nature ecology & evolution

ARTICLES

https://doi.org/10.1038/s41559-019-1085-x

Insect herbivory reshapes a native leaf microbiome

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Insect herbivory is pervasive in plant communities, but its impact on microbial plant colonizers is not well-studied in natural systems. By calibrating sequencing-based bacterial detection to absolute bacterial load, we find that the within-host abundance of most leaf microbiome (phyllosphere) taxa colonizing a native forb is amplified within leaves affected by insect herbivory. Herbivore-associated bacterial amplification reflects community-wide compositional shifts towards lower ecological diversity, but the extent and direction of such compositional shifts can be interpreted only by quantifying absolute abundance. Experimentally eliciting anti-herbivore defences reshaped within-host fitness ranks among *Pseudomonas* spp. field isolates and amplified a subset of putatively phytopathogenic *P. syringae* in a manner causally consistent with observed field-scale patterns. Herbivore damage was inversely correlated with plant reproductive success and was highly clustered across plants, which predicts tight co-clustering with putative phytopathogens across hosts. Insect herbivory may thus drive the epidemiology of plant-infecting bacteria as well as the structure of a native plant microbiome by generating variation in within-host bacterial fitness at multiple phylogenetic and spatial scales. This study emphasizes that 'non-focal' biotic interactions between hosts and other organisms in their ecological settings can be crucial drivers of the population and community dynamics of host-associated microbiomes.

For many organisms, attack by multiple enemies is inevitable and often occurs sequentially during the lifetime of individual hosts. Prior attack can alter host phenotypes and change how future attacks unfold, often generating cascading effects at larger spatial and temporal scales[1–4]. Given the large effects of co-infection on host health and the population dynamics of their parasites, explicitly studying co-infection is becoming increasingly common[4–6]. But rarely has this perspective been extended to studies of diverse host-associated microbial communities ('microbiomes'). Instead, microbiome studies tend to focus on effects of host genotype or abiotic variation on microbiome diversity patterns[7–11]. This has left a major gap in our understanding of how host colonization from non-microbial enemies impacts the population biology of host-associated microbial taxa.

With regard to plants, there is tremendous interest in understanding the structure and function of the microbiome both for applied purposes, such as engineering growth promotion and disease resistance[12,13], and as model systems for host–microbial symbioses more generally. Insect herbivory represents a pervasive threat to plants in wild and agricultural settings alike[14]. Herbivory alters plant phenotypes through tissue damage and induction of plant defences, which can change the susceptibility of plants to attack by insects[15] as well as microorganisms[16,17]. Thus, factors that influence the impact of herbivores on hosts will likely affect the colonization and growth of plant-associated microorganisms. While insect herbivores[14] and plant-associated microorganisms[18] have clear effects on plant phenotypes and fitness, they are generally considered independently. Our study addresses this gap by explicitly considering how patterns of abundance and diversity of leaf-colonizing (endophytic) bacterial taxa are altered in the presence of insect herbivory and by exploring the associations among herbivory, bacterial infection and plant fitness in a native forb (*Cardamine cordifolia*, Brassicaceae; 'bittercress').

We first used marker gene sequencing (16S ribosomal RNA) coupled with paired leaf culturing to establish and validate sequence-based estimates of absolute bacterial load in host tissue. By elucidating a relationship between bacterial load and the sequence counts of bacteria- versus host-derived 16S (Box 1), our approach enabled standard 16S marker gene sequencing to quantitatively reveal variation in abundance distributions of entire suites of bacterial taxa across hosts with and without prior herbivory by the specialist leaf-mining insect *Scaptomyza nigrita* (Drosophilidae). We then assessed the extent of co-clustering between microbial abundance and intensity of insect herbivory at the plant patch scale across our natural study populations and related microbe–herbivore co-aggregation to fruit set, a component of plant fitness. In parallel, we examined variation in sensitivity to inducible plant defences against chewing herbivores among 12 genetically diverse, bittercress-derived isolates of *Pseudomonas* spp. bacteria. We did so by experimentally infecting accessions of native bittercress in which prior herbivory was simulated by exogenously pre-treating plants with the plant defence hormone jasmonic acid (JA). JA induces canonical defences against chewing herbivores in plants[17], including bittercress[19].

Our experiments reveal that insect herbivory, via induction of plant defences, can modify endophytic bacterial diversity patterns by amplifying naturally prevalent and potentially phytopathogenic bacterial taxa within a native plant host. This mechanism may be at least partly responsible for the strong positive association between herbivory and endophytic bacterial abundance within leaf microbiomes seen under field conditions. Crucially, the patterns and degree of bacterial abundance variation we found cannot be explained by traditional compositional analysis of high-throughput marker gene sequencing, which masks the extent and direction of within-host variation in bacterial load. By linking marker gene counts to an absolute standard, our study shows how insect herbivory associates with variation in bacterial loads at leaf and patch scales within a natural plant population. More generally, this work highlights the importance of (1) accounting for prevalent but 'non-focal' biotic interactions that hosts have with other colonizers in their natural contexts and (2) using detection and analytical approaches to quantify these effects on components of microbial fitness.

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Box 1: Devising an estimator of bacterial load from 16S data

Defining the estimator

To establish an estimator of bacterial load using 16S sequence data, we hypothesized that the composition of the sequencing data, in terms of host- versus bacteria-derived 16S reads, may provide information about the underlying density of bacteria. This occurs, we reasoned, because DNA templates of the two sources compete as targets during the amplification reaction and biases towards one or the other will accrue exponentially. By this logic, the logarithm of the relative abundance of bacteria–host 16S counts captures information about the density of bacterial cells in the starting material. Accordingly, for each sample, we calculated the following estimator

$$\gamma = \ln(r_B/r_H)$$

where r_B and r_H are the read counts of bacteria- and curated host-derived 16S counts for a given sample, respectively; r_B can be calculated at any taxonomic level, ranging from the single bacterial amplicon sequence variant (bASV) to all of the bacteria present in the sample, by summing the sequence counts at the desired taxonomic scale.

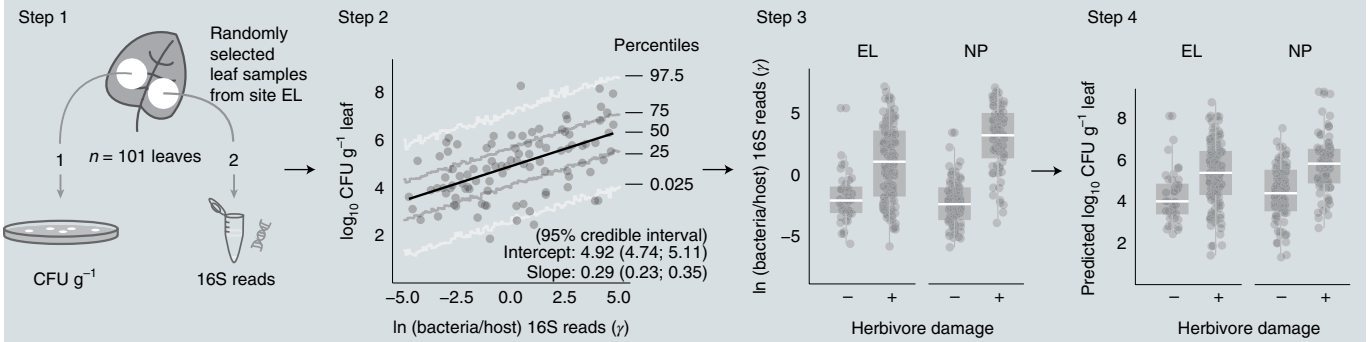
Validating and deploying the estimator

Step 1: Collect paired tissue samples and enumerate bacteria independently. We validated this estimator empirically by examining the relationship between γ and an independent measure of bacterial abundance in leaf tissues derived from bacterial culturing of a subset ($n = 101$) of the samples from the EL study. These samples were surface-sterilized, homogenized and plated on non-selective King's B media to enumerate bacterial colony-forming units (CFU) per g starting leaf material, following Humphrey et al.[20]. This approach is appropriate because a majority of bacterial taxa typically found to colonize leaf tissues can be cultivated in the laboratory on rich media[20,43].

Step 2: Quantify relationship between 16S data and directly observed bacterial load. We then estimated the slope and intercept of the relationship between observed $\log_{10}$ CFU g^{-1} leaf tissue (hereafter log CFU) and the predictor variable γ for this sample set using a Bayesian linear regression, which allowed us to directly incorporate uncertainty in model fit into downstream analyses. We found a clear positive association between γ and log CFU (see figure), validating our usage of γ as an estimator of absolute bacterial abundance in leaf tissues.

Step 3: Model relationship between observed γ and herbivore damage. We then deployed the validated estimator to test whether bacterial abundance as measured by γ was elevated in insect-damaged plant tissues. To begin, we modelled how γ varied across herbivore-damaged and undamaged leaves for various bacterial taxa. The example in the Box figure below shows that the distributions of γ calculated for all bacteria are elevated in herbivore-damaged bittercress tissues sampled from both sites EL and NP.

