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Building pyramids against the evolutionary emergence of pathogens

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Abstract

Mutations allowing pathogens to escape host immunity promote the spread of infectious diseases in heterogeneous host populations and can lead to major epidemics. Understanding the conditions that slow down this evolution is key for the development of durable control strategies against pathogens. Here we use theory and experiments to compare the efficacy of three strategies for the deployment of resistance: (i) a *mixing* strategy where the host population contains two single-resistant genotypes, (ii) a *pyramiding* strategy where the host carries a double-resistant genotype, (iii) a *combining* strategy where host population is a mix of a single-resistant genotype and a double-resistant genotype. First, we use evolutionary epidemiology theory to clarify the interplay between demographic stochasticity and evolutionary dynamics to show that the *pyramiding* strategy always yields lower probability of evolutionary emergence. Second, we test experimentally these predictions with the introduction of bacteriophages into bacterial populations where we manipulated the diversity and the depth of immunity using a CRISPR-Cas system. These biological assays confirm that pyramiding multiple defenses into the same host genotype and avoiding combination with single-defense genotypes is a robust way to reduce pathogen evolutionary emergence. The experimental validation of these theoretical recommendations has practical implications for the optimal deployment of resistance varieties in agriculture and for the design of durable vaccination strategies.

Keywords: evolutionary emergence, epidemiology, demographic stochasticity, host resistance, CRISPR, bacteriophage, virus, infectious disease control.

34 Introduction

35 The spread of pathogen epidemics is driven by the composition of host populations and, in
36 particular, by the fraction f_R of resistant hosts. Larger values of f_R generate “herd immunity” in
37 well-mixed populations because a randomly chosen susceptible host is expected to be
38 surrounded by many resistant neighbors. Since resistant hosts cannot be successfully
39 infected, their presence shields susceptible individuals from the risk of being infected and
40 reduce the spread of a given pathogen. In fact, the epidemic is expected to stop growing when
41 $f_R > 1 - 1/R_0$, where R_0 refers to the basic reproduction ratio of the pathogen. This
42 theoretical framework provides key guidelines for the deployment of control measures like
43 vaccination [1,2] or the deployment of resistant varieties of crops in agriculture [3,4].

44 The efficacy of these control strategies, however, is challenged by the potential
45 acquisition of escape mutations allowing the pathogen to infect a resistant host. Whether those
46 variants will appear, establish, and spread depend on multiple evolutionary forces, including
47 the composition of the host population. For instance, a larger fraction f_R of resistant hosts is
48 expected to reduce the growth rate of the wild-type pathogen and, consequently, to limit the
49 influx of escape mutations. But a larger fraction f_R of resistant hosts is also expected to
50 increase the fitness benefit associated with an escape mutation. This will increase the
51 probability of establishment of a given mutation (i.e. lower risk of stochastic extinction when
52 rare) and it will also increase the speed at which this variant will spread. The balance between
53 these two opposite effects may thus result in a higher risk of pathogen adaptation for
54 intermediate frequency of resistance. Hence, a better understanding of the influence of the
55 host population composition on pathogen adaptation may help identify more durable control
56 strategies.

57 Many theoretical studies have explored complex ecological scenarios to evaluate the
58 impact of various strategies for the deployment of host resistance genotypes across space and
59 time in agriculture [3,5–14]. In particular, several studies contrasted the efficacy of *mixing*
60 multiple single host resistant genotypes with the efficacy of *pyramiding* multiple resistant genes
61 within a single genotype. Earlier models did not incorporate demographic feedbacks or any
62 influence of demographic stochasticity and focused on the long-term deterministic evolutionary
63 outcomes. Under these conditions the *mixing* strategy can outperform the *pyramiding* strategy
64 because the former strategy can prevent the spread of pathogens carrying multiple escape
65 mutations [5,6]. More recent studies challenged this guidance and relied on simulation models
66 that highlight the importance of epidemiology, demographic stochasticity, and spatial structure
67 on both the epidemiology and the evolution of the pathogen [9–11,14,15]. Taken together, the
68 available theoretical literature may appear confusing because distinct studies make different
69 recommendations on the optimal strategy for the deployment of resistance against a pathogen

70 [16]. This confusion stems from the different assumptions of the models (e.g. with or without
71 demography, with or without stochasticity) but also on the different optimality criteria used to
72 identify the most effective pathogen control strategies (e.g. no evolution of multi-escape
73 mutations, minimal disease incidence) [8,17,18]. Besides, experimental studies needed to
74 evaluate the durability of control strategies against pathogens are notoriously difficult to carry
75 out, particularly in agriculture [8,13,19]. This lack of experimental validation does not help to
76 elucidate the pros and cons of distinct deployment of resistance strategies.

77 Here we develop a joint theoretical and experimental approach to analyse the durability
78 of different strategies for the deployment of host resistance. We focus on a very specific
79 quantity to evaluate the efficacy of a control strategy: the probability of pathogen emergence
80 with (or without) adaptation. This quantity provides a relevant measure of control efficacy
81 because it accounts for both short-term (epidemiological time scale) and long-term
82 (evolutionary time scale) dynamical processes [20]. Experimental measurements of
83 evolutionary emergence, however, are challenging because the stochastic nature of pathogen
84 extinction requires a large number of replicate populations to measure the probability of
85 emergence. These experiments require also the ability to manipulate the composition of the
86 host population and to track the evolution of the pathogen population. These hurdles can be
87 overcome by studying the evolutionary emergence of virulent bacteriophages in bacterial
88 populations that use the adaptive CRISPR immunity to prevent phage infections.

89 CRISPR-Cas adaptive immune systems are widespread among many bacteria and
90 archaea. A CRISPR immunity phenotype is genetically encoded by a so-called CRISPR locus
91 (Clustered Regularly Interspaced Short Palindromic Repeats)—an array of short repetitive and
92 unique nucleotide sequences (“repeats” and “spacers”, respectively). “Spacers” are derived
93 from (foreign) genetic elements, such as viral genomes, and provide immunity to re-infection
94 based on recognition (via a short CRISPR RNA) and cleavage (by a Cas nuclease) of the
95 cognate sequence (known as “protospacer”). Crucially, a virus can adapt to CRISPR-Cas
96 immunity if it acquires escape mutations in the protospacer targeted by the host [21–23].

