Soil microbiota and herbivory influence the structure of plant-associated microbial communities in tomato plants

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Abstract

Plant-associated microbiomes are key to shaping many aspects of plant biology, including their fitness, ecology, and evolution. While several studies focused on testing the effect of different factors (e.g., plant genotype, soil, stressors) on the assembly of plant microbiomes, we still know a little about the possible mechanisms that drive these effects. In this study we tested whether soil microbial community and herbivory influence the microbial community of tomato plants, and whether their influence in different plant compartments is driven by active (via plant) or passive (via microbial spillover) mechanisms. We grew our plants in soils hosting three different microbial communities, we covered (or not) the soil surface to prevent (or allow) passive microbial spillover between compartments, and we exposed them (or not) to herbivory by *Manduca sexta*. Results suggest that the soil-driven effect was detectable regardless of soil coverage, suggesting that this effect might be mediated by the plant. On the other hand, the herbivore-driven effect on the rhizosphere microbiota was detectable only when the soil cover was absent, suggesting that this effect might be driven by microbial spillover. These results promote understanding of the drivers of plant microbiome assembly, their relative strength, and the mechanisms behind them.

Keywords: microbiome; metabarcoding; 16S; microcosm

Introduction

It is well established that each plant compartment (e.g., roots, leaves, flowers, fruits) associates with a distinct microbiome, and its structure depends on plant genotype (Wassermann *et al.*, 2019; Malacrinò *et al.*, 2022), developmental stage (Chaparro *et al.*, 2014; Hu *et al.*, 2020), geography (Coleman-Derr *et al.*, 2016; Ware *et al.*, 2021), soil microbiome (Zarraonaindia *et al.*, 2015; Malacrinò, Karley, *et al.*, 2021), biotic interactions (Solís-García *et al.*, 2020; Cui *et al.*, 2021; French *et al.*, 2021; Malacrinò, Wang, *et al.*, 2021), abiotic stressors (Xu *et al.*, 2018; Vescio *et al.*, 2021; Yu *et al.*, 2022), and several other factors. While this checklist of influences is important to understand the dynamics of plant-microbe interactions, only a few studies have focused on testing the relative strength and direction of different factors on the structure and diversity of plant microbiomes. For example, Tkacz et al. (2020) show that soil has a stronger effect than plant species in shaping the plant microbiome belowground. Similar results were obtained by our previous study (Malacrinò, Karley, *et al.*, 2021), where we found that the plant microbiota in different compartments (rhizosphere soil, roots, leaves) was influenced more strongly by soil microbial diversity than plant genotype or herbivory. Yet, we still know little about the possible mechanisms that can generate this soil-driven effect on the microbial communities at different plant compartments.

In our previous study (Malacrinò, Karley, *et al.*, 2021) we speculated that the soil- and herbivory-driven effects might be mediated by changes in plant metabolism/physiology. This was based on the observation that, although soil and herbivory altered the microbial communities in all compartments (rhizosphere, root, leaves, herbivores), the overlap of microbial taxa between compartments was low and not different from an overlap generated by random chance. Conversely, Hannula et al. (2019) found that the soil microbial community did not influence the root and leaf microbiome composition, but altered the herbivore-associated microbiota. Interestingly, this effect was observed when insects were feeding on potted plants but not when they were feeding on detached leaves. Thus, the authors suggested that caterpillars might actually acquire their microbiome directly from soil. While the two studies are different in terms of both plant (potato vs. dandelion) and herbivore models (*Macrosiphum euphorbiae* - sap feeding vs. *Mamestra brassicae* - chewing), they suggest that the soil-driven effect on plant- and herbivore-associated microbiota might be mediated by different mechanisms: via changes in plant metabolism/physiology (active mechanism) or by microbial spillover between compartments (passive mechanism). Similarly, the effect driven by herbivores can be generated by active mechanisms (changes in plant metabolism) or by passive mechanisms (e.g., transfer of microorganisms between compartments via excrements or honeydew). Although both soil- and herbivory-driven effects on the plant and herbivore microbiota have been previously reported (Hannula *et al.*, 2019; Humphrey and Whiteman, 2020; Tkacz *et al.*, 2020; French *et al.*, 2021; Malacrinò, Karley, *et al.*, 2021), to the best of our knowledge, no previous study tested whether this effect is driven by active (via plant) or passive (via microbial spillover) mechanisms.

With the aim to fill this knowledge gap, we here test the effects of soil microbiome composition and herbivory on the rhizosphere, root, leaf, and herbivore microbiota in tomato plants. Considering the results from previous studies, we hypothesize that soil microbial community will drive a stronger effect than herbivory on plant microbiota in all compartments. We hypothesize that the effect of soil and herbivory on plant microbiomes is driven by plant-

mediated mechanisms. To test this idea, we grew plants isolating the above-ground portion of the plant from potential spillover from soil. Thus, if our hypothesis is true, we should observe a soil- or herbivory-driven effect on the plant microbiome regardless of the presence of soil surface cover.

Methods

Experimental design

Here we tested our hypotheses using a full factorial design, growing tomato plants (*Solanum lycopersicum* L. variety Moneymaker, Urban Farmer, Indianapolis, IN, USA) in microcosms inoculated with three different microbial communities. To test whether the soil-driven effect on leaves and herbivore microbiota is driven by the plant or by the passive spillover of microorganisms from soil, the soil surface of half the plants was covered with a neoprene disk (Fig. S1), while the other half were left uncovered. Also, within each group, we exposed half the plants to herbivory by *Manduca sexta* (Great Lakes Hornworm, Romeo, MI, USA) while the other half served as control. Each combination of soil inoculum (n = 3), coverage (n = 2), and herbivory (n = 2) was replicated 8 times, for a total of 96 plants.

Microcosm setup

Soil to be used as inoculum was collected from three different fields at the Marion Campus of The Ohio State University (40.574 N, 83.088W, Marion, OH, USA). To ensure our microcosms hosted three different microbial communities, we sampled soils from three fields with different levels of disturbance: prairie (restored prairie left undisturbed for the past 45 years), field margins (uncultivated area at the border between the prairie and an agricultural field), and agricultural soil (collected in a field sown with soybean and subjected to a corn-soybean rotation). From each field, soil was collected at 5-10 cm of depth, sieved to 3 cm to remove large debris, homogenized, and stored at 4°C. Sterile background soil (see below) was prepared by mixing sand and soil collected from the field (ratio 2:1, the soil portion was generated by mixing the three inocula in equal proportion), 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.

Microcosms were set up in 600 mL experimental deepots (Stewe & Sons Inc., Tangent, OR, USA). At the bottom and top of each pot we added 100 mL of sterile background soil to reduce the risk of microbial contamination between pots. Between the layers of sterile background soil, we added 400 mL of soil inoculum. Each soil inoculum was made by mixing 220 mL of sterile background soil and 60 mL of each of the three soils collected in the field (180 mL in total) of which one was added without any manipulation, while the other two were autoclaved as reported above. In this way, we controlled for physical and chemical differences between pots, which only differed in terms of their alive microbial community. Seeds were germinated on sterilized coir 2 weeks before the experimental setup. After pots were filled with the respective soil inoculum, a single tomato seedling was transplanted into each pot. If the pot was assigned to the "covered" group, the soil surface was covered with a black neoprene disk (Fig. S1), being careful to not damage the seedling. Plants were then randomized into two blocks and left to grow in an insect-screened greenhouse with an average temperature of 25°C and a photoperiod of 16 h of light and 8 h of darkness.

Five weeks after the experimental setup, plants assigned to the herbivory treatment (*n* = 48) were exposed to herbivory by a single 2nd instar larva of *M. sexta*. All plants were screened with a microperforated plastic bag that allowed transpiration while preventing larvae to escape. After 1 week, larvae were collected, flash-frozen in liquid nitrogen, and stored at -80°C. Microperforated plastic bags were removed, and plants were left to grow for another week. From each pot, we collected 3 punch-holes from different randomly-selected leaves, before transferring the shoot to a paper bag. Roots were cleared from the bulk soil, and from each plant we collected ~25mg of rhizosphere soil. Roots were then carefully washed, and after collecting ~25 mg of roots, they were transferred to a paper bag. All samples for DNA extraction were immediately flash-frozen in liquid nitrogen, while shoots and roots were transferred to an oven (60°C) for 1 week before being used to measure biomass. Larvae were individually dissected to remove the intestine, which was transferred to a 2 mL tube and stored at -80°C before DNA extraction, while the carcass was transferred to pre-weighed 2 mL tubes and dried in an oven for 1 week before being weighed.

DNA extraction, library preparation, and sequencing

DNA extraction and library preparation was performed according to our previous study (Malacrinò, Karley, *et al.*, 2021). Briefly, each sample was lysed in an extraction buffer, and total DNA was extracted using a phenol:chloroform protocol. After quality check, from each sample we prepared libraries targeting the bacterial 16S ribosomal RNA (rRNA) gene with primer pair 515f/806rB (Caporaso *et al.*, 2012). Amplifications were also carried out on DNA extracted from soil inoculum, and nontemplate controls where the sample was replaced with nuclease-free water in order to account for possible contamination of instruments, reagents, and consumables used for DNA extraction. After this first PCR, samples were purified (Agencourt AMPure XP kit, Beckman and Coulter) and used for a second short-run PCR to ligate Illumina adaptors. Libraries were then purified again, quantified using a Qubit spectrophotometer (Thermo Fisher Scientific Inc.), normalized using nuclease-free water, pooled together, and sequenced on an Illumina NovaSeq 6000 SP 250PE flow cell at the Genomic Sciences Laboratory of the North Carolina State University (Raleigh, NC, USA).

Raw reads processing and data analysis

Paired-end reads were processed using the DADA2 v1.22 (Callahan *et al.*, 2016) pipeline implemented in R v4.1.2 (R Core Team, 2020) to remove low-quality data, identify ASVs and remove chimera. Taxonomy was assigned using the SILVA v138 database (Quast *et al.*, 2013). Data was analyzed using R v4.1.2 with the packages *phyloseq* (McMurdie and Holmes, 2013), *vegan* (Dixon, 2003), *DESeq2* (Love *et al.*, 2014), and *lme4* (Bates *et al.*, 2014).

The diversity of microbial communities was estimated for each sample using Faith's phylogenetic diversity index (Faith, 1992), and tests were performed by fitting a linear-mixed effect model specifying compartment (rhizosphere soil, roots, leaves, herbivore), soil inoculum (agricultural, margin, prairie), coverage (covered and control), herbivory (present and absent), and their interactions as fixed factors, and block as a random effect. The package *emmeans* was used to infer pairwise contrasts (corrected using false discovery rate, FDR). Similarly, we tested the influence of the same factors on the structure of bacterial microbiomes in our system using a multivariate approach. Distances between pairs of

samples, in terms of community composition, were calculated using an unweighted Unifrac matrix, then visualized using a NMDS procedure. Differences between sample groups were inferred through permutational multivariate analysis of variance (PERMANOVA, 999 permutations), specifying compartment, soil inoculum, coverage, herbivory, and their interactions as fixed factors, and using the factor "block" to stratify permutations. Pairwise contrasts were inferred using the package *RVAideMemoire*, correcting p-values for multiple comparisons (FDR).

Biomass data analysis

Biomass data were analyzed in R v4.1.2 (R Core Team, 2020). Shoot, root, and insect carcass dry biomass data were individually fit to linear-mixed effect models specifying soil inoculum (agricultural, margin, prairie), coverage (covered and control), herbivory (present and absent), and their interactions as fixed factors, and block as a random effect. The package *emmeans* was used to infer pairwise contrasts (corrected using false discovery rate, FDR).

Results

Microbial community composition

Across all the samples, we identified 1793 bacterial taxa (Fig. S2). The rhizosphere soil was characterized by a higher abundance of the bacterial genera *Pseudomonas* (20.01%), *Sphingobium* (17.82%), unidentified rhizobia (10.21%), and unidentified bacterial taxa (10.56%). Roots hosted a wider proportion of unidentified rhizobia (21.25%), unidentified bacterial taxa (17.92%), *Stenotrophomonas* (11.45%), and *Actinoplanes* (10.02%). Leaves were mostly associated with unidentified rhizobia (17.01%), *Sphingobium* (16.22%), and *Actinoplanes* (13.71%). The microbiota of herbivores was mostly represented by unidentified bacterial taxa (19.67%) and *Pseudomonas* (17.65%).