Step 4: Transform results for γ into predicted bacterial load via parameters from Step 2. Finally, we used posterior draws of parameters from the Step 2 model to predict how variation in γ translates into predicted bacterial load as expressed in log CFU. Elevated γ in herbivore-damaged tissues translates into higher bacterial loads when predicted based on the relationship between γ and log CFU derived in Step 2. Further details on how we specified and estimated models, as well as how we incorporated parameter uncertainty throughout this approach, can be found in Methods: Quantifying and modelling bacterial abundance patterns.



Results

Bacterial loads are amplified in insect-damaged leaf tissues. We devised an estimator of bacterial absolute abundance (γ) from 16S sequence data by statistically calibrating information on 16S counts from host versus bacteria to a culture-based standard of bacterial load (Box 1). Using this approach, we found that bacteria within herbivore-damaged leaves at our field sites (see Methods) exhibited local population sizes several doublings greater compared with the bacteria found in undamaged leaves (Fig. 1a,b; median ±95% credible interval of posterior predicted additional doublings: 2.5 [2.1; 3.9] site Emerald Lake [EL]; 4.5 [3.6; 5.3] site North Pole Basin [NP]). This result, rooted in sequence data, is further validated by the parallel observation that damaged leaves showed higher bacterial loads than undamaged leaves via culturing of the $n = 101$ calibration set (Supplementary Fig. 5; mean difference of 3.7 bacterial doublings [1.8–5.6, 95% credible interval on mean difference]; Welch's unequal variance two-sample t-test, $t = 3.86$, $P < 0.001$), which is quantitatively consistent with a prior result from a parallel and independent culture-based study in this system[20].

Herbivore-associated bacterial amplification is both community-wide and taxon-specific. We then capitalized on the high taxonomic resolution and sampling depth afforded by amplicon sequencing to examine shifts in the abundance and distribution of diverse bacteria within the bittercress leaf microbiome. We constructed exact bacterial amplicon sequence variants (bASVs) from our 16S data and estimated how bASVs from each bacterial family varied across damaged and undamaged leaves (see Methods). The within-host density of bacterial bASVs from several bacterial families was elevated in herbivore-damaged leaves compared with undamaged leaves (Fig. 1). For most bacterial families, the relative increase in within-host density with herbivory was greater

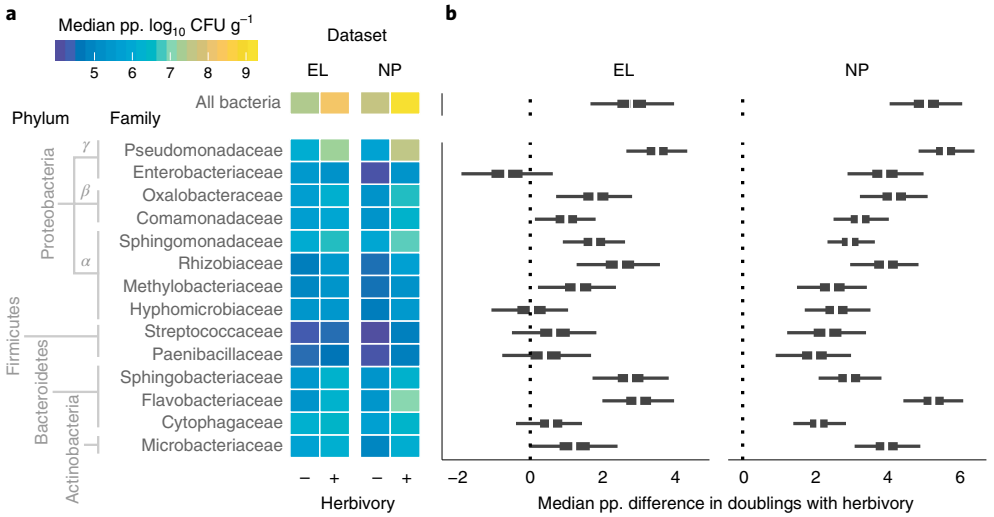


Fig. 1 | Pervasive increases in endophytic bacterial load in herbivore-damaged leaves. a,**b**, Posterior predicted (pp.) infection intensity of bacterial amplicon sequence variants from the 14 most prevalent bacterial families show variation in the extent of elevated growth in herbivore-damaged leaf tissue. **a**, Heatmap shows median predicted $\log_{10}$ bacterial abundance (colony-forming units (CFU) per g starting leaf material) from 200 posterior simulations of the best-fitting model of each bacterial family separately (see Methods). **b**, Boxplots showing median (white), 95% (thin) and 50% (thick) quantiles of the posterior predicted median difference in the number of bacterial cell divisions (that is, doublings) achieved in herbivore-damaged leaves compared with undamaged leaves, for sites EL and NP separately.

at site NP than at site EL (Fig. 1b). This was largely because several taxa showed lower baseline loads in undamaged leaves at site NP (Extended Data Fig. 1). In contrast, bacterial loads for all families were similar for damaged leaves at both sites (Extended Data Fig. 1). Pseudomonadaceae was the most abundant taxon across all leaves and also showed the greatest fold increase under herbivory (Fig. 1).

Several statistical models of abundance (γ; see Methods) at the level of bacterial family showed support for bASV-level differences in intercept and slope values (Supplementary Table 2), including Pseudomonadaceae and Sphingomonadaceae. Two individual bASVs in particular drove family-level patterns in these clades (Supplementary Fig. 6), which together comprised ~20% of all sequencing reads across the two sample sets. Within the Pseudomonadaceae, the bASV labelled `Pseudomonas3` was the single most abundant bASV across both field sites, and it falls within the putatively phytopathogenic *P. syringae* clade (Supplementary Fig. 7). We previously showed that *P. syringae* strains can be pathogenic, induce chlorosis and reduce leaf photosynthetic function in bittercress[20]. Thus, a major component of the signal of elevated bacterial load in the presence of insect herbivory comes from putatively phytopathogenic genotypes within the group *P. syringae*.

Compositional shifts in leaf bacterial communities under herbivory. When the absolute bacterial abundance patterns described above were analysed in a compositional framework, we detected differences in overall community structure and ecological diversity between damaged and undamaged leaves. Specifically, we found lower evenness (J'; Fig. 2b) in damaged leaves, indicating a stronger skew towards a smaller number of bacterial taxa: Pseudomonadaceae comprise an even greater proportion of the population in damaged leaves owing to their already-high average abundance in undamaged leaves and large fold increase under herbivory. Family-level relative abundances differed in terms of Shannon–Jensen (S–J) divergence (that is, β-diversity) between damaged versus undamaged leaves (Fig. 2c). These observations indicate that amplification of bacteria in herbivore-damaged leaves can produce community-wide signatures of reduced within-host diversity and elevated between-host diversity at broad taxonomic scales.

Plant defences against chewing herbivores enhance growth of putative phytopathogens *in planta*. We then directly addressed how inducible plant defences against chewing herbivores impacted the within-host fitness of a suite of bittercress-derived endophytic bacteria from the family Pseudomonadaceae. Plant pre-treatment with the plant defence hormone JA caused clear alterations in within-host growth of five of the 12 *Pseudomonas* spp. strains tested (Fig. 3a), with the most pronounced changes resulting in between two-and-a-half and five additional doublings of two phylogenetically distinct *P. syringae* isolates (20A and 02A; Supplementary Table 3). Increased within-host density of these two strains can account for differences in total *Pseudomonas* spp. abundance, as well as differences in abundance patterns summed at the level of bacterial clade (*P. syringae* versus *P. fluorescens*; Fig. 3b). By recapitulating the elevated *P. syringae* and family-wide increased abundance under herbivory seen in our field studies, this greenhouse result highlights that induction of plant defences against chewing herbivores is one potential mechanism whereby insect herbivory could lead to amplification of bacterial taxa within the bittercress leaf microbiome.

Notably, two *Pseudomonas* spp. strains (22B and 20B) exhibited markedly decreased within-host fitness in JA-treated compared with mock-treated leaves (Fig. 3a,b). Such herbivore-driven fitness variation among *P. syringae* is undetectable when only considering larger taxonomic scales of genus or family (Fig. 3), where genotypes which *increase* in local abundance contribute to an overall signature of elevated taxon-wide abundance as measured by lower resolution tools (for example, 16S sequencing). Thus, induction of plant defences against chewing herbivores leads to the amplification and numerical dominance of a narrow subset of the *P. syringae* community within this host population (Fig. 3c). Such changes result in compositional shifts towards decreased Shannon evenness in JA-treated leaves (Fig. 3d) and an overall community-wide divergence with mock-treated leaves (Fig. 3e).

