution of the effective net growth rate ψ across all drug combinations measured in checkerboard assays. Each panel corresponds to one drug pair, and each cell represents a single concentration condition. Colors indicate the median estimate of ψ across bootstrap resamples. Symbols indicate whether ψ differs significantly from zero: stars denote significantly positive growth, diamonds significantly negative growth. These effective growth rates are inferred from time-resolved luminescence trajectories as described in the Methods..

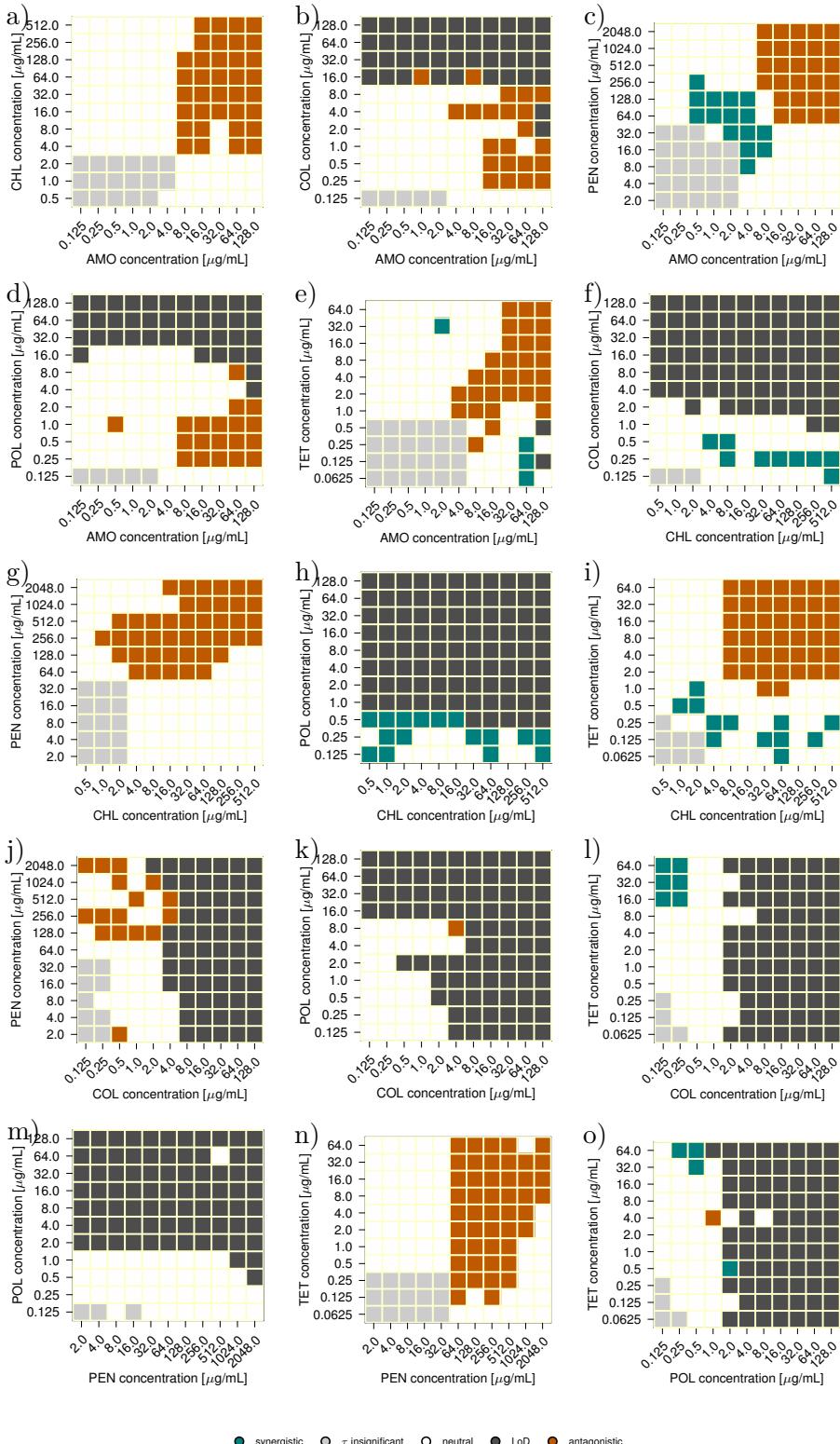
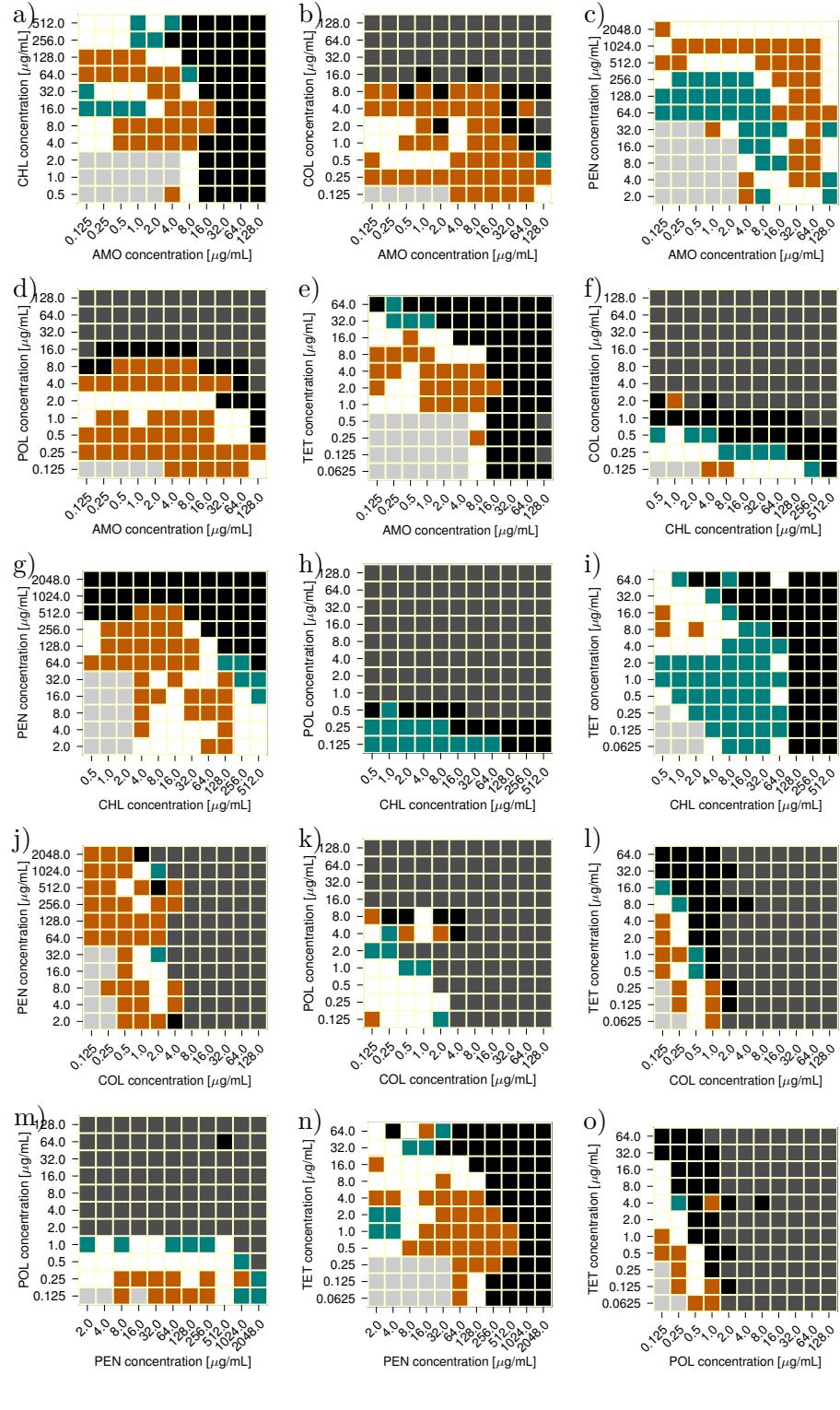


