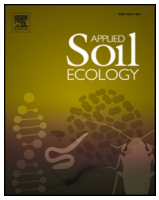
Applied Soil Ecology 190 (2023) 105012

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil



Pinpointing the distinctive impacts of ten cover crop species on the resident and active fractions of the soil microbiome

Sara G. Cazzaniga [a,b], Lize Braat [a,c], Sven van den Elsen [a], Carin Lombaers [b], Johnny Visser [d], Lia Obinu [a,e], Jose G. Maciá-Vicente [f], Joeke Postma [b], Liesje Mommer [g], Johannes Helder [a,*]

[a] Laboratory of Nematology, Wageningen University & Research, P.O. Box 8123, 6700 ES Wageningen, the Netherlands
[b] Biointeractions and Plant Health, Wageningen University & Research, P.O. Box 16, 6700 AA Wageningen, the Netherlands
[c] Plant Breeding, Wageningen University & Research, P.O. Box 384, 6700 AJ, Wageningen, The Netherlands
[d] Field Crops, Wageningen University & Research, Edelhertweg 1, 8219 PH Lelystad, the Netherlands
[e] Department of Agricultural Sciences, University of Sassari, Viale Italia 39A, 07100 Sassari, Sardinia, Italy
[f] Plant Ecology and Nature Conservation Group, Wageningen University & Research, P.O. Box 47, 6700 AA Wageningen, the Netherlands
[g] Forest Ecology and Forest Management Group, Wageningen University & Research, P.O. Box 47, 6700 AA Wageningen, the Netherlands

ARTICLE INFO

Keywords:
Rhizosphere
Marigold
Brassicaceae
Fabaceae
Poaceae
Boraginaceae

ABSTRACT

Cover crops are used in agriculture to minimize soil erosion, prevent nutrient leaching and increase soil organic matter content. Cover crops can also be grown to stimulate the soil microbial community to improve soil biological conditions. Despite their widespread use, little is known about the impact of different cover crop species on the composition and activity of the soil microbiome. Here we investigate the effect of distinct cover crop species on the rhizosphere microbiome and characterize both the resident (ribosomal (r)DNA-based) and the potentially active (rRNA-based) fractions of the bacterial, fungal, protist and metazoan communities in the cover crops rhizosphere. We conducted a field experiment using 70-l bottomless containers in which we grew ten monocultures of commonly used cover crop species belonging to five plant families, and an unplanted control treatment (fallow). The total DNA and RNA were extracted from soil and the bacterial, fungal, protistan and metazoan communities were characterized using Illumina MiSeq sequencing. We found that all cover crop species significantly impacted the resident and the potentially active microbial communities in their rhizospheres. Cover crops exerted distinct selection strengths on the native microbial communities. For individual cover crops, the impacts on the resident and the potentially active microbial communities differed while showing similar overall tendencies. Oilseed radish (Brassicaceae) was shown to provoke the strongest microbial shifts, in part attributable to a promotion of the bacterial family Pseudomonadaceae and a repression of Microascaceae in the rhizosphere. Lentil (Fabaceae) induced a widespread stimulation of fungal taxa, including Trichocomaceae and fungal members of the Glomerales order, whereas black oat and hybrid ryegrass (both Poaceae) gave rise to relatively mild changes in the soil microbial communities. Analyses of rRNA-based rhizobiome data revealed that, except for phacelia, all cover crops induced an increase in microbial network complexity as compared to the fallow control. Data presented here provide a broad baseline for the effects of cover crops on four organismal groups, which may facilitate future cover crop selection to advance soil health.

1. Introduction

From the 1960s onwards, agricultural intensification has led to higher and more stable crop yields with a fraction of the labour inputs previously needed (Normile and Mann, 1999). However, intensive agriculture also carries negative effects on soil health, including degradation of the physical, chemical and biological properties of soils (Banerjee et al., 2019; Tsiafouli et al., 2015). A recent report states that 60–70 % of the soils within the European Union are classified as unhealthy as a result of current agricultural practices (Veerman et al., 2020). Reconsideration and adjustments of these practices are needed to reverse this undesirable phenomenon. It is noted that 'soil health' is a broad term, which has been defined as "the continued capacity of soil to function as a living ecosystem that sustains plants, animals, and people"

* Corresponding author.
E-mail address: hans.helder@wur.nl (J. Helder).

https://doi.org/10.1016/j.apsoil.2023.105012
Received 8 February 2023; Received in revised form 16 May 2023; Accepted 10 June 2023
Available online 21 June 2023

(https://www.nrcs.usda.gov/wps/portal/nrcs/main/soils/health/). Here we will use this term in a narrower sense focusing on the capacity of soil biota to sustain plant growth and development.

A number of soil management practices have been shown to be effective in improving soil health while maintaining acceptable crop production levels (Eyhorn et al., 2019; Schrama et al., 2018; Vukicevich et al., 2016). Among these, cover cropping - the cultivation of fast-growing non-economic plants between the harvest of the main crop and the sowing of the next main - is implemented to minimize nutrient leaching and soil erosion, and to increase the soil organic matter content (Blanco-Canqui et al., 2015; Kaye and Quemada, 2017; Wick et al., 2017). Cover crops also may have a positive effect on the biological condition of soils as they boost and shift the activity and abundance of soil microbes (Kim et al., 2020; Vukicevich et al., 2016; Wick et al., 2017). Potential downsides of cover crops include their potential function as reservoirs for pests and pathogens as cover crops may facilitate them to bridge a non-favourable period in their life cycle (Bakker et al., 2016; Walder et al., 2017), and their action as weeds in the next cropping season (Wayman et al., 2015). However, as long as these risks are mitigated, many plant species can be considered for cover cropping.

Most of the currently used cover crops belong to the plant families Poaceae, Brassicaceae and Fabaceae (Griffiths et al., 2022; Vukicevich et al., 2016), and a trend toward further diversification has been observed including members of the families Boraginaceae and Asteraceae (*e.g.*, Elhakeem et al., 2021). Cover crops of the same plant family tend to have similar ecosystem functions, *e.g.,* grasses typically decrease soil density, brassicas increase microporosity, and legumes promote aggregate stability (Hudek et al., 2022; Tribouillois et al., 2015), and these characteristics co-determine cover crop choice.

Plants exert a selective effect on soil bacterial and fungal communities, and the altered rhizobiome might result in improved plant nutrient uptake and increased pathogen suppression (Berendsen et al., 2012; Doornbos et al., 2012). Such a combination of increased levels of plant-absorbable nutrients as well as an improved resistance against biotic and abiotic stresses is here labelled as microbiome-mediated soil health promotion. During vegetative growth, up to 40 % of the carbon fixed by plants is released into the rhizosphere through root exudates (Bais et al., 2006; Bonkowski, 2004) which directly modulates the microbial community associated with the roots (Badri and Vivanco, 2009; Berendsen et al., 2012). The steering of the local microbiome by plants is largely dictated by the composition of these exudates (Berg and Smalla, 2009; Pascale et al., 2019), which, in turn, is largely determined by plant phylogeny. Although representatives of plant families show similarities in root exudate composition, even at species and subspecies levels genotype-specific rhizodeposits have been reported (Micallef et al., 2009; Schlaeppi et al., 2014; Yeoh et al., 2017). As previously reported for several crops and model plants (Cloutier et al., 2023; Tkacz et al., 2015; Turner et al., 2013; Uksa et al., 2014), also the selection strength, *i.e.*, the extent by which plants shape their rhizosphere by promoting and/or repressing fractions of the soil microbiome, varies per plant species. To the best of our knowledge, selection strengths of individual cover crops on the soil microbiome has never been compared.

Next to bottom-up selection by plant exudates, the bacterial and fungal communities in the rhizosphere are co-shaped by the top-down selection due to the activity of major consumers of these communities, protists and metazoans (Gao et al., 2019; Mielke et al., 2022). In temperate agricultural systems, primary consumers-biomass in the top layer of arable fields is typically 40 to 100 times smaller than the bacterial and fungal biomass (Pausch et al., 2018). In the rhizosphere, the increased abundance and activity of bacteria and fungi attracts bacterivorous protists and metazoa, including bacterial- and fungal-feeding nematodes (Bonkowski, 2004). Here, trophic interactions become a driving force co-determining the microbiome assembly and activity (Gao et al., 2019). Therefore, major categories of primary consumers, protists and metazoans, should be taken along to achieve a proper understanding of the shaping of the rhizobiome.

Although rhizosphere communities have been characterized for a substantial number of plant species (Fitzpatrick et al., 2018; Yadav et al., 2018), our knowledge of the microbial signatures of cover crop species is rather crude. Previous studies, such as the ones by Bacq-Labreuil et al. (2019), Finney et al. (2017) and Gkarmiri et al. (2017) illustrate the ability of cover crop species to affect the soil microbiome assembly and activity, and in particular how different functional groups, such as arbuscular mycorrhiza and saprophytic fungi, respond to the presence of different cover crop species. However, most studies present data at a high taxonomic level, consider one or a few cover crop species only and are seldomly focused on the rhizosphere microbiome.

A substantial part of the soil microbial community is known to be dormant (Fierer, 2017). This 'microbial seed bank' as it was referred to by Lennon and Jones (2011) may comprise up to 80 % of the cells and about 50 % of the taxa in bulk soils and is here referred to as the resident fraction. Taking along the active fraction of the soil microbiome is informative as this fraction is responsible for its actual ecological functioning. Ribosomal DNA (rDNA) and rRNA-based community profiling are used to characterize the resident and active microbial fractions, respectively, but regarding the latter, some caution is justified. Dormant soil biota might harbour high numbers of ribosomes, as for instance was shown for spores of several *Bacillus* species (Filion et al., 2009). Hence, it is preferred to refer to rRNA-based communities as potentially active fractions, rather than active fractions (Blazewicz et al., 2013). Recent studies underlined the relevance of including the potentially active fractions of the microbiome (Bay et al., 2021; Harkes et al., 2019; Ofek et al., 2014).