Putative phytopathogens are aggregated in highly herbivore-damaged plant patches. We then analysed how `Pseudomonas3`, a highly abundant individual bASV within the *P. syringae* group, varied across bittercress plant patches in relation to the level of herbivory on those plant patches. At site NP, we found a highly

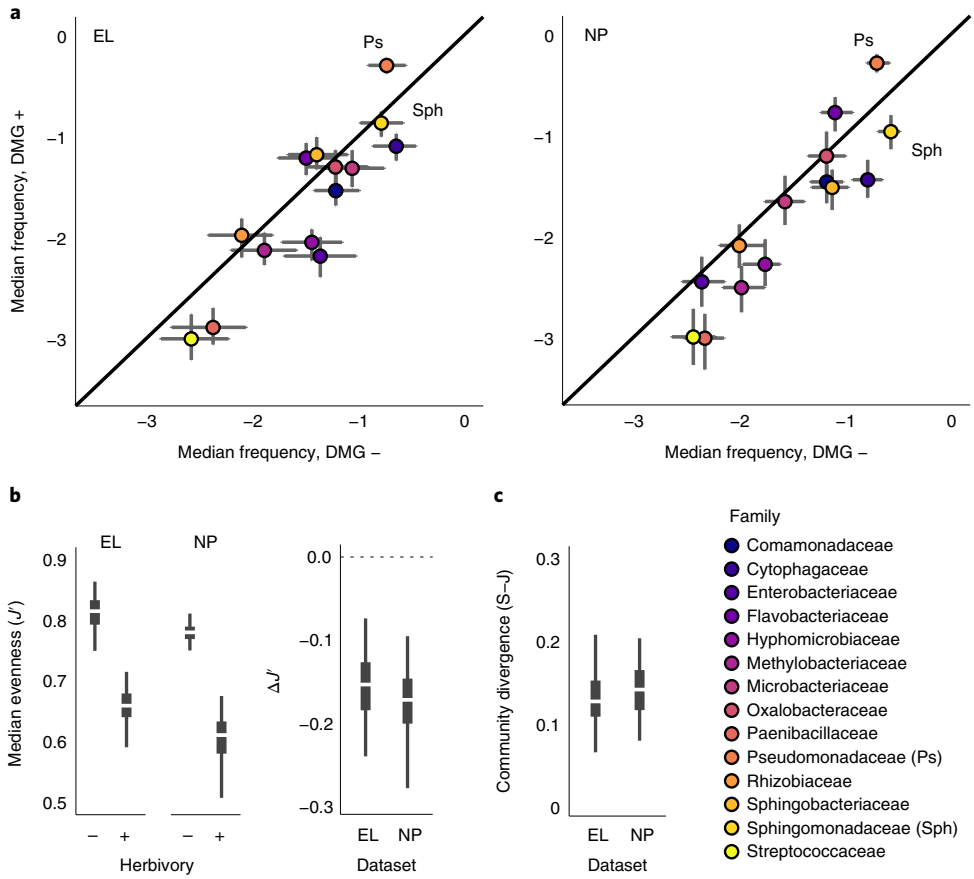


Fig. 2 | Herbivore-damaged leaves harbour compositionally diverged microbiomes with reduced ecological diversity shifted heavily towards Pseudomonadaceae. a, The $\log_{10}$ relative abundance of each family in undamaged (*x*-axis; DMG –) versus damaged (*y*-axis; DMG +) samples shows skew towards Pseudomonadaceae (Ps) and relative reductions of abundance among most other taxa at both study sites, including *Sphingomonas* (Sph), which shows a ~twofold increase in number of doublings in herbivore-damaged leaves. Error bars represent 95% predicted median frequency intervals calculated from posterior simulations of bacterial abundance across damaged and undamaged leaf classes. **b**,**c**, Compositional changes from the amplification of already abundant taxa (for example, Pseudomonadaceae) produces reduced community-level evenness (*J′*) shown in **b** and leads to compositional divergence (that is, *β*-diversity) between damaged and undamaged leaves at both study sites (**c**). Boxplots represent the median (white), 95% (thin) and 50% (thick) quantiles of each statistic calculated from the posterior predicted median relative frequency data depicted in **a**.

aggregated (that is, right-skewed) distribution of herbivore loads across plant patches (Fig. 4a, top marginal density plot). This aggregated herbivore distribution across plant patches results in a predicted 50-fold enrichment of local density of Pseudomonas3 in the most-damaged compared to the least-damaged plant patch (Fig. 4). Analysed in a more general framework, over half the predicted Pseudomonas3 population is harboured in just one-fifth of the plant patches in the bittercress population at our study site NP (Extended Data Fig. 2).

Herbivore–bacteria co-aggregation is associated with lower plant fitness. At site NP, bittercress patches with higher herbivore intensity showed lower reproductive success, with the most damaged patches estimated to produce half as many fruits as patches with no herbivore damage (Fig. 4b and Supplementary Table 4). Plants with more insect damage tend to have higher levels of bacterial infection. Thus, standing variation in fruit set is closely associated with levels of co-aggregation of these natural plant enemies across our sample within this native bittercress population.

Causes of herbivore aggregation in natural plant populations. Although not the primary focus of our study, our field experiments were also designed to test how mid-season pre-treatment with the exogenous plant defence hormones JA or salicylic acid (SA) impacted plant attack rates by *S. nigrita* (see Methods). JA-induced bittercress can locally deter adult *S. nigrita* and reduce larval feeding rates[19]. SA treatment canonically induces defences against biotrophic microbial colonizers and often pleiotropically suppresses plant defences against chewing herbivores[17], including *S. nigrita*[20]. Thus, treatment with either plant defence hormone has the potential to modify the foraging behaviour of *S. nigrita*.

By the end of the growing season, we observed that the degree of herbivore aggregation among host plants at site NP varied extensively across plant patches at site NP (Extended Data Fig. 3a,b) and reflected little statistical signature of causation by the early-season hormone treatments with either JA or SA. Estimates for both SA and JA treatment coefficients were elevated above the mock/control condition, but the posterior distribution for both hormone effects overlapped zero (lower fourth percentile <0 for JA; lower 15th percentile <0 for SA; Supplementary Table 5 and Extended Data Fig. 3c). Thus, while prior plant exposure to JA, and possibly also SA, may cause elevated *S. nigrita* herbivory at the patch scale, standing variation in *S. nigrita* herbivory arising stochastically or from unmeasured factors at site NP dominates over any causal effects of our population-level defence hormone manipulation (Extended Data Fig. 3). Additionally, these early-season plant defence hormone treatments showed no discernible effects on distributions of γ for overall or family-wise bacterial abundance (Extended Data Fig. 4).

