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From the root of variation: A metabolomics perspective to plant soil-feedback

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Chapter 4

Temporal dynamics of plant-soil feedbacks on soil microbiomes, leaf metabolomics and plant-insect interactions

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Manuscript

Abstract:

1. Most plant-soil feedback (PSF) studies condition the soil for a short period of time only before initiating the feedback phase. However, the strength and even the direction of the PSFs may change depending on the time of conditioning.
2. We collected soil from monocultures of six plant species, growing in 200 L containers outdoors and examined temporal changes in the PSF for one of the species, *Jacobaea vulgaris.* Soil was collected every two months and genetically identical *J. vulgaris* clones were grown in sterilized bulk soil inoculated with 10% live soil in a climate-controlled growth chamber. Each time we measured nutrient availability in the soil, plant biomass and the performance of a generalist insect herbivore (*Mamestra brassicae*). At two, eight and twelve months we analysed the foliar metabolic composition of the test plants with ^{1}H-Nuclear magnetic resonance spectroscopy (NMR) and the bacterial and fungal community composition and abundance in the monoculture soils.
3. Plant growth and the strength of PSF varied over the course of the experiment but at each time point, *J. vulgaris* produced less biomass in its own soil than in the soils conditioned by other species, and most biomass when grown in 100 % sterilized soil. Metabolomic profiles in the test plants varied mainly with collection time. Performance of *M. brassicae* differed among soils but only after soils had been conditioned for twelve months. PSFs could not be explained by nutrient availability in the monoculture soils. Bacterial and fungal communities changed over time, and *J. vulgaris* biomass was related to composition of bacterial and fungal communities but only at twelve months. Furthermore, after twelve months, metabolomic composition could be explained by the fungal community composition in the soil.
4. *Synthesis.* Our results show that for *J. vulgaris*, the direction of the PSF does not change with time of conditioning but that the magnitude of the PSF varies over time. Leaf metabolomes are sensitive to plant-induced changes in the soil but the temporal dependency on these changes in the soil remain difficult to predict.

Key Words: Ecometabolomics, Insect herbivory, *Mamestra brassicae*, Plant-herbivore interactions, Plant-soil interactions, Soil legacy effects, Temporal variation.

Introduction

Soil is a medium in which plants grow, and greatly impacts the plant that grows in it, as well as other plant-associated organisms. For example, plant biomass, foliar metabolomes and the performance of herbivorous insects all depend on the microbial community that is present in the soil (Heinen, Sluijs, Biere, Harvey, & Bezemer, 2018; Huberty et al., 2020b; Zhu et al., 2018). However, plants also impact biotic and abiotic parameters in the soil, such as the microbial community. Via their effects on the soil, plants can influence the performance of other plants that grow later in this soil (Plant-soil feedback (PSF); Bever, 1994; Van der Putten et al., 2013). While many studies have provided evidence for PSFs, the temporal dynamics of PSF processes are still poorly understood (Bezemer, Jing, Bakx-Schotman, Bijleveld, & Bijleveld, 2018; Hawkes, Kivlin, Du, & Eviner, 2013; Lepinay et al., 2018).

Most knowledge of PSFs is based on greenhouse studies in which soil is conditioned by a plant for several weeks only. The impact of a plant on the soil microbial community can change considerably over time (Hannula et al. 2019a), but how the duration of conditioning by the first plant affects the performance of the succeeding plant and the herbivores on the plant is not well-known. If pathogens accumulate over time in the soil, the negative soil-mediated effect on another plant may increase over time (Luo et al., 2019), but this could also result in temporal variation in the defence induction patterns of plants (Heil & Bostock, 2002). This could lead to more pronounced metabolomic changes in plants in response to soil conditioning over time, and to stronger responses of insect herbivores to soil conditioning. In this study, we collected soil from monocultures every two months over the course of a year. We measured microbial composition and nutrient availability in these soils, and examined the temporal changes in, and relationships between PSFs, leaf metabolomics and herbivory, using identical clones of a common forb, *Jacobaea vulgaris*.

Soil properties that are important for plant growth such as nutrient availability and the composition of soil microbial communities all change over time. This could be due to changes in the climate, but also due to plant-mediated effects. Rhizodeposition patterns, for example, change with the age of the plant (Dechassa & Schenk, 2004) and the chemical composition of roots can vary over time due to changes in plant phenology or environmental temperatures (Huang, Bont, Hervé, Robert, & Erb, 2020). In PSF experiments that start with bare soil, over

time, root biomass will increase, and therefore the root surface that is in contact with the soil increases. This suggests that the influence of the plant on soil nutrients and microbial communities in the soil will increase over time (Latz, Eisenhauer, Scheu, & Jousset, 2015; Micallef, Channer, Shiaris, & Colón-Carmona, 2009). We therefore expect that the strength of PSFs will become stronger with increasing time of conditioning. Such temporal effects on biomass have been reported for the duration of short (two to eight weeks) conditioning periods (Lepinay et al., 2018). However, how these effects change over the course of a year, and how this influences on plant metabolomes and plant-herbivore interactions is unknown.

Previous work has shown that the growth and chemical composition of the plant *J. vulgaris* depends on microbial characteristics of the soil in which the plant grows (Bezemer et al. 2006; Joosten et al. 2009, Kostenko et al. 2012; Bezemer et al. 2013, Huberty et al. 2020a). This species exhibits a strong negative conspecific feedback, and generally grows better in soil conditioned by grasses than in soil conditioned by forbs (Van de Voorde et al. 2012; Wubs and Bezemer 2018). Aphids and caterpillars feeding on the foliage of the plant also respond to soil legacies and this has been shown to be related to changes in plant chemical composition (Kostenko et al. 2012; Kos et al. 2015). As the metabolic changes in *J. vulgaris* are related to changes in the soil (Huberty et al. 2020a) and plant- mediated effects on the soil during the conditioning phase change over time (Hannula et al. 2019a), we expect that plant-herbivore interactions on the succeeding plant will also be time-dependent.

In this study we test in a controlled set-up how inoculation with soil from six monocultures (one conspecific and five heterospecific soils) over time influences growth and chemical composition of genetically identical clones of *J. vulgaris,* and the performance of a generalist herbivore, *M. brassicae*. The soils were collected every two months after establishment of the monocultures, and at each time point abiotic properties of the soil were determined. Furthermore, *J. vulgaris* was grown each time in identical sterilized bulk soil inoculated with 10% monoculture soil and in 100% sterilised soil in a growth cabinet. Metabolic profiles of plants were analysed at two, eight and twelve months and related to existing analyses of the bacterial communities of the same soils (Hannula et al. 2019a) using correlation approaches. Furthermore, the abundance of bacteria and fungi in the soil was determined with qPCR.

We address the following questions (i) Does the influence of PSF on plant biomass, herbivore performance and metabolomic profiles get stronger with increasing age of the conditioning

plant community? ii) Does the sign and magnitude of PSF change over time? iii) Do temporal changes in potential causal agents of PSF (microbes, nutrients) explain the observed soil-plant-insect patterns?

Material & Methods

Jacobaea vulgaris is a biennial herb which is native to Europe and Asia and invasive in North America, Australia and New Zealand (Bain, 1991). *J. vulgaris* can grow in diverse habitats such as sand dunes, woodlands and grasslands and therefore has the capacity to grow in a broad range of soils (Bezemer, Harvey, Kowalchuk, Korpershoek, & van der Putten, 2006). *J. vulgaris* growth is negatively affected by growing in its own soil than in soils conditioned by other plant species, likely caused by an accumulation of soil pathogens in its own soil (Bezemer, Harvey, Kowalchuk, Korpershoek, & van der Putten, 2006; Van de Voorde, Van der Putten, & Bezemer, 2011; Wubs & Bezemer, 2016) . Furthermore, the microbial composition of the soil was shown to influence the concentration and composition of secondary metabolites such as pyrrolizidine alkaloids and primary metabolites such as amino acids (Kos, Tuijl, De Roo, Mulder, & Bezemer, 2015; Kostenko, van de Voorde, Mulder, van der Putten, & Bezemer, 2012; Wang et al., 2019) and the resistance of *J. vulgaris* to herbivores (Kostenko et al., 2012).

Monocultures

Thirty containers (48 cm x 80 cm x 50 cm), were filled with 200 L soil on the 3rd and 4th of April 2017. The soil was sieved through a 32 mm sieve in order to remove large stones and roots. The soil originated from a grassland near Lange Dreef, Driebergen, The Netherlands (52° 02´ N, 5° 16´ E) and is described as holtpodzol, sandy loam (84% sand, 11% silt, 2% clay, ~3% organic matter, 5.9 pH, 1.15 g N kg^{-1}, 0.06 g P_2O_5 kg^{-1}, 0.94 g K kg^{-1}). To inoculate the soil with a diverse microbial community, the soil in each container was topped with 5 cm of soil collected from a natural grassland "De Mossel" (Natuurmonumenten, Ede, The Netherlands, 52° 04´ N, 5° 45´ E) which was sieved through a 10 mm sieve. These soils are characterized as holtpodzol, sandy loam (94% sand, 4% silt, 2% clay, ~5% organic matter, 5.2 pH, 1.06 g N kg^{-1}, 0.08 g P_2O_5 kg^{-1} , 0.74 g K kg^{-1}).

Seeds of the species used in this experiment were sown in steamed potting soil and grown for 3 weeks in a greenhouse (70% relative humidity, light/dark 16/8h, 21/16 °C) and with supplemented light from 400 W metal halide lamps (225 µmol m-2 s^{-1} photosynthetically active radiation, 1 lamp per 1.5 m^2). All seeds originated from Cruydt-Hoeck (Nijberkoop, The Netherlands), except for *Jacobaea vulgaris*, which were collected in the mossel area in 2014. The plant species we used were three grasses, *Holcus lanatus, Festuca ovina, Alopecurus pratensis,* and three forbs, *Hypochaeris radicata, Jacobaea vulgaris, Taraxacum officinale.*

The seedlings were planted at a density of 100 seedlings per container on the 1st of May 2017. Plants that died were replaced within the first two weeks. Seedlings from other species were weeded out regularly. For each plant species 5 containers were established. The containers were placed in a randomized block design in a common garden at the Netherlands Institute Of Ecology (NIOO-KNAW) in Wageningen, the Netherlands (51° 59´ N, 5° 40´ E). The containers were watered regularly during the summer months.

Soil sampling

Every two months, four soil cores (0.7 cm diameter, 10 cm depth) were taken from each container. The soil was mixed and large pieces of roots were removed. Details about the sampling dates are provided in the supporting information (Supporting information Table S4.1). Photos of the monocultures at each sampling point are presented in Supporting information Fig. S4.1 of the supporting information (Supporting information Fig. S4.1). After the final sampling point (May 2018) total aboveground biomass was removed from each container, dried at 40°C and total shoot biomass per container was determined (Supporting information Fig. S4.2). Due to a technical failure of the growth cabinet used for the plant growth experiment (see below) in January 2018 the six-month sampling event was excluded from further analysis.

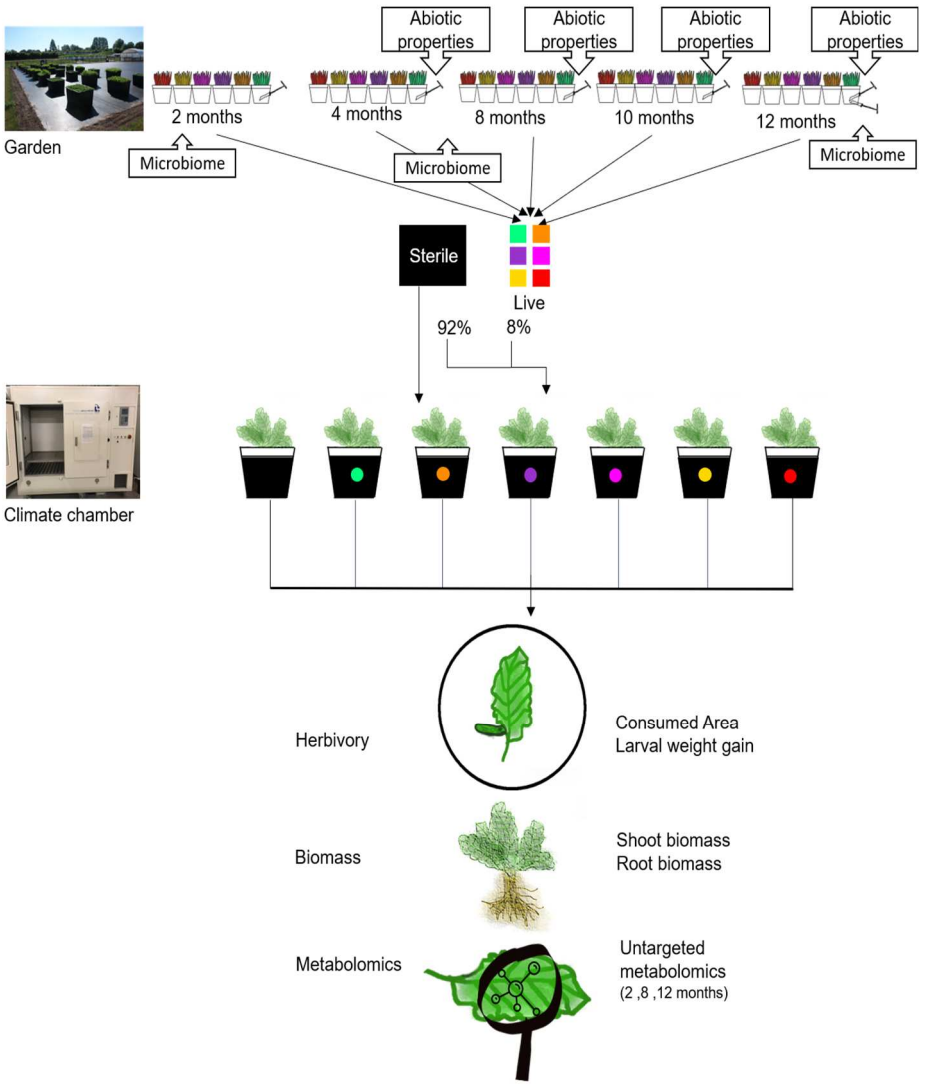

Figure 4.1 Conceptual framework of the experimental design: Soils were conditioned in large containers by monocultures by six plant species in a common garden over the course of one year. Soil samples were collected five times and soil abiotic characteristics were measured. The soil was mixed with sterilized bulk soil and *J. vulgaris* was grown in a climate chamber in different rounds. After 6 weeks of growth one leaf was clipped and used for a detached leaf assay with *Mamestra brassicae*, biomass of shoot and roots was determined and leaf metabolomics analysis was done for the 2, 8 and 12 months rounds. Samples to determine fungi and bacteria communities in the soil were taken at 2, 6, 12 months. Samples to quantify bacteria and fungi were taken at 1, 2, 3, 4, 5, 6, 9, 10, 12.