Here, we present a field experiment in which we characterized the impact of ten commonly used cover crops (including representatives from five distinct plant families) on both the resident and the potentially active fractions of the bacterial, fungal, protistan and metazoan communities in the rhizosphere. We hypothesised that the presence of cover crops would induce changes in the composition, activity and interactions of the rhizosphere microbial community as compared to the fallow control. Furthermore, we expected to see cover crop-specific, and - to a lesser extent - plant family-specific effects on the resident and potentially active microbial fractions of all four main organismal groups. The question of the overall impact of individual cover crops on the soil microbiome was addressed both quantitatively and qualitatively: (1) do cover crop species differ in the extent by which microbial taxa in the rhizosphere are promoted and/or repressed? and (2) do cover crop species differ in the kind of microbial taxa that they promote and/or repress in the rhizosphere? Lastly, we generated microbial networks on the basis of the active fractions of the organismal groups and (3) asked ourselves whether cover crops had differential effects on the level of associations between organismal groups.

A better understanding of the specific microbial signatures of cover crops will contribute to cover crop applications beyond the current, general, scope. The insights presented in this paper might be considered a first step toward the selection of cover crops to steer the soil microbial community in such a way that they contribute to the restoration of soil health.

2. Materials and methods

2.1. Experimental design

A field experiment was carried out at the Wageningen University and Research experimental farm 'Vredepeel', located in the southeast of the Netherlands. The experiment consisted of 11 treatments, including ten cover crop species and an unplanted control (fallow), each replicated eight times. Cover crop treatments included widely used cover crop cultivars, as well as a prospective oilseed radish cultivar referred to as E1039. Bottomless containers (70 l; ∅ 55 cm, height 43 cm) were randomly positioned in eight blocks, and hence the total experiment included 88 containers. These were dug into the field in such a way that

there was no height difference between the soil surface in the buckets and the surrounding soil. The containers were filled with topsoil (20 cm) originating from subplots of a nearby long-running field experiment 'Soil Health Experiment' (SHE) (Korthals et al., 2014). Each block of containers was filled with the topsoil originating from one of the SHE subplots. Half of the SHE subplots were managed following organic practices and the other half following conventional practices until 2017. It is noted that all subplots received the same soil management in the two years before this field experiment (a single application of cattle slurry per year) (field management data provided in Suppl. Table 1). Barley was the last main crop grown on all plots between March and June 2019. Barley crop remains were incorporated into the soil at the beginning of June 2019, and the topsoil was collected from the field at the end of June 2019 to be transferred to the containers for this experiment. In each block, ten containers were sown with single cover crop cultivars (Table 1), and one container was kept fallow. Cover crop seeds were sown at the end of July 2019 (sowing densities shown in Table 1), and weeds were removed manually during the duration of the experiment. In 14 containers cover crop growth was negatively affected by drought in the late summer of 2019 (weather data provided in Suppl. Table 1) and excluded from the experiment. Thus, 74 of the original 88 containers were sampled at the end of the experiment (list in Suppl. Table 3).

2.2. Soil sampling

Soil samples were collected on the 3rd of October 2019, approximately two and a half months after the sowing of cover crops. For this, 2–20 plants (depending on the plant size and root system) were randomly collected from each container, uprooted and shaken to discard non-rhizosphere soil. Plant samples were transported to the nearby laboratory, where rhizosphere soil was collected by brushing off the soil adhering to the roots (see Suppl. Table 2 for number of plants used, and average and total plant dry weight). Fallow soil from the control container was collected with an auger (15 mm ∅ × 20 cm depth). Three cores were sampled for each fallow container, and after thorough mixing and sieving (mesh size 5 mm), a subsample of 10 g was collected. Rhizosphere and fallow soil samples were transferred to clean Ziplock plastic bags, snap-frozen in N_2 (l), kept on dry ice during transport and subsequently stored at −80 °C at the Laboratory of Nematology.

2.3. Nucleic acids extraction and sequencing

Soil total DNA and RNA were extracted simultaneously following a protocol optimised for 2 g soil (Harkes et al., 2019). This extraction method comprises bead beating, precipitation of humic acids with an ammonium aluminium sulphate solution, and phenol-chloroform extraction. cDNA was synthesised from the extracted RNA using a Maxima First Strand cDNA Synthesis Kit for RT-PCR (Fermentas, Thermo Fisher Scientific Inc., USA) following the manufacturer's instructions. In preparation for the first step of the library construction, DNA and cDNA samples were diluted to 1 ng μl^{-1} and 0.1 ng μl^{-1}, respectively. Following Harkes et al. (2019), the library was generated in a two-step PCR procedure. The first step consisted of the amplification of organismal group-specific 16S and 18S rRNA regions. To this end, locus-specific primers extended with an Illumina read area and an appropriate adapter were employed that targeted the V4 region of 16S of bacteria, and the V9, V7-V8, V5-V7 of 18S of protozoa, fungi and metazoa, respectively (Suppl. Table 4). For PCR amplification, 3 µl of the diluted samples were used as templates. PCR was carried out with the following temperature profile: 3 s at 95 °C; followed by 39 cycles of 10 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C; and a final extension step of 5 s at 72 °C. All reactions in the first PCR step were done in triplicate, and PCR products were pooled per sample and organismal groups. The second PCR step was performed using 40× dilutions of the amplicons from the first PCR as templates. The second PCR was used to attach the sample-specific Illumina index combination, used for multiplexing the samples upon pooling, and the Illumina sequencing adapter to the amplicons of the first PCR. The following temperature profile was employed: 3 s at 95 °C; followed by 10 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C; and a final extension step of 5 s at 72 °C. A random selection of products of the first and second PCR steps was checked on an agarose electrophoresis gel to ensure that the amplifications were successful in producing amplicons of the expected size. Lastly, PCR products were pooled together and sent out for sequencing to Useq (Utrecht Sequencing Facility, Utrecht, The Netherlands). Illumina MiSeq sequencing was performed using a 2 × 300 bp V3 kit.

2.4. Pre-processing of the sequencing data

Sequencing data were demultiplexed and subset into the four organismal groups based on their locus-specific primer sequences, using a custom Python script. Sequencing reads were pre-processed in QIIME2 (Bolyen et al., 2019). Read picking was carried out with the QIIME2 DADA2 denoising algorithm (Callahan et al., 2016) separately for each organismal group. For bacteria, protists and fungi, paired-end reads were merged and used to generate amplicon sequence variants (ASVs) (Callahan et al., 2017). For metazoa, only the forward reads were used to generate ASVs due to the short average read length of the reverse reads.

Taxonomic assignment of ASVs was carried out using the q2-feature-classifier plugin and *classify-sklearn* function (Pedregosa et al., 2011), with organismal-group-specific pre-trained reference databases. For bacteria, fungi and metazoa, the non-redundant SILVA database (Glöckner et al., 2017) (*silva-138-ssu-nr99-seqs-derep-uniq*, version 138, 99 % identity criterion) was pre-trained to generate amplicon-region specific classifiers. For the taxonomic assignment of protists, the pr2 reference database (Guillou et al., 2012) was used to build the pre-trained amplicon-region-specific classifier. QIIME2 output files were imported in R as 'phyloseq objects' with the function *import_qiime* of the phyloseq package (v1.34.0) (McMurdie and Holmes, 2013). The

Table 1
Details of the cover crop species used in this study, including taxonomic affiliation, the origin of seeds, and sowing density. A selection of the most commonly used cover crop species was made on the basis of several relevant papers including Bacq-Labreuil et al. (2019), Hooks et al. (2010), Wick et al. (2017), Zhang et al. (2022) and Zukalová and Vasak (2002).

Common name	Family	Species	Cultivar	Company	Sowing density kg/ha
Vetch	Fabaceae	*Vicia sativa*	Amelia	Joordens Zaden (NL)	125
Lentil		*Lens culinaris*	Eston	Joordens Zaden (NL)	120
Oilseed Radish	Brassicaceae	*Raphanus sativus var. oleiformis*	Terranova	Joordens Zaden (NL)	30
Oilseed Radish		*Raphanus sativus* var. *oleiformis*	E1039	Joordens Zaden (NL)	30
Black oat	Poaceae	*Avena strigosa*	Pratex	PH Petersen (DE)	80
Tall fescue		*Festuca arundinacea*	Firecracker	Barenbrug (NL)	35
Hybrid ryegrass		*Lolium hybridum*	Daboya	Vandinter Semo (NL)	45
Phacelia	Boraginaceae	*Phacelia tanacetifolia*	Beehappy	DSV-zaden (NL)	10
Borage		*Borago officinalis*	Wild type	Nebelung (DE)	50
Marigold	Asteraceae	*Tagetes patula*	Ground control	Takii Europe (NL)	8

phyloseq package was also used to process the phyloseq objects prior to the statistical analysis. Unassigned ASVs and ASVs assigned to chloroplasts and mitochondria as well as to non-target organisms were filtered out using the function *subset_taxa*. Samples with an unacceptably low number of reads (<1000) were filtered out using the *prune_samples* function. In the R package metagMisc (v0.0.4) (Mikryukov, 2018) the function *phyloseq_filter_prevalence* was used to filter out singletons and ASVs with <10 reads in the whole dataset.

2.5. Microbiome diversity and composition

ASV diversity and richness of each cover crop rhizosphere and fallow were determined for each organismal group using rarefied ASV tables. Rarefying was performed in the phyloseq R package using the function *rarefy_even_depth* with the options 'without replacement' and 'to the minimum library size'. The function *alpha* of th microbiome R package (v.1.12.0) (Lahti and Sudarshan, 2012–2019) was used to calculate Observed, Chao1 and Shannon diversity and richness metrics. The non-parametric Kruskal Wallis and Wilcoxon post-hoc tests with Bonferroni correction for multiple testing were used to pinpointing significant differences in alpha diversity scores among cover crop species.