Fig. S3.4 Bliss interaction classification based on bootstrap inference of treatment effects. For each concentration pair, treatment effects were resampled to obtain a distribution of the Bliss interaction index μ . Cells are colored according to statistical significance: teal indicates synergistic interactions (95% confidence interval entirely below zero), orange indicates antagonistic interactions (95% confidence interval entirely above zero), and white indicates no significant deviation from additivity. All estimates are based on effective growth rates ψ inferred from checkerboard experiments.¹⁰¹

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10 Fig. S3.5 Loewe interaction classification based on bootstrap inference of treatment effects. For each concentration pair, bootstrap samples were used to compute a distribution of the fractional inhibitory concentration index (FICI). Teal indicates significant synergy (95% confidence interval entirely below 1), orange indicates significant antagonism (95% confidence interval entirely above 1), and white indicates no significant deviation from Loewe additivity. All estimates are derived from effective growth rates ψ obtained from checkerboard assays..

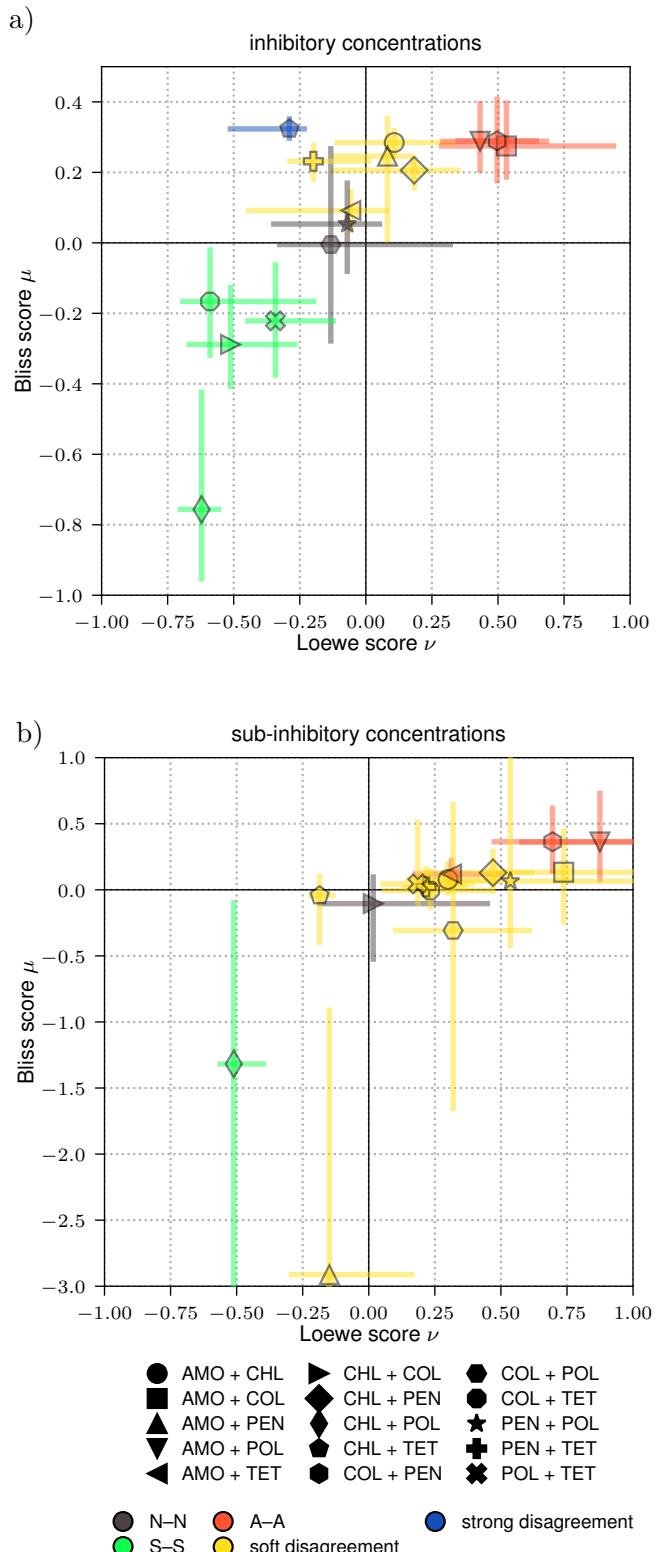


Fig. S3.6 Comparison of Loewe and Bliss interaction scores in two growth regimes. (a) Inhibitory (super-MIC) concentrations. (b) Sub-inhibitory (sub-MIC) concentrations. Each point represents 10³ drug combination with error bars indicating bootstrap confidence intervals. Colors encode agreement or disagreement between Loewe and Bliss classifications.

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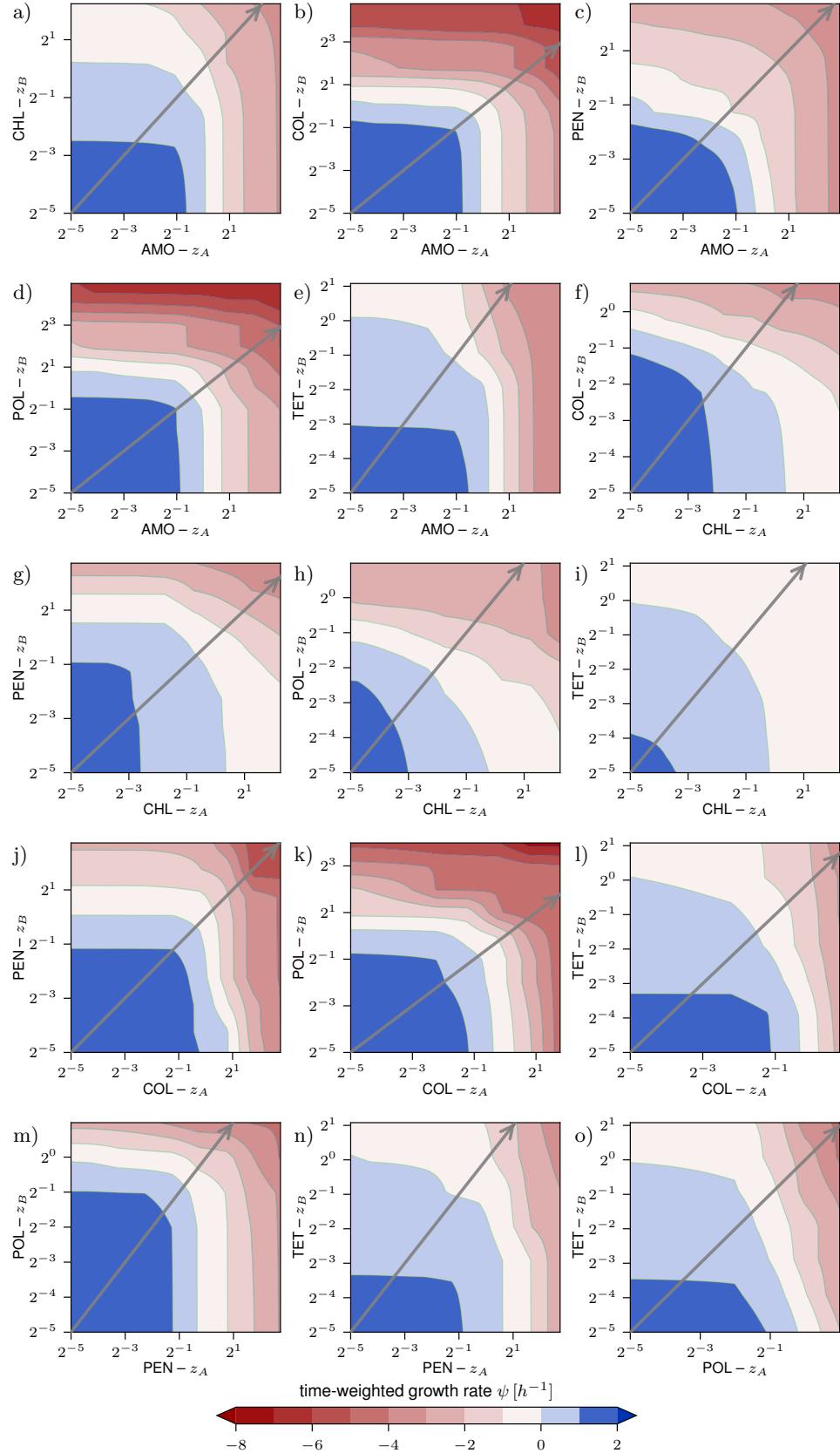