Soil abiotic characteristics

Every two months, the soil abiotic characteristics were determined for each soil, starting at four months. For each soil, a subsample was dried (40°C) and sieved through a 2 mm mesh. 30 ml of 0.01 M $CaCl_2$ was added to 3 g of soil and the mixture was shaken for 2 hours at 250 rpm and centrifuged at 3000 rpm for 5 minutes. 15 ml of the supernatant was filtered through a Whatman Puradisc Aqua 30 syringe filter with cellulose acetate membrane. To measure soil extractable nutrients (i.e., Fe, K, Mg, P, S, Zn), 12.87 ml of this filtrate were transferred to a 15 ml tube and 130 µL HNO_3 were added, vortexed and analysed by inductively coupled plasma - optical emission spectrometer (ICP-OES, Thermo Scientific iCAP 6500 Duo Instrument with axial and radial view and CID detector microwave digestion system). The remaining of the filtrate was transferred to a 15 ml tube and pH was determined. NO2+NO3 and NH4, were measured on a QuAAtro Autoanalyzer (Seal analytical, Mequon, Wisconsin, USA). For each sampling round, five samples from the sterilized bulk soil from the bag that was used for the plant growth experiment (see below) was also analysed except for the first round of analyses (4 months).

Soil bacterial and fungal communities

During the course of the experiment soil samples were collected every month from each container for molecular identification of the bacterial and fungal communities. These samples have been sequenced and the results have been published elsewhere (Hannula et al. 2019a). Here we use the data from soil samples collected at 2, 6, 12 months this coincided with the two of the three sampling rounds for which leaf metabolomics were analysed (See below). For one of the rounds of the feedback experiment (8 months) the soil microbial sampling of 6 months was used as there was no sampling at 8 months (Supportive information Table S4.1). A detailed description of the collection of the soils and the data processing is found in the Supplementary methods and in Hannula et al 2019a. The quantity of bacteria and fungi was determined by qPCR against a known standard as described in Hannula et al. 2019a. Here we use data from all time points.

Plant soil feedback test

For the PSF tests we used clonal *J. vulgaris* grown in tissue culture for 17 years and formerly collected from Meijendel, the Netherlands. Per time point we asexually propagated a total of 60 *J. vulgaris* plants on MSO medium with 100 mg/L benzylaminopurine (BAP) in a climate room (16h: 8 h light: dark photoperiod, 20°C). After 4 weeks of growth they were individually put on MSO medium without BAP for 10 days to form roots. Time intervals of growth on the two different media were the same for all time points. For each time point 35 equally sized (approximately 4 cm) *J. vulgaris* plantlets were selected for the PSF experiment and one plantlet was planted per pot.

The plants were grown in pots filled with 50 g (dry weight) of soil collected from the containers mixed with 410 g sterilized bulk soil. To calculate how much wet soil from each sample was needed, a subsample was weighed, dried (40 °C) and soil moisture was determined. After collection and prior to filling the pots, the soil samples were kept for 3 days at 4°C. The bulk soil consisted of homogenized soil that was used to fill the containers (Lange Dreef, Driebergen) that was gamma-irradiated (>25 Kgray). For each pot, the soil was mixed separately. At each round, five pots were filled with 100% bulk soil and served as sterile control. To allow the microbial community to establish before proceeding with the planting, the pots were put in a climate chamber without light for 7 days (16°C, humidity 70 %).

The PSF experiment was carried out in the growth cabinet each round to assure uniform conditions (16h: 8h light: dark photoperiod, 21, 16°C, humidity 70 % and PAR 290 µmol/m^2/s). During the six months round, the lights in the cabinet were accidentally switched off for an unknown period of time. Therefore, the resulting data from this sampling round were not included in further analyses. Plants were watered twice a week. To ensure similar soil moisture in all pots each time we weighed each pot and added water until it reached a weight of 505 gram. Water was autoclaved to avoid introduction of microbes through the water. After 6 weeks of growth the plants were harvested. For this, plants were carefully removed from the pots and roots were washed. After that the two largest leaves of each plant were cut and fresh weight was recorded. For the time points at which metabolomic profiles were measured, the largest leaf was immediately wrapped in aluminium foil and then flash-frozen in liquid nitrogen and stored at -80°C. The second largest leaf was always used for a non-choice

bioassay using the caterpillar *M. brassicae* (see below). The roots were cut off the shoots and wrapped in aluminium foil and flash frozen in liquid nitrogen. The remainder of the shoots were put in a bag and frozen at -80°C. All frozen samples were lyophilised for 7 days and then stored in an exicator with silica gel upon use.

Herbivory assay

The stem of the second largest leaf of each plant was wrapped in wet cotton wool and parafilm to keep the leaf hydrated. *Mamestra brassicae* (Lepidoptera: Noctuidae) eggs were obtained from the University of Wageningen, the Netherlands and upon hatching the caterpillars were reared on artificial diet (as described in Hannula et al., 2019b). For each round, we recorded the biomass of each of the 35 L2 caterpillars prior to placing them individually on one of the leaves in a petri dish (100 x 15 mm). The petri dishes were randomly ordered and placed in a climate chamber with no light, 20°C, humidity 70 %. After 4 days the caterpillars were weighed again and leaf consumption of each leaf was assessed by drawing the consumed area of every leaf on an acetate sheet and scanning the sheet with an Epson STD4800. Food consumption (in mm^2) was then accessed with the program WinFOLIA (Version: 2016b Pro, Regent Instruments Canada Inc.). Mean weight gain per day of *M. brassicae* was also calculated. A few leaves desiccated (1 for 4 months, 1 for 8 months, 3 for 12 months). These samples were excluded from analysis.

Metabolomics ^1H NMR Analysis

Metabolomic profiling was performed using leaf samples from the two, eight- and twelve-month conditioning rounds. For the metabolomics analysis the samples were extracted according to an adapted version of the protocol described by Kim et al. (2010). In short, the lyophilised leaf was ground with a metal ball bearing in a TissueLyser (Retsch Mixer Mill MM 400) for 3 min at 30 s^{-1}. Of this powder 20 mg was weighed and extracted with 600 µl of Methanol-d_4, sonicated for 10 min and centrifuged for 10 min at 13.000 ppm. 250 µl of supernatant was transferred to an NMR tube (103.5 × 3 mm, inside-ø 2.24 ± 0.05mm).

We used a Bruker AV-600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany), operating at a frequency of 600.13 MHz to record the ^1H NMR spectra. CH_3OH-d_4 was used as internal lock and ^1H NMR spectra were recorded with pulse width (PW) = 30 ° (11.3 µs), Relaxation

delay (RD) = 1.5 sec and 128 scans with 10 min and 26 sec acquisition time with 0.16 Hz/point. To reduce the signal of H_2O frequency during the recycle delay we used a presaturation sequence. FIDs were Fourier transformed by a line broadening of 0.3 Hz.

We manually baseline corrected the spectra and calibrated them to the solvent at 0.60 ppm before phasing them in TOPSIN (v.3.0. Bruker). The data was bucketed with scaling to total intensity and a bucket width of 0.04 ppm in AMIX software (v. 3.9.12 Bruker BioSpin GmbH, Reinstetten, Germany). This is a pre-processing step which is often used in metabolomics to reduce the effect of small shifts of signals between samples (Kim, Choi, & Verpoorte, 2010). The residual signals from the solvents in regions between 4.70 – 4.90 ppm and 3.32 – 3.28 ppm were excluded. Pre-processing led to a data matrix with 246 buckets per sample. Each bucket contains the intensity of the signal from the NMR within the range of the buckets and corresponds directly to the molar level of a compound leading to a signal in this region of the NMR. Molecules which have more than one H atom will therefore lead to signals in several buckets across the NMR spectra. The chemical environment of the H atom is defined by the neighbouring atoms and determines the chemical shift and the splitting pattern of a signal.

Statistical analysis

All analyses were performed in R Studio (RStudio Team, 2016) using the packages "vegan" (Oksanen et al., 2018) and "mixomics" (Cao et al., 2020). Networks were constructed in R Studio and processed in Cytoscape version 3.7.2 (Shannon et al., 2003). Heatmaps were created with metaboanalyst (Chong et al., 2018).

Dry weight of shoots and roots, weight gain of *M. brassicae* and the total area eaten were analysed each with a two-way ANOVA with the factors "Time" and "Monoculture". Since each round a new experiment was started and the individuals measured were not the same, time was not included as a repeated measure factor in these analyses. Data were then analysed per round. Plants grown only in sterile soil were excluded from the dataset for this analysis. For each round we reran the ANOVA including the 100% sterilized soil, and tested with a Dunnett-t test if the monoculture soils significantly from the sterile soil. All assumptions of ANOVA were fulfilled.

Soil abiotic characteristics were analysed each with an ANOVA with the factors "Time" and "Monoculture". For that the soil the samples were always taken from the same replication unit, the containers, and therefore time was included as a repeated factor in this analysis. Data were then analysed per round. Plants grown only in sterile soil were excluded from the dataset for this analysis. For each round we reran an ANOVA including the 100% sterilized soil, and tested with a Dunnett-t test if the monoculture soils significantly from the sterile soil. All assumptions of ANOVA were fulfilled.

Metabolomic analysis

We visualised metabolomic changes in the leaves with Principal component analyses (PCA), one PCA was constructed for all data, followed by individual PCA for each of the three rounds separately. Statistical significance was inferred from a permutational analysis of variance (PERMANOVA) based on Bray Curtis dissimilarities with the factors "Monoculture", "Time" and "Shoot biomass". Then separate PERMANOVAs for each round, with the factors "Monoculture", and "Shoot biomass" were conducted. For these analyses the metabolome data were normalised by the control by dividing for each sample the intensity of each bucket by the mean intensity of that bucket for plants grown in sterilized soil. Permutations were set to 999. A heatmap showing the 40 signals within the buckets, which differed the most between the plants grown in the different soils (tested with ANOVA) was constructed for each of the three rounds. For the eight months round only two of the five *J. vulgaris* plants grown in conspecific soil survived. Therefore, *J. vulgaris* soil was excluded for this round in the heatmap.

Bacterial and fungal community analysis

Sequences of bacteria and fungi were analysed with the PIPITS and the Hydra pipeline (Gweon et al., 2015; Mattias de Hollander, 2017). The data was filtered before being used for statistical analysis. An abundance threshold of 0.01 and a persistence of 10 was used. Algorithms were used to identify OTUs. For bacteria and fungi, we used SILVA (Quast et al., 2013) UNITE (Nilsson et al., 2019) respectively. Changes in the composition of the fungal and bacterial community were depicted with a PCA. Statistical significance was inferred from a permutational analysis of variance (PERMANOVA) based on Bray Curtis dissimilarities with the factors "Monoculture" and "Time". Data were then analysed per round with the factor

"Monoculture". Bacterial and fungal copies (qPCR) were each analysed with an ANOVA with the factors "Time" and "Monoculture". Then they were each analysed per sampling round with an ANOVA with the factor "Monoculture". Fungal copies were log transformed to meet the assumption of normality.

Relationship between soil characteristics and plant and herbivore responses

The relationship between the composition of soil abiotic characteristics and features measured in the plant (shoot & root biomass, *M. brassicae* weight gain per day and damage) was analysed with redundancy analysis (RDA). The relationship between the metabolome and the abundance of bacteria/fungi was measured each with and RDA. Soil characteristics were standardised prior to the analysis. Univariate variables measured in the plant (shoot & root biomass, *M. brassicae* weight gain per day and damage) and abundance of bacteria and fungi in the soil was analysed with a Pearson correlation.

The relationship between fungal community, bacterial community, soil abiotic characteristics, and leaf metabolome composition was analysed with co-inertia analysis. Significances were tested with a permutation test with 999 permutations. For these analyses the filtered OTUs were used.

Circos plots were constructed to display correlations between bacteria, fungi and the NMR buckets which differed between the monocultures. For bacteria and fungi the OTUs were labelled to the finest taxonomic rank known. When possible, the buckets were assigned to chemical groups (sugars, phenolic compounds, TCA related compounds, aliphatic). Compounds that could be identified from the NMR spectra were assigned by chemical shift and splitting pattern. Buckets which could not be assigned to a chemical group were labelled with their chemical shift (ppm). To make the circos plots, the 40 variables in each measured community (bacteria, fungi, metabolome) that were most influential in a sparse Partial Least Squares (sPLS) were selected and correlated to each other. Only correlations with Pearson correlation coefficients higher than 0.8 were plotted. These correlations use the latent components as proxy (González, Cao, Davis, & Déjean, 2012). The components were set to two components each. This was done per round and for each monoculture soil. Correlation circle plots were constructed to display the correlation of the selected variables and the components of the sPLS.

To further explore the relationship between the metabolome of the plant and the bacterial and fungal communities in the soil, relevance network analyses were carried out. A sparse sPLS with regression (three sPLS components, 20 variables each) was run and then a network was constructed for all rounds and for each round separately.

In a last step, correlation networks were constructed. This approach is different from the approach described before since all variables are included in the analysis whereas in other analysis only variables selected through sPLS were included. For the correlation networks, all the variables of all measured communities (bacteria, fungi, metabolome) were correlated between and within each other.

Only correlations with Pearson correlation coefficients higher than 0.9 were extracted and plotted as a network in Cytoscape. All the above methods were also carried out testing the relationship between soil abiotic characteristics and the metabolome but there were no strong correlations (all pairwise comparisons were lower than 0.5) and therefore these data are not shown.

Results

Biomass

Shoot and root biomass varied greatly over time and between monoculture soils (Table 4.1, Fig. 4.2). Shoot and root biomass was lowest in soil collected after eight months, but this was also true for plants grown in sterile soil (Fig. 4.2). In the eight and ten month rounds, biomass did not differ between monocultures (Fig. 4.2, Table 4.1). Over time, *J. vulgaris* consistently showed poor growth on its own soil, compared to growth on other soils. Biomass did not vary much among the soils of the five other species. Overall, biomass was highest in 100% sterilized soil.