Differences in microbial community structure across cover crops were calculated by constructing dissimilarity matrices with the Bray-Curtis Distance metric on the non-rarefied normalised ASV tables. Normalisation was carried out on the ASV tables using the Cumulative Sum Scaling (CSS) method (*cumNorm* function from R package metagenomeSeq v. 1.32.0) (Paulson et al., 2013). The results of the distance metrics were visualized in Principal Coordinate Analysis (PCoA) graphs for each organismal group, built with the function *plot_ordination* implemented in the phyloseq R package. The factors explaining the dissimilarities among the microbiomes of the different treatments were tested using PERMANOVA (*adonis2* function from the vegan package, Oksanen et al. (2013). The factors included in the PERMANOVA were 'subplot' (to account for block effects), 'nucleic acid' (to account for the difference between DNA and cDNA), and 'treatment' (to account for the effect of the cover crop treatments). To make comparisons between cover crop's rhizosphere and fallow and across cover crop's rhizospheres, pairwise PERMANOVAs (pairwise adonis) were carried out based on Bray-Curtis multivariate distances with Benjamini-Hochberg correction for multiple testing and 999 permutations. The R^2 values resulting from the pairwise contrasts of each cover crop species *versus* the fallow control, were used as a proxy for the selection strength exerted by individual cover crops on the native microbial community at the level of the rhizosphere.

The taxonomic composition of the cover crop's microbiome was visualized with stacked bar plots, generated using the function *plot_composition* of the microbiome R package. The plots show the relative abundance per cover crop treatment of the most abundant microbial families (bacteria and fungi) or orders (metazoa and protists), while taxa represented by <2 % of all reads were grouped in the "Other" category.

2.6. Differential abundance analyses of rhizospheric microorganisms by ANCOM-BC

A Differential Abundance analysis (DA) of microbial taxa was performed with analysis of compositions of microbiomes with bias correction (ANCOM-BC) in R (ANCOMBC R package) (Lin and Peddada, 2020). The test aimed to pinpoint the differential effect of each cover crop on the fallow soil in the generation of each of the cover crops' rhizospheres. Cover crops species and block were the covariates of interest, while the fallow soil (control) was used as the reference level. ANCOM-BC was performed at the family level for bacteria and fungi, and at the order level for protists and metazoa. To do this, AVS tables were agglomerated to the desired taxonomic level in with *tax_glom* function of the phyloseq R package. The beta coefficients resulting from the linear regression indicated depletion (negative values) or enrichment (positive values) of the differentially abundant taxa in the rhizosphere of each cover crop compared to the fallow. Results of the ANCOM-BC test allowed us to generate rhizospheric profiling per each cover crop species. Rhizospheric profiling were plotted in heatmaps, and associated dendrograms were generated based on the Euclidean distance. Dendrograms indicated the distance between cover crop species based on the value of beta coefficients as a function of the abundance of each differentially abundant taxon.

2.7. Microbial networks based on potentially active rhizospheric communities

Network analyses were performed to pinpoint the correlations among members of the active microbiome fraction in the rhizosphere of the ten cover crops and the fallow treatment. A co-occurrence analysis was carried out with SparCC (Friedman and Alm, 2012) for each treatment in R software (*sparcc* function from SpiecEasi R package (Kurtz et al., 2021). To run SparCC, the ASV tables and the taxonomy tables were agglomerated to family (bacteria and fungi) or order (protists) levels to reduce the network complexity. For each SparCC analysis, the statistical significance of the inferred correlations was assessed by computing a bootstrap value (function sparccboot of SpiecEasi R package). Statistically significant ($p < 0.05$) SparCC correlations with a value of >0.6 or <−0.6 were included into the network analyses. The visualization of the network was initiated in R with the igraph package (Csardi and Nepusz, 2006), and then transferred to Cytoscape SW (Shannon, 2003) with the function createNetworkFromIgraph of the RCy3 R package (Gustavsen et al., 2019). Network statistics were analysed in Cytoscape. Higher values for network characteristics such as numbers of nodes and edges and average number of neighbours were considered a proxy for network complexity. Furthermore, the number of positive and negative connections across the different organismal groups, *i.e.*, bacteria, fungi, and protists was calculated in the form of a ratio (across groups connections/total connections) for fallow and cover crops rhizosphere to infer the effect of cover crops on the multitrophic interactions alone.

3. Results

3.1. Sequencing results

The sequencing of 74 DNA and 74 cDNA rhizosphere samples from ten cover crop species and the bulk soil (fallow) treatment resulted in a total of 24,823,255 reads from four organismal groups, *i.e.*, bacteria, fungi, protists and metazoa. After pre-processing and filtering, 6,082,172 (2,886,425 DNA and 3,195,747 cDNA) reads were retained and used for further analyses, of which 3,252,176 belonged to bacteria, 868,312 to fungi, 1,475,652 to protists and 486,032 to metazoa, with a median of 30,431 reads per DNA and 26,754 per cDNA sample. Samples featuring <1000 reads were filtered out from each organismal group's dataset (Table 2). Due to a high number of samples with a low number of reads in the metazoan cDNA dataset, the analyses of the metazoan community were based on DNA data only. 6631 ASVs were assigned to bacterial taxa, 258 to fungal taxa, 1812 to protistan taxa and 82 to metazoan taxa. For bacteria and fungi, the taxonomic resolution allowed for investigation of the communities up to the family level, whereas protists were mainly studied at the order level, as only a minority of ASVs were assigned to lower taxonomic levels. The metazoan community was only studied at the order level.

3.2. Factors affecting the rhizosphere community composition

The microbiome composition of the resident (DNA) and potentially active (cDNA) microbial communities significantly differed. PERMANOVA showed that the factor 'nucleic acid type' explained 12 % of the overall variation in both the bacterial and the fungal communities ($p <$

Table 2
PERMANOVA analysis with Bray-Curtis dissimilarity metric to assess the variation explained by cover crop treatment on the resident (DNA) and active (cDNA) communities separately, and on each of the four organismal groups.

Organismal group	Resident community (DNA)			Active community (cDNA)		
	R^2[a]	n[b]	p-Value	R^2[a]	n[b]	p-Value
Bacteria	0.40	73 (−1)	0.001	0.40	74	0.001
Fungi	0.44	74	0.001	0.51	70 (−4)	0.001
Protists	0.37	73 (−1)	0.001	0.37	74	0.001
Metazoa	0.33	68 (−6)	0.001	ND[c]		

[a] R^2 = fraction of the variation explained by the experimental factor cover crop.
[b] n = the number of samples included for the test. Between brackets, the number of samples removed due to low sequencing coverage is given. Dissimilarity is significant for *p*-values < 0.01.
[c] ND = not determined, metazoa community was only assessed at the DNA level (resident community).

0.001), and 13 % in the protist's community ($p < 0.001$) (Suppl. Fig. S1, Suppl. Table 5). Hence, resident microbial communities as well as the potentially active fractions thereof were analysed separately. In the next coming sections, we simplified and shortened the term 'potentially active fractions of the microbial community' to 'active fractions' solely to facilitate readability. The effect of blocks was significant (PERMANOVA < 0.001) but limited as it explained only 7.2, 6.4 and 8.4 % of the variation across the bacteria, fungal and protistan communities. For the metazoan community, however, the block effect was more prominent, as it accounted for 17.9 % of the overall microbiome variation (Suppl. Table 5).

Cover crop species had a major effect on the assembly of both the resident and active fractions of the rhizosphere microbiome for all four organismal groups, explaining 33 to 51 % (PERMANOVA, $p < 0.001$) of the overall variation (Table 2, Fig. 1). The smallest effect was observed for the resident metazoan community, whereas the strongest impact was detected for the active fraction of the fungal community (Table 2). Both oilseed radish cultivars showed the strongest effect on microbial communities across all cover crop species, as shown by clearly separate data clusters in PCoA ordinations (Fig. 1). In contrast, black oat, hybrid ryegrass, and marigold induced relatively mild shifts, as indicated by the proximity of these samples to the fallow control in the PCoAs (Fig. 1). These patterns were supported by the pairwise PERMANOVA analyses (Table 3). Table 3 shows levels of contrast between resident and active fractions of the bacterial and fungal communities in the rhizosphere of individual cover crops as compared to the microbial community