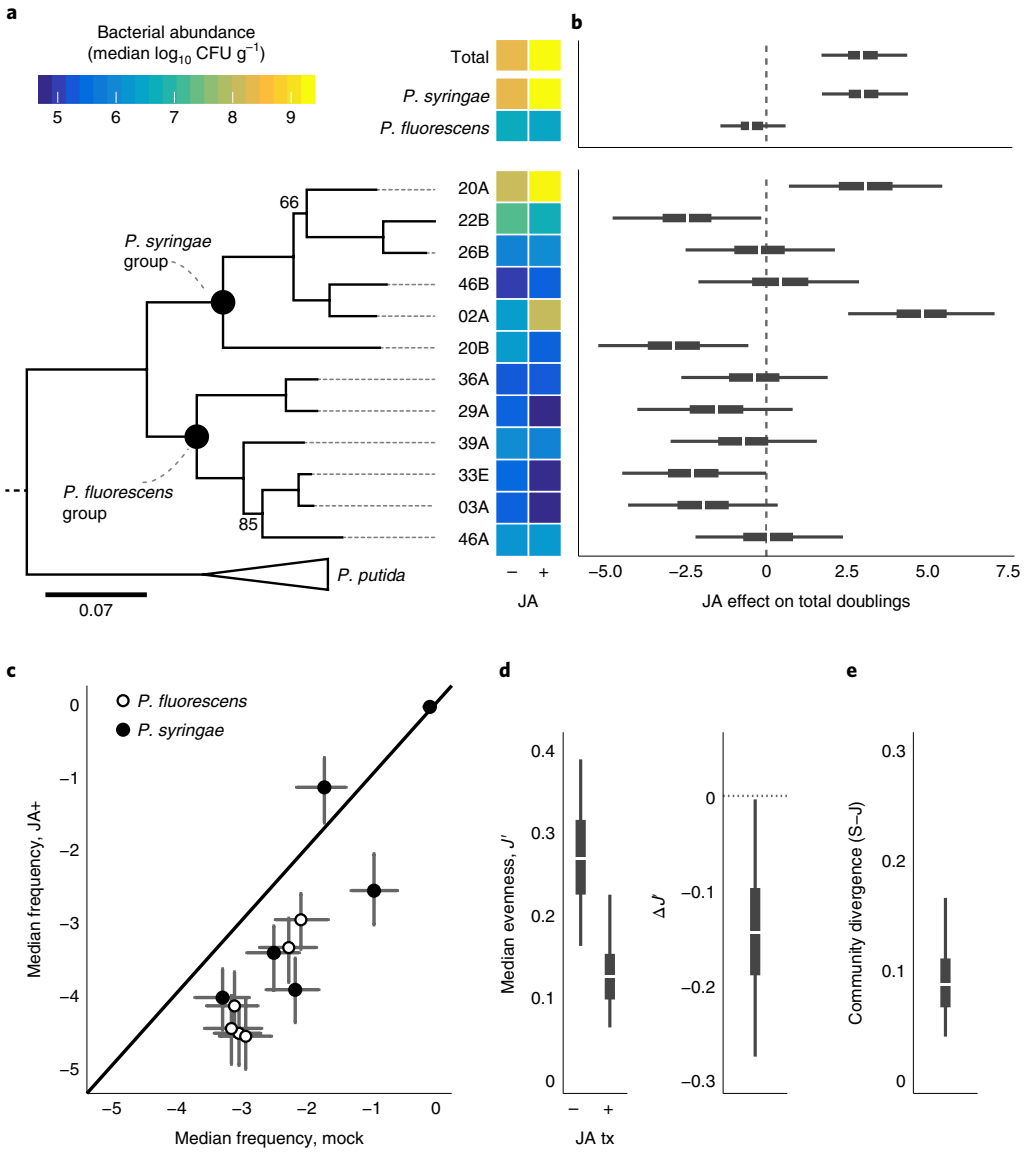


Fig. 3 | Eliciting plant defences against chewing herbivores alters within-host performance of putative phytopathogens. a, Experimental infections with 12 *Pseudomonas* spp. strains, concurrently isolated from EL study site[20], reveal strain-to-strain variation in growth under mock-treated and jasmonic acid (JA) induced plants. Heatmap shows median $\log_{10}$CFU g^{-1} surface-sterilized plant tissue 2 d post inoculation. Maximum likelihood phylogeny of strains estimated with four housekeeping loci (2,951 base pairs)[20]. **b**, The median (white), 95% (thin) and 50% (thick) quantiles of the posterior difference between the number of bacterial doublings attained by bacteria growing in JA- versus mock-treated leaves (see Supplementary Methods). **c**, Compositional analysis of relative abundances (±95% posterior interval) calculated from **a** reflect decreased evenness (J'; **d**) in JA-treated plant tissues, leading to overall community-level divergence (**e**). **d**,**e**, Boxplots of the median (white), 95% (thin) and 50% (thick) quantiles from 200 posterior simulations of abundance from the model results depicted in **b**.

Discussion

Overview. Here we show that insect herbivory is strongly associated with bacterial abundance and diversity within a native plant microbiome using field and greenhouse experiments. We provide evidence that activation of plant defences against chewing herbivores is at least one causal mechanism whereby such within-host amplification of leaf-colonizing bacteria can occur. Specifically, the growth of most bacterial taxa found in the leaf microbiome of native bittercress was amplified in plant tissues damaged by the specialist herbivore *S. nigrita* (Fig. 1) at two separate sub-alpine field sites. These ecological effects were only detectable by linking sequence-based bacterial quantification to an external standard of absolute abundance (Box 1), rather than relying on compositional analysis as is commonly done with studies of both plant and animal microbiomes (but see Vandeputte et al.[21]).

The bacterial clades most altered under herbivory include strains from groups well-known for causing plant disease (*P. syringae*) and our follow-up experimental work in the greenhouse showed that inducing plant defences against chewing herbivores in bittercress was sufficient to cause similar degrees of amplification of putatively phytopathogenic *P. syringae* genotypes in leaf tissues. Amplification of specific *P. syringae* genotypes can largely account for species- and family-level patterns seen in our field studies, which has coarser taxonomic resolution. Overall, these experiments suggest that *S. nigrita* herbivores may play a larger role than previously appreciated in promoting the within-host growth of particular bacterial

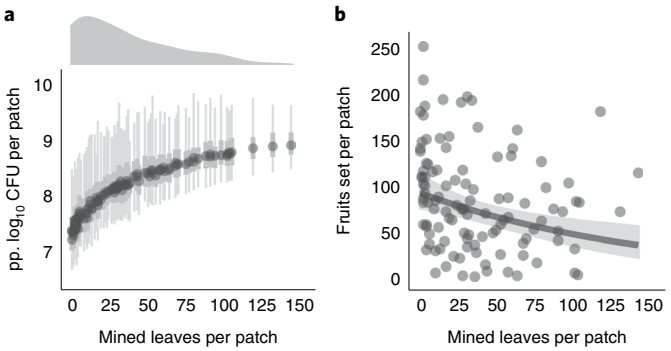


Fig. 4 | Co-infection by herbivores and phytopathogenic bacteria is aggregated across plant populations and is associated with lower plant reproduction. a, Median, 95% and 50% quantiles of 200 posterior simulations of predicted bacterial load across plant patches ($n = 110$ at site NP; $n = 16$ stems sampled per patch). Density plot above *x*-axes exhibits right-skewed (that is, aggregated) distribution of herbivore damage at the plant patch level. **b**, Patch-level herbivory (and thus co-infection intensity) is associated with decreased fruit set in this native plant population. Plotted are raw fruit set data summed at the patch level ($n = 16$ stems per patch), with marginal effects slope (and its 95% credible interval) plotted after accounting for average plant height (see Supplementary Table 4).

genotypes or pathovars in bittercress, although the causal nature of the role of herbivores was not gleaned from the field portion of this study. Given that the majority of plants face herbivore attack to some degree[14,22], it is possible that our results generalize across plant–microbe systems.

Herbivore-inducible plant defences can amplify putative phytopathogens. The mechanisms governing the growth-promoting effects of insect herbivory on leaf-colonizing bacteria are potentially numerous. Leaf damage itself can release nutrients, alleviating resource constraints for bacteria while also creating routes for colonization of the leaf interior from the leaf surface[23,24]. Plant defences induced by chewing herbivores could directly or indirectly alter interactions with bacteria independent of the physical effects of plant tissue damage. It is known that JA-dependent anti-herbivore defences can suppress the subsequent activity of signalling pathways responsive to bacterial infection[17], allowing bacteria (including strains of *P. syringae*) to reach higher densities within JA-affected leaf tissues[25]. *S. nigrita* can trigger JA-dependent host defences in bittercress[19] and this form of defence signalling is widely conserved among diverse plant groups[17]. Released from top-down control, a diversity of resident microbes may then proliferate as defences are more strongly directed against herbivory, which may manifest in community-wide patterns of abundance changes as noted in our study (Fig. 1).

The hypothesis that anti-herbivore defences pleiotropically increase bacterial growth is consistent with results from our greenhouse experiment. We found that the within-host fitness of several strains of putatively phytopathogenic *P. syringae* increased within bittercress leaves pre-treated with JA compared with mock-treated leaves (Fig. 2). Although our experiment is consistent with this plausible mechanism by which herbivores can facilitate bacterial growth within plants, it does not identify the proximal mechanism(s) responsible for these effects. JA induction may have instead stressed the plants or triggered expression of traits unrelated to defence per se, reducing basal tolerance to infection by a subset of the bacterial community. Such net effects of JA induction on bacterial abundance are also likely influenced by underlying constitutive levels of genetic resistance and/or tolerance to herbivory, traits which often exhibit quantitative variation within and among plant populations[26]. The role of host genetic variation in mediating the impacts of herbivore attack on microbial plant colonization is an open avenue of future research.

Finally, several other abundant bacterial groups (for example, Sphingomonadaceae and Flavobacteriaceae) exhibited amplified abundance under insect herbivory in our field studies (Fig. 1). Functional studies examining finer-scale variation among genotypes of these relatively less well-studied bacterial groups would be highly fruitful for establishing a more general understanding of the mechanistic basis of plant–microbe interactions in the context of inducible defences against chewing herbivores.

Herbivore distributions can alter the spatial patterning of plant disease. The impact of insect herbivory on phyllosphere bacteria can be observed at several spatial scales. Herbivore damage was highly clustered on a subset of hosts (Fig. 4), which is a pattern consistent with other plant–herbivore systems[27] as well as many host–macroparasite systems more generally[28]. An accompanying effect of aggregated herbivore damage is the enrichment of bacterial infection on a subset of the host population (Fig. 4 and Extended Data Fig. 2), which alters the spatial structure of growth and potentially also the transmission of plant-colonizing microorganisms. Uncovering the temporal dynamics of how herbivore aggregation precedes or follows microbial attack—or whether the two colonizers cyclically amplify one another—will require more controlled studies that manipulate the timing and density of herbivory itself.

Regardless of the precise mechanisms resulting in such herbivore–microbe co-aggregation, plant patches with the highest levels of co-aggregation had substantially (~50%) lower reproductive success compared with minimally damaged plant patches (Fig. 4b). Although we cannot identify the cause of lower plant fitness from our study, the co-aggregation of these distinct plant colonizers may at least partly explain it. Whether these plants were more stressed to begin with or achieved lower fruiting success because of their infestation with herbivores and phytopathogens cannot be resolved without future studies which isolate the causal effects of single and multiple infection on plant fitness.