Table 4.1. Results of ANOVA testing the effects of monoculture soil (*Holcus lanatus, Festuca ovina, Alopecurus pratensis, Hypochaeris radicata, Jacobea vulgaris, Taraxacum officinale*) and the different rounds (2,3,8,10,12). Further the effect of monoculture soils for each round on shoot and root biomass of *Jacobaea vulgaris* and larval weight gain and food consumption of *Mamestra brassicae* was tested for each round separately. F-values, degrees of freedom (df) and P-values are presented. Significant P-values are presented in bold.

Round	Factor	Shoot	Root	Larval weight gain	Consumed area
	Round(R)	$F_{(4,120)}$=42.16, **P<0.001**	$F_{(4,120)}$=34.62, **P<0.001**	$F_{(4,98)}$=62.03, **P<0.001**	$F_{(4,98)}$=49.14, **P<0.001**
	Monoculture (M)	$F_{(5,120)}$= 5.39, **P<0.001**	$F_{(5,120)}$= 7.55, **P<0.001**	$F_{(5,98)}$=1.34, P=0.254	$F_{(5,98)}$=0.06, P=0.998
	R*M	$F_{(20,120)}$= 0.71, P=0.80	$F_{(20,120)}$= 0.64, P=0.87	$F_{(20,98)}$=1.16, P=0.30	$F_{(20,98)}$=1.38, P=0.15
2	M	$F_{(5,24)}$= 3.12, **P=0.026**	$F_{(5,24)}$= 2.96, **P=0.032**	$F_{(5,24)}$=1.38, P=0.27	$F_{(5,24)}$=0.89, P=0.50
4	M	$F_{(5,24)}$= 2.65, P=0.048	$F_{(5,24)}$= 5.15, **P=0.003**	$F_{(5,23)}$=0.65, P=0.66	$F_{(5,22)}$=2.25, P=0.09
8	M	$F_{(5,24)}$= 0.41, P=0.84	$F_{(5,24)}$= 0.50, P=0.77	$F_{(5,14)}$=0.62, P=0.69	$F_{(5,11)}$=0.43, P=0.81
10	M	$F_{(5,24)}$= 0.77, P=0.58	$F_{(5,24)}$= 0.45, P=0.81	$F_{(5,22)}$=2.41, P=0.07	$F_{(5,22)}$=0.12, P=0.99
12	M	$F_{(5,24)}$= 2.55, **P=0.050**	$F_{(5,24)}$= 3.44, P=**0.017**	$F_{(5,22)}$= 2.67, **P=0.049**	$F_{(5,19)}$=0.67, P=0.65

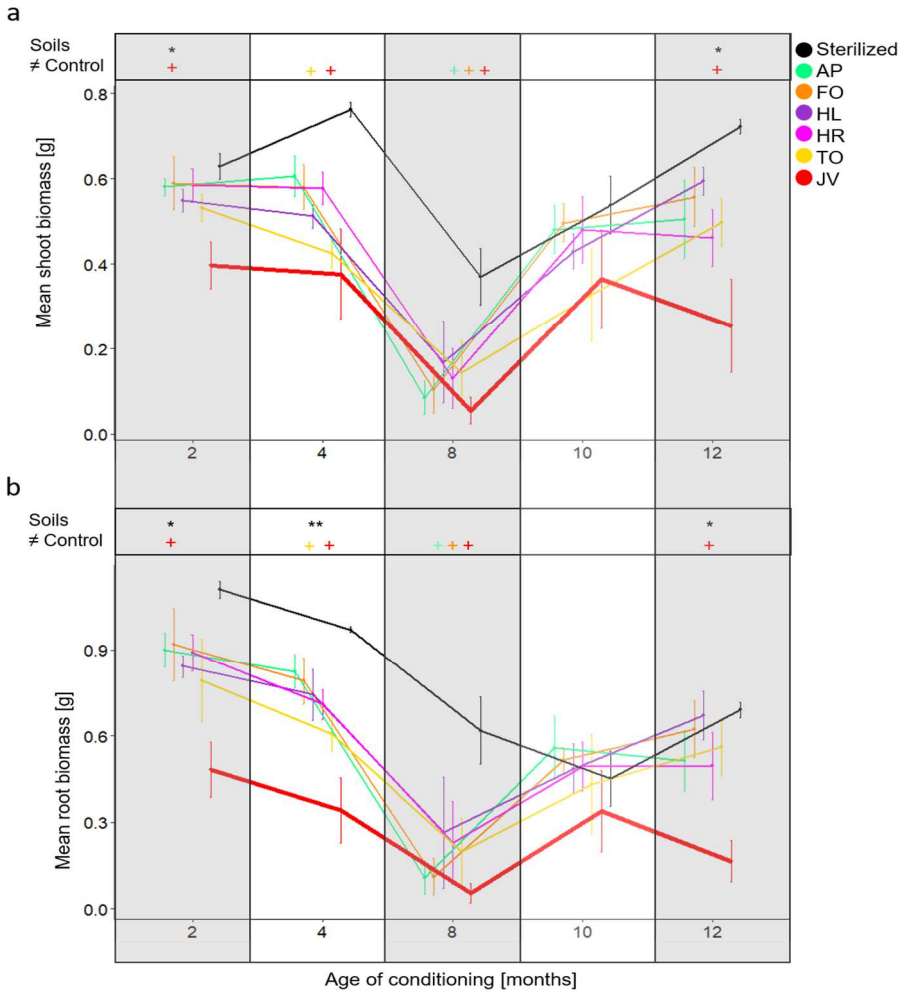

Figure 4.2 Mean (± SE) shoot (a) and root (b) biomass of *Jacobaea vulgaris* in monoculture soils collected over the course of one year (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , TO= *Taraxacum officinale, JV= J. vulgaris* and in 100% sterilized soil (control). For each round, results of an ANOVA testing the effects of monocultures are also depicted in the figure (*p<0.05, **p<0.01). Plants grown in sterilized soil were excluded for this analysis. A Dunnet post hoc test, following a separate ANOVA was used for the comparison of each treatment with the100% sterilized soil was tested (P<0.05 is depicted as + with the colour indicating the species).

Herbivory

Weight gain and food consumption of *M. brassicae* varied greatly between rounds (Table 4.1) but there was no effect of monoculture soil (Fig. 4.3) except on weight gain in the 12 months round (Table 4.1).

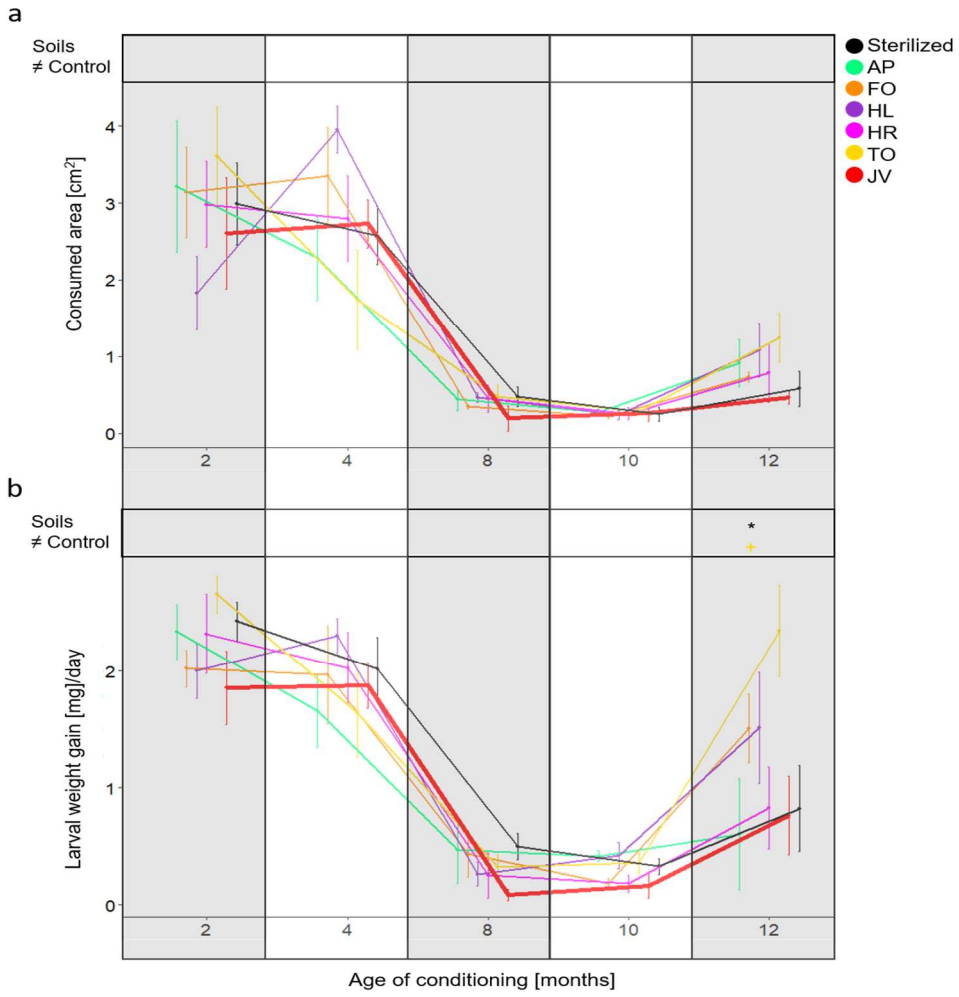

Figure 4.3 Mean (± SE) area consumed (a) and larval weight gain (b) of *M. brassicae* larvae on leaves of *Jacobaea vulgaris* grown in monocultures soils (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , TO= *Taraxacum officinale, JV= J. vulgaris*) and in 100% sterilized soil (control) in each round. For each round, results of an ANOVA testing the effects of monocultures are also depicted in the figure (*p<0.05). Plants grown in sterilized soil were excluded for this analysis. A Dunnet post hoc test, following a separate ANOVA was used for the comparison of each treatment with the100% sterilized soil was tested (P<0.05 is depicted as + with the colour indicating the species).

Leaf metabolomes

The leaf metabolomes differed strongly among the three sampling rounds (Table 4.2 Fig. 4.4). The two month and eight month sampling rounds were separated most clearly along the first PCA axis, while the two and twelve month rounds were more similar in composition along the first axis but were separated along the second axis (Fig. 4.4). Separate PCAs for each round show that there is considerable variation in metabolome composition, but that the metabolomes of plants grown in sterilized soil varied less than those of plants grown in conditioned soils (Supporting information Fig. S4.3). The results of the overall PERMANOVA confirmed that metabolomes differed significantly between rounds - and were related to plant biomass, but did not differ between monoculture soils (Table 4.2). However, separate PERMANOVAs per round revealed that the soil effects were lower in the two month round than in the later rounds (measured as higher R^2; Table 4.2). The effect of total plant biomass on metabolomes also increased over time (Table 4.2). Phenolic compounds varied most strongly between the monoculture soil treatments, but formic acid was the only compound that responded consistently among rounds (Supporting information Fig. S4.4).

The variation in the composition of the metabolome was significantly related to the consumed area by caterpillars in the eight month round (Table S4.2).

Table 4.2. Results of permutational multivariate analysis of variance (PERMANOVA) testing the effect of monoculture soil (*Holcus lanatus, Festuca ovina, Alopecurus pratensis, Hypochaeris radicata, Jacobaea vulgaris, Taraxacum officinale*), round (2, 8, 12 months) and total plant biomass on the leaf metabolome of *J. vulgaris*. Further the effect of monoculture soils and plant biomass on the metabolome was tested for each round separately. The analyses are based on Bray-Curtis distances. Permutations were set to 999. Pseudo F-values, degrees of freedom (df), explained variance (R^2) and P-values are presented. Significant P-values are presented in bold.

Round	Factor	F	R^2	P
	Round (R)	$F_{(2,39)}$=30.37	0.35	**0.001**
	Monoculture (M)	$F_{(5,39)}$= 0.78	0.02	0.58
	Total Biomass (B)	$F_{(1,39)}$=35.83	0.21	**0.001**
	M*R	$F_{(10,39)}$= 1.26	0.07	0.28
	M*B	$F_{(5,39)}$= 1.28	0.04	0.27
	R*B	$F_{(2,39)}$= 1.58	0.02	0.21
2	M	$F_{(5,18)}$= 0.75	0.12	0.63
	B	$F_{(1,18)}$= 1.70	0.05	0.19
	M*B	$F_{(5,18)}$= 1.61	0.26	0.15
8	M	$F_{(5,5)}$= 7.05	0.29	**0.006**
	B	$F_{(1,5)}$=57.15	0.47	**0.001**
	M*B	$F_{(5,5)}$= 4.81	0.20	**0.023**
12	M	$F_{(5,16)}$= 1.68	0.22	0.16
	B	$F_{(1,16)}$=11.42	0.29	**0.001**
	M*B	$F_{(5,16)}$= 0.62	0.08	0.77

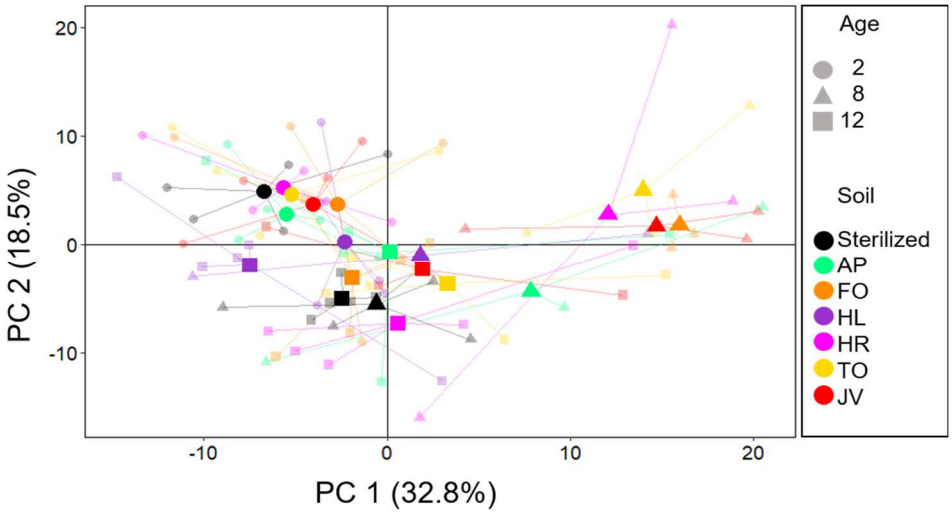

Figure 4.4 Principal component analysis (PCA) depicting the composition of the metabolome of *Jacobaea vulgaris* grown on in monoculture (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , TO= *Taraxacum officinale,* JV= *J. vulgaris* and in 100% sterilized soil (control) in the different rounds (2 months circles, 8 months triangles, 12 months squares). Centroids connected to the sample scores of the replicates for the first two axes of an unconstrained principle component analysis (PCA) are presented. The percentage explained variance by each axis is also depicted. PCAs depicting metabolomic composition at each round are presented in Figure S4.3 of the supporting information.