Soil inoculum drives the structure and diversity of plant microbiota

Microbiota structure

We first tested the influence of compartment (rhizosphere soil, roots, leaves, herbivore), soil inocula (agricultural, margins, prairie), herbivory (present, absent), and coverage (present, absent) on the structure of plant-associated microbiomes. Results (Tab. S1) suggest that compartment explains most of the variation. To further test whether soil inocula, herbivory, and coverage have a differential effect within each compartment, we ran separate PERMANOVA models for rhizosphere soil, roots, leaves, and herbivores (Tab. 1). Soil inocula influenced the structure of microbiota in all compartments ($p < 0.005$; Fig. 1), and within these models was always the factor explaining most of the variation (3.3 - 14.3%; Tab. 1). Herbivory explained only a minor portion of the variation (1.2 - 1.3%; Tab. 1), and had an effect only on the structure of root bacterial microbiota (Fig. 2). Soil cover also explained very little of the variation (1.7 - 3.2%; Tab. 1; Fig. 3).

Thus, after verifying the occurrence of a soil-driven effect on plant- and herbivore-associated microbiota, we then tested whether these effects are driven by microbial spillover between compartments or occur via the host plant. Results show that both soil inoculum and cover influenced the leaf microbiota, but the interaction between these factors did not show a significant effect ($p = 0.3$; Tab. 1), suggesting that the soil-driven effect occurred regardless of the presence of soil cover. Similarly, the herbivore-associated microbiome was solely influenced by soil inoculum ($p = 0.001$; Tab. 1) and no effect was driven by soil cover ($p = 0.2$; Tab. 1).

In addition, we tested whether the herbivory-driven effect on the rhizosphere and roots microbiota is driven by active or passive mechanisms. In roots, all three factors (soil inoculum, herbivory, coverage) influences the structure of the bacterial microbiota (Tab. 1). Post-hoc comparisons clarified that there was a main effect of coverage on the bacterial microbiota of roots only when herbivores were absent (Tab. S3), while herbivory did not influence the root microbiota (Tab. S4). When comparing the rhizosphere microbiota of plants exposed to herbivory and control plants, we did not find differences when soil was covered ($p > 0.05$), while we found differences when soil was not covered ($p < 0.05$; Tab. S4).

Table 1. PERMANOVA models testing the effect of soil inoculum (S; agricultural, margin, prairie), herbivory (H; present, absent), coverage (C; present, absent), and all their interactions on the structure of plant bacterial microbiota for each compartment. Values in bold represent $p < 0.05$.

		Rhizosphere			**Roots**			**Leaves**			**Herbivore**		
Factors	**df**	R^2	F	p	R^2	F	p	R^2	F	p	R^2	F	p
S	2	0.036	1.90	**0.004**	0.143	8.78	**0.001**	0.033	1.62	**0.005**	0.091	2.15	**0.001**
H	1	0.013	1.37	0.135	0.013	1.61	**0.029**	0.012	1.26	0.161	-	-	-
C	1	0.018	1.97	**0.019**	0.032	3.95	**0.001**	0.017	1.67	**0.019**	0.025	1.21	0.204
S x H	2	0.052	2.74	**0.001**	0.031	1.91	**0.001**	0.022	1.09	0.299	-	-	-
S x C	2	0.024	1.27	0.126	0.044	2.69	**0.001**	0.021	1.07	0.300	0.051	1.18	0.151
H x C	1	0.017	1.87	**0.025**	0.021	2.57	**0.001**	0.014	1.41	0.095	-	-	-
S x H x C	2	0.039	2.08	**0.001**	0.028	1.74	**0.003**	0.021	1.01	0.460	-	-	-

Microbiota diversity

We also tested the effects of compartment, soil inoculum, herbivory, and coverage on the diversity of bacterial communities (Tab. S2), using Faith's phylogenetic diversity index as a

metric. We found a significant compartment x soil inoculum x herbivory x coverage effect ($p < 0.001$; Tab. S2), and conducted post-hoc contrasts comparing covered and uncovered plants (Tab. S5) and comparing plants exposed to herbivory and controls (Tab. S6). However, there were no consistent patterns, but rather effects that were likely driven by specific combination of compartment, soil inoculum, and herbivory or coverage.

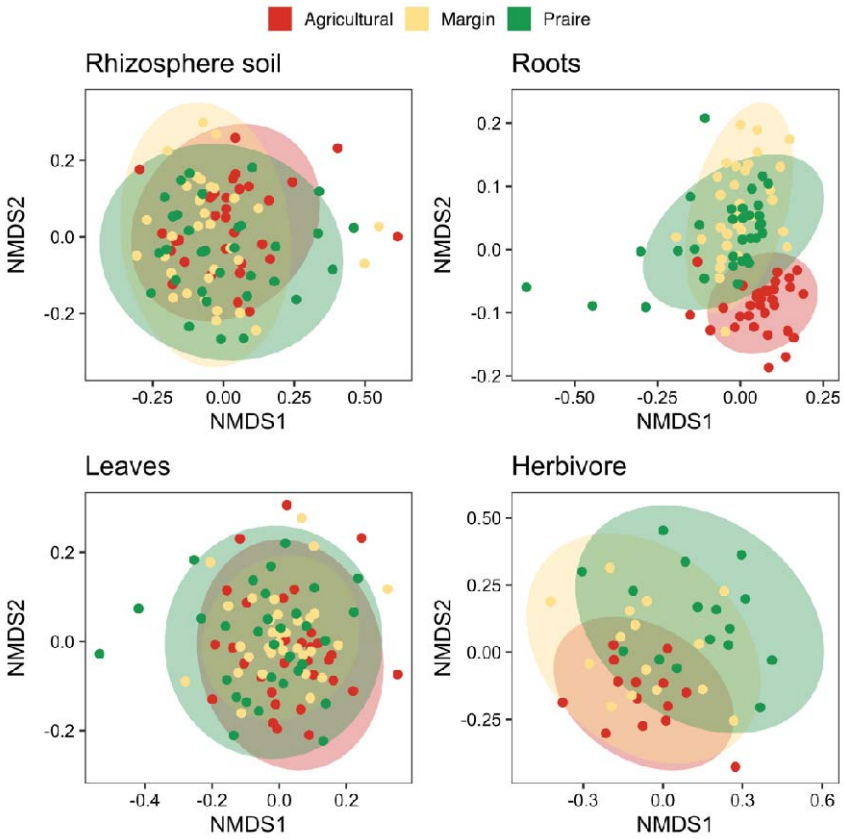

Figure 1. NMDS plots of bacterial community Unifrac distance matrix for each compartment. Points and 95% CI ellipses are coloured by soil inoculum.

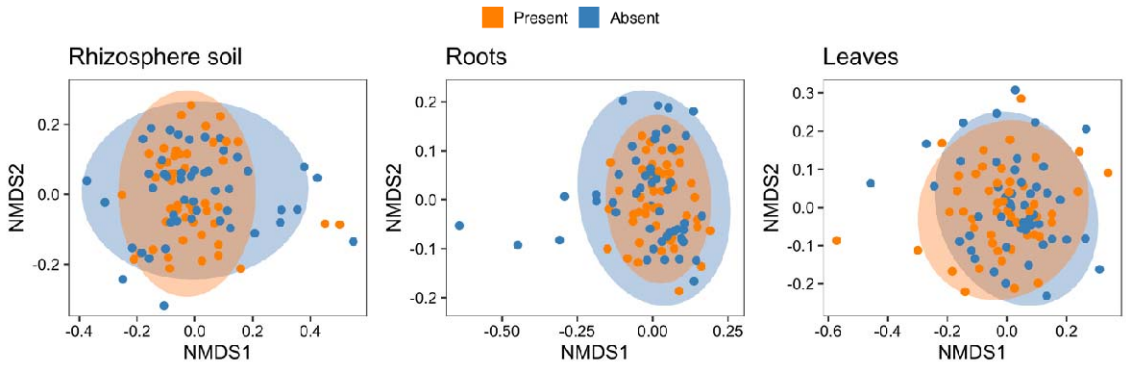

Figure 2. NMDS plots of bacterial community Unifrac distance matrix for each compartment. Points and 95% CI ellipses are coloured by presence or absence of herbivore.

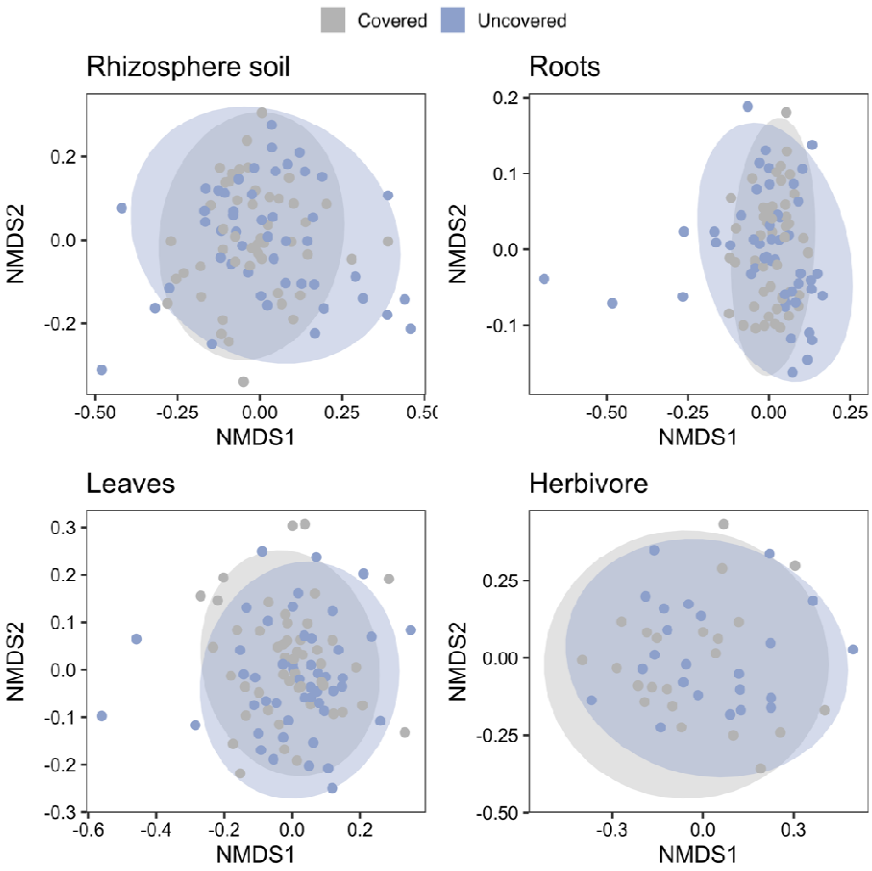

Figure 3. NMDS plots of bacterial community Unifrac distance matrix for each compartment. Points and 95% CI ellipses are coloured by presence or absence of soil cover.

Biomass

We found that both herbivory (χ^2 = 11.62, p < 0.001) and coverage (χ^2 = 48.17, p < 0.001) had an effect on shoot biomass (Fig. S3). Specifically, we found a higher biomass of control plants compared to those exposed to herbivory (p < 0.001), and a higher biomass in plants which soil surface was covered compared to those not covered (p = 0.001). Similarly, root biomass was influenced by soil inoculum (χ^2 = 8.86, p = 0.011), herbivory (χ^2 = 20.32, p < 0.001), and coverage (χ^2 = 6.26, p = 0.012; Fig. S3). Specifically, we found a higher root biomass in plants grown on agricultural soil compared to those grown on prairie soil (p = 0.01), and marginally higher also compared to field margin soil (p = 0.06). We also found a greater root biomass in plants grown in pots with a soil cover compared to uncovered pots (p < 0.001), and a higher biomass in plants not exposed to herbivores compared to those exposed to *M. sexta* (p = 0.01). No effect of soil inoculum (χ^2 = 0.74, p = 0.68) or cover (χ^2 = 0.71, p = 0.39) was detected on insect biomass.

Discussion

In this study we tested the influence of soil microbial community and herbivory in structuring the plant microbiome, and whether the soil- or the herbivory-driven effect is mediated by

passive microbial spillover or actively via the host plant. Our results suggest that, in our system, soil microbial community influences the plant microbiome composition both below- and above-ground, and that the effect aboveground is not due to passive microbial transfer between the two compartments. This bottom-up effect can be detected up to the herbivore-associated microbial communities. We also found that herbivory drives an effect on rhizosphere microbiota, but this can be detected only as long as soil surface is not covered, suggesting that this effect is driven by passive microbial spillover between compartments.

Soil-driven effect

Within each compartment, we found that soil inoculum was the most common factor driving the structure of bacterial communities. We found a similar result in our previous study (Malacrinò, Karley, *et al.*, 2021), where we observed differences between a high- and a low-diversity microbial inoculum on the microbiota of plants and herbivores. In this study we used soil from three different origins as inoculum to generalize our results, and we observed differences between each soil inoculum at each compartment. As in our previous study (Malacrinò, Karley, *et al.*, 2021), we also found a soil-driven effect on the herbivore microbiota, despite using a different host plant (tomato vs potato) and herbivores with different feeding strategies (chewing vs sap-feeding). A strong soil driven has also been shown in *Arabidopsis thaliana*, *Medicago truncatula*, *Pisum sativum*, and *Triticum aestivum* (Tkacz *et al.*, 2020), grapevine (Zarraonaindia *et al.*, 2015), and dandelion (Hannula *et al.*, 2019). In contrast to these studies, we asked whether this influence is driven by a passive microbial spillover between soil and the other compartments, or whether the soil microbiome exerts an effect on plants that results in changes to the herbivore microbiome.

Our study shows that soil microbiome composition also shapes the microbial community associated with herbivores via an active mechanism. Our results show that the soil microbial community influences the herbivore microbiome community. If the passive hypothesis is true, then we would expect to see an effect of soil coverage driven by the transfer of microbes between compartments. We saw no influence of soil coverage in shaping the microbial communities at these compartments. If the active hypothesis is true, then we would expect no difference in herbivore microbiome composition between soil coverage treatments. Our results, thus, support the active (via plant) hypothesis. The active mechanism behind this pattern could be changes in plant metabolism or physiology that influenced the leaf metabolite or physical composition, and therefore might have altered the diet of *M. sexta* leading to changes in microbiome composition (Colman *et al.*, 2012; Malacrinò, 2022).

Herbivory-driven effect

Herbivory driven changes in microbiome composition could also be plant mediated or passive, and our data contributes to parsing out support for these hypotheses as well. Other studies have also shown that herbivory drives changes in shoot (Humphrey and Whiteman, 2020), root (Ourry *et al.*, 2018), and rhizosphere (Kong *et al.*, 2016; French *et al.*, 2021; Malacrinò, Wang, *et al.*, 2021) microbiome composition. These studies posit that changes in composition are driven by changes in metabolites, physiology, and root exudates. Humphrey & Whiteman (2020) postulate that the effects of herbivory on plant-associated microbial communities might be mechanistically explained by changes in plant metabolism/physiology. By contrast, we found the influence of herbivory on the rhizosphere microbiome disappeared

when soil was covered. Differences between our study and previous studies could also be due to the different herbivore guilds (chewing versus sucking) or host plant species.

Finally, we also found that the plant biomass was influenced by our treatments. As expected herbivory reduced biomass. Unexpectedly soil coverage influenced biomass. We speculate that this effect may be due to increases in soil temperatures due to the black cover. Our soil covers may have behaved similarly to black plastic mulch which increases soil temperatures and plant biomass (Ibarra *et al.*, 2001).

Our results suggest that soil microbial community composition drives herbivore microbial community composition via active mechanisms whereas in this system herbivory drives changes in the rhizosphere microbial community passively. This study contributes to understanding the assembly of plant microbiome compartments and their response to external factors. This is of high priority if we want to enable the manipulation of plant microbiomes. The fact that soil microbial community drives such a strong effect on plant microbiomes might enable the use of soil microbiomes to enhance specific microbial functions or plant traits. Similarly, the strong effect driven by soil microbiome on herbivores in this and other systems suggest possibilities for steering soil microbial communities to negatively influence insect pests. Thus, management of the soil microbiome could promote food security and safety, restoration of damaged environments, and reservation of endangered ecosystems.

Data availability

Raw data is available at NCBI SRA under Bioproject XXX (will be added after acceptance). The code used to process and analyze the data is available on GitHub XXX (will be added after acceptance).

Conflicts of interests

Authors do not declare any conflict of interest.

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