The pharmacodynamic inoculum effect from the perspective of bacterial population modeling

Desiree Y. Baeder¹ and Roland R. Regoes¹

¹Institute of Integrative Biology, ETH Zurich, Universitätsstrasse 16, 8092 Zürich, Switzerland

1 Abstract

2 The quantitative determination of the effect of antimicrobials is essential for
 3 their clinical application, for our microbiological understanding of pharma-
 4 codynamics, and for predictive mathematical modeling. It is well-established
 5 that the effect of antimicrobials depends on the bacterial inoculum size used
 6 in assays — a phenomenon called *inoculum effect*. The commonly used phar-
 7 macodynamic (PD) function that captures the antimicrobial effect, however,
 8 is derived from assays conducted with one bacterial inoculum size only. Here,
 9 we used the mathematical multi-hit model, which describes antimicrobial ac-
 10 tion mechanistically, to quantify the effect of the inoculum on the PD param-
 11 eters. Based on our quantitative analysis, we propose an extended PD func-
 12 tion that incorporates the inoculum effect. The extended PD function could
 13 mimic the long-term population dynamics for reversibly binding antimicro-
 14 bials, but failed when antimicrobials were also assumed to be degraded. We

15 further show that, in simulation with competing antimicrobial-sensitive and
 16 -resistant bacteria, neglecting the inoculum effect leads to an overestimation
 17 of the competitive ability of the resistant strain. Our work emphasizes that
 18 the PD function — and in general any efficacy measure, such as the MIC —
 19 should include information about the inoculum size on which it is based, and
 20 ideally account for the inoculum effect.

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

23 Introduction

24 Efficacy of antimicrobials against bacteria is quantified using time-kill curves
 25 and is subsequently captured in so-called pharmacodynamic (PD) functions,
 26 also known as E_{max} and Zhi models (1, 2, 3). A time-kill curve captures how
 27 a bacterial population changes over time in presence of a given concentration
 28 of an antimicrobial (Fig. 1a). During exponential growth, time-kill curves are
 29 approximately linear on a logarithmic scale. Thus, the slopes of these log-
 30 linear time-kill curves describe the change of the bacterial population over
 31 time and are a direct measure of the bacterial net growth rate. The PD func-
 32 tion returns the net growth rate for any given antimicrobial concentration.
 33 The parameters of the PD function are estimated based on several time-kill
 34 curves differing in the antimicrobial concentration, but with the same initial

35 bacterial population size (bacterial inoculum) (1, 4, 5, 6, 7, 8).

36 A more widespread but less informative way to express efficacy of an-
 37 timicrobials is the minimum inhibitory concentration (MIC). The MIC is the
 38 lowest drug concentration that prevents visible bacterial growth (Fig. 1b).
 39 For concentration-independent antimicrobials (steep PD curve), the MIC is
 40 comparable to the antimicrobial concentration that results in a time-kill curve
 41 with the slope of zero (8). In this case, one of the four parameters of the
 42 PD function, which we call MIC_{PD} , is equivalent to the MIC. Although it is
 43 recognized that the MIC might change due to change in bacterial inoculum
 44 size (see for example the MIC protocol by Wiegand *et al.* (9)), both the
 45 CLSI and the EUCAST recommendation to measure the MIC is based on
 46 a standard bacterial inoculum of 5×10^5 CFU/ml only (10, 11). Thus, as
 47 in the case of time-kill curves, standardization of the inoculum neglects any
 48 effect on efficacy of antimicrobials due to changes in the bacterial inoculum.

49 Both experimental and theoretical work has put forward hypotheses ex-
 50 plaining why the inoculum size matters when determining the efficacy for
 51 antimicrobials targeting exponentially growing bacteria. Maybe the most in-
 52 tuitive explanation is that the amount of antimicrobials is finite in the system
 53 and a decrease in the amount of free antimicrobials changes the efficacy of the
 54 antimicrobial (12, 13, 14). The amount of antimicrobials decreases over time
 55 because of binding processes. Assuming mass action kinetics, the number of
 56 free antimicrobial molecules is reduced proportional to bacterial cell density,
 57 which results in changes in the efficacy of the antimicrobial. In addition to

this binding kinetic effect, bacteria can produce extracellular enzymes that further decrease the concentration of antimicrobials. For example, β -lactam antibiotics are degraded by β -lactamases (15, 16, 17, 18, 19, 20, 21, 22), and antimicrobial peptides can be degraded by proteases (23, 24, 25). The higher the bacterial population, the more enzymes are produced and thus the higher the change in antimicrobial efficacy.

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 (20). Since then, the inoculum effect has been measured experimentally for a variety of combinations of bacterial species and antibiotics (13, 17, 18, 21, 22, 26, 27, 28). Recently, the inoculum effect became of interest in the field of antimicrobial peptides (AMPs) (29, 30). It is common to determine the inoculum effect in terms of the change in the MIC with inoculum size (13, 17, 22, 26, 27, 29). To our knowledge, the inoculum effect has not yet been determined using the more comprehensive PD function approach. In addition to its experimental quantification in the wet lab, the inoculum effect has been explored with mathematical models. These models either comprise a mechanistic description of the interaction between antimicrobial molecules and bacterial cells (12, 14, 28, 31, 32) or are based directly on MIC measurements (13). In the latter case, the inoculum effect was determined by measuring the MIC at a variation of bacterial inoculum sizes. The measured effect was then incorporated by extending the PD function for the MIC_{PD} parameter.

81 Here we use mathematical modeling as a tool to explore how bacterial
 82 inoculum size affects all four parameters of the PD function. The advantage
 83 of employing mathematical models is the possibility to determine the efficacy
 84 of an antimicrobial for various bacterial inoculum sizes and to explore mech-
 85 anisms of the inoculum effect individually and in combination, which would
 86 be challenging experimentally. We adopt a mathematical framework called
 87 the multi-hit model, which is based on the idea that antimicrobial molecules
 88 hit, i.e. bind to, cellular targets (33). In this approach, complex chemical
 89 reactions and molecular pathways are summarized in a simple mechanistic
 90 structure. This framework enables us to simulate bacterial population dy-
 91 namics, from which we can infer the pharmacodynamic curve. We already
 92 used the multi-hit model in previous work (34) to describe the action of
 93 AMPs and a similar, albeit more antibiotic-specific, approach was used to
 94 model classic reaction kinetics of antibiotics (12).

95 A mechanistic explanation for the inoculum effect has already been pro-
 96 vided by Abel zur Wiesch *et al.* (12). In their model, binding of molecules
 97 decreases the pool of free antimicrobials and thus the antimicrobial efficacy.
 98 Here, we use the multi-hit model to extend this analysis and apply the results
 99 to the PD function. With the multi-hit framework, we are able to ask how the
 100 changes in the inoculum size affects the pharmacokinetics of antimicrobials
 101 and how this in turn affects the PD curve. We show that reversible binding
 102 alone can explain the inoculum effect. Further, we quantify the inoculum
 103 effect in terms of change in PD parameter values with increasing inoculum

size. In a next step, we analyze how the PD curve changes due to adding degradation of antimicrobial molecules by bacterial enzymes and illustrate how well the PD function predicts long-term population dynamics. Last, we apply our findings to the population dynamics of competing bacterial strains and predict how the inoculum size influences competition outcomes when a resistant strain is introduced.

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 (33). In the model, bacteria are killed as soon as a certain number of antimicrobial molecules hit the cell. The modeling framework allowed us to keep track of the number of bacteria and antimicrobial molecules over time. We assumed that the interaction occurs in a constant volume. Hence, the amounts of bacteria and antimicrobial molecules are equivalent to their concentration.

Model predictions on pharmacokinetics and pharmacodynamics

First we used our multi-hit modeling framework to investigate if an inoculum effect emerges simply as a result of concentration-dependent interactions between bacteria and antimicrobials and their binding kinetics. To this end,

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.

our basic model assumed reversible binding of antimicrobial molecules to target sites on the bacterial cells. In the model, antimicrobials were recycled back into the system once a bacterial cell died. As a consequence, only the amount of free antimicrobials, but not their total amount, decreased during the time course.