Bacteria and fungi

Bacterial composition in the soil differed strongly between the three rounds but also between the different monocultures (Supporting information Fig. S4.5a Table S4.3). Fungal composition also differed strongly between monocultures and differed between the rounds (Supporting information Fig. S4.5b Table S4.3). The abundance of bacteria in the soil peaked at 6 months for five of the six species, but one month earlier in *J. vulgaris* monocultures. (Supporting information Fig. S4.6a, Table S4.4). Fungal copy numbers were higher in *T. officinale* and *J. vulgaris* monocultures after 6 and 12 months (Fig. S4.6b, Table S4.4).

Soil abiotic properties

Organic matter; iron (Fe) and sulphur (S) varied over time (Supporting information Fig. S4.7, Table S4.5). Potassium and to a lesser extent magnesium and phosphate availability was higher in monoculture soils of *J. vulgaris* and to a lesser for *T. officinale* soil than in soil conditioned by the other plant species (Supporting information Fig. S4.7; Table S4.5).

Relationships between soil and plant characteristics over time

Bacterial composition in the soil explained metabolome changes for the eight month round and this was true for fungi for the twelve month round (Table 4.3). The composition of both the bacterial and the fungal community significantly explained root biomass at the twelve month round and for the fungal community this was also true for shoot biomass. Soil abiotic characteristics did not explain metabolomic composition but were significantly related to larval weight gain after eight months and leaf consumption by *M. brassicae* after ten months (Table 4.3).

Table 4.3. Relationships between properties measured in the plant (shoot and shoot biomass, metabolome and insect performance) and soil characteristics (soil abiotic properties, bacterial composition, fungal composition, bacteria abundance and fungi abundance) overall and for each round. The relationships with metabolomes were analysed with coinertia analysis. For the coinertia analysis the RV coefficient and the significance tested with permutation tests (999 permutations) is presented. Relationships between univariate variables (abundance data) were analysed with Pearson correlations. The correlation coefficient is presented. All other relations were analysed with redundancy analysis (RDA). For the RDAs the explained variance and the significance tested with permutation tests (999 permutations) are displayed. *, ** indicate significant effects in the tests at $P < 0.05$; $P < 0.01$, respectively. Significant p-values are presented in bold.

Round	Variable	Abiotic characteristics	Bacteria community	Fungi community	Bacteria abundance	Fungi abundance
	Shoot biomass (SB)	**6.26*** **	**1.55*** **	**1.52** **	**0.18***	**-0.17***
	Root biomass (RB)	**4.11*** **	**2.62*** **	**1.81*** **	0.12	**-0.18***
	Metabolome (M)	0.14	**0.15***	0.11	0.02	0.03
	Larval weight gain (WG)	1.86	**1.89** **	**2.57*** **	0.15	-0.03
	Consumed area (CA)	0.90	**1.83***	**7.25 *** **	**0.27***	-0.14
2	SB	6.40 [a]	3.40	4.16	0.27	-0.15
	RB	5.97 [a]	3.10	4.42	0.33	-0.09
	M	0.14 [a]	0.42	0.43	0.03	0.01
	WG	1.05 [a]	3.53	3.68	-0.09	0.01
	CA	0.09 [a]	3.35	3.57	0.23	0.06
4	SB	6.05			-0.24	**-0.44***
	RB	10.45			-0.48	-0.44
	M					
	WG	2.61			-0.17	0.01
	CA	3.79			-0.08	-0.22
8	SB	3.42	7.46 [b]	7.06 [b]	0.03	-0.03
	RB	2.73	7.37 [b]	7.02 [b]	-0.01	0.08
	M	0.26	**0.52*** [b]	0.45 [b]	0.03	0.06
	WG	**15.98***	6.81 [b]	5.44 [b]	-0.17	0.26
	CA	11.93	6.05 [b]	6.76 [b]	0.07	0.01
10	SB	12.48			-0,21	0.08
	RB	5.38			-0.22	0.10
	M					
	WG	2.62			0.08	0.15
	CA	**13.64***			0.08	0.05
12	SB	5.16	3.94	**5.27** **	0.01	-0.10
	RB	6.46	**4.08***	**4.64***	-0.01	-0.10
	M	0.18	0.35	**0.39***	0.03	0.07
	WG	6.06	3.66	4.07	-0.15	0.19
	CA	6.45	4.10	4.00	-0.23	0.12

[a] Soil abiotics were not measured after 2 months and data collected for 4 months were used.
[b] Communities were measured in soil after 6 months of conditioning

The links between soil microbial communities and metabolomes varied between the three sampling rounds (Fig. 4.5). After two months we observed positive correlations between soil fungi, bacteria and metabolic compounds selected through sPLS. After eight months the number of positive correlations increased, while after twelve months there were fewer positive correlations and also negative correlations between fungi or bacteria and the metabolome appeared. The identity of metabolic compounds correlated with microbes varied between the rounds. However, in each round, changes in compounds related to the TCA-cycle such as malate and malic acid were linked to OTUs of bacteria and fungi. The patterns also varied between monocultures (Supporting information Fig. S4.8). Notably, there are more negative correlations between fungal OTUs and the metabolome for forbs than for grasses. While for most species the OTUs of fungi and the bacteria were linked to metabolome changes, for *H. lanatus* soil there were no links between changes in bacteria and the metabolome. For most monoculture soils there were more strong correlations between the metabolome and bacteria than between metabolome and fungi.

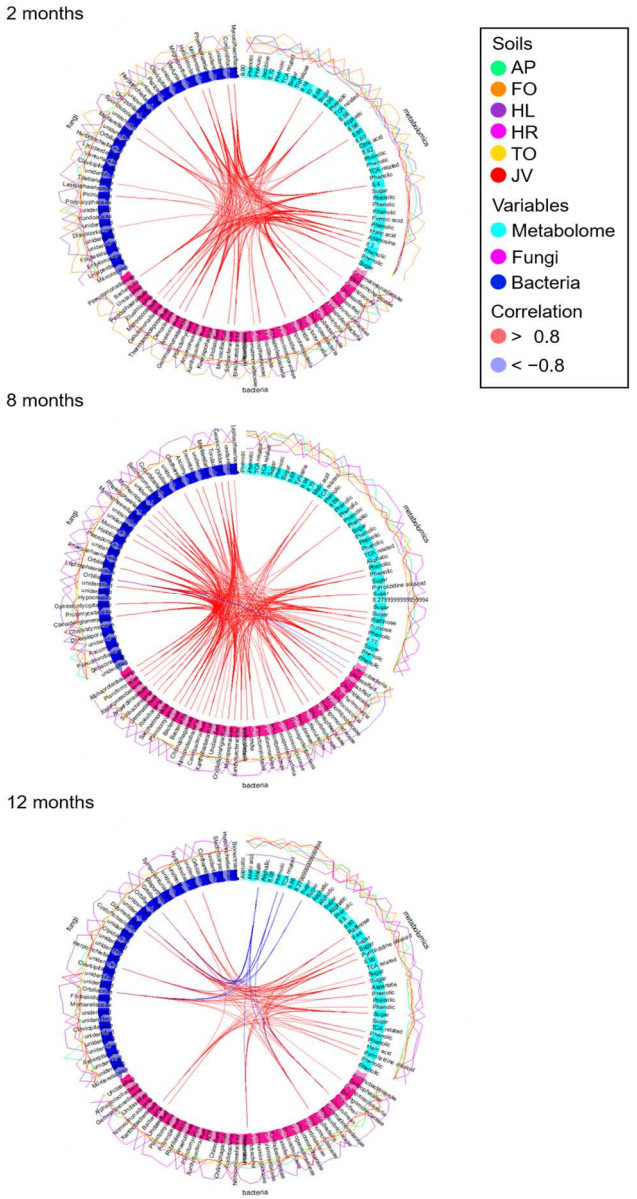

Figure 4.5 Circos plots visualising the correlation between bacterial and fungal communities in the soil and metabolomic changes in *Jacobaea vulgaris* plants grown in these soils for each round. Displayed are correlations that are higher than 0.8 (red) or lower than -0.8 (blue). Lines outside the circles show the concentration of each compound in the plants in six monocultures. For the bacteria and the fungi, the abundance of each OTU in each monoculture is displayed. Correlations within each measured community (metabolome, bacteria, fungi) are not depicted. Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , JV= *J. vulgaris*, TO= *Taraxacum officinale*.

Bacterial and fungal communities in the soil were strongly linked in all rounds, as depicted by the positive correlations in the correlation network (Fig. 4.6). In general, there were more strong positive correlations between the metabolome and bacterial OTUs than fungi. Strongest correlations were found after eight months. The correlations between fungi, bacteria and metabolome varied between monoculture soils (Supporting information Fig. S4.9). Notably, the number of negative correlations between bacteria and fungi in the soil and the metabolome was highest in soil of *J. vulgaris*. For soils conditioned by all species except *H. radicata* there were more positive correlations between the metabolome and bacteria than fungi.

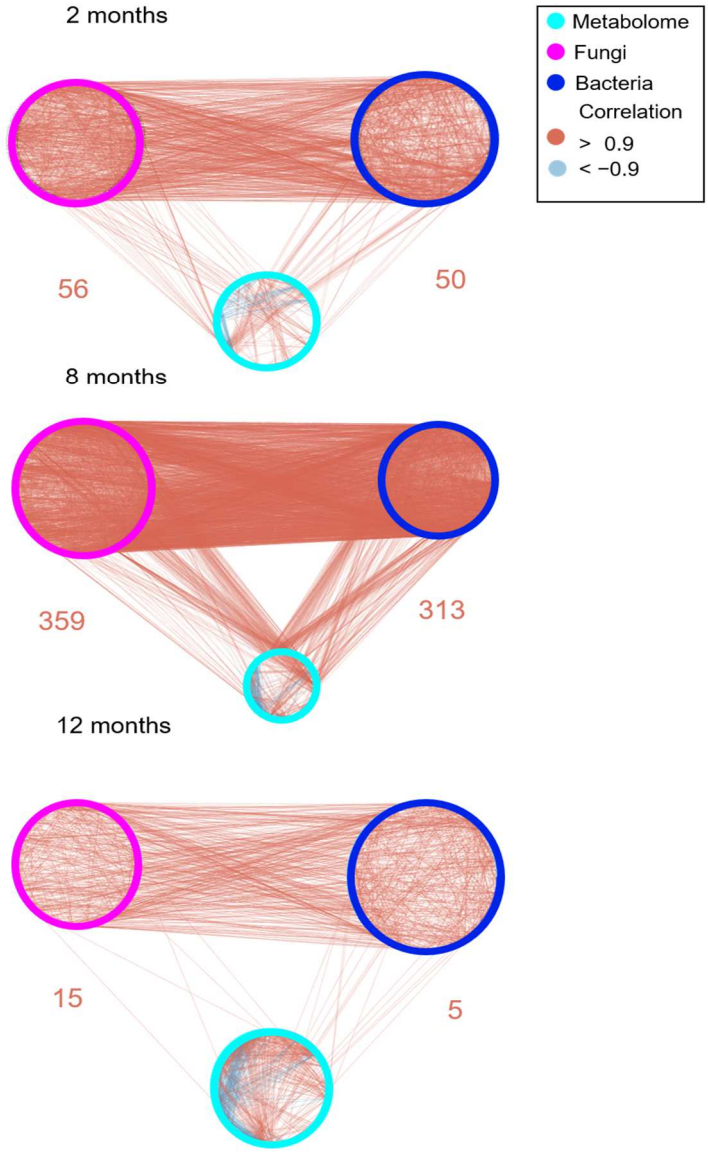

Figure 4.6 Correlation network between metabolomes fungi and bacteria for each round. Displayed are Pearson correlations between and within the metabolome of *Jacobaea vulgaris* and the bacterial and fungal communities in the soil. Depicted are correlations with a coefficient higher than 0.9 (red) or lower than -0.9 (blue).

We further investigated how changes in the metabolome are correlated to changes in the bacterial and fungal community in the soil using relevance networks (Supporting information Fig. S4.10, S4.11). These networks showed similar patterns as the circus plots and correlation networks. Fungal and bacterial links to metabolomic changes are strongest after eight months. The relevance networks were also constructed between the metabolomic changes and changes in soil abiotic characteristics in the soil. However, correlations between the metabolome and the abiotics were low and therefore none of the coefficients passed the threshold of 0.6.

Discussion

In this study we examined the growth pattern of *J. vulgaris* grown in sterilized soil and in soils conditioned by a conspecific and five heterospecific plant species for two, four, eight, ten and twelve months. Furthermore, we tested metabolomic changes in shoots of *J. vulgaris* caused by soils that were conditioned for different lengths of time and by different plant species. This enabled us to examine if soil legacies, in addition to influencing the growth of plants, also affects their chemical composition. At the same time, we tested if these potential metabolic changes lead to changes in the performance of the generalist herbivore, *M. brassicae*. Simultaneously we linked the potential causes of these metabolomics changes to bacterial and fungal communities, as well as the nutrients, in the soils which were used as inoculates. Our findings highlight two important aspects. First, although the magnitude of soil effects on biomass varies between different rounds of the same experiment, the patterns are generally very similar. Second, the influence of PSF on the metabolome is less stable between the different rounds of the experiment and is related to the time of conditioning. Metabolomic changes in the plants are linked stronger to microbial and fungal communities in the soils after eight months of conditioning than after two and twelve months of conditioning. Below these findings will be discussed in more detail.