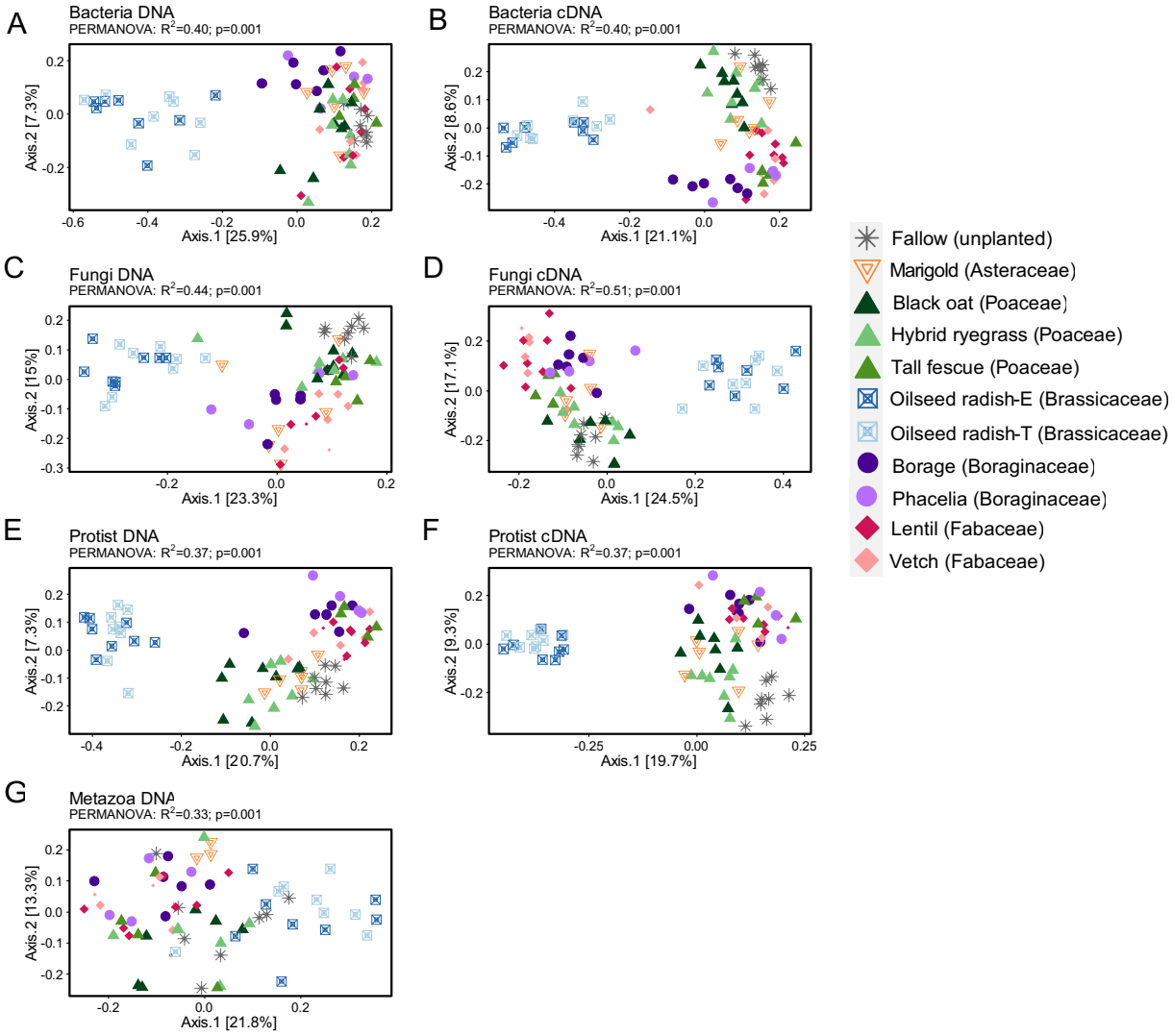


Fig. 1. Principal Coordinate Analysis (PCoA) of CSS normalised ASV data. Dissimilarity matrix built on Bray-Curtis metric and plotted separating ASVs based on cover crop treatment. Samples appear separated along the axes as cover crop treatment accounted for 19–26 % of variation along the principal PCoA axis and explained 33–51 % of the dissimilarity among sample groups (PERMANOVA, $p \leq 0.001$).

Table 3
Significant (adjusted $p < 0.05$) R^2 values based on comparisons between microbial communities in cover crop rhizospheres and fallow bulk soil calculated by pairwise-PERMANOVA based on Bray-Curtis distance with 999 permutations and Benjamini-Hochberg (BH) correction for multiple testing. This table is an excerpt from Supplementary Table 6 where all comparisons are shown. Cover crops are presented in order of their increasing effect on the resident bacterial community.

Cover crops	Contrasts with fallow					
	Resident bacteria	Active bacteria	Resident fungi	Active fungi	Resident protists	Active protists
Hybrid ryegrass	0.12	0.13	0.18	0.23	0.14	0.15
Black oat	0.13	0.16	0.12	0.17	0.16	0.20
Marigold	0.14	0.16	0.31	0.36	0.15	0.20
Lentil	0.16	0.24	0.35	0.46	0.16	0.21
Vetch	0.20	0.21	0.36	0.43	0.18	0.23
Phacelia	0.20	0.27	0.29	0.41	0.25	0.25
Tall fescue	0.21	0.29	0.25	0.32	0.26	0.26
Borage	0.27	0.34	0.35	0.44	0.23	0.25
Oilseed radish T	0.42	0.37	0.42	0.49	0.36	0.43
Oilseed radish E	0.44	0.41	0.52	0.51	0.35	0.40

assembly in the fallow control. As can be seen in Fig. 1 and based on the R^2 values presented in Table 3, the extent by which plants shape their rhizobiome, hereafter referred to as selection strength, is cover crop species-specific. Oilseed radish-E and -T (Brassicaceae) showed the highest selection strength on all three organismal groups (R^2 values: 0.36–0.52, Table 3). Hybrid ryegrass and black oat generally had the lowest selection strength on all organismal groups (R^2 values: 0.12–0.23). It is noted that tall fescue, another representative of the Poaceae, had a selection strength close to the one of phacelia (Table 3). On average, the impact of cover crops on the active fraction of the microbial communities was stronger than on the resident community. This was most prominent for the fungal community with average (for all cover crop species studied) R^2 values of 0.32 and 0.38 for the resident and the active communities respectively.

3.3. Impact of cover crops on the microbial richness and diversity in the rhizosphere

To assess the effects of individual cover crops on the ASV richness and diversity, three diversity indices were used (Observed, Shannon and Chao1). All three indices identified vetch, tall fescue, lentil, phacelia and borage as cover crops associated with resident and active bacterial diversities that exceeded the diversity in the fallow controls. The two oilseed radish cultivars showed alpha diversities lower than the fallow controls (Suppl. Table 7-A, B).

As compared to the bacterial communities, cover crops had a milder effect on the fungal richness and diversity indices. The four representatives of the families Fabaceae and Boraginaceae, vetch, lentil, borage and phacelia, as well as tall fescue (Poaceae), were associated with more diverse resident and active fungal communities compared to fallow by the indices Observed and Chao1 (Wilcoxon post-hoc with Bonferroni correction $p < 0.05$). Both for the resident and the active fractions, the Shannon index did not reveal significant differences between the fallow control and the aforementioned cover crops (Suppl. Table 7-C, D).

Also for the protists, vetch, lentil, borage and phacelia, as well as tall fescue were often associated with microbiomes showing a higher diversity than the fallow control. However, the Shannon index pointed to the absence of significant differences in the resident protistan communities between most cover crop species (except for black oat and the two oilseed radish cultivars) and the fallow control. Regarding the active protist fractions, the Shannon index did not reveal a significant difference between the fallow control and any of the cover crops (Suppl. Table 7-E, F). Concerning the resident metazoan community, borage and vetch were associated with the most diverse resident community (Suppl. Table 7-G).

3.4. Impact of cover crops on the relative abundances of microbiota in the rhizosphere

In Fig. 2 the relative abundances of microbiota in the rhizosphere are shown for each of the ten cover crop species, as well as for the fallow control. A selected number of results will be detailed below.

3.4.1. Bacteria

Numerous rare bacterial taxa ('other (<2 %)' in Fig. 2) were present in the rhizosphere of all cover crops tested. In the case of hybrid ryegrass, tall fescue and lentil, this category comprised >50 % of both the resident and the active bacterial community. Among the bacterial families with abundances >2 %, striking differences were observed between the individual cover crop species. Pseudomonadaceae were highly stimulated in the rhizosphere of the two oilseed radish cultivars and borage (Fig. 2A, B). Among the 43 Pseudomonadaceae ASVs, 42 belonged to the genus *Pseudomonas*, and the remaining could not be assigned to a genus. Moreover, Moraxellaceae and Rhizobiaceae were abundantly present in the rhizospheres of the two oilseed radish cultivars. On average Moraxellaceae accounted for 8.4 % of the resident and 7.6 % of the active community, and Rhizobiaceae were amply represented in the resident and active communities (respectively 7.2 % and 5.0 %). Regarding the active fraction of the bacterial communities, Blrii41 (order Polyangiales) was most abundant in the hybrid ryegrass rhizosphere (9.8 %). This bacterial family was activated by cover crops belonging to Poaceae, Asteraceae and Fabaceae, and not by representatives of the Brassicaceae and Boraginaceae. It is noted that Oxalobacteraceae were activated by all cover crops, while the active members of this bacterial family made up <2 % in the fallow control (See Suppl. Table 8 for details on the composition of the bacterial communities).

3.4.2. Fungi

As compared to the bacterial community, the fraction 'other (<2 %)' is relatively small in the fungal communities (Fig. 2-C, D). The fungal family Mortierellaceae was abundantly present in the rhizospheres of all cover crops tested, as well as in the fallow control. The relative abundances of this fungal family ranged from 9.2 % (active community of borage) to 53 % (resident community of fallow control). Notably, the representation of this fungal family in the resident community tended to exceed its relative presence in the active community. An opposite pattern was observed for the Microascaceae; although present within the resident community of most cover crops, they constituted a larger part of the active fraction of the fungal community. At the level of individual cover crops, the rhizosphere of borage showed the highest abundance of Plectosphaerellaceae (58.1 % and 53.8 % in the resident and active communities, respectively) ($p < 0.05$). The two oilseed radish cultivars stood out as their rhizospheres were highly enriched in Olpidiaceae (on average 28.7 % of the resident community). It is noted that the presence of this fungal family was much lower in the active fraction (on average

Fig. 2. Microbial composition at the family level of the rhizosphere of the cover crops. A) bacterial resident community, B) bacterial active community; C) Fungal resident community; D) fungal active community; E) protists resident community; F) protists active community; G) metazoa resident community (at order level). All taxa accounting for <2 % or relative abundance were grouped as "Others (<2 %)".

5.5 %). An opposite trend was observed for the Orbiliaceae, active representatives of this fungal family were amply present in the rhizosphere of the two oilseed radish cultivars (on average 8.5 %), while the relative abundance in the resident community was <2 % (see Suppl. Table 9 for details on the composition of the fungal communities).

