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Agrichemicals boost the effects of antibiotics on antibiotic resistance evolution

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Antibiotic resistance is medicine's climate change: caused by human activity, and resulting in more extreme outcomes. Resistance emerges in microbial populations when antibiotics act on phenotypic variance within the population. This can arise from either genotypic diversity (resulting from a mutation or horizontal gene transfer), or from 'adaptive' differences in gene expression due to environmental variation. Adaptive changes can increase fitness allowing bacteria to survive at higher concentrations of the antibiotic. They can also decrease fitness, potentially leading to selection for antibiotic resistance at lower concentrations. There are opportunities for other environmental stressors to promote antibiotic resistance in ways that are hard to predict using conventional assays. Exploiting our observation that commonly used herbicides can increase or decrease the minimum inhibitory concentration (MIC) of different antibiotics, we provide the first comprehensive test of the hypothesis that the rate of antibiotic resistance evolution under specified conditions can increase, regardless of whether a herbicide increases or decreases the antibiotic MIC. Short term evolution experiments were used for various herbicide and antibiotic combinations. We found conditions where acquired resistance arises more frequently regardless of whether the exogenous non-antibiotic agent increased or decreased antibiotic effectiveness. This "damned if you do/damned if you don't" outcome suggests that the emergence of antibiotic resistance is exacerbated by additional environmental factors that influence competition between bacteria. Our work demonstrates that bacteria may acquire antibiotic resistance in the environment at rates substantially faster than predicted from laboratory conditions.

Classification: Biological sciences / evolution

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Short title: Herbicide evolved antibiotic resistance

Abstract

Antibiotic resistance is medicine's climate change: caused by human activity, and resulting in more extreme outcomes. Resistance emerges in microbial populations when antibiotics act on phenotypic variance within the population. This can arise from either genotypic diversity (resulting from a mutation or horizontal gene transfer), or from 'adaptive' differences in gene expression due to environmental variation. Adaptive changes can increase fitness allowing bacteria to survive at higher concentrations of the antibiotic. They can also decrease fitness,

potentially leading to selection for antibiotic resistance at lower concentrations. There are opportunities for other environmental stressors to promote antibiotic resistance in ways that are hard to predict using conventional assays. Exploiting our observation that commonly used herbicides can increase or decrease the minimum inhibitory concentration (MIC) of different antibiotics, we provide the first comprehensive test of the hypothesis that the rate of antibiotic resistance evolution under specified conditions can increase, regardless of whether a herbicide increases or decreases the antibiotic MIC. Short term evolution experiments were used for various herbicide and antibiotic combinations. We found conditions where acquired resistance arises more frequently regardless of whether the exogenous non-antibiotic agent increased or decreased antibiotic effectiveness. This "damned if you do/damned if you don't" outcome suggests that the emergence of antibiotic resistance is exacerbated by additional environmental factors that influence competition between bacteria. Our work demonstrates that bacteria may acquire antibiotic resistance in the environment at rates substantially faster than predicted from laboratory conditions.

Significance

Neither reducing the use of antibiotics nor discovery of new ones may be sufficient strategies to avoid the post-antibiotic era. This is because bacteria may be exposed to other non-antibiotic chemicals that predispose them to evolve resistance to antibiotics more quickly. Herbicides are examples of some of the most common non-antibiotic chemicals in frequent global use. In some combinations the herbicides we tested made bacteria phenotypically resistant to higher concentrations of antibiotics, while in other combinations bacteria became susceptible at lower antibiotic concentrations. In both cases the herbicides worked with antibiotics to accelerate genotypic resistance evolution. Unfortunately, antibiotic resistance may increase even if total antibiotic use is reduced, and new ones are invented, unless other environmental exposures are also controlled.

Key words antibiotic resistance, evolution, adaptive resistance, herbicide, minimum selective concentration

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57 **Abbreviations** MIC, minimum inhibitory concentration; MSC, minimum selective
58 concentration; NOEL, no observable effect level

Introduction

As fundamental tools for infection control, antibiotics underpin diverse human systems ranging from hospital care to concentrated animal feeding operations through to crop and pollinator disease management. The loss of this tool due to antibiotic resistance will result in higher mortality and morbidity, but also deny access to many routine medical procedures for risk of subsequently untreatable infections (Teillant et al. 2015; Thomas et al. 2014). Antibiotic resistances also threaten agricultural productivity (Stockwell & Duffy 2013; Van Boeckel et al. 2015). Despite over a half century of warning, neither science nor innovation has managed to invent us away from the threat of a post-antibiotic era.

One stewardship strategy is using them less so that we might use them for longer (CDC 2013; Collingnon et al. 2016). If bacteria almost never encounter antibiotics at concentrations high enough to harm them, there would be little opportunity for resistant variants to emerge and establish. Based on this, it has been suggested that judicious use of antibiotics that keeps most antibiotic exposures to less than the minimum inhibitory concentration (MIC) should preserve antibiotic susceptibility in bacteria (Andersson & Hughes 2014). In practice, the lowest concentration of antibiotic leading to the evolution of resistance in a given environment, the so called minimum selective concentration (MSC), can be much lower than the MIC (Fig. 1) (Andersson & Hughes 2014; Baquero et al. 1998b; Hermsen et al. 2012).

Variation in antibiotic responses can be caused by either genetic or physiological differences between individual bacteria. The toxic effect of an antibiotic may occur at different concentrations for different individuals because some have *acquired* genes or alleles through mutation or horizontal gene transfer (i.e. change in genotype). Also, organisms can have *innate* differences between them, e.g. due to differences in permeability.

