The pharmacodynamic inoculum effect from the perspective of bacterial population modeling

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Synopsis

Background The quantitative determination of the effects of antimicrobials is essential for our understanding of pharmacodynamics and for their rational clinical application. However, common pharmacodynamic measures of antimicrobial efficacy, such as the MIC and the pharmacodynamic function, fail to capture the observed dependence of efficacy on the bacterial population size — a phenomenon called *inoculum effect*.

Objectives We aimed to assess the relationship between bacterial inoculum size and pharmacodynamic parameters, and to determine the consequences of the inoculum effect on bacterial population dynamics under treatment with antimicrobials.

Methods We used the mathematical multi-hit model to quantify the effect of the inoculum on the pharmacodynamic parameters. The model describes antimicrobial action mechanistically, which allowed us to test various hypotheses concerning the mechanistic basis of the inoculum effect.

Results Our model showed that the inoculum effect can arise from the binding dynamics of antimicrobial molecules to bacterial targets alone and does not require enzymatic degradation of antimicrobials. With enzymatic degradation, however, the inoculum effect is more pronounced. We propose to include the inoculum effect when measuring antimicrobial efficacy,

i. e. to extend the pharmacodynamic function with the inoculum effect. This extended pharmacodynamic function mimiced simple long-term population dynamics well. More complex scenarios were only captured with the mechanism-based multi-hit model. In simulations with competing antimicrobial-sensitive and -resistant bacteria, neglecting the inoculum effect lead to an overestimation of the competitive ability of the resistant strain.

Conclusions Our work emphasizes that the pharmacodynamic function — and in general any efficacy measure, e.g. the MIC — should include information about the inoculum size on which it is based, and ideally account for the inoculum effect.

Keywords: inoculum effect, pharmacodynamic function, multi-hit model, antimicrobial efficacy, MIC, bacterial population dynamics

Introduction

The efficacy of antimicrobials against bacteria is commonly quantified using time-kill curves (Fig. 1a). The slopes of log-linear time-kill curves describe the change of the bacterial population over time and are a direct measure of the bacterial net growth rate. The net growth rate and its dependence on the antimicrobial concentration can be captured by the so-called pharmacodynamic (PD) function.[1–3] The parameters of this PD function, however, depend on the initial bacterial population size in the time-kill curve experi-

ments.[4,5]

A more widespread, but less informative, way to express efficacy of antimicrobials is the minimum inhibitory concentration (MIC) (Fig. 1b). Although it is recognized that the MIC might change with bacterial inoculum size (see for example the MIC protocol by Wiegand *et al.*[6]), both the CLSI and the EUCAST recommendations to measure the MIC are based on a single standard bacterial inoculum of 5×10^5 CFU/ml.[7,8] This standardization of the inoculum yields an estimate of the MIC that cannot be extrapolated to other bacterial population sizes.

Changes in efficacy of antibacterial drugs caused by changing the initial bacterial inoculum size are summarized under the term *inoculum effect* (Fig. 1). The inoculum effect was first described in the early 1940s,[9] and was observed for a variety of combinations of bacterial species and antimicrobials more recently.[4,10–18] In addition to its experimental establishment, the inoculum effect has been explored with mathematical models.[4,13,19–23] These experimental and theoretical studies have put forward hypotheses explaining the inoculum effect that fall into two broad categories. The first category of hypothesis attribute the inoculum effect to the binding kinetics of antimicrobials to bacterial targets that leads to larger reduction of antimicrobials with higher bacterial population sizes.[4,20,21] The second invokes the secretion enzymes at high bacterial inocula, e.g. β-lactamases[9,10,14–16,24–26] and proteases.[27–29]

In this study, we used mathematical models as a tool to investigate these

competing hypotheses about the inoculum effect, and to derive a quantitative understanding of its extent. Specifically, we use the so-called multi-hit model[20,30,31] that describes the binding of antimicrobial molecules to targets on bacterial cells, to address if the binding kinetics by itself already leads to an inoculum effect, and to what extent it influences the PD parameters. We further investigate how the inoculum effect biases the competition between antimicrobial-sensitive and -resistant strains.

Methods

Multi-hit model

Throughout this paper, we used the multi-hit model[20,30,31] that describes a bacterial population consisting of N_{tot} cells. The key idea of this model is that targets on bacterial cells are "hit", i. e. bound, by antimicrobial molecules, and a bacterial cell dies if a threshold number of targets has been hit. Formally, the bacterial population in the multi-hit model is structured according to how many antimicrobials have hit the cell (Fig. 2). We denote the number of bacteria that have i antimicrobial molecules adsorbed to cellular targets by N_i. The number of absorbed molecules varies from cell to cell, with $0 \leq i \leq n$. Thus, we have a total of $n+1$ classes and $N_{tot} = \sum_{i=0}^{n} N_i$.

The rate of change of each subpopulation is described by the following

ordinary differential equation:

$$\begin{aligned} \frac{dN_i}{dt} &= \alpha_{i-1}AN_{i-1} - \alpha_i AN_i + \\ &\quad \mu_{i+1}(i+1)N_{i+1} - \mu_i iN_i + \\ &\quad \Omega(N_i) - d_i N_i \end{aligned} \tag{1}$$

Here, the parameters α_i and μ_i, $0 \leq i \leq n$, denote binding and unbinding rate constants. The term $\Omega(N_i)$ in equation 1 summarizes replication processes, which we modeled according to Abel zur Wiesch *et al.*[20] The parameter d_i, $0 \leq i \leq n$, describes the death rate constant.

Antimicrobials are assumed to be bactericidal only in this model. The pharmacodynamics is incorporated into the multi-hit model by introducing a threshold number of antimicrobials z that need to be bound to bacterial targets on an individual cell for the cell to die. In our basic model, this threshold z is equal to the amount of total targets, $z = n$ and once it is reached, the rate of change of this subpopulation N_z is described with

$$\begin{aligned} \frac{dN_z}{dt} &= \alpha_{z-1}AN_{z-1} - \alpha_z AN_z + \\ &\quad \Omega(N_z) - d_z N_z \end{aligned} \tag{2}$$

Here, $d_z > d_i$.

The multi-hit model allows us to keep track of the amount of antimicrobials A. In the basic model, there is no loss of antimicrobials in the system,

although they can be unbound or bound. The kinetics of the number of unbound antimicrobial molecules A is described by:

$$\frac{dA}{dt} = \sum_{i=0}^{n}(\mu_i i N_i + d_i i N_i - \alpha_i A N_i) \tag{3}$$

Note that, even though the variables of the multi-hit model denote numbers of cells and antimicrobial molecules, they are equivalent to concentrations because we assume a constant volume of $V = 1ml$.

Variations in the Multi-hit framework

In the basic model (equation 1–3), we assumed the case of complete multi-hit,[30] with $z = n$, which maybe best describes antimicrobials that need to hit a large fraction of the existing targets, for example AMPs.[32] Moreover we assumed that the total amount of antimicrobials is stable and that antimicrobial molecules are recycled back into the system when a cell dies.

We considered variants of the multi-hit model by relaxing some of the assumptions above: (i) not all target need to be hit $z < n$, which leads to non-effective binding, (ii) non-reversible binding, (iii) unspecific binding (i.e. binding to non-targets) in combination withnon-reversible binding, leading to molecules attaching non-reversibly to targets and non-targets alike, and (iv) enzymatic degradation. For details and model equations, see Supplement S1.