3.4.3. *Protists and metazoa*

The resident and active protistan communities showed a high relative abundance of rare taxa (Fig. 2-E, F, category 'other (<2 %)'). Among the protist taxa present at higher relative abundances (>2 %), the Cercomonadidae and Pythiaceae were shown to be present and active in the rhizospheres of all cover crops tested. With relative abundances of 27.1

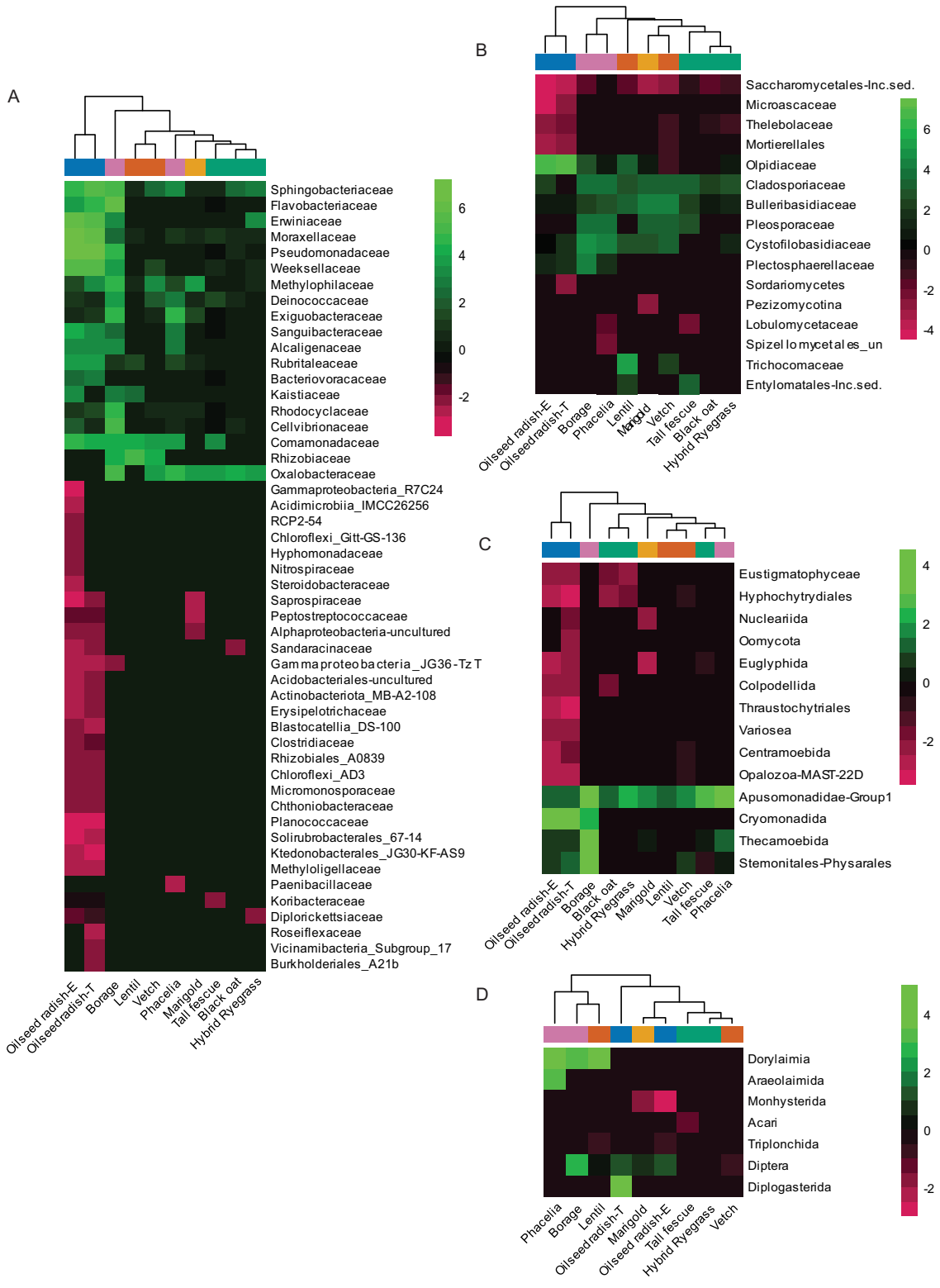


Fig. 3. Heatmap of the beta coefficient assigned to the differentially abundant taxa pinpointed with ANCOM-BC for A) resident bacterial families, B) resident fungal families, C) resident protist orders, D) resident metazoan orders, E) active bacterial families, F) active fungal families, G) active protists orders. Beta coefficients $>$ 0 (shades of green) indicate that the average abundance of the taxon in the cover crop treatment is higher than in the reference (fallow soil). Beta coefficients $<$ 0 (shades of red) indicate that the average abundance of the taxon in the cover crop treatment is lower than in the reference (fallow soil). The dendrograms per column and row were calculated based on Euclidean distance. The colours above the heatmap represent the plant family of each cover crop cultivar: orange = Asteraceae, green = Poaceae, blue = Brassicaceae, purple = Boraginaceae, and red = Fabaceae. For improved figure readability, for bacteria, fungi and protists only taxa with beta coefficients higher than 3 and lower than −2 are represented. The complete heatmaps are available in Supplementary Fig. S2.

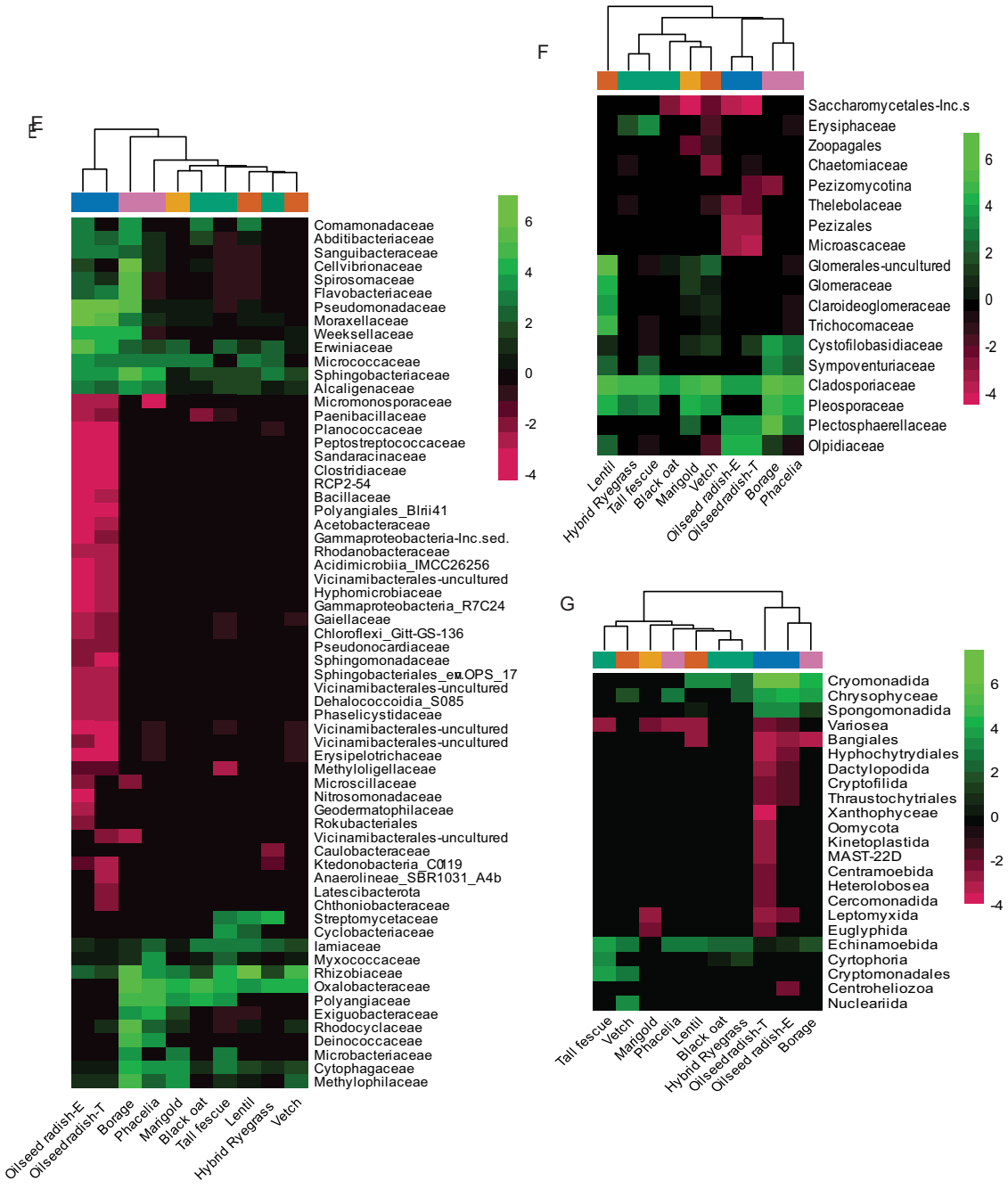


Fig. 3. (*continued*).

% and 37.7 % in the resident communities of borage and phacelia, the oomycete family Pythiaceae was dominantly present in the rhizosphere of Boraginaceae (Fig. 2-E, F). In the case of the two oilseed radish cultivars, the Rhogostomidae (order Cryomonadida) were remarkably well represented in both the resident (on average 40.6 %) and the active (on average 35.5 %) fraction of the protistan community. The Vahlkampfiidae were abundant in the resident community of all cover crops rhizosphere, but they made up <2 % of the active protist fraction. An opposite trend was observed for the Nucleariidae and Sandonidae, which were abundant in the active community of all cover crops rhizosphere but underrepresented in the resident community.

Analysis of the resident metazoan community in the rhizosphere of cover crops revealed the dominance of nematodes (seven of the most abundant orders shown in Fig. 2-G). Rhabditida (17.7–46 %), opportunistic bacterivores, were abundantly present in the rhizosphere of all cover crops. Diplogasterida (bacterivores) were present in remarkably high abundances in the rhizosphere of the two oilseed radish cultivars (on average 50 %). Representatives of the orders Tylenchida and Areolaimida were commonly present in all cover crops but less so in the two oilseed radish cultivars (~3 % *versus* ~ 20–30 %) (See Suppl. Table 10 for details on the composition of protist and metazoan communities).