Our results show that generally patterns of PSF on plant biomass stayed the same independently of age of the conditioning community. Biomass of *J. vulgaris* grown in soils collected at different times differed. In general, *J. vulgaris,* except after ten months of conditioning, showed the highest biomass above and belowground in 100% sterilized soil and the lowest in its conspecific soil. This increased biomass for plants grown in sterile soil was also found in multiple other studies (Joosten, Mulder, Klinkhamer, & Van Veen, 2009; Kos,

Tuijl, De Roo, Mulder, & Bezemer, 2015a; Wang et al., 2019; Xue, Bezemer, & Berendse, 2018). Furthermore, similar to our findings other studies showed that *J. vulgaris* has a negative conspecific feedback (Bezemer et al., 2018; Van de Voorde, Van der Putten, & Bezemer, 2011). Our results now show that PSF is remarkably consistent, even if the conditioning plant community is aging. The strength of PSF moderately differs depending on the age of the community but the overall patterns stay the same over the course of a year. This is an important finding for PSF research since it highlights that the time of conditioning is not a major factor influencing the outcome of PSF experiments. We also showed that differences in the root biomass were more pronounced than differences in the shoot biomass. Biomass of the *J. vulgaris* roots decreased throughout the different rounds over the course of a year and did not reach the same biomass as in the first round of the experiment. Contrary to that, the shoot biomass of *J. vulgaris* in the feedback phase lowered until the age of 8 months and after that did reach the same magnitude again than in the beginning of the experiment. These findings strengthen the view that root biomass is more sensitive to PSF than shoot biomass. Other PSF studies also report this stronger effect of PSF on root than on shoot biomass (Kos et al., 2015a; Wang et al., 2019). This is probably caused by the proximity of the roots to the soil and therefore a more direct influence of the soil microbes on the roots than on the shoots. The species-specific differences of conditioned soils were less pronounced at the sampling at which the community was eight or ten months old, this might be because this sampling took place in early January and March. During winter, the functions of the microbial communities in the soil might get more similar since decomposition processes are getting dominant due to litter material which accumulated on the surface of the soil in the previous months. This could also be an explanation for the stronger links between the microbiome and the metabolome of *J. vulgaris* that we show in this study. In our study it is not possible to disentangle seasonal from time effects and future studies should address these points with long term experiments over multiple years.

We did not find any significant difference in the performance of *M. brassicae* feeding on the plants grown in differently conditioned soils except for the plants grown in soil inoculated with soil conditioned by *T. officinale* for twelve months, on which the daily weight gain was higher than on the plants in other experimental rounds. However, we did not find a relation between the metabolome composition and the herbivore performance after twelve months. Multiple studies have found effects of PSF on insect performance (Badri, Zolla, Bakker, Manter, &

Vivanco, 2013; Bezemer et al., 2013; Heinen, Biere, & Bezemer, 2019). In another study the abundance of the specialized aphid *Aphis jacobaeae* for example showed large differences on *J. vulgaris* grown on 10 differently conditioned soils (Kos et al., 2015b). However, a generalist aphid species (*Brachycaudus cardui*) did not show these differences in abundance due to PSF. This shows that the outcome of PSF studies investigating the herbivore reaction to PSF is highly variable depending on the used insect species, plant species and conditioning species. This might also be the reason that we did not find strong PSF effects on insect performance in our present study. Furthermore, a recent study showed that the biomass of *M. brassicae* fed on *T. officinale* grown in different soil legacies did differ if *M. brassicae* was feeding directly on the plant however in a detached leaf assay this was not the case (Hannula, et al., 2019a). This showed that the insects are directly affected by the soil microbiome. In our present study we only tested herbivore performance in a detached leaf assay and might therefore have missed legacy effects on insects which require contact with the soil.

The metabolome of *J. vulgaris* changed between the different rounds of the experiment and this occurred in conditioned soil but also in sterilized soil. While the metabolome of plants grown in sterilized soil was similar in the eight- and twelve-month sampling rounds, it was different at the two-month round. These differences can have various explanations. *J. vulgaris* might, although kept under the same conditions in a climate chamber without seasons, still have an imprinted seasonal rhythm which might lead to differences between seasons. To our knowledge there is no study testing these seasonal changes of metabolomes in plants growing in a controlled environment. However, some studies have demonstrated that in plants grown in natural conditions, abiotic properties are the best predictors of metabolomes (Peters, Gorzolka, Bruelheide, & Neumann, 2018; Sampaio, Edrada-Ebel, & Costa, 2016). Second, all sterilized soil used in the feedback rounds was irradiated at the beginning of the experiment and soil nutrient levels changed profoundly between the experimental rounds, but those changes do not match the patterns seen in the metabolome changes.

The causal agents for the observed PSF are mainly changes in community composition of bacteria and fungi of the soil over time. Soil abiotic conditions did not explain the patterns that were observed. We investigated the changes in the microbiome (bacteria and fungi) of the soils that were used as inoculates in the present study over the course of one year in Hannula et al. 2019a. In that study we could show that changes in bacterial communities were mainly

explained by the time since the establishment of the plant community. Changes in fungal communities were mainly related to the plant species grown in the soil and their functional group (grass/forb). In our present study the biomass of *J. vulgaris* differed between the different rounds of experiments over time but in most rounds the species-specific effects on biomass stayed similar. Combined with the results of our previous study this points out that the metabolomic changes due to soils are mainly related to changes in bacterial communities in the soil which are mainly influenced by time. Our network analysis shows that the links between the metabolomic changes are stronger with bacterial OTUs than with fungal OTUs. PSF effects on biomass that vary but show similar patterns over time In our earlier study fungal communities in the soil mainly changed due to the plant species that was grown in the soil before. It could therefore be that the PSF effects on biomass are mainly triggered by fungi in the soil. Evidence for this in the present study however is weak since the fungal community of the soil only has a significant effect on the growth of the plants after twelve months of conditioning. However, at this time, the bacterial community also influenced plant biomass. We further found that the abundance of bacteria was highest six months after establishment of the communities. We found a similar pattern in the biomass responses of *J. vulgaris* with a drop in biomass after eight months of growth. Although the bacterial abundances were already lower again at eight months, the increase at six months could have led to changes in the soil that still influenced the growth of the plants at eight months. Higher bacterial abundances might go along with higher rates of exudation of the plants in order to maintain their microbial communities in the soils, this potential peak of exudation of the plants might have led to a chemical legacy in the soils, which we did not measure in this experiment, that led to a decrease of the biomass of *J. vulgaris* after eight months. Since the containers from which the soil samples in each round were collected were outside, we were not able to control for differences in herbivore loads of the different plant species in the different containers. A previous study has shown that above and belowground herbivory can influence soil legacies and the response of *J. vulgaris* to them (Wang et al., 2019). Therefore, it is possible that those potential differences in herbivore loads between species and the different rounds of experiments did enhance or decrease the effect of the inoculum on *J. vulgaris*.

In conclusion, we show that legacy effects of six different soils on above- and belowground biomass of a common focal plant differ in magnitude over time, but the overall response patterns stay remarkably similar over time. Interestingly, we find that plant metabolomic

responses to soils are highly inconsistent and became more apparent in the later sampling rounds. This indicates that the effects of soil legacies on plant metabolomic profiles become more pronounced when the soil is conditioned for a longer period of time, which is in stark contrast with the relatively consistent responses in plant biomass. This strongly suggests that soil legacy effects may have farther-reaching impacts than plant growth alone. As plants are the primary resources for most organisms, soil legacy effects, through plant-metabolomic processes, may have lasting impacts higher in the ecological food web.

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Author contributions

M.H and T.M.B designed and planned the experiment. R.H, K.S, R.J, S.E..H and M.H carried out the experiments and kept the containers running for 1 year. M.H processed the metabolomics samples and analysed the results with T.M.B and Y.H.C. S.E.H. processed and analysed the sequencing data. M.H wrote the first version of the manuscript and all co-authors critically added to the manuscript.

Supporting Information

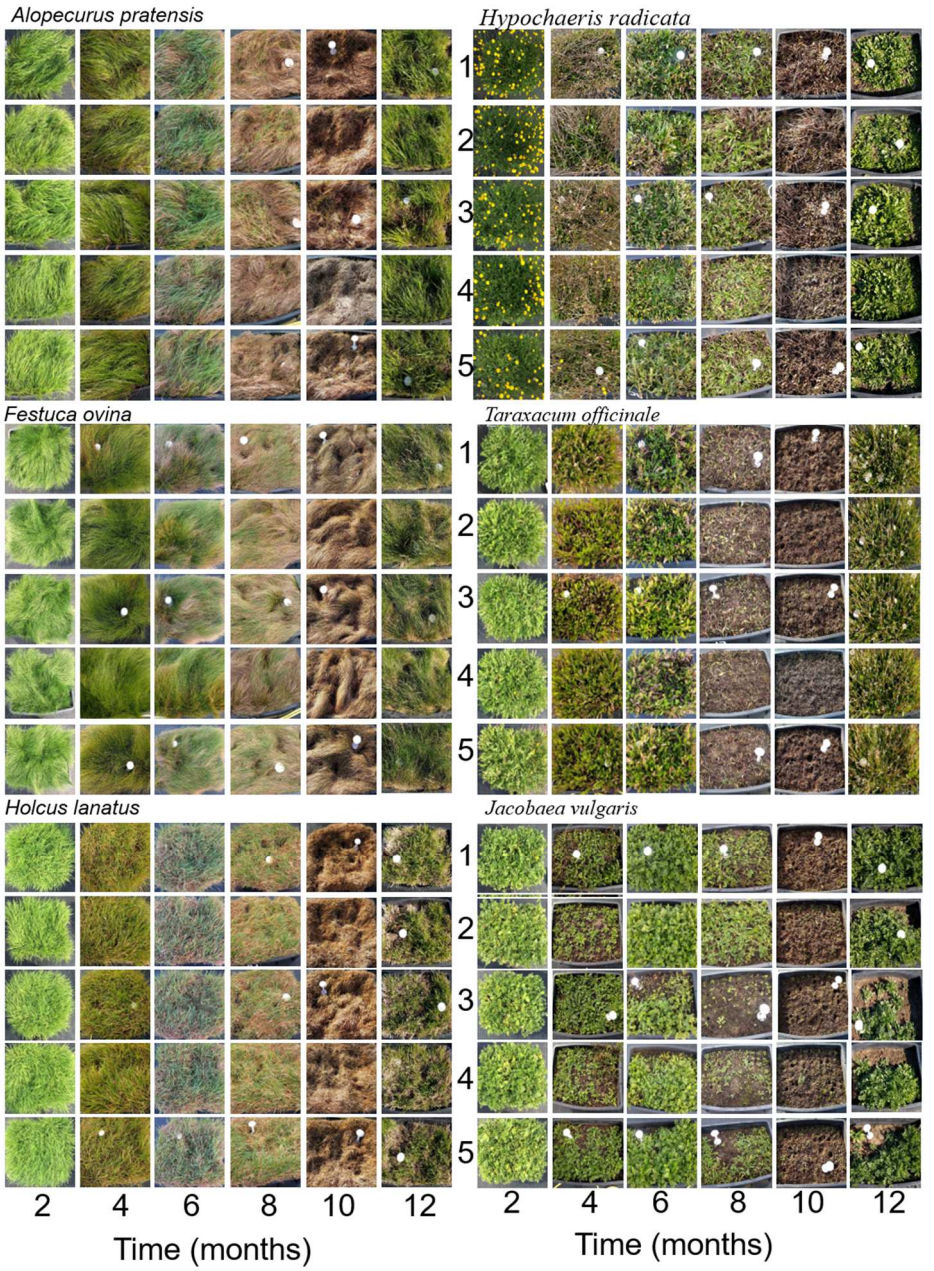

Figure S4.1 Photos depicting temporal aboveground changes in the monoculture vegetations. Numbers from 1-5 refer to the different replicates. The white marks in some of the photos are sensors which measured temperature and soil moisture.

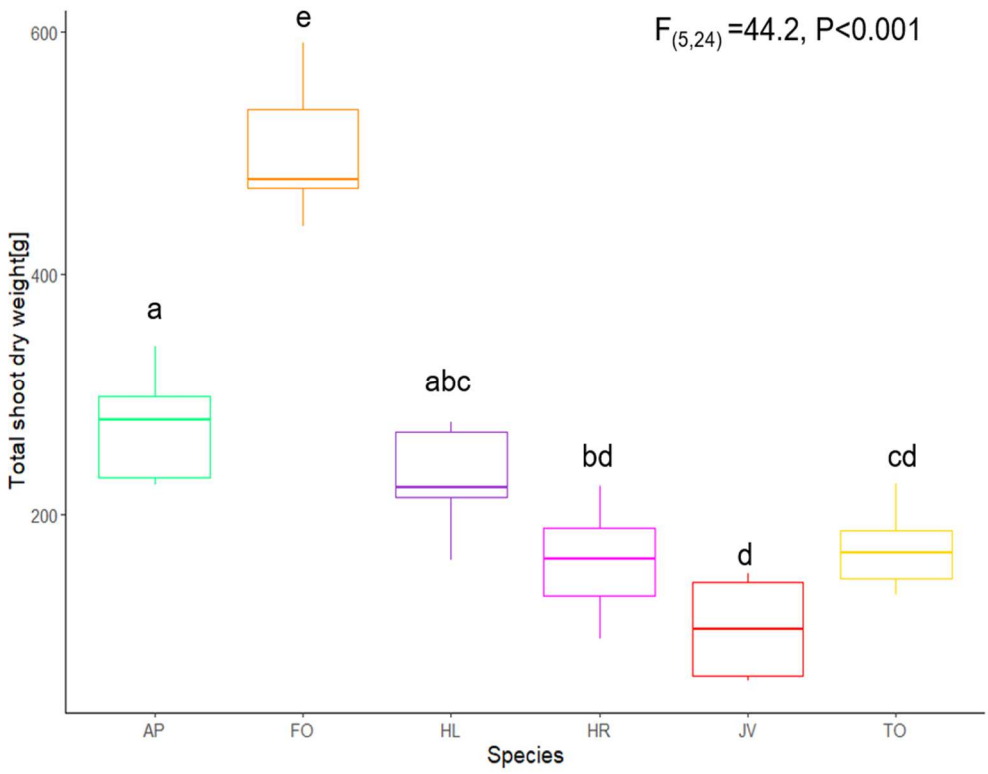

Figure S4.2 Mean shoot dry weight (±SE) of the different monocultures after 12 months (15 May2018). Tukey box-and-whisker plots display the median (horizontal line), the quartiles (box) of the data and the whiskers show all variation. Different letters above the bars represent significant differences ($p<0.05$) a Tukey HSD posthoc test. Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale*, JV= *Jacobaea vulgaris*. Results of a One-Way ANOVA are also presented.

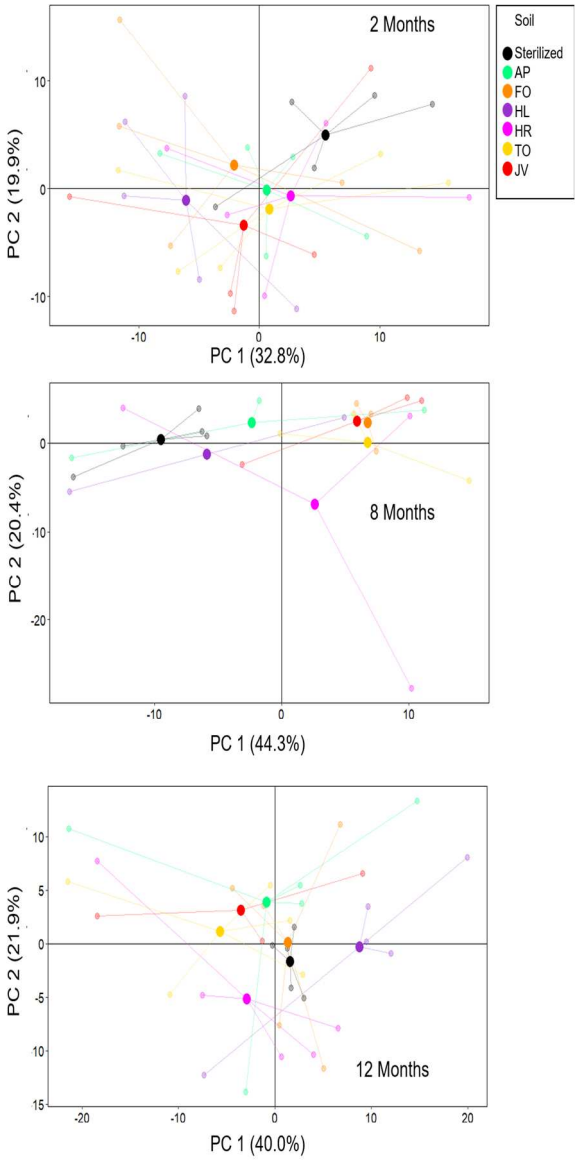

Figure S4.3 Composition of the metabolome of *Jacobaea vulgaris* grown in different soils (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale,* JV *J. vulgaris,* and 100% sterilized soil (control) at the three rounds. Centroids connected to the sample scores of the replicates for the first two axes of an unconstrained principle component analysis (PCA) are presented. The percentage explained variance by each axis is also depicted.

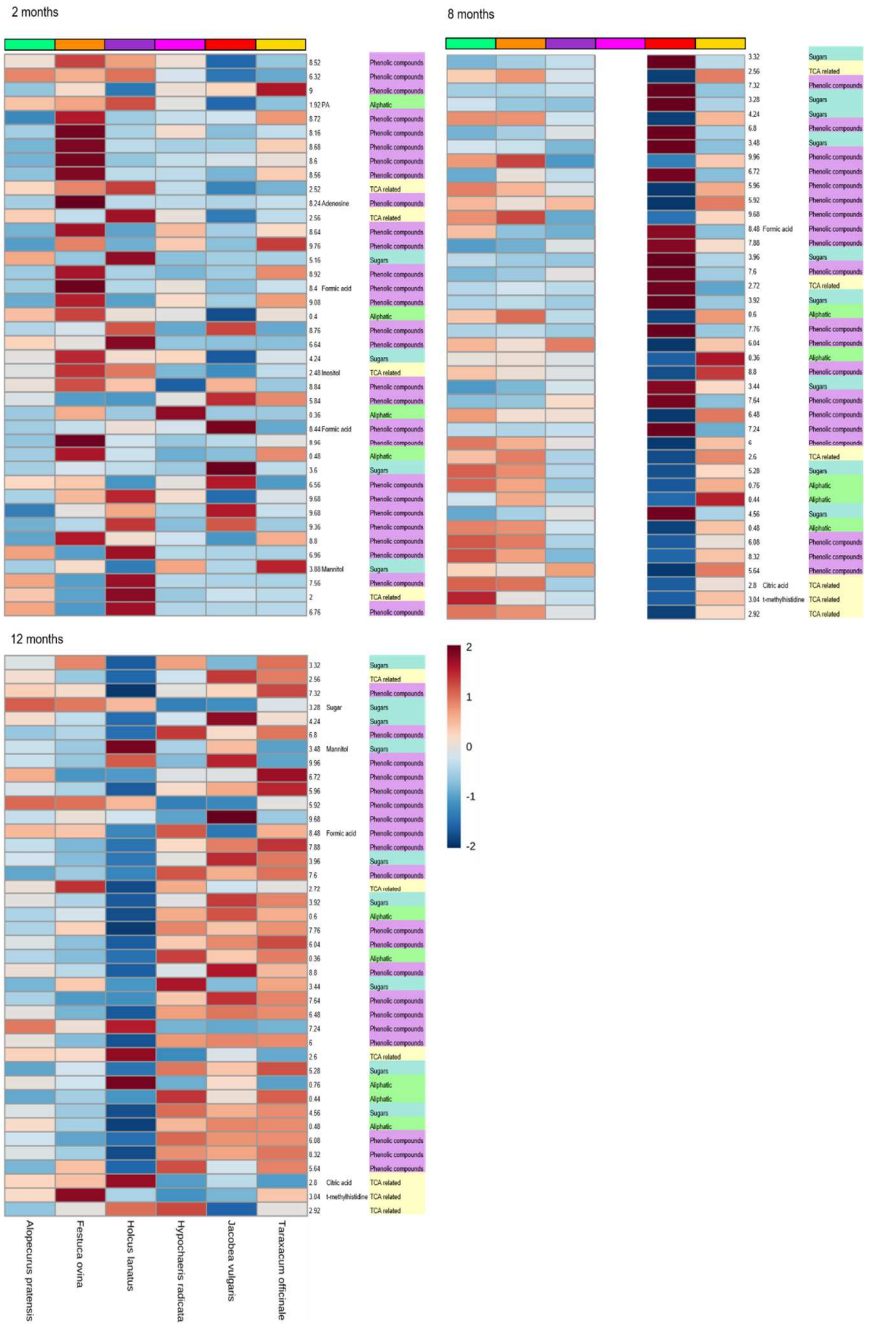

Figure S4.4 Heatmap showing the mean normalised intensity of the 40 buckets (rows) per timepoint (2, 8, 12 months) which varied most among the soils marked by colours above the heatmaps and indicated at the bottom of the heatmaps. Buckets were associated to compounds if possible and compound classes. The same compound classes are marked in the same colour.

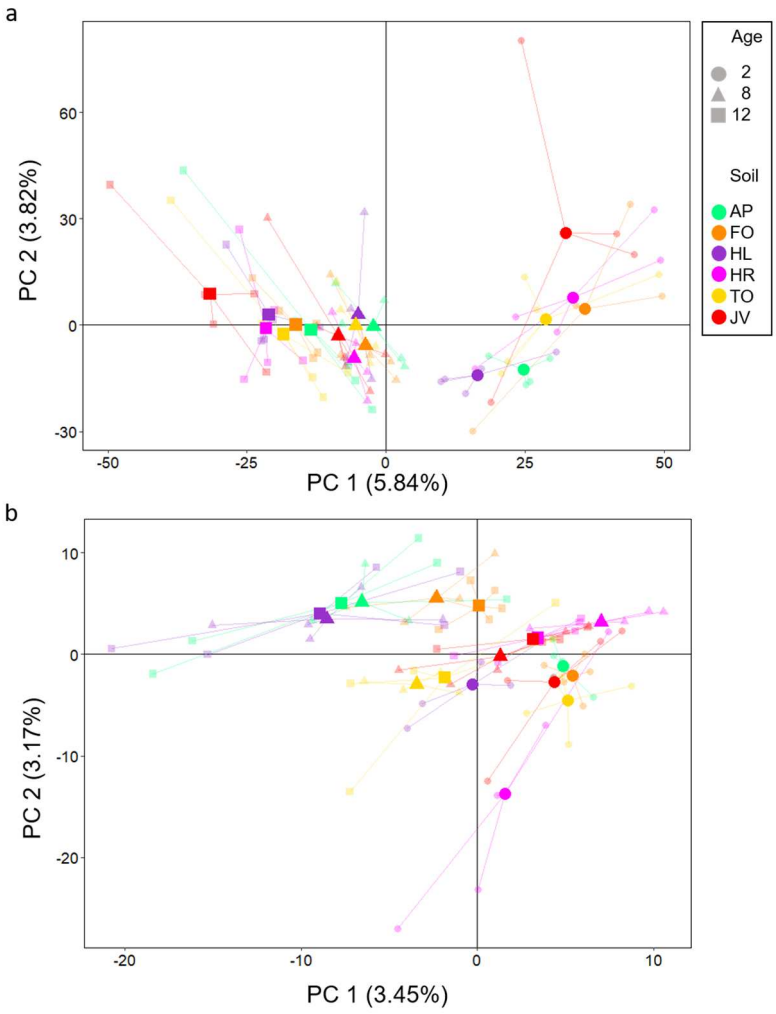

Figure S4.5 Composition of the bacterial a) and fungal b) communities in monoculture soils (AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata* , TO= *Taraxacum officinale,* JV= *Jacobaea vulgaris*) at the three rounds (different symbol shapes). Centroids connected to the sample scores of the replicates for the first two axes of an unconstrained principle component analysis (PCA) are presented. The percentage explained by each axis is also depicted.

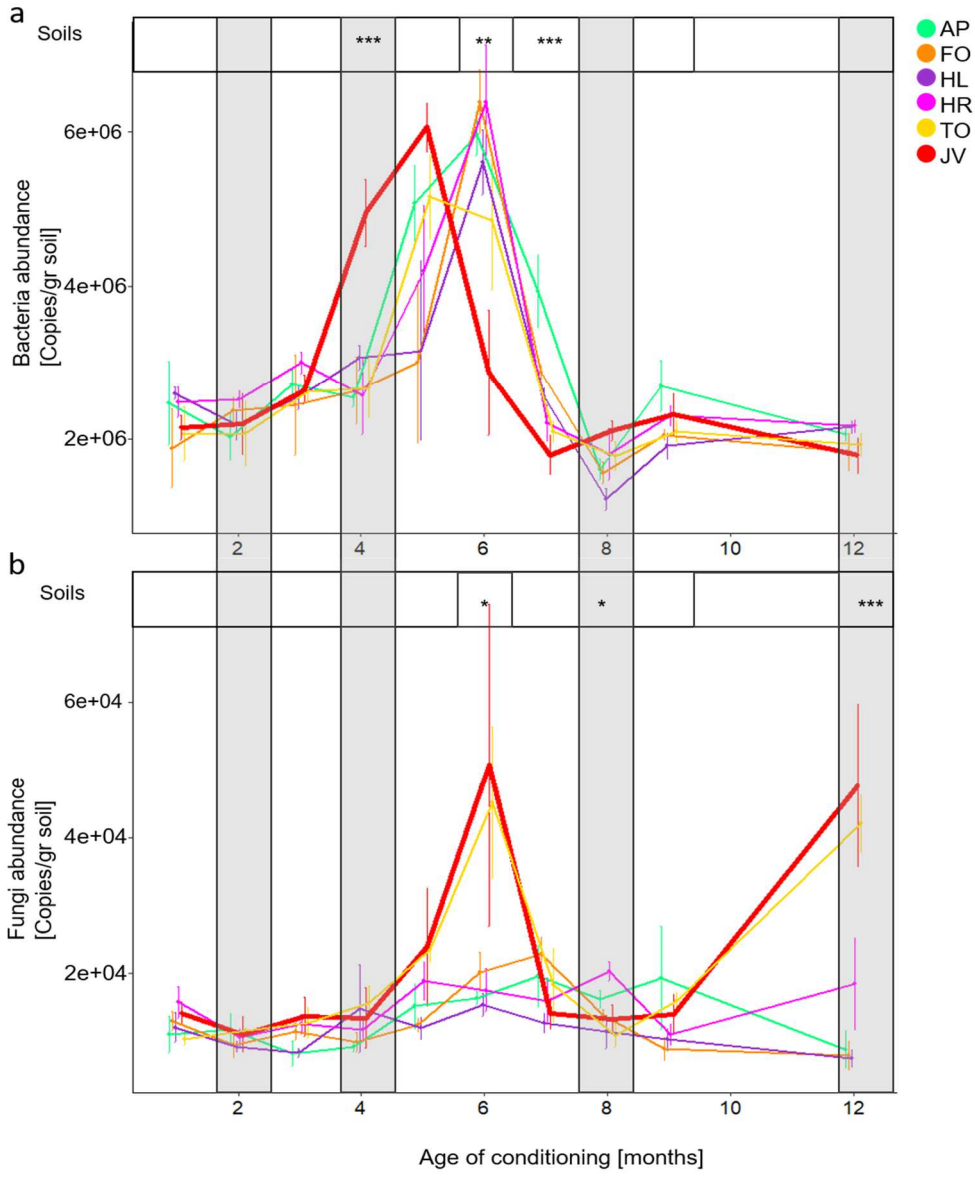

Figure S4.6 Mean temporal changes in bacterial (a) and fungal (b) abundance in the monoculture soils (copies per g of soil ± SE). AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale* . *JV* = *Jacobaea vulgaris* at different timepoints (x-axis). Samples were collected monthly for nine months and after 12 months. For information, significance from ANOVA (Table S5) are depicted above the figures. *p<0.05, **p<0.01, ***p<0.001. Grey parts of the figures indicate sampling times that match with the PSF experiment.