Fig. S3.7 Effective net growth rate surfaces ψ for all drug combinations. Each panel shows the median effective growth rate estimated from time-resolved luminescence trajectories across the concentration grid. Colors indicate the magnitude of ψ , with negative values corresponding to net killing and positive values to net growth. All panels share a common color scale to allow direct comparison across drug pairs.

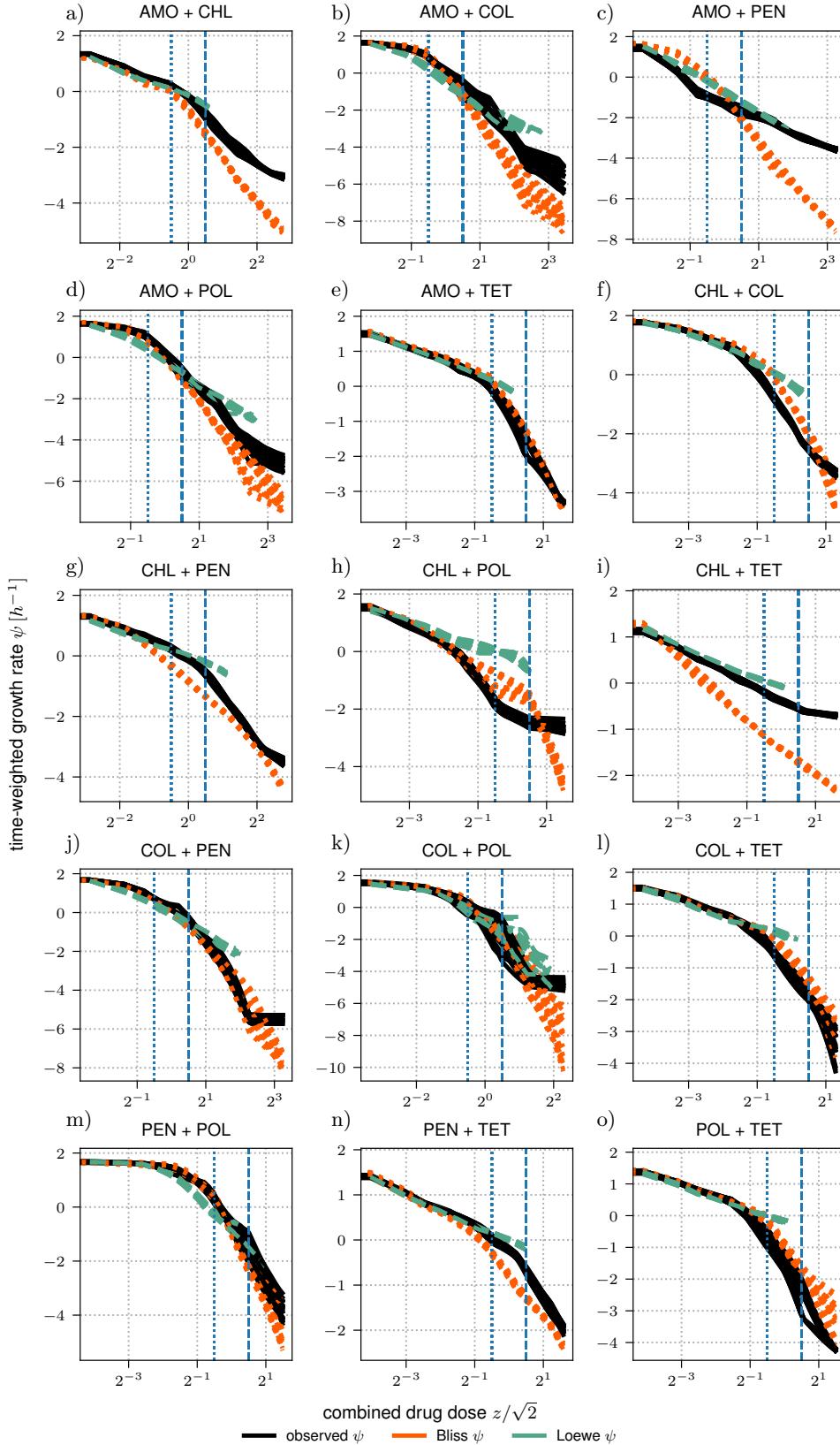
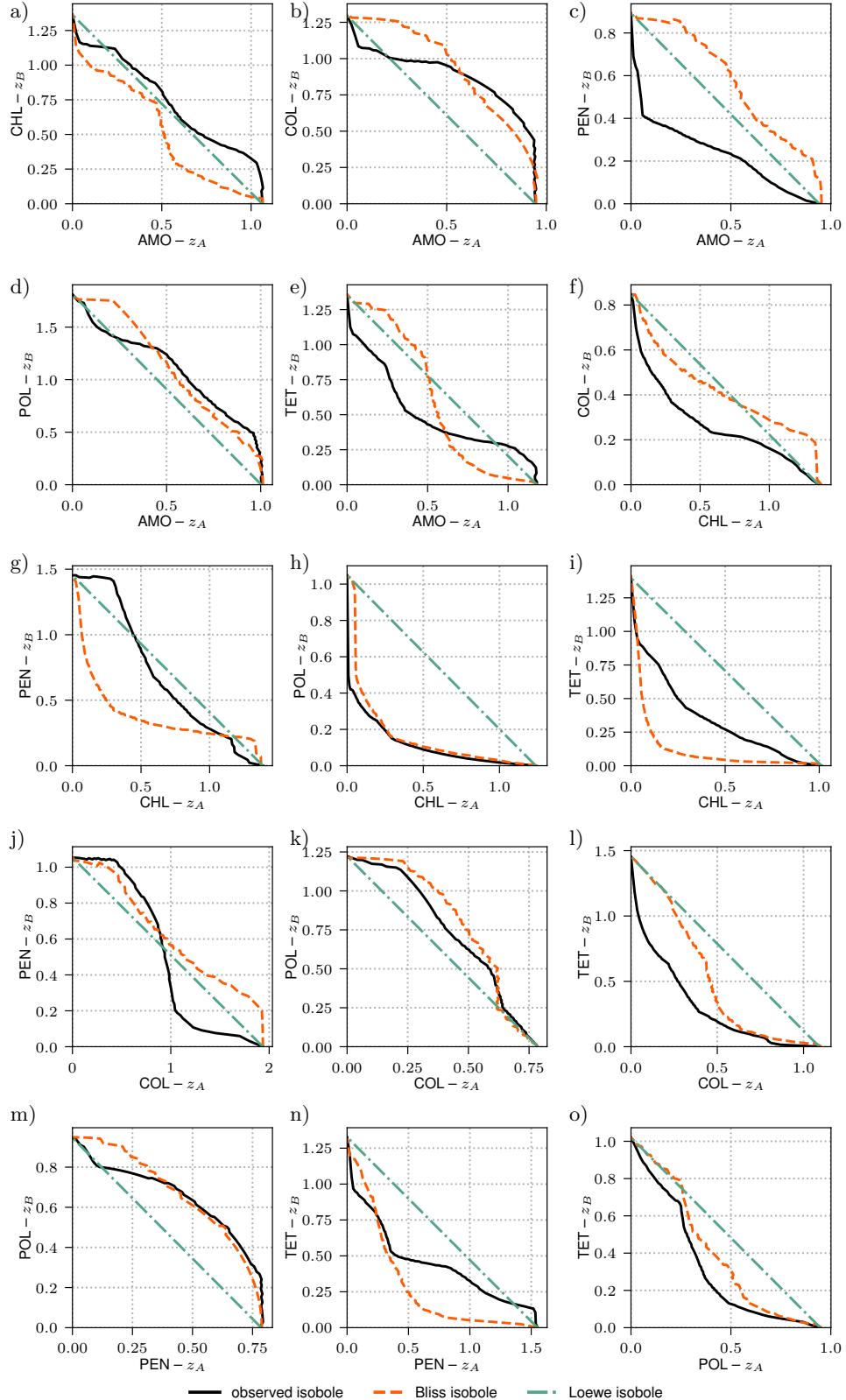