Pharmacodynamic function

To investigate how the efficacy of antimicrobials changes with the inoculum in our multi-hit model simulations, we used the pharmacodynamic (PD) function[1–3] as a measure of antimicrobial efficacy. Specifically, we calculated the bacterial net growth rate ψ on basis of the antimicrobial concentration A at the start of the simulation ($A(t = 0) = A_0$), with

$$\psi(A_0) \quad = \quad \psi_{max} - e(A_0) \tag{4}$$

In this common conceptualization of PD effects, the net growth rate ψ is divided into the growth rate of the bacteria in absence of the antimicrobials ψ_{max} and an effect $e(A_0)$ that depends on the concentration of the antimicrobials. Note that we use the concentration A_0, because throughout the simulation, A varies due to binding and degradation.

The effect $e(A_0)$ is further parameterized by the concentration, MIC_{PD}, that leads to a zero net growth rate, $\psi(A_0 = MIC_{PD}) = 0$, the minimum net growth rate that can be obtained with antimicrobials $\psi_{min} = \psi(A_0 \to \infty)$, and a parameter that characterizes the steepness of the pharmacodynamic relationship, κ:

$$\psi(A_0) \quad = \quad \psi_{max} - \frac{(\psi_{max} - \psi_{min})(A_0/MIC_{PD})^{\kappa}}{(A_0/MIC_{PD})^{\kappa} - \psi_{min}/\psi_{max}} \tag{5}$$

Here, the MIC_{PD} is a approximation of the MIC if the antimicrobial is

concentration independent.[33]

Alternatively, the PD function can be defined in terms of the parameters ψ_{max}, the maximum effect E_{max}, with $E_{max} = \psi_{max} - \psi_{min}$, the dose that results in half of the maximum effect A_{50}, with $\psi(A_{50}) = E_{max}/2$, and κ.[1] Combining the two definitions of the PD function allows the calculation of the slope parameter κ based on estimates of the other PD parameters:

$$\kappa = \frac{\log\left(-\frac{\psi max}{\psi min}\right)}{\log\left(\frac{MIC_{PD}}{A_{50}}\right)} \tag{6}$$

To estimate the PD parameters, we simulated time-kill curves for which we varied the antimicrobial concentration (Fig. S1). In a first step, the net growth rate was calculated based on the simulated time-kill curves using the relationship:

$$\psi(A) = \frac{log(N_{tot}(t_1)) - log(N_{tot}(t_0))}{t_1 - t_0} \tag{7}$$

These net growth rates were then used to estimate the PD parameters. The steepness parameter κ was calculated according to equation 6.

The estimates of the PD function parameters differed by inoculum. This dependence of the PD parameters were captured by an extended PD function, $\psi(A_0, N_{inoc})$. Specifically, the inoculum effect was quantified as slope m of

the function

$$A_{0,\psi=y}(N_{inoc}) \quad = \quad mN_{inoc} + c, \tag{8}$$

with $y = 0$ and $y = E_{max}/2$ in the case of $\psi(A_0 = MIC_{PD})$ and $\psi(A_0 = A_{50})$, respectively.

Pharmacokinetic function

To model bacterial population dynamics over longer time periods, we used the pharmacokinetic (PK) function as described in Regoes *et al.*[1] that addresses decay of antimicrobial molecules. In short, the initial antimicrobial concentration A_0 is assumed to decay with a rate k_A, and reset to A_0 every 8 hours, resulting in the following PK function:

$$A(t) \quad = \quad A_0 e^{k_A(t-p)} \tag{9}$$

with $n \times 8h \leq t \leq (n+1) \times 8h$.

Population model

To investigate the agreement between the bacterial population dynamics predicted by the common parameterization of antimicrobial efficacy with PD functions and those predicted by the more detailed multi-hit model, we incorporated the extended PD function and the pharmacokinetic function into

a population model:

$$\frac{dN_{tot}}{dt} = \psi_{max} N \left(1 - \frac{N}{K}\right) - e(A_0, N_{inoc})N \tag{10}$$

Here, K is the carrying capacity. We used this framework before in.[34]

Competition

To describe competition between an antimicrobial-sensitive and an antimicrobial-resistant strain, we extended the basic multi-hit model with a second strain. The number of sensitive and resistant cells are denoted by S and R, respectively. In our simulations, strain R is assumed to have a 10 times lower attachment rate , which could correspond to surface modification on resistant bacterial cells — a common resistance mechanism against AMPs.[27] For more details and model equations, see Supplement S2.

Implementation

The multi-hit model was implemented in R[35] (www.r-project.org; version 3.4.0). We solved the deterministic multi-hit model numerically with the package deSolve.[36] For plotting, we used the following packages: sfsmisc[37] and RColorBrewer.[38] The code is available upon request.

Results

We developed a mathematical model to describe the interaction between a bacterial population and antimicrobials. The model is based on the multi-hit concept of antimicrobial action,[30] according to which bacteria are killed as soon as a certain number of antimicrobial molecules hit the cell. This modeling framework describes the interaction between antimicrobial molecules and bacterial cells in more detail than common population biological models, and thus allowed us to investigate the determinants of the inoculum effects and predict quantitatively how the inoculum affects the pharmacodynamics.

Binding kinetics alone generates an inoculum effect

First, we investigated if an inoculum effect emerges simply as a result of concentration-dependent interactions between bacteria and antimicrobials and their binding kinetics. To that end, we used the basic multi-hit model that assumes reversible binding of antimicrobial molecules to target sites on the bacterial cells.

We found that an inoculum effect emerged. In our simulations, a bacterial population starting at a high inoculum grew over time, while a smaller initial population exposed to the same amount of antimicrobials declined (Fig. 3a). The explanation of the effect can be found in the concentration of free antimicrobial molecules that is sensitively affected by the bacterial inoculum (Fig. 3b), resulting in a lower efficacy if the inoculum is high.

Consistent with this explanation, the inoculum effect was abrogated if the free antimicrobial concentration was assumed to be constant independent of any binding processes (Fig. S2). Thus, only the binding of antimicrobial molecules — even though it was assumed reversible — caused an inoculum effect. Allowing for degradation of antimicrobials by extracellular enzymes in our model exacerbated the inoculum effect (Fig. 3), but was not necessary.

Inhibitory concentrations increase linearly with inoculum, but with a proportionality constant < 1

This inoculum effect can be quantitatively captured. Specifically, we studied how the inoculum affects the parameters of the *pharmacodynamic function* We considered the effect of the inoculum on the pharmacodynamic function that describes relationship between the net growth rate of bacterial population and the antimicrobial concentration (see Methods). Performing *in silico* time kill experiments, we estimated the pharmacodynamic functions for different inocula (Fig. 4a), and found that the efficacy across all antimicrobial concentrations is lower for larger inocula.

The most important efficacy parameter, the pharmacodynamic parameter MIC_{PD} increases linearly with the inoculum (Fig. 4b). However, the proportionality constant is less than one, meaning that a doubling of the inoculum does not lead to a doubling of number of antimicrobial molecules required to halt bacterial population from growing, and thus does not lead

to a doubling of the MIC_{PD}. We found a similar linear increase of another common efficacy measure, the A_{50} (see Methods for definition and Fig 4c). We also found that these two efficacy parameters increased linearly with the number of hit required to kill a bacterial cell, z (Fig. 4b, 4c) and that the increase was steeper when we allowed for degradation Fig. S3. Furthermore, the steepness parameter κ did not increase linearly, but saturated (Fig. 4d). These results hold for different modes modes of binding (Fig. S4).

