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Cite this article: Lucas JM, Gora E, Salzberg A, Kaspari M. 2019 Antibiotics as chemical warfare across multiple taxonomic domains and trophic levels in brown food webs. *Proc. R. Soc. B* **286**: 20191536.
http://dx.doi.org/10.1098/rspb.2019.1536

Received: 30 June 2019
Accepted: 30 August 2019

Subject Category:
Ecology

Subject Areas:
ecology, microbiology, environmental science

Keywords:
invertebrate, community assembly, biodiversity, decomposer food web

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.4657808.

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Antibiotics as chemical warfare across multiple taxonomic domains and trophic levels in brown food webs

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Bacteria and fungi secrete antibiotics to suppress and kill other microbes, but can these compounds be agents of competition against macroorganisms? We explore how one competitive tactic, antibiotic production, can structure the composition and function of brown food webs. This aspect of warfare between microbes and invertebrates is particularly important today as antibiotics are introduced into ecosystems via anthropogenic activities, but the ecological implications of these introductions are largely unknown. We hypothesized that antimicrobial compounds act as agents of competition against invertebrate and microbial competitors. Using field-like mesocosms, we tested how antifungal and antibacterial compounds influence microbes, invertebrates, and decomposition in the brown food web. Both antibiotics changed prokaryotic microbial community composition, but only the antibacterial changed invertebrate composition. Antibacterials reduced the abundance of invertebrate detritivores by 34%. However, the addition of antimicrobials did not ramify up the food web as predator abundances were unaffected. Decomposition rates did not change. To test the mechanisms of antibiotic effects, we provided antibiotic-laden water to individual invertebrate detritivores in separate microcosm experiments. We found that the antibiotic compounds can directly harm invertebrate taxa, probably through a disruption of endosymbionts. Combined, our results show that antibiotic compounds could be an effective weapon for microbes to compete against both microbial and invertebrate competitors. In the context of human introductions, the detrimental effects of antibiotics on invertebrate communities indicates that the scope of this anthropogenic disturbance is much greater than previously expected.

1. Introduction

For forest detritivores, food exists in ephemeral patches across the forest floor [1]. These patchy resources are colonized and consumed by bacteria, fungi and invertebrates. The resulting inter-taxon competition for resources, like leaf litter and carrion, influences the diversity and abundance of the forest floor assemblage (i.e. the *brown food web*; [2–6]). While previous work has demonstrated that microorganisms deter animals [7], how these microorganisms compete with organisms that are orders of magnitude larger is poorly resolved. Here, we explore how one competitive tactic, antibiotic production, can structure the composition and function of a neotropical brown food web [8].

The primary hypothesis for why microbial organisms produce antibiotics is as a means of competition among microbial taxa [9,10]. Antibiotic production is an important aspect of microbial life history as it is widespread and has evolved in many microbial taxa (e.g. *Streptomyces*, *Peniclillium*, *Cephalosporium Bacillus*; [11–14]). In particular, microbiota in the phyla Actinobacteria have developed

a plethora of bioactive secondary metabolites [15] and associated antibiotic resistance [16,17]. In the laboratory, these antibiotic-producing organisms can deter and kill competing microbes [10], but these studies are often restricted to simplified systems that are not representative of complex communities *in situ*. Consequently, how these antimicrobial compounds shape natural microbial communities and related ecological processes remains unclear.

A complementary hypothesis is that microbes secrete compounds typically considered antimicrobials to deter animal competitors [2]. A diversity of soil invertebrates—diplopods, isopods, collembola and oribatid mites—compete with microbes by consuming microbe-covered detritus [18,19]. Some invertebrates digest only the microbial turf, whereas others rely on a gut microbiome to digest the detritus itself [2,20]. In either scenario, microbes inhabiting detritus are often killed or disrupted by detritivorous invertebrates and thus microbiota may experience strong selection to kill or deter invertebrates [2]. However, this long-standing hypothesis remains largely untested in natural invertebrate communities [10,11,21,22].

There are two distinct mechanisms for how microbial organisms may use antimicrobials to avoid being harmed by grazing invertebrates: (i) by decreasing food resources through the suppression of surrounding microbial turf [19], or (ii) by attacking the invertebrates' own mutualistic endosymbionts [23–25]. If the latter is the case, antimicrobial compounds and particularly antibacterials should target detritivorous invertebrates that rely strongly on gut endosymbionts [26,27]. By contrast, invertebrate predators in brown food webs should suffer less from antimicrobials as they neither compete with microbiota nor consume poisonous antibiotic compounds. However, over time, and with sustained shifts in the primary consumer population, predatory invertebrates may also be influenced by antibiotic compounds.

These potential mechanisms focus on the evolutionary history of antibiotic compounds as a defence against invertebrate competitors, but, in the Anthropocene, large-scale antibiotic additions are increasing the frequency of antibiotic-invertebrate interactions. Human activities introduce antibiotics to natural ecosystems via a variety of pathways (e.g. livestock waste, improper medicine disposal, waste water), and the ecological impacts of these introductions are largely undescribed [28,29]. Human-introduced antibiotics are derived from natural production pathways and it is likely that anthropogenic introductions are as much as or more than localized natural production levels. However, natural levels of antibiotic production are largely unknown; thus, it is unclear how natural concentrations of antibiotics compare to anthropogenic introductions. Here, we characterize the effects of antibiotic compounds on invertebrate community composition and ecosystem function using a range of dosages based on typical anthropogenic additions.