The decrease in the amount of free antimicrobials was dependent on the bacterial inoculum N_{inoc} (Fig. 2a). The higher the bacterial inoculum, the more antimicrobial molecules hit bacteria due to the mass-action type binding kinetics in our model, and the fewer antimicrobial molecules were free to hit in the following time step. The decrease in the amount of free antimicrobials due to initial binding to bacteria in the inoculum, in turn, changed the bacterial population dynamics. A bacterial population starting at a high bacterial inoculum grew over time, while a population exposed to the same amount of antimicrobials but starting at a low bacterial inoculum declined (Fig. 2b). Thus, binding of antimicrobial molecules — even though it was reversible — caused an inoculum effect, i.e. the antimicrobial decreased in efficacy with increasing bacterial inoculum.

Figure 2: Inoculum size affects pharmacokinetics and pharmacodynamics. PD functions are estimated for a range of inoculum sizes. Increasing the bacterial inoculum size from 5×10^5 (blue) to 5×10^7 (orange) changes the efficacy of antimicrobials (b) due to reversible reduction of the number of free antimicrobials (a). (c) We determined the efficacy for varying inoculum sizes (N_{inoc}) with $z = 10$, resulting in a PD plane (note that in contrast to (d)-(f), 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 (b) are marked as dots in the corresponding colors. In (d)-(f) 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 (d) corresponds to the bold solid line in (c) and the bold black line in (e) correspond the bold dashed lines in (c). Parameters not mentioned here are listed in Table S1.

141 This inoculum effect altered the parameter of the pharmacodynamic (PD)
 142 function. The PD function describes the net growth rate of bacterial popula-
 143 tion for a given antimicrobial concentration and is a tool to describe efficacy
 144 of an antimicrobial. Here, we compared parameterized PD functions that are
 145 based on different inoculum sizes N_{inoc} with the PD plane (Fig. 2c). The PD
 146 plane shows that the shape of the PD function changed with increasing in-
 147 bacterial inoculum. To illustrate the magnitude of change, we plotted the net
 148 growth rates of the population dynamics pictured of Fig. 2b in the PD plane
 149 (Fig. 2c). The net growth rate of a population based on a small inoculum size
 150 (blue point) was negative, while it was positive for a populations based on a
 151 large inoculum (orange point). Note that both populations were exposed to
 152 the same amount of antimicrobials at the beginning of the simulation (A_0).
 153 Without restricting A_0 , the inoculum effect vanishes (Fig. S2). The exam-
 154 ple illustrates that the PD function and therefore the PD parameters are
 155 dependent on the bacterial inoculum.

156 In terms of specific PD parameters, both the MIC_{PD} , the amount of
 157 antimicrobials that results in zero growth, and A_{50} , the antimicrobial con-
 158 centration that results in half of the maximum effect, increased with in-
 159 creasing inoculum (Fig. 2d, 2e). The increase was linear and increased for
 160 increasing number of targets needed to kill a cell (z) (Fig. 2d, 2e). Superflu-
 161 ous binding according to the fractional single hit model, i.e. antimicrobials
 162 can hit more than z targets, increased the inoculum effect, as well as non-
 163 reversible binding, and non-targeted binding combined with non-reversible

binding (Fig. S3). The steepness of the curve, parameterized with the PD parameter κ , increased non-linearly with increasing inoculum size (Fig. 2f). The maximum effect of the antimicrobial E_{max} is defined by the parameters ψ_{max} , the maximum growth rate in absence of the antimicrobial ($\psi_{max} = \psi(A = 0)$), and ψ_{min} , the minimum net growth rate for $A \rightarrow \infty$. In previous work (34) we showed that ψ_{max} is only dependent on replication and death, parameterized by the multi-hit parameter b and d , respectively, with ($\psi_{max} = b - d$). We also showed that ψ_{min} is solely determined by the death rate of the zombie stages (d_z) (34). This means, both PD parameters ψ_{max} and ψ_{min} and therefore also the parameter E_{max} were independent of any binding processes and the bacterial inoculum (Fig. S4b, S4c).

Quantifying the inoculum effect

We quantified the inoculum effect in terms of the PD function by incorporating how the PD parameters change due to changes in the bacterial inoculum. We already determined that both MIC_{PD} and A_{50} increased linearly with increasing bacterial inoculum in our basic multi-hit model. The linear increase is a direct measure of the inoculum effect and therefore, we used the linear relationship $A_{0,\psi}(N_{inoc}) = mN_{inoc} + c$ to quantify the inoculum effect.

This linear relationship describes how many antimicrobial molecules A_0 we need to achieve an effect, specified in terms of net growth rate ψ , for a given bacterial inoculum size N_{inoc} . The slope m in this relation measures the inoculum effect. The inoculum effect increased with increasing number

186 of hits needed to kill a cell (z), and was higher when we assumed superfluous
 187 binding, non-reversible binding, and non-reversible and non-targeted binding
 188 compared to the basic model of reversible binding (Table 1). Moreover, it was
 189 higher for A_{50} than for MIC_{PD} in all the above cases. While the intersection
 190 c was influenced by the number of hits needed to kill a cell (Table 1) it
 191 is per definition independent of the inoculum effect. The slope m and the
 192 intersection c describe how many antimicrobial molecules we need to achieve
 193 a given pharmacodynamic efficacy. With these two parameters, we can also
 194 describe how many more antimicrobial molecules we need to achieve the same
 195 pharmacodynamic efficacy when we increase the bacterial inoculum x -fold:

$$\frac{A_0(xN_{inoc})}{A_0(N_{inoc})} = \frac{mxN_{inoc} + c}{mN_{inoc} + c} < x \text{ for } c > 0 \quad (1)$$

196 Thus, the increase in antimicrobials required to achieve the same effect on
 197 an x -fold increased inoculum is, according to our model, less than x -fold.

198 The PD parameter κ was calculated in terms of the PD parameters
 199 MIC_{PD} and A_{50} . The bacterial inoculum size N_{inoc} affected the shape and
 200 position of the pharmacodynamic curve, with the PD parameter MIC_{PD} ,
 201 A_{50} and κ . Temporary binding of antimicrobial molecules alone was enough
 202 to result in a perceivable inoculum effect and the inoculum effect was in-
 203 tensified if we assumed more complex binding processes. In agreement with
 204 this result, the inoculum effect was abrogated if the free antimicrobial con-
 205 centration was assumed to be constant independent of any binding processes

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. 2d - 2f.

z	n	binding	MIC_{PD}		A_{50}	
			$c_{MIC_{PD}}$	$m_{MIC_{PD}}$	$c_{MIC_{PD}}$	$m_{A_{50}}$
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

(Fig. S2). Using our results, we were able to describe the net growth of bacteria for given N_{inoc} and A :

$$\psi(A_0, N_{inoc}) = \psi_{max} - \frac{E_{max} \left(\frac{A_0}{A_{50}(N_{inoc})} \right)^{\kappa(N_{inoc})}}{1 + \left(\frac{A_0}{A_{50}(N_{inoc})} \right)^{\kappa(N_{inoc})}} \quad (2)$$

$$= \psi_{max} - \frac{(\psi_{max} - \psi_{min}) \frac{A_0}{MIC_{PD}(N_{inoc})}^{\kappa(N_{inoc})}}{\frac{A_0}{MIC_{PD}(N_{inoc})}^{\kappa(N_{inoc})} - \frac{\psi_{min}}{\psi_{max}}} \quad (3)$$

with

$$A_{50}(N_{inoc}) = m_{A_{50}} N_{inoc} + c_{A_{50}} \quad (4)$$

$$MIC_{PD}(N_{inoc}) = m_{MIC_{PD}} N_{inoc} + c_{MIC_{PD}} \quad (5)$$

$$\kappa(N_{inoc}) = \frac{\log \left(-\frac{\psi_{max}}{\psi_{min}} \right)}{\log \left(\frac{MIC_{PD}(N_{inoc})}{A_{50}(N_{inoc})} \right)} \quad (6)$$

209 Degradation enhances the inoculum effect

210 Next, we extended our basic model to integrate proteolytic degradation of
 211 antimicrobials by extracellular enzymes. Bacteria that are able to express
 212 extracellular enzymes have often been used to explore the inoculum effect
 213 (17, 18, 19, 20, 21, 22). Assuming that each cell produces enzymes, the
 214 amount of enzymes is dependent on the bacterial density. In contrast to
 215 reversible binding, which we assumed in our basic model, degradation of
 216 antimicrobials decreased the number of antimicrobials irreversibly, i.e. the
 217 total number of antimicrobials in the system decreased.

218 Degradation of antimicrobial molecules in addition to reversible binding
 219 enhanced the effect the bacterial inoculum has on the dynamics of the an-
 220 timicrobials. While in the basic model the amount of free antimicrobials
 221 was only reduced due to binding, degradation decreased the amount of free
 222 antimicrobials further (Fig. 3a). The reduction of antimicrobials in turn en-
 223 abled higher bacterial population sizes (Fig. 3b) compared to the basic model.
 224 Not surprisingly, quantification of the inoculum effect based on PD param-
 225 eters showed that the inoculum effect was stronger assuming degradation on
 226 top of reversible binding compared to reversible-binding only: The slopes
 227 of MIC_{PD} and A_{50} , $m_{MIC_{PD}}$ and $m_{A_{50}}$, were higher compared to reversible
 228 binding (Fig. 3d, 3e). Again, due to the assumptions in the model, the size
 229 of the PD curve, i.e. parameter ψ_{max} , ψ_{min} , and therefore E_{max} were not
 230 affected by the inoculum (Fig. S5a, S5b).

Figure 3: Degradation enhances the inoculum effect. (a) Antimicrobials are affected by degradation depending on the (b) bacterial population size. Here, we use two examples of bacterial inoculum sizes: low (blue) and high (orange). Changes in the dynamics of antimicrobials due to degradation depend on the initial bacterial inoculum size. In general, degradation (purple, dashed line) increases the number of molecules needed to achieve (c) $\psi(MIC_{PD}) = 0$ and (d) $\psi(MIC_{A_{50}}) = E_{max}/2$ compared to reversible binding only (black line). All parameters used for the simulations are listed in Table S1.

231 Evaluation of the extended PD function

232 The PD function $\psi(A_0)$ describes efficacy based on initial dynamics in the
 233 system and has been used to extrapolate long term bacterial population
 234 dynamics, e.g. (1, 13, 35, 36, 37, 38, 39). Here, the underlying assumption
 235 is that the long-term bacterial population dynamics can be predicted on the
 236 basis of the initial short-term dynamics. This assumption is only valid if, *at*
 237 *any time during the time-kill process*, the net replication rate of a bacterial
 238 population of size N can be approximated by $\psi(A_0, N)$ that has been derived
 239 from the initial decline. This may not be the case because at later times the
 240 bacterial cells will have bound antimicrobial molecules, resulting in a state
 241 more susceptible to the antimicrobial effect. For these reasons, we asked if
 242 the extended PD function $\psi(A_0, N_{inoc})$ can be used to describe long term
 243 dynamics.

244 The multi-hit model enabled us to analyze if the short term population
 245 dynamics captured in the extended PD function predict long term dynamics.
 246 For this, we parameterized the extended PD function $\psi(A_0, N_{inoc})$ based on
 247 short term population dynamics from the multi-hit model. The multi-hit was
 248 then extended with a carrying capacity to account for density dependent
 249 reduction in growth and used to simulate long term population dynamics.
 250 The long term population dynamics from the multi-hit model were compared
 251 to predictions from a simple population model that relies on the extended
 252 PD function.

253 Fig. 4 (solid lines) shows that the two approaches are in reasonable agree-

ment 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 (4, 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 inoculum 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.

Predicting the outcome of bacterial competition

We used the multi-hit model to ask how the inoculum effect influences competition and, more specifically, how the bacterial inoculum affects the competitiveness of a recently mutated, more resistant strain. To answer this question, we extended the multi-hit framework to two bacterial strains, strain S and strain R . Strain R is more resistant to the antimicrobial than strain S due to a $10 \times$ lower attachment rate α . Note that production of extracellular enzymes is also a mechanism of bacterial resistance, but here we tried to avoid complex population dynamics to be able to compare the results of the multi-hit

Figure 4: 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 22). (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. S6. 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. 3a, 3b. (b) We predicted long term dynamics based on $\psi(A_0, N_{inoc})$ with a simple population model (equation 22). All parameters used for the simulations are listed in Table S1.

276 model with the dynamics based on the PD function. By extending the basic
277 model by a strain R , we were able to simulate competition under complex
278 pharmacokinetics (PKs).

279 We were interested if a resistant strain that is introduced in the system
280 can take over the population within the simulation time. Strain R was not
281 able to outcompete strain S at low antimicrobial concentrations (Fig. 5a)
282 due to an implemented cost of resistance. At intermediate concentrations, R
283 took over the population (Fig. 5b) and at high concentrations, both strains
284 were killed (Fig. 5c). These examples illustrate that the outcome of com-
285 petition depended on antimicrobial dose given (A_0) and we asked how both
286 antimicrobial concentration at the beginning of the simulation and bacterial
287 inoculum size change the outcome of competition.

288 We therefore extended our analysis for a broad spectrum of antimicrobial
289 input concentrations and bacterial inoculum sizes (Fig. 5d). Here the range of
290 antimicrobial concentrations for which the resistant strain can take over the
291 population was dependent on the bacterial inoculum N_{inoc} . The higher the
292 bacterial inoculum the smaller was the range of antimicrobial concentrations
293 in which the resistant strain took over. These results were qualitatively
294 reproduced with the extended PD function (Fig. S7).

295 For comparison, we simulated the competition between the two strains
296 in a model that does not display the inoculum effect. To parameterize such
297 a model, we determined the PD function $\psi(A_0)$ for $N_{inoc} = 5 \times 10^5$. As
298 expected, without the inoculum effect, the competitiveness of R did not

299 depend on the inoculum (Fig. 5e), and was overestimated, especially at high
300 inoculum sizes (compare Fig. 5e and Fig. 5d).

Figure 5: 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.

Discussion

The inoculum effect has been demonstrated experimentally for a number of bacterial strains and antimicrobials (13, 17, 18, 21, 22, 26, 27, 29, 30, 40, 41). In most cases the MIC (13, 17, 22, 26, 27, 29) was used to determine and quantify the inoculum effect. Udekwa *et al.* (13) demonstrated how to use MIC measurements to integrate the inoculum effect as change in MIC_{PD} in the PD function. However, they assumed that other PD parameters, among other things κ , were not affected by the inoculum effect.

In this work, we analyzed the inoculum effect based on mathematical modeling of bacterial population dynamics. We investigated whether the bacterial inoculum size has an impact on the efficacy of antimicrobials as measured by the PD function. A mechanism-based mathematical framework called multi-hit model (33) enabled us to explore the effect of increasing the inoculum size. Our findings are consistent with Abel zur Wiesch *et al.* (12): due to the kinetics of antimicrobials binding to bacterial cells, larger amounts of antimicrobials are required to attain the same efficacy when the bacterial inoculum size is increased.

We examined this inoculum effect quantitatively: the inoculum affected the PD parameters MIC_{PD} , A_{50} , and κ . We showed that a change in efficacy cannot only be achieved by varying the antimicrobial concentration, but also achieved by varying the bacterial inoculum. Formally, the MIC_{PD} , A_{50} , and κ increased with increasing inoculum size. Consequences of the inoculum ef-

fect may be altered outcome of survival and resistance evolution experiments,
which we have previously shown are dependent on the PD parameters (37).