97 This natural microbial system offers a unique opportunity to study the dynamics of viral
98 adaptation in heterogeneous host populations: (i) many replicates can be carried out
99 simultaneously using bacteria and phages in 96-well plates [20]; (ii) CRISPR immunity
100 provides a very convenient way to manipulate both the *diversity* of host immunity (different
101 bacteria derived from the same population can carry different “spacers” in their CRISPR array
102 [20,24]) and the *depth* of host immunity (multiple “spacers” can be stacked within the CRISPR
103 array of the same multiresistant bacterium [25]); (iii) the most common mechanism of phage
104 adaptation to CRISPR-based immunity is well documented: virulent phages escape CRISPR
105 resistance through mutation in their target sequence (the “protospacer”) [21–23,26].

106 In the next sections we present the theoretical framework used to compute the
107 probability of evolutionary emergence of pathogens after being introduced in a heterogeneous
108 host population. We use this model to understand the effect of multiple factors on the fate of
109 the pathogen: (i) the number of viruses introduced, (ii) the proportion of resistant hosts, (iii) the
110 diversity and the depth of immunity of resistant hosts. This allows us to contrast the influence
111 of different strategies of resistance deployment on the probability of pathogen evolutionary
112 emergence. In a second step, we manipulated the heterogeneity of bacterial CRISPR immunity
113 to experimentally test the validity of our theoretical predictions on the evolutionary emergence
114 of phage populations.

115

116 **Materials and Methods**

117 **Theory**

118 Pathogen emergence is defined as the ability to escape early extinction and thus to initiate an
119 epidemic after the introduction of a small quantity of pathogens in the host population. This
120 probability of emergence depends both on the host (e.g. proportion and diversity of resistant
121 hosts) and the pathogen (e.g. inoculum size, viral mutation rate, genetic composition) [20,27–
122 29]. For a pathogen to emerge, we assume that the host population contains a fraction
123 $(1 - f_R)$ of individuals fully susceptible to the pathogen while the remaining fraction f_R of the
124 population is resistant. Resistance is assumed to be perfect (a virus cannot infect a resistant
125 host without a matching escape mutation) but we previously showed in [20] how it is possible
126 to expand this analysis to imperfect immunity. Among the resistant hosts, we consider three
127 alternative scenarios (**Figure 1**):

128 (i) a *mixing* scenario, in which the resistant fraction of the population is a mix of two single-
129 resistance genotypes (A+B) aiming at distinct pathogen *target sites* (a target site is defined
130 here as a region of the pathogen genome recognized by immune effectors and where a
131 mutation or a deletion may allow escape recognition by host immunity). We allow the
132 frequency of the two resistant hosts to vary and f_A refers to the frequency of the resistant
133 host A among the resistant hosts ($f_B = 1 - f_A$ is the frequency of the resistant host B
134 among the resistant hosts);

135 (ii) a *pyramiding* scenario, in which the resistant fraction of the population is monomorphic with
136 a double-resistance genotype (AB);

137 (iii) a *combining* scenario, in which the resistant fraction of the population results from a mix of
138 single-resistance genotypes (say A) and a double-resistance genotype (AB). We allow the
139 frequency of the two resistant hosts to vary and f_A refers to the frequency of resistant host
140 A among the resistant hosts ($f_{AB} = 1 - f_A$ is the frequency of the resistant host AB among

141 the resistant hosts). Note that the *pyramiding* scenario is a limit case of the *combining*
142 scenario when $f_A = 0$.

143 Even if host resistance is assumed to be perfect the pathogen can evade recognition by
144 acquiring escape mutations in the corresponding immunity target sites. Therefore, a pathogen
145 with escape mutation i can infect a fraction $(1 - f_R) + f_R P_i$ of the total host population, where
146 $P_i = \sum_{h \in \{A, B, AB\}} f_h P_i^h$ is the fraction of the resistant hosts that can be infected by the pathogen
147 with genotype i and P_i^h measures the ability of the pathogen genotype i to infect of the host
148 genotype $h \in \{\emptyset, A, B, AB\}$. In the *pyramiding* scenario, pathogens with less than 2 escape
149 mutations can only infect a fraction $(1 - f_R)$ of the total host population and only pathogens
150 with 2 escape mutations can infect all the hosts. In the *mixing* and in the *combining* scenario,
151 the fitness of a single escape mutant depends on the identity of the single-resistance genotype
152 in the host population and the composition of the resistant population (see **supplementary**
153 **information**).

154 We further assume a classic birth-death process to model the epidemiological
155 dynamics where a host infected with a pathogen that does not carry escape mutations spreads
156 this pathogen in a fully susceptible host population at rate b and dies at rate d . Host resistance
157 prevents infection and may thus affect the effective birth rate, but without affecting d . Whereas
158 escape mutations may allow the pathogen to infect a larger fraction of the host population, they
159 also carry a fitness cost c which causes pathogens with i escape mutations to reproduce at
160 rate $b_i = b(1 - c)^i$. The probability of acquiring an escape genotype $i \in \{A, B, AB\}$ by
161 mutation is noted μ_i and may vary among target sites. Note that different genetic changes in
162 the protospacer could allow the virus to escape recognition by the CRISPR-Cas immunity [30].
163 This increases the mutation rate towards an escape phenotype, but we do not need to
164 distinguish these different mutations to model the dynamics of the virus. Crucially, the
165 acquisition of 2 escape mutations requires two independent changes of the phage genome,
166 which is expected to occur at a much smaller rate than the rate of single escape mutations:
167 $\mu_{AB} \approx \mu_A \mu_B \ll \mu_A, \mu_B$. For the sake of simplicity, we assume that escape mutations are fixed
168 and cannot revert to the ancestral types. These reversions to the wild-type target are expected
169 to have a negligible effect on the probability of evolutionary emergence when the target site
170 mutation rate remains small [20,31].

171 We detailed in the **supplementary information** how we compute the probability of
172 emergence (with or without viral evolution) after the introduction of an inoculum of V phage
173 particles in a heterogeneous bacterial host population. We also derive approximations for the
174 probability of evolutionary emergence inspired from models of evolutionary rescue. Those

175 approximations help to contrast the effects of the composition of the host population on the
176 risk of evolutionary emergence.