Most studies of antibiotic effects at the community level use simplified laboratory environments (e.g. [11,22]; but see [10,21]), but the structure of soil communities potentially influence antibiotic effectiveness [30]. We attempt to close this gap and enhance realism using mesocosms constructed in the field from microbial and invertebrate communities residing in tropical soil and leaf litter. We test the hypothesis that compounds typically deemed as antimicrobial are generalized chemical weapons that act as agents of competition against invertebrates and microbiota. We expect antibacterial and antifungal compounds to shift the composition of both microbial and invertebrate communities on the tropical forest floor. In particular, we predict that Actinobacteria will benefit and increase in relative abundance with the addition of antibiotics, given their ability to produce antibiotic compounds [15] and their associated antibiotic resistance [16,17]. We also predict that antibiotics decrease the abundance of detritivorous soil invertebrates, but not their predators, as detritivorous invertebrates are most likely to compete with microbiota and be targeted by microbial chemical defences. Finally, we hypothesize that antibiotics regulate ecosystem-level processes and predict slower decomposition rates in antibiotic-laden environments owing to the disruption of the natural decomposer community.

2. Methods

This study was conducted in 2014 in a seasonally wet tropical forest on Barro Colorado Island (BCI) in Panama (9°09′ N, 79° 051″ W). Samples were collected in June of 2014, which is during the Panamanian wet season. This study site is described in depth by Leigh [31].

(a) Field sampling

We constructed mesocosms (see below for details) using soil and leaf litter collected from two regions of BCI that were separated by 1 km (9.1601 N, 79.84085 W and 9.15608 N, 79.84902 W). By sampling these distinct locations, we addressed whether the effects of antibiotic additions were consistent despite differences between the initial microbial and invertebrate communities (PERMANOVA microbes: pseudo-$F_{1,90} = 4.48$, $p < 0.001$, invertebrate: pseudo-$F_{1,94} = 8.59$, $p < 0.001$, electronic supplementary material, figure S1). We collected the top 2–3 cm of soil from each location, along with the layer of coarse and fine leaf litter covering this soil. Leaf litter was placed in shaker sifters with 1 cm^2 metal mesh to separate the coarse leaf litter from the finer siftate. Each material type (soil, litter, siftate) was homogenized within locations to ensure similar initial mesocosm communities. To establish an initial profile (time 'zero') of the microbial communities, DNA was extracted from representative soil and siftate in each region using Zymo Xpedition™ soil/faecal extraction kits (Zymo Research, Irvine, CA, USA). These kits are designed to extract and stabilize DNA in the field, ensuring minimal DNA degradation during sampling. Samples were maintained at −40°C until subsequent processing could occur.

(b) Antibiotic treatments

Treatments were a synthetic antifungal (Captan, Bionide Chemical, Oriskany, NY, USA), a natural, broad-spectrum antibacterial (streptomycin sulfate, Fischer Scientific, Grand Island, NY, USA), or controls (deionized H_2O). We chose these specific antibiotics based on two criteria: (i) their ability to reduce target taxa (fungi or bacteria) and (ii) their innocuousness towards non-target taxa (NCBI PubChem Compound Database, [32]. Streptomycin binds to the S12 protein of the bacterial 30S ribosomal subunit, inhibiting protein synthesis, leading to cell death (NCBI PubChem Compound Database [32], National Cancer Institute). Additionally, we chose streptomycin as our antibacterial because this compound is naturally produced by the widespread and abundant soil bacteria, *Streptomyces griseus* [33]. Captan is a synthetically produced, non-specific thiol reagent that inhibits respiration of fungi (RED: [34]). We chose Captan as we currently know of no naturally produced and publicly available antifungal that does not have indirect effects on other taxonomic groups.

Antibiotics were dissolved in 15 ml of deionized H_2O and evenly applied to mesocosm environments on the first day of the experiment at one of three dosage levels: 1.5 mg g^{-1}, 3 mg g^{-1} or

6 mg g^{-1} for the antibacterial or 12.5 ug g^{-1}, 25 ug g^{-1}, 50 ug g^{-1} for the antifungal. Dosage levels were based on previous studies documenting levels of anthropogenic introduction [35,36] and dosage-lethality studies [27,37–39]. Owing to the potentially ephemeral and varied production of antibiotics, to our knowledge, there are no studies that provide consistent and discrete measurements of the concentration of antibiotic compounds naturally produced in soils. There are however, multiple studies demonstrating the widely variable concentrations of antibiotics that occur in waste water runoff or via agricultural manure inputs [40–42]. For example, streptomycin has a very poor absorption rate, with 50–60% of their dosage excreted in urine [43]. This can result in streptomycin excretion rates of 1 g day^{-1} in human urine, or 21 mg day^{-1} in dog urine that can freely enter into aquatic or terrestrial environments. Therefore, while our dosages cannot be compared to natural production levels as these data are not available, their concentrations are comparable to widespread anthropogenic introductions.