Innate resistance can also dependent upon genes expressed or repressed conditionally, resulting in increased efflux or decreased influx of antibiotics and overall lower intracellular antibiotic concentrations (Fernandez & Hancock 2012). Such genes or expression induction thresholds may differ between species, and individuals within a species may phenotypically differ depending on whether or not they expressed those genes before being inhibited by the antibiotic. This resistance through changes in gene expression is also known as an *adaptive* (change in phenotype) response. It can be triggered by antibiotics and other chemical toxins or

environmental cues (Blair et al. 2015; Palmer & Kishony 2013; Sanchez-Romero & Casadesus 2014).

Populations of adaptively resistant bacteria can in time produce variants with acquired resistance to even higher concentrations of antibiotic (Cohen et al. 1989; Gustafson et al. 1999; Shen et al. 2011). This raises the possibility that environmental stimuli that cause phenotypic antibiotic resistance variation between individuals could be hotspots for evolution of acquired antibiotic resistance.

In earlier studies, we found that herbicides could induce adaptive changes in how bacteria respond to antibiotics (Kurenbach et al. 2017; Kurenbach et al. 2015). Some herbicides and antibiotic combinations increased the antibiotic MIC (Fig. 1A) and some lowered it (Fig. 1B), while others did not change the MIC but did alter survival at below-MIC concentrations (Fig. 1C). Here we exploit those observations to test the novel hypothesis that both increases *and* decreases in MIC caused by exposure to herbicides can lead to an increase in the rate of acquired resistance evolution in populations of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium.

Materials and Methods

Media

Strains and plasmids are listed in Table 1. Bacteria were grown in standard rich growth medium, LB (Lennox) (Invitrogen) at 37°C and supplemented with ampicillin (Amp, AppliChem), chloramphenicol (Cam, Sigma) ciprofloxacin (Cip, Pentex), streptomycin (Str, Sigma), tetracycline (Tet, Sigma), or nalidixic acid (Nal, Sigma) as appropriate. Liquid cultures were grown with aeration (180 rpm), and plates were incubated in plastic bags to avoid drying out. Commercial herbicide formulations were Kamba^{500} (Nufarm, NZ) containing 500 g/L dimethyl salt of dicamba, and Roundup Weedkiller (Monsanto, Australia) containing 360 g/L isopropylamine salt of glyphosate. Herbicide concentrations are reported in parts per million acid equivalent (ppm ae) to allow for comparison with other formulations. Antibiotic and herbicide concentrations used are specified in the main text or in figure legends.

Plasmid constructs

Plasmid pAH14 was created by removing the *Hin*dIII and *Bam*HI fragment of pBR322 and inserting *cat* from pACYC184 at the *Pst*I site within *bla*. The resulting traits for this plasmid were Cam^R, Amp^S, Tet^S. pAH11 was created by insertion of *cat* from pACYC184 into the *Eco*RI and *Not*I sites of RSF1010, resulting in plasmid-determined traits Cam^R, Str^S.

Culturing conditions

E. coli or *S. enterica* were grown in 10 mL liquid LB medium containing Cip, herbicide, both, or neither. Initial densities were ca. 10^5 cfu/mL for the former treatment and ca. 10^6 cfu/mL (*E. coli*) and 10^5 cfu/mL (*S. enterica*) for the latter three. Cultures were grown for 24 hrs. For the experiments described in the section "Acquired resistance frequencies increase due to herbicide-induced increases in MIC" the experiment was abandoned if cultures containing Cip in the absence of herbicide were visibly turbid at this point. Here, the chosen Cip concentration was above MIC, and growth was therefore interpreted as the presence of a resistant mutant in the starting culture. Herbicide and antibiotic concentrations used are detailed in Table 2 and Table 3. Cultures were then diluted as above in the same conditions and incubated again for 24 hrs. A 10-fold dilution in LB without herbicides or antibiotics followed to ensure observed effects were not due to herbicide induced adaptive responses. Titers (cfu/mL) were determined at the end of each incubation step on both LB and on Cip at an initially non-permissive concentration (ca. 2x MIC) (Dan et al. 2016). The frequency of Cip resistant mutants and the number of generations was calculated.

Determination of resistance levels

Individual colonies of strains grown on solid LB medium were used to inoculate 100 µL of liquid LB in a 96 well plate. The plate was incubated with aeration to saturation before ca. 4 µL samples were stenciled onto LB plates containing the appropriate antibiotics. Plates were incubated at 37°C for 18 hrs. Strains were scored positively for growth if growth was tangible.

Competition experiments

Isogenic strains of *E. coli* differing only in the MIC phenotype (i.e. high vs. low) and an additional selection marker encoded on a low copy number plasmid were co-incubated in liquid LB medium containing herbicide, antibiotic, both, or neither and grown to saturation before dilution by a factor of 10^3 in the same conditions. The antibiotic concentration chosen was below

NOEL for both strains. The titer of each culture was determined by plating on non-selective medium after each incubation step. After 5 rounds of incubation, the ratio of strains was determined by selecting for the second – competition irrelevant – marker.

Natural selection was defined as the difference in the exponential growth rate of the two strains (van den Bosch et al. 2014). Under this interpretation, the change in the proportion of resistant individuals per unit of time is the logistic curve (Mallet 2012) with the explicit solution $p=e^{st}/(c+e^{st})$, where t=time, p=proportion of resistant individuals, s=strength of selection, c=constant describing the initial proportion of resistant individuals, $c=(1/p_0)-1$. The constant p_0 is the proportion of resistant individuals at t_0 (the start of the experiment). t is defined as the number of generations, using the generation time for the more resistant strain. By rearranging this formula, the strength of selection is $s= \ln(pc/(1-p))/t$.

Growth curves

Growth curves were established at 37°C in liquid LB medium supplemented with herbicide, antibiotic, both, or neither using a FLUOstar Omega microplate reader (BMG LABTECH, Germany). The OD_{600} was determined every 6 min for 16 hrs and averaged over five replicates. Cultures were started at densities of ca. 10^6 cfu/mL. Antibiotic and herbicide concentrations used are detailed in the legend of Suppl. Fig. S1.

Statistical analysis

R was used for all statistical analyses (R Core Team 2013). For mutant frequency experiments (changes to MIC), an ANOVA was used to analyze the randomized complete block design, using each independent experiment as a block with presence/absence of antibiotic/herbicide as levels. Residuals were used to test for normality and equality of variances and log transformed data where appropriate. Tukey's HSD test was used to determine which treatments were significantly different from each other.

For mixed culture experiments (changes to MSC), two sets of analyses were performed. First, we determined whether adding antibiotics increases the strength of selection at different herbicide concentrations. Second, we determined whether adding herbicides increases the strength of selection at different antibiotic concentrations. Each set of questions was tested using contrasts performed with the glht function in the multcomp package (Hothorn et al. 2008) with a two-sided

alternative and sequential Bonferroni procedure. These contrasts were fit to an ANOVA model treating each combination of herbicide and antibiotic as a treatment category. Residual plots confirmed that the assumptions of normality and equality of variance were met.

Statistical analysis for growth curves

To estimate carrying capacity (k), a logistic growth model on the raw data using non-linear least squares (nls) was fit in R. Differences in population growth between strains in different treatments were estimated by estimating r, the intrinsic growth rate, of each strain. Data sets were log transformed and the slope of the growth curve between t=48 mins and t=150 mins was measured. A visual inspection of the plots revealed that before t=48 mins graphs were not linear. After t=150 mins, growth slowed as cells were entering stationary phase.

We tested for differences between the two strains in both r and k by calculating contrast in an ANOVA (aov). Residual plots were used to test for violations of assumptions for ANOVA. Assumptions of normality and equal variances were met in all data sets. In two data sets there were a small number of outliers. These had small influences on parameter estimates and were hence not removed. Contrasts between treatments were calculated using the glht package in R using the sequential Bonferroni correction.

Results

Acquired resistance frequencies increase due to herbicide-induced increases in MIC

Exposing either *E. coli* or *S. enterica* to the herbicide formulation Roundup increased the MIC of the fluoroquinolone antibiotic ciprofloxacin, as did exposing *S. enterica* to the herbicide Kamba. This was due to adaptive changes induced by herbicide exposure (Kurenbach et al. 2015). These combinations were used to test the hypothesis that the opportunity afforded by reproduction of bacteria exhibiting adaptive resistance to normally lethal concentrations of antibiotic was sufficient for the population to evolve higher frequencies of resistant genotypes.

The rate of acquired resistance mutations in populations of *E. coli* or *S. enterica* (all strains described in Table 1) was measured over the course of about 25 generations in a standard rich growth medium, liquid LB, or LB medium containing the appropriate herbicide (below the No Observable Effect Level, NOEL) with or without ciprofloxacin supplementation (Table 2). The

ciprofloxacin concentration was the same in all cultures to which it was added but below MIC for bacteria in cultures simultaneously exposed to herbicide. The bacteria were then transferred to solid medium supplemented with high levels of ciprofloxacin (≈ 2x MIC) and no herbicide, which was permissive only to the growth of variants with acquired resistance.

Genetic variants able to grow on high concentrations of ciprofloxacin after 25 generations in LB medium, with or without herbicide supplementation, arose at the same frequency. This indicated that the herbicides were not mutagens at these concentrations. In a separate standard test of mutagenicity (Funchain et al. 2001), bacteria were exposed to herbicides and plated on the antibiotic rifampicin. No difference in resistance frequencies were observed (data not shown).

Consistent with our prediction that herbicide-induced adaptive resistance allowed rare spontaneously arising ciprofloxacin resistant mutants to increase a culture's MIC, populations of bacteria with continuous exposure to herbicide and antibiotic had significantly higher numbers of ciprofloxacin resistant variants. The frequency ranged from 10^2 times higher for the combination of Cip+Kamba+*S. enterica* to 10^5 times higher for Cip+Roundup+*E. coli* (Table 2).

Cip resistant colonies were isolated at the end of each experiment from all treatments. We determined MICs for 56 *S. enterica* isolates (isolated on 0.05 µg/mL Cip): 27 isolates from Kamba+Cip and 29 isolates from Roundup+Cip treatments. The parental strain and 2 isolates from each LB, Kamba, and Roundup treatments, also isolated on 0.05 µg/mL Cip, were included as controls.

The parental strain and 6 evolved isolates did not grow at 0.07 µg/mL, a concentration just above the selection concentration. We observed MICs of 0.1 µg/mL Cip for 21 isolates and 0.2 µg/mL Cip for 28 isolates. Only 7 isolates displayed higher MICs (2 from Kamba+Cip, 4 from Roundup and Cip, and 1 from LB), the highest being 1.25 µg/mL Cip reached by one isolate isolated from a Roundup+Cip culture. Importantly, we observed no correlation between level of resistance and original treatment, which indicates that there are no qualitative differences between the ciprofloxacin resistant variants arising in the different treatments.

Herbicide-induced adaptive changes in MIC also change MSC

Antibiotic resistant bacteria are becoming a fixed part of many environments despite the concentration of antibiotics often being very low (Hermsen et al. 2012). We hypothesized that exposure to some herbicides can shift the MSC to lower antibiotic concentrations leading to competition between individuals with different physiological responses and thus providing an environment in which genotypically resistant bacteria evolve.

Mixed cultures of *E. coli* were created to represent pre-existing antibiotic resistance heterogeneity within natural environments. The phenotypic differences were created using bacteria carrying isogenic plasmids with different alleles of *tetA* (Tet^{high}, Tet^{low}, Table 1). The MIC for tetracycline of Tet^{low} was 1.5 µg/mL and for Tet^{high} it was 125 µg/mL.

Tet^{high} and Tet^{low} were competed in liquid LB, LB+Tet, and LB+Tet+Roundup media for about 42 generations. This herbicide lowers the antibiotic MIC (Fig. 1B). The same concentration of Roundup was used in combination with various concentrations of tetracycline, all of which were below the MIC of Tet^{low}. Roundup reduced tetracycline MIC for Tet^{low} to 0.5 µg/mL, but growth of Tet^{high} was not affected for tetracycline concentrations up to 50 µg/ml, far above levels used in this experiment.

The MSC, i.e. the concentration of tetracycline at which one strain started to dominate the culture, was determined from the calculated selection coefficients. A positive coefficient indicated selection for the more resistant strain. The selection coefficient was significantly different for competitions in environments supplemented with ≥0.1 µg/mL tetracycline compared to competitions in LB medium (Fig. 2). Mixed cultures in LB+Tet+Roundup had significantly different selection coefficients compared to the LB competitions beginning at a tetracycline concentration of 0.05 µg/mL.

A reverse experiment was performed using Kamba instead of Roundup. This herbicide increases the MIC of Tet^{low} from 1.5 to 3 µg/mL. When Kamba was part of the environment, the selection coefficient was significantly different from the competition in LB medium at a tetracycline concentration ≥0.5 µg/mL, compared to ≥0.1 µg/mL in its absence (Fig. 2). In both cases, MIC for the Tet^{low} strain and MSC moved in the same direction.

Herbicide-induced changes in MSC can occur without a change in MIC

E. coli cultures in medium supplemented with Kamba grew faster at some sub-lethal concentrations of ciprofloxacin, but Kamba did not change the MIC (Fig. 1C) (Kurenbach et al. 2015). To determine if exogenous chemicals that alter fitness at sub-lethal antibiotic concentrations - but do not change the MIC - have an impact on the evolution of resistance, we measured the frequency at which acquired ciprofloxacin resistance arose during culture in the combination of Cip+Kamba+*E. coli*.

The mutation rate of an *E. coli* monoculture was measured over the course of about 25 generations in a standard rich growth medium, liquid LB, and in LB supplemented with Cip, Kamba, or Cip+Kamba. The experiment was similar to those described in the section "Acquired resistance frequencies increase due to herbicide-induced increases in MIC", but here the ciprofloxacin concentration was below MIC. The bacteria were then transferred to solid medium supplemented with high levels of ciprofloxacin (≈ 2x MIC) and no herbicide, which was permissive only to the growth of variants with acquired resistance.

The frequency of more resistant variants was the same for cultures grown in LB, LB+Kamba or LB+Cip+Kamba (for both treatment combinations $p > 0.2$; Table 3). Therefore the ciprofloxacin MSC was not reached under any of these conditions.

The frequency of more resistant variants recovered from the LB+Cip culture condition was about 10^5 times higher than the other three culture conditions ($p < 10^{-4}$ for all treatment combinations), indicating that the ciprofloxacin concentration met or exceeded the MSC. The addition of Kamba neutralized the selective effect of ciprofloxacin by shifting the MSC to a higher level without changing the MIC.

Herbicide-induced changes to MSC are herbicide concentration-dependent

Pairs of isogenic strains of *E. coli* (Table 1) with either different streptomycin (Str^{high}, Str^{low}) or tetracycline (Tet^{high}, Tet^{low}) MICs were competed for about 40 generations. The frequency of each strain after competition was measured. The concentration of the appropriate antibiotic did not vary between cultures, but the concentration of herbicide did. Combinations were chosen that lead to a decrease in MIC with the chosen herbicide, namely Str+Kamba, and Tet+Roundup.

There was no differential in the fitness of paired strains when they were co-cultured in standard rich medium (Fig. 3). In cultures containing antibiotic but not supplemented with herbicide neither strain had a fitness advantage, indicating that the antibiotic concentration was below MSC. Likewise, the herbicide alone did not affect fitness except for a statistically non-significant effect at the highest tested concentration of Roundup.

In each test, the combination of antibiotic and herbicide reduced MSC. The more resistant strain invariably increased relative to the isogenic competitor in a herbicide dose-dependent manner when both antibiotic and herbicide were in the environment. Selection coefficients were statistically significantly different from LB medium at 183 parts per million acid equivalent (ppm ae) Kamba and 311 ppm ae Roundup.

The observed fitness differential was due to the faster reproductive rate of the Str^{high} and Tet^{high} strains in environments with sub-lethal concentrations of antibiotic for both strains in each pair. This was shown by measuring the growth rate of the strains in monoculture rather than in competition (Supplementary Fig. S1). No differences were observed for the monoculture growth rate (r) of matched isogenic pairs in LB medium, LB+antibiotic, or LB+herbicide ($p>0.1$ for all combinations; Supplementary Fig. S1A and C). Only in the combined LB+herbicide+antibiotic treatment did strains with higher MICs have shorter generation times in monoculture than their lower MIC counterparts ($p=1.6x10^{-5}$ (Kamba+Str) and $p=4.7x10^{-10}$ (Roundup+Tet)).

Significant differences in the carrying capacity of the environments for all treatment combinations were observed for the matched strains exposed to Kamba+Str measured in monoculture. The high MIC strain achieved higher final optical densities ($p<2x10^{-16}$; Supplementary Fig. S1B). In contrast, the Str^{low} strain population grew to higher final optical densities in LB, LB+Tet, and LB+Roundup treatments. In cultures with Roundup+Tet, Tet^{high} and Tet^{low} had similar densities ($p=0.24$, Supplementary Fig. S1D).

Discussion

In this study we report that when bacteria are simultaneously exposed to herbicides and antibiotics, mutants with higher levels of resistance can evolve. In some cases, resistance evolved 100,000 times faster.

Herbicides can increase the MIC of some antibiotics. At what otherwise would be a lethal concentration of the antibiotic, the bacteria can continue to reproduce. Each reproductive event has a low but steady potential of producing a variant daughter with a higher MIC. We found that these strains have a fitness advantage and accumulate differentially to their low MIC cousins.

Herbicides can also decrease the MIC of some antibiotics. At what otherwise would be a concentration of antibiotic below the MSC, too low to have an effect on the fitness of two bacteria differing in their MICs, we found that the bacteria with the higher MICs replaced the bacteria with lower MICs. The shift in MSC seemed to be of a similar magnitude as the shift in MIC observed for the lower MIC strain.

Finally, herbicides can also alter survival potential at some antibiotic concentrations but not change the concentration to which the entire population is innately susceptible. In the case presented here, MSC was shifted by the herbicides, but MIC was not. The herbicide mitigated the selective pressure caused by the antibiotic, and no genotypes with higher resistance levels established in the population. Although not tested, it is likely that in the reverse case, when MSC is lowered by a substance, antibiotic resistance may arise at higher frequencies.

Our research shows that manufactured chemical products such as the herbicides can have a complex effect on the evolution of antibiotic resistance. They did not replace antibiotics, but could accelerate resistance evolution. Herbicides that reduce the MSC will be more effective at stimulating resistance evolution at the lower ends of the antibiotic concentration gradient, while herbicides that increase the MIC will be more effective at stimulating resistance evolution at the higher ends.

Infections by antibiotic resistant bacteria are increasingly common, with the human and economic costs also increasing (Friedman et al. 2016). As a consequence of the rise of resistant pathogens, combination treatments using a non-antibiotic to increase an antibiotic's effectiveness have been suggested to help preserve the usefulness of antibiotics (Allen et al. 2014; Schneider et al. 2017; Wright 2016). Indeed, various combinations are in clinical use (Worthington & Melander 2013). While this might improve or enable treatment of an infection, our results indicate that decreasing bacteria's survival by making an antibiotic more potent may increase the development of resistance by creating more environments where adaptive pressure is present.

Herbicides and different ingredients in product formulations can have antimicrobial activities, with some being more potent antimicrobial agents than are others (Kurenbach et al. 2017). For example, Roundup was more toxic than Kamba to the bacteria that we used. The low level toxicity is likely how they induce adaptive resistance, a source of phenotypic heterogeneity (Kurenbach et al. 2015). In addition, biocidal agents that are mutagens may introduce genotypic heterogeneity. At the concentrations used in this work, the herbicides did not appear to be mutagenic.

Antibiotic and herbicide gradients may be viewed as two environmental dimensions concentrating competition between bacteria of different genotypes and physiotypes. The particular concentrations of antibiotic and herbicide at any point of intersection of the gradients above a threshold level increases the frequency of the most fit strains and species, amplifying any linked or associated traits in the more fit competitor. While antibiotics can do this without herbicides (Andersson & Hughes 2014; Baquero et al. 1998a; Denamur & Matic 2006), the herbicides used in this study increase the range of concentrations under which the antibiotic affects the evolution of resistance.

The concentrations of herbicides and antibiotics we used were below recommended application levels and hence within relevant environmental ranges, suggesting that what we observe has the potential to occur in many places where the two biocides are found together. Simultaneous herbicide and antibiotic exposures are common. Herbicides are used in agriculture, where spray drift or walking through treated fields exposes farm livestock and pets, which may be on therapeutic or prophylactic antibiotics. Most ingested antibiotic is not metabolized and thus excreted (Chee-Sanford et al. 2009), becoming mixed with soil as crop fertilizer which *in situ* may be subsequently sprayed with herbicide. Microbes from these mixes may be carried by blow- and house-flies (Zurek & Ghosh 2014). Likewise honeybees may be exposed to herbicide spray or residues as they forage and return to an antibiotic-treated hive. Additionally, herbicides are used in urban environments for purposes like gardening and lawn care, including parks and roadsides (Atwood & Paisley-Jones 2017). Worldwide, herbicide use was approximately 1×10^9 kilograms in 2012 with up to 2×10^8 kilograms of the active herbicidal ingredients glyphosate, 2, 4-D and dicamba used in the US in 2012 (Atwood & Paisley-Jones 2017).

Other chemicals also have been shown to cause adaptive resistance and to increase resistance frequencies (Egeghy et al. 2012; Gustafson et al. 1999; Levy 2001). Non-antibiotic prescription medicines and food emulsifiers select antibiotic resistant gut bacteria (Kurenbach et al. 2017; Maier et al. 2018). Approximately 8 million manufactured chemical substances are currently in commerce (Egeghy et al. 2012; Shen et al. 2011). According to the US Environmental Protection Agency, annual production of each of the top 3,000 chemicals is greater than 6 x 10^{11} kilograms/year (EPA 2008). They are not regulated for effects on antibiotic resistance and not tested for such effects.

The susceptibility of bacteria to antibiotics must be seen as a non-renewable resource, one that requires careful stewardship worldwide (Amabile-Cuevas 2016; Heinemann & Kurenbach 2017). Evidence that antibiotic resistance evolution is influenced by exposure of bacteria to a wide range of substances may require us to make changes in how we manage both antibiotics and other manufactured and widely distributed chemical products. This is because many facets of the extrinsic environment induce adaptive changes, a complexity frequently ignored in standard studies of resistance. As our results show, complex effects of exposures to non-therapeutic chemicals may undermine strategies to preserve the effectiveness of antibiotics through altering just their use. To our knowledge, there has been no attempt to systematically test common chemicals to which pathogenic bacteria are chronically exposed for effects on antibiotic resistance.

Conclusions

Neither reducing the use of antibiotics nor discovery of new ones may prevent the post-antibiotic era. This is because bacteria may be exposed to other non-antibiotic chemicals that predispose them to evolve resistance to antibiotics more quickly. Herbicides are examples of some of the most common non-antibiotic chemicals in frequent global use. More research is necessary to see to what extend other different manufactured chemicals may contribute to this effect. Moreover, depending on how the manufactured chemicals are used, or how they move through the waste stream, there may be combinatorial effects caused by mixtures of different products. Future work should take into account likely combinations as well as different ways that microbes could be exposed to chemical products.

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Figure 1(on next page)

Effects of herbicides on bacterial responses to antibiotics.

Herbicides can change responses to antibiotics. Solid curves indicate survival as a function of only antibiotic concentration, intersecting the x-axes at the MIC. Dashed curves show herbicides (A) increasing the MIC, (B) decreasing the MIC, and (C) having no effect on MIC but altering population survival at antibiotic concentrations below-MIC. The hypothetical MSC is the point of divergence of solid and dashed curves and the area in gray illustrates all antibiotic concentrations where the herbicide changes the response to antibiotics.

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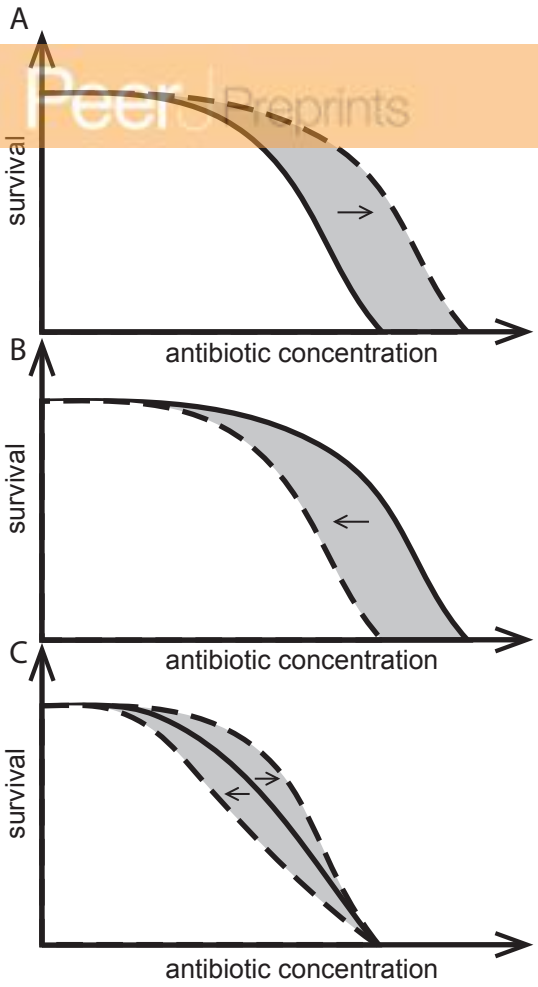

Figure 2(on next page)

Competition between strains of different potential to resist tetracycline.

Competition between tetracycline resistant (AH201, Tet^{high}) and susceptible (AH214, Tet^{low}) strains in different concentrations of tetracycline with or without Kamba (K) or Roundup (R). Kamba was used at 1830 ppm ae, Roundup at 311 ppm ae. Selection coefficients are presented as a function of tetracycline concentration, with positive values indicating a selection for the more resistant strain. Values are averages of three independent experiments; error bars are SEM (standard deviation/√n). * indicate the lowest tetracycline concentrations for each treatment where selection coefficients were significantly different from the -Tet/-herbicide treatment.

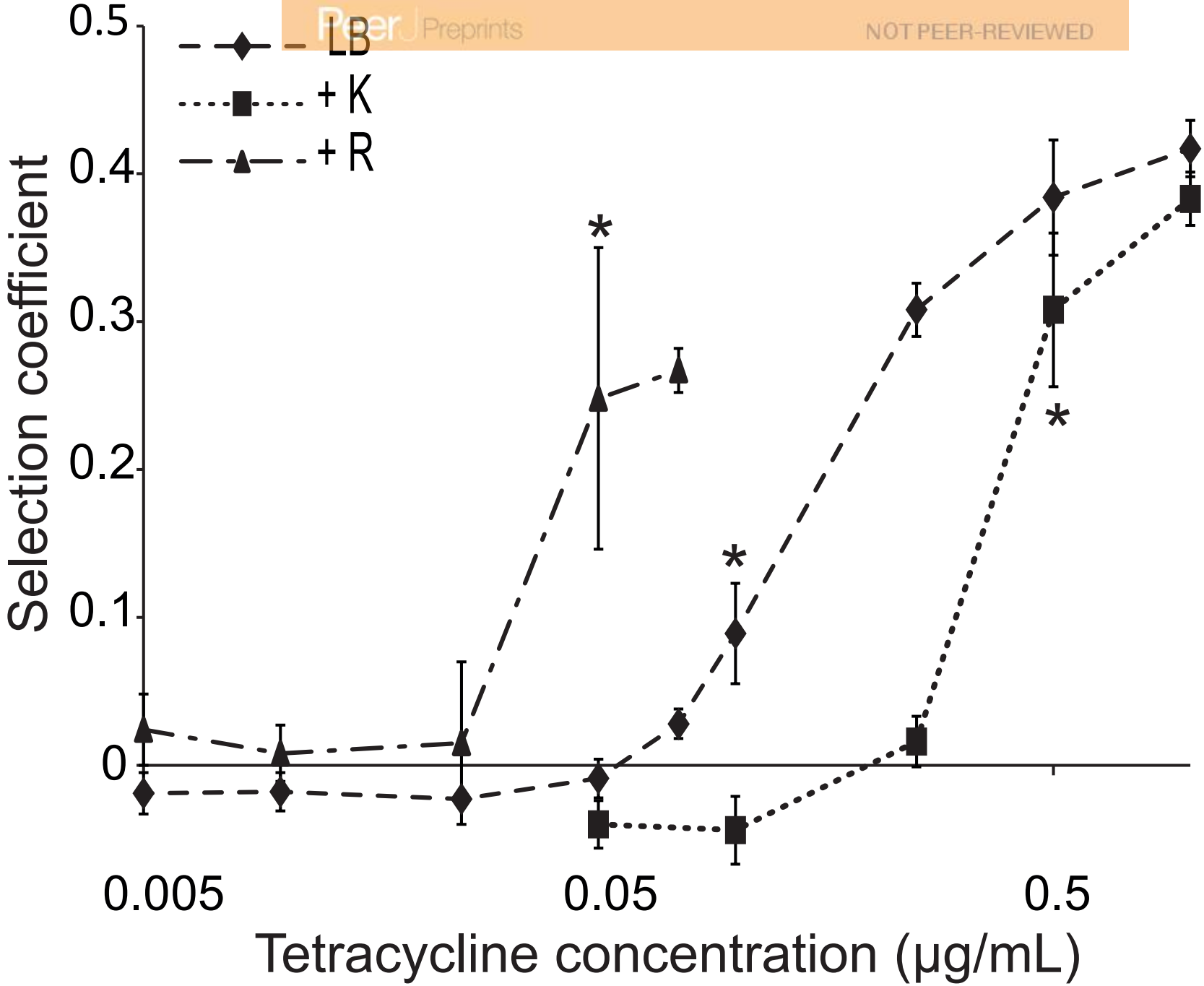

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Figure 3(on next page)

Dose-response to two herbicides with (●) or without (▲) antibiotic (used at a single concentration).

A positive selection coefficient indicates selection for the strain with a higher MIC. Results are averages of 3 independent experiments. Error bars are SEM (standard deviation/√n). (A): Kamba+Str, using strains AH204 (Str^{high}, MIC_{Str}: 200µg/mL) and AH211 (Str^{low}, MIC_{Str}: 1µg/mL); (B): Roundup+Tet, using strains AH201 (Tet^{high}, MIC_{Tet}: 125µg/mL) and AH214 (Tet^{low}, MIC_{Tet}: 1.5µg/mL). Concentrations of antibiotics were 0.25µg/mL for Str and 0.05µg/mL for Tet. Asterisks indicate contrasts where addition of antibiotic significantly changed the strength of selection, with *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

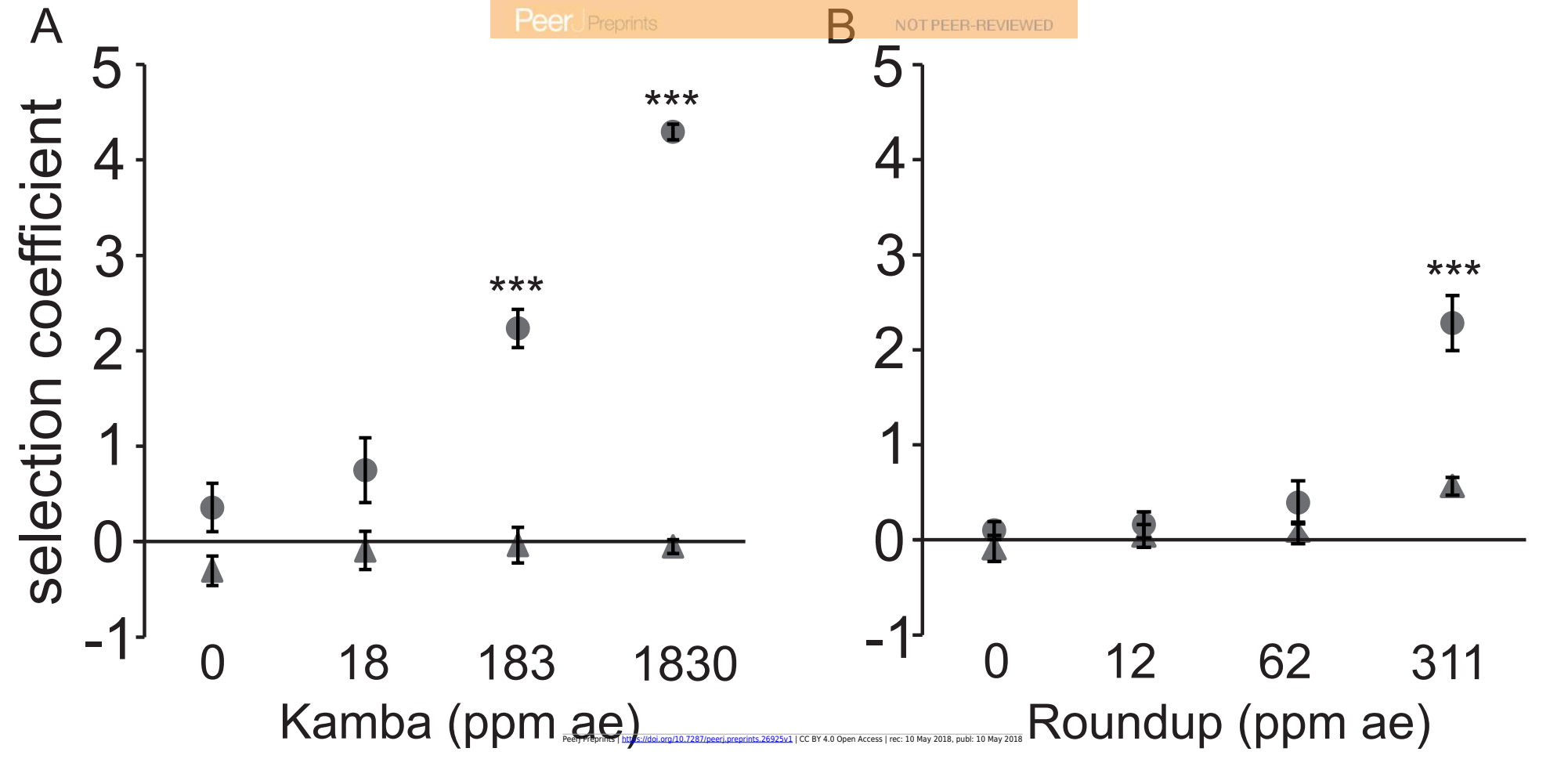

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Table 1(on next page)

Bacteria and plasmids

Bacteria	Genotype/comments	Relevant antibiotic resistance level	Reference/ source
E. coli			
BW25113	wild-type. F^-, λ^-, Δ*(araD-araB)567*, *ΔlacZ4787(::rrnB-3), rph-1,* Δ*(rhaD-rhaB)568, hsdR514*		(41)
SB21	*hsdS*, *leuB6*, *thr*		(42)
AH201 (Tet^{high})	SB21 (pBR322)	Tet: 125 μg/mL	This study
AH214(Tet^{low})	SB21 (pAH14)	Tet: 1.5 μg/mL	This study
JB436	SB21 Nal^R	Nal: 60 μg/mL	(43)
AH204 (Str^{high})	JB436 (RSF1010)	Str: 250 μg/mL	This study
AH211 (Str^{low})	SB21 (pAH11)	Str: 1 μg/mL	This study
S. enterica sv Typhimurium			
SL3770	LT2, pyr^+, *rfa*		(44)
Plasmids			
pBR322	Amp^R, Tet^R	Amp: 100 μg/mL, Tet: 125 μg/mL	(45)
pAH14	pBR322 derivative, Cam^R, Amp^S, Tet^S	Cam:20 μg/mL	This study
RSF1010	Str^R	Str: 250 μg/mL	(46)
pAH11	RSF1010 derivative, Cam^R, Str^S	Cam: 20 μg/mL, Str: 1 μg/mL	This study

1

2

Table 2 (on next page)

Mutant frequency per generation

Frequencies of acquired resistance were determined with or without herbicide and Cip exposure. Results are averages of at least 4 independent experiments. Brackets show SEM (standard deviation/√n). Cip concentration used for final plating: 0.05 µg/mL for *S. enterica* and 0.06 µg/mL for *E. coli*. 1,250 ppm ae Roundup or 1830 ppm ae Kamba were used. [a]Herbicide+antibiotic mutant frequency is significantly different from LB but not from herbicide treatment, for all other combinations herbicide+antibiotic is significantly different from both LB and herbicide treatments. [b]*S. enterica* experiments were conducted concurrently, using the same LB controls for both assays. *E. coli* was strain BW25113. [c]$P < 0.01$ [d]$P < 0.001$

1

	LB	LB+ Herbicide	LB+ Herbicide +Cip
S. enterica			
Kamba	$3.57x10^{-6}$	$2.01x10^{-4}$	$1.30x10^{-2}$
	$(1.27x10^{-6})^{b}$	$(1.95x10^{-4})$	$(1.29x10^{-2})^{c}$
Roundup	$3.57x10^{-6}$	$2.91x10^{-5}$	$2.79x10^{-2}$
	$(1.27x10^{-6})^{b}$	$(2.47x10^{-5})$	$(1.71x10^{-2})^{ac}$
E. coli			
Roundup	$1.80x10^{-9}$	$1.97x10^{-10}$	$2.72x10^{-5}$
	$(1.62x10^{-9})$	$(5.46x10^{-11})$	$(2.67x10^{-5})^{d}$

2

Table 3(on next page)

Ciprofloxacin resistant variants of *E. coli* BW25113 per generation

a) p-value for comparison between the treatment condition and LB medium. Kamba was used at 1830 ppm ae, Cip was 0.025 µg/mL in liquid and 0.06 µg/mL in solid media.

1

Treatment	Resistant variants / generation	SEM	p-value[a]
LB	$7.5x10^{-9}$	$3.9x10^{-9}$	na
Kamba	10^{-7}	$6.3x10^{-8}$	0.22
Cip	$1.2\ x10^{-3}$	$6.5x10^{-4}$	$2.4x10^{-6}$
Kamba+Cip	$5x10^{-7}$	$4.5x10^{-7}$	0.27

2