177

178 **Experiments**

179 We used the Gram-positive bacterial strain *Streptococcus thermophilus* DGCC 7710 which is
180 susceptible to the virulent phage 2972. We also used three CRISPR-resistant clones (also
181 referred as bacteriophage-insensitive mutants: BIMs) that were derived from *S. thermophilus*
182 DGCC 7710 and differ only in their CRISPR arrays (**Tables S1** and **S2**). Two of these clones
183 carried a single additional spacer (strains A and B) targeting the genome of phage 2972, while
184 the remaining clone carried a combination of these two spacers (strain AB) precisely obtained
185 using the approach developed by Hynes et al. [25]. The addition of a single spacer in the
186 CRISPR1 array of *S. thermophilus* DGCC 7710 provides a robust resistance against infection
187 by the wild-type virulent phage 2972 [21–23] (**Table S2**). The rate at which phage 2972
188 acquires a mutation allowing to escape CRISPR immunity was found to be approximately equal
189 to $2.8 \cdot 10^{-7}$ mutations/locus/replication [32]. The acquisition of a single escape mutation may
190 or may not yield significant fitness costs for the phage [22,32].

191 We monitored the dynamics of the phage population after introducing an inoculum of *V*
192 viruses in each well of a 96-well plate containing 200 μL of replicate bacterial populations with
193 a proportion $f_R = 90\%$ of resistant cells and $1 - f_R = 10\%$ of susceptible cells. This virus
194 inoculum was sampled from a lysate obtained after amplifying a single plaque of the wild-type
195 phage 2972 on *S. thermophilus* DGCC 7710 (the initial frequency of single and double escape
196 mutants was estimated in **Table S3**: $3.7 \cdot 10^{-6}$ and $3.7 \cdot 10^{-6}$ against single resistance A and
197 B, respectively, but we did not detect double escape mutants). We manipulated the
198 composition of the resistant bacterial population to produce three experimental treatments to
199 test the predictions of the theoretical model (**Figure 1**): (i) *mixing* (strains A and B in equal
200 frequency), (ii) *pyramiding* (only strain AB), (iii) *combining* strains A and AB in equal frequency
201 (combining A) or *combining* strains B and AB in equal frequency (combining B). After an
202 overnight incubation (22 h) we quantified the abundance and the evolution of the phages after
203 spotting a fraction of each replicate (2 μL) on a lawn of the different bacterial strains to
204 measure: (i) the presence/absence of phages using a lawn of susceptible cells (ii) the
205 presence/absence of escape mutations in the phage population using lawns of single-
206 resistance bacteria (A or B) and a lawn of double-resistance bacteria (AB) [20,32].

207 We used logistic regression models with the presence/absence on susceptible bacteria
208 (or on resistant bacteria) as the response variable as a function of the inoculum size and the
209 composition of the host population (see **supplementary information**).

210

211 Results

212 Emergence and evolutionary emergence

213 We derive the probability of pathogen emergence after the introduction of an inoculum of V
214 pathogens. This inoculum is sampled from a population where some phage genotypes may
215 already carry escape mutations: p_i refers to the frequency of genotype $i \in \{\emptyset, A, B, AB\}$. In the
216 following we focus mainly on scenarios where the frequencies of preexisting escape mutations
217 remain low (i.e. $p_\emptyset \approx 1$). **Figure 2** shows the effect of the inoculum size and the frequency of
218 resistance on pathogen emergence under different deployment strategies.

219 In the absence of pathogen mutations ($p_\emptyset = 1$ and $\mu = 0$) the probability of pathogen
220 emergence is equal to $P_E = 1 - (f_R + 1/R_0)^V$ when $f_R + 1/R_0 > 1$, where $R_0 = b/d$ is the
221 basic reproduction ratio of the pathogen [20]. As indicated with a dashed line in **Figure 2**, this
222 probability of pathogen emergence drops rapidly with the increase in the proportion of resistant
223 hosts and pathogen emergence becomes impossible when $f_R > 1 - 1/R_0$. Note that this
224 threshold is independent of the deployment strategy because they all share the same value of
225 f_R .

226 However, the pathogen population may avoid extinction through the acquisition of
227 escape mutations. The term *evolutionary emergence* refers to these situations where
228 emergence is consecutive to pathogen evolution [28,29]. In **Figure 2** we compare the
229 probabilities of evolutionary emergence in a symmetric scenario where $f_A = 1/2$ for increasing
230 values of f_R (**Figure 2A**) and V (**Figure 2B**). Crucially, the probability that the pathogen adapts
231 to host resistance depends on the deployment of host resistance strategies and the *pyramiding*
232 treatment always yields lower probability P_{EE} of evolutionary emergence. Indeed, in both the
233 *mixing* and the *combining* treatments, the presence of a single-resistance genotype provides
234 a “stepping stone”, allowing the virus to recover the ability to grow in the host population after
235 the acquisition of a single escape mutation. Besides, the acquisition of this first escape
236 mutation may allow the pathogen to acquire later on the ability to escape both types of
237 resistance. The lower probability to acquire both escape mutations at the same time explains
238 the step-like shape of the probability of emergence in **Figure 2** (see also **Figure S1**). As
239 expected, preexisting mutations always increase the probability of pathogen emergence and
240 allow the pathogen population to escape extinction even in the extreme case where $f_R = 1$
241 and no fully susceptible hosts are present in the host population (**Figure 2A**).

242 We can generalize these results for asymmetric scenarios where $f_A \neq 1/2$.
243 Interestingly, variations of f_A have different effects in the mixing and combining treatments
244 (**Figure 3**). In the *mixing* treatment, the probability of emergence is minimized when f_A is close

245 to $1/2$ and thus when the amount of diversity is maximized in line with the effect of diversity
 246 discussed in Chabas et al [20]. In the *combining* treatment, the risk of emergence is minimized
 247 when $f_A = 0$ because this is the case where all the resistant hosts carry two resistances (i.e.
 248 *pyramiding* treatment).