(c) Mesocosm design and sampling

We created seven mesocosms per treatment for each source location, for a total of 96 mesocosms (2 locations × 2 antibiotics × 3 dosages × 7 replicates + 7 controls per location). Mesocosms consisted of 1 l containers with extra fine mesh on the top and bottom to aid in containment of organisms and drainage of stagnant water. Each container consisted of 600 g of equal parts by volume of soil, siftate and coarse leaf litter. Mesocosms were kept at ambient temperatures under a covered platform in the field with natural 12 h light-dark cycles and 15 ml of distilled water were added weekly to simulate rainfall for a total of 21 days. Each mesocosm contained a litter bag containing pre-weighed 9 cm grade P8 filter paper (Fisher Scientific, Hampton, NH, USA) to monitor rates of cellulose decomposition. We used standardized cellulose substrates to assess changes in decomposition because cellulose is common to all plant litter and decomposes during the short duration of our experiments. However, we note that it is not necessarily representative of all plant litter decomposition.

After 21 days, we destructively harvested all mesocosms and tested their pH (Mettler Toledo, Columbus, OH, USA). We ran our experiment for 21 days to limit sampling DNA from microbes killed by the antibiotics; 21 days has been shown to be sufficient time for non-viable DNA to be eliminated from soils [44]. We extracted and stabilized DNA from an equal mixture of siftate and soil to assess mesocosm microbial communities as outlined above for initial time-point samples. Microbial samples were then maintained at −40°C until subsequent processing could occur. After microbial sampling, the mesocosms were placed into Berlese funnels for 48 h to extract all living invertebrates. Invertebrates were preserved in 95% EtOH and identified at least to Class using assignments made by Clay *et al.* [45] in our same study system.

(d) Microbial community analysis

All microbial samples were extracted and analysed using the protocol outlined by Lucas *et al.* [46]. Briefly, samples were disrupted and stabilized using Xpedition™ Lysis/Stabilization solution and bashing beads (Zymo Research, Irvine, CA, USA). We then extracted DNA according to the manufacturer's protocol (Zymo Soil/Fecal Xpedition™ mini kit). We amplified the V2 region of the 16S rRNA gene using primers S-D-Arch-0519-a-S-15/S-D-Bact-0785-b-A-18 [47]. These primers are specifically designed to capture bacterial and archaeal sequences. The S-D-Arch-0519-a-S-15 primer was modified to include a 16 bp M13 sequence to enable the attachment of a unique 12 bp 'barcode'. Barcoded polymerase chain reaction (PCR) products were cleaned and equimolar amounts of each uniquely barcoded PCR product were pooled for sequencing on an Illumina MiSeq instrument (2 × 250 bp PE V2).

(e) Sequence processing

All 16S sequencing reads were analysed and demultiplexed using QIIME [48]. We removed sequencing reads that contained errors in the barcoded region, ambiguities, homopolymers (greater than six nucleotides in length), or an average quality score of less than 25. Primer sequences were trimmed, and chimeric sequences were eliminated using USEARCH (v.6.1) and the 'gold' reference database [49]. Then sequences were clustered into *de novo* operational taxonomic units (OTUs) at 97% similarity. Microbial taxonomic classification was assigned via the SILVA reference database release 119 [50] using the PyNAST aligner. Prior to all statistical analyses, we rarefied (randomly subsampled) each sample to an even sequencing depth of 2500 reads $sample^{-1}$ (electronic supplementary material, figure S2); note that all sequences do not meet their asymptote. All sequencing data are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.2cs8d7f) [51].

(f) Survival tests for key detritivores

We ran a pair of experiments to assess the mechanism by which antibiotics decrease invertebrate survivorship. Both experiments used identical 60 mm diameter Petri dish microcosms with water provided via a cotton ball soaked in distilled water (controls) or antibiotic-laden water with the same middle concentrations as the mesocosm experiment (3 mg g^{-1} streptomycin or 25 ug g^{-1} Captan). However, in one experiment the microcosm was entirely sterile (no food resources and no microbiota; hereafter the *sterile microcosms*) and in the second experiment the invertebrates were provided with 5 g soil and leaf litter as food and a natural source of microbiota (hereafter the *soil microcosms*). The soil microcosm experiment was run in August 2014, while the sterile microcosm experiment was run in June 2016. The intention of these survival tests was to assess whether the antibiotic compound alone could impact invertebrate survival. By conducting one of these experiments in sterile environments, we were able to separate the effects of antibiotic effects from lower food availability (i.e. decrease in microbial abundance).

Both experiments were conducted on two key detritivore taxa, isopods and diplopods (hereafter millipedes), because they responded strongly in the mesocosm experiment. We collected 180 millipedes and 180 isopods from the field (30 organisms $treatment^{-1}$ $experiment^{-1}$). We then placed one organism inside each microcosm and monitored their survival over time. Isopods were monitored for one week in both the sterile and soil microcosms, whereas millipedes were monitored for longer time periods owing to their greater survivability in captivity. Specifically, we monitored millipedes for six weeks in the sterile mesocosms and five weeks in the soil mesocosms. The survival status of the individual organisms was recorded daily for isopods and weekly for millipedes.