What drives the highly skewed pattern of herbivory among host plants? Plant populations often display a patchwork of defensive phenotypes influenced by plant abiotic stress, variation in the underlying defensive alleles or by defence induction from prior herbivore or microbial attack. While plant defences can shape herbivore attack rates in the laboratory and over wide spatial extents[26], less is known about how patterns of defence induction impact the population dynamics of insect herbivores[27,29]. Many insects are deterred by anti-herbivore defences, but some specialist herbivores use these same cues as attractants due to detoxification mechanisms which confer resistance to such defences[30]. *S. nigrita* uses anti-herbivore plant defences to locate hosts but also avoids high concentrations of defensive compounds when given the option[19]. Thus, the joint expression of positive chemotaxis towards JA-inducible compounds, coupled to aversion of high levels of JA-responsive defensive chemistry, may influence where herbivory becomes concentrated among plants in native bittercress populations.

Results from our field hormone treatments using JA and SA both showed elevated patch-level *S. nigrita* leaf miner damage compared with mock-treated patches (Extended Data Fig. 3a,b). However, the statistical signature of these treatment effects was not clear enough to confidently conclude that our field trials substantially altered natural patterns of herbivory by this specialist, given the high degree of stochastic or unexplained variation in herbivore damage we observed across plant patches (Extended Data Fig. 3c and Supplementary Table 5). Discovering the biotic and abiotic factors structuring herbivory patterns in natural host populations thus remains a challenge in this system[31] as well as many others[29], and we have not attempted to solve this problem in the present study.

Nonetheless, our study suggests that predicting herbivore distributions may be key for understanding population-level distributions of plant-associated bacteria. Regardless of its causes, insect herbivore damage can be readily measured and incorporated into plant microbiome studies to help reveal the drivers of variation in microbiome abundance and diversity within plant communities.

Quantifying bacterial loads is crucial for understanding the ecology of the microbiome. The patterns of abundance variation among bacterial taxa across leaf types, when distilled into a community-level compositional metric, showed decreased ecological diversity (that is, evenness) in damaged versus undamaged leaves, resulting in overall compositional divergence between sample sets (Figs. 2,3). This results from particular taxa undergoing larger absolute changes in abundance than other taxa, which leads to stronger skews in the composition of the community calculated on the relative scale. Compositional analysis on its own would preclude inference of the direction or magnitude of changes in bacterial abundance[32], even though this is of primary interest to researchers exploring the microbiome and its impact on host fitness[21,33,34]. Compositional methods are thus poorly suited to studies of the population biology of microbiome-dwelling bacterial taxa when bacterial load varies or when microbial fitness is a desired response variable.

Direct bacterial quantification[21], as well as controlled DNA spike-ins[35], can correct for biases and ambiguities inherent to compositional analyses. Our study provides an additional framework for enabling standard high-throughput 16S sequencing approaches to provide quantitative measures of bacterial abundance when canonical approaches (for example, quantitative PCR) are infeasible due to host organelle contamination. Rather than being discarded, 16S read counts derived from host organelles—once curated—can provide an internal reference population against which the proportionality of other taxa can be measured[36]. While we have established the usefulness of our estimator of bacterial load (γ) using a paired culture-based experiment, this need not be the only way. Bacterial culturing is an intrinsically noisy means of enumerating bacteria, due to dilution and/or counting noise. In addition, chemically mediated antagonism or facilitation among bacterial species or strains can cause over- or under-detection of particular combinations of taxa on agar plates[37]. These limitations will no doubt set a lower limit to the resolution of biological effects one is capable of detecting with culture-dependent methods. Testing the generality of our approach across other plant–microbe systems, and with other means of enumerating bacteria in samples, is therefore important.

Conclusions. Our study emphasizes that large effects on the population biology of *P. syringae*, and many other lineages of leaf-colonizing bacteria, may stem from the action of insect herbivores. Important biotic interactions such as herbivory are absent from the classic 'disease triangle' of plant pathology. The role of insect herbivores in *P. syringae* epidemiology—and plant–microbiome relations in general—has been underappreciated. Variation in bacterial abundance across samples, and the implications of relative abundance changes for bacterial fitness, are not easily detectable via compositional analyses applied to 16S data, which typically do not use external or internal standards. Thus, studies aiming to decipher why plant microbiomes differ in structure or function should endeavour to quantify bacterial loads to retain this important axis of variation as a focal response variable, while also considering additional biotic interactions commonly encountered by the hosts under study.

Methods

Field studies of herbivore–bacteria co-infection. We surveyed herbivore damage arising from *S. nigrita* (Supplementary Fig. 1f,g) in replicate 0.5 m^2 plots of native bittercress along transects in sub-alpine and alpine streams near the Rocky Mountain Biological Laboratory (RMBL) in each of two years (2012, Emerald Lake [EL], $n = 24$ plots; 2013, site North Pole Basin [NP], $n = 60$ plots; Supplementary Fig. 1a–d). Our analysis of the impact of hormone treatments on *S. nigrita* foraging patterns was previously published for site EL[20] and we implemented a similar approach for site NP in this study. Full experimental design details are given in the Supplementary Methods and are depicted in the schematic in Supplementary Fig. 1e.

By the end of the growing season, when herbivory and bacterial infection had run their course, we determined *S. nigrita* leaf miner damage status of all leaves (both sites) as well as fruit set (site NP only) produced on each of the focal bittercress stems (stem-level sample size $n = 768$, site EL; $n = 1920$, site NP). At both sites we collected leaf tissue in a randomized manner (see Supplementary Methods) to quantify the abundance and diversity of bacteria that had colonized the leaf interior.

Amplicon sequencing of bacteria in leaf tissues. We quantified bacterial abundance in leaf tissues using next-generation amplicon sequencing of the bacterial 16S rRNA locus using the Illumina MiSeq platform. To enrich our samples for endophytic bacteria, we surface-sterilized all samples prior to DNA extraction, which achieved a reliable reduction of bacterial abundance as detected by our 16S analysis approach (Supplementary Fig. 2). Subsequently, we extracted DNA from the 192 leaf discs (~0.8 cm^2) from site EL and 192 tissue pools from site NP (four discs per pool) and amplified bacterial 16S following published protocols[38] (see Supplementary Methods). We amended this protocol by including peptic nucleic acid (PNA) PCR clamps into reaction mixtures to reduce amplification of host chloroplast- and mitochondria-derived 16S, following Lundberg et al.[39]. This was highly effective at reducing the proportion of host-derived 16S reads per library in our sample sets (Supplementary Fig. 3).

We then used `DADA2`[40] to error-correct, trim, quality-filter and merge the paired-end sequencing reads that passed error thresholds off the sequencer. Of the approximately 4 million raw reads, ~90% were retained following quality control via `DADA2` (Supplementary Fig. 4) and these reads were then delineated into exact amplicon sequencing variants (ASVs). The 16S reads from bittercress chloroplast or mitochondria were manually curated and summed into 'host-derived' for comparison with bacteria-derived 16S (see Supplementary Methods).

Quantifying and modelling bacterial abundance patterns. To quantitatively assess how herbivore damage relates to abundance and diversity of microbial plant colonizers, we required a link between 16S counts and bacterial load. We therefore devised and validated an estimator (γ) of the abundance of bacterial ASVs within host tissues (Box 1). Using γ as an empirically validated estimator of absolute bacterial load in leaf tissues, we constructed a two-stage modelling approach to estimate bacterial load across our complete sample set.

We first fitted and compared a series of increasingly flexible Bayesian regression models to estimate how γ varies as a function of herbivore damage in leaves (see Supplementary Methods). When calculating γ, we took r_B at the bASV-level for all bASVs within each of the 14 most abundant bacterial families, together comprising >95% of total bASV counts in the datasets. We then took the candidate best stage-1 model, heuristically defined as the model with the lowest leave-one-out Bayesian information criterion[41], and used it to generate $n = 200$ replicate sets of simulated response values ($\hat{\gamma}$) predicted by the model parameter's fit to the original data.

In the next stage, we used this distribution of $\hat{\gamma}$ as an input predictor variable to the model we fitted between our observed γ and observed log CFU (Box 1). This allowed us to report bacterial abundance estimates, based initially on 16S count data, on the scale of predicted log CFU per unit leaf mass—a more directly interpretable measure of within-host fitness. Rather than point estimates, we sampled intercept, slope and residual error parameters from their joint posterior distribution of the calibration CFU model for each data point independently. Specifically, this has the effect of incorporating uncertainty in the fit between observed log CFU and γ such that downstream predictions are not overly biased by the precise value of any regression slope estimate, which may itself arise from peculiarities in the action of PNA during amplification of 16S. Overall, this two-stage modelling approach was designed to incorporate uncertainty in the model fit for γ as well as in the relationship between observed γ and observed log CFU. The endpoint of this approach is 200 sets of posterior predicted log CFU values for each sample in the dataset, which formed the basis of downstream calculations of bacterial abundance variation, as well as ecological diversity (Shannon evenness, J') and similarity (Shannon–Jensen divergence, S–J) in and between damaged and undamaged leaf sets, respectively (see Supplementary Methods).

