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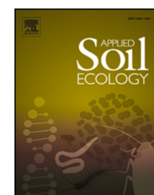

Cover crop mixtures do not assemble markedly distinct soil microbiotas as compared to monocultures in a multilocation field experiment

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ABSTRACT

Cover crops are used in cropping systems to enhance ecosystem services, such as soil resilience to erosion or microbial activity. Different cover crops are selected to steer specific processes, but whether cover crop mixtures have an added value over monocultures remains debated. Here, we investigated if cover crop mixtures accumulate soil microbiotas distinct from those of monocultures, potentially leading to more varied microbially-driven soil functions. We performed a field experiment at three locations in the Netherlands, each including nine cover crop monocultures, five- and eight-species mixtures, and a fallow control. After three months, we measured cover crop biomass and profiled soil bacterial, fungal, and arbuscular mycorrhizal fungal communities via amplicon sequencing. The different crop monocultures produced similar biomass across all three locations, and mixtures had average productivity compared to monocultures. The diversity and community structure of soil microbial communities was primarily determined by the geographical location, and then by cover crop treatment at each location. Although the cover crop species affected the soil microbiome differently, cover crop mixtures did neither increase microbial diversity nor the overall community differentiation compared to monocultures. Our results suggest that mixing cover crop species does not significantly influence microbially-driven soil functions, at least in short-term crop rotations.

1. Introduction

The use of cover crops in arable farming has increased steadily over the past decade (Lamichhane and Alletto, 2022). This practice offers both economic and environmental benefits, as cover crops facilitate the reduction of chemical inputs while providing ecosystem services that sustain production and improve the environmental sustainability (Fan et al., 2020). Historically, leguminous cover crops were predominantly grown between cash crop cycles to improve soil nutrition (Kaspar and Singer, 2011; Kramberger et al., 2009), but current practices have expanded the diversity of plant taxa and management techniques to profit from multiple ecosystem services, often concurrently (Blanco-Canqui et al., 2015). Today, a range of cover crop species belonging to multiple plant families are used to achieve specific services, such as reducing soil erosion, suppressing weed growth, improving soil fertility, or promoting beneficial soil organisms (Kaye and Quemada, 2017; Kim et al., 2020; Vukicevich et al., 2016). Furthermore, there is interest in planting mixtures of cover crop species, either during or in between cash crop cycles, as a means to achieve multiple ecosystem functions within each growing season (multifunctionality) and reduce the impact of

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variable environmental conditions (Elhakeem et al., 2021; Finney et al., 2016).

Because different cover crop species provide distinct ecological services (Kaspar and Singer, 2011; Li et al., 2020; Ramírez-García et al., 2015), combining them in mixed stands may enhance the multifunctionality of crops and their resilience to changing environmental conditions (Abdalla et al., 2019; Blanco-Canqui et al., 2015; Gfeller et al., 2018; Li et al., 2020). Several studies support this notion for services that directly rely on the crops' biomass accumulation, such as nutrient retention by plant organs or increasing the quantity and quality of organic matter entering the soil (Couëdel et al., 2018b; Elhakeem et al., 2021; Finney et al., 2016). In comparison, much less is known about the effects of cover crop mixtures on the composition of soil microbial communities, which are crucial in driving soil biotic processes that improve soil health and support long-term agricultural productivity (Eisenhauer et al., 2012). If cover crop mixtures accumulate a more diverse soil microbiota than monocultures, they might also enhance overall soil functioning and provide additional services beyond those provided by individual plant species.