Fig. S3.8 Polar pharmacodynamic curves for all drug combinations. For each drug pair, we show one-dimensional cuts through the ψ -surface splines at $\phi = 45^\circ$ (equal mixing in units of zMIC), plotting ψ as a function of the combined dose z . The blue dotted line marks $z = 1/\sqrt{2}$, corresponding to both single-drug doses being at 0.5 zMIC, and the blue dashed line marks $z = \sqrt{2}$, corresponding to both being at 1 zMIC. Solid curves show the median surface-derived ψ , with corresponding Bliss- and Loewe-based predictions evaluated along the same trajectory.

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Fig. S3.9 Isoboles of constant effective growth rate ψ derived from the interpolated response surfaces. Each panel shows curves of equal ψ in concentration space, illustrating deviations from linear additivity. Bending toward the origin indicates synergistic interactions, whereas outward bending indicates antagonism.

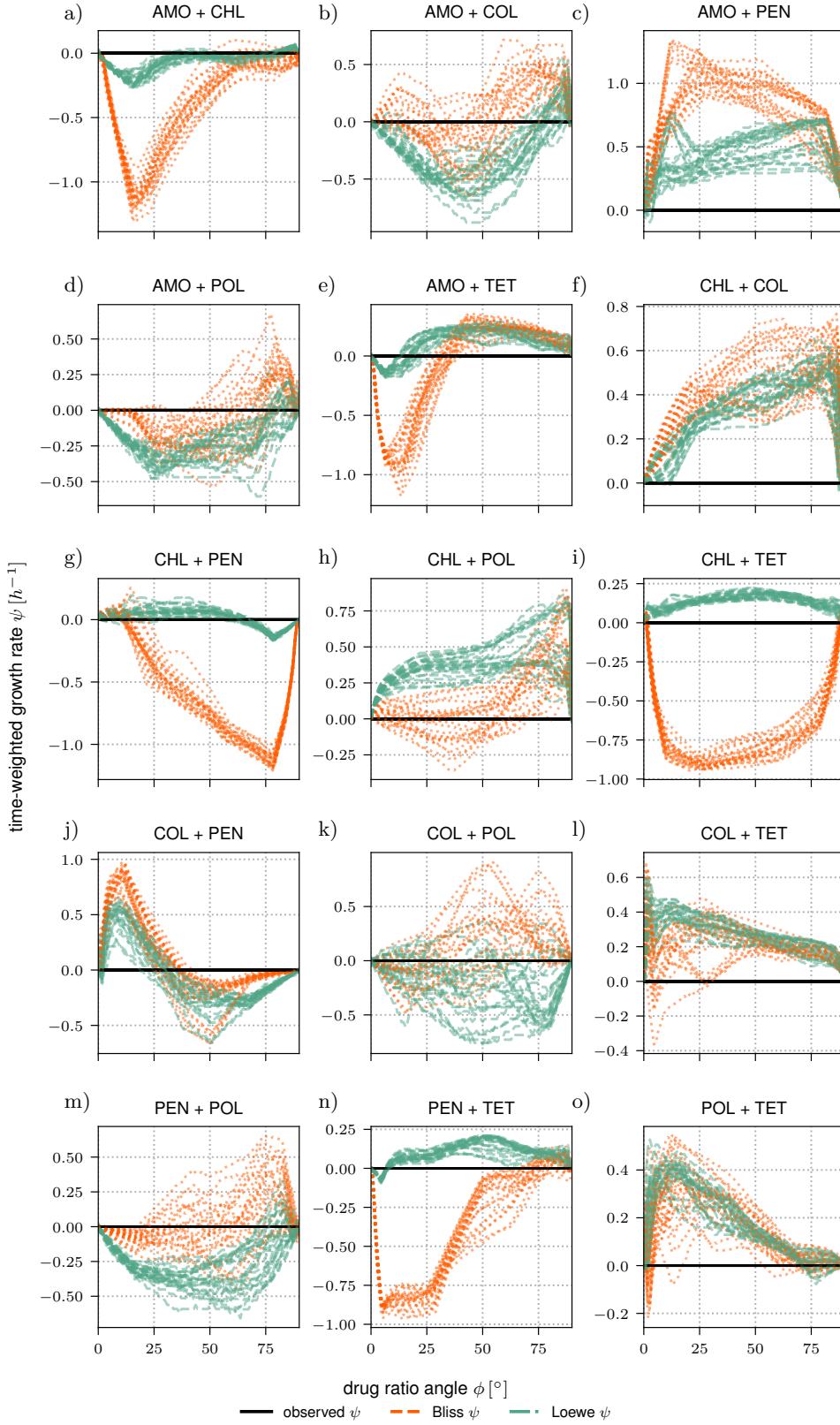


Fig. S3.10 Angular interaction profiles for all drug combinations. For each drug pair, we extract isoboles (paths of constant ψ) from the consensus ψ -surface and evaluate Bliss- and Loewe-based predictions for ψ along these paths (z, ϕ), plotted as a function of the mixing angle ϕ . Each combination is shown for one sub-inhibitory isobole, the MIC isobole, and one inhibitory isobole.

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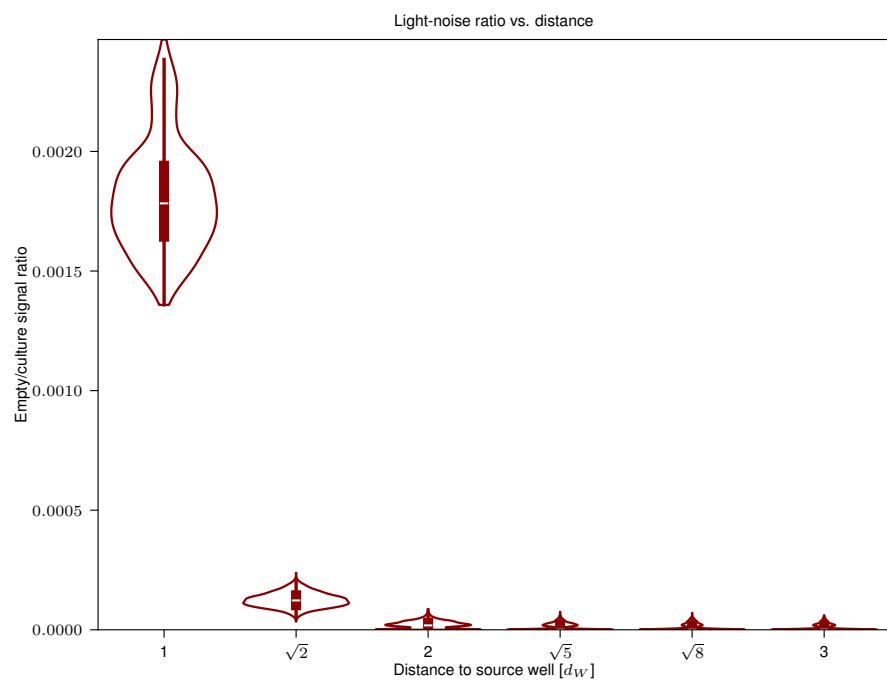


Fig. S3.11 Light noise distribution (shows the fraction of the original light intensity arriving in neighbouring wells; dependent on the distance to the source well; in well lengths d_w .