Population-level analysis of herbivore–bacteria co-aggregation. We assessed how patch-level variation in herbivory correlated with bacterial infection intensity at the field-scale by focusing on the most highly abundant bASV at both field sites (`Pseudomonas3`). Leaf-level abundance of this individual bASV was predicted using the approach described above. We then summed the predicted abundance (on the linear scale) of bacteria across leaves within each plant patch and used these predicted bacterial sums to calculate the cumulative proportion of the total

Pseudomonas3 population harboured by plant patches with differing levels of herbivory, which portrays the extent of co-aggregation of herbivores and bacteria across the host population.

Experimental infections *in planta*. We directly examined how inducible plant defences against chewing herbivores impacted within-host bacterial performance using field-derived accessions of bittercress plants and their bacterial colonizers. We randomized the selection of six focal strains from within each of the two dominant *Pseudomonas* clades (*P. syringae* and *P. fluorescens*) represented in our endophytic strain collection from bittercress[20]. We infected each strain into a single leaf on each of $n = 32$ distinct bittercress clones that had been randomized to be pre-treated 3 d prior with JA (1 mM; Sigma) or a mock solution. Bittercress clones were originally isolated as rhizomes from various sites within 2 km of the RMBL in 2012[42] and were regrown in the greenhouse at University of Arizona for up to 12 months prior to use. Two days post-infection, we sampled, sterilized, homogenized and dilution-plated leaf discs onto non-selective rich King's B media, following Humphrey et al.[20]. We compared bacterial abundance ($\log_2$) between treated and untreated samples using Gaussian Bayesian regression models. We subsequently used posterior predicted abundances as the basis for considering how herbivore-inducible defences impact the composition and diversity of this *Pseudomonas* community at different taxonomic levels (see Supplementary Methods).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All sequence data has been deposited to the NIH Sequence Read Archive (SRA) under accession SUB5541275 and can also be accessed via BioProject ID PRJNA587302 (http://www.ncbi.nlm.nih.gov/bioproject/587302). Saved model objects are available for download from Dryad Digital Repository[44].

Code availability

Code to reproduce all analyses, figures and tables is available at https://github.com/phumph/coinfection.

Received: 19 May 2019; Accepted: 12 December 2019;
Published online: 27 January 2020

References

1. Lloyd-Smith, J. O., Poss, M. & Grenfell, B. T. HIV-1/parasite co-infection and the emergence of new parasite strains. *Parasitology* **135**, 795–806 (2008).
2. Laine, A.-L. Context-dependent effects of induced resistance under co-infection in a plant-pathogen interaction. *Evol. Appl.* **4**, 696–707 (2011).
3. Tollenaere, C., Susi, H. & Laine, A.-L. Evolutionary and epidemiological implications of multiple infection in plants. *Trends Plant Sci.* **21**, 80–90 (2016).
4. Karvonen, A., Jokela, J. & Laine, A.-L. Importance of sequence and timing in parasite coinfections. *Trends Parasitol.* **35**, 109–118 (2018).
5. Halliday, F. W., Umbanhowar, J. & Mitchell, C. E. Interactions among symbionts operate across scales to influence parasite epidemics. *Ecol. Lett.* **20**, 1285–1294 (2017).
6. Susi, H., Barrès, B., Vale, P. F. & Laine, A.-L. Co-infection alters population dynamics of infectious disease. *Nat. Commun.* **6**, 5975 (2015).
7. Horton, M. W. et al. Genome-wide association study of *Arabidopsis thaliana* leaf microbial community. *Nat. Commun.* **5**, 5320 (2014).
8. Bulgarelli, D. et al. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* **488**, 91–95 (2012).
9. Bodenhausen, N., Bortfeld-Miller, M., Ackermann, M. & Vorholt, J. A. A synthetic community approach reveals plant genotypes affecting the phyllosphere microbiota. *PLoS Genet.* **10**, e1004283 (2014).
10. Edwards, J. et al. Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl Acad. Sci. USA* **112**, E911–E920 (2015).
11. Wagner, M. R. et al. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nat. Commun.* **7**, 12151 (2016).
12. Finkel, O. M., Castrillo, G., Herrera Paredes, S., Salas González, I. & Dangl, J. L. Understanding and exploiting plant beneficial microbes. *Curr. Opin. Plant. Biol.* **38**, 155–163 (2017).
13. Orozco-Mosqueda, M. D. C., Rocha-Granados, M. D. C., Glick, B. R. & Santoyo, G. Microbiome engineering to improve biocontrol and plant growth-promoting mechanisms. *Microbiol. Res.* **208**, 25–31 (2018).
14. Maron, J. L. & Crone, E. Herbivory: effects on plant abundance, distribution and population growth. *Proc. Biol. Sci.* **273**, 2575–2584 (2006).
15. Agrawal, A. A. Induced responses to herbivory and increased plant performance. *Science* **279**, 1201–1202 (1998).
16. Bressan, M. et al. Exogenous glucosinolate produced by *Arabidopsis thaliana* has an impact on microbes in the rhizosphere and plant roots. *ISME J.* **3**, 1243–1257 (2009).
17. Thaler, J. S., Humphrey, P. T. & Whiteman, N. K. Evolution of jasmonate and salicylate signal crosstalk. *Trends Plant Sci.* **17**, 260–270 (2012).
18. Wagner, M. R. et al. Natural soil microbes alter flowering phenology and the intensity of selection on flowering time in a wild *Arabidopsis* relative. *Ecol. Lett.* **17**, 717–726 (2014).
19. Humphrey, P. T. et al. Aversion and attraction to harmful plant secondary compounds jointly shape the foraging ecology of a specialist herbivore. *Ecol. Evol.* **6**, 3256–3268 (2016).
20. Humphrey, P. T., Nguyen, T. T., Villalobos, M. M. & Whiteman, N. K. Diversity and abundance of phyllosphere bacteria are linked to insect herbivory. *Mol. Ecol.* **23**, 1497–1515 (2014).
21. Vandeputte, D. et al. Quantitative microbiome profiling links gut community variation to microbial load. *Nature* **551**, 507–511 (2017).
22. Turcotte, M. M., Davies, T. J., Thomsen, C. J. M. & Johnson, M. T. J. Macroecological and macroevolutionary patterns of leaf herbivory across vascular plants. *Proc. Biol. Sci.* **281**, 20140555 (2014).
23. Hirano, S. S. & Upper, C. D. Bacteria in the leaf ecosystem with emphasis on Pseudomonas syringae-a pathogen, ice nucleus, and epiphyte. *Microbiol. Mol. Biol. Rev.* **64**, 624–653 (2000).
24. Lindow, S. E. & Brandl, M. T. Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* **69**, 1875–1883 (2003).
25. Cui, J. et al. Pseudomonas syringae manipulates systemic plant defenses against pathogens and herbivores. *Proc. Natl Acad. Sci. USA* **102**, 1791–1796 (2005).
26. Züst, T. et al. Natural enemies drive geographic variation in plant defenses. *Science* **338**, 116–119 (2012).
27. Underwood, N., Anderson, K. & Inouye, B. D. Induced vs. constitutive resistance and the spatial distribution of insect herbivores among plants. *Ecology* **86**, 594–602 (2005).
28. Shaw, D. J. & Dobson, A. P. Patterns of macroparasite abundance and aggregation in wildlife populations: a quantitative review. *Parasitology* **111**, S111–S127 (1995).
29. Karban, R. & Baldwin, I. T. *Induced Responses to Herbivory* (Univ. Chicago Press, 1997).
30. Wittstock, U. et al. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc. Natl Acad. Sci. USA* **101**, 4859–4864 (2004).
31. Alexandre, N. M. et al. Habitat preference of an herbivore shapes the habitat distribution of its host plant. *Ecosphere* **9**, e02372 (2018).
32. Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V. & Egozcue, J. J. Microbiome datasets are compositional: and this is not optional. *Front. Microbiol.* **8**, 2224 (2017).
33. Falony, G. et al. Population-level analysis of gut microbiome variation. *Science* **352**, 560–564 (2016).
34. Raes, J. Editorial overview: it's the ecology, stupid: microbiome research in the post-stamp collecting age. *Curr. Opin. Microbiol.* **44**, iv–v (2018).
35. Stämmler, F. et al. Adjusting microbiome profiles for differences in microbial load by spike-in bacteria. *Microbiome* **4**, 28 (2016).
36. Lovell, D., Pawlowsky-Glahn, V., Egozcue, J. J., Marguerat, S. & Bähler, J. Proportionality: a valid alternative to correlation for relative data. *PLoS Comput. Biol.* **11**, e1004075 (2015).
37. Foster, K. R. & Bell, T. Competition, not cooperation, dominates interactions among culturable microbial species. *Curr. Biol.* **22**, 1845–1850 (2012).
38. Thompson, L. R. et al. A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* **551**, 457–463 (2017).
39. Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D. & Dangl, J. L. Practical innovations for high-throughput amplicon sequencing. *Nat. Methods* **10**, 999–1002 (2013).
40. Callahan, B. J. et al. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).
41. Vehtari, A., Gelman, A. & Gabry, J. Practical Bayesian model evaluation using leave-one-out cross-validation and WAIC. *Stat. Comput.* **27**, 1413–1432 (2017).
42. Humphrey, P. T. et al. Heritable plant phenotypes track light and herbivory levels at fine spatial scales. *Oecologia* **187**, 427–445 (2018).
43. Lebeis, S. L. et al. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* **349**, 860–864 (2015).
44. Humphrey, P. T. & Whiteman, N. K. Dryad Data from: Insect herbivory reshapes a native leaf microbiome. (Dryad Digital Repository, 2019); https://doi.org/10.5061/dryad.qz612jm95

Acknowledgements

P.T.H. and N.K.W. gratefully acknowledge funding from the National Science Foundation (Grant Nos. DEB-1309493 to P.T.H. and DEB-1256758 to N.K.W.), the National Institute of General Medical Sciences of the National Institutes of Health (Grant No. R35GM119816 to N.K.W.), as well as the Rocky Mountain Biological Laboratory. We are indebted to field assistance provided by H. Briggs, K. Cromwell, A. Koning, L. Anderson, K. Niezgoda, D. Picklum and N. Alexandre; bioinformatics advice from T. K. O'Connor; and laboratory assistance from H. Pyon and A. Abidov. We thank our contacts at Argonne National Laboratory, S. Owens and J. Koval, for their technical expertise and support.