Plants gradually accumulate specific soil microbiotas through root exudation and the modification of soil organic matter and abiotic conditions that select for particular microbes (Andreo-Jimenez et al., 2015; Bais et al., 2006, 2004; Fanin et al., 2021; Nguyen, 2009). These include microbial groups with direct beneficial and detrimental effects on plant growth, such as arbuscular mycorrhizal fungi (AMF) and pathogens (Blanco-Canqui et al., 2015; Wen et al., 2017; Wick et al., 2017), as well as microbes that exert indirect effects on plant performance by driving soil processes like nutrient cycling, litter decomposition, or carbon sequestration (van der Heijden et al., 2008). Recent studies have shown the capacity of individual cover crop species to shape the soil microbiome, uncovering significant and prolonged effects of species of various plant families in promoting or suppressing specific microbial groups (Cazzaniga et al., 2023a, 2023b). It remains to be determined whether these plant-specific changes in the soil microbiota result in distinct microbial communities when different cover crop species are cultivated together. Importantly, investigations should determine if these changes precede the usual practice of incorporating cover crop biomass into soil before the cash crop cycle, which may specifically boost decomposer microorganisms (Liu et al., 2021) but mask subtler shifts in microorganisms more reliant on living hosts, such as AMF or other endophytes. Moreover, it is important to explore whether these effects extend beyond the rhizosphere of specific plants into the bulk soil, where microbial activity affects a greater soil volume.

Besides their numerous benefits, the use of cover crops raises concerns regarding the potential risk of pathogen introduction and carry-over to subsequent cash crops (Agtmaal et al., 2017; Bakker et al., 2016; Walder et al., 2017). Specific cover crops may serve as reservoirs for certain pathogens, creating favorable conditions for pathogen survival, proliferation, and transmission. Due to lower individual plant species density, cover crop mixtures may have the potential to mitigate the buildup and spread of soil-borne pathogens through a phenomenon known as pathogen dilution (Keesing et al., 2006; Keesing and Ostfeld, 2021). In this process, non-host plants can limit pathogen spread, thus reducing the incidence and severity of diseases in neighboring plants. While this effect has been observed in biodiverse grasslands and intercropping systems (Ampt et al., 2022; Maciá-Vicente et al., 2023; Wang et al., 2017), it remains to be seen whether cover crop mixtures can reduce the populations of soil-borne pathogens relative to those in monospecific stands.

Here, we conducted a field experiment to compare the effects of cover crop mixtures with those of monocultures on both plant stand productivity and the soil microbiome (bacteria and fungi). To increase the generalizability of our findings, we replicated this field experiment at three locations in the Netherlands, assessing the consistency in the microbiota selection by different plant groups across diverse environmental conditions. We tested the hypotheses that cover crop mixtures (1) produce more biomass than monocultures, (2) accumulate a taxonomically (and functionally) distinct and more diverse soil microbiota, and (3) reduced populations of soil-borne pathogens.

2. Materials and methods

2.1. Experimental design

We conducted the experiment at three distant locations at the premises of three cover crop seed companies distributed latitudinally across 220 km in the Netherlands (Fig. 1a): DSV Zaden located in Ven-Zelderheide (DSV), Joordens Zaden in Kessel (Joordens), and Vandinter Semo in Scheemda (Vandinter). At each location, we established one repetition of the experiment, each with 12 treatments consisting of one fallow treatment without plant cover, nine different cover crop monocultures from eight plant species in five families, and two cover crop species mixtures including combinations of five and eight species (Table 1). We established the experimental plots at all three locations on fields with sandy soils, although there was clear variation in several soil properties and crop history across locations. Table S1 shows the details for each location concerning soil properties, recent crop histories, exact dates of sowing and sampling, and phenological stage of the cover crop species at the time of sampling.

We selected the plant species and mixtures based on the diverse and complementary services they provide, and because they all are suitable for planting before the same cash crop (potato), as indicated by the cover crop seed producing companies. The 8-species mixture was designed to match a commercial cover crop mixture containing the individual species included in the experiment (Terralife® Solarigol TR, DSV Zaden), while the 5-species mixture was a derivative thereof leaving out the species with the lowest seed quantities in the 8-species mix.