Formally, we found the following relationship between the efficacy parameters and the inoculum N_{inoc}:

$$MIC_{PD}(N_{inoc}) = m_{MIC_{PD}} N_{inoc} + c_{MIC_{PD}} \tag{11}$$

$$A_{50}(N_{inoc}) = m_{A_{50}} N_{inoc} + c_{A_{50}} \tag{12}$$

Estimates for the intercept and slope parameters, c and m, of these linear relationship are shown in Table 1. With these two parameters, we can also describe how many more antimicrobial molecules we need to achieve the same pharmacodynamic efficacy when we increase the bacterial inoculum x-fold:

$$\frac{MIC_{PD}(xN_{inoc})}{MIC_{PD}(N_{inoc})} = \frac{mxN_{inoc} + c}{mN_{inoc} + c} \quad < \quad x \text{ for } c > 0 \tag{13}$$

The same relationship holds for the A_{50}, but, of course, with A_{50}-specific estimates for c and m. Thus, the increase in antimicrobials required to achieve the same effect on an x-fold increased inoculum is, according to our

model, less than x-fold and thus the proportionality constant is less than one.

The parameters characterizing the net bacterial growth rate in absence of antimicrobials is independent of the inoculum (Fig. S5a). Also the maximum effect that can be achieved with an antimicrobial, E_{max}, and the related minimum net growth rate, ψ_{min}, are independent of the inoculum (Fig. S5b, S5c). This is a result of the fact that these parameters are not affected by the binding of antimicrobial molecules to targets on the bacterial cells.[31]

The pharmacodynamic function captures only short-term dynamics without enzymatic degradation

The PD function $\psi(A_0)$ has been used to predict the long term bacterial population dynamics from antimicrobial effect on the initial net growth rate.[1,4,5,34,39–41] The underlying assumption in this approach is that the long-term bacterial population dynamics can be predicted on the basis of the initial short-term dynamics. This assumption is only valid if, *at any time during the time-kill process*, the net replication rate of a bacterial population of size N can be approximated by $\psi(A_0, N)$ that has been derived from the initial decline. We asked if an extended PD function $\psi(A_0, N_{inoc})$ adequately captures the long term dynamics.

To this end, we incorporated the quantitive effects of the inoculum on

each PD parameter into the following extended pharmacodynamic function:

$$\psi(A_0, N_{inoc}) = \psi_{max} - \frac{E_{max}\left(\frac{A_0}{A50(N_{inoc})}\right)^{\kappa(N_{inoc})}}{1+\left(\frac{A_0}{A50(N_{inoc})}\right)^{\kappa(N_{inoc})}} \tag{14}$$

$$= \psi_{max} - \frac{(\psi_{max}-\psi_{min})\frac{A_0}{MIC_{PD}(N_{inoc})}^{\kappa(N_{inoc})}}{\frac{A_0}{MIC_{PD}(N_{inoc})}^{\kappa(N_{inoc})} - \frac{\psi_{min}}{\psi_{max}}} \tag{15}$$

In this expression, $MIC_{PD}(N_{inoc})$, $A50(N_{inoc})$, and $\kappa(N_{inoc})$ denote the effects on inoculum on these PD parameters described in the previous subsection.

Next, we compared the long term population dynamics from the multi-hit model to predictions from a simple population model that relies on the extended PD function. Fig. 5 (solid lines) shows that the two approaches are in reasonable agreement assuming reversible binding only (basic model). Although the extended PD function underestimated the pharmacodynamic efficacy of the antimicrobial for low antimicrobial concentrations, the overall dynamical behavior of the system could be captured.

In contrast, the dynamics based on the multi-hit model, in which enzymatic degradation was allowed, differed fundamentally from the dynamics predicted with the pharmacodynamic function at low bacterial inoculum (5, blue, dashed lines). Degradation decreased antimicrobials over time and the population was eventually able to increase in size. This effect was more pronounced at low bacterial inoculum sizes because at high bacterial inocu-

lum sizes, the antimicrobial had already only little effect in the basic model. The extended PD function therefore captured simple bacterial population dynamics while only the mechanism-based multi-hit model could describe more complex population dynamics.

Inoculum effect alters the outcome of bacterial competition

To shed light on the effects of the inoculum on the competition between bacterial strains, we extended the multi-hit model framework by an additional resistant strain R. We assume that strain R carries a fitness cost and is hence not able to outcompete strain S at low antimicrobial concentrations (Fig. 6a). At intermediate concentrations, R outcompetes strain S (Fig. 6b). Lastly, at high concentrations, both strains are killed (Fig. 6c). The antimicrobial concentrations that mark the transitions between these three dynamical outcomes define the so-called *mutant selection window* (MSW).[42,43]

To investigate how the inoculum affects the size of the MSW, we conducted simulations across a range of inocula. In these simulations we also included a time frame of 7 days and repeated doses of the antimicrobial, to mimic antimicrobial usage in a treatment scenario. We therefore use the term "treatment MSW". The difference between the MSW and the treatment MSW is that the MSW is based on time-kill curves with a single input of antimicrobials, while the treatment MSW is based on time-kill curves in

treatment regimes.

We find that the treatment MSW shrinks and even vanishes for increasing inocula (Fig. 6d). Qualitatively similar results hold for simulations based on the extended PD function (Fig. S8). Simulating bacterial competition with a model that does not capture the inoculum effect, in contrast, left the MSW unaffected (Fig. 6e).

Discussion

In this study, we investigated how the bacterial inoculum affects the pharmacodynamics of antimicrobials with mathematical models. We were able to derive quantitative predictions for the inoculum effect not only on the MIC, but on multiple pharmacodynamic parameters. In addition to paving the way to a more comprehensive quantitative understanding of pharmacodynamic relationships, our predictions give rise to many experimentally testable hypotheses that we detail below.

Udekwu *et al.*[4] previously showed how the inoculum effect on the MIC can be integrated into the PD function. We extended these efforts to the additional pharmacodynamic parameters: the antibiotic concentration at which 50% of the maximum effect is obtained, A_{50}, and the parameter that describes the steepness of the pharmacodynamic relationship, κ. We found that the inoculum affected the PD parameters MIC_{PD} and A_{50} linearly, and the parameter κ non-linearly, resulting in an inoculum effect that does not

simply scale with the number of bacterial cells and antimicrobial molecules in the system. For example, to obtain the same effect on a, say, 100-fold increased bacterial population, we require less than 100-fold increase in the number antimicrobial molecules. [1]. This finding directly contradicts the argument that the inoculum effect is an artifact[26] arising from simple scaling. We could show that to achieve the same antimicrobial effect for a higher inoculum always requires a smaller increase in the number of antimicrobial molecules than a simple scaling would suggest. How much smaller depends on the specific parameters of the bacteria, antimicrobials and assay system. Interestingly, this means that, on a per-molecule level, antimicrobials become more effective with increasing inoculum.

The mechanistic underpinning of the inoculum effect in our simulations is that an increase in the bacterial inoculum results in higher depletion of free antimicrobials. We call this the *numbers game*:Binding, even if it is reversible, takes molecules out of the pool of free antimicrobial molecules, which in turn means that there are fewer molecules available for binding in the next time step. For larger bacterial inocula, the antimicrobial depletion by binding is increased, resulting in the inoculum effect. This numbers game results in an inoculum effect has already been observed in mathematical modeling studies.[20,30] Experimental studies that investigate the possibility and extent

[1] If we increase the bacterial inoculum from 5×10^5 to 5×10^7, we only need a 9-fold increase in antimicrobial molecules to achieve the same pharmacodynamic efficacy. These calculations are based on equation 13 and parameter values of slope m and intersection c are listed in Table 1 (reversible binding, $n = z = 10$).

of an inoculum effect arising from the numbers game alone, however, are rare.[4,18,20] Often, the inoculum effect is measured in systems where additional effects, e.g. non-targeted binding[44] or enzymatic degradation,[10–12,14–16,24,45–47] occur. These effects strongly increased the complexity of the population dynamics in our simulations. We therefore suggest to conduct experimental studies that address the inoculum effect first in systems that are governed only by a simple binding kinetics of antimicrobials to their targets. Such studies will form the basis of a quantitative understanding of the inoculum effect, and will provide valuable baselines for studies of systems with more complexity.