(g) Statistical analysis

We performed community analyses using PRIMER-E (PRIMER v. 7.0.13, [52]). We compared invertebrate and microbial (bacterial and archaeal) community composition using PERMANOVA (9999 permutations; [53]). We included treatment as the fixed effect, dosage as a fixed effect nested within treatment, location as a random effect, and the interaction between location and treatment. We performed stepwise model reduction and dropped non-significant interaction and nested effects. We also tested for differences in beta diversity among treatments using PERMDISP (9999 permutations; [53]). PERMDISP tests calculate within treatment dissimilarity in community composition (i.e. compositional beta diversity) and then compare the magnitude of dissimilarity among treatments. We visualized these patterns using non-metric multidimensional scaling (NMDS). These

analyses were based on Bray-Curtis dissimilarity (after the log(x + 1) transformation) and weighted UniFrac distance [54] for invertebrates and microbes, respectively. We repeated these analyses for two subsets of the invertebrate communities: detritivores and predators. We used the Bonferroni correction to account for multiple hypothesis testing and report alpha when less than 0.05.

We further compared treatment effects on specific taxa, decomposition, and pH using a combination of linear models and effect size analyses. We compared alpha diversity, defined as observed OTUs for microbes and number of orders for invertebrates, among treatments using a series of two-sample non-parametric t-tests and Monte Carlo permutations to calculate p-values. We used the effect size Cohen's d [55] to quantify the direction and magnitude of the change in abundance for bacterial phyla in response to antibiotic addition as described in Kaspari *et al*. [56]. Values of Cohen's $d \geq |0.5|$ represent a 'medium' effect size, while values larger than $|0.8|$ are considered to be 'large'. Therefore, we only report phyla that responded with Cohen's d values $\geq |0.5|$. We also ran linear mixed effect models using the package *lme4* in R statistical software to test for treatment effects on the abundance of specific invertebrate taxa, invertebrate richness, decomposition rates and pH levels ([57], v. 0.99.903; [58]). For each model, we included treatment as a fixed effect, dosage as a fixed effect nested within treatment, and litter/soil source as a random effect. We performed nested model reduction and sequentially dropped non-significant terms according to Akaike information criterion (AIC) values and likelihood ratio test p-values (electronic supplementary material, tables S1 and S2). We log-transformed abundance data to meet model assumptions and we examined model residuals to confirm appropriate fit. To test for differences in survival across antibiotic treatments, isopods and millipedes were tested with the SurvDiff function, followed by a log-rank *post hoc* test for pairwise differences, using the *survival* package in R [59]. Kaplan–Meier survival curves were generated using the *survminer* package in R [60].

3. Results

Antibiotics changed the composition of prokaryotic and invertebrate assemblages (PERMANOVA microbes: pseudo-$F_{2,94} = 2.41$, $p = 0.002$, invertebrate: pseudo-$F_{2,94} = 1.77$, $p = 0.033$; figures 1 and 2), but did not change pH or cellulose decomposition rates. The composition of both invertebrate and prokaryotic communities changed with antibacterial addition relative to controls (PERMANOVA pairwise microbes: pseudo-$t = 1.84$, $p = 0.004$, invertebrate: pseudo-$t = 1.61$, $p = 0.01$). However, antifungal additions only affected the prokaryotic, not invertebrate, communities (PERMANOVA pairwise: microbial: pseudo-$t = 1.65$, $p = 0.005$; invertebrate: pseudo-$t = 1.29$, $p = 0.095$). The effects of antibiotics were unaffected by dosage (PERMANOVA microbes: pseudo-$F_{4,90} = 0.99$, $p = 0.513$, invertebrates: pseudo-$F_{4,90} = 1.25$, $p = 0.167$) and persisted despite substantial differences in initial community composition between the two source locations (PERMANOVA microbes: pseudo-$F_{1,90} = 4.48$, $p < 0.001$, invertebrate: pseudo-$F_{1,94} = 8.59$, $p < 0.001$). Despite community-level changes, antibiotics had no effect on soil pH (dAIC = 2.1, $\chi^2_2 = 1.90$, $p = 0.39$; electronic supplementary material, table S1) nor rates of cellulose decomposition (dAIC = 3.66, $\chi^2_2 = 0.33$, $p = 0.84$; electronic supplementary material, table S1) after 21 days. Thus, antibiotics shifted community composition but did not change a key ecosystem function of brown food webs.

Bacterial phyla responded strongly to both the antibacterial and antifungal treatments (electronic supplementary material, figure S3), with some differences at the family and genus levels (electronic supplementary material, figure S4).

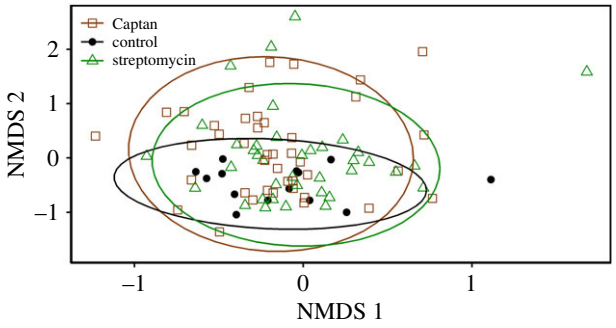

Figure 1. NMDS representation of microbial communities in mesocosms treated with antifungal (Captan, brown squares), antibacterial (streptomycin, green triangles) or controls (deionized H_2O, black circles). Microbial dissimilarity matrices were calculated using sequence-based weighted UniFrac distances. Although community composition was similar between antibacterial and antifungal treatments, community composition of these treatments differed from controls (PERMANOVA: $F_{3,96} = 1.84$, $p = 0.02$). (Online version in colour.)