3.5. *Differential abundance analysis of rhizosphere microbiomes*

In the next step, an analysis of compositions of microbiomes with bias correction (ANCOM-BC, Lin and Peddada, 2020) was used to determine the rhizospheric profiling of the resident and active taxa of each cover crop compared to the fallow soil. Beta coefficients, a quantitative measure for differential abundances, ranged from −4.4 (Microascaceae under oilseed radish) to 7.2 (Pseudomonadaceae under oilseed

radish) indicating that, overall, the stimulation of taxa in the rhizosphere by the cover crops was stronger than the repression. As this analysis concentrates on changes in abundances rather than abundances *per se,* the heatmaps generated with the beta-coefficient values include taxa present in low abundance that were lumped under the category 'other (<2 %)' in Fig. 2. On the other hand, taxa that were shown to be present in the rhizosphere of all cover crops in relative abundances comparable to fallow, such as the bacterial family Sphingomonadaceae, are not included in Fig. 3.

For all organismal groups, most taxa that were significantly affected at the DNA level (resident community) were often also influenced at the cDNA level (active community). Taking into account all bacterial taxa that were significantly stimulated or repressed, 69 % was affected at both DNA and cDNA levels. For fungal and protist communities these levels of communality were respectively 78 % and 78 % (Suppl. Tables 11, 12, 13). In by far most cases, the directionality of the changes was identical; most often a taxon repressed at the DNA level was also repressed at the cDNA level, and the same holds for stimulated microbiota. Nevertheless, a few exceptions were observed: the bacterial family Iamiaceae was significantly repressed by borage at the DNA level and stimulated at the cDNA level, and Nitrosomonadaceae were stimulated by oilseed radish (cultivar -E), while its activity was repressed (Suppl. Table 11).

3.5.1. Bacteria

ANCOM-BC identified 175 and 177 differentially abundant families among the resident and active bacterial communities, respectively (a selection of the most enriched and most depleted families is presented in the heatmap in Fig. 3-A, E, the complete heatmap representing all the differentially abundant families is provided as Suppl. Fig. 2-A, D). The two oilseed radish cultivars showed bacterial profiles that were most deviant from the fallow controls. Despite a few cultivar-specific changes, the two oilseed radishes clustered together in dendrograms (Fig. 3-A, E). In the rhizosphere of the oilseed radishes, the number of repressed bacterial taxa exceeded the number of promoted taxa for both the resident and the active bacterial communities. Planococcaceae (both in resident and active communities) and Bacillaceae (in the active community) were among the most repressed families, while Pseudomonadaceae, Moraxellaceae and Erwiniaceae resided among the most enriched families (both in resident and active communities). Borage showed a bacterial profile exceptionally distinct from the fallow soil, with a high number of differentially enriched families. Among these, the resident and active fractions of the Flavobacteriaceae, Cellvibrionaceae and Sphingobacteriaceae were strongly promoted as compared to fallow controls (in all cases beta coefficients > 5). It clustered separately from the oilseed radish and the other cover crops in the profiling of the resident rhizospheric taxa, while clustered together with phacelia in the profiling of the active taxa (Fig. 3-A, E).

3.5.2. Fungi

ANCOM-BC pinpointed 44 and 46 differentially abundant fungal families in respectively the resident and active communities of the cover crop rhizospheres (see Suppl. Table 12 for ANCOM-BC results on the fungal community, and Suppl. Fig. 2-B, E, F for the full heatmaps). Regarding the resident community, the two oilseed radish cultivars showed a high number of differentially abundant fungal families. Most striking is the strong repression of the representatives of the fungal family Microascaceae and the order Saccharomycetales, and the stimulation of members of the protistan family Olpidiaceae. Apart from a mild repression of Saccharomycetales in the resident rhizospheric profiling resident, the impact of lentil on members of the fungal community was invariably positive (Fig. 3-B, F). The stimulating effect was most notable for the fungal family Trichocomaceae and the fungal order Glomerales. In Fig. 3-F, this order is represented by 'Glomerales-uncultured', 'Glomeraceae' and 'Claroideoglomeraceae'. For both representatives of the Boraginaceae, stimulation of the Cystofilobasidiaceae and the Pleosporaceae were observed both in the resident and the active fraction of the fungal community (Fig. 3-B, F). The fungal family Cladosporiaceae is exceptional as its members were stimulated by almost all cover crops in both the resident and active communities (only exception oilseed radish cultivar -T) (Fig. 3-B, F). Members of the different plant families clustered more closely together than with members of other families, with the exception of lentil and vetch which displayed a sharp distance, especially at the level of the active profiling.

3.5.3. Protists and metazoa

As main consumers of primary decomposers, protists and metazoans were indirectly affected by cover crops. ANCOM-BC analyses revealed that 46 and 48 protist orders were differentially abundant in the resident and active rhizosphere communities, respectively. The two oilseed radish cultivars had the broadest impact on the resident and active protistan communities, followed by borage (Suppl. Table 13 and Suppl. Fig. 2-C, F for the complete heatmap of the protistan community). These cover crops clustered together in the active profiling, while they have a reduced distance in the resident profiling. Cryomonadida was relatively most enriched in the oilseed radish and borage rhizosphere. With a beta coefficient of 3.9, the resident community of the borage rhizosphere was particularly enriched in members of the Stemonitales-Physarales (plasmodial slime moulds), while for all other cover crops abundances lower than 2 % were detected (Suppl. Table 13).

ANCOM-BC of the metazoan community identified 7 differentially abundant metazoan orders out of 79 (Suppl. Table 11). The highest number of differentially abundant metazoan orders was found for marigold (Fig. 3-D). The nematode order Diplogasterida was exclusively enriched in the rhizosphere of the oilseed radish cultivar -T, and Monhysterida was depleted in oilseed radish cultivar -E and marigold, while Araeolaimida were enriched in the rhizosphere of phacelia (Fig. 3-G). It is noted that the sample size (2 g of rhizosphere soil) is low for metazoa, and the shifts reported here require confirmation by the analysis of more and larger subsamples.

3.6. Associations within and between organismal groups in cover crop rhizospheres

Network analyses were performed to map potentially positive and negative connections within and between the active fractions of the bacterial, fungal and protist communities in the rhizosphere of cover crops, and in bulk soil for the fallow control (Fig. 4). Metazoa could not be included in these analyses due to the low number of reads in the metazoan cDNA dataset. The network of the fallow soil featured 59 nodes and 55 edges and 2.3 average number of neighbours (for other network characteristics see Suppl. Table 14). Among the cover crops tested, only phacelia's rhizosphere showed a lower network complexity (32 nodes, 26 connections, 2.17 avg. neighbours) (Fig. 4). The highest level of network complexity was induced by vetch (141 nodes, 468 edges, 7.4 avg. neighbours). Generally, the highest number of connections was found between bacteria and protists, followed by connections between bacteria and fungi. A relatively low level of connectivity was detected between fungi and protists. Marigold was exceptional as the highest number of connections was found between bacteria and fungi. Fallow, phacelia, tall fescue and vetch had a majority of negative interactions between organismal groups (Fig. 4, dotted lines), while for all other cover crops, the majority of the interactions were positive (Fig. 4, solid lines).

4. Discussion

4.1. Cover crop species exert different selection strengths on the rhizosphere microbiome

All ten cover crops characterized in this study exerted significant effects on the soil rhizobiome, and the kind of effects was shown to be

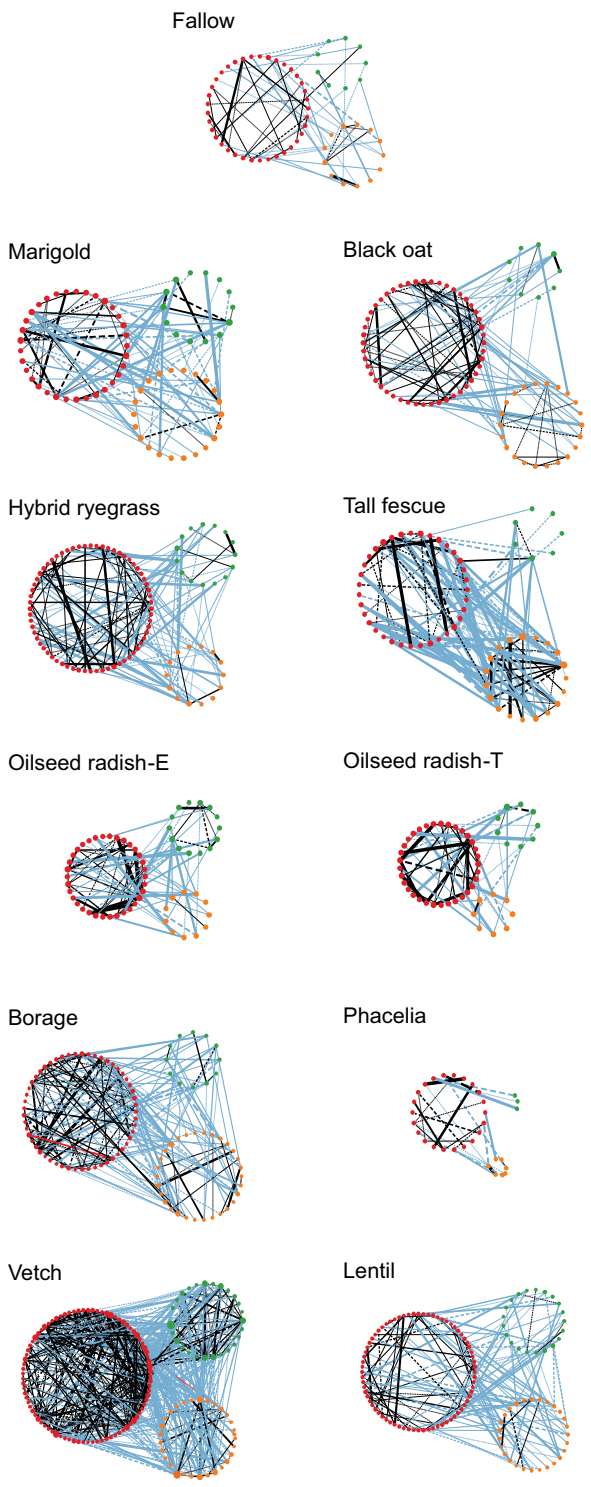


Fig. 4. Network co-occurrence analysis of the active rhizosphere microbiome. Each node represents a bacterial, fungal or protist taxon. The red, green and orange nodes represent respectively bacterial families, fungal families, and protist orders. The node size is scaled based on the number of connections per node. The width of the connections (edges) represents the strength of the SPARCC correlation. Only significant ($p < 0.05$) correlations with values > 0.6 (positive correlations, solid line) and < -0.6 (negative correlations, dashed line) were retained for analysis. The light blue edges indicate the interactions between different organismal groups (bacteria-fungi, fungi-protists, bacteria-protists), while black edges are used to indicate connections within members of the same organismal group.