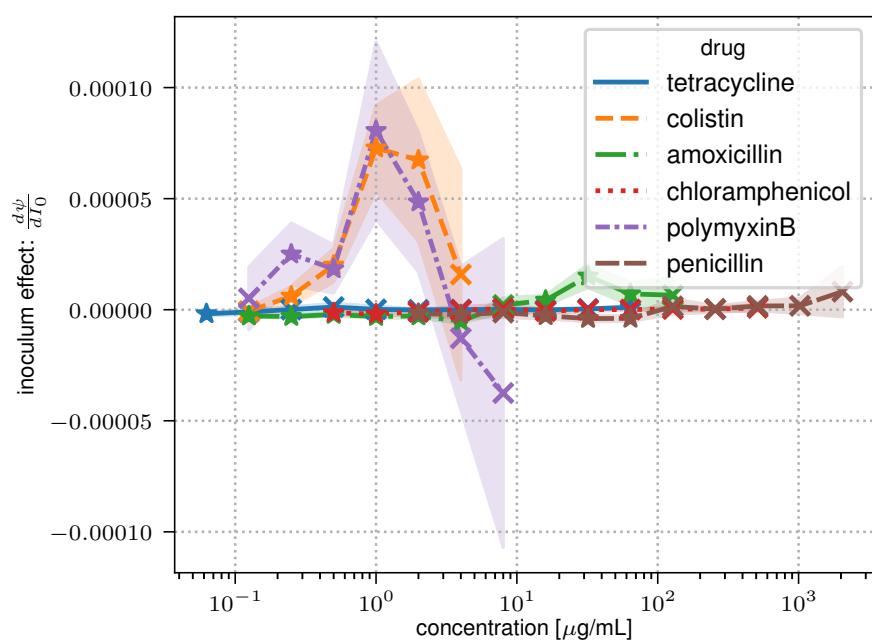


Fig. S3.12 Slope of the inoculum effect ($d\psi/dI_0$) as a function of drug concentration. For each drug and concentration, slopes ($d\psi/dI_0$) are obtained by regressing the observed net growth rates across inocula. Crosses denote concentrations with non-significant inoculum effects, whereas stars indicate statistically significant effects.

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Concluding Remarks

Gjini2021: - Drug interactions can shape resistance evolution. - Difficult to draw general conclusion (e.g. synergistic combinations always accelerate resistance”)

- Drug interactions are very confusing. - we dont know which interaction we want. we dont know how to define them. and they change over dosing and ratios. it seems very pointless to proceed from here. What we need instead are within and between host pkpd models that we can parameterize with exp data of the kind i generated. based on these we can set goals; like minimizing prob that resistance emerges, or maximizing the number of uninfected patients.

...

It is clear that antimicrobial resistance (AMR) will remain a significant health crisis for the foreseeable future, underscoring the importance of understanding the evolutionary forces shaping bacterial population dynamics under antibiotic pressure. This thesis addressed several interlinked questions centered around how antimicrobial treatment affects microbial populations at both within- and between-host scales. Our attempt to answer these questions inevitably led to new insights and further inquiries: How should antibiotics be deployed when the resistance phenotype of the infecting pathogen is unknown? Is combination therapy consistently superior to alternative strategies in suppressing resistance? How does horizontal gene transfer, especially mediated by plasmids, alter the evolutionary landscape compared to purely chromosomal resistance? What are the pharmacodynamic implications of combining antibiotics? And finally, how can we reliably quantify bacterial population dynamics under inhibitory conditions to adequately explore these drug interactions?

In the following sections, we revisit these core questions and summarize the key insights gained throughout this thesis.

4.1 Is combination therapy the best treatment strategy?

Determining whether combination therapy is the optimal regimen is not straightforward, because the very definition of therapeutic “success” remains contested and aspects such as tolerability are rarely built into current models and population level success is not always the best outcome for each individual. In Chapter 2 we therefore focused on three population-level outcomes that also benefit individual patients, well knowing that these do not cover the full complexity: (i) prevention of double resistance, (ii) suppression of single resistance, and (iii) reduction of the overall infection burden.

Using an in-vitro hospital-ward model we tested these endpoints under three scenarios. In the prevention setting (no pre-existing double resistance) and the containment setting (double resistance already present), both parametrised with realistic basic reproduction numbers (\mathcal{R}_0), combination consistently achieved the lowest levels of single and double resistance; mixing and cycling performed nearly as well, whereas monotherapies allowed single resistance to accumulate. A sensitivity analysis based on a stochastic model calibrated to the experiments confirmed the dominance of combination across a wide parameter range.

Because double resistance appeared only rarely in those realistic scenarios, we created an intentionally extreme emergence scenario in which every incoming patient carried a single-resistant strain. Even under this strong selection pressure, combination therapy generated the fewest new double-resistant infections, while the untreated control produced the most.

At first sight these findings contradict two common reservations: (i) that combination inevitably selects pre-existing double resistance, and (ii) that using more drugs must create more resistance. In our containment scenario small minorities of double-resistant bacteria indeed persisted for long periods—even without treatment—so their eradication seems unlikely under any regimen. Crucially, however, the practical challenge is to prevent new double-resistant strains, and here combination outperformed all alternatives. Likewise, the correlation between total antibiotic use and resistance [Gregory2018] does not hold when both drugs are applied simultaneously to block plasmid transfer: in our experiments ‘‘more drugs’’ meant less resistance.

The mechanistic explanation hinges on the contrast between purely vertical (chromosomal) and horizontally transferable (plasmid) resistance. For chromosomal genes the probability of double resistance scales with mutation rate and the number of bacteria carrying single resistances.

For plasmids it scales with the probability that A- and B-resistant bacteria meet and conjugate. Combination therapy tackles both facets: it reduces the prevalence of single-resistant infections across the ward and suppresses plasmid exchange within super-infected hosts, whereas withholding treatment maximises both processes.

Admittedly, it is not surprising that our in-vitro results align with theoretical predictions: both approaches share the same core assumptions and omit major real-world complexities such as host heterogeneity, variable pharmacokinetics, microbiome interactions, and treatment tolerability. Nevertheless, the experimental-epidemiology framework offers a compelling middle ground. By using real bacterial strains and clinically relevant antibiotics, it adds biological realism that pure modelling lacks, yet it still controls key confounders and remains far more affordable and ethically straightforward than large-scale clinical trials.

What I take away from this work is that the observation that more antibiotic use leads to more resistance should not discourage appropriate treatment. While this may hold at large scales, it does not necessarily apply in individual settings like hospitals. There, it is more important to treat effectively—especially to eliminate resistant bacteria. Combination therapy does this most reliably. Used appropriately, antibiotics can sometimes reduce resistance rather than promote it.

4.2 Can luminescence serve as a proxy for net growth?

To analyse drug interactions on a large scale, we adopted the bioluminescence assay of [1] and tested its suitability at inhibitory concentrations in Chapter 3.

To understand when luminescence can approximate net growth, we formalized the dynamics of light emission in simplified equations. In this framework, the change in light intensity approximates: (i) the change in cell number, if the per-cell light emission (cell-specific luminosity) remains constant, and (ii) the change in biomass, if the cell-specific luminosity scales with cell mass. In our experiments, we observed that the change in light intensity consistently matched the estimated change in biomass more closely than the estimated change in cell number, making (ii) the better assumption. This means we can typically use the change in luminescence as a proxy for biomass change, but only approximates cell number when changes in cell size are slow compared to changes in population size.

Conducting this study also highlighted the importance of knowing what each assay actually mea-

sures. CFU estimates the fraction of culturable cells and is sensitive to loss of culturability or antibiotic carry-over; OD combines signals from both live and dead biomass; and luminescence assays respond to changes in cell-specific luminosity. These distinctions are crucial when interpreting decline rates based on these read-outs, as each method implies a different operational definition of “death.”

These insights provided the basis for Chapter 4, where luminescence enabled us to quantify the population dynamics of antibiotic combinations across sub- and super-MIC ranges.

4.3 Can interaction data from sub-MIC concentrations be extrapolated to the killing range?

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4.4 General concluding note

This thesis explores the selection pressures shaping antimicrobial resistance evolution within hosts and across host populations.

Throughout, I have tried to be explicit about the limitations of the models and assays employed. Rather than dwelling on individual data points, I have focused on the patterns that persist across systems. Some of the most valuable lessons arose from serendipitous observations masquerading as obstacles; learning to pause and analyse such “nuisances” proved more fruitful than pushing past them.

Methodologically, I hope the insights gained with bioluminescence assays will assist others who wish to quantify population dynamics at inhibitory drug levels. At the same time, the pitfalls encountered with both CFU counting and bioluminescence should serve as a cautionary tale.

We found supporting evidence that, in concordance with results for chromosomal resistance, the rise of plasmid-mediated resistance can best be faced using combination therapy. Any strategy, however, must ultimately be validated in clinical settings, where host heterogeneity, patient-specific pharmacokinetics, and pathogen evolution can substantially alter outcomes.

While it is still not clear whether combination therapy is categorically superior to alternative strategies, our work on the pharmacodynamics of drug combinations demonstrates that specific drug interactions can influence population dynamics, and that interactions at inhibitory

concentrations are not always identical to those at sub-inhibitory levels. Considering this in future studies—by explicitly distinguishing synergistic from antagonistic combinations—could help clarify the inconclusive outcomes of current study designs.

However, the “perfect” treatment strategy alone will not curb AMR. While this thesis focused on uninformed (empirical) therapy, the informed treatment guided by rapid diagnostics and real-time genomic surveillance is preferable and As point-of-care sequencing and resistance-gene panels mature, these systems will become more available in the future. At the population level, relatively simple and scalable public-health interventions have already demonstrated their impact. England’s national infection-control programme—including mandatory MRSA reporting, patient screening, isolation protocols, and care bundles—was associated with a 97% reduction in ICU-acquired MRSA bloodstream infections between 2007 and 2012 [2]. Israel’s centrally coordinated outbreak response, led by the Ministry of Health and involving mandatory case reporting, patient cohorting, and active oversight, achieved an approximately 80% decline in the incidence of carbapenem-resistant *K. pneumoniae* within two years [3]. Conjugate vaccine roll-outs offer a complementary success story: following the introduction of PCV7 in the United States, penicillin-nonsusceptible invasive pneumococcal disease declined by over 80% in children under two, and by nearly 50% in adults aged 65 [4].

Together, these examples demonstrate that coordinated, evidence-led action—often straightforward to implement—can profoundly reshape the trajectory of antimicrobial resistance evolution.

4. CONCLUDING REMARKS

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