Soil microbial diversity impacts plant microbiota more than herbivory

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Abstract

Interactions between plants and microbiomes play a key role in ecosystem functioning and are of broad interest due to their influence on nutrient cycling and plant protection. However, we do not yet have a complete understanding of how plant microbiomes are assembled. Here, we tested and quantified the effect of different factors driving the diversity and composition of plant-associated microbial communities. We manipulated soil microbial diversity (high or low diversity), plant species (*Solanum tuberosum* or *Solanum vernei*), and herbivory (presence or absence of a phloem-feeding insect *Macrosiphum euphorbiae*), and found that soil microbial diversity influenced the herbivore-associated microbiome composition, but also plant species and herbivory influenced the soil microbiome composition. We quantified the relative strength of these effects and demonstrated that the initial soil microbiome diversity explained the most variation in plant- and herbivore-associated microbial communities. Our findings strongly suggest that soil microbial community diversity is a driver of the composition of multiple associated microbiomes (plant and insect), and this has implications for the importance of management of soil microbiomes in multiple systems.

Keywords

rhizosphere; phyllosphere; bacteria; fungi; metabarcoding; *Solanum*; *potato aphid*

Introduction

Microbiomes can be considered an extension of the plant genome (Berg *et al.*, 2014; Schlaeppi and Bulgarelli, 2015; Rosenberg and Zilber-Rosenberg, 2016; Levy *et al.*, 2018). While their functional importance has been widely dissected in the last two decades of microbiome research, how plant microbial communities assemble, respond to environmental stimuli, and interact with their host remains to be determined (Cordovez *et al.*, 2019; Saikkonen *et al.*, 2020). In addition, research has rarely examined or compared multiple drivers and, to the best of our knowledge, no study has tested the relative strength of different drivers of microbial community composition.

There have been a number of studies identifying individual factors that drive the microbiome composition of plants and their associated organisms and environments. It is well established that plant microbiota structures mainly by plant compartment (e.g. different plant organs, and diverse between endosphere and ectosphere) (Trivedi *et al.*, 2020). Plant genotype and developmental stage have been shown to influence the composition of both plant and soil microbiomes (Wagner *et al.*, 2016). Soil microbiome composition has also been shown to shape plant microbiome composition (Cordovez *et al.*, 2019), and plant pathogens and herbivory produce compositional shifts in plant-associated microbial communities (Lareen *et al.*, 2016). However, we still know little about the relative strength of these factors in shaping plant microbiomes.

Soil provides microbial inoculum and sets the conditions for both plant and microbial growth (Schlaeppi *et al.*, 2014). While different plant tissues can develop distinct microbiomes, soil provides an important reservoir of microbial inoculum for both the phyllosphere and rhizosphere (Bai *et al.*, 2015). The overlap of soil and plant microbiota has been found in a variety of plants, for example *Saccharum officinarum* (de Souza *et al.*, 2016), *Boechera stricta* (Wagner *et al.*, 2016), *Vitis vinifera*

52 (Zarraonaindia *et al.*, 2015; Mezzasalma *et al.*, 2018) and in the biofuel crops *Panicum virgatum* and
53 *Miscanthus x giganteus* (Grady *et al.*, 2019). This suggests that soil microbial communities can
54 represent a major factor shaping plant microbiomes in both above and belowground plant
55 compartments. Furthermore, it has been shown that soil microbial community can influence the
56 feeding behaviour of insect herbivores (Badri *et al.*, 2013). However, only one study to our knowledge
57 has reported that soil microbial community can directly influence both the aboveground microbiota
58 of plants (*Taraxacum officinale*) and the microbiota of an insect herbivore (*Mamestra brassicae*),
59 showing overlap between the microbial communities of the insect and soil (Hannula *et al.*, 2019).
60 However, it was unclear whether the influence of soil microbiome on the caterpillar's microbiome
61 was due to passive transfer (e.g. microbe dispersal when watering) or an active colonisation
62 mechanism (Hannula *et al.*, 2019). Thus, there is potential for the influence of soil microbial
63 communities on plant-associated microbial communities to extend beyond their plant host.

64 Plant species and genotype also contribute to the composition of multiple plant-associated
65 microbiota (Turner *et al.*, 2013). Plant microbial communities assemble differently in different organs,
66 and their composition varies according to plant phylogeny (Dastogeer *et al.*, 2020). Plant traits (tissue
67 morphology and physicochemical properties) and resources might drive these plant-genotype effects
68 on plant microbiota (Fitzpatrick *et al.*, 2018; Dastogeer *et al.*, 2020). For example, an analysis of 30
69 species of angiosperms revealed differences in the diversity and composition of root microbiomes
70 across plant species (Fitzpatrick *et al.*, 2018). Similarly, the rhizosphere microbiota of wheat, maize,
71 tomato and cucumber each had unique microbial communities (Ofek *et al.*, 2014), and leaf and root
72 microbial communities of *Agave* species clustered according to the host plant species (Coleman-Derr
73 *et al.*, 2016). Furthermore, several studies reported that host plant identity is an important factor in
74 the assembly of insect herbivore-associated microbial communities (Colman *et al.*, 2012; Malacrinò,

75 2018). For example, host plant species influenced the composition of the microbiome associated with
76 the herbivores *Ceratitis capitata* (Malacrinò *et al.*, 2018) and *Thaumetopoea pytiocampa* (Strano *et*
77 *al.*, 2018). Therefore, plant species identity has an effect on the communities of microorganisms in
78 the rhizosphere, living in the different plant organs, and even within plant herbivores.

79 Only a few studies have tested the effects of herbivory on plant microbiomes. For example,
80 whitefly infestation of pepper plants led to an increased proportion of Gram-positive bacteria in the
81 rhizosphere (Yang *et al.*, 2011), and aphid herbivory on pepper plants increased the abundance of
82 *Bacillus subtilis* and decreased that of the pathogen *Ralstonia solanacearum* in roots (Lee *et al.*, 2012).
83 While whitefly herbivory shifted the rhizosphere microbiome composition in pepper plants (Kong *et*
84 *al.*, 2016), there was no effect of aphid herbivory on the rhizosphere microbiota of *Brassica oleracea*
85 var. *capitata* (O'Brien *et al.*, 2018). Herbivore attack may alter plant root exudates which, in turn,
86 promote the assembly of beneficial rhizosphere microbiota (Hu *et al.*, 2018; Rolfe *et al.*, 2019). Thus,
87 herbivory can alter plant and rhizosphere microbiomes, but the relative impact of herbivory versus
88 plant species or initial soil diversity on plant-associated microbiomes has not been investigated.

89 To date most studies have focused on one or two drivers of microbiome composition (e.g., soil
90 microbiota, plant species or herbivory) and, to the best of our knowledge, no study has tested the
91 comparative strength of multiple biotic drivers expected to shape above- and belowground
92 microbiomes *in vivo*. This represents a major gap in our understanding of the relative importance of
93 factors determining microbiome assembly. Here we ask how plant microbiome composition is shaped
94 by three different major drivers of plant-associated microbial communities — soil microbial diversity,
95 plant species, and herbivory — and whether they have equal impact on plant-associated
96 microbiomes. By manipulating insect herbivory (presence/absence), plant species identity and soil
97 microbial diversity in a microcosm system, and by quantifying their effects on both bacterial and

fungal plant-associated microbiome composition, we tested the relative strength of these three biotic factors in shaping rhizosphere, plant (root and shoot), and herbivore microbiomes. As outlined above, there is evidence of overlaps between soil and plant microbiota, thus we hypothesize that soil microbial diversity is the major driver structuring plant microbiomes at different compartments. Previous studies reported that, within the same plant species, geographical location greatly contributes to assemble the plant microbiota (Christian *et al.*, 2016; Lin *et al.*, 2020). Similarly, there is evidence that soil microbial community can influence herbivores (Badri *et al.*, 2013) and their microbiota (Hannula *et al.*, 2019). Thus, we hypothesize that plant species and herbivory will impact plant-associated microbial communities, but their magnitude would be lower than soil microbial diversity.

Methods

Experimental design

In this study, we used a 2 × 2 × 3 factorial design to test our hypothesis. We grew two *Solanum* species (*Solanum tuberosum* and *Solanum vernei*) in soil with different microbial diversities: high diversity and low diversity (see below). To evaluate the effects of herbivory on plant and rhizosphere microbiota, we infested plants (within each 'soil' × 'plant species' combination) with two clonal lines of the polyphagous aphid species *Macrosiphum euphorbiae* (potato aphid); uninfested plants served as a control. Each treatment combination of plant species (n=2), soil microbial diversity (n=2), and aphid clonal line and presence/absence (n=3) was replicated five times, involving 60 plants in total.

Study System

Solanum tuberosum (genotype TBR-5642) and *Solanum vernei* (genotype VRN-7630) seeds were obtained from the Commonwealth Potato Collection at The James Hutton Institute (Dundee, Scotland, UK). Seeds were germinated in steam-sterilized coir, and then transplanted to the experimental pots after 3 weeks.

We used two aphid clones of *M. euphorbiae* (AK13/08 and AK13/18) previously collected in the field (James Hutton Institute, Dundee, UK — 56.457 N, 3.065 W) and reared for several generations on excised leaves of *Solanum tuberosum* (cv. Desirée) in ventilated cups at 20 °C and 16:8 h (light:dark).

All inoculum was prepared from soil collected from an uncultivated field at the James Hutton Institute (56.457 N, 3.065 W) (Bennett *et al.*, 2016; Karley *et al.*, 2017), sieved to 3 cm to remove rocks and large debris, and homogenized. The high diversity inoculum consisted of whole soil, and half of the high diversity inoculum was steam sterilized by autoclaving at 121 °C for 3 h, allowing it to cool for 24 h and then autoclaving it again at 121 °C for a further 3 h. The low diversity inoculum was prepared by blending 50 ml of high diversity inoculum with twice the volume of water, filtering the solution through a 38 µm sieve, and vacuum filtering the collected solution through a Whatman filter paper no. 1 (Bennett *et al.*, 2011). Half of the filtrate was autoclaved at 121°C for 20 min. When preparing the experimental pots, those assigned to the high-diversity treatment were inoculated with high-diversity inoculum (whole soil) and autoclaved low-diversity inoculum (filtrate), while those assigned to the low-diversity treatment were inoculated with low-diversity inoculum and autoclaved high-diversity inoculum. This process allowed us to homogenize the abiotic inputs across our pots while varying the microbial community. For further details please see the section below and Figure S2 (Supplementary material).

The filtration process eliminated larger soil microbes, such as arbuscular mycorrhizal (AM) fungi, from the low diversity inoculum. We found significant differences in microbial phylogenetic diversity (see below) between high and low diversity inoculum (Faith's phylogenetic diversity index 38.31±3.66 for high diversity soil, 8.76±5.94 for low diversity soil; $F_{1, 10} = 22.05$, $P < 0.001$) which also translated into different microbial diversities in the rhizosphere (see Results below).

Microcosm setup

Experimental pots (1 L) were assembled as depicted in Fig. S2 (Supplementary material). We added 100 ml of sterile background soil to the bottom and top of each pot to reduce the risk of microbial contamination between pots when watering. Sterile background soil was prepared by mixing Sterilized Loam (Keith Singleton, Cumbria, UK) and sand (ratio 1:1), autoclaving this mixture at 121 °C for 3 h, allowing it to cool for 24 h and then autoclaving it again at 121 °C for a further 3 h. Between the layers of sterile background soil, we added a mix of 100 ml live or sterile high diversity inoculum (10% of the pot volume) and 700 ml of sterile background soil. Pots assigned to the high diversity treatment were filled with live high diversity inoculum and received 1 ml of sterile low diversity inoculum, while pots assigned to the low diversity treatment were filled with sterile high diversity inoculum and received 1 ml of live low diversity inoculum. In this way, we controlled for physical and chemical differences between pots, which only differed in terms of their microbial community. One potato seedling was transplanted into each pot, pots were randomized into two blocks, and left to grow in an insect-screened greenhouse with an average temperature of 25 °C and 16:8 h (light:dark) photoperiod.

Five weeks after transplanting, two apterous adult aphids of *M. euphorbiae* clone AK13/08 were added to 20 plants, two apterous adult aphids of clone AK13/18 were added to 20 plants, and

20 plants were left uninfested. All plants were screened with a microperforated plastic bag (Sealed Air, UK) that allowed transpiration while preventing aphid escape. Three weeks following infestation, we collected from each pot five aphids, leaves, roots and rhizosphere soil (≈500 mg each) and stored them at -80 °C. The five aphids were randomly collected from five different leaves on each plant. Leaf samples were also randomly collected from each plant, being careful to not sample from leaves infested by aphids. Rhizosphere soil was sampled from soil still adhering to the roots after removing loose soil, released by vigorously shaking roots and then sampled. Roots were carefully washed with tap water before collecting a random sample of root tissue. Aphid infestation was scored using a 0–5 scale of severity (scale 0 = no aphids, scale 1 = between 1 and 250 aphids, scale 2 = between 251 and 500 aphids, scale 3 = between 501 and 750 aphids, scale 4 = between 751 and 1,000 aphids, scale 5 = more than 1,000 aphids).

DNA extraction, Illumina Miseq libraries preparation and sequencing

Samples were crushed in an extraction buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) using three 1 mm Ø stainless steel beads per tube, with the aid of a bead mill homogenizer set at 30 Hz for 5 min (TissueLyzer II, Qiagen, UK). Total DNA was extracted using phenol/chloroform, and it was subsequently checked for quantity and quality with a Nanodrop 2000 (Thermo Fisher Scientific Inc., USA). We conducted a metabarcoding analysis for both bacterial and fungal communities of leaves, roots and rhizosphere soil, and bacterial communities of aphids. Bacterial communities were characterized by targeting the 16S rRNA gene with primers 515f/806rB (Caporaso *et al.*, 2012). Fungal communities were analysed by amplifying the fungal ITS2 region of the rRNA with primers ITS3-KYO/ITS4 (Toju *et al.*, 2012). Amplifications were also carried out on DNA extracted from soil inoculum, and non-template controls where the sample was replaced with nuclease-free water in

188 order to account for possible contamination of instruments, reagents and consumables used for DNA
189 extraction (see Supplementary material).

190 PCR reactions were performed in a total volume of 25 µl, containing about 50 ng of DNA, 0.5
191 µM of each primer, 1X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, USA) and nuclease-free water.
192 Amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Germany) set at 95 °C for
193 3 minutes, 98 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, repeated 35 times, and ended with 10
194 minutes of extension at 72 °C. Reactions were carried out in technical triplicate, in order to reduce the
195 stochastic variability during amplification (Schmidt *et al.*, 2013), and a no-template control in which
196 nuclease-free water replaced target DNA was utilized in all PCR reactions (see Supplementary
197 material). We also PCR-tested all root samples for the presence of AM fungi using specific primers
198 (Lee *et al.*, 2008), finding presence of AM fungi just in plants grown on high-diversity treatment soil.

199 Libraries were checked on agarose gel for successful amplification and purified with Agencourt
200 AMPure XP kit (Beckman and Coulter, CA, USA) using the supplier's instructions. A second short-run
201 PCR was performed in order to ligate the Illumina i7 and i5 barcodes and adaptors following the
202 supplier's protocol, and amplicons were purified again with Agencourt AMPure XP kit. Libraries were
203 then quantified through Qubit spectrophotometer (Thermo Fisher Scientific Inc., USA), normalized
204 using nuclease-free water, pooled together and sequenced on an Illumina MiSeq platform using the
205 MiSeq Reagent Kit v3 300PE chemistry following the supplier's protocol.

206

207 ***Raw reads processing***

208 De-multiplexed forward and reverse reads were merged using PEAR 0.9.1 algorithm using
209 default parameters (Zhang *et al.*, 2014). Data handling was carried out using QIIME 1.9 (Caporaso *et*
210 *al.*, 2012), quality-filtering reads using default parameters, binning OTUs with a 97% cut-off and

discarding chimeric sequences using VSEARCH (Rognes *et al.*, 2016). Singletons and OTUs coming from amplification of chloroplast DNA were discarded from the downstream analyses. Within the ITS2 dataset, all non-fungal OTUs were discarded using ITSx (Bengtsson-Palme *et al.*, 2013). Taxonomy was assigned to each OTU through the BLAST method by querying the SILVA database (v. 132) for 16S (Quast *et al.*, 2012), and UNITE database (v. 8.0) for ITS2 (Nilsson *et al.*, 2019).

Data analysis

Data analysis was performed using R statistical software 3.5 (R Core Team, 2013) with the packages *phyloseq* (McMurdie and Holmes, 2013), *vegan* (Dixon, 2003) and *picante* (Kembel *et al.*, 2010).

Core microbiota. The core microbiota was identified separately for each compartment using the package *ampvis2* (Andersen *et al.*, 2018), considering an OTU as a member of the core microbiota if it was retrieved at a relative abundance of >0.1% in more than 50% of samples. We also tested if the number of core OTUs shared by pairs of compartments was greater/less than an overlap generated by random chance. To do so, for each compartment we draw a group of random OTUs from all those identified in that compartment, in a quantity equal to the number of observed OTUs for that compartment (i.e., if for a compartment we observed 10 core OTUs, we drew 10 random OTUs from the pool of OTUs identified in that compartment). Then, we calculated the number of overlapping random OTUs between all pairs of compartments. We did this separately for bacteria and fungi, and we repeated it 10,000 times. Then, for each pair of compartments, we selected the highest number of random overlapping OTUs among the 10,000 permutations and we tested it against the number of observed overlapping OTUs for that pair of compartments using a χ^2 test.

233 *Phylogenetic diversity*. We selected Faith's phylogenetic diversity index (Faith, 1992) to
234 estimate the diversity of microbiotas in our system because, in contrast to other indices, it takes into
235 account the phylogenetic relationship between taxa within the community. Comparison of diversity
236 indices among groups was performed by fitting a linear mixed-effects model, separately for bacterial
237 and fungal community, specifying *compartment* (i.e., rhizosphere soil, root, leaf, aphid), *soil*
238 *treatment*, *plant species*, *herbivory* (and their interactions) as fixed factors and *aphid clonal line* and
239 *block* as a random effect (Table 1). We also ran separate analyses for plant (rhizosphere soil, root,
240 leaf) and aphids obtaining comparable results (Tab. S1 and S2). The use of *aphid clonal line* as a
241 random variable in the mixed-effects model allowed for the control of differences in the performance
242 of aphid clonal lines. Models were fitted using the *lmer*() function under the *lme4* package (Bates *et*
243 *al.*, 2015) and the package *emmeans* was used to infer pairwise contrasts (corrected using False
244 Discovery Rate, FDR).

245 *Community structure.* We analyzed the effects of treatment factors (*compartment*, *soil*
246 *treatment*, *plant species*, *herbivory* and their interactions) on the structure of the microbial
247 communities using a multivariate approach. Distances between pairs of samples, in terms of
248 community composition, were calculated using a unweighted Unifrac matrix, and then visualized
249 using a Canonical Analysis of Principal Coordinates (CAP) procedure (Anderson and Willis, 2003).
250 Differences between sample groups were inferred through PERMANOVA multivariate analysis (999
251 permutations stratified at the level of *block* and *aphid clonal line*). The use of *aphid clonal line* for
252 stratification in PERMANOVA allowed for the control of differences in the performance of aphid clonal
253 lines. We also ran separate analyses for plant (rhizosphere soil, root, leaf) and aphids obtaining
254 comparable results (Tab. S1 and S2).

Soil diversity vs. plant species vs. herbivory driven effects. Which is strongest? We assessed the impact of soil treatment, plant species and herbivory for each OTU using the R package *DESeq2* (Love *et al.*, 2014). Using this package, we calculated the effect of each factor (herbivory, plant species, and soil microbial diversity) to the abundance of OTU (expressed as abs $\log_2$ Fold Changes) in each plant compartment. We first built a model using *compartment* (leaves, roots and rhizosphere), *soil treatment*, *herbivory* and *plant species* as factors. Then, we extracted the appropriate contrasts (*Low diversity*/*High diversity* for soil treatment, *S. vernei*/*S. tuberosum* for plant species and *Herbivore*/*No herbivore* for herbivore treatment) for each compartment (leaves, roots and rhizosphere). From each contrast, we used the absolute log2 Fold Change values (*ashr* shrinked (Stephens, 2017)) for each OTU to quantify the impact of soil, plant and herbivore treatments on the microbiota in each compartment. Comparisons of absolute log2 Fold Change values were performed by fitting a linear mixed-effects model, specifying *compartment*, *treatment* (herbivory, plant or soil) and their interaction as fixed factors and *OTU identity* as a random effect, and using the package *emmeans* to infer contrasts (FDR corrected).

Aphid infestation. We tested whether the aphid infestation levels were influenced by soil microbial diversity by fitting a cumulative link mixed model using the *ordinal* R package (Christensen, 2015), specifying *soil treatment*, *plant species*, and their interaction as fixed factors and *block* and *aphid clonal line* as a random effect.

Results

Dataset summary and community composition

Overall, we identified 43,879 bacterial and 4,713 fungal OTUs. The analysis of the core microbiota resulted in identifying 150 core bacterial OTUs and 26 fungal OTUs (Fig. 1). We identified

278 81 bacterial and 19 fungal OTUs as the core microbiota of rhizosphere soil, where the bacterial
279 community was largely dominated by uncultured taxa (50.6%), *Ramlibacter* (10.1%), *Pseudomonas*
280 (9.1%), *Massilia* (8.6%) and *Chitinophaga* (6.1%), while the fungal community was mostly represented
281 by *Peziza* (44.9%), unidentified fungi (18.1%), *Humicola* (9.1%), *Mortierella* (8.8%), *Penicillium* (8.6%),
282 *Mucor* (5.5%) and *Trichoderma* (4.9%). The core microbiota of roots was represented by 74 bacterial
283 and 16 fungal OTUs. Root tissues were mainly associated with *Flavobacterium* (39.4%) and uncultured
284 bacterial taxa (23.7%), while *Peziza* (22.9%), uncultured taxa (18.1%) and *Fusarium* (11.1%) dominated
285 the fungal community. Leaf core microbiota was represented by 20 bacterial and 6 fungal OTUs, with
286 a higher abundance of *Stenotrophomonas* (24.1%), *Candidatus* Hamiltonella (20.9%), *Flavobacterium*
287 (10.5%) and *Pedobacter* (9.4%), while fungi were mainly represented by *Cladosporium* (42.3%),
288 *Penicillum* (34.3%) and *Peziza* (13.6%). Aphids were mainly associated with *Buchnera* (64.1%) and
289 Candidatus *Hamiltonella* (35.8%). We identified 5 bacterial OTUs (*Flavobacterium* – 2 OTUs,
290 *Citrobacter*, *Pedobacter*, *Terrimonas*) shared between all compartments, and 4 fungal OTUs
291 (*Cladosporium*, *Humicola*, *Penicillium* and *Peziza*) shared between leaves, roots and rhizosphere. For a
292 more detailed analysis of the core microbiota, please see the Supplementary material.

293 To gain understanding on the overlap of core OTUs between compartments, we tested the
294 number of observed core OTUs overlapping between two compartments against a the number of
295 OTUs overlapping between two compartments due to random sampling. We found that the overlap of
296 bacterial core OTUs between pairs of plant compartments was always greater than random chance (P
297 < 0.05). Aphids also showed a higher number of shared bacterial core OTUs with leaves than random
298 chance (8 OTUs, $\chi^2 = 5.4$; $P = 0.01$), but not with roots (7 OTUs, $\chi^2 = 2.7$; $P = 0.09$) and rhizosphere soil
299 (5 OTUs, $\chi^2 = 2$; $P = 0.15$). In fungi, we saw that the OTUs shared between leaves and roots ($\chi^2 = 1.3$; P
300 $= 0.25$) or rhizosphere soil ($\chi^2 = 0.5$; $P = 0.47$) were not different than those shared by chance, while

301 OTUs shared between roots and rhizosphere soil were greater than those shared by chance (χ^2 = 6.2;
302 P = 0.01).
303

304 ***Phylogenetic diversity***

305 For bacterial communities, we found a significant compartment × soil treatment × plant
306 species interaction (Tab. 1). In all plant compartments (leaves, roots, rhizosphere) we found a higher
307 phylogenetic diversity in *S. vernei* than in *S. tuberosum* when plants were grown on low-diversity soil
308 treatment, and no differences between the two plant species were found when plants were grown on
309 high-diversity soil treatment (Supplementary material Tab. S3). Plant species did not influence aphid
310 bacterial diversity when they were exposed to plants grown on high- or low-diversity soil treatment
311 (Supplementary material Tab. S3). In fungal communities we found a significant effect of the factor
312 "plant species", reporting a higher diversity in *S. vernei* than *S. tuberosum* plants (P = 0.001, Tab. 1),
313 although we did not find any significant interaction with other factors.

314 We found a significant compartment × soil treatment × herbivory interaction in both bacterial
315 and fungal communities. Post-hoc contrasts show a higher leaf bacterial diversity in aphid-infested
316 plants compared with uninfested control plants when they were grown on low-diversity soil
317 treatment (Supplementary material, Tab. S4). Root bacterial and fungal communities, in both soil
318 treatments, had higher diversity values in infested plants compared with uninfested control plants
319 (Supplementary material, Tab. S4). In the rhizosphere we observed differences between infested and
320 uninfested plants in both bacterial and fungal community diversity of plants grown on high-diversity
321 soil treatment, while this difference was found just in the fungal community of plants grown on low-
322 diversity soil treatment (Supplementary material, Tab. S4).

We found phylogenetic diversity of the aphid microbiota was highest in the low diversity treatment (Figure S3), which mirrored differences in aphid infestation levels (χ^2=8.19, df=1, *P*=0.004; mean infestation scores: 3.10±0.23 for high diversity soil, 2.45±0.28 for low diversity soil).

Microbial community composition

The multivariate analysis (i.e., the PERMANOVA) reported a significant compartment × soil treatment × plant species interaction (Tab. 1). Post-hoc contrasts showed differences between *S. vernei* and *S. tuberosum* in the structure of leaf, root and rhizosphere bacterial and fungal communities when plants were grown on low-diversity soil treatment (Fig. 2a-f, Tab. S5). In high-diversity soil treatment, only root fungal communities differed between *S. vernei* and *S. tuberosum* (Fig. 2e, Supplementary material Tab. S5). On the other hand, differences between low-diversity and high-diversity treatments were found in both *S. vernei* and *S. tuberosum* in root and rhizosphere communities (both bacterial and fungal, Fig. 2b-c and 2e-f, Supplementary material Tab. S6). In leaves, differences between soil treatments were found just in the bacterial community of *S. vernei* (Fig. 2a, Supplementary material Tab. S6). We also found a significant compartment × herbivory interaction (Tab. 1), with herbivory influencing bacterial communities in all compartments, but fungal community just in leaves and roots (Tab. 2). Our multivariate analysis demonstrated that the strongest driver of rhizosphere, root and leaf microbial community structure was soil diversity treatment (Tab. 2) for both bacterial and fungal communities. Indeed, bacterial and fungal communities responded to soil diversity treatment and plant species across all compartments (roots, rhizosphere and leaves) (Table 2, Fig. 2a-f). Based on the variation explained by each factor included in the model, soil microbial diversity was the most important factor shaping the microcosm's microbiota in all compartments

(Table 2). The variation explained by soil microbial diversity tended to decrease when moving across compartments from rhizosphere to leaves and aphids (Table 2).

Soil diversity vs. plant species vs. herbivory driven effects. Which is strongest?

We answered this question in two ways, focusing on the single factors included in our design (soil treatment, plant species, herbivory). First, as discussed above, using the variation explained by each of our predictor variables in our PERMANOVA model, we determined that the predictor that explained the most variation was the initial soil community diversity. Both soil diversity, plant species and herbivory influenced bacterial and fungal assemblies in our system. Soil treatment explained ≈30% (rhizosphere), ≈20% (root), and ≈7% (leaf) of variation in microbiome community composition (Table 2). However, the variance in community composition explained by both plant species and herbivory (3–5%) was always lower than the variance explained by the soil treatment. Furthermore, soil treatment explained ≈8% of variation in aphid microbiota (Table 2). This suggests that the soil-driven effect is stronger than the other effects in our system.

Second, to investigate in more detail which factor (soil, plant, herbivore) had a stronger influence on plant microbiome composition we examined the magnitude of change in abundance for each OTU (absolute $\log_2$ Fold Changes) in relation to soil treatment, plant species and herbivory. For both bacterial and fungal communities, and in all compartments, the changes produced by soil treatment were greater than those produced by herbivory and plant species ($\chi^2_{bacteria}$=23331.3 and χ^2_{fungi}=1055, df=2, P<0.001, Fig. 3), with the only exception being the leaf fungal community, where no differences were found between the three factors (Fig. 3b). Also, in all cases, there was no difference between the changes produced by herbivory and those produced by plant species (Fig. 3). The analysis of changes in the abundance of OTUs in aphids revealed that soil diversity treatment had

368 a greater influence than plant species in shaping aphid bacterial communities (χ^2=766.8, df=1,
369 P<0.001; abs($\log_2$FoldChange)$_{soil}$ = 1.7±0.1 and abs($\log_2$FoldChange)$_{plant}$ = 0.37±0.1). Collectively, these
370 results demonstrate that the strongest effect on microbial taxa in the rhizosphere, roots, shoots, and
371 aphid herbivores is driven by the initial soil community diversity.

372 # Discussion

373 Here we test the influence of multiple drivers (and their interaction) on plant microbiome
374 diversity and composition, and we show that, of all the drivers tested, soil microbial diversity had the
375 greatest influence on the microbial community composition of rhizosphere, roots, leaves, and even
376 aphid herbivores. Thus, we correctly hypothesized that soil microbial diversity drives changes in plant
377 and herbivore microbiota and that this effect would be much stronger than plant species or
378 herbivory. This influence of soil microbial diversity correlated with aphid abundance on infested
379 plants. Furthermore, we showed herbivory and plant species also affect the microbiome community
380 composition of leaves, roots and rhizosphere, but their effects are weaker than those driven by soil
381 diversity. We also observed that the response of plant microbiome to herbivory or plant species
382 differs according to soil treatment.

383 Plant and insect microbial diversity was influenced by soil microbiota and interactions between
384 soil microbiota and plant species and herbivory. When we quantified the relative contribution of the
385 main effects (soil, plant species, and herbivory), soil treatment was the strongest driver of plant
386 related microbiota composition. Soil community composition is well-known to influence plant
387 microbial composition (Bulgarelli *et al.*, 2012; Schlaeppi *et al.*, 2014; Bai *et al.*, 2015; Zarraonaindia *et*
388 *al.*, 2015; de Souza *et al.*, 2016; Wagner *et al.*, 2016; Mezzasalma *et al.*, 2018; Grady *et al.*, 2019), but
389 here we reveal that soil produces a stronger effect when compared to other factors (i.e. plant species

390 and herbivory). Soil represents a major reservoir of microbial inoculum for plants (Zarraonaindia *et*
391 *al.*, 2015; Trivedi *et al.*, 2020), especially belowground. However, we also observed that the effect
392 driven by soil is a function of plant species, likely because the intrinsic characteristics of each plant
393 species can modulate specific changes in the diversity and composition of microbial communities at
394 each compartment (Turner *et al.*, 2013; Dastogeer *et al.*, 2020).

395 Interestingly, we found that soil microbial diversity influences the phylogenetic diversity and
396 structure of aphid microbiota. The influence of the soil microbial diversity on aphid bacterial
397 communities and aphid infestation level could potentially be explained through two, non-mutually
398 exclusive, mechanisms: (i) translocation of microbes from the rhizosphere through or on the plant and
399 (ii) changes in plant physiology and/or metabolome. Although leaves are physically separated from
400 roots, their microbiomes can still interact at interfaces such as the stem, and microbial translocation
401 could occur due to active and passive mechanisms (Bai *et al.*, 2015; Wagner *et al.*, 2016). A recent
402 study comparing the microbiota of caterpillars feeding on detached leaves and intact plants found the
403 microbiota of caterpillars that fed on intact plants had a similar community composition to the soil
404 microbiota (Hannula *et al.*, 2019) suggesting direct (splashing of soil microbiota on leaves) or indirect
405 (movement through the plant) microbial translocation. However, our data does not show this pattern,
406 as the core microbiome belowground is different from shoots and herbivores, few OTUs are common
407 to all compartments in the system, and the overlap of core OTUs between aphids and belowground
408 compartments is not greater than random chance. The aphids in our system employ a different
409 feeding strategy (sap-feeding) compared to the caterpillars (chewing) in the previous study (Hannula
410 *et al.*, 2019), and chewing herbivores may have an increased likelihood of environmental uptake of
411 microbes (Paniagua Voirol *et al.*, 2018). We thus find it unlikely that microbes in our system were
412 translocated through or on the plant to the herbivore.

413 The second potential mechanism is through changes in plant physiology. Many soil microbes
414 are able to modulate plant nutrient intake, or prime plant defences (Martinez-Medina *et al.*, 2016).
415 The composition of belowground microbial communities can alter plant metabolism (Li *et al.*, 2019),
416 which in turn influences herbivore fitness (Mason *et al.*, 2019). Our low diversity treatment lacked
417 large microbes including arbuscular mycorrhizal fungi, a group well known to prime plant defences
418 (Jung *et al.*, 2012; Bennett *et al.*, 2018), although aphids are less susceptible to changes in defenses
419 primed by arbuscular mycorrhizal fungi (Koricheva *et al.*, 2009). Thus, the changes we observed in the
420 aphid microbiome could be due to changes in host plant physiology and metabolome, for example
421 triggering of plant defences which has been shown to decrease the diversity of plant-associated
422 microbial communities (Kniskern *et al.*, 2007). The higher aphid abundance on plants grown in the
423 high diversity soil provides indirect evidence for such changes in plant biochemical composition, as
424 does a previous study showing increased aphid suitability as a host for parasitoids when feeding on
425 *Solanum* plants grown with AM fungi from the same site (Bennett *et al.*, 2016). While no previous
426 study found a relationship between soil microbial diversity and aphid infestation, previous studies
427 show that soil microbiota can influence insect feeding behaviour through changes in the plant
428 metabolome (Badri *et al.*, 2013), which can represent the likely mechanism driving our observations.
429 While a clear consumer-driven effect was observed on the plant microbiome in our study, it
430 was a weaker effect than soil microbial diversity. Thus, herbivory plays a less significant role in
431 determining plant microbiome composition. The herbivory-driven effect on the bacterial community
432 composition of the roots and rhizosphere could be driven by changes in plant physiology (e.g. defence
433 activation, carbon metabolism) and root exudation due to aphid feeding activity (Züst and Agrawal,
434 2016; Hoysted *et al.*, 2018). Herbivory has been shown to alter the types of organic compounds
435 released at the root surface leading to changes in the composition of rhizosphere microbial

436 communities (Lareen *et al.*, 2016; Hu *et al.*, 2018). Previous research has shown that *Bemisia tabaci*
437 (whitefly) herbivory can alter the rhizosphere microbiome of pepper plants (Kong *et al.*, 2016), and
438 artificial induction of plant defences (Hein *et al.*, 2008), or their deactivation (Kniskern *et al.*, 2007),
439 has been shown to shape rhizosphere microbial communities. In our study we focused on a sap-
440 feeding insect, which is known to trigger specific physiological responses in plants. Thus, further
441 research is needed to test if different herbivores with different feeding strategies are able to drive
442 changes in the microbiota thriving in different plant compartments.

443 Plant species was also a predictor shaping the microbiome community composition of the
444 rhizosphere, plants, and herbivores in our study. Plant species is known to be a strong driver of root
445 and rhizosphere microbiota because plant species differ in morphology, chemistry and relationship
446 with microorganisms (Trivedi *et al.*, 2020). *Solanum* plants are known to produce toxic glycoalkaloids
447 as chemical defense against herbivory (Altesor *et al.*, 2014), and these compounds could also shape
448 the microbiota associated with the plant. The strength of this effect might be context-dependent due
449 to the interaction with the soil microbial composition (Bulgarelli *et al.*, 2013; Fierer, 2017). This might
450 explain why we observed a greater impact of soil treatment than plant species in the belowground
451 microbiotas in our study. Also, it might explain why we found differences in the bacterial community
452 between plant species just in one soil treatment. It has been previously shown that soil represents a
453 reservoir for leaf microbial communities and that phyllosphere habitat selects for specific members
454 (Grady *et al.*, 2019), which partially explains our observation that the impact of soil microbial diversity
455 was greater than plant species on the phyllosphere bacterial and fungal communities. The differences
456 in the microbiome composition of aphids feeding and reproducing on the two different plant species
457 is not surprising, as it is well known that the identity of the host plant is a major factor in shaping
458 insect-associated microbial community composition (Colman *et al.*, 2012; Malacrinò, 2018). The fact

459 that seeds were not surface-sterilized might be a caveat of our study, potentially influencing the
460 structure of plant microbiome differently for each species. However, previous studies show that even
461 surface-sterilized seeds show differences in their endophytic microbiome according to the plant
462 genotype (Walitang *et al.*, 2018; Raj *et al.*, 2019; Liu *et al.*, 2020), and our results show a major effect
463 driven by soil microbial community composition, thus we are confident that our results were not
464 biased by not surface-sterilizing seeds.

465 By quantifying and comparing the relative strength of multiple biotic drivers of microbial
466 community composition, our work contributes to a more comprehensive understanding of the factors
467 determining the outcome of plant–microbe–insect interactions, and how plant-associated
468 microbiomes assemble and respond to resource- and consumer-driven effects. Thus, if understood
469 and managed correctly, these interactions have potential to be applied in natural and managed
470 systems to improve food security and safety, or the success of ecological restoration efforts.

Declarations

Availability of data and material

Raw data is available at the NCBI SRA database under accession number PRJNA557499.

Competing interests

We declare that none of the authors have competing financial or non-financial interests.

Authors' contributions

All authors designed the experiment. AM and AEB performed the greenhouse experiment. AM prepared library and performed data analysis. AM wrote the first draft of the manuscript and all authors contributed substantially to revisions.

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Tables

Table 1. Models testing the effect of compartment (aphids, leaves, roots, rhizosphere soil), soil treatment (high diversity, low diversity), plant species (*S. tuberosum*, *S. vernei*), herbivory (infested, control) and their interaction on the phylogenetic diversity (linear mixed-effect model) and taxonomical structure (PERMANOVA) of bacterial and fungal communities.

	Bacterial community					**Fungal community**				
		Phylogenetic diversity		**PERMANOVA**			**Phylogenetic diversity**		**PERMANOVA**	
Factors	**df**	χ^2	**P**	**F**	**P**	**df**	χ^2	**P**	**F**	**P**
Compartment (Cp)	3	843.62	**<0.001**	23.39	**0.001**	2	895.91	**<0.001**	12.9894	**0.001**
Soil treatment (S)	1	2.19	0.13	13.26	**0.001**	1	225.62	**<0.001**	14.906	**0.001**
Plant species (P)	1	18.11	**<0.001**	2.37	**0.001**	1	10.67	**<0.01**	1.9625	**0.003**
Herbivory (H)	1	34.66	**<0.001**	2.52	**0.001**	1	47.21	**<0.001**	3.7912	**0.001**
Cp × S	3	37.23	**<0.001**	5.67	**0.001**	2	252.44	**<0.001**	5.7238	**0.001**
Cp × P	3	8.18	**0.04**	1.51	**0.005**	2	1.66	0.43	1.3138	**0.03**
S × P	1	23.6	**<0.001**	1.69	**0.025**	1	3.74	0.05	1.7088	**0.014**
Cp × H	2	16.51	**<0.001**	1.61	**0.007**	2	20.7	**<0.001**	2.0339	**0.001**
S × H	1	0.01	0.9	1.25	0.11	1	5.61	**0.01**	1.2592	0.107
P × H	1	2.26	0.13	1.17	0.18	1	0.16	0.68	1.1442	0.217
Cp × S × P	3	12.57	**<0.01**	1.42	**0.005**	2	0.28	0.86	1.34	**0.029**
Cp × S × H	2	6.86	**0.03**	1.28	0.07	2	12.38	**<0.01**	1.107	0.211
Cp × P × H	2	1.9	0.38	1.04	0.31	2	1.18	0.55	1.0136	0.402
S × P × H	1	2.59	0.1	1.13	0.20	1	1.33	0.24	1.1739	0.152
Cp × S × P × H	2	0.25	0.87	1.05	0.29	2	0.84	0.65	1.1249	0.186

683 **Table 2**. Analysis of the effects of soil treatment (high diversity, low diversity), plant species (*S.*
684 *tuberosum*, *S. vernei*), herbivory (infested, control) on the bacterial and fungal community
685 taxonomical structure for each compartment (aphids, leaves, roots and rhizosphere) performed
686 through PERMANOVA (Unifrac distance matrix). Analyses were run separately for each compartment
687 within each community. Values in bold represent $P<0.05$.

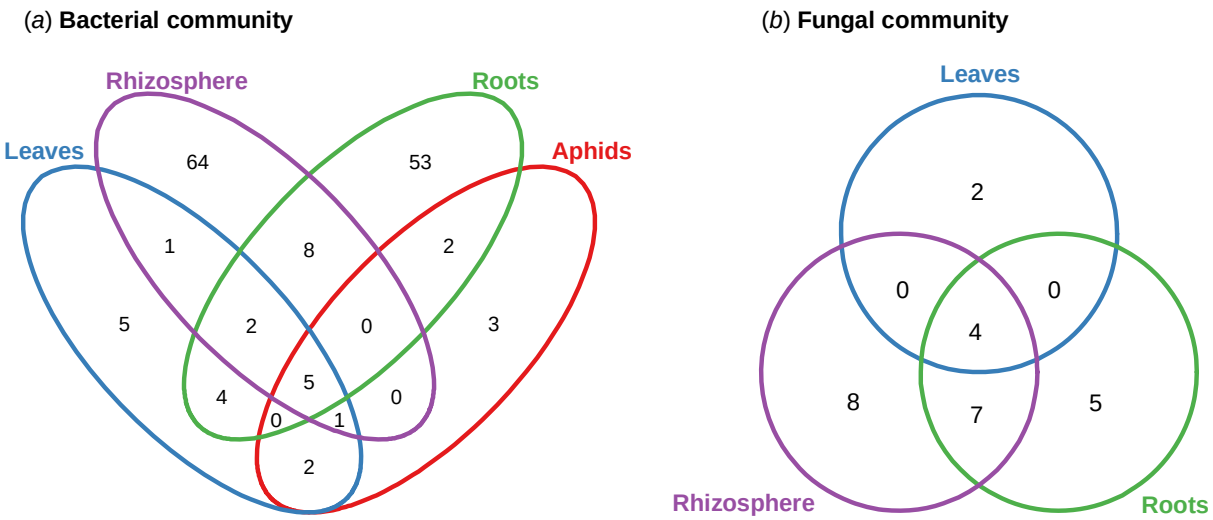
688

	Aphids		**Leaves**		**Roots**		**Rhizosphere**	
Bacterial community	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*
Soil treatment	0.08	**<0.01**	0.07	**<0.01**	0.16	**<0.01**	0.28	**<0.01**
Plant species	0.03	**0.01**	0.03	**<0.01**	0.03	**<0.01**	0.02	**0.04**
Herbivory	-	-	0.03	**<0.01**	0.04	**<0.01**	0.02	**0.04**
	Aphids		**Leaves**		**Roots**		**Rhizosphere**	
Fungal community	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*
Soil treatment	-	-	0.06	**<0.01**	0.21	**<0.01**	0.31	**<0.01**
Plant species	-	-	0.02	0.26	0.03	**<0.01**	0.02	0.17
Herbivory	-	-	0.05	**<0.01**	0.03	**0.01**	0.02	0.07

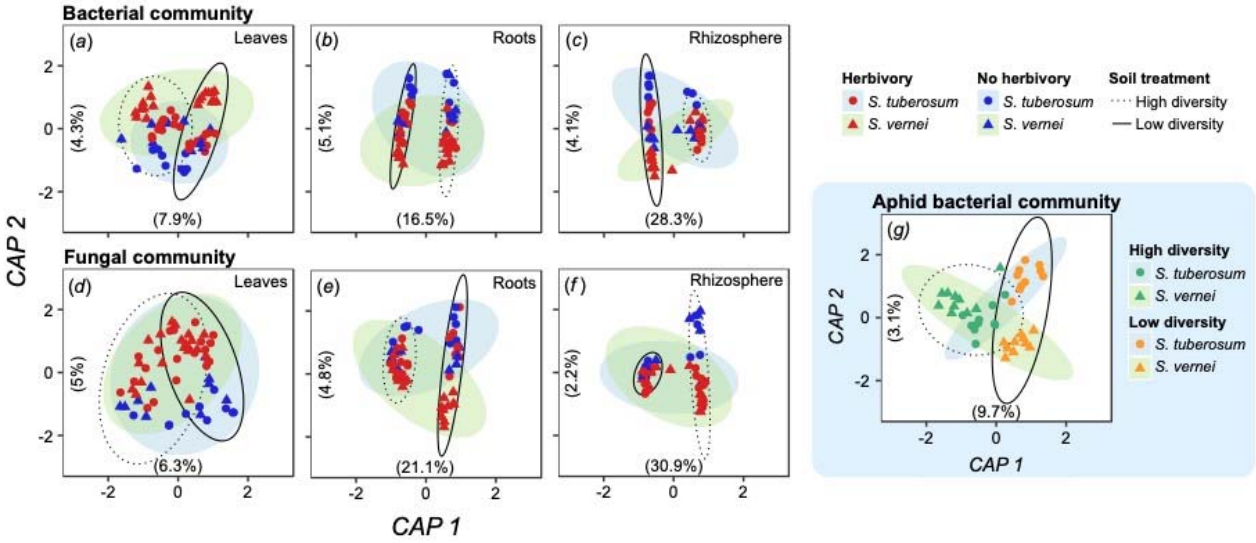
689

Figure captions

Figure 1. Core bacterial (A) and fungal (B) OTUs shared between compartments. Core OTUs were identified among those with relative abundance of >0.1% in more than 50% of samples.



695 **Figure 2.** Canonical Analysis of Principal coordinates (CAP) analysis of bacterial and fungal
696 communities Unifrac distance matrix for each compartment. We report the response of bacterial (*a*-*c*)
697 and fungal (*d*-*f*) communities to soil microbial diversity, plant species and herbivory in leaves (*a*, *d*),
698 roots (*b*, *e*) and rhizosphere soil (*c*, *f*). Aphid bacterial community (*g*) responded to both plant species
699 and soil microbial diversity. For each graph, percentages in parentheses inside each graph along the
700 axes report the variance explained by the respective axis.



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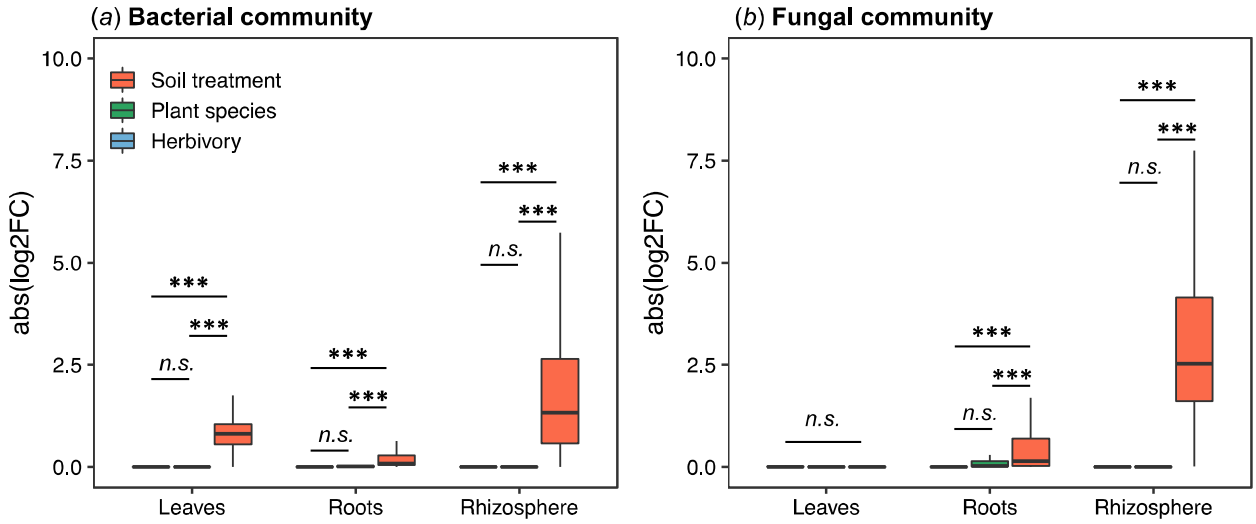
702

703 **Figure 3.** Magnitude of changes in abundance for each OTU (absolute $\log_2$ Fold Changes). For each
704 compartment (leaves, roots and rhizosphere) we investigated the response of single OTUs to soil
705 microbial diversity (red), plant species (green) and herbivory (blue), for (*a*) bacterial and (*b*) fungal
706 communities. Comparisons were tested using a linear mixed-effect model, and contrasts were
707 extracted using the function *emmeans*. *** P<0.001.



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Supplementary material

Supplementary material. Supplementary results, tables and figures.