At each site, the experimental design followed a randomized block design with four replicates. Replicates consisted of 8 × 1.5 m plots placed immediately adjacent to each other in parallel rows making up the blocks, which were separated by 1 m from each other (Fig. 1b). We sowed the plots in early August 2021 (Table S1) to the recommended densities for each species (Table 1). Maintenance of plots was restricted to periodic removal of unsown species by hand, without additional watering or fertilization.

2.2. Collection of soil samples

We collected bulk soil samples from each plot in mid-October 2021, three months after sowing the cover crops (Table S1), to analyze the soil microbiome associated with each cover crop treatment. At each plot, we took one soil sample by pooling together 20 subsamples, collected with an auger (∅ 1.5 cm, core length 20 cm) along the longitudinal length of the plot, excluding 0.5 m from the edges (Fig. 1c). After thorough mixing, subsamples (6–8 g) were snap-frozen in liquid nitrogen, transported on dry ice to the laboratory the same day, and stored at −80 °C until further processing. This resulted in a total of 144 soil samples (12 treatments × 4 blocks × 3 sites).

2.3. Biomass measurements

We estimated total above- and below-ground biomass production by the different cover crop treatments in late October 2021 (Table S1). To estimate above-ground biomass, we collected all biomass (>1 cm above soil surface) in three 0.25 m^2 square subplots (delimited by a 0.5 × 0.5 m frame) randomly positioned across the area of each plot, but at least 0.5 m away from the plot edges (Fig. 1c). For the below-ground biomass, we collected 6 soil cores to a depth of 30 cm in each of the three subplots also used for determining above-ground biomass, using a wide auger (∅ 8 cm). We pooled all the soil cores collected from a single plot and washed away the soil to collect the roots. We dried the above-ground and below-ground plant material at 70 °C for two days to estimate

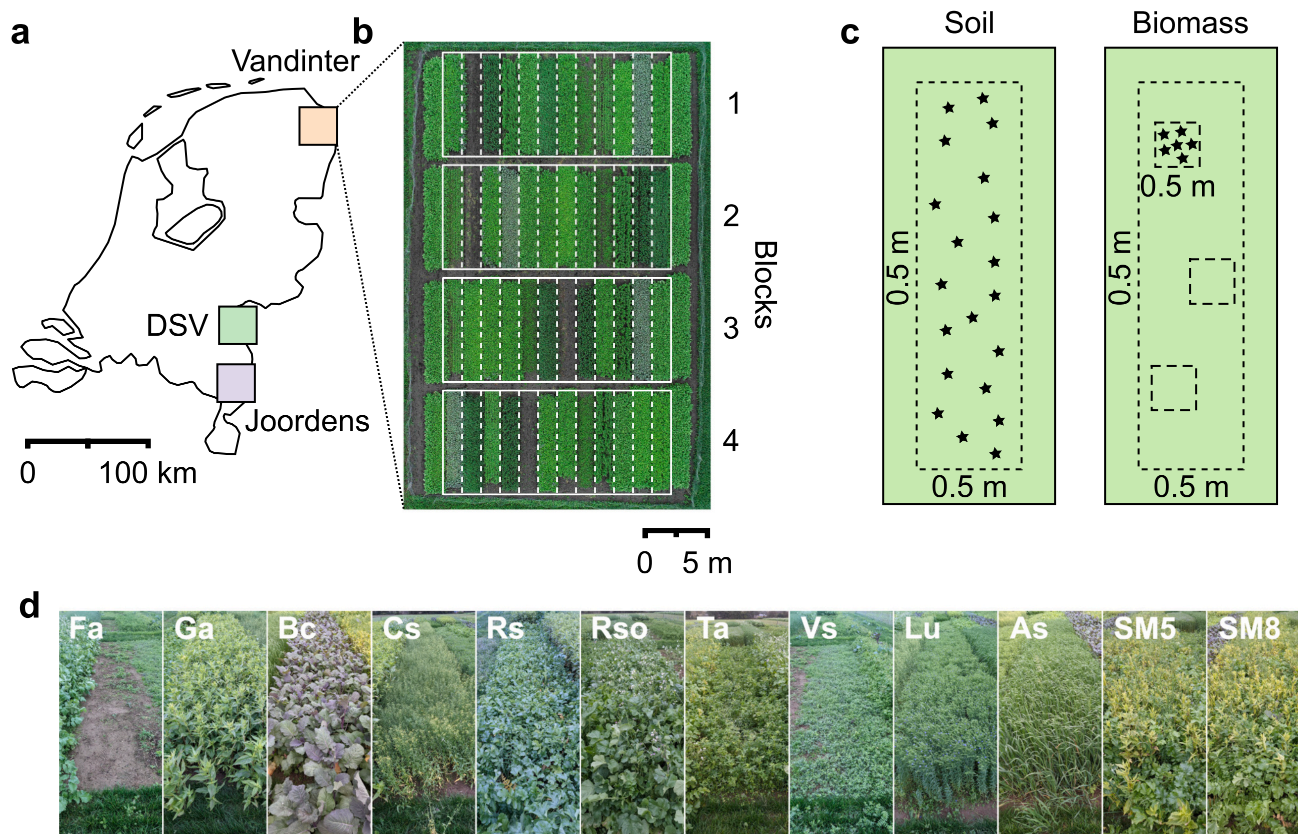

Fig. 1. Study locations and design. (a) Map of the Netherlands showing the location of the three sites where the experiment was performed: DSV Zaden in Ven-Zelderheide (DSV), Joordens Zaden in Kessel (Joordens), and Vandinter Semo in Scheemda (Vandinter). (b) Drone photograph taken at Vandinter on September 29, 2021. The solid white lines delimit the four experimental blocks, and the dashed lines the 12 cover crop treatments within each block. (c) Diagrams representing the strategies for collecting soil samples along the plot length, and above-ground and below-ground biomass at three sub-plots. The stars illustrate the position of soil cores taken for collecting soil and root biomass. The diagrams are not to scale. (d) Photographs illustrating the status of the cover crop treatments in one block at the DSV location on the day of soil sampling. Abbreviations are provided in Table 1.

Table 1
Details of the cover crop species used in the experiment, and inclusion in the two mixed culture treatments.

				Seed quantities sown (kg ha^{-1})		
Species code	Species	Plant family	Origin[a]	Monocultures	SM5[b]	SM8
Fa	- (fallow)	–	–	0	–	–
Ga	*Guizolia abyssinica*	Asteraceae	DSV	10	5.8 (12 %)	6.6 (15 %)
Bc	*Brassica carinata*	Brassicaceae	DSV	14	–	0.9 (2 %)
Cs	*Camelina sativa*	Brassicaceae	DSV	7	–	0.4 (1 %)
Rs	*Raphanus sativus*	Brassicaceae	DSV	25	8.2 (17 %)	6.2 (14 %)
Rso	*Raphanus sativus* ssp. *oleiferus*	Brassicaceae	Vandinter	25	–	–
Ta	*Trifolium alexandrinum*	Leguminosae	DSV	30	–	2.6 (6 %)
Vs	*Vicia sativa*	Leguminosae	DSV	100	19.2 (40 %)	15.4 (35 %)
Lu	*Linum usitatissimum*	Linaceae	DSV	35	4.3 (9 %)	3 (7 %)
As	*Avena strigosa*	Poaceae	DSV	70	10.6 (22 %)	8.8 (20 %)

[a] Company that provided the seed batches for the experiment.
[b] SM5, 5-species mixture; SM8, 8-species mixture. In the mixtures, the percentage of each species' seeds respect to the total weights is provided within parentheses.

biomass gravimetrically and extrapolated the biomass productivity to kg ha^{-1}.

2.4. Soil microbiome profiling

We extracted total soil DNA from 2 g of the composite soil samples using a custom protocol (Harkes et al., 2019), and quantified it using a Qubit 4 fluorometer (Thermo Fisher Scientific, USA). We used 3 ng of DNA from each sample to amplify the bacterial V4 region of the 16S rDNA and the fungal rDNA internal transcribed spacer 2 (ITS2) region using the primer pairs 515F/806R (Caporaso et al., 2012) and fITS7/ITS4 (Ihrmark et al., 2012), respectively, including adapter sequences at the 5′ ends for multiplex indexing. All PCR reactions were run in 20 μl volumes using the Phusion Hot Start II Polymerase (Thermo Fisher Scientific). We attached sample-specific Illumina indexes to amplicons following Cazzaniga et al. (2023a, 2023b), pooled the samples in one library for bacteria and one for fungi, quantified them with a KAPA Library Quantification Kit (Roche, Switzerland), and sent them out for sequencing on a Illumina MiSeq platform (2 × 300 bp, V3 chemistry) at Useq (Utrecht Sequencing Facility, Utrecht, the Netherlands).

In addition to bacteria and fungi, we profiled the soil communities of AMF by nested amplification of a 18S rDNA gene fragment using the

primer pairs AML1/AML2 (Lee et al., 2008) and AM1/NS31 (Öpik et al., 2008). The first PCR was set up in 25 μl reactions comprising 200 μM of dNTPs, 0.5 mM of each primer, and 0.02 U μl^{-1} of Q5® High-Fidelity DNA Polymerase (New England Biolabs) in 1× Q5 reaction buffer, and 2 μl of template DNA. Thermal cycles consisted of a first denaturation step at 94 °C for 3 min, thirty cycles of denaturation at 94 °C for 45 s, hybridization at 58 °C for 45 s, and elongation at 72 °C for 45 s and a final elongation step at 72 °C for 10 min. Since the PCR bands were clear and no primer dimer or excessive dNTPs were visible on 1 % agarose electrophoresis gels, 1 μl was directly used for the second PCR using the same reaction conditions as the first, but in a reaction volumes of 50 μl. PCR products were purified with the ZR-96 DNA Clean-Up Kit (Zymo Research, CA, USA), quantified using a PicoGreen assay (Thermo Fisher Scientific), and sent to the Beijing Genomics Institute (Beijing, China) for library preparation and sequencing on a iSeq-100 platform.

2.5. Bioinformatics workflow

We used the DADA2 pipeline (Callahan et al., 2016) in R v4.3.0 (R Core Team, 2023) to assemble, quality-filter, and cluster the sequence reads into amplicon sequence variants (ASVs; Callahan et al., 2017) with specific parameters for each organismal group (Table S2). To taxonomically assign the ASVs, we first compared all sequences against a local copy of NCBI GenBank using BLASTN v2.12.0+ to discard those not matching the target group, and then assigned them to taxa by comparisons against the SILVA v132 (Quast et al., 2013), the UNITE v9.0 (Kõljalg et al., 2005), and the MaarjAM VT release 2019 (Öpik et al., 2010) reference databases for bacteria, fungi, and AMF, respectively, using the Naïve Bayesian Classifier tool of MOTHUR v1.47.0 (Schloss et al., 2009; Wang et al., 2007).

The sequence data generated are available from the NCBI Sequence Read Archive under BioProject number PRJNA1144384, and the scripts with the code used to process and annotate the sequence reads are available at https://github.com/jgmv/MiSeq_process.