Author contributions

P.T.H. and N.K.W. designed the study. P.T.H. carried out the study and analysed the data. P.T.H. and N.K.W. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41559-019-1085-x.

Supplementary information is available for this paper at https://doi.org/10.1038/s41559-019-1085-x.

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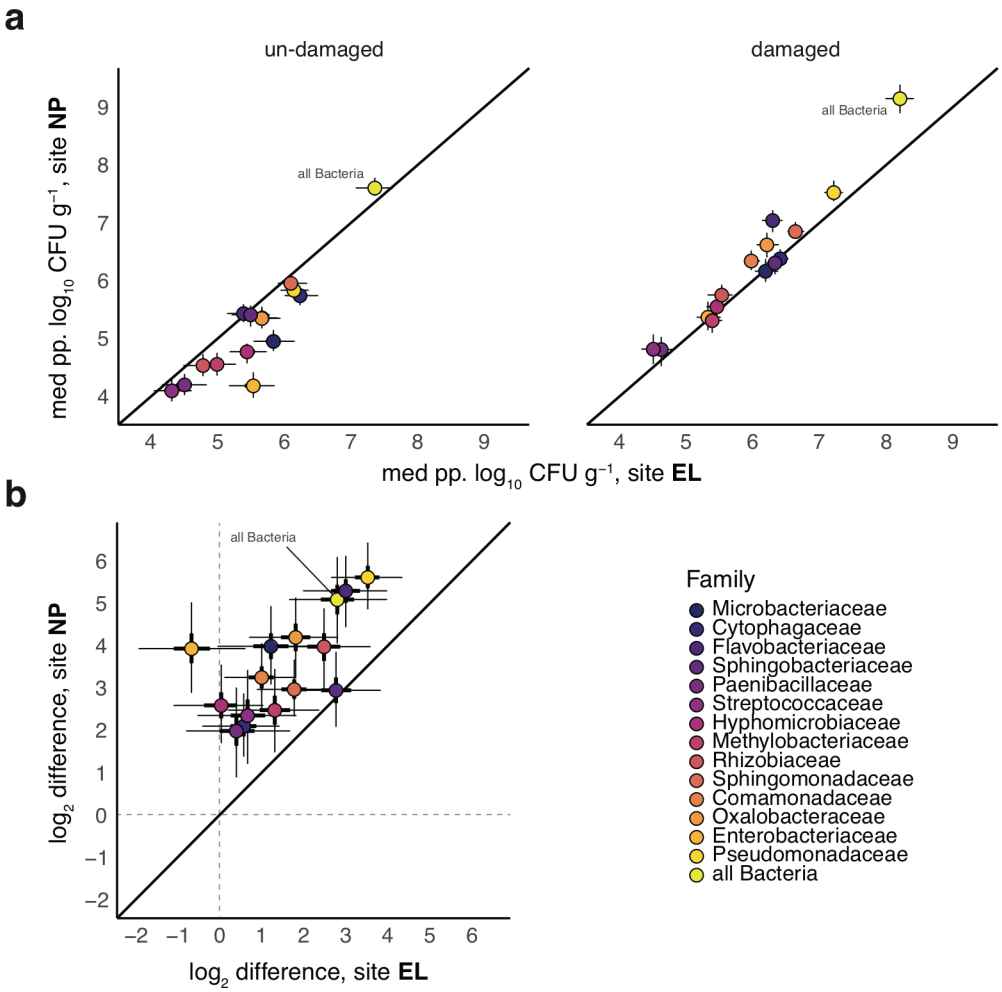
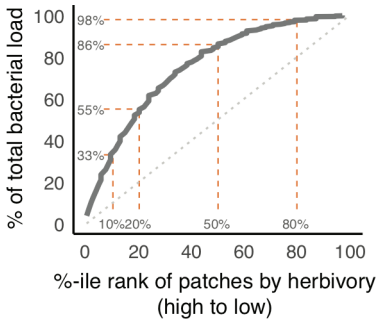
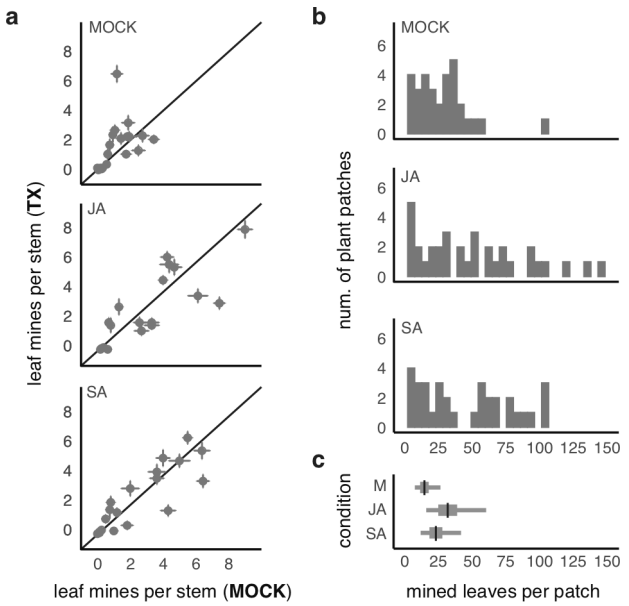


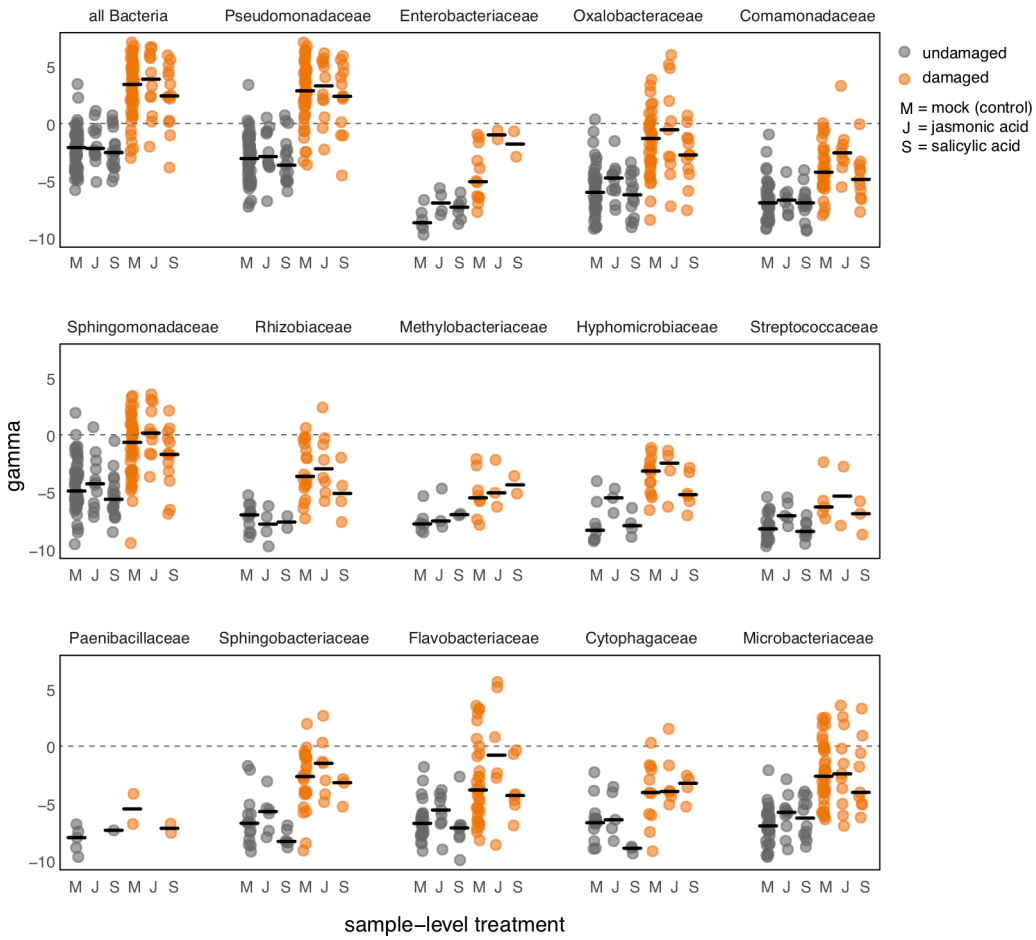
Extended Data Fig. 1 | Correspondence between predicted bacterial abundance and herbivory effects between sites EL and NP. a. Plotted are median (circles) ± 95% posterior distributions of predicted abundance for bacterial bASVs summed at the family level for sites EL (*x*-axis) and NP (*y*-axis). **b**. Comparison between the magnitudes of $\log_2$-fold differences between damaged and undamaged leaves at sites EL (*x*-axis) and NP (*y*-axis). Middle 50%-ile and 95%-iles of median effects (circles) are depicted by thick and thin bars, respectively. On both plots, we also show data summed across all taxa in the dataset ('all Bacteria').