249 The influence of host composition on the probability of evolutionary emergence can be
 250 captured within the framework of evolutionary rescue models. This framework is relevant as
 251 soon as $f_R > 1 - 1/R_0$ because the wild-type virus is doomed to go extinct when the
 252 proportion f_R of resistant hosts leads to a negative growth rate of the wild-type virus population.
 253 We derive approximations for the probability of evolutionary emergence under the assumption
 254 that the viral mutation rate is small (**supplementary information**). In the symmetric scenario
 255 (i.e. $f_A = 1/2$) this yields:

$$\begin{aligned}
 \text{Mixing: } P_{EE}^M &\approx 2V\mu \left(1 - \frac{d}{b(1-c)(1-f_R/2)}\right) + O(\mu^2) \\
 \text{Combining: } P_{EE}^C &\approx V\mu \left(1 - \frac{d}{b(1-c)(1-f_R/2)}\right) + O(\mu^2) \\
 \text{Pyramiding: } P_{EE}^P &\approx O(\mu^2)
 \end{aligned} \tag{1}$$

256 This approximation captures both the effect of a larger inoculum size and the effect of treatment
 257 on P_{EE} illustrated in **Figure 2**. Note that larger inoculum sizes are also expected to increase
 258 P_{EE} via the introduction of pre-existing mutants, not modelled in (1). The above approximation
 259 is particularly useful to discuss the effect of the composition of the host population. In particular,
 260 in the *mixing* strategy the P_{EE} is expected to be twice larger than in the *combining* strategy in
 261 the symmetric scenario. And both these strategies are expected to have higher P_{EE} than the
 262 *pyramiding* strategy because μ_{AB} is assumed to be much smaller than μ_A and μ_B .

263

264 **Experiments**

265 Increasing the size V of the virus inoculum increased the ability to observe the presence of
 266 phages on fully susceptible bacterial populations (Type II Anova: LR Chi-square =3744.2, df=1,
 267 $P < 2.2 \cdot 10^{-16}$) and reached its maximal value when $V > 10^3$ (**Figure S2**). We found an effect of
 268 host treatment on the probability to detect phages on fully susceptible bacteria which is difficult
 269 to interpret because it interacts with the inoculum size (**supplementary information**).
 270 Importantly, note that this treatment effect is not due to pathogen evolution since pathogen
 271 evolution is not detectable when $V < 10^3$.

272 It is tempting to equate our measure of the presence of phages on susceptible bacteria
 273 with the probability of emergence P_E . Yet, as soon as the wild-type phages start to replicate,

274 the proportion of susceptible bacteria is expected to drop and f_R is expected to be ≈ 1 after
275 the overnight culture. So, the presence/absence of phage on susceptible bacteria may actually
276 result from the detection of some of the phages that have been inoculated but did not adsorb
277 to a host cell yet. In the following, we prefer to focus on the analysis of the presence/absence
278 of phage able to replicate on different types of resistant hosts (i.e. host A, B or AB) because it
279 provides an unambiguous measure of the probability of pathogen adaptation to host immunity.

280 Our analysis of the probability of the phage to adapt to at least one type of resistance
281 confirms our predictions on the effect of inoculum and host composition (**Figures 4 and 5**). In
282 particular, we recover the predicted relationship $P_{EE}^M > P_{EE}^C > P_{EE}^P$ when we focused on the
283 *Combining B* treatment (i.e. a combination of strains B and AB): Tukey HSD test, $P_{EE}^M -$
284 $P_{EE}^{CB}=0.78$, $z=3.08$, $P=0.011$; $P_{EE}^{CB} - P_{EE}^P=2.67$, $z=9.40$, $P<0.001$. However, we find no
285 significant differences between the probabilities of viral evolution in the *Mixing* and in the
286 *Combining A* treatments (i.e. a combination of strains A and AB): Tukey HSD test, $P_{EE}^M -$
287 $P_{EE}^{CA}=0.12$, $z=0.49$, $P=0.96$. This suggests that the probability for a virus of acquiring an escape
288 mutation against resistance A is higher than against resistance B. Note that the expected
289 twofold increase in the probability of viral evolution in the *Combining* treatment relative to the
290 *Mixing* treatment (see equation (1)) lies in the 95% confidence intervals we compute: P_{EE}^M/P_{EE}^{CA}
291 $= 1.13 [0.60;2.13]$; $P_{EE}^M/P_{EE}^{CA} = 2.17 [1.14;4.15]$ (**Figure 5B**, red dashed line).

292 Interestingly, similar treatment effects were found when we analysed the ability of
293 phage 2972 to acquire both escape mutations (**Figure S4**). In particular, we found that the
294 *Mixing* treatment was most favourable for the emergence of double escape mutations (Tukey
295 HSD test, $P_{EE_2}^M - P_{EE_2}^{CA}=1.75$, $z=6.71$, $P<0.001$; $P_{EE_2}^M - P_{EE_2}^{CB}=0.84$, $z=3.39$, $P=0.0039$; $P_{EE_2}^M -$
296 $P_{EE_2}^P=1.78$, $z=6.81$, $P<0.001$), even if none of the bacteria carry both resistance in this
297 treatment. This effect likely results from the sequential acquisition of multiple mutations, which
298 is facilitated in the mixing treatment. In other words, the *mixing* strategy is far less durable than
299 the *pyramiding* strategy. Besides, as predicted by our theoretical model, the probability of
300 evolutionary emergence under the *combining* strategy falls in between the two other strategies
301 and confirms that the presence of single-resistant genotypes speeds up the acquisition of
302 escape mutations and promotes evolutionary emergence even when some hosts are
303 multiresistant.

304

305 Discussion

306 In this study, we have explored the influence of several factors such as pathogen life history
307 traits (birth and death rates), mutation rates, pathogen initial inoculum size, fraction and depth
308 of host resistance, on the ultimate fate of a pathogen introduced in a heterogeneous host

309 population. In particular, we showed that larger inoculum size favors the emergence and the
310 adaptation of the pathogen to the host population because of two main effects. First, larger
311 inoculum size increases the probability of the introduction of a preexisting escape mutation
312 which further increases the evolutionary potential of the pathogen population. Second, even in
313 the absence of preexisting mutations in the inoculum, a larger inoculum size of the wild-type
314 pathogen provides more opportunities for the emergence of escape genotypes by mutation.