In addition to determining if the binding kinetics of antimicrobials alone results in an inoculum effect, we suggest experiments that assess the more qualitative prediction of the multi-hit modeling framework we presented here. For example, we showed that the more targets are needed to be hit to kill a cell (z), the more pronounced the inoculum affect was, quantified with the PD parameters MIC_{PD}, A_{50}, and κ. This result suggests that the size of the inoculum effect increases with the number of antimicrobial molecules are needed to kill a cell. In the case of antimicrobial peptides, this number varies from 80-800 in the case of protegrin[48] to $10^6 - 10^7$ in the case of PMAP-23.[32] With everything else being equal, we therefore expect a more pronounced inoculum effect for PMAP-23 than for protegrin. Similar predictions can be easily derived for other antimicrobials if quantitative parameters characterizing their mechanisms of action are known. Generally, the quantitative

relationships between the mechanisms of action of an antimicrobial, its pharmacodynamics, and the size of the inoculum effect represent a promising area of future theoretical and experimental studies.

The inoculum effect has been demonstrated experimentally for a number of bacterial strains and antimicrobials.[4,10–12,14–18,20,45,46] In most cases, the MIC was used to determine and quantify the inoculum effect.[4,10–12,14,17] Our comprehensive analysis of the inoculum's effect on all pharmacodynamic parameters showed that the steepness parameter κ increases. As a consequence, the inoculum effect is stronger for A_{50} than for MIC_{PD}, and determining the effect on A_{50} represent the most sensitive measure of the inoculum effect. Based on this observation, we recommend to not only quantify the MIC, but the whole pharmacodynamic relationship, and to use optical density (OD) measurements with caution because they do not allow the estimation of the pharmacodynamic relationship for antibiotic concentrations above the MIC_{PD}.

The absence of the inoculum effect has been reported repeatedly.[26,49] Our results shed light on the experimental settings that render the establishment of the inoculum effect most feasible. First, in our simulations the inoculum effect did not manifest itself in certain regions of the PD plane: for very small and very high antimicrobial molecule numbers, the inoculum effect was absent. Second, the establishment of the inoculum effect on the MIC is impaired because of the uncertainties arising from the doubling of the antimicribial concentration that is required according to the standard protocol

for MIC determination. To give a specific example: according to the standard protocol, "real" values of the MIC of 9 and 15 $\mu g/ml$ would be both estimated as 16 $\mu g/ml$; as a consequence, an inoculum effects increasing the MIC from 9 to 15 $\mu g/ml$ would not be detected. Lastly, in many studies an arbitrary threshold of an 8-fold increase of the MIC for a 100-fold higher bacterial inoculum is used to define the inoculum effect,[6,10,14–16] clouding inoculum effects of smaller extent. Rather than setting such a cutoff, we suggest to determine the MIC_{PD} or, even better, the A_{50} from adequately powered time-kill experiments.

Most surprisingly, we found that the inoculum effect can have unexpected and wide-ranging consequences for the evolutionary dynamics of bacterial populations under drug treatment. Our simulation of bacterial competition between drug-sensitive and -resistant strains revealed that the competitiveness of drug-resistant strain is overestimated when the inoculum effect is not considered. We found that "treatment MSW" — an estimation of the mutant selection window (MSW)[42,43] under drug treatment — shrinks for higher bacterial inoculum sizes. With regard to competition experiments between drug-sensitive and -resistant bacterial strains, such as those presented in e. g.,[43] our analysis predicts the selection coefficients to decrease with the inoculum. Hence the inoculum effect is generally expected to impact bacterial survival and evolution *in vitro*, within infected hosts, and potentially even on the epidemiological level.

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Transparency declaration

We have no competing interests.

Authors contributions

R.R.R. and D.Y.B. initiated this project, conceived the model, analyzed the results and drafted the manuscript. D.Y.B. wrote the code in R.

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Figures and Tables

Table 1: Results of fitting linear function $x = m_x N_{inoc} + c_x$ with $x = MIC_{PD}$ and $x = A_{50}$ to the simulated data. Reversible binding with $z = 1, 2, 10$ is also plotted in Fig. 4b - 4d.

			MIC_{PD}		A_{50}	
z	n	binding	$c_{MIC_{PD}}$	$m_{MIC_{PD}}$	$c_{MIC_{PD}}$	$m_{A_{50}}$
1	1	reversible	2514516	0.167	8284221	0.258
2	2	reversible	6234471	0.728	14665506	0.812
10	10	reversible	37675418	6.508	61533236	6.577
10	39	reversible	37368958	6.672	61618874	6.902
10	10	non-reversible	36111621	9.081	62435986	9.944
10	10	non-reversible, non-targeted	57313826	9.416	111901448	11.08

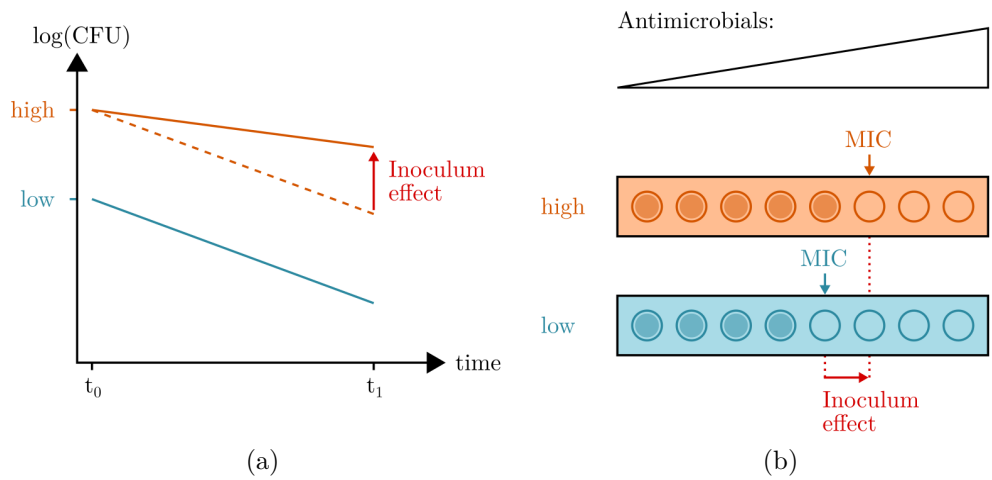


Figure 1: The inoculum effect changes efficacy of antimicrobials. Two common methods to quantify the efficacy of antimicrobials are (a) time-kill curves and (b) MIC measurements. (a) Time-kill curves illustrate the change in population size over time in presence of antimicrobials. The bacterial inoculum is the bacterial population size N at $t_0 = 0h$ ($N_{inoc} = N(t = 0)$). The slope of the curve results from fitting a log-linear function to the population dynamics. The slope of time-kill curve representing dynamics of bacteria starting at a high inoculum size (orange) is higher than dynamics of bacteria starting at a low inoculum (blue). In the absence of an inoculum effect, the slope is independent of the inoculum (dashed orange line). (b) Alternative to measuring time-kill curves, efficacy is typically determined with the MIC. An inoculum effect is observed if increasing the inoculum size from low (blue) to high (orange) increases the MIC.

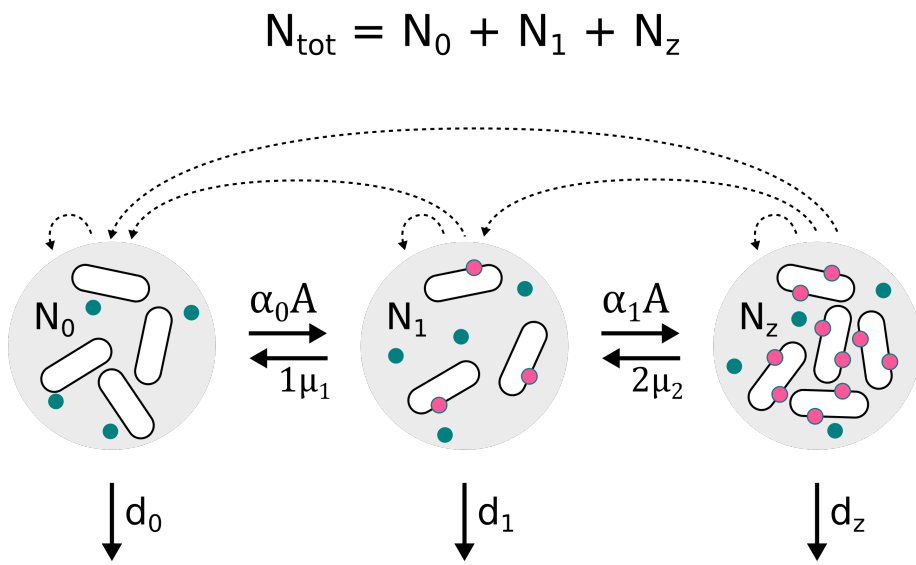
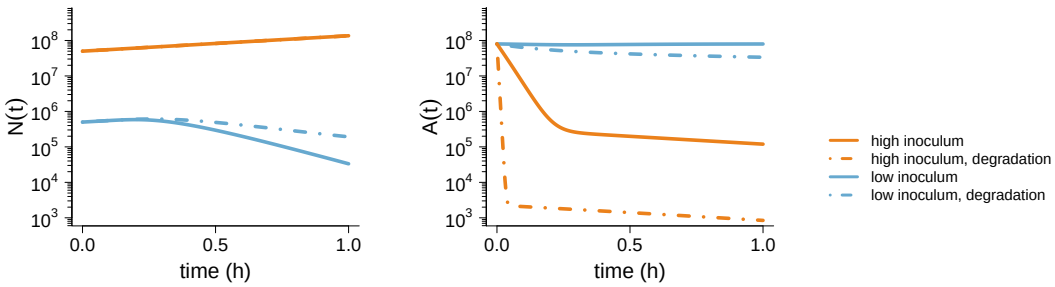


Figure 2: Schematic representation of the basic multi-hit model with three classes ($n = 2$). At every time point the bacterial population N_{tot} can be divided in n subpopulations based on the number of antimicrobials that have hit the bacterial cells. A hit is the mathematical simplification of adsorption and subsequent downstream processes connected to binding of one antimicrobial molecule to a bacterial target site and is described with the rate α. The parameter μ is the detachment of an antimicrobial from the bacterial target. We assumed that finding the bacterial cell is the rate limiting step for an antimicrobial molecule.[50] This is appropriate if the number of targets are in abundance, e.g. when antimicrobial peptides adsorb to the cell membrane of bacterial cells. For antimicrobials that act within the cell, a hit rate dependent on the number of free targets is more appropriate (see[20]). Each class N_i gains bacterial cells from the class N_{i-1} with the term $\alpha_{i-1}AN_{i-1}$ and a bacterium dies with an increased death rate d_z if two antimicrobial molecules have hit the cell, i.e. $z = 2$. Therefore, $N_{tot} = N_0 + N_1 + N_2$, with $N_2 = N_z$. A is the number of free antimicrobial molecules, here indicated in blue, antimicrobials that have hit a cell are indicated in pink. The rates of the ODE (equation 1) are indicated with arrows and bacterial replication with dashed arrows.



(a) (b)

Figure 3: (a) Increasing the bacterial inoculum size from $5 * 10^5$ (blue) to $5 * 10^7$ (orange) changes the efficacy of the antimicrobial due to (reversible, solid lines) reduction of the number of free antimicrobials (b). The dashed lines show the dynamics in a system with degradation of antimicrobials. Parameters not mentioned here are listed in Table S1.

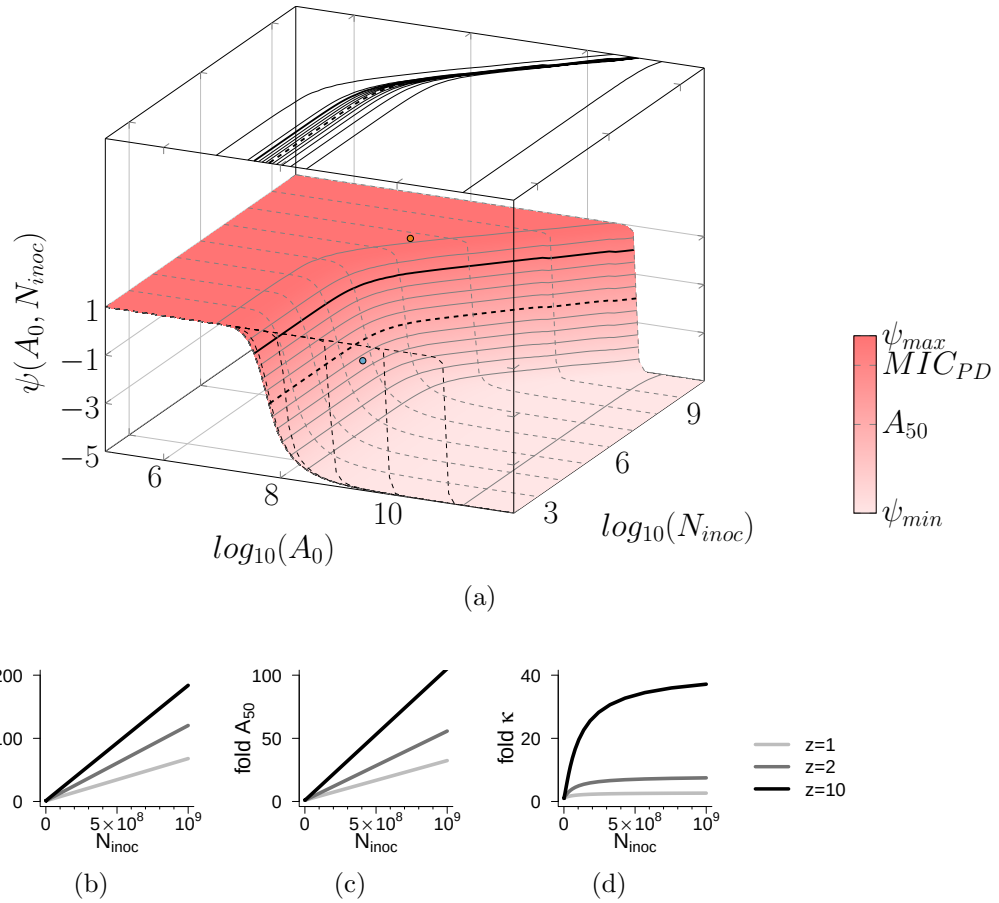
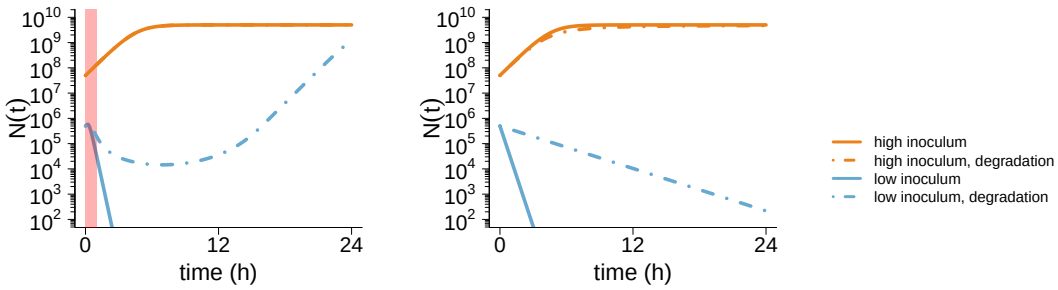


Figure 4: Inoculum size affects pharmacodynamics. (a) We determined the efficacy for varying inoculum sizes (N_{inoc}) with $z = 10$, resulting in a PD plane (note that in contrast to (b)-(d), the inoculum effect here is illustrated on a log_{10}-log_{10} scale). In the PD plane, solid gray contour lines show combinations of antimicrobials and inoculum sizes with the same net growth rate, both in the PD plane and projected above the PD plot. MIC_{PD} and A_{50} are indicated as bold solid and dashed line, respectively. For a number of inoculum sizes, we plotted the PD curve as dashed contour lines along the antimicrobial molecule number axis and again projected the contour lines, this time in front of the plot. The slopes of Fig. 3a (solid lines). are marked as dots in the corresponding colors. In (b)-(d) we plotted the PD parameters MIC_{PD}, A_{50}, and κ against the bacterial inoculum for varying number of hits needed to kill a bacterial cell (z). The bold black line in (b) corresponds to the bold solid line in (a) and the bold black line in (c) correspond the bold dashed lines in (a). Parameters not mentioned here are listed in Table S1.



(a) (b)

Figure 5: Dynamics of bacterial population $N(t)$ within the first 24h after exposure to antimicrobials were simulated with (a) the multi-hit model and (b) a population model that incorporates $\psi(A_0, N_{inoc})$ (equation 10). (a) We compared dynamics without extracellular proteins (solid lines) and with extracellular proteins (dashed lines) as well as dynamics for low (blue) to high (orange) initial bacterial population sizes N_{inoc}. For the dynamics of $A(t)$ and $E(t)$, see Fig. S7. The red area marks the time frame ($0h \leq t \leq 1h$) used to determine the net growth rate $\psi(A_0, N_{inoc})$, on which (b) is based on. For a higher resolution of this time frame, but without carrying capacity, see Fig. 3. (b) We predicted long term dynamics based on $\psi(A_0, N_{inoc})$ with a simple population model (equation 10). All parameters used for the simulations are listed in Table S1.

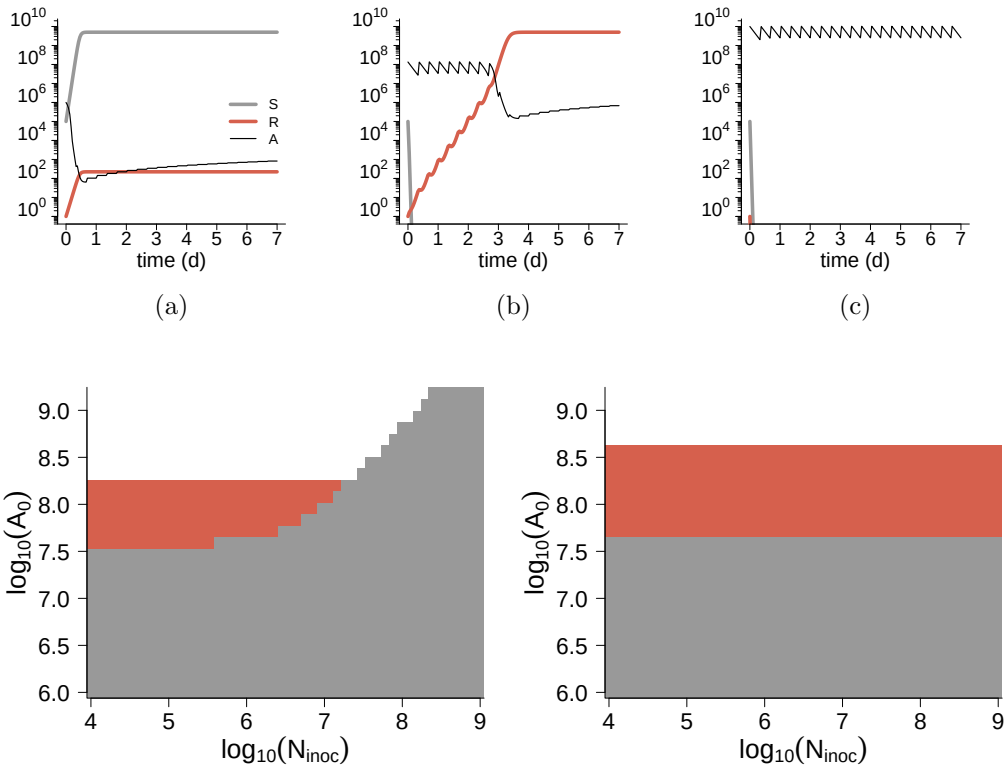


Figure 6: The inoculum effect influenced competition outcomes. Antimicrobial doses A_0 changed the outcome of competition: (a) S won when exposed to low antimicrobial doses, (b) R won at intermediate antimicrobial doses and (c) bacteria went extinct at high antimicrobial doses. (d) In an additional dimension, the inoculum effect changed the outcome of competition (gray: S is more abundant, red: R is more abundant, white: extinction ($S, R < 1$) at the end of the simulation). (e) For comparison, we used the PD function to simulate competition without inoculum effect.

Supplement

S1 Variations in the multi-hit framework: Details

In the case of (i) superfluous binding, we implemented the fractional multi-hit model by Hedges.[30] In this model, $z < n$, which means binding to targets is possible although the cell already reached the zombie stage. The fractional multi-hit model describes cases like AMPs that kill by forming pores into the membrane of bacteria.[48] Here, a cell has more targets, i.e. places where a pore can be formed in the membrane, than pores needed to kill a cell.

In the case of (ii) non-reversible binding, we set $\mu = 0$ and assumed that antimicrobial molecules are not recycled back into the system after cell death. Examples of non-reversible binding are binding of the antibiotics streptomycin and kanamycin to bacterial ribosomes.[23] This results in

$$\frac{dA}{dt} = \sum_{i=0}^{n} -\alpha_i A N_i \tag{16}$$

In the case of (iii) non-reversible and non-targeted binding, we assumed that in addition to non-reversible binding, molecules attach irreversibly to leaked cell content of dead cells. In our model, dead cells leak l targets per dying cell for non-targeted binding. L is the total number of targets available

for non-targeted binding with AMPs with the rate α_d.

$$\frac{dL}{dt} = l\sum_{i=0}^{n} d_i N_i \tag{17}$$

$$\frac{dA}{dt} = \sum_{i=0}^{n}(-\alpha_i A N_i - \alpha_d A L) \tag{18}$$

As in the cases (ii) and (iii), enzymatic degradation (case (iv)) decreases the amount of antimicrobials in the system. We assumed that all bacterial cells produce enzymes E with the production rate p and that enzymes degrade antimicrobial molecules with the rate d_E:

$$\frac{dE}{dt} = p\sum_{i=0}^{n} N_i - d_E E \tag{19}$$

In the presence of antimicrobials, enzymes in our model catalyze antimicrobial degradation in a two step process. First, an enzyme binds to an antimicrobial molecule to form the complex ($E.A$), then the bound enzyme catalyzes the degradation of the antimicrobial, resulting in enzymes and products: $\mathrm{E} + \mathrm{A} \underset{r_2}{\overset{r_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{A} \xrightarrow{r_3} \mathrm{E} + \mathrm{P}$

$$\frac{dE}{dt} = p\sum_{i=0}^{n} N_i - d_E E - r_1 EA + r_2 E.A + r_3 E.A \tag{20}$$

$$\frac{dE.A}{dt} = r_1 EA - r_2 E.A - r_3 E.A \tag{21}$$

$$\frac{dA}{dt} = \sum_{i=0}^{n}(\mu_i i N_i + d_i i N_i - \alpha_i A N_i) - r_1 AE + r_2 E.A \tag{22}$$

S2 Cometition

The competition between a sensitive strain S and a resistant strain R is described with:

$$\begin{aligned}
\frac{dS_i}{dt} &= \alpha_{i-1}AS_{i-1} - \alpha_i AS_i + \\
&\quad \mu_{i+1}(i+1)S_{i+1} - \mu_i iN_i + \\
&\quad \Omega(S_i) - d_i S_i
\end{aligned} \tag{23}$$

$$\begin{aligned}
\frac{dR_j}{dt} &= \alpha_{j-1}AR_{j-1} - \alpha_j AR_j + \\
&\quad \mu_{j+1}(j+1)R_{j+1} - \mu_j jR_j + \\
&\quad \Omega(R_j) - d_j R_j
\end{aligned} \tag{24}$$

$$\begin{aligned}
\frac{dA}{dt} &= \sum_{i=0}^{n}(\mu_i iS_i + d_i iS_i - \alpha_i AS_i) + \\
&\quad \sum_{j=0}^{n}(\mu_j iR_j + d_j jR_j - \alpha_i AR_i)
\end{aligned} \tag{25}$$

Here, i and j describe the number of targets hit on a sensitive and resistant bacterial cell, respectively, with $0 \leq i, j \leq n$. To model realistic long term population dynamics, we also included a carrying capacity, as it is done in:[20]

$$\Omega(N_i) = \left(2\sum_{x=i}^{n}(f_{x,i}b_x N_x) - b_i N_i\right)\left(1 - \frac{N_{tot}}{K}\right) \tag{26}$$

We simulated population dynamics in a window of 7 days.

S3 Tables

Table S1: Variables, indices and parameters of the multi-hit model. If not mentioned elsewhere, the parameter values used for the simulations are listed here. All rates are in $\frac{1}{h}$.

Variables	Definition
N_i	Number of bacteria that are hit by i antimicrobial molecules at time t.
N_{tot}	Total number of bacteria at time t, with $N_{tot} = \sum_{i=0}^{n} N_i$.
N_{inoc}	Number of bacteria at time $t = 0$, with $N_{inoc} = N_{tot}(t = 0)$.
A	Number of free antimicrobial molecules at time t, with $A_0 = A(t = 0)$.
E	Number of extracellular bacterial enzyme molecules at time t. We used $E(t = 0) = N_{inoc}p/(b_0 - d_0 - d_E)$.
$E.A$	Number of enzyme-antimicrobial complexes at time t, with $E.A(t = 0) = 0$.
S	Number of sensitive bacterial cells at time t.
R	Number of resistant bacterial cells at time t, with $R(t = 0) = 1$.
L	Number of targets for non-targeted binding at time t, with $L(t = 0) = 0$.

Indices	Definition
n	Total number of possible hits.
i,j	Number of hits, with $0 \leq i, j \leq n$. For the simulations, $n = 10$
z	Number of hits needed to kill a bacterial cell, with $1 \leq z \leq n$. If not mentioned, $z = 10$ is used.

Parameters	Definition	Value
t	time-point of the simulation, if not mentioned, $t_0 = 0h$ and $t_1 = 1h$ are used to determine the net growth rate of the bacterial population.	
b_i	Replication rate of bacterial cells in class N_i, with $i < z$.	1
b_z	Replication rate of bacterial cells in class N_i, with $i \geq z$.	0
d_i	Death rate of bacterial cells in class N_i, with $i < z$	0
d_z	Death rate of bacterial cells in class N_i, with $i \geq z$.	5
α_i	Hit rate of antimicrobial molecules with $0 \leq i \leq n$.	5×10^{-7}
α_n	Hit rate of antimicrobial molecules in the case all targets are hit.	0
μ_i	Detachment rate of antimicrobial molecules with $0 \leq i \leq n$.	0.1
r_1	Association rate of extracellular enzymes to antimicrobial molecules.	10^{-7}
r_2	Disassociation rate of $E.A$ complexes.	5×10^{-11}
r_3	Rate of catalysis.	5×10^{-4}
p	Production rate of bacterial enzymes.	50
d_E	Decay rate of bacterial enzymes.	0.1
α_d	Rate of non-targeted binding.	5×10^{-7}
l	Amount of intracellular targets of a bacterial cell.	20
K	Carrying capacity of the bacterial population	5×10^{9}

S4 Figures

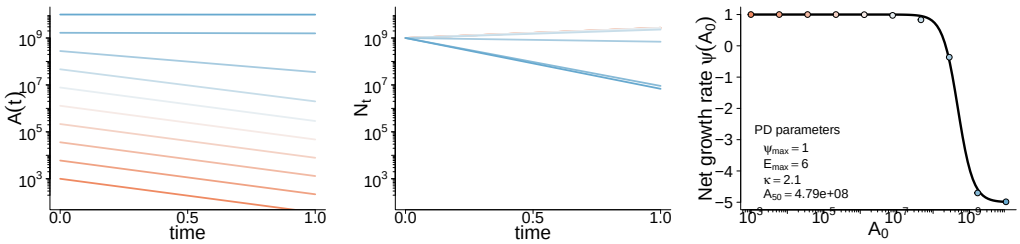


Figure S1: Example of an estimation of PD parameters for $N_{inoc} = 109$. The estimation of PD parameters is based on the time-kill curves (middle panel) that are simulated with varying concentrations of A at time $t = 0$, A_0 (left panel), but always for the same inoculum concentration (middle panel). The slope was estimated for the time frame from $t_0 = 0$ to $t_1 = 1$h. Resulting, the parameter for $\psi(A_0)$ (right panel) are estimated for the given bacterial inoculum.

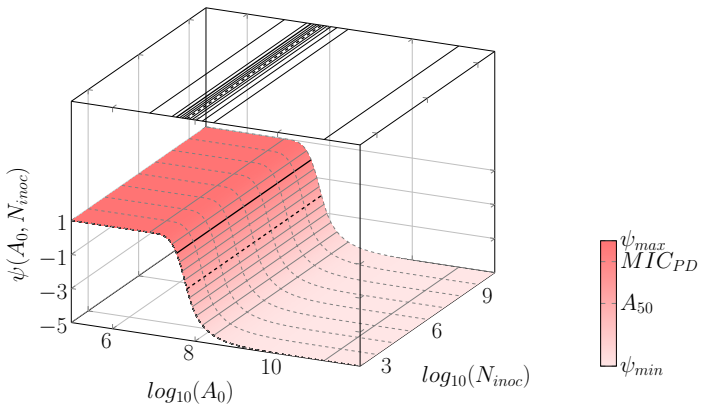
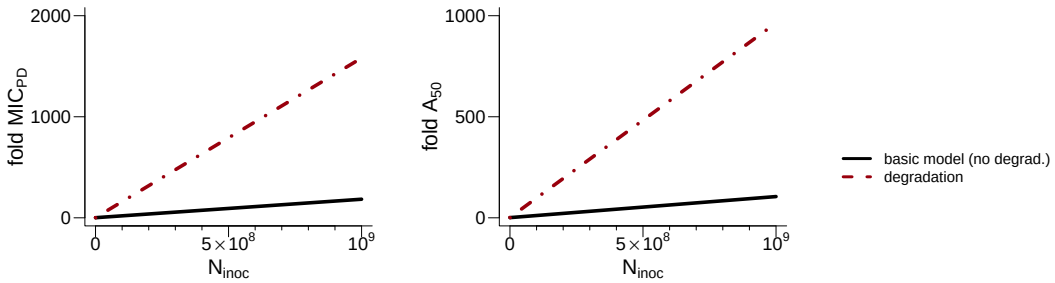
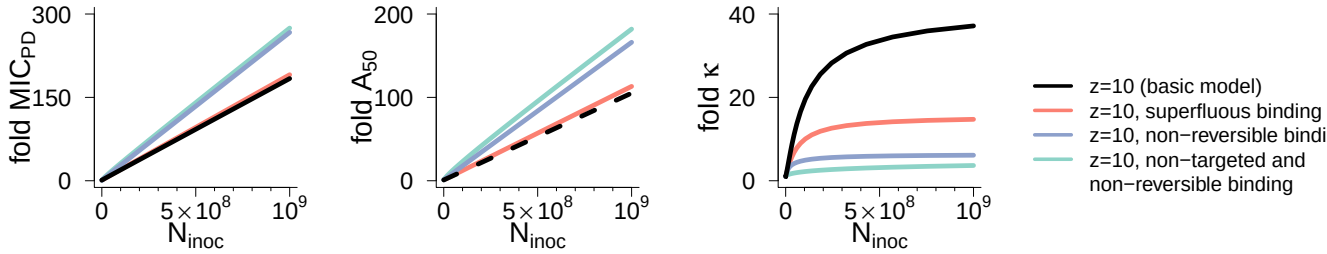


Figure S2: PD plane without inoculum effect due to fixed number of free antimicrobials. Here, we assumed $\frac{dA}{dt} = 0$. Parameters not mentioned here are listed in Table S1.



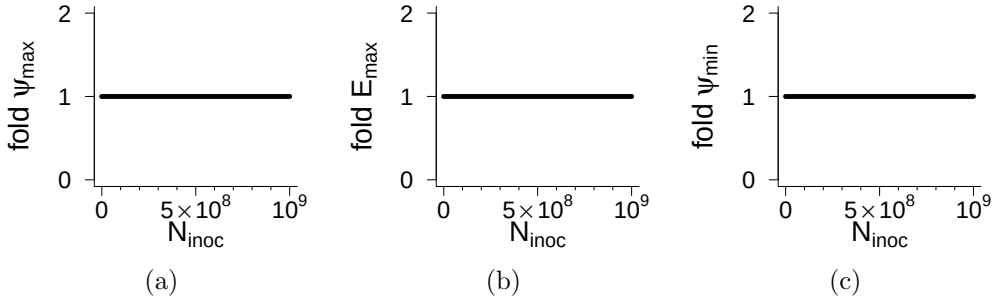
(a) (b)

Figure S3: Degradation enhances the inoculum effect. Degradation (purple, dashed line) increases the number of molecules needed to achieve (a) $\psi(MIC_{PD}) = 0$ and (b) $\psi(MIC_{A_{50}}) = E_{max}/2$ compared to reversible binding only (black line). For other PD paramters, see Fig. S6. All parameters used for the simulations are listed in Table S1.



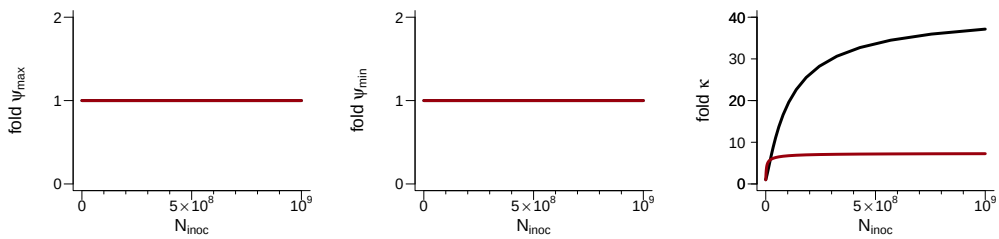
(a) (b) (c)

Figure S4: PD parameters (a) MIC_{PD}, (b) A_{50}, and (c) κ for superfluous, non reversible and non-reversible and non-targeted binding compared to reversible binding ($z = 10$, as in Fig. 4b- 4d). Note that all model variations shown here do not result in an change of the PD parameters E_{max}, ψ_{max}, and ψ_{min}. All parameters used for the simulations are listed in Table S1.



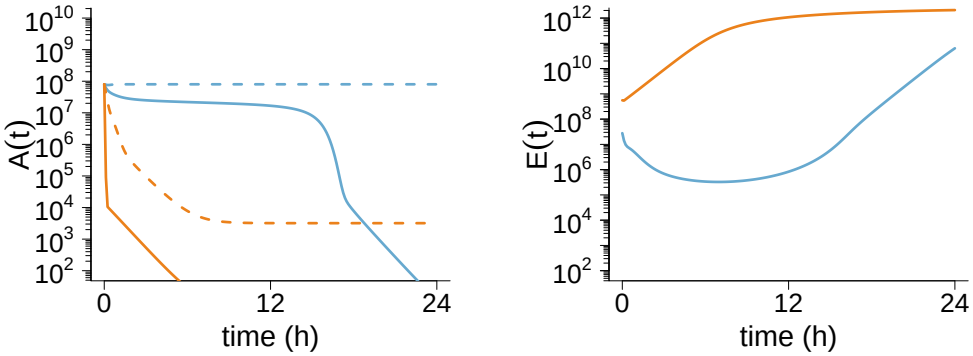
(a) (b) (c)

Figure S5: Changing the bacterial inoculum does not change PD parameters (a) ψ_{max}, (b) E_{max}, and (c) ψ_{min}. Note that all model variations shown in Fig. 4b- 4d result in no effect on these PD parameters. All parameters used for the simulations are listed in Table S1.



(a) (b) (c)

Figure S6: Effect of degradation on PD parameters (a) ψ_{max}, (a) ψ_{min}, and κ. All parameters used for the simulations are listed in Table S1.



(a) (b)

Figure S7: Initial dynamics are differ fundamentally from long-term dynamics. The figures show the dynamics of (a) the amount of antimicrobial molecules ($A(t)$), (b) bacterial population size $N(t)$, and (c) extracellular proteins ($E(t)$) within the first $24h$ after exposure to antimicrobials. Here, we compared dynamics with extracellular proteins (solid lines) and dynamics of the basic model (dashed lines) as well as dynamics for a low (blue) and a high (orange) initial bacterial population size. In (b), we marked the time frame typically used to determine the net growth rate $\psi(A_0)$. All parameters used for the simulations are listed in Table S1.

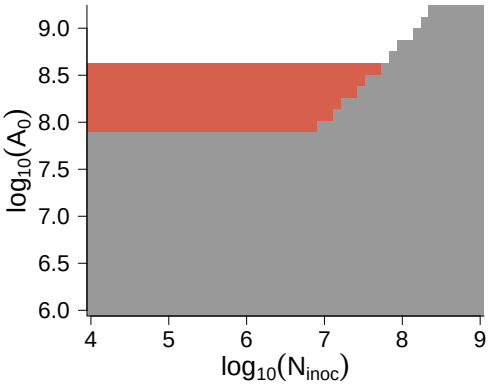


Figure S8: The effect of the bacterial inoculum on outcome of competition with the extended PD function (gray: S is more abundant, red: R is more abundant, white: extinction at the end of the simulation). All parameters used for the simulations are listed in tab. S1.