However, contrary to our prediction, the relative abundance of Actinobacteria did not increase in either treatment (Cohen's d: antifungal −0.43, antibacterial 0.04), though we did see such changes in some genera within this phylum (e.g. *Rubrobacteraceae*, *Virgisporangium*; electronic supplementary material, figure S4). Differences in prokaryotic community composition were not, however, accompanied by changes in alpha diversity (dAIC = 0.5, $\chi^2_2 = 3.52$, $p = 0.17$; electronic supplementary material, table S1) or beta diversity (PERMDISP: pseudo-$F_{2,95} = 2.1$, $p = 0.2$; figure 2).

The antibacterial treatment shifted detritivorous invertebrate community composition but not beta diversity (PERMANOVA: pseudo-$F_{2,94} = 2.65$, $p = 0.005$, $\alpha = 0.025$, PERMDISP: pseudo-$F_{2,95} = 0.60$, $p = 0.622$, $\alpha = 0.025$). Overall, the antibacterial decreased detritivore abundance by an average of 34% (electronic supplementary material, table S2 and figure 3); annelida, oribatid mites, millipedes and isopods decreased the most (67%, 39%, 39% and 72%, respectively, electronic supplementary material, table S2 and figure 3). The antifungal decreased annelida and isopods by 59% and 75%, respectively (electronic supplementary material, table S2 and figure 3). Amphipods had marginally significant responses to antibiotics (electronic supplementary material, table S2). As predicted, predatory invertebrates (e.g. Aranea, Pseudoscorpion) did not respond to either antibiotic (PERMANOVA: pseudo-$F_{2,95} = 1.07$, $p = 0.384$, $\alpha = 0.025$; PERMDISP: pseudo-$F_{2,95} = 0.30$, $p = 0.791$, $\alpha = 0.025$; electronic supplementary material, table S2 and figure S5). Invertebrate alpha diversity was lower in antibiotic-treated mesocosms (dAIC = 4.31, $\chi^2_2 = 8.31$, $p = 0.02$, electronic supplementary material, figure S6), though invertebrate beta diversity did not differ (PERMDISP pseudo-$F_{2,95} = 0.76$, $p = 0.56$; figure 2). Dosage did not influence the abundance of any taxa (all dosage effects: dAIC < 1.22, $\chi^2_4 = 9.22$, $p > 0.07$).

(a) Distinguishing antibiotic direct from indirect effects

In both sterile and soil microcosms, antibiotics decreased isopod survival (generalized linear model (GLM) sterile: $\chi^2_2 = 7.5$, $p = 0.02$; soil: $\chi^2_2 = 13.0$, $p = 0.001$; figure 4*c*). Antibacterials decreased isopod survival rates in both microcosms types (log-rank *post hoc* test: $p < 0.002$; figure 4*a*,*c*), but antifungals only decreased survival in sterile microcosms (log-rank

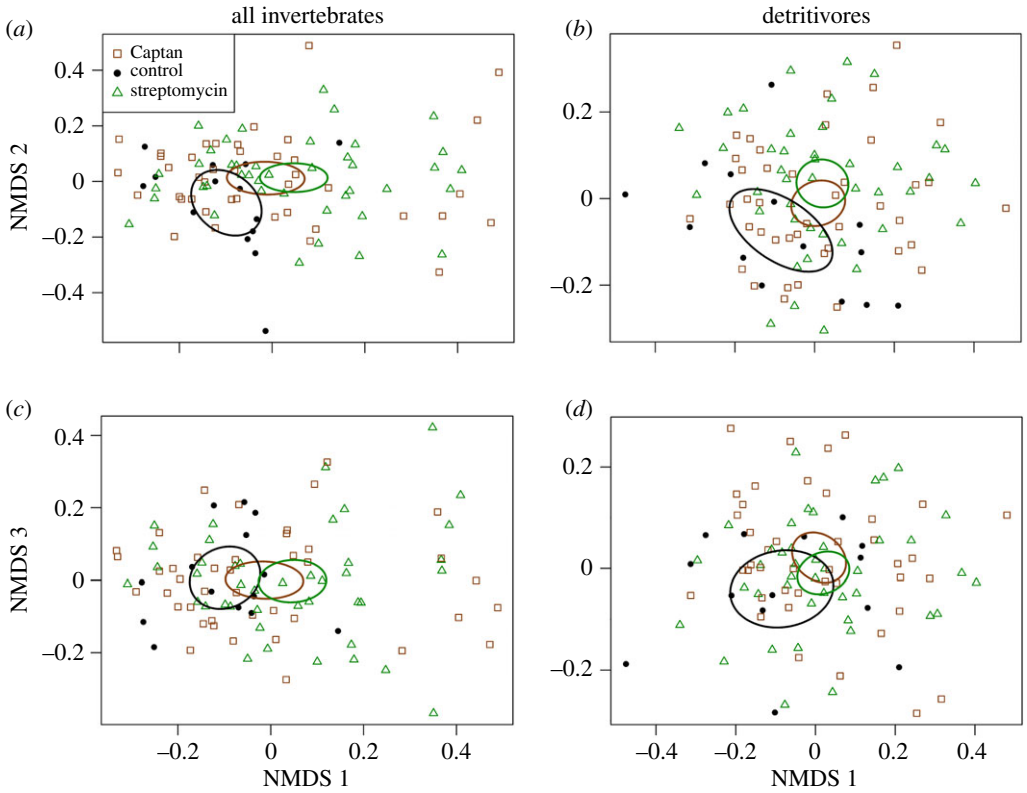

Figure 2. NMDS representation of the (*a*,*c*) whole invertebrate community and (*b*,*d*) detritivore community in mesocosms treated with antifungal (Captan, brown squares), antibacterial (streptomycin, green triangles) or controls (deionized H_2O, black circles). Invertebrate dissimilarity matrices were calculated using Bray–Curtis dissimilarity distances. Although community composition was similar between antibacterial and antifungal treatments, community composition of these treatments differed from controls for invertebrates as a whole (PERMANOVA: pseudo-$F_{2,94} = 1.77$, $p = 0.033$). These differences were driven by changes in the detritivore invertebrates (PERMANOVA: pseudo-$F_{2,94} = 2.65$, $p = 0.005$), but not the predatory invertebrates (PERMANOVA: pseudo-$F_{2,94} = 1.07$, $p = 0.384$). (Online version in colour.)

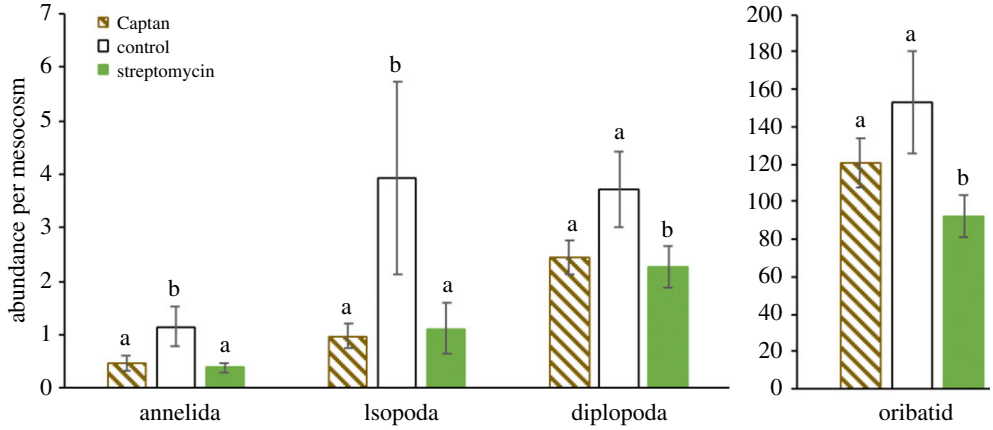

Figure 3. Average abundance per mesocosm of invertebrate detritivores that responded to additions of antifungal (Captan, brown dashed bars, $n = 42$) and/or antibacterial (streptomycin, green solid bars, $n = 42$) antibiotics versus controls (deionized H_2O, black open bars, $n = 14$). Error bars represent standard error and, when different, letters above bars represent significant differences in abundance. Oribatid mites are presented with a separate *y*-axis owing to their higher abundance. See the electronic supplementary material, table S2 for statistical results. (Online version in colour.)

post hoc test sterile: $p = 0.02$, soil: $p = 0.19$; figure 4*a*). Isopod survival rates did not differ between antibiotic treatments in sterile microcosms (log-rank *post hoc* test: $p = 0.59$; figure 4*a*), but antibacterials decreased survival more than antifungals in the soil microcosms (log-rank *post hoc* test: $p = 0.04$; figure 4*b*).

Millipedes exhibited more complex responses. In sterile microcosms, millipedes had lower survival in the presence of antibacterials as compared to control groups, but were not significantly affected by antifungals (GLM: $\chi^2_2 = 6.7$, $p = 0.04$; figure 4*b*). By contrast, when raised in soil microcosms, millipedes did not have lower survival in antibiotic treatments compared to controls, but millipedes in antifungal treatments had lower survival than millipedes in antibacterial environments (GLM: $\chi^2_2 = 7.4$, $p = 0.02$, log-rank *post hoc* test: $p = 0.045$; figure 4*d*).

4. Discussion

Here, we demonstrate that the effects of antibiotic compounds ramify across multiple taxonomic domains within the decomposer food web (figures 1 and 2). Given the rise in anthropogenic

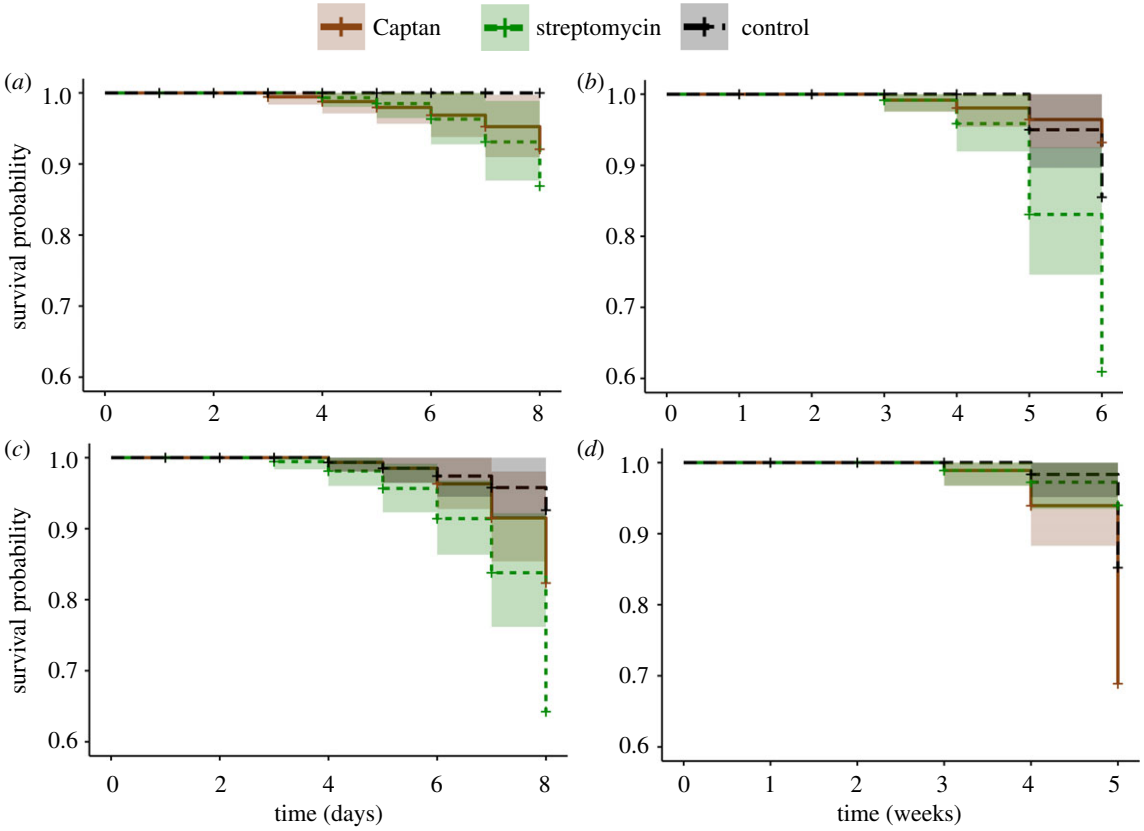

Figure 4. Survival probabilities (with 95% confidence intervals) of (*a*,*c*) isopods and (*b*,*d*) millipedes when raised in antibiotic-laden microcosms, shown as Kaplan—Meier survival curves. Sterile environment tests are depicted in (*a*,*b*), while soil environments are depicted in (*c*,*d*). Antifungals are noted by solid brown lines, antibacterials are noted by dotted green lines, and controls are noted by dashed black lines. Note that the *x*-axis is in days for isopods and weeks for millipedes. (Online version in colour.)

introductions of antibiotic compounds, these results suggest that the scale and scope of anthropogenic effects on microbial and invertebrate communities are underestimated. The significant suppression of key detritivorous invertebrates by antibacterials—and their failure to effect predatory taxa—provides initial support for Janzen's hypothesis [2] that antibiotics produced by free-living microbes target animal competitors. This suggests that antibiotics have the potential to significantly alter the structure of the soil's brown food webs.

(a) Antibiotic dosage and anthropogenic effects

Surprisingly, we detected no dosage effects in this tropical soil ecosystem. The minimum dosage was sufficient to generate all changes in microbial and invertebrate community composition; (however, see [37]). This suggests that even small amounts of these active compounds have large ramifying effects on microbial and invertebrate community composition. Our additions were equal to or less than concentrations of anthropogenic additions, demonstrating that anthropogenic antibiotic introductions can have major effects on soil food webs across taxonomic domains [61]. With the increasing use of antibiotics to promote livestock growth [62] and the excretion rates of antibiotics ranging from 17% to over 90% of the original dosage [28], brown food webs in agricultural ecosystems are particularly threatened. Whether soil food webs in agricultural landscapes adapt to the presence of antibiotics is an important next step. Our results also have implications for natural ecosystems. At large and small spatial and temporal scales, it is likely that the production of antibiotics in the natural environment varies, underlining the importance of documenting antibiotic effects across dosages. Although natural concentrations of antibiotic compounds are underexplored, the consistent responses of detritivores regardless of dosage demonstrates the potential for effective antagonistic activity of antibacterial compounds against competitors in natural settings.

(b) Antibiotics suppress invertebrate detritivores but not predators

We provide initial evidence in semi-natural mesocosms that even small quantities of antibiotics could allow microbes to antagonistically compete with macroorganisms. This supports Janzen's [2] long-standing hypothesis on inter-Kingdom competition and demonstrates a mechanism via which microbes can exhibit bottom-up control on invertebrate assemblages. The invertebrate taxa that were most affected—millipedes and isopods—are common in tropical environments, play important roles in the decomposition process, and are probably in constant competition with microbial taxa [2,63]. By contrast, we did not observe a change in cellulose decomposition with antibiotic treatment. We acknowledge that cellulose is only one component of plant matter and it is possible that antibacterial effects decreased the decomposition of more complex plant matter. Therefore, describing the mechanism by which antibiotics impact these taxa and other plant materials is fundamental to understanding community assembly and ecosystem processes.

The differential responses of invertebrates to the antibacterial versus antifungal treatments support the hypothesis that

natural antibacterials target the endosymbionts of invertebrate detritivores. The invertebrate community only responded to the antibacterial treatment despite similar effects of both antibiotics on the relative abundance of environmental microbes. If decreased food availability was the main driver of invertebrate suppression, then we would expect similar responses to both the antifungal and antibacterial treatments. Instead, the decreased survivorship of invertebrates was probably caused by the disruption of their internal microbiomes. Indeed, millipedes and isopods use endosymbiotic microbes for digestion [20,64,65] and exhibit increased mortality when their endosymbionts are disrupted [66].

Results from our sterile microcosms further support the microbiome disruption hypothesis. Antibacterials decreased isopod and millipede survival in sterile environments, demonstrating that the antibiotic compounds alone can disrupt invertebrates. This is similar to the work of Zhu *et al*. [67], who found that antibiotics disrupted the microbiome of the collembola *Folsomia candida* leading to a decrease in growth in non-soil environments. However, when we raised millipedes in soil microcosms without competitors, their survival was not affected by antibiotic compounds. We suggest two working hypotheses for this interesting result. First, large, long-lived millipedes may have been able to consume and replenish their endosymbiotic community. Second, without competition, millipedes may have been able to forage for enough high-quality food resources to survive for the duration of the study.

Some of our invertebrates (e.g. dipteran larvae, springtails) did not respond to antibiotic treatments. If, as we suggest here, antibiotics attack invertebrates via endosymbionts, then these taxa may have less active or more antibiotic-resistant microbial symbionts. Wide-ranging studies of detritivore microbiomes do not yet exist. However, recent work has highlighted the limited importance of invertebrate endosymbionts in some taxa [68]. Hammer *et al*. [69] demonstrated that *Manduca sexta* caterpillar survivorship was unchanged when their microbiome was eliminated by antibiotic compounds. Similarly, Baguer and colleagues [27] failure to show antibiotic effects for collembola and enchytraeids may signal gastrointestinal tracts that, like *M. sexta*, do not require endosymbiotic support. We suggest that antibiotic experiments like ours can provide an initial community-level assessment of endosymbiotic reliance, filling a problematic information gap on invertebrate microbiomes [68].

(c) Prokaryotic communities were altered by antibiotic addition

Prokaryotic community composition shifted in response to both antifungal and antibacterial treatments in a fashion similar to a study in northwestern temperate rainforest [32]. However, contrary to our prediction, the antibiotic-producing phyla Actinobacteria did not thrive. This result, combined with the negative responses of some bacterial taxa to the antifungal treatment, highlights the complex and contingent matrix of mutualistic and antagonistic relationships that exist within forest floor communities [70].

(d) Caveats, conclusions and future directions

One major limitation of studies of antibiotic compounds comes from our fundamental lack of understanding of these compounds in natural environments. Studies documenting natural antibiotic concentrations and their influence in field-based conditions are necessary to fully develop our understanding of the ecology of antibiotics. Additionally, it is possible that the antibiotics we used in this experiment had direct effects on the invertebrate organisms that responded. While we chose these compounds because of their specificity for only their target taxa (i.e. bacteria or fungi), it is possible that these compounds are toxic to invertebrates in ways that have yet to be determined. Previous studies demonstrate that Captan can harm aquatic organisms [71], and decrease worm reproduction rates [39], though it was found to be non-toxic to mature worms [39] and bees [72]. Similarly, high doses of streptomycin have been shown to decrease blood pressure and cause respiratory depression in mammals (NCBI PubChem Compound Database), but documented ecotoxicology reports on invertebrates are lacking. A direct effect on invertebrates would not negate our findings as streptomycin is a naturally occurring compound that could be directly disrupting invertebrates *in situ*.

Despite interspecific differences, the substantial impacts of the naturally produced antibacterial (streptomycin) suggest that naturally occurring antibiotic compounds can contribute to the patchiness often observed in microbial and invertebrate litter communities [73,74]. Experimental manipulations of antibiotics and the endosymbiotic communities of invertebrates are necessary to confirm the mechanism by which antibiotics disrupt key detritivores. Additionally, further field tests are necessary to determine the relative importance of these patterns in the ephemeral landscape of hyper-diverse forest floors. Finally, our study provides insight into the potential ramifications of anthropogenic introductions of antibiotics and demonstrates a need for similar investigations in human systems.

Data accessibility. All data have been submitted to the Dryad Digital Repository: https://doi.org/10.5061/dryad.2cs8d7f [51].

Authors' contributions. J.M.L., E.G., A.S. and M.K. conceived the ideas and designed methodology; J.M.L. and A.S. collected the data; J.M.L. and E.G. analysed the data. All authors contributed critically to the drafts and gave final approval for publication.

Competing interests. The authors declare no conflict of interest.

Funding. This work was funded by the National Science Foundation (grant no. GRF- 2014170874) and the Smithsonian Tropical Research Institute's Short-term Fellowship to J.M.L.

Acknowledgements. We thank Michael Weiser, Brian Bill, Bradley Stevenson and Karl Roeder for their comments on the project design and previous versions of the manuscript. We thank the OMRF for sequencing microbial communities and Stevenson Laboratory at OU for providing laboratory support. We also thank the staff on BCI, Panama for logistical support during the field portion of this project.

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