315 Our theoretical analysis yielded clear predictions on the effect of the host composition
316 on the probability of evolutionary emergence of a pathogen: *pyramiding* is the most effective
317 way to reduce the risk of pathogen adaptation, even in the presence of preexisting escape
318 mutants in the pathogen inoculum (**Figure 2**). The worst strategy is the fully asymmetric *mixing*
319 strategy (e.g. $f_A = 1$) because it takes only a single escape mutant to exploit the whole host
320 population. The fully symmetric *mixing* strategy is better than the asymmetric *mixing* strategy
321 because, as shown by previous studies, higher host diversity reduces the probability of
322 evolutionary emergence [20,24,33–35]. The efficacy of the *combining* treatment is intermediate
323 and is very sensitive to the relative proportion of single and multiple resistances. In particular,
324 we showed that the overlap between the resistance genes carried by single- and double-
325 resistant host genotypes in the *combining* treatment may greatly enhance the risk of
326 evolutionary emergence because escaping single-resistance may provide a “stepping stone”
327 towards the acquisition of multiple escape mutations. We show in the supplementary
328 information how these theoretical predictions can be generalized with more than two resistance
329 genotypes (equations S11 and S12). In particular, we confirm that larger resistance diversity
330 decreases the probability of evolutionary emergence in the *mixing* strategy [20], and the
331 durability of the *pyramiding* strategy increases with the accumulation of resistances.

332 Our experimental results confirmed both the positive effect of larger inoculum size and
333 the hierarchy in the efficacy of different host treatments on the probability of pathogen
334 adaptation. Note, however, that our model oversimplifies several features of the pathogen
335 dynamics taking place in our biological experiments. First, we modeled viral growth as a “birth-
336 death” process while the reproduction of a virulent phage follows a “burst-death” cycle. The
337 burst-death process is expected to alter the variance associated with the reproduction event
338 and may thus alter the predictions of the evolutionary outcome [36], but see [20] for a
339 comparison between these two ways to model pathogen dynamics. Second, we assumed the
340 fraction of the different host genotypes to be constant throughout the experiment. This is a very
341 rough approximation because the fraction of the susceptible hosts will drop relatively rapidly
342 when the wild-type genotype spreads. Consequently, the fraction f_R of resistant hosts is
343 expected to increase rapidly through time. Similarly, the relative fraction of the different types
344 of hosts is expected to vary with time after the emergence of single escape mutations that will
345 exploit specifically a fraction of these resistant hosts. Yet, the good match between our

346 theoretical predictions and our experimental results suggests that the conclusions of our model
347 are robust to the specific details of the epidemiology of the pathogen.

348 Our conclusions are also consistent with a review of the available empirical studies on
349 the durability of different crop protection programs which concluded that *pyramiding* is the most
350 durable strategy [8]. For instance, the durability of wheat cultivars was associated with the
351 pyramiding of multiple resistant genes [7]. A few experimental studies have tracked the
352 evolution of pathogens over several generations and demonstrated the beneficial impact of the
353 *pyramiding* strategy. A study on the evolution of plant-parasitic nematode showed that the use
354 of pyramided genotypes protected the plant-crop over several years [37]. Another study on
355 transgenic broccoli plants indicated that the expression of multiple *Bacillus thuringiensis* (Bt)
356 toxins hampered the epidemiology and evolution of a major insect pest, the diamondback moth
357 (*Plutella xylostella*) [38–40]. In addition, this latter study revealed the detrimental effect of
358 combining the same resistance genes in different plant varieties for the durability of resistance.
359 Indeed, as in our *combining* strategy, the advantage of using plant genotypes containing two
360 dissimilar Bt toxin genes for resistance management may be compromised if they share similar
361 toxins with single-gene plants that are deployed simultaneously.

362

363 **Conclusions and broader implications**

364 Microbes carrying CRISPR-Cas immunity against virulent bacteriophages provide ideal
365 biological models to obtain experimental measures of the probability of pathogen emergence
366 and evaluate their ability to escape host resistance under different control strategies [20].
367 Besides, the specificity of CRISPR-Cas immunity to bacteriophages is very similar to the
368 classical gene-for-gene model of specificity driving the coevolution between many plants and
369 their pathogens. Our biological experiments confirm our theoretical predictions on the influence
370 of (i) the resistance strategy and (ii) the initial dose of the pathogen. In particular, we find that
371 the *pyramiding* strategy is a more effective way to reduce the evolutionary emergence of the
372 pathogen. These microbiological assays confirm that exposing pathogens to a mix of different
373 host genotypes carrying a low number of resistance genes facilitate the adaptation of the
374 pathogen because it provides multiple routes (with slower slopes) towards complex pathogen
375 genotypes carrying multiple escape mutations. This result does not conflict with the positive
376 effect of host diversity for the reduction of pathogen evolutionary emergence [20,41]. But for a
377 given amount of host resistance diversity, the present study shows that stacking this diversity
378 in a limited number of genotypes is a more effective strategy than using a mixture of single-
379 resistance host genotypes to prevent pathogen emergence. The success of the pyramiding
380 strategy may explain why many microbes carry several genes coding for distinct defence
381 systems in their genome [42,43]. While these genomic defence islands may provide immunity

382 against a wide variety of diverse phages, they may also limit the emergence and evolution of
383 bacteriophage variants, thereby increasing the persistence of microbes in various ecosystems.

384 While our results are relevant for several areas, including for crop management in
385 agriculture [8,37,44] as well as in food fermentation [45], they may also hold for the
386 management of drugs and vaccines. In HIV, for instance, the success of the combination
387 therapy is arguably due to the use of the *pyramiding* strategy where the patient is treated
388 simultaneously with multiple drugs [46–48]. A similar conclusion was reached with a theoretical
389 model that explored alternative treatment strategies against bacteria as a combination therapy
390 (*pyramiding*) outperforms other ways to use available antibiotics [49–51]. In malaria, the use
391 of artemisinin-containing combination therapies (*pyramiding*) is also believed to provide a way
392 to slow the spread of antimalarial drug resistance [52–55]. These results suggest also that the
393 use of phage cocktails in phage therapy is likely to be more effective because the pathogenic
394 bacteria will have difficulties to evolve resistance against multiple phages [56,57].

395 The durability of vaccines may also be explained by the *pyramiding* effect. Unlike
396 therapeutic drugs, some vaccines often elicit multiple immune responses against several
397 pathogen targets and this could explain why resistance to vaccines evolves usually more
398 slowly than drug resistance [58]. The recognition of the value of immune diversity could lead
399 to new vaccination strategies. For example, the deployment of different vaccines among
400 different individuals to create a mosaic of vaccination has the potential to outperform
401 conventional vaccination [59]. Moreover, several studies demonstrated that combining multiple
402 immune response to different epitopes can increase significantly the efficacy of vaccination
403 [60–64]. The rise of mRNA vaccines [65,66] may facilitate the development of such new
404 generation of multivalent vaccines that could use the *pyramiding* effect to increase their
405 durability.

406 *Pyramiding* multiple defenses in the same host may thus provide a durable strategy for
407 both prophylactic and therapeutic control of infectious diseases. The recognition of the value
408 of *pyramiding* is ancient [67] but we hope our work clarifies the complex interplay between
409 demography, evolution, and stochasticity. The influence of many other factors remains to be
410 investigated. For instance, our model does not account for the change in the composition of
411 the host population after the start of a viral epidemic. Time-inhomogeneous branching process
412 models could be developed to better understand the influence of these epidemiological
413 feedbacks on evolutionary emergence. In addition, the importance of epistasis in fitness
414 among the escape mutations carried by the pathogen is expected to affect the probability of
415 emergence (see **Figure S1**). Patterns of epistasis are also likely to have an impact on the
416 influence of pathogen recombination [68,69]. Yet, our model does not allow for coinfections
417 and, consequently, does not allow for recombination. The influence of genetic recombination
418 on the robustness of the *pyramiding* effect remains to be investigated. Finally, our joint

419 theoretical and experimental study could be extended to explore a wider range of deployment
420 strategies in space and time [48,70–73]. This approach could thus be used to identify and to
421 test the durability of new strategies to limit the emergence and the evolution of pathogens.

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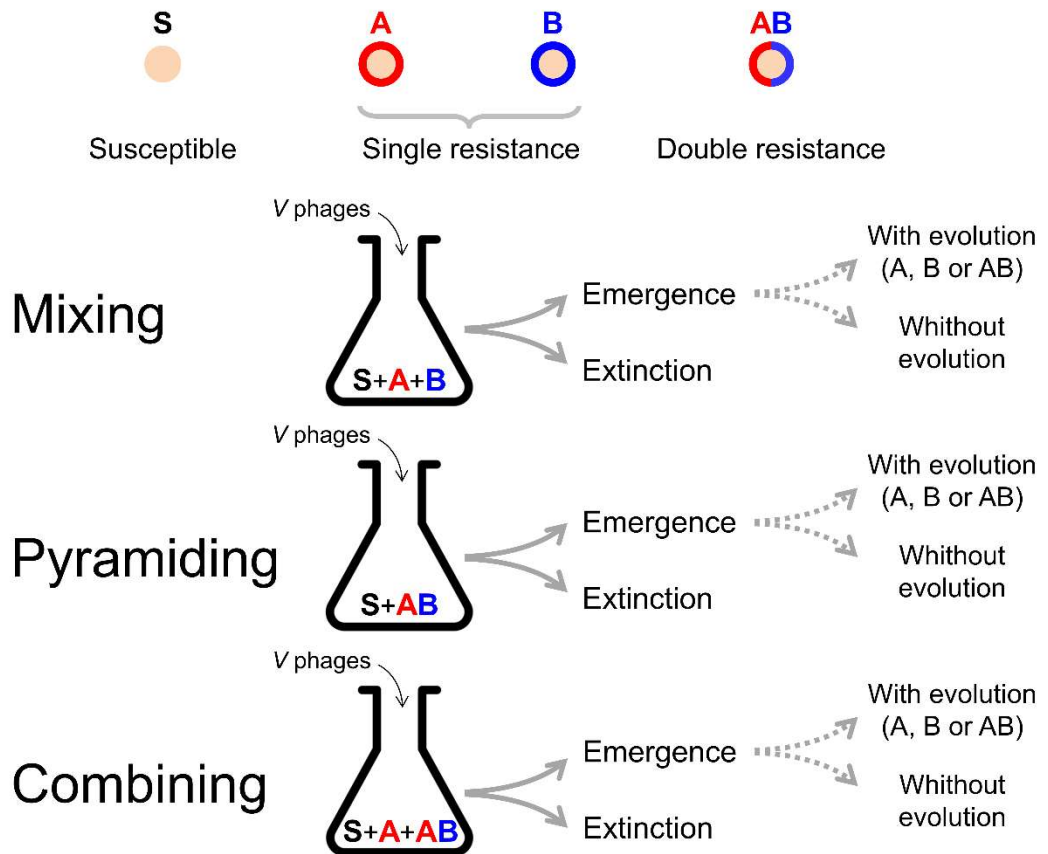
423 **Data and Code Availability**

424 Data has been deposited in zenodo: <https://zenodo.org/records/8114168>

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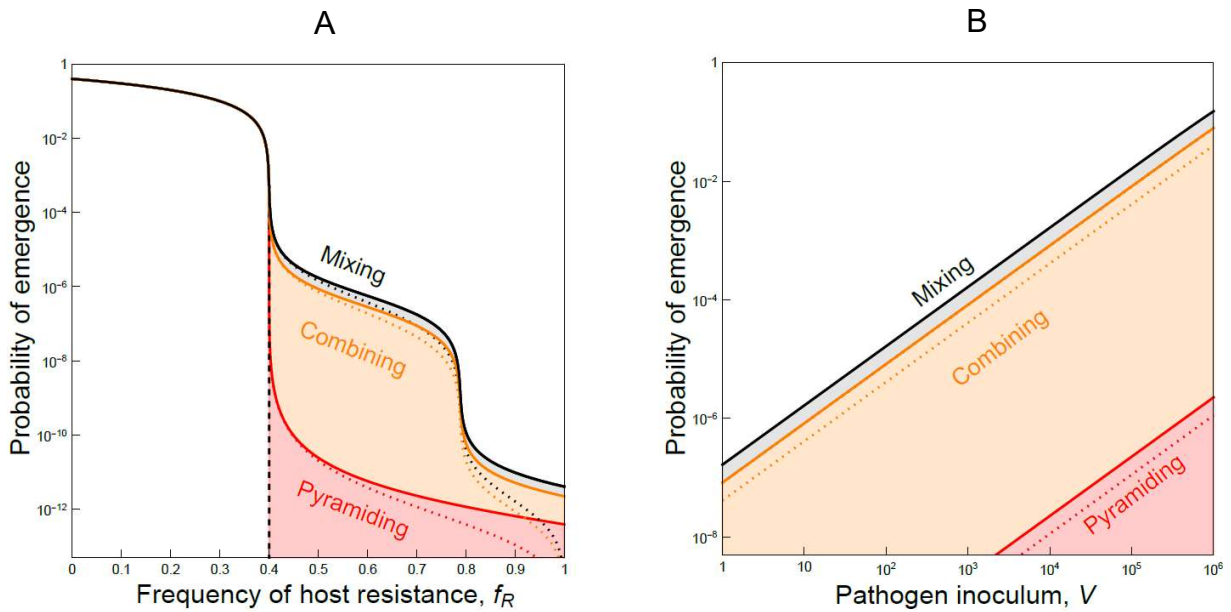
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434 **Figure 1: Schematic representation of the *mixing*, *pyramiding*, and *combining***
 435 **scenarios.** In each scenario the host population is a mix of a proportion $(1 - f_R)$ of susceptible
 436 bacteria (S) and a proportion f_R of resistant bacteria (A, B, and AB). In our experiment we used
 437 $f_R = 0.9$. The composition of the population of resistant bacteria differs between the *mixing*
 438 (1:1 mix of two single-resistant hosts A+B), the *combining* (1:1 mix of a single-resistant host,
 439 A (combining A) or B (combining B), and a double-resistant host AB), and the *pyramiding* (a
 440 double-resistant host AB) scenarios. After the inoculation of V phages the viral population may
 441 either go extinct or produce an epidemic. The virus epidemic may either result from the
 442 replication of the ancestral virus (no evolution) or in the additional replication of phage
 443 genotypes carrying escape mutations against A, B, or AB. We carried out these experiments
 444 in 96-well plates that allowed us to replicate our inoculation experiment in 96 host populations
 445 (each replicate population was $200 \mu L$). After an overnight incubation the cultures (22 h) we
 446 measured (i) the occurrence of phage epidemics (i.e., emergence) by plating a fraction ($2 \mu L$)
 447 of each replicate population on a lawn of sensitive cells (*S. thermophilus* DGCC 7710) and (ii)
 448 the presence of escape phage mutants (i.e., evolutionary emergence) by plating a fraction (2
 449 μL) of each replicate population on a lawn of singly resistant (A or B) or doubly resistant (AB)
 450 bacteria (see **supplementary information**).

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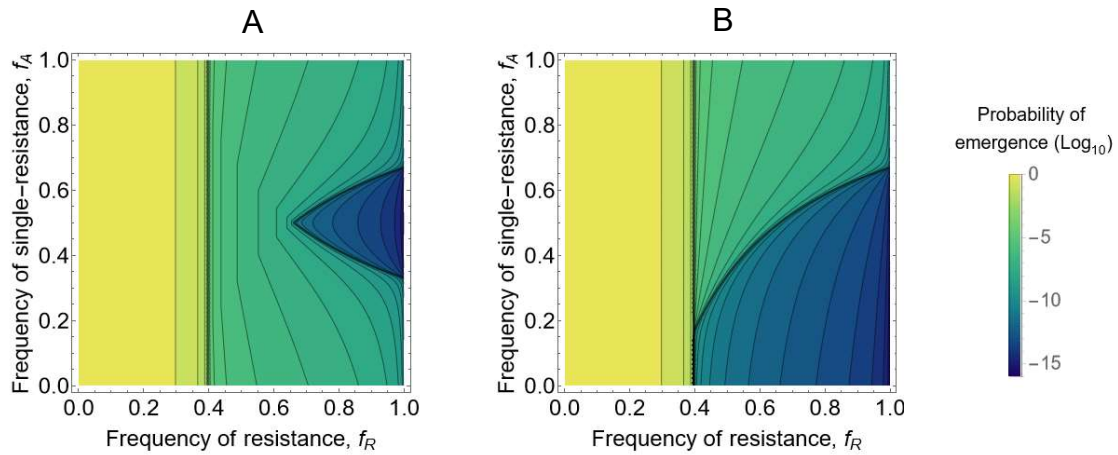


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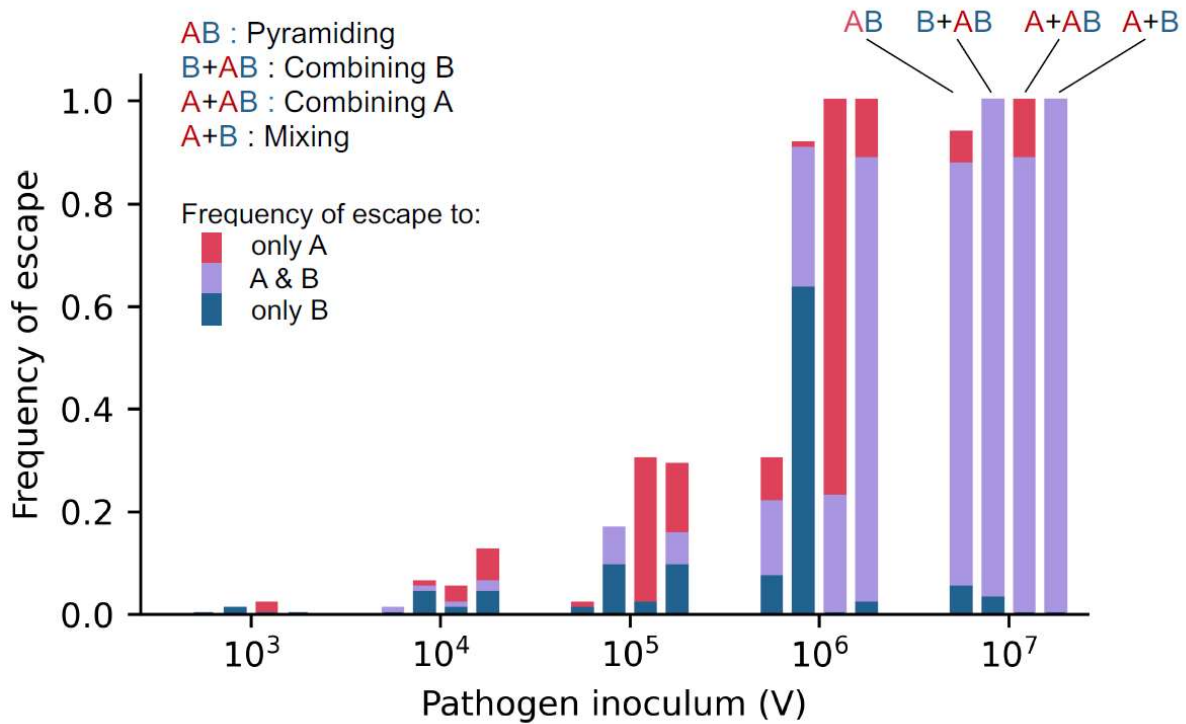
455 **Figure 2: Theoretical predictions for pathogen emergence under the *mixing* (black),**
 456 ***combining* (orange), and *pyramiding* (red) scenarios.** In (A) we show the effect of f_R , the
 457 fraction of resistant hosts in the host population, on the probability of pathogen emergence
 458 after the introduction of a single virus ($V = 1$) with or without preexisting mutations (full or
 459 dotted lines, respectively). The probability of emergence in the absence of pathogen evolution
 460 is indicated with the dashed black line. The color shading refers to *evolutionary emergence* in
 461 the different scenarios (i.e., pathogen emergence resulting from pathogen evolution). In (B) we
 462 show the effect of V , the size of the virus inoculum, on the probability of pathogen emergence
 463 with or without preexisting mutations when $f_R = 0.7$. Other parameter values: $b = 1.66$, $d = 1$,
 464 $c = 0.01$, $\mu = 10^{-6}$, $p_\emptyset = 1 - p_A - p_B - p_{AB}$, $p_A = p_B = 10^{-6}$, $p_{AB} = 10^{-12}$ (without preexisting
 465 mutations: $p_\emptyset = 1$, $p_A = p_B = p_{AB} = 0$).

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469 **Figure 3: Theoretical predictions for pathogen emergence under (A) the *mixing* and (B)**
 470 **the *combining* scenarios.** We show the effect of f_R , the fraction of resistant hosts in the host
 471 population, and f_A , the fraction of a single-resistant host in the resistant host population on the
 472 probability of pathogen emergence after the introduction of a single virus ($V = 1$) and without
 473 preexisting mutations ($p_\emptyset = 1$, $p_A = p_B = p_{AB} = 0$). Other parameter values: $b = 1.66$, $d = 1$,
 474 $c = 0.1$, $\mu = 10^{-6}$.
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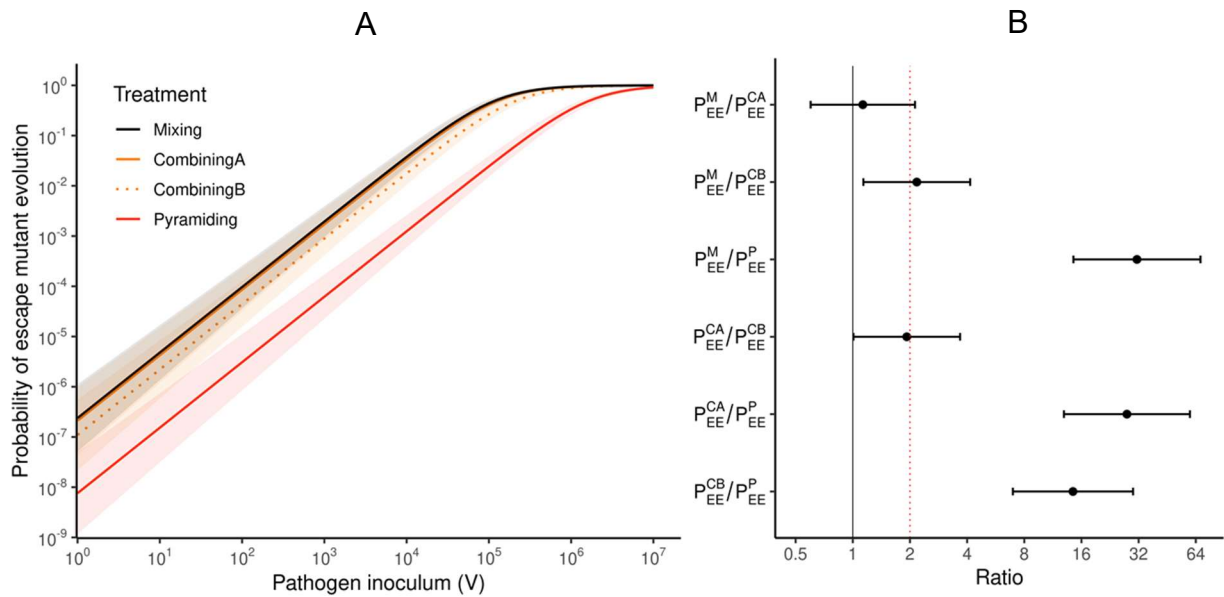
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479 **Figure 4: Probability of pathogen evolutionary emergence for different inoculum dose**
 480 **(V) and for different resistance treatments (mixing, combining, and pyramiding) in**
 481 **biological assays.** We plot the proportion of populations (among the 96 experimental
 482 replicates) that resulted in a virus amplification on different resistant hosts. For each pathogen
 483 inoculum size V we show measures of pathogen evolutionary emergence for the four
 484 experimental treatments (from left to right): (i) Pyramiding AB, (ii) Combining: B+AB, (iii)
 485 Combining: A+AB, (iv) Mixing: A+B. The size of the colored bars measure to the frequency of
 486 emergence of pathogens which could infect only resistant hosts A (red), only resistant hosts B
 487 (blue) and both A and B (purple).

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Figure 5: Probability of evolutionary emergence is higher in the *mixing* treatment and lowest in the *pyramiding* treatment in biological assays. (A) We plot here the estimation of the probability of evolutionary emergence (i.e., the probability to evolve at least one escape resistance) against the inoculum size V and the resistance treatment. The model can be written as $\text{logit}(P_{EE}^T) \sim a_T \log(V) + b_T$, where the slope parameter is the same for all treatments (see **supplementary information**). The lines indicate the prediction of the statistical model for the different treatments and the shaded areas show 95% confidence interval. (B) We compare the estimated values of b_T (i.e. the probability of evolutionary emergence of treatment T for an inoculum $V = 1$) for all pairs of treatment and we plot $e^{b_{T1} - b_{T2}} \approx P_{EE}^{T1} / P_{EE}^{T2}$. The error bars show 95% confidence interval and the red dashed line refers to a two-fold difference in the probability of emergence. This two-fold effect is expected when we compare the mixing and the combining treatments (P_{EE}^M / P_{EE}^{CA} and P_{EE}^M / P_{EE}^{CB} , see equation (1)).

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