Extended Data Fig. 2 | Plant patches with high herbivory harbor a disproportionate fraction of the estimated population of the most abundant *P. syringae* bASV. Scaling of percentile rank (high to low) of patch-level herbivore load with total population-level percentage of bacterial propagules present in the sampled patch. At site NP, the top 20% of plant patches with the most herbivory harbor > 50% of bacterial propagules in the plant population.



Extended Data Fig. 3 | Effects of hormone pretreatment effects on levels of *S. nigrita* herbivory in bittercress populations at site NP. a. For each plot separately, plotted is the average (± 1 std error) leaf mines per stem calculated at the patch level (n=16 stems per patch) for mock-treated (x-axis) versus hormonetreated (y-axis) patches. The three panels represent plots assigned to each of the three conditions: mock (that is, control), jasmonic acid (JA) or salicylic acid (SA). **b**. Histograms of patchlevel leaf miner damage broken down by patch-level treatment. **c**. Marginal effects for estimates of patch-level treatment on total mined leaves per patch (see table S5 for statistical results). Black bars are posterior means, while thick and thin bars comprises middle 50%- and 95%-iles of posterior distributions of model terms marginalized over all other parameters.



Extended Data Fig. 4 | Hormone pre-treatment (Mock, JA, or SA) five weeks prior to sampling does not leave a clear signature in the distribution of γ across samples for undamaged (gray) or herbivore damaged (orange) leaf samples. For each of the 14 most abundant bacterial families, in addition to all Bacteria, we have plotted the distributions of raw γ for damaged and undamaged samples across mock- (M), JA-, or SA-treated plant patches. Black bars represent medians for the respective distribution, and data points are slightly jittered along the x-axis. Systematic differences in γ can be easily seen between damaged versus undamaged sample classes, whereas no systematic differences can be seen between the different hormone treatment classes within each damage class. If any effects of hormone treatments are indeed present, they do not constitute a discernible feature of these data, supporting the choice to devote minimal attention to this aspect of our experiment.

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☐	☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐	☒ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software or code was used to collect data.
Data analysis	All analyses were performed with custom scripts were written by PTH which leveraged statistical and computational features from published, open-source packages using the R platform for statistical computing. All scripts and raw data are deposited at https://github.com/phumph/coinfection/

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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- A description of any restrictions on data availability

All sequence data has been deposited to the NIH Sequence Read Archive (SRA) under accession SUB5541275 and can also be accessed via BioProject ID PRJNA587302 (http://www.ncbi.nlm.nih.gov/bioproject/587302). Saved model objects are freely available for download from Dryad digital repository (https://doi.org/10.5061/dryad.qz612jm95). Code to reproduce all analyses, figures, and tables can be found at https://github.com/phumph/coinfection.

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Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Field studies took place in each of two years (2012, site EL; 2013, site NP) in native populations of bittercress (Cardamine cordifolia; Brassicaceae). We established plots along transects and monitored herbivore damage by the specialist herbivore Scaptomyza nigrita (Drosophilidae), fruit set, and a variety of plant phenotypic characteristics (e.g., number of leaves, stem height). We randomized the selection of leaf tissue from each plant patch for subsequent bacterial quantification in order to compare how bacterial diversity and abundance varied in relation to insect herbivory. We also randomized plant plots to early-season treatment with exogenous plant hormones to test how such treatments affected patterns of S. nigrita herbivory in the wild. Parallel experimental studies used field-derived bittercress accessions grown in the greenhouse for up to 1 year prior to being randomized to receive plant hormone pre-treatments (mock versus defense hormone induction). We subsequently infected plants with 12 natural bittercress isolates of Pseudomonas spp. bacteria to directly test how inducible plant defenses against chewing herbivores impacted within-host fitness of bittercress-derived endophytic bacteria.
Research sample	A research sample constituted a leaf (site EL) or a pool of four leaves (site NP) sampled randomly with respect to stem and leaf position, from each plant plot, used for bacterial detection via 16S rRNA marker gene amplification and next-generation sequencing. A sample for the greenhouse experiment was a single leaf from which we cultured bacteria on rich laboratory medium.
Sampling strategy	For field studies, we determined a sample size of n=20 plant plots per treatment condition (n=3 conditions = 60 plots total, site NP) as the maximally feasible sample size for this type of experiment given our personnel and logistical constraints. A previous field study using fewer plots (n=8 per condition) detected differences in S. nigrita damage rates on plants pre-treated with various plant hormones compared to mock-treated plants. Thus, a sample size ~2.5X larger we believed would be sufficient for carrying out a separate and independent study. Our greenhouse study used all available C. cordifolia accessions that had been propagated in the greenhouse at University of Arizona.
Data collection	Data was hand-collected by PTH as well as various field assistants under the supervision of PTH (see Acknowledgments). All electronic data entry from hand-written data-sheets was manually checked twice. Paper data sheets from field studies have been scanned and archived by PTH.
Timing and spatial scale	July 2012--August 2012 for site EL; July 2013--August 2013 for site NP. 16S sequence data was collected during Fall of 2014. Spatial scale of EL study was 0.5 km transect; NP study was ~1 km transect. Detailed maps of plots and larger regional context are provided in supplemental information.
Data exclusions	We excluded 16S taxonomic units which were sampled in only a single library and which had fewer than 10 total 16S counts across the dataset.
Reproducibility	Field and greenhouse studies used maximal sample sizes afforded by time, personnel, and resources. All studies (e.g., field study at site NP; greenhouse study) were completed once due to logistical constraints. All code and raw data has been provided in order to facilitate replication of statistical analyses.
Randomization	For field studies at sites EL and NP, plant plots were randomly assigned to condition (n=3 conditions) using random number generators, and each of the two patches per plot were randomly assigned to receive the actual treatment or a paired mock control treatment via drawing a Bernoulli random variable with equal odds. For leaf-level sampling, we randomly sampled a single damaged and undamaged leaf position from each of n=4 stems per patch (site NP) using digital random number generators deployed in the field. Samples were later processed in blocks where treatments and plots were distributed evenly across blocks, and blocks were randomized for processing order. For the greenhouse study, plants were randomized to treatment groups (n=2) just prior to treatments, and leaves on plants were randomized to be infected with one of each of the bacterial strains (or mock controls). Plants from similar initial collection sites were matched for randomization across treatment conditions.
Blinding	Lead author PTH and all field assistants were blind to the treatment conditions of each plant plot during data recording/entry and sample collection. The key for plot ID-to-treatment mapping was revealed only after data entry prior to analysis. For amplicon sequencing, all samples were included on the same sequencing run and the IDs of samples were sufficiently vague to not reveal treatment and/or herbivory status to those conducting the amplicon sequencing. As the presence of herbivore damage on a leaf sample was not possible to mask, the order in which samples were processed was thoroughly interspersed.

Did the study involve field work? ☒ Yes ☐ No

Field work, collection and transport

Field conditions	Field studies were conducted in alpine and sub-alpine riparian habitats in the West Elk Mountains of southwestern Colorado,

Field conditions	USA. Further descriptions can be found in the Supplemental Methods. Bittercress plants were growing in stream beds containing flowing meltwater through mid-August 2012 and 2013.
Location	All location details (e.g., latitude, longitude, and elevation) are supplied as maps in the Supplemental Material.
Access and import/export	Our study employed a low-impact sampling of plant tissues and non-destructive monitoring of plant phenotypes and reproductive fitness. All study procedures had received prior approval from a multi-personnel research committee at the Rocky Mountain Biological Laboratory, which enforces a very high standard for minimal impact on native plants, animals, and abiotic features of the environment.
Disturbance	Our field crew minimally disturbed riparian plant communities by ensuring all footing was positioned in streams and not on top of vegetation. No field supplies were left behind following the conclusion of field studies.

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Materials & experimental systems

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☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging