# Model-informed design of antibiotic therapy against antimicrobial resistance

Tandar, S.T.

# Chapter 4

# Interspecies interactions alter the antibiotic sensitivity of *Pseudomonas aeruginosa*



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# Abstract

Polymicrobial infections are infections that are caused by multiple pathogens and are common in patients with cystic fibrosis (CF). Although polymicrobial infections are associated with poor treatment responses in CF, the effects of the ecological interactions between co-infecting pathogens on antibiotic sensitivity and treatment outcome are poorly characterized. To this end, we systematically quantified the impact of these effects on the antibiotic sensitivity of *Pseudomonas aeruginosa* for nine antibiotics in medium conditioned by thirteen secondary cystic fibrosis-associated bacterial and fungal pathogens through time-kill assays. We fitted pharmacodynamics models to these kill curves for each antibiotic-species combination and found that interspecies interactions changing the antibiotic sensitivity of *P. aerugonosa* are abundant. Interactions that lower antibiotic sensitivity are more common than those that increase it, with generally more substantial reductions than increases in sensitivity. For a selection of co-infecting species, we performed pharmacokinetics-pharmacodynamics modelling of *P. aerugonosa* treatment. We predicted that interspecies interactions can either improve or reduce treatment response to the extent that treatment is rendered ineffective from a previously effective antibiotic dosing schedule and vice versa. In summary, we show that quantifying the ecological interaction effects as pharmacodynamic parameters is necessary to determine the abundance and the extent to which these interactions affect antibiotic sensitivity in polymicrobial infections.





# Importance

In CF patients, chronic respiratory tract infections are often polymicrobial, involving multiple pathogens simultaneously. Polymicrobial infections are difficult to treat as they often respond unexpectedly to antibiotic treatment, which might possibly be explained because co-infecting pathogens can influence each other's antibiotic sensitivity, but it is unknown to what extent such effects occur. To investigate this, we systematically quantified the impact of co-infecting species on antibiotic sensitivity, focusing on *P. aeruginosa*, a common CF pathogen. We studied for a large set co-infecting species and antibiotics whether changes in antibiotic response occur. Based on these experiments, we used mathematical modeling to simulate the response of *P. aeruginosa* to colistin and tobramycin treatment in the presence of other co-infecting bacteria. This study offers comprehensive data on altered antibiotic sensitivity of *P. aeruginosa* in polymicrobial infections, serves as a foundation for optimizing treatment of such infections, and consolidates the importance of considering co-infecting pathogens.

# Introduction

Patients with cystic fibrosis (CF) suffer from chronic lung infections[1]. polymicrobial infections (PMIs), *i.e.*, infections involving multiple microbial species simultaneously, are common in patients with CF[2–5] (**Fig. 1A**). *Pseudomonas aeruginosa* is the most common pathogen in CF-PMIs in adults, but a variety of other microbial species have been found to co-infect[6,7], potentially complicating CF treatment. For some of these co-infecting species, such as *Staphylococcus aureus*, *Haemophilus influenzae* and *Burkholderia cepacia*, the impact on the course of the infection has been well-established[8,9]. For other co-infecting species, such as *Achromobacter xylosoxidans*, *Streptococcus pneumoniae* and *Ralstonia mannitolilytica*, it remains unclear to what extent these organisms contribute to disease progression in chronic CF-associated respiratory tract infections[10–15].



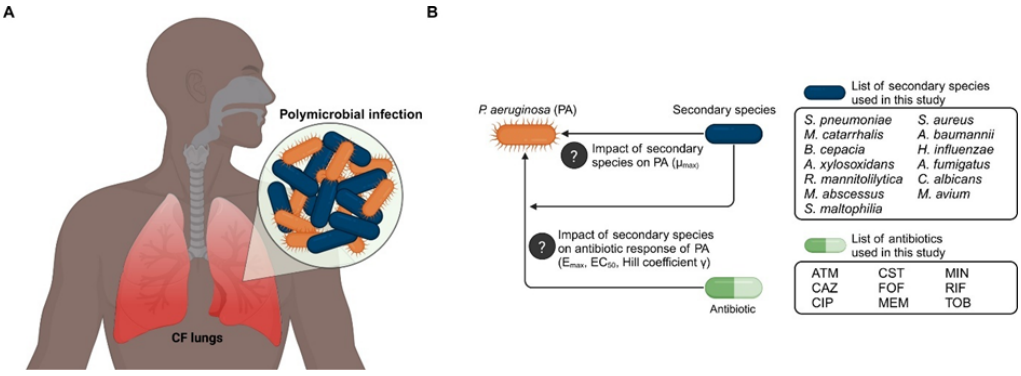


**Fig. 1 | Polymicrobial infections in CF patients.** **A.** Polymicrobial infections in CF patients are common. **V.** A schematic representation of the routes of so far unexplored impact of secondary species in CF-PMI and an overview of species and antibiotics used in this study. ATM=aztreonam, CAZ=ceftazidime, CIP=ciprofloxacin, CST=colistin, FOF=fosfomycin, MEM=meropenem, MIN=minocycline, RIF=rifampicin, TOB=tobramycin.

Antibiotic treatment of CF-PMIs is notoriously difficult. CF-PMIs are rarely fully cleared, requiring long-term antibiotic treatment to suppress exacerbations[16–18]. PMIs can respond unexpectedly to antibiotic treatments. Treatment response may differ from what is expected from initial antibiotic sensitivity tests of single species of the CF-PMIs[19,20]. It is unclear if the presence of multiple pathogenic species in CF-PMIs alter antibiotic efficacy as compared to mono-microbial infections, and to what extent such effects should be considered in treatment guidelines. So far, limited *in vitro* data has shown that species in PMIs can interact with potential effects on antibiotic sensitivity[21–25].

Due to a lack of systematic data on these interspecies interactions, the general impact of such interactions on antibiotic treatment outcome in CF-PMIs remains unknown. In this context, obtaining specific understanding of the effects of interspecies interactions on antibiotic pharmacodynamics (PD) is essential. Determining changes in minimum inhibitory concentrations (minimum inhibitory concentration (MIC)) is not sufficient to evaluate the potential impact of interspecies interactions on treatment response, as the MIC is a composite metric which combines changes in antibiotic sensitivity and growth rate, at one time point[26]. In contrast, when expressing the impact of interspecies interactions as changes in PD parameters, the effect of interspecies interactions may be specifically attributed to specific PD parameters. These parameters include an-

tibiotic sensitivity ($EC_{50}$), maximum antibiotic effect ($E_{max}$), the sensitivity of pathogen kill rate (Hill exponent), and changes in pathogen fitness in the absence of antibiotic ($\mu_{max}$)[27], which can then be used as part of pharmacokinetics-pharmacodynamics (PK-PD) analyses.

In this study we aimed to systematically determine the impact of a large set of relevant CF-associated pathogens on the fitness and PD of *P. aeruginosa* for a range of antibiotics (**Fig. 1B**). To this end, we cultured *P. aeruginosa* in medium conditioned by each secondary species separately as a proxy for the presence of a co-infecting species (**Fig. 2A**). In this conditioned medium (CM), we performed antibiotic time-kill studies for *P. aeruginosa* for different combinations of antibiotics and secondary species, which enabled assessment of changes in the PD response (**Fig. 2A**). To evaluate the impact of interspecies interactions on antibiotic treatment schedules of *P. aeruginosa*, we performed PK-PD modelling for selected antibiotic-secondary species combinations. Together, these results give guidance on the potential impact of interspecies interactions for antibiotic treatment strategies for CF-PMIs.



# Materials and Methods

***Strains***

The focal pathogen strain used in this study was PAO1-Xen41, a bioluminescent *P. aeruginosa* strain encoding a single chromosomal copy of the *luxCDABE* operon (PerkinElmer). Strains representative of the 13 CF-associated secondary species that were used in the pairwise interaction assays together with *P. aeruginosa* PAO1-Xen41 included *A. xylosoxidans* DSM2402, *Acinetobacter baumannii* DSM30007, *Aspergillus fumigatus* DSM819, *B. cepacia* DSM7288, *Candida albicans* DSM1386, *H. influenzae* DSM44196, *Mycobacterium abscessus* DSM44196, *Mycobacterium avium* ATCC700898, *Moraxella catarrhalis* DSM9143, *R. mannitolilytica* DSM17512, *S. aureus* DSM346, *Stenotrophomonas maltophilia* DSM21257, and *S. pneumoniae* DSM14377 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures).

***Preparation of CM***

The focal pathogen *P. aeruginosa* was cultured in medium conditioned by each secondary species (**Fig. 2A**). The CM was used to approximate the presence of a CF-associated secondary species in order to assess the contact-independent interaction between *P. aeruginosa* and each of the secondary species. Preparation of the CM started by culturing each secondary species on Mueller-Hinton agar (MHA) with species-specific supplements and culture conditions (Table S1). All secondary species were incubated at 37°C for 24 hours, with the exception of *M. abscessus* and *S. pneumoniae*, which were allowed to grow for 48 hours, and *M. avium*, which was allowed to grow for 5 days as they have longer doubling time. After incubation on agar medium, suspensions of 0.5 MacFarland (approximately $1.5 \times 10^8$ colony forming unitscolony forming units (CFU) (CFU)/mL) were made in 0.9% (w/v) saline. This suspension was then diluted down to 3000 CFU/mL. Multiple tubes containing 40 mL of cation-adjusted Mueller-Hinton broth (CAMHB) with species-specific supplements were inoculated with 1 mL of the 3000 CFU/mL cell suspension. All species were cultured at 37°C with agitation at 150 rotations per minute (rpm) for 48 hours, except for *M. abscessus* and *M. avium*, which were cultured for 96 hours. Each culture tube was subsequently centrifuged (4654 g; 15 minutes) and filter-sterilized (0.22 µm) to remove secondary species from the CM. CM

nutrients were replenished by adding sterile 10-times concentration CAMHB containing the respective species-specific medium supplements (10% by volume).

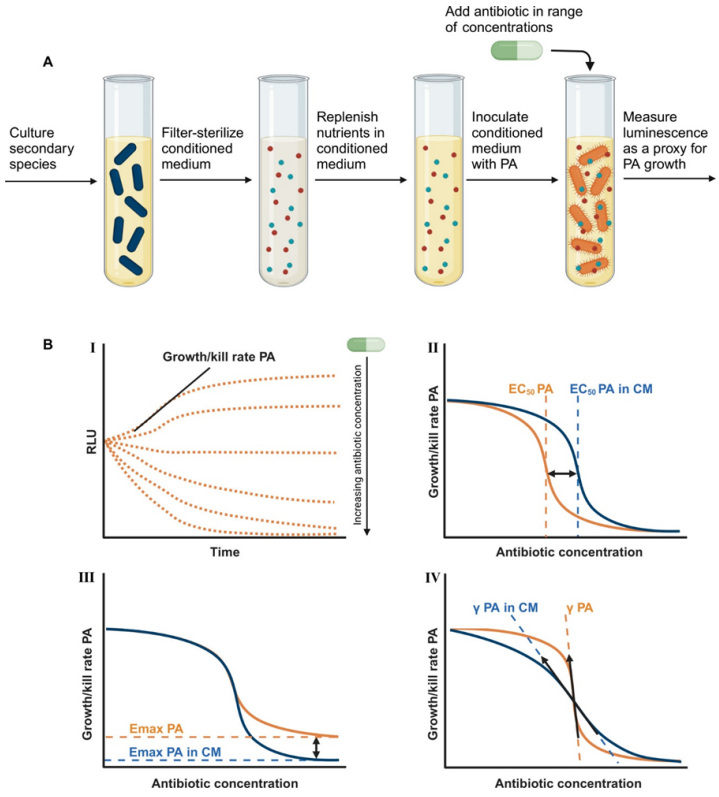


**Fig. 2 | Schematic overview of experimental set-up and PD analysis. A.** Preparation of CM and exposure of *P. aeruginosa* in CM to antibiotics. The secondary species was cultured in cation adjusted Mueller-Hinton (MH) broth allowing for the release of toxins and/or other metabolites inside the medium. Then the CM was filter-sterilized in order to remove the secondary species but not their toxins and/or other metabolites, and then nutrients were replenished. This replenished CM was used to culture *P. aeruginosa*, allowing us to determine the growth dynamics by measuring luminescence over time. **B.** I) Determination of $\mu_{max}$ by log-linear regression on exponential growth phase. II-IV) Visual representation of representative changes in PD parameters $EC_{50}$, $E_{max}$ and $\gamma$ expressed as changes in Hill equation, comparing conditioned and non-conditioned media

### *Antibiotics*

Antibiotic preparation was done in advance of all the experiments according to the manufacturers' recommendation with aztreonam, ceftazidime, ciprofloxacin, colistin, fosfomycin, meropenem, minocycline, rifampicin and tobramycin being dissolved in their respective solvents, aliquoted and stored at the specific storage conditions required. Before each experiment, antibiotic stock solutions were diluted in the CM required to obtain the desired range of concentrations.

***Time-kill experiments***

Time-kill experiments of *P. aeruginosa* (**Fig. 2A**) were performed for all antibiotics in medium conditioned with each secondary species. The time-kills were performed in 96-well plates (250 µL culture volume) with a *P. aeruginosa* starting cell density of approximately $5 \times 10^6$ CFU/mL. Time-kill plates were incubated aerobically at 37°C with agitation at 150 rpm. The number of viable bacterial cells over time was estimated by measuring luminescence signal and relative light units (RLU) hourly for 20 hours (BMG Fluostar Omega; gain 3800; interval 1.36 seconds).

***Pharmacodynamic analysis***

We first computed the maximum growth rate for each specific combination of an antibiotic, secondary species, and drug concentration by log-linear regression on the exponential growth/kill phase. For this purpose, we developed a phase selection script, which automatically determines the exponential growth/kill phase (Fig. S1). In order to quantify the PD parameters that display the antibiotic sensitivity of *P. aeruginosa*, concentration-effect relationships (**Eq. 1**) were fitted for the individually estimated growth rates against the corresponding antibiotic concentrations for each co-infecting species-antibiotic combination:

$$E = E_{\max} \times \frac{C^{\gamma}}{C^{\gamma} + EC_{50}^{\gamma}} \qquad \textbf{Eq. 1}$$

Here, $E$ represents the growth/kill rate of *P. aeruginosa*, $C$ represents the drug concentration, $E_{max}$ the maximum effect, $EC_{50}$ the drug concentration to achieve a half-maximum drug effect and the Hill exponent $\gamma$ represents the steepness of the dose-response curve. The fitting procedure was performed using the 'drc' package[28] in R. The effect of secondary pathogen on PD parameters $E_{max}$, $EC_{50}$, and $\gamma$ (**Fig. 2B**) was quantified as its fold-change (FC) to its respective value estimated in non-CM. We considered $log2(FC) > 0.5$ to indicate an increase in the specific PD parameter and $log2(FC) < -0.5$ to indicate a decrease. The impact of co-infecting pathogens on the fitness of *P. aeruginosa* was determined by dividing the maximum growth rate $\mu_{max}$ in the absence of drugs in CM by the $\mu_{max}$ in the absence of drugs in a non-CM, obtaining the FC and determining whether there was an increase or decrease in fitness in the same way as the PD parameters.

***Mathematical PK-PD modelling***

Mathematical PK-PD model-based analysis was performed to evaluate the impact of selected secondary species on the clinical outcome of *P. aeruginosa* treatment with either colistin or tobramycin. First, published population pharmacokinetics (PK) models for colistin and tobramycin were implemented to describe drug concentration in the lung over the course of antibiotic treatment, using the standard treatment regime from these studies[29,30]. Simulated colistin dosing involved a 160 mg loading dose (inhalation) followed by 160 mg maintenance doses (intravenous; 30 minutes infusion) with an interval of 8 hours. Simulated tobramycin dosing involved a constant intravenous (bolus) administration of 139.65 mg tobramycin with an 8-hours interval. We simulated the treatment for a typical individuals with the PK clearance parameter were increased with one standard deviation of the mean from the patient population for colistin, reflecting the sub-population of patients with increased drug clearance and therefore reduced colistin exposure, for tobramycin the standard clearance parameters were used. A tobramycin

lung-plasma partition coefficient of 0.12 was used to calculate lung concentration based on the plasma concentration (**Eq. 2**)[31]. This conversion was not necessary for colistin, as the model already provided the lung PK directly.

$$C_{\mathrm{TOB,lung}} = 0.12 \times C_{\mathrm{TOB,plasma}} \qquad \textbf{Eq. 2}$$

A population-limited growth model was used to describe the growth behavior of *P. aeruginosa* in different antibiotic conditions (**Eq. 3**).

$$\frac{dN}{dt} = \mu_{\mathrm{observed}} \times \left(1 - \frac{N}{N_{\mathrm{max}}}\right) \times N \qquad \textbf{Eq. 3}$$

Here, $N_{max}$ is the maximum cell density of *P. aeruginosa* in the epithelial lining fluid (ELF) of the lung and was fixed to $9 \times 10^{10}$ CFU/mL based on the observed carrying capacity of our the experimental system. Here, $N$ represents *P. aeruginosa* cell density in CFU/mL at a given time in the simulation and is set at $5 \times 10^{6}$ CFU/mL at time 0 according to the starting cell density of our time-kill experiments. The observed growth rate $\mu_{observed}$ was calculated using **Eq. 4**.

$$\mu_{\mathrm{observed}} = \mu_{\mathrm{max}} - E \qquad \textbf{Eq. 4}$$

Bacterial growth kinetic- and drug effect parameters estimated from the experimental data were used to determine the effect of the selected co-infecting species on the drug response of *P. aeruginosa* expressed as changes in $\mu_{observed}$. To evaluate the impact of secondary species, we selected several species with a variable effect on PD parameters to illustrate their potential impact. All PK-PD parameters, details of antibiotic dosing schedules, and other variable values can be found in Table S2.

# Results

***Secondary species CM altered antibiotics PD against P. aeruginosa***

We plotted the measured RLU over time to obtain growth/kill curves of *P. aeruginosa* in medium conditioned with each secondary species. This was performed in absence of antibiotics and in the presence of multiple concentrations of each antibiotic. All raw growth/kill curves that we obtained this way can be found in Supplementary Material A.

To assess the impact of a secondary species on antibiotic PD parameters for *P. aeruginosa*, we determined the growth/kill rate of *P. aeruginosa* for a range of antibiotics and antibiotic concentrations in medium conditioned by each of the CF-associated species. We then derived concentration-effect curves by plotting the growth/kill rates over the corresponding antibiotic concentrations. This allowed us to compare concentration-effect curves for conditioned and non-CM for the same antibiotic-secondary species combination. All secondary species are capable of altering each of the PD parameters, but it depends on the antibiotic which PD parameters are affected and whether they are increased or decreased. To illustrate how the changes in growth/kill curves correspond to changes in the concentration-effect curves, we selected representative examples for meropenem (**Fig. 3A-B**) and ciprofloxacin (**Fig. 3C-D**). We observed similar changes for a variety

of antibiotic-secondary species combinations (Supplementary Material B). For instance, the growth rate of *P. aeruginosa* in non-CM was decreased compared to medium conditioned with either *A. baumannii*, *A. xylosoxidans*, or *S. maltophilia* during treatment with 2 mg/L meropenem (**Fig. 3A**), visible in the corresponding concentration-effect curves (**Fig. 3B**) as a shift to the right, denoting an increase in $EC_{50}$. Conversely, the growth rate of *P. aeruginosa* in non-CM was increased compared to medium conditioned with either *A. xylosoxidans*, *B. cepacia* or *S. aureus* during treatment with 0.063 mg/L ciprofloxacin (**Fig. 3C**), visible as a shift to the left (**Fig. 3D**), denoting a decrease in $EC_{50}$. While for meropenem this does not appear to result in a large change in the steepness of the concentration-effect curves (Hill exponent $\gamma$), for ciprofloxacin, the presence of a co-infecting species leads to a reduction in steepness compared to the control. Changes in $E_{max}$ are also visible as an upward or downward shift in the lower asymptote for both antibiotics. These examples illustrate how the PD parameters used cover the full range of antibiotic responses.



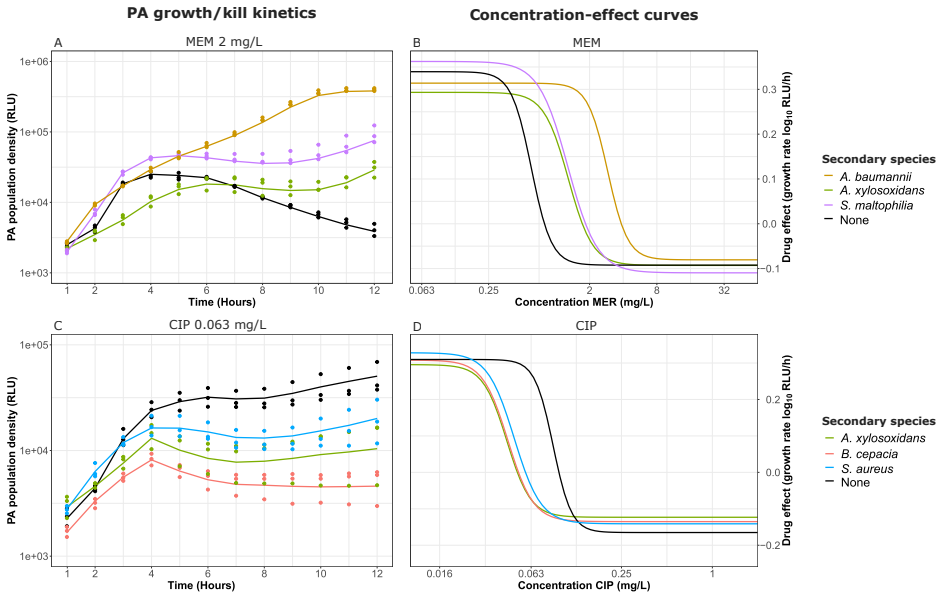


**Fig. 3 | Representative examples of the translation of growth/kill kinetics to the concentration-effect curves. A.** *P. aeruginosa* growth/kill kinetics in the presence of 2 mg/L meropenem in conditioned and non-conditioned medium. **B.** Concentration-effect curves of *P. aeruginosa* in the presence of meropenem in conditioned and non-conditioned medium. **C.** *P. aeruginosa* growth/kill kinetics in the presence of 0.063 mg/L ciprofloxacin in conditioned and non-conditioned medium. **D.** Concentration-effect curves of *P. aeruginosa* in the presence of ciprofloxacin in conditioned and non-conditioned medium. MEM = meropenem, CIP = ciprofloxacin.