plant species and - to some extent - plant family dependent. The characterization of the resident and active fractions of the rhizosphere communities of the cover crops revealed distinct levels of selection strengths by the cover crops on the rhizosphere microbiome. The two cultivars of oilseed radish affected the rhizosphere microbiome most distinctly. Both oilseed radish cultivars sharply suppressed a wide range of microbial taxa, in particular bacterial and protistan microorganisms, and, at the same time, strongly promoted a smaller subset. This trend observed on the individual taxa through ANCOM-BC is accompanied by the alpha diversity results, which indicated a lower richness and evenness in the rhizosphere (especially of) bacterial community of oilseed radish. It is noted that members of the Brassicaceae plant family, including oilseed radish, produced a category of secondary metabolites called glucosinolates. The release of these metabolites and their biocidal hydrolysis products in the rhizosphere by living roots was demonstrated for canola (Choesin and Boerner, 1991) and mustard roots (Schreiner and Koide, 1993) and directly impacted the rhizobiome (Bressan et al., 2009). We suggest that the release of glucosinolates and their breakdown products might have contributed to the observed high selection strength of both oilseed radish cultivars.

Borage and phacelia (both Boraginaceae) had an overall strong effect on all organismal groups. Borage belongs to the Boraginaceae subtribe Boragininae. Other representatives of this subtribe, members of the genus *Nonea,* were demonstrated to secrete tricetin derivatives, a rare type of flavone (Wollenweber et al., 2002). Assuming that borage secretes similar types of flavones, it would be worth investigating whether this category of secondary metabolites is responsible for the observed effects. As compared to borage, phacelia showed an overall milder impact on the rhizobiome.

Lentil and vetch (both Fabaceae) had a remarkably strong impact on the fungal community. A stimulating effect of legumes on the fungal community abundance and diversity has been reported before and was associated with the relatively abundant release of amino acids, sugars and flavonoids in the rhizosphere (Isobe et al., 2001; Turner et al., 2013; Zhou et al., 2017). At the DNA level lentil and vetch induced comparable shifts in the microbiome, but at the RNA level substantial differences were observed between the two legumes. Although vetch and lentil are closely related plant species - both belong to the same tribe within the family Fabaceae (Fabeae) - vetch produces at least two ß-cyanoalanines that are not produced by lentil (Thavarajah et al., 2012). The more widespread repression of fungal taxa in the vetch rhizosphere might relate to these toxic substances, although it is unknown whether they are secreted in the rhizosphere.

This study included three representatives of the Poaceae, and it was remarkable to see that tall fescue had a stronger impact on the rhizobiome than black oat and hybrid ryegrass. Tall fescue showed a more widespread stimulation of rhizosphere microbiota and only a few taxa were repressed. Black oat belongs to the Poaceae subtribe Aveninae, while hybrid ryegrass and tall fescue both reside in the subtribe Loliinae, and thus are phylogenetically related. Hence, there is no phylogenetic rationale that could explain why tall fescue had a stronger impact on its rhizobiome than the two other poaceous cover crop species. However, tall fescue tends to have thicker and deeper root systems as compared to ryegrasses (Cheng et al., 2016). Because root traits impact the release of root exudates (Saleem et al., 2018), this may co-explain why tall fescue exerted a selection strength that exceeded one of the two other poaceous cover crops under investigation.

4.2. Compositional changes underlying differential selection strengths

Whereas an overview of all microbial taxa associated with individual cover crops was shown in Fig. 2, the heat maps presented in Fig. 3 focuses on microbial taxa that are significantly stimulated or repressed by one of multiple cover crop species. Generally, the absolute values of the positive beta coefficients representing the level of promotion of individual taxa, exceeded the level of repression, as represented by negative

beta coefficients. So overall, the stimulating effect of cover crops on microbial life was stronger than their repressing effects. Here we highlight five cover crop-induced shifts that were particularly remarkable. Three examples involve soil microorganisms that were shown to be promoted by at least one of the cover crops investigated here, while two examples are given of cover crop-repressed microorganisms.

The increased abundance of the Pseudomonadaceae bacterial family in the rhizosphere of some cover crops was remarkable. This bacterial family constituted up to 37.9 % and 15.5 % of the oilseed radish and borage bacterial community in the rhizosphere and yielded beta coefficients up to 6.9. Strong plant-induced stimulation of the bacterial family Pseudomonadaceae has been reported before. In a field experiment with four crops including canola (*Brassica napus*), the *Brassica* species was shown to strongly stimulate endophytic *Pseudomonas* representatives over multiple years and locations (Cordero et al., 2020), and - to a lesser extent - Pseudomonadaceae in the rhizosphere. The bacterial family Pseudomonadaceae harbours plant pathogens, beneficial species that can act as biological control agents (Weller et al., 2002) as well as plant growth-promoting rhizobacteria (*e.g.*, Lugtenberg and Kamilova, 2009). It should be noted that levels of Pseudomonadaceae *per se* were reported not to be a good indicator of general disease suppressiveness in the transition study toward organic production (Marzano et al., 2015). Hence, more detailed monitoring is needed to assess the potential suppression-related implications of the observed stimulation of representatives of the family Pseudomonadaceae.

Two arbuscular mycorrhizal fungal (AMF) families, Glomeraceae and Claroideoglomeraceae, showed an increased presence and/or activity in the rhizosphere of some of the cover crops tested. Previously, the presence of Claroideoglomeraceae was linked to mechanically disturbed soils, while Glomeraceae were more abundant in undisturbed habitats (Moora et al., 2014). The cover crops characterized here were grown in mechanically disturbed soil. Lentil, and to a lesser extent marigold and vetch, exclusively induced enrichment of Claroideoglomeraceae. However, at the cDNA level, we observed an increased activity of both Glomeraceae and Claroideoglomeraceae for lentil, and less prominently for vetch and marigold. Our results do not contradict the results of Moora et al. (2014) as this study focused on Glomeromycota and was performed at the DNA level only. There was no AMF signal to be expected for the Brassicaceae (non-host for AMF, Cosme et al., 2018), but we cannot explain why no increased AMF presence or activity compared to fallow soil was observed in the rhizosphere of the Poaceae included in this study.

The fungal family Cladosporiaceae showed an elevated presence in the rhizobiome in nine out of ten cover crops tested, but the most striking was the increase in its activity. This was most explicitly observed for borage, phacelia, lentil and vetch (beta coefficients >5). The fungal family Cladosporiaceae harbours seven genera, the genera *Davidiella* and *Cladosporium* being by far the most widespread ones. *Davidiella* is most often found on aboveground tissues (Longley et al., 2020), whereas *Cladosporium* representatives are present both above- and belowground (Bensch et al., 2012). The genus *Cladosporium* harbours 189 described, mostly saprophytic, species (Sandoval-Denis et al., 2016a), and next to saprobes, this genus comprises above- and belowground endophytes and plant pathogens. Uncharacterized *Cladosporium* members were recently detected in the rhizobiome of maize (Zhao et al., 2021) and also – in a non-agricultural setting – in the rhizosphere of giant goldenrod (Harkes et al., 2021). We hypothesize that soil-borne, saprophytic and/or pathogenic *Cladosporium* species are responsible for the observed increased presence and activity in the rhizosphere of nearly all cover crops rhizosphere compared to fallow.

The bacterial family Planococcaceae was identified as the most strongly repressed bacterial family, and the repression was almost exclusively observed in the oilseed radish rhizospheres (next to a mild repression by hybrid ryegrass). The family Caryophanaceae/Planococcaceae is a polyphyletic bacterial family with >100 species classified within 13 genera (Gupta and Patel, 2020). Recently, *Planococcus* was observed as an endophyte in sugar beet (*Beta vulgaris*) roots and in higher relative abundances in its rhizosphere (Li et al., 2020). However, no ecological explanation could be given for this shift. The absence of any known characteristics exclusive to all bacterial members of the family *Planococcaceae* (Gupta and Patel, 2020) makes it impossible to assess the ecological impact of *Planococcaceae* in the rhizosphere.

With high beta-coefficients, Microascaceae belonged to the strongest repressed fungal taxa among all cover crops, and this repression was only observed for oilseed radish. Microascaceae currently accommodate a morphologically heterogeneous group of fungi, comprising saprophytic and plant pathogenic species (Sandoval-Denis et al., 2016b). Fungal members of the Microascaceae family inhabit niches in association with different kinds of bark beetles, *Petriella* and *Petriellopsis* are associated with soil, dung and compost (Lackner and de Hoog, 2011). We hypothesize that the toxicity of isothiocyanates associated with Brassicaceous plants (Bressan et al., 2009) may suppress this fungal family in the oilseed radish rhizosphere.