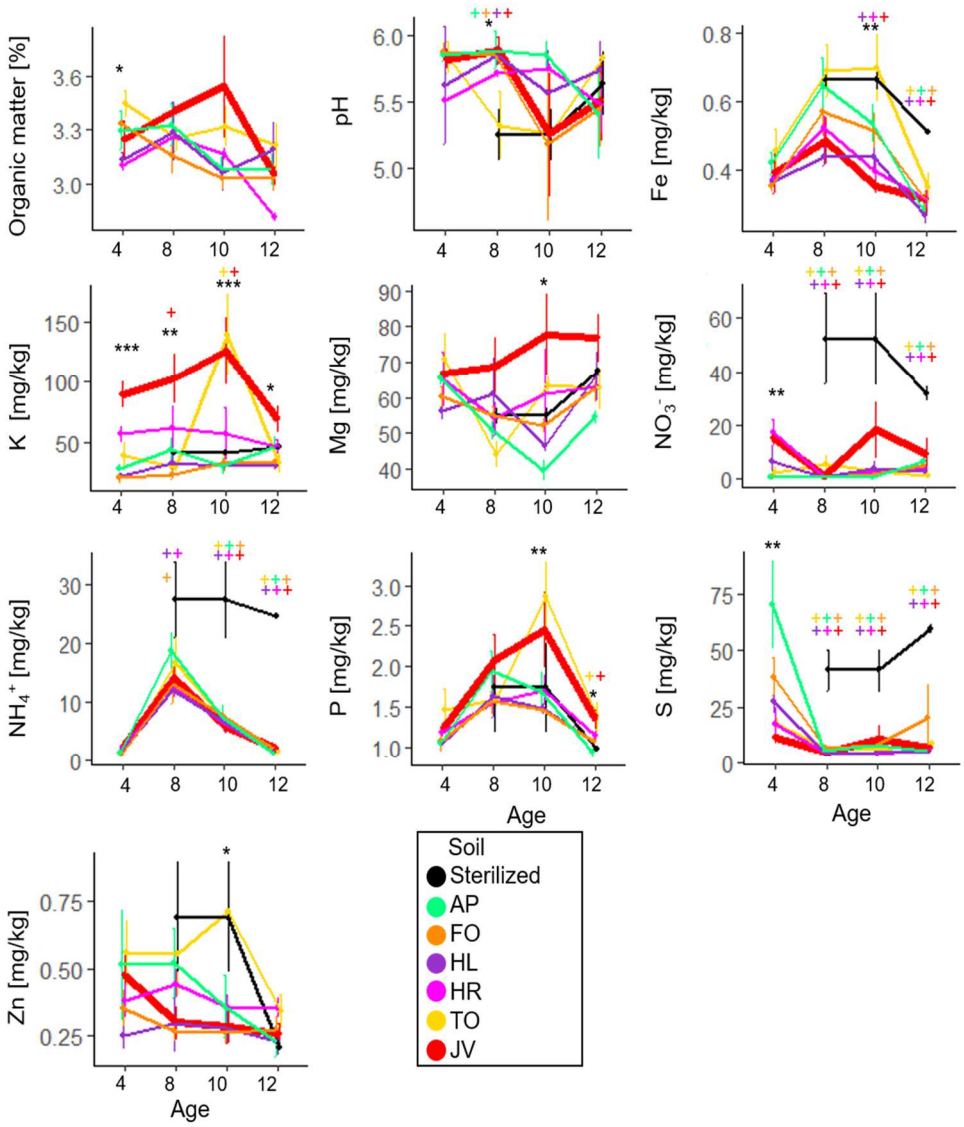

Figure S4.7 Soil abiotic properties measured over the course of the experiment. Points show the mean concentration [±SE] for the 5 containers per plant species (depicted by colours). Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale*, JV= *Jacobaea vulgaris*. Control (100% sterilised) soil is depicted in black. Results of an ANOVA testing for differences between the conditioning species are indicated by asterisks * p<0.05, **p<0.01, ***p<0.001. Significant differences between the control and monoculture soil based on a Dunett post hoc are depicted as + with the colour indicating the species.

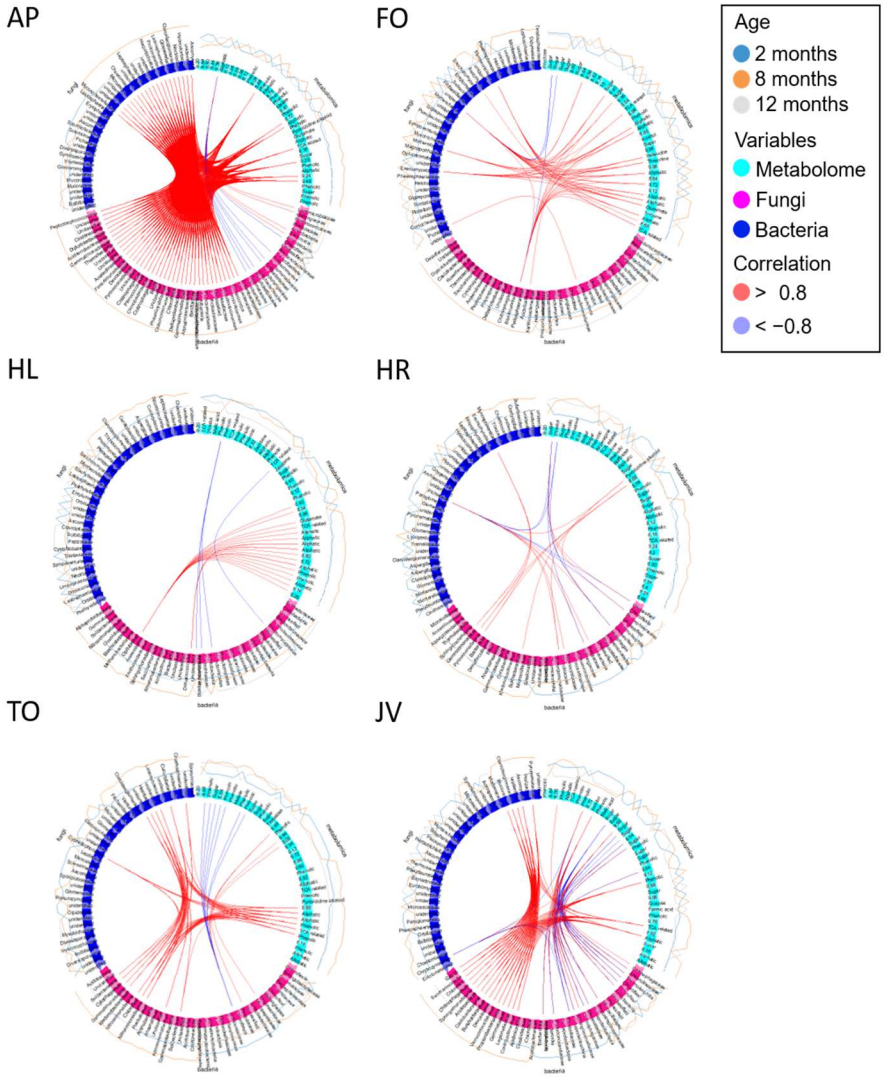

Figure S4.8 Circos plots visualising the correlation between bacterial and fungal communities in the soil and metabolomic changes in the plants grown in these soils for each monoculture. Displayed are correlations that are higher than 0.8 (red) or lower than -0.8 (blue). Lines outside the circles show the concentration of each compound in the plants in the three rounds. For the bacteria and the fungi, the abundance of each OTU in each round is displayed. Correlations within each measured community (metabolome, bacteria, fungi) are not depicted. Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale*, JV= *Jacobaea vulgaris*.

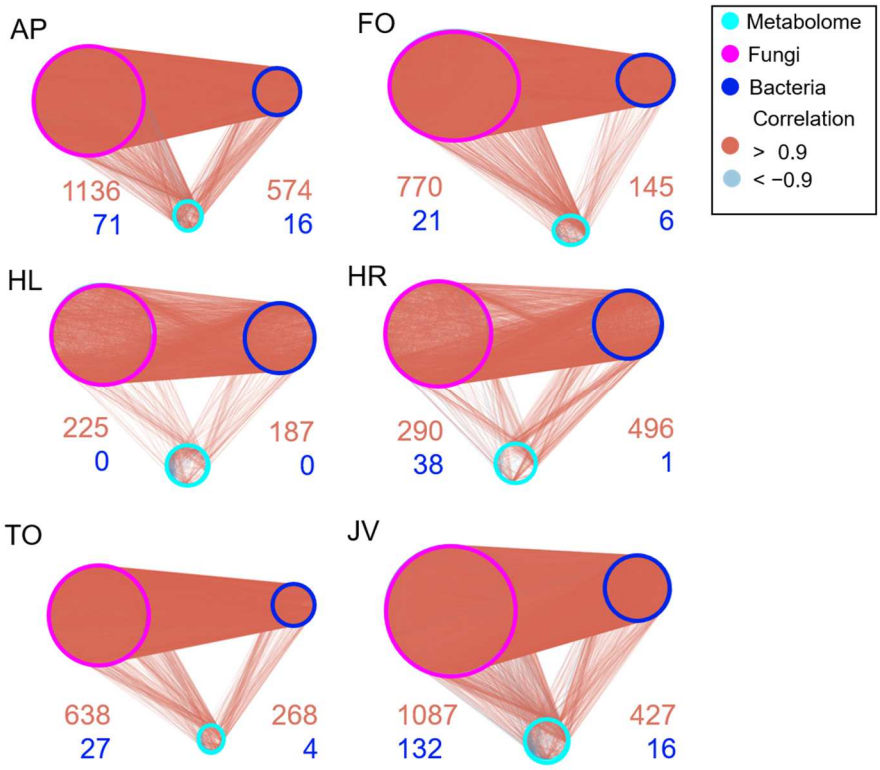

Figure S4.9 Correlation networks between bacteria and fungi in the soil and the metabolome of *Jacobaea vulgaris* for each monoculture separately. The networks are based on data from the three rounds (2,8,12 months) combined. Depicted are only correlations with correlation coefficients higher than 0.9 or lower than -0.9. Blue lines indicate negative correlations with correlation coefficients lower than -0.9 and red lines depict positive correlation with correlation coefficients higher than 0.9. Numbers depict the number of positive and negative correlations between bacteria or fungi and the metabolome. Plant species abbreviations are AP= *Alopecurus pratensis*, FO= *Festuca ovina*, HL=*Holcus lanatus*, HR= *Hypochaeris radicata*, TO= *Taraxacum officinale*, JV= *Jacobaea vulgaris*.

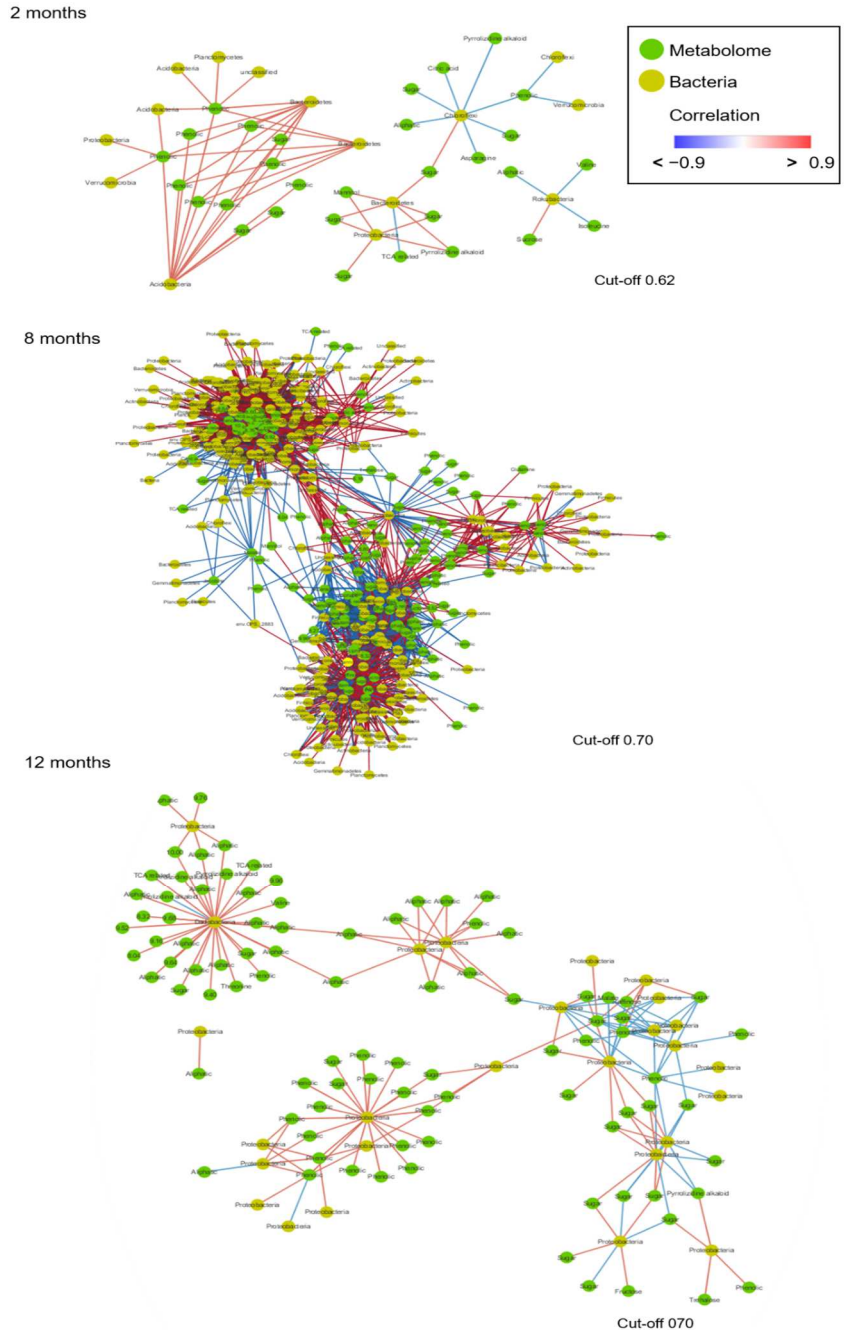

Figure S4.10 Relevance network visualising bacteria in the soil and compounds within the plant. The features used in this network were selected through Partial Least Squares (sPLS). Networks were constructed for each round separately. Each network displays correlations higher (red) or lower (blue) than the indicated cut offs (blue).