In our basic model, the reason for the inoculum effect is that an increase
in the bacterial inoculum results in higher depletion in the pool of free an-
timicrobials, we call this a "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, This numbers game has already been observed with mathemati-
cal modeling (12, 33), however the experimental validation of the inoculum
effect based on the numbers game alone is sparse (12, 13, 30) with the focus
being mainly on enzymatic degradation (for an overview, see (15)). We argue
that studies that experimentally measure the inoculum effect based on the
numbers game are vital for fully understanding the impact of the inoculum
effect.

Moreover, not only the numbers game in itself but also how variations
of the numbers game affect the efficacy of antimicrobials should be tested
experimentally. For example, our simulations show that the more targets are
needed to be hit to kill a cell (z), the more pronounced is the inoculum effect
on the PD parameters MIC_{PD} , A_{50} , and κ . This result suggest that the
inoculum effect is stronger the more 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 (42) to $10^6 - 10^7$ in the case of PMAP-23 (43).
With everything else being equal, we therefore expect a more pronounced

346 inoculum effect for PMAP-23 than for protegrin.

347 In a next step, we determined the effect of enzymatic degradation in addi-
 348 tion to the binding effects. The inoculum effect is often measured experimen-
 349 tally in systems with enzymes present that degrade antimicrobial molecules,
 350 for example β -lactamases (17, 18, 21, 22, 26, 27, 40, 41, 44). When allowing
 351 for enzymatic degradation in our simulations, the inoculum effect, quantified
 352 as increase in the pharmacodynamic parameters MIC_{PD} and A_{50} , was more
 353 pronounced. In these simulations, bacterial dynamics also increased in com-
 354 plexity, in particular at low bacterial concentrations, which influenced the
 355 accuracy of the extended PD function $\psi(A, N_{inoc})$ to predict the bacterial
 356 population dynamics.

357 Similar to the effects of enzymatic degradation, non-targeted binding en-
 358 hanced the inoculum effect in our simulations. Non-targeted binding was
 359 implemented as binding to intracellular components of dead cells. Dead
 360 cells were assumed to leak intracellular content that posed alternative tar-
 361 gets for antimicrobial molecules. As a result, the inoculum effect was more
 362 pronounced: the increase of the pharmacodynamic parameters MIC_{PD} and
 363 A_{50} was steeper with non-targeted binding than in the basic model. In ac-
 364 cordance with our modeling results, experiments showed that non-targeted
 365 binding decreased the amount of antimicrobial peptide molecules available
 366 to kill bacteria (45).

367 To investigate the influence of the inoculum effect on bacterial competi-
 368 tion, we extended the basic multi-hit model by a resistant strain R . We mod-

eled resistance as a decrease in the hit rate α , which corresponds to resistance due to target modification (23). Simulation of bacterial competition revealed that the competitiveness of the resistant strain is overestimated when the inoculum effect is not considered. The range of antimicrobial concentration, in which R can take over is a rough estimate of the mutant selection window (MSW) (46, 47), which we call "realized MSW". The difference between the MSW and the realized MSW is that the MSW is based on time-kill curves with a single input of antimicrobials, while the realized MSW is based on time-kill curves in which the antimicrobials are repeatedly inputted. In our simulations, the estimated MSW becomes smaller for higher bacterial inoculum sizes. This means that, while a higher population size of the sensitive strain S increases the probability of resistance emergence, more sensitive bacteria in the presence of antimicrobials also decreases the competitiveness of the emerged resistant strain by reducing antimicrobial efficacy. We expect the selection coefficient in competition experiment, e.g. (47), to decrease with increasing inoculum. Moreover, this unexpected feature of the inoculum effect might be of relevance for epidemiological problems, for example co-existence of drug resistant and sensitive bacteria (48, 49).

The absence of the inoculum effect has been reported repeatedly (19, 50). In our system, the inoculum effect was not perceivable in certain regions of the PD plane: for very small and very high antimicrobial numbers, the inoculum effect was absent. In addition, the inoculum effect on the MIC might be challenging to detect because of inherent uncertainties in determining the

MIC by using doubling dilutions. As a concrete example, if, by increasing the inoculum, the "real" value of the MIC increases from 9 to 15 $\mu g/ml$, the measured MIC would be in both cases be 16 $\mu g/ml$. Furthermore, 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 with MIC measurements (9, 17, 18, 21, 22), clouding inoculum effects of smaller extent. More precise determination of the bacterial population size, e.g. with flow cytometry, could help to precisely quantify the inoculum effect.

In addition, it has been argued that the inoculum effect is an artifact (19). The core of this argument is that, to obtain the same pharmacodynamic efficacy, increasing the bacterial inoculum requires a proportionate increase in the amount of antimicrobials. Following this line of thought, keeping the ratio of antimicrobials to bacteria ($\frac{A_0}{N_{inoc}}$) constant should always result in the same pharmacodynamic efficacy. In our simulations, however, the ratio of antimicrobials to bacteria ($\frac{A_0}{N_{inoc}}$) does not predict efficacy. Rather, to obtain the same effect on a 100-fold increased bacterial population, we require less than 100-fold increase in antimicrobial molecules. For example, 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 and the ratio $\frac{A_0}{N_{inoc}}$ decreases from 80 to 7¹. Thus, with increasing inoculum, each antimicrobial molecule becomes actually more effective.

---

¹ Calculations are based on equation 1 and parameter values of slope m and intersection c are listed in Table 1 (reversible binding, $n = z = 10$).

413 The scale of the inoculum effect depends on the binding mechanisms
 414 of the antimicrobial, the target hit number z , and of the effect size of the
 415 antimicrobial that we are interested in. The latter one gives us an clue where
 416 to best measure the inoculum effect: The PD plane (Fig. 2c) shows that the
 417 inoculum effect is best measured at concentrations close to the A_{50} , because
 418 at low and very high concentrations, we do not expect to see an inoculum
 419 effect. More precisely, our quantitative analysis shows that κ increases, which
 420 means that the inoculum effect is stronger for A_{50} than for MIC_{PD} . This
 421 means that the effect of A_{50} is most suitable to measure the inoculum effect.
 422 Based on this observation, we recommend to not only quantify the MIC , but
 423 the whole PD function curve and to use optical density (OD) measurements
 424 with caution, since with OD measurements, only concentrations up to the
 425 MIC_{PD} can be measured.

426 In summary, we investigated the effect of the bacterial inoculum on the
 427 efficacy of antimicrobials in a simple conceptual model that describes the
 428 attachment and detachment dynamics of antimicrobial molecules to target
 429 sites on the bacteria. We showed that the inoculum effect emerges from these
 430 simple dynamics. The quantitative aspect of this effect are surprisingly com-
 431 plex. In our mechanistic population model, the inoculum effect was caused
 432 solely by binding and degradation, but further mechanisms have been in-
 433 voked to explain the inoculum effect. The efficacy of antimicrobials can
 434 change because of bacterial density dependent change of abiotic factors. For
 435 example, pH (31), accessibility (23), susceptibility to antimicrobials (32, 51)

can all change with bacterial density. These factors likely play an important role *in vivo*, however studying the inoculum effect focuses mainly on empirical yet remains to be explored, with a few notable exceptions (23). Although we did not focus on these mechanisms, it is possible to extend the multi-hit framework to incorporate these explanations. These further mechanisms will exacerbate the inoculum effect that arises from the binding kinetics. We also showed that the inoculum effect influences the competitive dynamics between bacterial strains under treatment. We conclude that a comprehensive quantitative description of pharmacodynamics requires a detailed mechanistic population model and cannot be captured by simple pharmacodynamic functions (1, 13, 37, 39).

1 Methods

Multi-hit model

We used the multi-hit model introduced by Hedges (33) to explore bacterial population dynamics *in silico* in presence of antimicrobial peptides (AMPs). We have already applied a modified version of the multi-hit model in previous work (34) and a similar approach has been introduced by Abel zur Wiesch *et al.* (12) to describe effects of antibiotics.

The multi-hit model describes a bacterial population consisting of N_{tot} cells. In the presence of antimicrobials, bacterial cells are hit by antimicrobial molecules. The key idea of this population model is to categorize all

457 bacterial cells according to how many antimicrobials have hit the cell (Fig. 6).
 458 A hit is the mathematical simplification of adsorption and subsequent down-
 459 stream processes connected to binding of one antimicrobial molecule to a
 460 bacterial target site and is described with the rate α . The parameter μ is the
 461 detachment of an antimicrobial from the bacterial target. Bacteria that have
 462 i antimicrobial molecules adsorbed are categorized in class N_i . The number
 463 of absorbed molecules varies from cell to cell, with $0 \leq i \leq n$. Thus, we
 464 have a total of $n + 1$ classes and $N_{tot} = \sum_{i=0}^n N_i$. The rate of change of each
 465 subpopulation is described with the following ordinary differential equation:

$$\begin{aligned} \frac{dN_i}{dt} = & \alpha_{i-1}AN_{i-1} - \alpha_iAN_i + \\ & \mu_{i+1}(i+1)N_{i+1} - \mu_i i N_i + \\ & \Omega(N_i) - d_i N_i \end{aligned} \quad (7)$$

466 Each class N_i gains bacterial cells from the class N_{i-1} with the term $\alpha_{i-1}AN_{i-1}$.
 467 Here, we assumed that finding the bacterial cell is the rate limiting step for
 468 an antimicrobial molecule (52). This is appropriate if the number of targets
 469 are in abundance, e.g. when antimicrobial peptides adsorb to the cell mem-
 470 brane of bacterial cells. For antimicrobials that act within the cell, a hit rate
 471 dependent on the number of free targets is more appropriate (see (12)).

472 The term $\Omega(N_i)$ in equation 7 summarizes replication processes, which
 473 we modeled according to Abel zur Wiesch *et al.* (12). In short, cells of class
 474 N_i replicate with the replication rate b_i . During replication, x bound antimi-

475 crobial molecules of the mother cells are redistributed to the two daughter
476 cells according to a hypergeometric distribution:

$$\Omega(N_i) = 2 \sum_{x=i}^n (f_{x,i} b_x N_x) - b_i N_i \quad (8)$$

477 The parameter $f_{x,i}$ denotes the fraction of cells from class N_x that are redis-
478 tributed to N_i due to replication.

479 Bacteria in class N_i die with a natural death rate d_i and due to interaction
480 with the antimicrobial. We assume that the antimicrobial is bactericidal
481 only. The bactericidal effect becomes apparent in the zombie stage. Once a
482 threshold number of antimicrobial molecules has hit the cell ($i = z$), the cell
483 enters the zombie class. In the basic model, we assumed complete multi-hit
484 (33), with $z = n$. In the zombie class, bacteria are doomed to die and do so
485 with an increased death rate $d_z > d$.

486 The multi-hit model allows us to keep track of the amount of antimicro-
487 bials A . In the basic model, there is no loss of antimicrobials due to non-
488 reversible binding, non-targeted binding, or degradation, i.e. we assumed
489 that all antimicrobial molecules bound to a bacterium are recycled back into
490 the system after the death of this cell:

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

491 Here, the variables N and A are used in a term describing mass action ki-
492 netics. Therefore, N and A are typically concentrations of bacteria and

antimicrobial molecules, respectively (53). In the framework of the multi-hit model, it is however necessary to use the absolute amount of bacteria and antimicrobial molecules because we keep track of how many antimicrobial molecules have hit the bacterial cells (12). The volume of the system V is assumed constant, with $V = 1ml$. Resulting, the number of bacteria cells and antimicrobial molecules are equivalent to their concentration values.

Variations in the Multi-hit framework

We determined the multi-hit model based on the ODE system presented above. In the basic model (equation 7–9), we assumed the case of complete multi-hit (33), with $z = n$. The complete multi-hit model is appropriate for antimicrobials that need to hit a large fraction of the existing targets. An example of such a case are AMPs that kill according to the carpet model (43). 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 relaxed these assumptions with (i) superfluous binding, (ii) non-reversible binding, (iii) non-targeted and non-reversible binding, and (iv) enzymatic degradation.

In the case of (i) superfluous binding, we implemented the fractional multi-hit model by Hedges (33). In this model, $z < n$, i.e. 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 (42). Here, a cell has more targets, i.e. places where

Figure 6: 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. In this example, a bacterium dies with an increased death rate d_z if two antimicrobial molecules have hit the cell, i.e. $z = 2$. Therefore, $N_{\text{tot}} = N_0 + N_1 + N_2$, with $N_2 = N_z$. Antimicrobials that have hit a cell are indicated in pink. A is the number of free antimicrobial molecules, here indicated in blue. The rates of the ODE (equation 7) are indicated with arrows. For simplicity, bacterial replication according to equation 7, 8 is only indicated with dashed arrows.

515 a pore can be formed in the membrane, than pores needed to kill a cell.

516 In the case of (ii) non-reversible binding, we set $\mu = 0$ and assumed that
517 antimicrobial molecules are not recycled back into the system. Examples
518 of non-reversible binding are binding of the antibiotics streptomycin and
519 kanamycin to bacterial ribosomes (54). This results in

$$\frac{dA}{dt} = \sum_{i=0}^n -\alpha_i A N_i \quad (9)$$

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

$$\frac{dL}{dt} = l \sum_{i=0}^n d_i N_i \quad (10)$$

$$\frac{dA}{dt} = \sum_{i=0}^n (-\alpha_i A N_i - \alpha_d A L) \quad (11)$$

525 As in the cases (ii) and (iii), enzymatic degradation (case (iv)) decreases
526 the amount of antimicrobials in the system. Bacteria can for example produce
527 proteases, e.g. aureolysin (25), which catalyze hydrolysis of AMPs. We
528 assumed that all bacterial cells produce enzymes E with the production rate

529 p and that enzymes degrade antimicrobial molecules with the rate d_E :

$$\frac{dE}{dt} = p \sum_{i=0}^n N_i - d_E E \quad (12)$$

530 In the presence of antimicrobials, enzymes in our model catalyze antimi-
531 crobial degradation in a two step process. First, an enzyme binds to an
532 antimicrobial molecule to form the complex $(E.A)$, then the bound enzyme
533 catalyzes the degradation of the antimicrobial, resulting in enzymes and prod-

534 ucts: $E + A \xrightleftharpoons[r_2]{r_1} E \cdot A \xrightarrow{r_3} E + P$

$$\frac{dE}{dt} = p \sum_{i=0}^n N_i - d_E E - r_1 EA + r_2 E.A + r_3 E.A \quad (13)$$

$$\frac{dE.A}{dt} = r_1 EA - r_2 E.A - r_3 E.A \quad (14)$$

$$\frac{dA}{dt} = \sum_{i=0}^n (\mu_i i N_i + d_i i N_i - \alpha_i A N_i) - r_1 AE + r_2 E.A \quad (15)$$

535 Pharmacodynamic function

536 The pharmacodynamic (PD) function (1, 2, 3) describes the net growth rate

537 ψ for given antimicrobial concentration A that was put in the system at $t = 0$

538 $(A(t = 0) = A_0)$, with

$$\psi(A_0) = \psi_{max} - e(A_0) \quad (16)$$

$$\psi(A_0) = \psi_{max} - \frac{(\psi_{max} - \psi_{min})(A_0/MIC_{PD})^\kappa}{(A_0/MIC_{PD})^\kappa - \psi_{min}/\psi_{max}} \quad (17)$$

Here, the effect e describes the reduction of the net growth rate due to antimicrobial A_0 . The MIC_{PD} is the antimicrobial concentration A_0 which results in a net growth rate of zero: $\psi(A_0 = MIC_{PD}) = 0$. Note that the MIC_{PD} is a approximation of the MIC if the antimicrobial is concentration independent (8). Other parameters of the PD function are the maximum net growth rate in absence of a drug $\psi_{max} = \psi(A_0 = 0)$, the minimum net growth rate $\psi_{min} = \psi(A_0 \rightarrow \infty)$, and the slope parameter κ . Alternatively, the PD function is described with 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).

Together, the two definitions of the PD function allowed us to calculate the slope parameter κ based on estimations of ψ_{max} , ψ_{min} , MIC_{PD} , and A_{50} , with

$$\kappa = \frac{\log\left(-\frac{\psi_{max}}{\psi_{min}}\right)}{\log\left(\frac{MIC_{PD}}{A_{50}}\right)} \quad (18)$$

To estimate the PD parameter, 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, with

$$\psi(A) = \frac{\log(N_{tot}(t_1)) - \log(N_{tot}(t_0))}{t_1 - t_0} \quad (19)$$

These net growth rates were then used to estimate the PD parameters. Here,

we determined $\psi(A_0 = 0)$ to estimate ψ_{max} and $\psi(A_0 = 10^{15})$ to estimate ψ_{min} . E_{max} was calculated as $\psi_{max} - \psi_{min}$. The expressions $\psi_{max} - \frac{E_{max}}{2} = \psi(A_0 = x)$ and $0 = \psi(A_0 = y)$ were optimized for x and y to parameterize A_{50} and MIC_{PD} , respectively. Finally, we calculated κ according to equation 18.

Multiple such parameterized PD functions for varying bacterial inoculum sizes were used to ascertain the change in PD parameters due change in the bacterial inoculum. These changes in parameters were implemented in an extended the PD function, $\psi(A_0, N_{inoc})$. The inoculum effect was quantified as slope m of the function

$$A_{0,\psi=y}(N_{inoc}) = mN_{inoc} + c, \quad (20)$$

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). In short, antimicrobials are in the system with the concentration A_0 . Antimicrobial molecules decay with the decay rate k_A . Without binding, the pharmacokinetic (PK) function is

$$A(t) = A_0 e^{k_A(t-p)} \quad (21)$$

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

574 Population model

575 The PD function and the pharmacokinetic function were implemented in a
576 population model to estimate population dynamics over time, with

$$\frac{dN_{tot}}{dt} = \psi_{max}N \left(1 - \frac{N}{K}\right) - e(A_0, N_{inoc})N \quad (22)$$

577 Here, K is the carrying capacity and ψ_{max} is used as an estimate of the
578 replication rate. We used this framework before in (37).

579 Competition

580 To describe competition between a sensitive strain S and a resistant strain
581 R , we extend the basic multi-hit model, with

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

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

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

582 Here, i and j describe the number of targets hit on a sensitive and resistant
583 bacterial cell, respectively, with $0 \leq i, j \leq n$. In our simulations, strain R had
584 a 10 times lower attachment rate ($\alpha_j = \alpha_i/10$) which reflects modification
585 in the surface of bacterial cells. This is a common resistance mechanism
586 against AMPs (23). To model realistic long term population dynamics, we
587 also included a carrying capacity, as it is done in (12):

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

588 We simulated population dynamics in a window of 7 days.

589 **Implementation**

590 The multi-hit model was implemented in R (55) (www.r-project.org; version
591 3.4.0) in RStudio (56) (version 1.0.143). We solved the deterministic multi-
592 hit model numerically with the package deSolve (57). For plotting, we used
593 the following packages: sfsmisc (58) and RColorBrewer (59). The code is
594 available upon request.

595 **Authors contributions**

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

598 **Competing interests**

599 We have no competing interests.

600 **Funding**

601 R.R.R. acknowledges the financial support of the ETH Zurich (ETH-41 15-2).

602 Acknowledgments

603 We thank Andrew Read for sparking our interest in the topic of inoculum ef-
604 fects, Pia Abel zur Wiesch, Sebastian Bonhoeffer, and Jens Rolff for reading
605 the manuscript, Bruce Levin for giving us valuable feedback on our discus-
606 sion, and João Pires for interesting discussions about the project.

607 Appendix

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

Figure S1: Example of an estimation of PD parameters for $N_{inoc} = 0$. 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 $\frac{dA}{dt} = 0$.

Figure S3: 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. 2d- 2f). 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.

Figure S4: Changing the bacterial inoculum does not change PD parameters (a) E_{max} , (b) ψ_{max} , and (c) ψ_{min} . Note that all model variations shown in Fig. 2d- 2f result in no effect on these PD parameters. All parameters used for the simulations are listed in Table S1.

Figure S5: 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.

Figure S6: Initial dynamics are different 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 S7: 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.

References

- [1] Roland R Regoes, Camilla Wiuff, Renata M Zappala, Kim N Garner, Fernando Baquero, and Bruce R Levin. Pharmacodynamic Functions: a Multiparameter Approach to the Design of Antibiotic Treatment Regimens. *Antimicrob. Agents Chemother.*, 48(10):3670–3676, 2004. ISSN 0066-4804. URL <http://aac.asm.org/cgi/doi/10.1128/AAC.48.10.3670-3676.2004>.
- [2] J Zhi, C H Nightingale, and R Quintiliani. A Pharmacodynamic Model for the Activity of Antibiotics Against Microorganisms under Nonsat-

- 617   urable Conditions. *J. Pharm. Sci.*, 75(11):1063–1067, 1986. ISSN 0022-
618   3549. URL <http://www.ncbi.nlm.nih.gov/pubmed/3102718>.
- 619   [3] A Nolting, T Dalla Costa, K H Rand, and H Derendorf. Pharma-
620   cokinetic-Pharmacodynamic Modeling of the Antibiotic effect of
621   Piperacillin in Vitro. *Pharm. Res.*, 13(1):91–96, 1996. ISSN 0724-8741.
622   URL <http://www.ncbi.nlm.nih.gov/pubmed/8668686>.
- 623   [4] Guozhi Yu, Desiree Y Baeder, Roland R Regoes, and Jens Rolff.
624   Combination Effects of Antimicrobial Peptides. *Antimicrob. Agents*
625   *Chemother.*, (January):AAC.02434–15, 2016. ISSN 0066-4804. URL
626   <http://aac.asm.org/lookup/doi/10.1128/AAC.02434-15>.
- 627   [5] Sunniva Foerster, Magnus Unemo, Lucy J Hathaway, Nicola Low, and
628   Christian L Althaus. Time-kill curve analysis and pharmacodynamic
629   modelling for in vitro evaluation of antimicrobials against neisseria gon-
630   orrhoeae. *BMC microbiology*, 16(1):216, 2016.
- 631   [6] Xuesong Wen, Ronette Gehring, Jim E Riviere, Brian V Lubbers,
632   Tara Nath Gaire, Breanna Fox, Victoria Quichocho, Victoriya V
633   Volkova, et al. Variation in fluoroquinolone pharmacodynamic parame-
634   ter values among isolates of two bacterial pathogens of bovine respiratory
635   disease. *Scientific reports*, 8(1):10553, 2018.
- 636   [7] Peter Ankomah and Bruce R Levin. Two-Drug Antimicrobial

- 637 Chemotherapy: A Mathematical Model and Experiments with *Mycobac-*
638 *terium marinum*. *PLoS Pathog.*, 8(1):e1002487, 2012. ISSN 1553-7374.
- 639 [8] Johan W Mouton and Alexander A Vinks. Pharmacoki-
640 netic/pharmacodynamic modelling of antibacterials in vitro and in vivo
641 using bacterial growth and kill kinetics. *Clinical pharmacokinetics*, 44
642 (2):201–210, 2005.
- 643 [9] Irith Wiegand, Kai Hilpert, and Robert EW Hancock. Agar and broth
644 dilution methods to determine the minimal inhibitory concentration
645 (mic) of antimicrobial substances. *Nature protocols*, 3(2):163, 2008.
- 646 [10] CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bac-*
647 *teria That Grow Aerobically; Approved Standard – Ninth Edition*. CLSI
648 document M07-A9, Wayne, PA: Clinical and Laboratory Standards In-
649 stitute, 2012.
- 650 [11] ISO. *Clinical laboratory testing and in vitro diagnostic test systems –*
651 *Susceptibility testing of infectious agents and evaluation of performance*
652 *of antimicrobial susceptibility test devices – Part 1: Reference method for*
653 *testing the in vitro activity of antimicrobial agents against rapidly grow-*
654 *ing aerobic bacteria involved in infectious diseases (Draft)*. ISO/DIS
655 20776-1:2018(E), Geneva, CH: International Organization for Standard-
656 ization, 2018.
- 657 [12] Pia Abel zur Wiesch, Sören Abel, Spyridon Gkatzis, Paolo Ocampo,

- 658 J. Engelstädter, Trevor Hinkley, Carsten Magnus, Matthew K Waldor,
659 Klas Udekwu, and Ted Cohen. Classic reaction kinetics can explain
660 complex patterns of antibiotic action. *Sci. Transl. Med.*, 7(287):287ra73–
661 287ra73, 2015. ISSN 1946-6234. URL [http://stm.sciencemag.org/
662 cgi/doi/10.1126/scitranslmed.aaa8760](http://stm.sciencemag.org/cgi/doi/10.1126/scitranslmed.aaa8760).
- 663 [13] Klas I Udekwu, Nicholas Parrish, Peter Ankomah, Fernando Baquero,
664 and Bruce R Levin. Functional relationship between bacterial cell den-
665 sity and the efficacy of antibiotics. *Journal of Antimicrobial Chemother-*
666 *apy*, 63(4):745–757, 2009.
- 667 [14] Pratik Bhagunde, Kai-Tai Chang, Renu Singh, Vandana Singh, Kevin W
668 Garey, Michael Nikolaou, and Vincent H Tam. Mathematical modeling
669 to characterize the inoculum effect. *Antimicrobial agents and chemother-*
670 *apy*, 54(11):4739–4743, 2010.
- 671 [15] Justin R Lenhard and Zackery P Bulman. Inoculum effect of β -lactam
672 antibiotics. *Journal of Antimicrobial Chemotherapy*, 74(10):2825–2843,
673 2019.
- 674 [16] Mark E. Rupp and Paul D. Fey. Extended spectrum β -lactamase (esbl)-
675 producing enterobacteriaceae. *Drugs*, 63(4):353–365, Feb 2003. ISSN
676 1179-1950. doi: 10.2165/00003495-200363040-00002. URL [https://
677 doi.org/10.2165/00003495-200363040-00002](https://doi.org/10.2165/00003495-200363040-00002).
- 678 [17] Kenneth S Thomson and Ellen Smith Moland. Cefepime, piperacillin-

- 679 tazobactam, and the inoculum effect in tests with extended-spectrum
680 β -lactamase-producing enterobacteriaceae. *Antimicrobial agents and*
681 *chemotherapy*, 45(12):3548–3554, 2001.
- 682 [18] Amos Adler, Ina Chmelnitsky, Yehuda Carmeli, et al. Effect of resis-
683 tance mechanisms on the inoculum effect of carbapenem in klebsiella
684 pneumoniae isolates with borderline carbapenem resistance. *Antimicro-*
685 *bial agents and chemotherapy*, 59(8):5014–5017, 2015.
- 686 [19] William A Craig, Sujata M Bhavnani, and Paul G Ambrose. The in-
687 oculum effect: fact or artifact? *Diagnostic Microbiology and Infectious*
688 *Disease*, 50:229–230, 2004.
- 689 [20] Itzhak Brook. Inoculum effect. *Reviews of infectious diseases*, 11(3):
690 361–368, 1989.
- 691 [21] Cheol-In Kang, Hyunjoo Pai, Sung-Han Kim, Hong-Bin Kim, Eui-
692 Chong Kim, Myoung-don Oh, and Kang-Won Choe. Cefepime and the
693 inoculum effect in tests with klebsiella pneumoniae producing plasmid-
694 mediated ampc-type β -lactamase. *Journal of Antimicrobial Chemother-*
695 *apy*, 54(6):1130–1133, 2004.
- 696 [22] Kenneth P Smith and James E Kirby. The inoculum effect in the era
697 of multidrug resistance: Minor differences in inoculum have dramatic
698 effect on minimal inhibitory concentration determination. *Antimicrobial*
699 *agents and chemotherapy*, pages AAC–00433, 2018.

- [23] Hwang-Soo Joo, Chih-Iung Fu, and Michael Otto. Bacterial strategies of resistance to antimicrobial peptides. *Phil. Trans. R. Soc. B*, 371(1695): 20150292, 2016.
- [24] Anna Ebbensgaard, Hanne Mordhorst, Michael Toft Overgaard, Claus Gyruup Nielsen, Frank Møller Aarestrup, and Egon Bech Hansen. Comparative evaluation of the antimicrobial activity of different antimicrobial peptides against a range of pathogenic bacteria. *PLoS One*, 10(12):e0144611, 2015.
- [25] Magdalena Sieprawska-Lupa, Piotr Mydel, Katarzyna Krawczyk, Kinga Wójcik, Magdalena Puklo, Bogusław Lupa, Piotr Suder, Jerzy Silber-ring, Matthew Reed, Jan Pohl, et al. Degradation of human antimicrobial peptide ll-37 by staphylococcus aureus-derived proteinases. *Antimicrobial agents and chemotherapy*, 48(12):4673–4679, 2004.
- [26] Masachika Saeki, Masaaki Shinagawa, Yuki Yakuwa, Shinya Nirasawa, Yuki Sato, Nozomi Yanagihara, and Satoshi Takahashi. Inoculum effect of high concentrations of methicillin-susceptible staphylococcus aureus on the efficacy of cefazolin and other beta-lactams. *Journal of infection and chemotherapy*, 24(3):212–215, 2018.
- [27] Branka Bedenić, Nataša Beader, and Živojin Žagar. Effect of inoculum size on the antibacterial activity of cefpirome and cefepime against klebsiella pneumoniae strains producing shv extended-spectrum β -lactamases. *Clinical microbiology and infection*, 7(11):626–635, 2001.

- 722 [28] Cheemeng Tan, Robert Phillip Smith, Jaydeep K Srimani, Katherine A
723 Riccione, Sameer Prasada, Meta Kuehn, and Lingchong You. The in-
724 oculum effect and band-pass bacterial response to periodic antibiotic
725 treatment. *Molecular systems biology*, 8(1):617, 2012.
- 726 [29] Judy N Chin, Michael J Rybak, Chrissy M Cheung, and Paul B Savage.
727 Antimicrobial activities of ceragenins against clinical isolates of resistant
728 staphylococcus aureus. *Antimicrobial agents and chemotherapy*, 51(4):
729 1268–1273, 2007.
- 730 [30] Filippo Savini, Vincenzo Luca, Alessio Bocedi, Renato Massoud,
731 Yoonkyung Park, Maria Luisa Mangoni, and Lorenzo Stella. Cell-density
732 dependence of host-defense peptide activity and selectivity in the pres-
733 ence of host cells. *ACS chemical biology*, 12(1):52–56, 2016.
- 734 [31] Jason Karslake, Jeff Maltas, Peter Brumm, and Kevin B Wood. Pop-
735 ulation density modulates drug inhibition and gives rise to potential
736 bistability of treatment outcomes for bacterial infections. *PLoS compu-
737 tational biology*, 12(10):e1005098, 2016.
- 738 [32] Jürgen B Bulitta, Jenny C Yang, Liliana Yohonn, Neang S Ly, Silvia V
739 Brown, Rebecca E D’Hondt, William J Jusko, Alan Forrest, and Brian T
740 Tsuji. Attenuation of colistin bactericidal activity by high inoculum of
741 pseudomonas aeruginosa characterized by a new mechanism-based pop-
742 ulation pharmacodynamic model. *Antimicrobial agents and chemother-
743 apy*, 54(5):2051–2062, 2010.

- 744 [33] A J Hedges. An Examination of Single-hit and Multi-hit Hypotheses
745 in Relation to the Possible Kinetics of Colicin Adsorption. *J. Theor.*
746 *Biol.*, 11(3):383–410, 1966. ISSN 0022-5193. URL <http://www.ncbi.nlm.nih.gov/pubmed/5967439>.
747
- 748 [34] Desiree Y Baeder, Guozhi Yu, Nathanaël Hozé, Jens Rolff, and Roland R
749 Regoes. Antimicrobial combinations: Bliss independence and loewe ad-
750 ditivity derived from mechanistic multi-hit models. *Phil. Trans. R. Soc.*
751 *B*, 371(1695):20150294, 2016.
- 752 [35] Bruce R Levin and Daniel E Rozen. Non-inherited antibiotic resistance.
753 *Nature Reviews Microbiology*, 4(7):556, 2006.
- 754 [36] Peter Ankomah and Bruce R Levin. Exploring the collaboration between
755 antibiotics and the immune response in the treatment of acute, self-
756 limiting infections. *Proceedings of the National Academy of Sciences*,
757 page 201400352, 2014.
- 758 [37] Guozhi Yu, Desiree Y. Baeder, Roland R. Regoes, and Jens Rolff. Pre-
759 dicting drug resistance evolution: insights from antimicrobial peptides
760 and antibiotics. *Proceedings. Biol. Sci.*, 285(1874):20172687, 2018.
- 761 [38] Irit Levin-Reisman, Irine Ronin, Orit Gefen, Ilan Braniss, Noam
762 Shoresh, and Nathalie Q Balaban. Antibiotic tolerance facilitates the
763 evolution of resistance. *Science*, page eaaj2191, 2017.

- [39] Bruce R Levin and Klas I Udekwu. Population dynamics of antibiotic treatment: a mathematical model and hypotheses for time-kill and continuous-culture experiments. *Antimicrobial agents and chemotherapy*, 54(8):3414–3426, 2010.
- [40] David S Burgess and Ronald G Hall II. In vitro killing of parenteral beta-lactams against standard and high inocula of extended-spectrum beta-lactamase and non-esbl producing klebsiella pneumoniae. *Diagnostic microbiology and infectious disease*, 49(1):41–46, 2004.
- [41] Esteban C Nannini, Martin E Stryjewski, Kavindra V Singh, Tom H Rude, G Ralph Corey, Vance G Fowler, and Barbara E Murray. Determination of an inoculum effect with various cephalosporins among clinical isolates of methicillin-susceptible staphylococcus aureus. *Antimicrobial agents and chemotherapy*, 54(5):2206–2208, 2010.
- [42] Dan Bolintineanu, Ehsan Hazrati, H Ted Davis, Robert I Lehrer, and Yiannis N Kaznessis. Antimicrobial mechanism of pore-forming protegrin peptides: 100 pores to kill e. coli. *Peptides*, 31(1):1–8, 2010.
- [43] Daniela Roversi, Vincenzo Luca, Simone Aureli, Yoonkyung Park, Maria Luisa Mangoni, and Lorenzo Stella. How Many Antimicrobial Peptide Molecules Kill a Bacterium? The Case of PMAP-23. *ACS Chem. Biol.*, 9(9):2003–2007, 2014. ISSN 1554-8929. URL <http://www.ncbi.nlm.nih.gov/pubmed/25058470><http://pubs.acs.org/doi/abs/10.1021/cb500426r>.

- 786 [44] Yu Mi Wi, Young Kyoung Park, Chisook Moon, Seong Yeol Ryu, Hyuck
787 Lee, Hyun Kyun Ki, Hae Suk Cheong, Jun Seong Son, Jin Seo Lee,
788 Ki Tae Kwon, et al. The cefazolin inoculum effect in methicillin-
789 susceptible staphylococcus aureus blood isolates: their association with
790 dysfunctional accessory gene regulator (agr). *Diagnostic microbiology*
791 *and infectious disease*, 83(3):286–291, 2015.
- 792 [45] F Savini, MR Loffredo, C Troiano, S Bobone, N Malanovic, TO Eich-
793 mann, L Caprio, VC Canale, Y Park, ML Mangoni, et al. Binding of
794 an antimicrobial peptide to bacterial cells: Interaction with different
795 species, strains and cellular components. *Biochimica et Biophysica Acta*
796 *(BBA)-Biomembranes*, page 183291, 2020.
- 797 [46] Karl Drlica and Xilin Zhao. Mutant selection window hypothesis up-
798 dated. *Clinical infectious diseases*, 44(5):681–688, 2007.
- 799 [47] Erik Gullberg, Sha Cao, Otto G Berg, Carolina Ilbäck, Linus Sandegren,
800 Diarmaid Hughes, and Dan I Andersson. Selection of resistant bacteria
801 at very low antibiotic concentrations. *PLoS pathogens*, 7(7):e1002158,
802 2011.
- 803 [48] Marc Lipsitch, Caroline Colijn, Ted Cohen, William P Hanage, and
804 Christophe Fraser. No coexistence for free: neutral null models for
805 multistrain pathogens. *Epidemics*, 1(1):2–13, 2009.
- 806 [49] Nicholas G Davies, Stefan Flasche, Mark Jit, and Katherine E Atkins.

- 807 Within-host dynamics shape antibiotic resistance in commensal bacte-
808 ria. *Nature ecology & evolution*, 3(3):440, 2019.
- 809 [50] Shingo Mizunaga, Tomoko Kamiyama, Yoshiko Fukuda, Masahiro Taka-
810 hata, and Junichi Mitsuyama. Influence of inoculum size of staphylococ-
811 cus aureus and pseudomonas aeruginosa on in vitro activities and in vivo
812 efficacy of fluoroquinolones and carbapenems. *Journal of Antimicrobial*
813 *Chemotherapy*, 56(1):91–96, 2005.
- 814 [51] Hidetada Hirakawa and Haruyoshi Tomita. Interference of bacterial cell-
815 to-cell communication: a new concept of antimicrobial chemotherapy
816 breaks antibiotic resistance. *Frontiers in microbiology*, 4:114, 2013.
- 817 [52] Carsten Magnus and Roland R Regoes. Restricted occupancy models
818 for neutralization of hiv virions and populations. *Journal of Theoretical*
819 *Biology*, 283(1):192–202, 2011.
- 820 [53] Hamish McCallum, Nigel Barlow, and Jim Hone. How should pathogen
821 transmission be modelled? *Trends in ecology & evolution*, 16(6):295–
822 300, 2001.
- 823 [54] Philip Greulich, Matthew Scott, Martin R Evans, and Rosalind J Allen.
824 Growth-dependent bacterial susceptibility to ribosome-targeting antibi-
825 otics. *Molecular systems biology*, 11(3), 2015.
- 826 [55] R Core Team. *R: A Language and Environment for Statistical Com-*

- 827   `puting`. R Foundation for Statistical Computing, Vienna, Austria, 2017.
828   URL <http://www.R-project.org>.
- 829   [56] RStudio Team. *RStudio: Integrated Development Environment for R*.
830   RStudio, Inc., Boston, MA, 2016. URL <http://www.rstudio.com/>.
- 831   [57] Karline Soetaert, Thomas Petzoldt, and R. Woodrow Setzer. Solving
832   Differential Equations in R: Package deSolve. *J. Stat. Softw.*, 33(9):1–25,
833   2010. ISSN 1548-7660. URL <http://www.jstatsoft.org/v33/i09/>.
- 834   [58] Martin Maechler et al. *sfsmisc: Utilities from "Seminar fuer Statis-*
835   *tik" ETH Zurich*, 2016. URL [https://CRAN.R-project.org/package=](https://CRAN.R-project.org/package=sfsmisc)
836   **sfsmisc**. R package version 1.1-0.
- 837   [59] Erich Neuwirth. *RColorBrewer: ColorBrewer Palettes*, 2014. URL
838   <https://CRAN.R-project.org/package=RColorBrewer>. R package
839   version 1.1-2.