### *Secondary species interactions often reduce antibiotic sensitivity*

To determine the impact of the interspecies interactions during CF on the antibiotic response of *P. aeruginosa*, we first focused on the $EC_{50}$. When a secondary species increases the $EC_{50}$ of an antibiotic, it means that a higher concentration of this antibiotic is needed to reach the same impact on *P. aeruginosa* compared to *P. aeruginosa* in monomicrobial infection. This, in turn, indicates that the species interaction leads to a

reduction in antibiotic sensitivity for *P. aeruginosa*. On the contrary, when a secondary species decreases the $EC_{50}$, this corresponds to an increase in antibiotic sensitivity for *P. aeruginosa*. Our results demonstrate that all secondary species lead to a change in $EC_{50}$ for the majority of the antibiotics tested, except for aztreonam, where none of the secondary species changed the $EC_{50}$ (**Fig. 4A**). For colistin, interactions only lead to an increase in $EC_{50}$, whereas for the other antibiotics, the impact of secondary species was bidirectional. Depending on the antibiotic, all secondary species caused either increases or decreases in $EC_{50}$, except for *A. baumannii*, *H. influenzae*, *M. avium* and *M. catarrhalis*, which only found to increase the $EC_{50}$. The impact of *R. mannitolilytica* and *S. aureus* was remarkably similar as both increased the $EC_{50}$ for minocycline and fosfomycin and decreased the $EC_{50}$ for ciprofloxacin and tobramycin. *S. maltophilia* and *A. xylosoxidans* most often caused a change in $EC_{50}$, for 7 out of the 9 antibiotics tested. The direction of the impact of *S. maltophilia* and *A. xylosoxidans* on the sensitivity of these antibiotics was also similar, with the exception of tobramycin where *S. maltophilia* did not alter the $EC_{50}$ and ceftazidime where *A. xylosoxidans* did not alter the $EC_{50}$. Overall, we showed that interactions that lead to an increase in $EC_{50}$ of *P. aeruginosa* were more commonly observed (41.9%) than interactions that lead to a decrease in $EC_{50}$ (12.8%) indicating that the antibiotic sensitivity of *P. aeruginosa* was more often reduced than increased in medium conditioned with other secondary CF-associated species (Fig. S2).



***Variation in Interaction-mediated changes in*** $EC_{50}$

In addition to changes in the $EC_{50}$ of *P. aeruginosa* due to its interaction with CF-associated secondary species, we observed changes in the $E_{max}$ and Hill exponent $\gamma$ highlighting the impact of these secondary species on the antibiotic response of *P. aeruginosa*. We showed that these PD parameters changed independently from each other and from the $EC_{50}$. Among others, we specifically showed that the $EC_{50}$ for rifampicin and colistin were altered by multiple interspecies interactions, but there were no changes in $E_{max}$ for these drugs. The opposite was the case for aztreonam, where there were no changes in the $EC_{50}$, but there were interaction-mediated changes in $E_{max}$ and Hill exponent $\gamma$. The distribution of the directions of the interspecies effects was also different for the $E_{max}$ and Hill exponent $\gamma$ compared to the $EC_{50}$. Interactions that increased the $E_{max}$ (14.5%) show that the maximum killing by the antibiotic increases during treatment, and *vice versa* for interactions that decreased the $E_{max}$ (12.8%) (**Fig. 4B**). *S. maltophilia* most often altered the antibiotic sensitivity by affecting the antibiotics' maximum effect ($E_{max}$) on the primary pathogen. *M. catarrhalis* was the only species that exclusively decreased the $E_{max}$. Interactions that increased the Hill exponent $\gamma$ (17.9%) show a stronger killing effect if antibiotic concentration increases during treatment (**Fig. 4C**). In contrast, interactions that decreased the Hill exponent $\gamma$ (20.5%) mean that a larger increase in antibiotic concentration is needed to increase the killing effect in co-infection compared to monomicrobial infection. Taken together, we showed that changes in PD parameters $E_{max}$ and Hill exponent $\gamma$ were more evenly distributed between increases and decreases than the changes in $EC_{50}$ (Fig. S2).

***CF-associated secondary species affected the*** $\mu_{max}$ ***of P. aeruginosa in the absence of antibiotics***

A shift in *P. aeruginosa* growth characteristics was also observed in the absence of antibiotics. To determine what the impact on CF-associated secondary species is on the $\mu_{max}$ of *P. aeruginosa*, we compared the $\mu_{max}$ of *P. aeruginosa* in non-CM to the

$\mu_{max}$ in CM, in the absence of any antibiotic. We showed that media conditioned by a secondary species were able to significantly alter the $\mu_{max}$ of *P. aeruginosa* (**Fig. 4D** and Table S3). Specifically, *A. xylosoxidans*, *A. baumannii*, *B. cepacia*, *M. abscessus* and *S. aureus* CM caused a decrease, whereas *A. fumigatus* and *H. influenzae* CM caused an increase in $\mu_{max}$. A decrease in $\mu_{max}$ indicates that growth rate of *P. aeruginosa* is lower when cultured in secondary species CM, whereas an increase indicates that the growth rate is higher, meaning that the population size of *P. aeruginosa* in a PMI might differ depending on which secondary species are present.

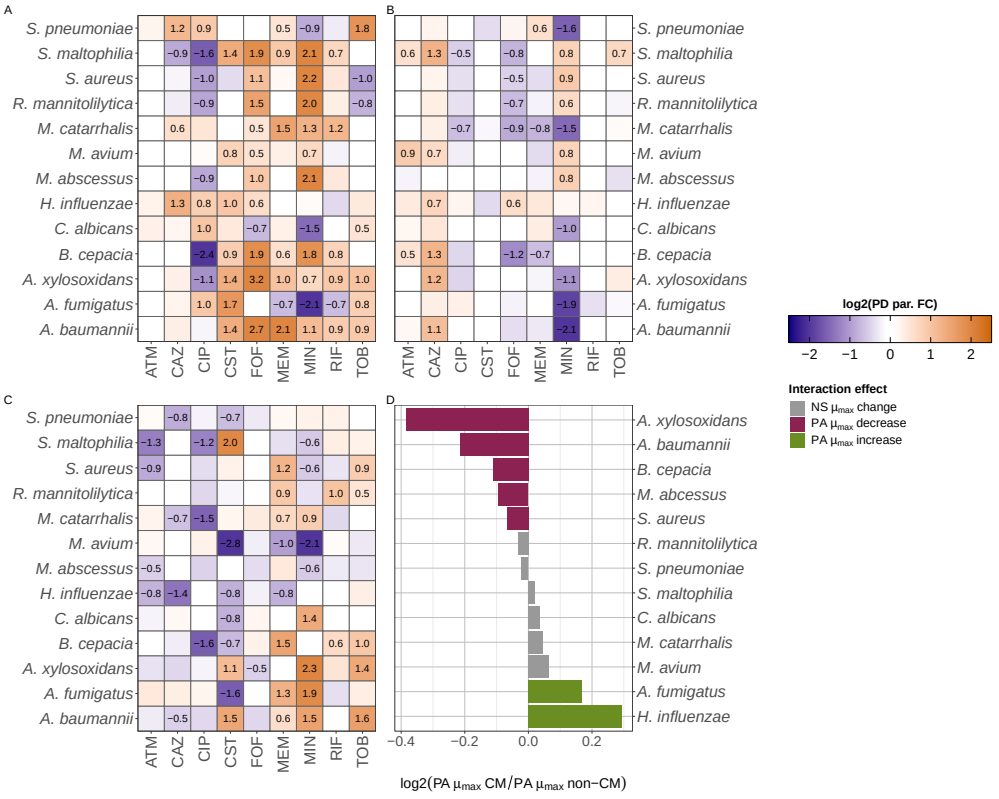


**Fig. 4 | Changes in PD parameters $EC_{50}$, $E_{max}$ and Hill exponent $\gamma$ and $\mu_{max}$ in the presence of a CF-associated secondary species. A.** Change in *P. aeruginosa*$EC_{50}$. **B.** Change in *P. aeruginosa*$E_{max}$. **C.** Change in *P. aeruginosa*Hill exponent $\gamma$. Cells in heatmaps A-C are colored when $log2(FC) > 0.15$ or $log2(FC) < -0.15$. Cells in heatmaps **A-C** display the value of the parameter changes where $|log2(FC)| > 0.5$. **D.** Change in *P. aeruginosa*$\mu_{max}$ in the absence of antibiotics. Significance ($p < 0.01$) was determined by performing a two-sample unpaired t-test for comparing the $\mu_{max}$ of *P. aeruginosa* in CM and non-conditioned medium (non-CM). NS=non-significant. Purple and green indicated significant decrease and increase in *P. aeruginosa*$\mu_{max}$, respectively. ATM=aztreonam, CAZ =ceftazidime, CIP=ciprofloxacin, CST=colistin, FOF=fosfomycin, MEM=meropenem, MIN=minocycline, RIF=rifampicin, TOB=tobramycin.

***Secondary species affect the outcome of colistin and tobramycin treatments against P. aeruginosa.***

To determine whether the observed impact of the secondary species on the PD parameters and $\mu_{max}$ affects the treatment outcome of *P. aeruginosa* in a CF-PMI, we simulated the effect of these observed changes on the *P. aeruginosa* eradication during treatment of a polymicrobial CF-lung infection. We focused on tobramycin and colistin, which are clinically relevant antipseudomonal antibiotics with narrow therapeutic windows (**Fig. 5**). The chosen array of secondary species caused both increasing and decreasing effects on the PD parameters and specifically on the $EC_{50}$, allowing us to explore the potential effects of interspecies interactions on antibiotic treatment. Colistin treatment (**Fig. 5A**) in without secondary species PD interaction effects lead to eradication at the end of the simulation (**Fig. 5B**) whereas tobramycin treatment (**Fig. 5C**) without secondary species PD interaction effects lead to a stable number of *P. aeruginosa* cell density (**Fig. 5D**). In simulations implementing PD interactions effects of *A. baumannii* or *A. xylosoxidans*, *P. aeruginosa* treatment failed with either antibiotic as the size of the initial infecting population of *P. aeruginosa* increased over the course of the simulated lung infection despite the antibiotic treatment. In contrast, *P. aeruginosa* was eradicated when treated with either antibiotic in simulations with *S. aureus* PD interactions effects. In the case of *P. aeruginosa* treated with colistin with *S. aureus* PD interaction effects, change in treatment outcome was observed albeit the relatively small change in antibiotic sensitivity ($\log_2(\text{FC}(EC_{50})) = -0.4$) when compared to the treatment outcome expected in the absence of interspecies interaction effects. For tobramycin, we also showed that *R. mannitolilytica* can lead to eradication with *P. aeruginosa*. Collectively, these results show that the impact of interspecies interactions on the antibiotic response of *P. aeruginosa* could alter treatment outcomes to the extent that treatment is rendered ineffective.

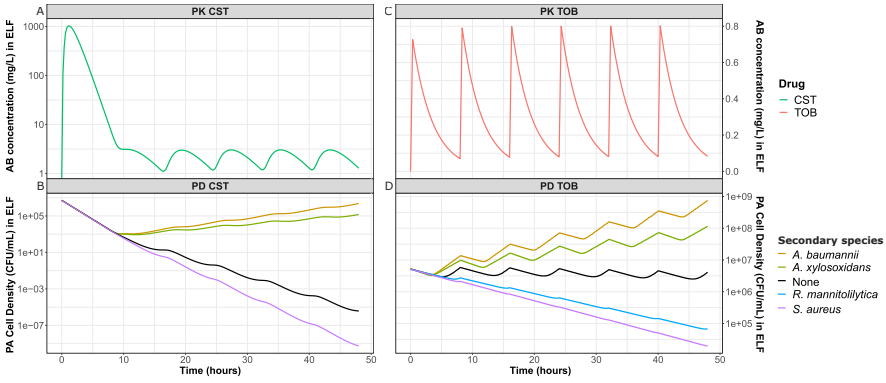


**Fig. 5 | PK-PD simulation of colistin and tobramycin treatment of *P. aeruginosa* with or without PD interaction effects of a secondary CF-pathogen. A.** PK simulation of colistin (CST) concentration in ELF. **B.** PD simulation of *P. aeruginosa* exposed to colistin in ELF. **C.** PK simulation of tobramycin (TOB) concentration ELF. **D.** PD simulation of *P. aeruginosa* exposed to tobramycin in ELF. Starting cell density in the ELF of *P. aeruginosa* is $5 \times 10^6$ CFU/mL for both tobramycin and colistin.

# Discussion

Chronic lung infections in CF patients are a characteristic example of PMIs[1], where contact-dependent and -independent pathogen-pathogen interactions are expected to alter the behaviors of the secondary species[32–34]. In this study, we established an experimental framework and a subsequent analysis pipeline to specifically investigate the contact-independent impact of any CF-associated secondary species on critical parameters for the antibiotic eradication of *P. aeruginosa* while approximating a CF-PMI. In particular, we performed high-throughput time-kill assays of *P. aeruginosa* in medium previously conditioned separately by each of the CF-associated secondary species for an extensive range of antibiotics and antibiotic concentrations, and we systematically determined changes in antibiotic response (PD parameters) and population size ($\mu_{max}$) of *P. aeruginosa*.



We showed that all secondary species included in our study are able to affect the antibiotic response of *P. aeruginosa* in an antibiotic-dependent manner and that a decrease was more common than an increase in sensitivity of *P. aeruginosa* (*i.e.*, an increase in $EC_{50}$). This was the case for the majority of the antibiotics to which we exposed *P. aeruginosa*, with the exception of aztreonam sensitivity where we observed no change, and ciprofloxacin sensitivity where an increase in sensitivity was more commonly observed. Similarly, interspecies interactions that occur in urinary tract infections (UTIs) were previously found to most commonly decrease the sensitivity of bacterial pathogens in UTIs against trimethoprim-sulfamethoxazole and nitrofurantoin[35]. In addition to the changes in antibiotic sensitivity, we showed that in absence of antibiotics, secondary species could either increase or decrease the $\mu_{max}$ of *P. aeruginosa*. Overall, we indicated that a decrease in sensitivity was more commonly observed, which is in accordance with the notion that interspecies interactions are more often of a competitive than a cooperative nature[35,36]. Our data demonstrates extensive impact of the secondary species on *P. aeruginosa* PD parameters and/or $\mu_{max}$. Given that we replenished the CM with nutrients after filtering out the secondary species, this observed contact-independent impact on *P. aeruginosa* is likely not due to nutrient depletion but due to metabolic by-products of these secondary species secreted in the CM. Future metabolomic analysis could shed light on which metabolites are secreted by the secondary species, possibly explaining which mechanisms are responsible for the interaction effects that we observed in this study.

Direct comparison with previous findings is complicated by the limited number of earlier studies that employed a range of methods to approximate interspecies interactions. Studies differ from each other in their choice of isolates, use different readouts and expose the species to each other through several methods in different lifestyles, such as direct co-culture or CM in planktonic or biofilm culture[37–42]. These differences in experimental setup might be the reason for contradicting study results. For example, one study found that 10% medium conditioned by a clinical isolate of *S. aureus* decreased tobramycin sensitivity for the reference strain PAO1 and clinical *P. aeruginosa* strains isolated from children with CF for which previous antibiotic eradication therapy had failed[43]. In contrast, a different study showed that planktonic and biofilm co-cultures of *S. aureus* ATCC25923 and *P. aeruginosa* in the presence of lung epithelial cells increased tobramycin sensitivity for three *P. aeruginosa* reference strains, but not for PAO1[44]. In our study, where we used a CM approach and a planktonic lifestyle, we found that *S. aureus* strain DSM346 increases the tobramycin sensitivity of *P. aeruginosa* (PAO1-Xen41). These examples highlight the relevance of applying

a high-throughput standardized workflow to enable the systematic exploration of the impact of interspecies interactions on *P. aeruginosa*'s antibiotic response.

Other studies determining the antibiotic response of *P. aeruginosa* in presence of other species used different experimental approaches, such as a direct co-culture, or determine the response of *P. aeruginosa* in biofilm models[23,40,45,46]. For many of these experimental approaches, it can often be challenging to obtain time course data in a high throughput fashion, meaning the output of these studies is often for one antibiotic concentration at a single time point, or time course data can only be obtained in a low throughput fashion. As our goal was to determine in detail the antibiotic concentration-effect profiles of *P. aeruginosa* in order to perform PD analysis, we decided to focus on measuring bacterial growth over time and for many antibiotics and antibiotic concentrations. This makes a biofilm or direct co-culture experimental model unsuitable for our study aims, where our CM approach does meet the study requirements. Promising antibiotic-species combinations identified in this study could serve as a starting point for further investigations of these interactions in more physiological conditions, such as *in vitro* assays involving artificial sputum medium, mixed biofilm coculture models, or *in vivo* models of PMIs. In addition, future studies may focus on the impact of higher order interactions between more than two species[2,3].



Our specific focus on identifying effects of species interactions on PD parameters instead of MIC is important, as this allows to obtain an understanding at the pharmacological mode of action of interspecies interactions. In addition, depending on the specific PD parameter which is altered, different adjustments of the antibiotic dosing schedules need to be implemented in order to improve treatment, underlining, in turn, the need for PD analysis based on antibiotic time kill-curves instead of lump sum methods such as MIC determination[26,27]. In our study, all species were found to alter the antibiotic response of *P. aeruginosa* for one or more of the PD parameters tested in an antibiotic-dependent manner. For some of these species, such as *A. baumannii*, *A. xylosoxidans* and *S. maltophilia*, the impact on the severity of lung infections in CF is poorly understood[47,48]. Our results suggest that those species can alter the antibiotic response of *P. aeruginosa* during treatment, thereby contributing to the understanding of how CF-PMIs progress over time.

To further evaluate the potential clinical implications of interspecies interactions on antibiotic treatment regimens, we simulated antibiotic treatment of *P. aeruginosa* with colistin or tobramycin implementing the PD effects of different secondary species, including *A. baumannii*, *A. xylosoxidans*, *S. aureus* or *S. maltophilia*. Our PK-PD simulations showed that the identified alterations in PD parameters induced by species interactions could have a clinically relevant impact, either potentiate treatment or leading to treatment failure. This is clinically relevant as colistin and tobramycin have narrow therapeutic windows, making the risk of underdosing likely when a pathogen-pathogen interaction results in decreased antibiotic sensitivity for *P. aeruginosa*[49,50]. Underdosing, in turn, not only leads to treatment failure, but may also lead to the development of antibiotic resistance[51]. On the other hand, when a pathogen-pathogen interaction results in increased *P. aeruginosa* sensitivity, this could mean that less antibiotic is necessary to obtain treatment success which could be beneficial for patients by minimizing the toxic effect of colistin and tobramycin. The use of PK-PD modeling strategies such as demonstrated for these case studies offer an important tool to help further translate *in vitro* PD data to the clinical situation and ultimately also offers the flexibility to further incorporate specific PD mechanisms[52,53].

In conclusion, our study provide a comprehensive quantitative overview on inter-

species interaction effects on the PD response of *P. aeruginosa* in the presence of CF-associated secondary species, and the potential to further translate such interaction effects to clinical dosing schedules through the use of PK-PD modeling. Our analyses demonstrate that the identified PD interaction effects have the potential to alter antibiotic treatment outcomes, consolidating the relevance of interspecies interactions on the antibiotic treatment of CF-patients with PMIs. Overall, our study provides the foundation for further studies on the role of interspecies interactions to optimize antibiotic treatment of CF-PMIs.

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# Supplementary Figures

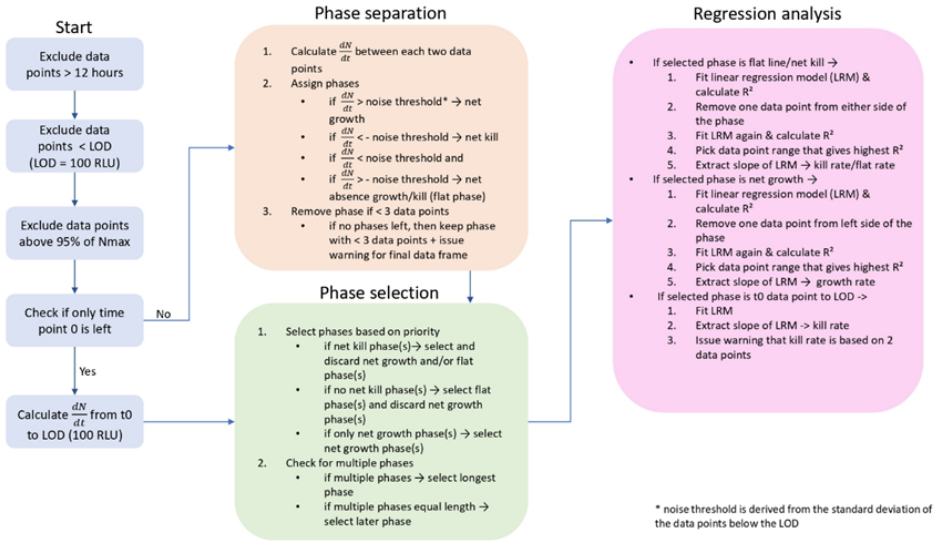



**Fig. S1 | Rationale and workflow of the growth phase selection algorithm.**

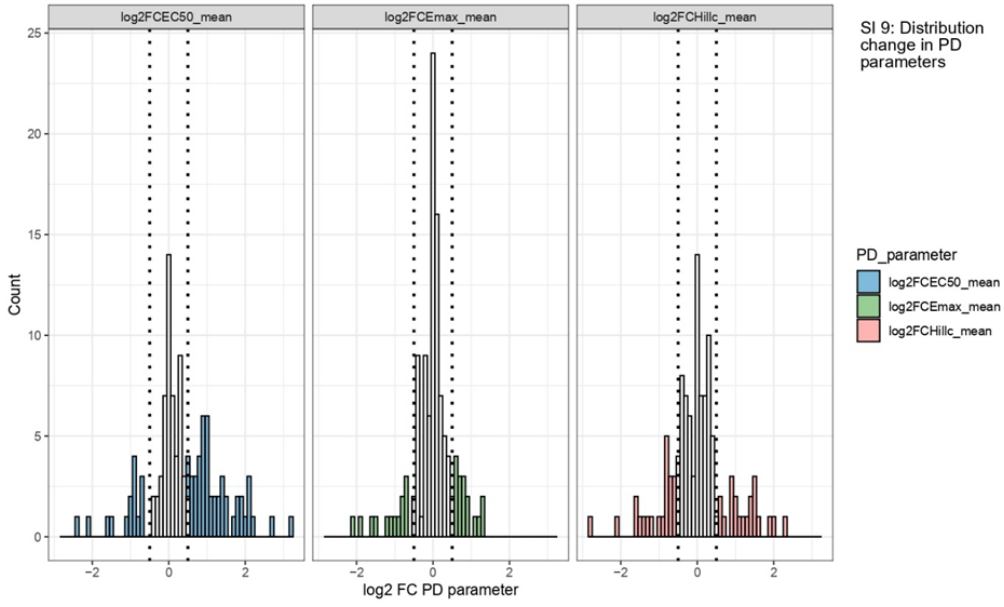



**Fig. S2 | Distribution of estimated antibiotic sensitivity parameters.**

# Supplementary Tables

**Table S1. Strain and culture conditions**

| Species | Strain | Source | Medium Supplements | Culture condition |
|---|---|---|---|---|
| *Achromobacter xylosoxidans* | DSM 2402 | DSMZ | None | Aerobic |
| *Acinetobacter baumannii* | DSM 30007 | DSMZ | None | Aerobic |
| *Aspergillus fumigatus* | DSM 819 | DSMZ | 10% glucose | Aerobic |
| *Burkholderia cepacia* | DSM 7288 | DSMZ | None | Aerobic |
| *Candida albicans* | DSM 1386 | DSMZ | 10% glucose | Aerobic |
| *Haemophilus influenzae* | DSM 10001 | DSMZ | 5% LHB<br>20 mg/L $\beta$-NAD | Aerobic<br>5% $CO_2$ |
| *Mycobacterium abscessus* | DSM 44196 | DSMZ | None | Aerobic |
| *Mycobacterium avium* | ATCC 700898 | ATCC | 10% Middlebrook ADC | Aerobic |
| *Moraxella catarrhalis* | DSM 9143 | DSMZ | 5% LHB<br>20 mg/L $\beta$-NAD | Aerobic<br>5% $CO_2$ |
| *Pseudomonas aeruginosa* | PAO1 Xen-41 | Perkin-Elmer | None | Aerobic |
| *Ralstonia mannitolilytica* | DSM 17512 | DSMZ | None | Aerobic |
| *Staphylococcus aureus* | DSM 346 | DSMZ | None | Aerobic |
| *Stenotrophomonas maltophilia* | DSM 21257 | DSMZ | None | Aerobic |
| *Streptococcus pneumoniae* | DSM 14377 | DSMZ | 5% LHB<br>20 mg/L $\beta$-NAD | Aerobic<br>5% $CO_2$ |

**Table S2. Value of PK parameters used for simulating colistin and tobramycin treatments**

| Antibiotics | Parameter | Description | Units | Typical value | Value Applied[a] |
|---|---|---|---|---|---|
| COL | $F_{aero}$ | Fraction of the dose that reaches the ELF | – | 0.09 | Typical value |
| | $CL_{IN\_CMS}$ | Clearance of CMS from lung to plasma | $\mu$L/min | 15.1 | 23.7 |
| | $CL_{OUT\_CMS}$ | Clearance of CMS from plasma to lung | $\mu$L/min | 6 | 13.68 |
| | $CL_{ps\_CMS}$ | Presystemic clearance of CMS to colistin | $\mu$L/min | 2.6 | 5.408 |
| | $V_{ELF}$ | Epithelial lining fluid volume | mL | 1.2 | Typical value |
| | $CL_{IN\_COL}$ | Clearance of colistin from lung to plasma | $\mu$L/min | 9.8 | 17.15 |
| | $CL_{OUT\_COL}$ | Clearance of colistin from plasma to lung | $\mu$L/min | 12.7 | 26.3 |
| | $V_{CMS}$ | Volume of distribution of CMS | Liters | 15.3 | 15.3 |
| | $CL_{R\_CMS}$ | Renal clearance of CMS | mL/min | 64.6 | 124.7 |
| | $CL_{NR\_CMS}$ | Nonrenal clearance of CMS | mL/min | 46.3 | Typical value |
| | $V_{COL}/f_m$ | Apparent volume of distribution of colistin | Liters | 13.7 | 13.7 |
| | $CL_{COL}/f_m$ | Apparent total clearance of colistin | mL/min | 53.1 | 73.3 |
| TOB | $CL$ | Proportionality constant relating creatinine clearance to drug clearance | $h^{-1}$ | 0.059 | Typical value |
| | $K_{12}$ | Transfer rate constant from compartment 1 to 2 | $h^{-1}$ | 0.012 | Typical value |
| | $K_{21}$ | Transfer rate constant from compartment 2 to 1 | $h^{-1}$ | 0.027 | Typical value |
| | $V_1$ | Central volume of distribution | $kg^{-1}$ | 0.327 | Typical value |
| | $F$ | Fraction of the dose that reaches the ELF | – | 12 | Typical value |

[a] Colistin clearance values applied in the simulations were adjusted to represent individuals with a higher colistin clearance (thus, lower exposure) based on the parameters' respective inter-individual variability (%IIV) of 0.57, 1.28, 1.08, 0.75, and 1.07 for $CL_{IN\_CMS}$, $CL_{OUT\_CMS}$, $CL_{ps\_CMS}$, $CL_{IN\_COL}$, and $CL_{OUT\_COL}$, respectively.

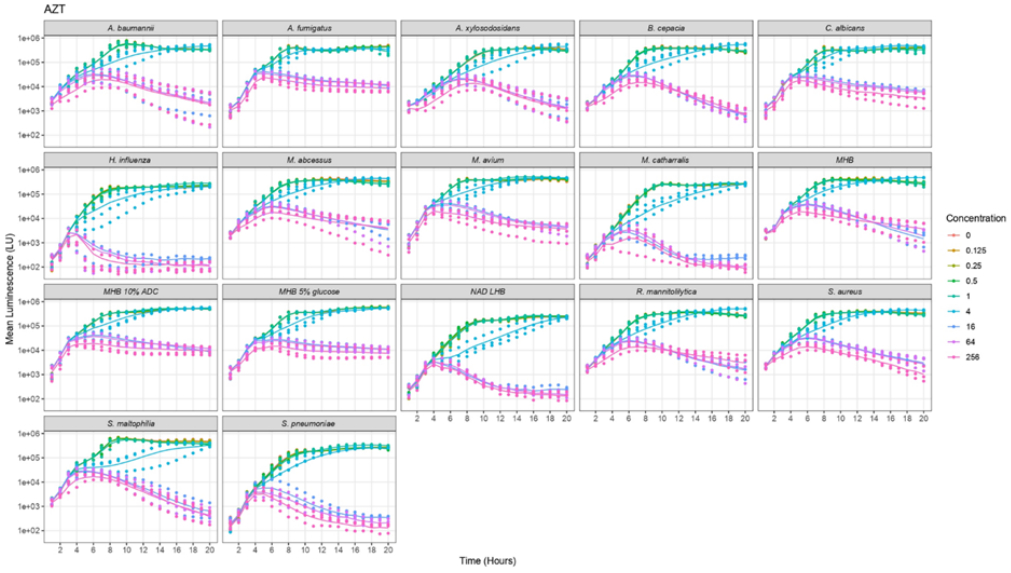
**Table S3. Growth rate of *P. aeruginosa* in different medium conditions.**

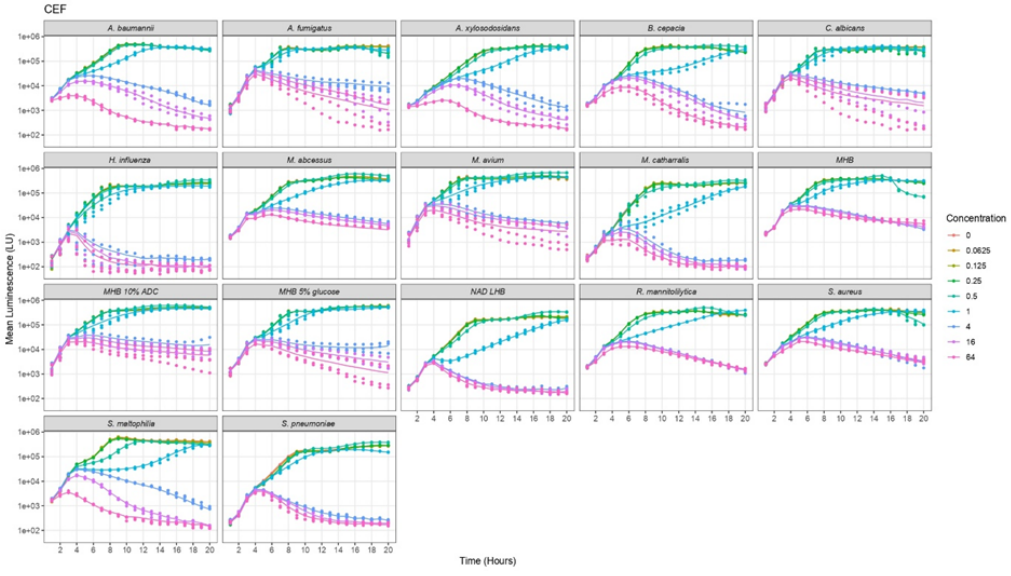
| Medium | Conditioned by species | Growth rate ($h^{-1}$) *Mean (SD)* | Growth rate ratio* *Mean (SD)* |
|---|---|---|---|
| **MH broth** | – | 0.35 (0.02) | – |
| | *A. baumannii* | – | 0.86 (0.06) |
| | *A. xylosoxidans* | – | 0.77 (0.08) |
| | *B. cepacia* | – | 0.93 (0.04) |
| | *M. abscessus* | – | 0.94 (0.07) |
| | *R. mannitolilytica* | – | 0.98 (0.04) |
| | *S. aureus* | – | 0.95 (0.07) |
| | *S. maltophilia* | – | 1.01 (0.06) |
| **MH broth + ADC** | – | 0.40 (0.03) | – |
| | *M. avium* | – | 1.04 (0.07) |
| **MH broth + glucose** | – | 0.33 (0.01) | – |
| | *A. fumigatus* | – | 1.12 (0.06) |
| | *C. albicans* | – | 1.03 (0.06) |
| **MH broth + LHB + NAD** | – | 0.38 (0.02) | – |
| | *H. influenzae* | – | 1.23 (0.07) |
| | *M. catarrhalis* | – | 1.03 (0.06) |
| | *S. pneumoniae* | – | 0.98 (0.08) |

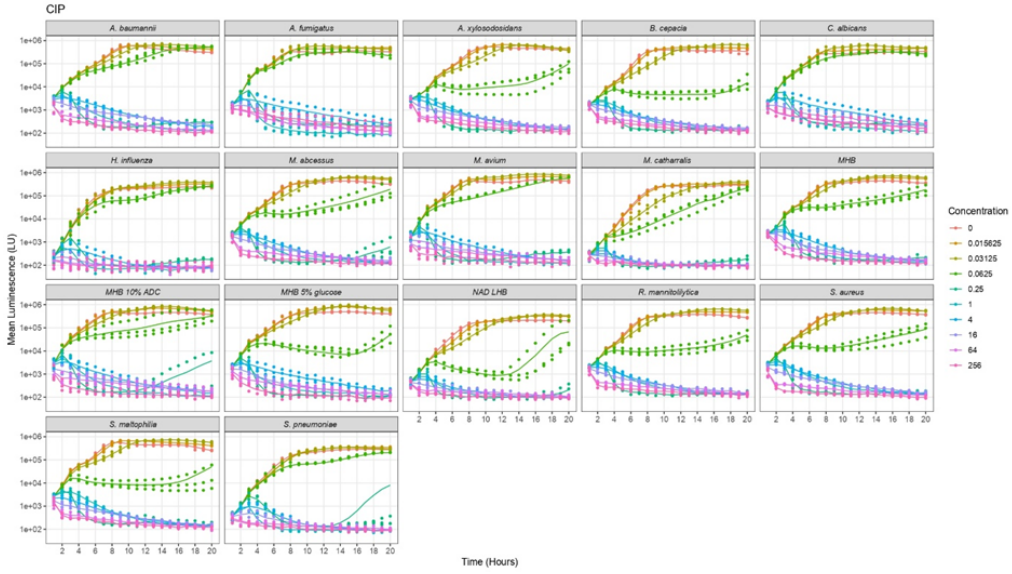
* Growth rate ratio to that measured in the respective unconditioned medium.

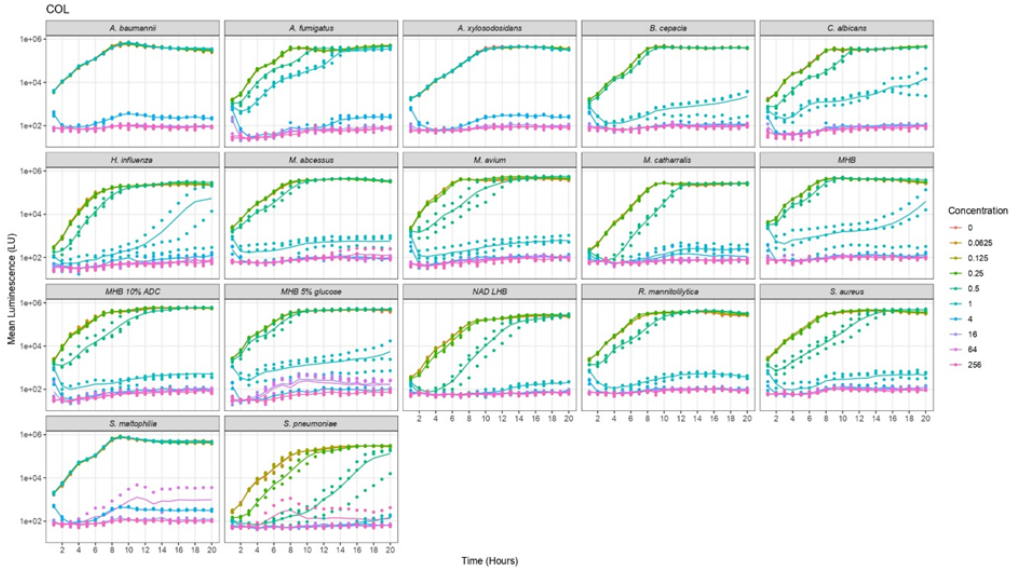
# Supplementary Material A

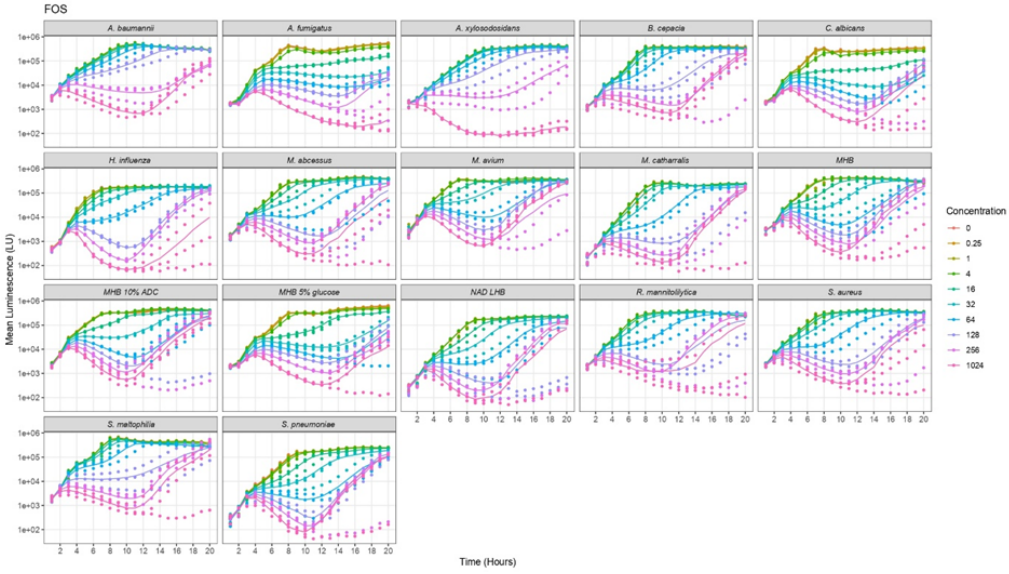
*Growth curve of different bacteria under varying antibiotic exposure*

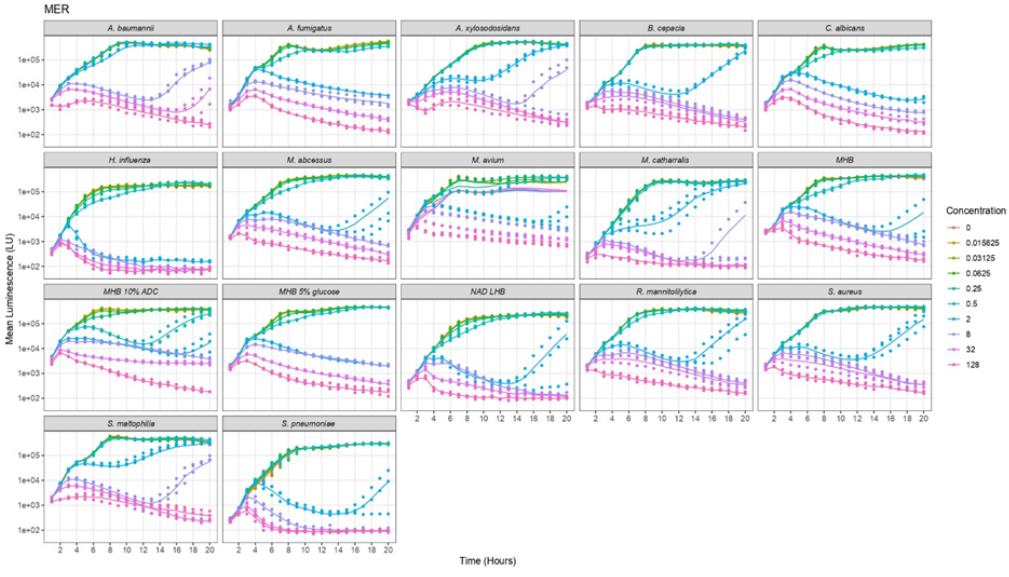


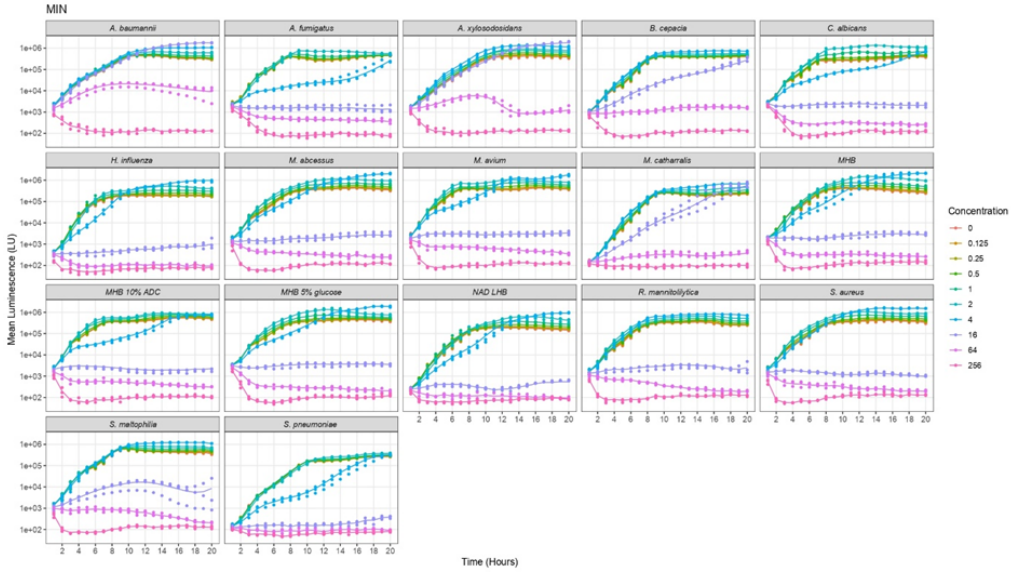



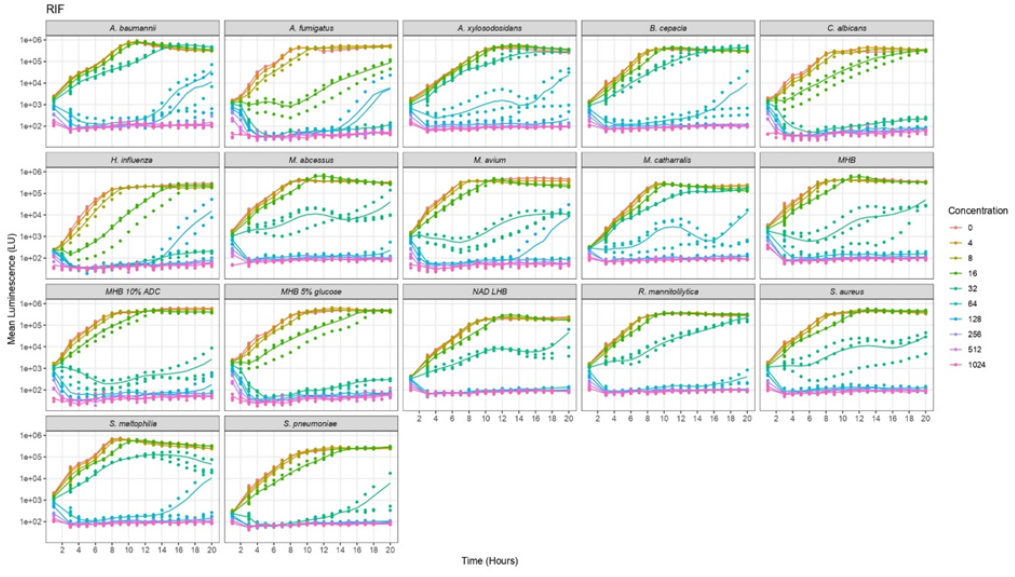

CIP
A. baumannii
A. fumigatus
A. xylosodosidans
B. cepacia
C. albicans
H. influenza
M. abcessus
M. avium
M. catharralis
MHB
MHB 10% ADC
MHB 5% glucose
NAD LHB
R. mannitolilytica
S. aureus
S. meltophilia
S. pneumoniae
Mean Luminescence (LU)
Time (Hours)
Concentration
0
0.015625
0.03125
0.0625
0.25
1
4
16
64
256

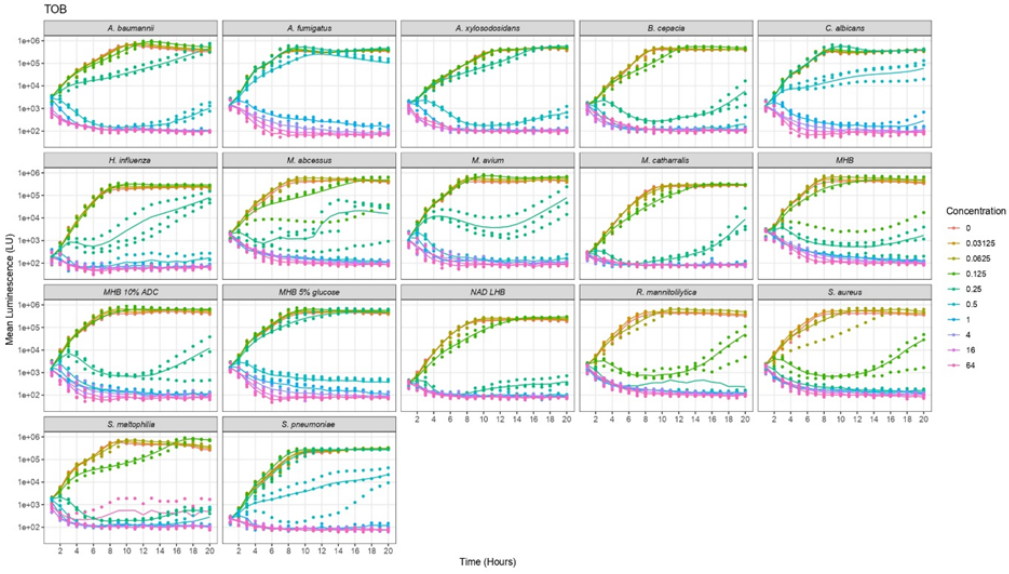

COL
A. baumannii
A. fumigatus
A. xylosodosidans
B. cepacia
C. albicans
H. influenza
M. abcessus
M. avium
M. catharralis
MHB
MHB 10% ADC
MHB 5% glucose
NAD LHB
R. mannitolilytica
S. aureus
S. meltophilia
S. pneumoniae
Mean Luminescence (LU)
Time (Hours)
Concentration
0
0.0625
0.125
0.25
0.5
1
4
16
64
256


FOS
A. baumannii
A. fumigatus
A. xylosodosidans
B. cepecia
C. albicans
H. influenza
M. abcessus
M. avium
M. catharralis
MHB
MHB 10% ADC
MHB 5% glucose
NAD LHB
R. mannitolilytica
S. aureus
S. meltophilia
S. pneumoniae
Mean Luminescence (LU)
Time (Hours)
Concentration
0
0.25
1
4
16
32
64
128
256
1024


MER
A. baumannii
A. fumigatus
A. xylosodosidans
B. cepecia
C. albicans
H. influenza
M. abcessus
M. avium
M. catharralis
MHB
MHB 10% ADC
MHB 5% glucose
NAD LHB
R. mannitolilytica
S. aureus
S. meltophilia
S. pneumoniae
Mean Luminescence (LU)
Time (Hours)
Concentration
0
0.015625
0.03125
0.0625
0.25
0.5
2
8
32
128

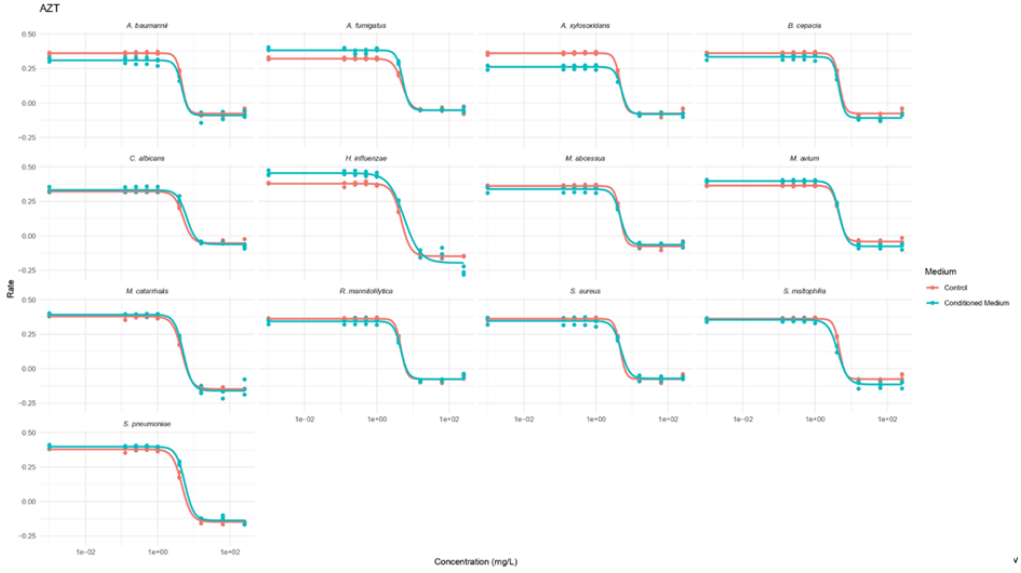

MIN
A. baumannii
A. fumigatus
A. xylosodosidans
B. cepacia
C. albicans
H. influenza
M. abcessus
M. avium
M. catharralis
MHB
MHB 10% ADC
MHB 5% glucose
NAD LHB
R. mannitolilytica
S. aureus
S. meltophilia
S. pneumoniae
Mean Luminescence (LU)
Time (Hours)
Concentration
0
0.125
0.25
0.5
1
2
4
16
64
256

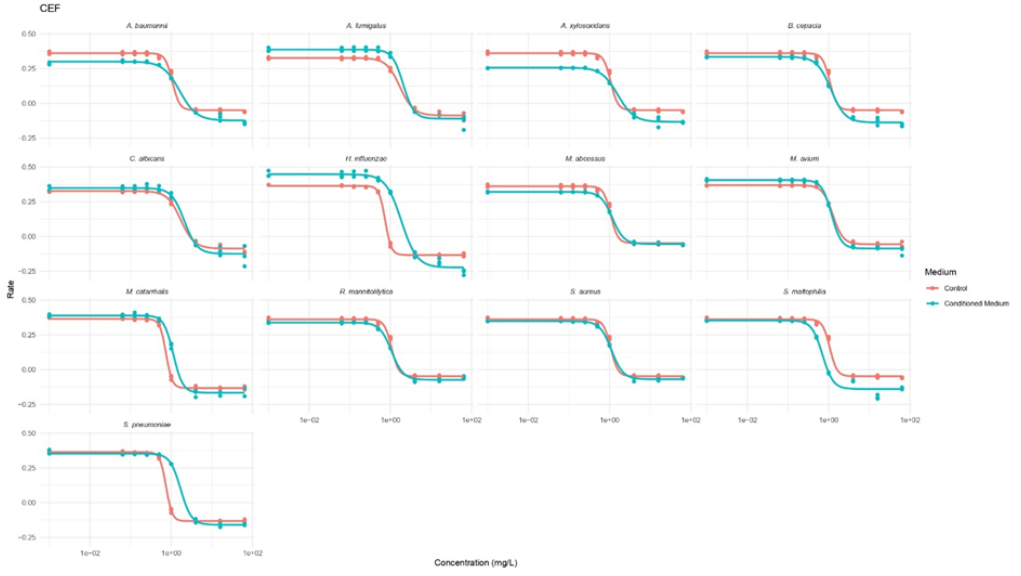

RIF
A. baumannii
A. fumigatus
A. xylosodosidans
B. cepacia
C. albicans
H. influenza
M. abcessus
M. avium
M. catharralis
MHB
MHB 10% ADC
MHB 5% glucose
NAD LHB
R. mannitolilytica
S. aureus
S. meltophilia
S. pneumoniae
Mean Luminescence (LU)
Time (Hours)
Concentration
0
4
8
16
32
64
128
256
512
1024

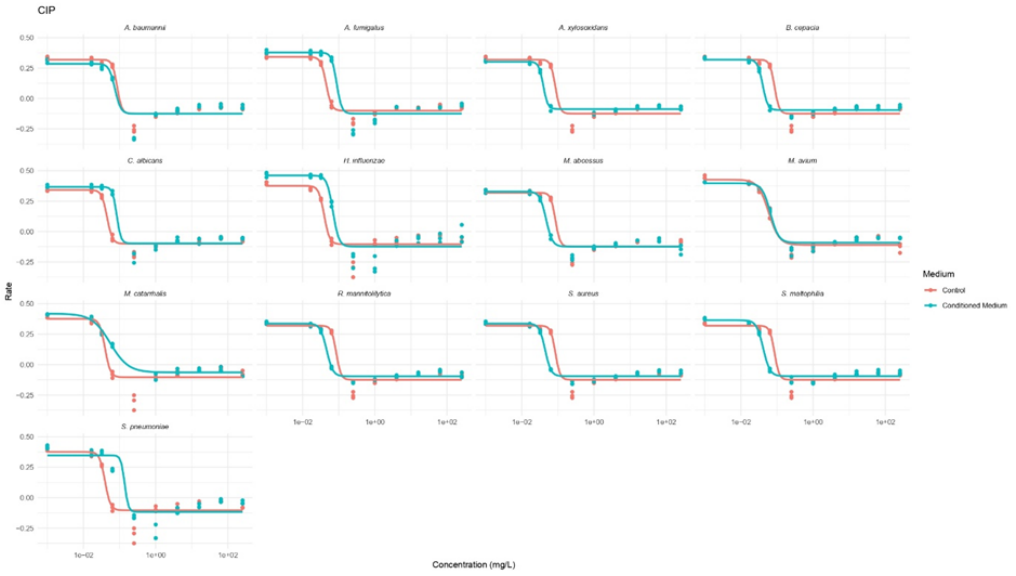

TOB
A. baumannii
A. fumigatus
A. xylosodosidans
B. cepecia
C. albicans
H. influenza
M. abcessus
M. avium
M. catharralis
MHB
MHB 10% ADC
MHB 5% glucose
NAD LHB
R. mannitolilytica
S. aureus
S. meltophilia
S. pneumoniae
Mean Luminescence (LU)
Time (Hours)
Concentration
0
0.03125
0.0625
0.125
0.25
0.5
1
4
16
64

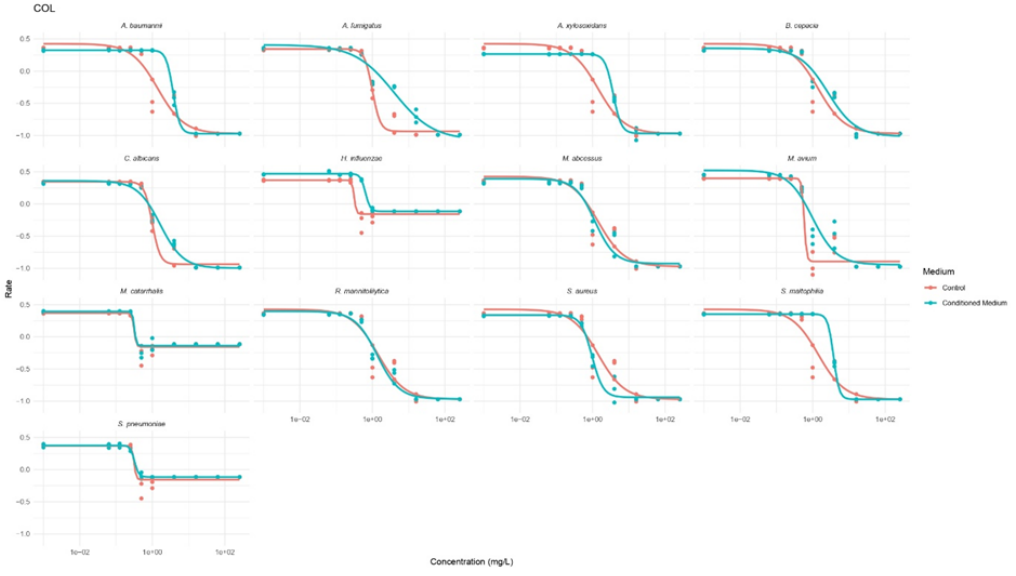
# Supplementary Material B

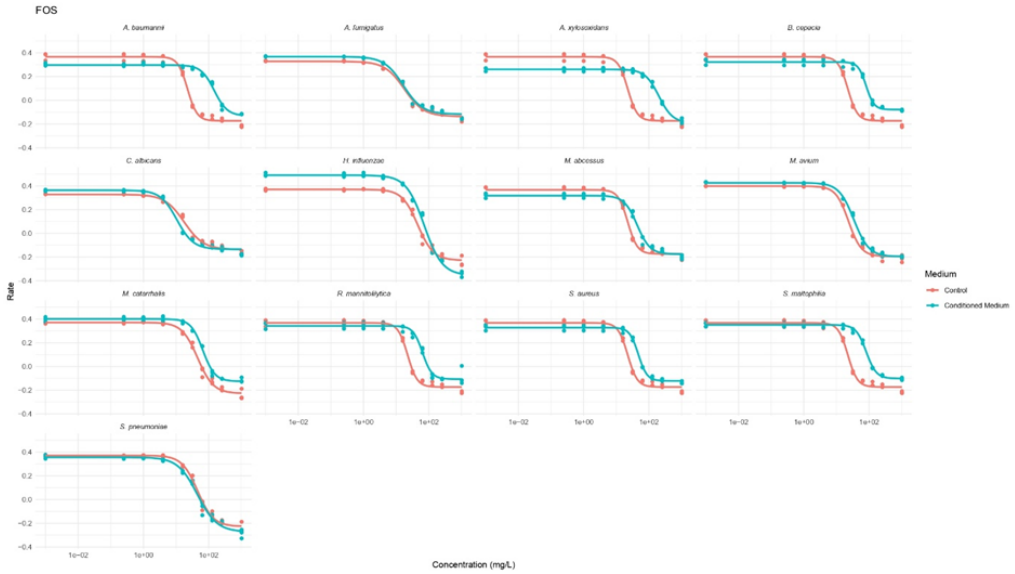
***Dose-response characteristics of different bacterial species***

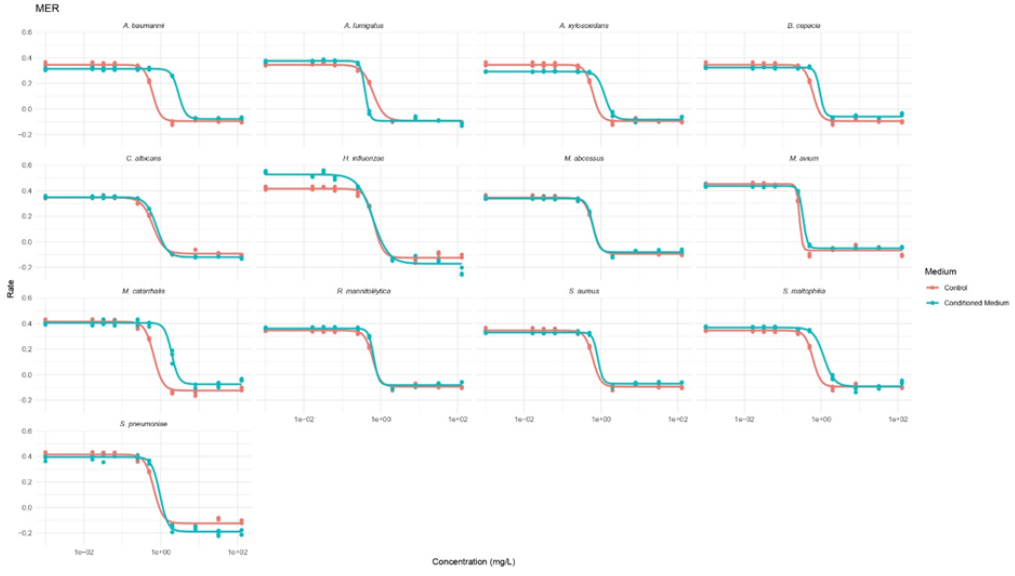


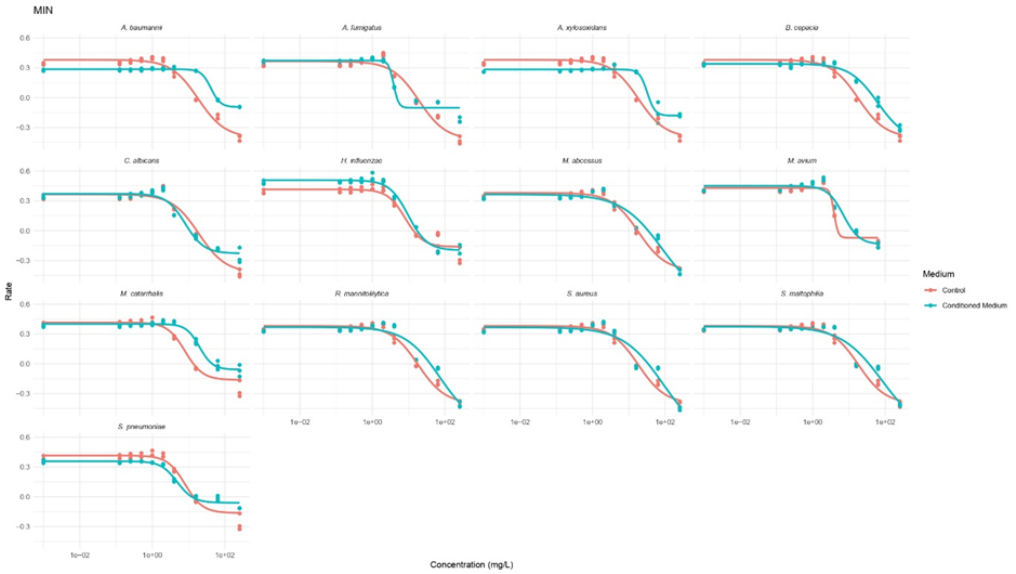



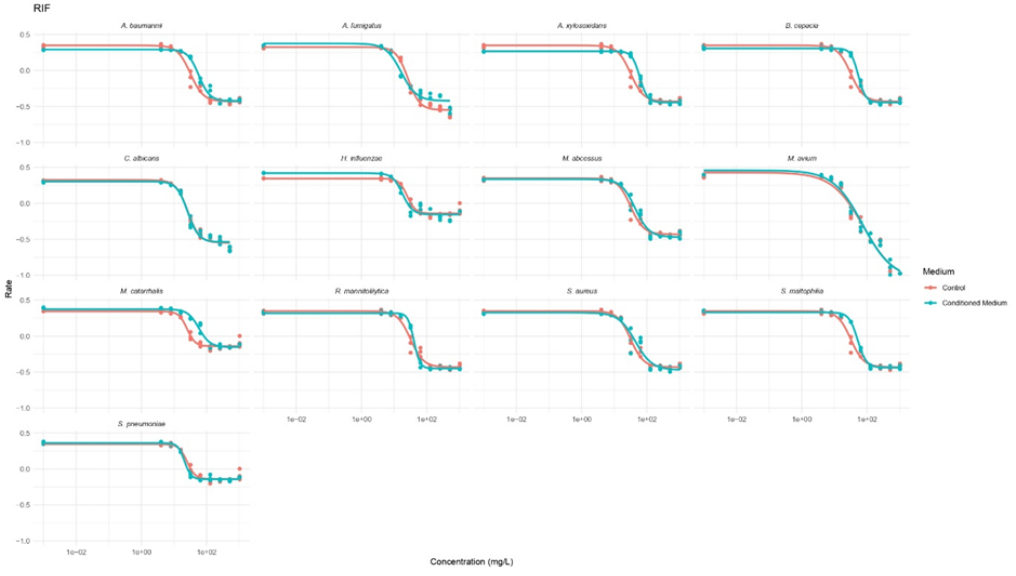

CIP
A. baumannii
A. fumigatus
A. xylosoxidans
B. cepacia
C. albicans
H. influenzae
M. abscessus
M. avium
M. catarrhalis
R. mannitolilytica
S. aureus
S. maltophilia
S. pneumoniae
Rate
Concentration (mg/L)
Medium
Control
Conditioned Medium

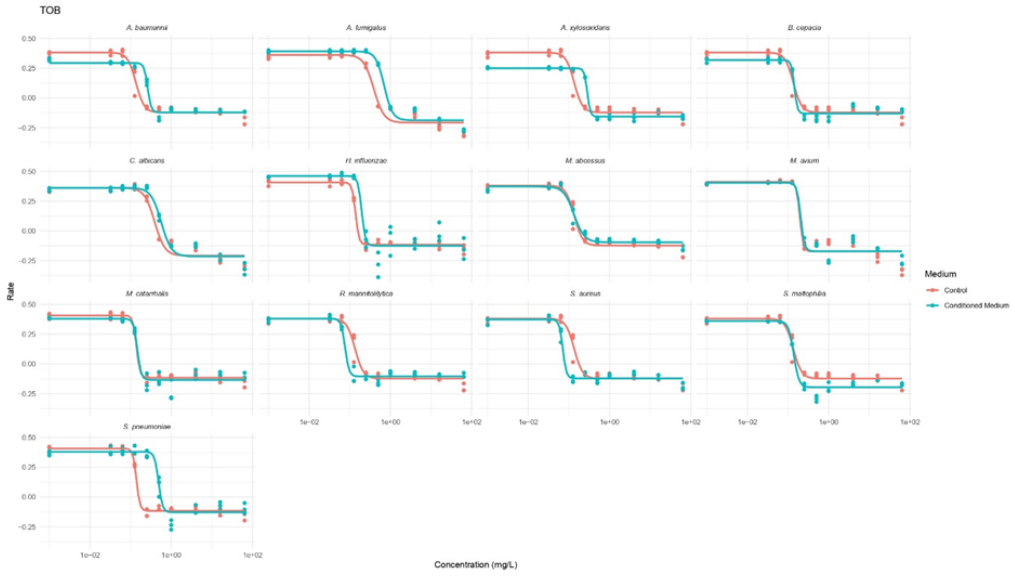

COL
A. baumannii
A. fumigatus
A. xylosoxidans
B. cepacia
C. albicans
H. influenzae
M. abscessus
M. avium
M. catarrhalis
R. mannitolilytica
S. aureus
S. maltophilia
S. pneumoniae
Rate
Concentration (mg/L)
Medium
Control
Conditioned Medium


FOS
A. baumannii
A. fumigatus
A. xylosoxidans
B. cepacia
C. albicans
H. influenzae
M. abcessus
M. avium
M. catarrhalis
R. mannitolilytica
S. aureus
S. maltophilia
S. pneumoniae
Rate
Concentration (mg/L)
Medium
Control
Conditioned Medium


MER
A. baumannii
A. fumigatus
A. xylosoxidans
B. cepacia
C. albicans
H. influenzae
M. abcessus
M. avium
M. catarrhalis
R. mannitolilytica
S. aureus
S. maltophilia
S. pneumoniae
Rate
Concentration (mg/L)
Medium
Control
Conditioned Medium


MIN
A. baumannii
A. fumigatus
A. xylosoxidans
B. cepacia
C. albicans
H. influenzae
M. abscessus
M. avium
M. catarrhalis
R. mannitolilytica
S. aureus
S. maltophilia
S. pneumoniae
Rate
Concentration (mg/L)
Medium
Control
Conditioned Medium


RIF
A. baumannii
A. fumigatus
A. xylosoxidans
B. cepacia
C. albicans
H. influenzae
M. abscessus
M. avium
M. catarrhalis
R. mannitolilytica
S. aureus
S. maltophilia
S. pneumoniae
Rate
Concentration (mg/L)
Medium
Control
Conditioned Medium


TOB
A. baumannii
A. fumigatus
A. xylosoxidans
B. cepacia
C. albicans
H. influenzae
M. abscessus
M. avium
M. catarrhalis
R. mannitolilytica
S. aureus
S. maltophilia
S. pneumoniae
Rate
Concentration (mg/L)
Medium
Control
Conditioned Medium