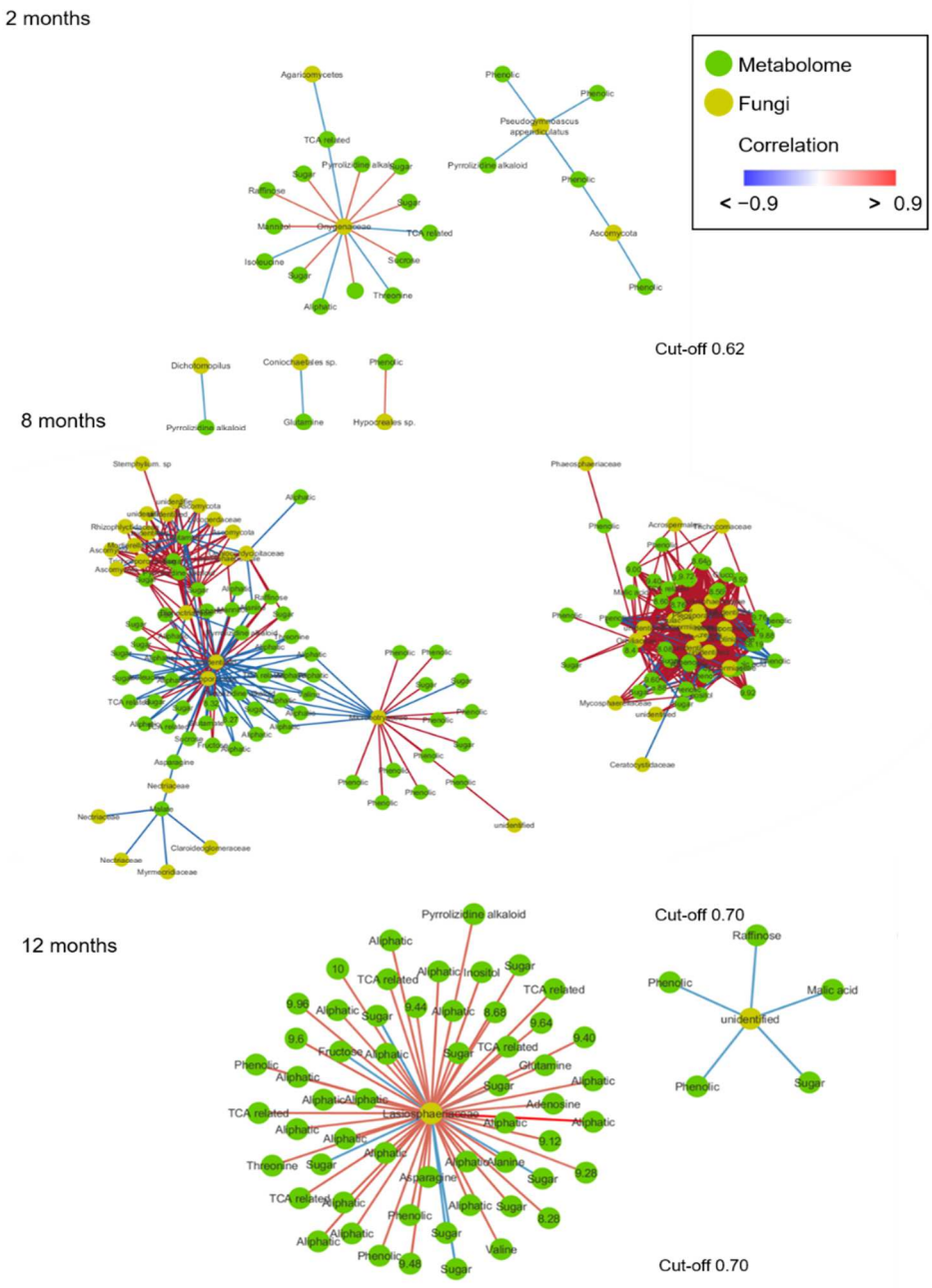

Figure S4.11 Relevance network visualising fungi in the soil and compounds within the plant. The features used in this network were selected through Partial Least Squares (sPLS) . Networks were constructed for each round separately. Each network displays correlations higher (red) or lower (blue) than the indicated cut offs (blue).

Table S4.1 Practical details about the experiment. Displayed are the dates of collection of the soil, planting of *Jacobaea vulgaris*, harvest for the plant soil feedback test and the collection dates of samples for microbial communities and abundance measurement.

	Plant soil feedback test			Microbial measurements	
Time	Collection of soil	Planting	Harvest	Collection of soil for microbial community	Collection of soil for abundance (qPCR)
1		No test		No sampling	01.06.2017
2	07.07.2017	17.07.2017	22.08.2017	03.07.2017	03.07.2017
3		No test		No sampling	03.08.2017
4	08.09.2017	20.09.2017	23.10.2017	No sampling	09.09.2017
5		No test		No sampling	09.10.2017
6		Discarded		07.11.2017	07.11.2017
7		No test		No sampling	07.12.2017
8	05.01.2018	12.01.2018	26.02.2018	No sampling	11.01.2018
9		No test		No sampling	06.02.2018
10	09.03.2018	16.03.2018	20.04.2018	No sampling	No sampling
12	06.05.2018	14.05.2018	18.06.2018	08.05.2018	08.05.2018

Table S4.2 Explained variance and the significance (permutation tests with 999 permutations) from a redundancy analysis (RDA) testing for the relationship between weight gain and consumed leaf area and the metabolome composition of *Jacobaea vulgaris* for all rounds together and for each of the three rounds separately. * indicates significance at P<0.05.

Round	Variable	Explained variance (%)
	Larval weight gain (WG)	2.04
	Consumed area (CA)	1.67
2	WG	2.97
	CA	4.27
8	WG	7.72
	CA	27.20*
12	WG	2.18
	CA	1.42

Table S4.3 Results of permutational multivariate analysis of variance (PERMANOVA) testing the effect of monoculture soil (*Holcus lanatus, Festuca ovina, Alopecurus pratensis, Hypochaeris radicata, Jacobaea vulgaris, Taraxacum officinale*) and round (2, 6, 12 months) on the bacterial and fungal composition of the soil. Further the effect of monoculture soils on the bacterial and fungal composition of the soil was tested for each round separately. The analyses are based on Bray-Curtis distances. Permutations were set to 999. Pseudo F-values, degrees of freedom (df), explained variance (R^2) and P-values are presented. Significant P-values are presented in bold.

		Bacteria			Fungi		
Round	Factor	F	R^2	P	F	R^2	P
	Round (R)	$F_{(2,68)}$=10.69	0.20	**0.001**	$F_{(2,69)}$=3.39	0.15	**0.001**
	Monoculture (M)	$F_{(5,68)}$= 1.81	0.08	**0.001**	$F_{(5,69)}$=5.28	0.09	**0.001**
	R*M	$F_{(10,68)}$= 1.13	0.10	0.10	$F_{(10,69)}$=1.83	0.16	0.10
2	M	$F_{(5,24)}$= 1.48	0.24	**0.001**	$F_{(5,24)}$=2.58	0.35	**0.001**
6	M	$F_{(5,23)}$= 1.37	0.23	**0.001**	$F_{(5,21)}$=3.02	0.42	**0.001**
12	M	$F_{(5,21)}$= 1.23	0.23	0.066	$F_{(5,24)}$=1.71	0.26	**0.001**

Table S4.4 Results of ANOVAs testing the effects of monoculture soil (*Holcus lanatus, Festuca ovina, Alopecurus pratensis, Hypochaeris radicata, Jacobaea vulgaris, Taraxacum officinale*) bacterial and fungal copies per gram of soil. Analysis were carried out for all rounds together and separately for each month. F-values, degrees of freedom (df) and P-values are presented. Significant P-values are presented in bold.

Time (months)	Variable	Bacteria (copies/gr soil)	Fungi (copies/gr soil)
	Round (R)	**$F_{(9,170)}$=12.93, P<0.001**	**$F_{(9,170)}$=4.43, P<0.001**
	Monoculture (M)	**$F_{(5,170)}$=3.08, P=0.002**	**$F_{(5,170)}$=4.34, P<0.001**
	R*M	**$F_{(45,170)}$=3.12, P<0.001**	$F_{(45,170)}$=1.24, P=0.17
1	M	$F_{(5,24)}$=0.64, P=0.68	$F_{(5,24)}$=1.04, P=0.42
2	M	$F_{(5,24)}$=0.41, P=0.84	$F_{(5,24)}$=0.40, P=0.84
3	M	$F_{(5,24)}$=0.38, P=0.86	$F_{(5,24)}$=1.89, P=0.13
4	M	**$F_{(5,24)}$=6.36, P<0.001**	$F_{(5,24)}$=0.54, P=0.74
5	M	$F_{(5,24)}$=2.27, P=0.08	$F_{(5,24)}$=2.57, P=0.05
6	M	**$F_{(5,24)}$=4.24, P=0.007**	**$F_{(5,23)}$=2.80, P=0.04**
7	M	**$F_{(5,24)}$=9.23, P<0.001**	$F_{(5,24)}$=1.14, P=0.37
8	M	$F_{(5,24)}$=2.36, P=0.071	**$F_{(5,24)}$=3.08, P=0.028**
9	M	$F_{(5,24)}$=1.85, P=0.14	$F_{(5,24)}$=1.54, P=0.21
12	M	$F_{(5,24)}$=1.04, P=0.42	**$F_{(5,24)}$=6.06, P<0.001**

Table S5 Results of ANOVA testing the effects of monocultures on soil abiotic characteristic for each. All rounds were analysed combined and for each round separately. Depicted are F and p values. The degrees of freedom were (5,24) for all separate tests. For the overall test the degrees of freedom were round 4, monoculture 5 and for the interaction 20 with residuals of 90. Significant p-values are presented in bold.

Factors		Round (R)	Monoculture (M)	R*M	M	M	M	M
Round					4	8	10	12
OM (%)	F	**2.84**	**4.46**	**2.91**	**3.04**	0.66	2.48	2.21
	p	**0.03**	**0.001**	**<0.001**	**0.030**	0.66	0.062	0.088
pH	F	0.811	**3.00**	0.74	0.51	**2.91**	0.72	0.44
	p	0.522	**0.02**	0.77	0.77	**0.035**	0.62	0.82
Fe (mg/kg)	F	**12.51**	**2.46**	1.46	0.94	2.41	**4.93**	0.83
	p	**<0.001**	**0.04**	0.12	0.47	0.067	**0.003**	0.54
K (mg/kg)	F	0.23	**2.46**	**3.22**	**14.66**	**5.48**	**5.92**	**3.86**
	p	0.92	**0.04**	**<0.001**	**<0.001**	**0.002**	**0.001**	**0.011**
Mg (mg/kg)	F	0.15	0.89	1.06	1.12	2.06	**3.45**	2.13
	p	0.96	0.49	0.40	0.38	0.11	**0.018**	0.097
NO_3^-(mg/kg)	F	1.23	**2.85**	**2.64**	**4.56**	1.01	2.23	0.72
	p	0.30	**0.02**	**<0.001**	**0.005**	0.44	0.086	0.62
NH_4^+(mg/kg)	F	0.23	**9.11**	0.75	1.83	1.44	0.36	2.15
	p	0.92	**<0.001**	0.76	0.15	0.25	0.87	0.095
P(mg/kg)	F	0.98	**4.87**	**1.66**	1.68	1.12	**4.12**	**3.32**
	p	0.42	**<0.001**	**0.06**	0.18	0.38	**0.008**	**0.020**
S(mg/kg)	F	**2.79**	**6.74**	**3.60**	**5.66**	2.20	0.95	0.96
	p	**0.03**	**<0.001**	**<0.001**	**0.002**	0.088	0.47	0.46
Zn(mg/kg)	F	0.25	0.73	0.578	1.11	1.64	**3.85**	1.28
	p	0.91	0.601	0.92	0.38	0.19	**0.011**	0.30

Supplementary methods

Per container, for molecular analysis, six soil cores were collected (12 cm deep, 7 mm diameter) and homogenized. After that the soil samples were stored immediately at -20°C until they could be processed. 0.75 g or soil per container was weighed and extracted with the PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany). Then the DNA quantity was evaluated with a Nanodrop spectrophotometer (Thermo Scientific, Hudson, NH, USA). After that we used approximately 100 ng of DNA for the PCR. We used the primers ITS4ngs and ITS3mix targeting the ITS2 region of fungi (Tedersoo et al., 2015) and the primers 515FB and 806RB (Apprill, McNally, Parsons, & Weber, 2015; Caporaso et al., 2012; Parada, Needham, & Fuhrman, 2016) targeting the V4 region of the 16S rRNA gene in bacteria. The presence of PCR product was checked with an agarose gel electrophoresis. The PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). With Nextera XT DNA library preparation kit set A & B (Illumina, San Diego, CA, USA), adapters and barcodes were added to the samples. Then the final PCR product was purified with AMPure beads and checked it with an agarose gel electrophoresis. It was then quantified with a Nanodrop spectrophotometer. Subsequently, the samples were equimolar pooled. All fungal samples (180) were pooled in one sequencing run. Bacterial samples were divided into two separate runs with 90 samples in each run. A mock community which contained ten fungal species as a control for the bioinformatics analysis, was used. Illumina MIseq PE 250 at McGill University and Genome Quebec Innovation Center was used to sequence libraries. Extraction negatives were used and further sequenced.

Quantitative PCR was used to determine the abundance of bacteria and fungi. qPCR was determined every month, up to 9 months and after 12 months (for sampling details see Supplementary Table S 4.1). To have similar amplification from all soils and to enhance the reaction T4 Gene 32 protein (Roche, Basel, Switzerland) was used. With a robot (Gorbett Research, Sydney, Australia) the reaction mixtures were dissolved in 20 µl volume that contained 0.3 µM of each primer, 0.25 µl T4 and 1.0-10.0 ng template DNA. The cycling conditions for fungi were: 40 sec at 95 °C, 1 min at 55 °C and 1 min at 72 °C and for bacteria 40 sec at 95 °C, 1 min at 53 °C and 1 min at 72 °C. Samples were analysed with a Rotor-Gene 3000 machine (Gorbett Research, Sydney, Australia). A serial dilution of plasmids extracted

from the Rotor-Gene 3000 machine was used as reference to calculate the copy numbers. Part of these data was already published in a previous paper of our group (Hannula, et al., 2019a).

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