4.3. Active and resident fractions of the microbiome communities

The nucleic acid type was the second most relevant variable in our analyses of cover crop-affected microbiomes explaining 12–13 % of the observed variation (Suppl. Table 5). The relevance of discriminating between the resident and the active fraction of the soil microbiome has been underlined before by, *e.g.*, Harkes et al. (2019), Ofek et al. (2014) and Bay et al. (2021). Cover crops generally had a stronger selection strength on the active rather than of the resident soil microbiome, suggesting that RNA-based analyses may better reflect the effect of the environmental influence on the microbiome community assembly (Bay et al., 2021). DNA-based analyses allow studying microbes present in the soil in a range of states (dead, dormant, and active), while RNA analyses allow for studying the potentially active fraction of the soil microbiome (Blagodatskaya and Kuzyakov, 2013). For some taxa presented in Fig. 2, this might be non-obvious as numerous low abundant taxa are residing in the category 'other (<2 %)'. It was remarkable, however, to see that 69–78 % of the microbial taxa affected at the DNA level were also affected at the cDNA level. Given that plants shape their rhizobiome to maximize their fitness (*e.g.*, Berendsen et al., 2012), it was not surprising that manipulations observed at DNA and cDNA levels predominantly showed the same directionality. Convergence between resident and active rhizospheric communities was also reported by Bay et al. (2021), who suggested that this is the result of the strong selective environment established by plants on the indigenous soil microbiome.

4.4. Cover crop-induced changes in microbial networks

The network analyses presented here are based on the active fractions of the bacterial, fungal and protist communities in the rhizosphere of individual cover crops, and they allowed us to compare cover crop-induced changes with the fallow control. These co-occurrence networks could be instrumental in pinpointing potential biological interactions (Hirano and Takemoto, 2019; Lupatini et al., 2014; Shi et al., 2016). The high connectivity between bacteria and protists, for example, may reflect the feeding preferences of protists toward bacteria, rather than fungi (Gao et al., 2019). Except for phacelia, all cover crops induced microbial networks that were more complex than the one of fallow control. This is in line with the principle of the 'rhizosphere effect', which implies that the plants' rhizosphere is a hotspot for microbial interactions (Pathan et al., 2020). Across cover crop rhizospheres, we observed great variation in the level of network complexity, as well as shifts in the interactions among organismal groups. These differences in the network complexity likely reflect the species-specific properties of the cover crops (Geisen et al., 2018; Santos et al., 2020). We hypothesised that the high complexity of the vetch microbiome might also be a result of the drought stress at the onset of this field experiment. In a ^{14}C labelling experiment, Sanaullah et al. (2012) reported that in the case of

legumes, drought stress had a significantly smaller negative effect on root exudation than it had on grasses. It should, however, be noted that for the other legume in this field trial, lentil, no similar effect was observed.

Co-occurrence networks are suitable for the generation of hypotheses about the biological meaning of observed negative or positive associations. It is worth noting that high network complexity (*e.g.*, high number of nodes and edges, number of neighbours, high modularity) has been associated with soil-borne pathogen suppression (Yang et al., 2017), enhanced nutrient-cycling (Wagg et al., 2019) and higher crop productivity (Tao et al., 2018). For this reason, the value of network analyses might be even greater when stressors or pathogens are present in the study stem.

Network analyses presented here are based solely on the active fractions of the rhizosphere microbiomes, whereas most soil microbiome studies concentrate on the resident microbial community. This choice is defendable as only non-dormant taxa can actively engage in biological interactions.

4.5. The inclusion of primary consumers in the characterization of cover crops rhizosphere

Bacterial and fungal communities in the cover crop rhizospheres are the result of the composition of the local microbial community in the soil, bottom-up control by the individual cover crops, and top-down regulation by primary consumers. The primary consumer community is here represented by protists and metazoa (mainly nematodes). It is noted that the primary consumer activity affects plant growth directly as grazing of the bacterial (and to a lesser extent fungal) biomass by protists results in the release of plant-available N (Clarholm, 1985; Xiong et al., 2020). Essentially the same holds for the impact of selective grazing by bacterivorous nematodes (Schratzberger et al., 2019). In this study, the importance of protists in the structuring of the rhizosphere microbiome assembly could be demonstrated by the high number of interactions between bacteria and protists in the network analysis and to a lesser extent between protists and fungi. In our study, the majority of interactions across organismal groups were positive, pointing at symbiotic and cooperative interactions (Jousset et al., 2008; Rossmann et al., 2020).

Large cover crop-specific shifts were observed both in the resident and the active fractions of the protistan community. One of the most striking examples is the strong activation of the protist order Cryomonadida (Cercozoa phylum) in the rhizospheres of the two oilseed radish cultivars as well as borage. Cryomonadida are known as amoeboid eukaryvores (Fiore-Donno et al., 2022). With slightly lower beta coefficient values, the stimulation of Cryomonadida was paralleled by a clear activation of Chrysophyceae, again mainly in both oilseed radish cultivars and borage (Suppl. Table 13). Chrysophyceae are predominantly unicellular, golden-brown algae that commonly occur in arable soils in temperate climate zones (Lentendu et al., 2014), and we hypothesize that the observed activation of Cryomonadida could be the result of a cover crop-specific stimulation of golden-brown algae in the rhizosphere. Nevertheless, this observation was not supported by the network analysis.

Concerning Metazoa, it should be mentioned that the subsamples analysed in this study, 2 g, might have been too small to get a proper representation of the metazoan community. A more complete representation of the nematode community would require an upscaling of the DNA and RNA extraction procedure described by Harkes et al. (2019). Evidently the subsample size depends on average size of members of an organismal group, as well as their spatial distribution. For bacteria and fungi, subsamples of 0.25–2.0 g is the golden standard (Wydro, 2022). This also holds for protists. For nematodes, traditionally 200 g of soil is used, this figure can be reduced till 100 g is case molecular detection methods are used (Wiesel et al., 2015). Rhizosphere soil has a much higher nematode density than bulk soil. Hence, 2 g samples can be informative, but will not provide a robust nematode community overview.

4.6. Experimental design

A significant effect of blocks was observed for all four organismal groups, and in particular for the metazoa (17 % *vs.* ~7 % in other organismal groups). This effect can largely be explained by the layout of the field experiment. Each block (≈4 m × 4 m) included 11 treatments, *viz.*, 10 cover crops and a fallow control. The eight blocks were positioned next to each other in a long rectangle (≈4 m × 60 m). This rectangle was positioned next to a maize field with a final crop height of about 3 m. As a result, there were slight differences in insolation between the individual blocks. This might have resulted in differences in soil temperature as this is mainly determined by ambient temperature and direct irradiation. However, as all treatments were represented in all blocks, this slight insolation gradient along the blocks could not have had a systematic effect on cover crop-induced changes in the soil microbiome.

5. Conclusions

Here we pinpointed the differential effects of ten cover crop species on both the resident and the active fractions of bacterial, fungal and protistan communities in the rhizosphere. Our results indicated that oilseed radish cover crops had the strongest effect on the rhizospheric microbial communities, together with borage. Vetch and, most explicitly lentil, had a strong stimulating effect on the fungal rhizosphere community, and similarly marigold influenced the fungal community more than the bacterial and protistan ones. Representatives of the Poaceae - especially black oat and hybrid ryegrass - had a remarkably mild impact on the soil microbiome. Hence, it is concluded that cover crops differ in the extent by which they manipulate the native microbiome in their rhizospheres. Subsequently, we investigated whether microbial taxa were promoted or repressed in a cover crop-specific manner. Differential abundance analyses revealed a range of cover crop-specific microbial shifts, and even a differential impact of two cultivars of the same cover crop species (oilseed radish) could be pinpointed. We conclude that individual cover crops affect soil microbial taxa in a cover crop genotype-specific manner. RNA-based network analyses revealed that most cover crops induced an increase in the microbial network complexity as compared to the fallow soil. However, the level of increase was shown to be cover crop species-specific. Based on microbial network parameters reflecting the level of network complexity, we conclude that individual cover crops had distinct effects on the degree of potential associations between the three main organismal groups, bacteria, fungi, and protists.

Overall, our data suggest that poaceous and fabaceous cover crops could be suitable for a general stimulation of soil microbiota, while members of the plant families Boraginaceae, and, most explicitly, Brassicaceae leave a relatively strong mark on the microbial community by promoting and repressing specific taxa of the native soil microbiome. The current dataset should be seen as a starting point for the application of specific cover crop species or mixtures thereof to steer the soil microbiome in a predictable direction to promote soil health and sustain healthy crop growth. Further studies should be aimed at determining to what extent the effects of cover crops on the soil microbiome persist over time and thus may affect the growth and development of the main crop.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Joeke Postma reports financial support was provided by Top Consortium for Knowledge and Innovation Horticulture & Starting Materials. Joeke Postma reports financial support was provided by Top Consortium for

Knowledge and Innovation Agri & Food. Liesje Mommer reports financial support was provided by Dutch Research Council VIDI grant.

Data availability

All sequences have been submitted to the NCBI database under BioProject ID PRJNA842568.

Acknowledgements

The authors would like to acknowledge the companies Barenbrug, DSV-zaden, Joordens Zaden and Vandinter Semo for their help in selecting and providing the cover crops seeds used in the experiment; Marc Kroonen, Harry Verstegen and the Vredepeel field team for setting up and maintaining the field experiment; Leendert Molendijk for the help in the experimental design. We also like to thank Joris van Steenbrugge for his help in the setting up of the bioinformatic pipelines; Paula Harkes for the guidance in the laboratory, Martijn Holterman, Francesco Garassino and Marco Albiero for helping with the soil sampling.

We acknowledge the Utrecht Sequencing Facility (USEQ) for providing sequencing service and data. USEQ is subsidized by the University Medical Center Utrecht and The Netherlands X-omics Initiative (NWO project 184.034.019).

Funding

This study was funded by the TKI project grants AF18085 and TU18150 of the Dutch Topsectors Agri&Food and Tuinbouw&Uitgangsmaterialen. LM is supported by NWO-VIDI grant 864.14.006.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2023.105012.

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