2.6. Quantification of soil-borne pathogens

We assessed the effect of the cover crop treatments on the soil populations of a selection of soil-borne pathogens using taxon-specific quantitative PCR (qPCR). Specifically, we targeted the economically important and widespread pathogens *Rhizoctonia solani* AG2–1, *R. solani* AG2–2IIIB, *R. solani* AG3, and *Verticillium dahliae* using the specific TaqMan-based qPCR assays developed by Budge et al. (2009), Woodhall et al. (2013), and Bilodeau et al. (2012). In brief, qPCRs were run in a Bio-Rad CFX 384 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using 10 μl reactions with the Premix Ex Taq™ (Perfect Real Time) qPCR kit (Takara Bio Europe SAS, Saint-Germain-en-Laye, France) containing 1 μl DNA template, 0.3 μM of each primer, and 0.1 μM of the specific probe. Cycling conditions were 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The Ct value for each reaction was assessed using the CFX Manager Software (Bio-Rad) with automatic thresholding. Standard curves were obtained from DNA extracts from pure cultures of each target fungus, using 10-fold serial dilutions ranging from 1000 pg to 10 fg, amplified in four replicates. qPCR slopes were between −3.3 and − 3.4, indicating amplification efficiencies close to 100 %. Quantification values were expressed in pg target DNA g^{-1} of soil, and log-transformed before statistical analyses.

2.7. Statistical analyses

We employed R v4.3.0 for all data analyses. We compared above- and below-ground biomass production, as well as total biomass production (the sum of above- and below-ground biomass) across cover-crop treatments for each location separately, using linear models followed by analysis of variance (ANOVA). We then assessed general patterns in productivity across locations by means of linear-mixed-effects models built with package LME4 v1.1–33 (Bates et al., 2014), with cover crop treatment as fixed effect, and the location, and block nested in location, as random intercept terms. We tested for significance of fixed term effects with Type II ANOVAs using Wald X^2 tests, implemented in package car v3.1–2 (Fox et al., 2021). We applied pairwise post hoc tests using Tukey's honestly significant difference (HSD) tests.

We analyzed the soil microbiome data using the same set of analyses for the bacterial, fungal, and AMF dataset, with small variations in each case. We first discarded samples with <5000 total reads in the bacterial dataset, and <1000 in the fungal and AMF datasets. As proposed by McMurdie and Holmes (2014), we did not rarefy the datasets, and instead accounted for differences in sequencing coverage by including it as a covariate, whenever necessary. We calculated ASV richness and Shannon diversity (H'; expressed as effective number of 'species' following Jost, 2006) using package vegan v2.6–4 (Oksanen et al., 2015), but used the latter as main descriptor of microbial diversity because of its lesser dependence on sequencing coverage. We compared microbial diversity across cover crop treatments within each location, and across locations, respectively, using linear and linear-mixed-effects models as described above for biomass data.

To analyze microbial community composition and structure, we standardized the relative ASV abundances using the Hellinger transformation (Legendre and Gallagher, 2001) and calculated Bray-Curtis dissimilarities among samples. We used principal coordinates analysis (PCoA) to visualize patterns in microbial community composition across locations and/or cover crop treatments, and tested for significant differences by permutational analysis of variance (PERMANOVA; McArdle & Anderson, 2001) using the function *adonis2()* in the R package vegan. We ran the latter with 999 permutations, but restricted permutations in the analyses encompassing the three locations using package permute v0.9–7 (Simpson et al., 2022) to account for interdependency among samples within each site. We visualized the taxonomic composition of communities using bar plots at different taxonomic levels, and Krona charts (Ondov et al., 2011).

We tested whether the treatments with cover crop mixtures promoted greater diversity in soil of ASVs with some degree of host specialization, as opposed to overall microbial communities. We first identified ASVs whose abundances were significantly increased in monocultures of cover crops of each plant family (Table 1), using multispecies generalized linear models (GLMs) built with package mvabund v4.2.1 (Wang et al., 2012). We ran this differential abundance analysis separately for each of the three locations because microbial communities differed greatly among locations. In each case, we only tested ASVs present in at least four samples to discard rare microbes with random occurrence, and included the factor block as a covariate in the GLM models to account for potential autocorrelation within locations. We considered ASVs to be specific for one plant family if it showed positive standardized interaction coefficients after a model selection with LASSO penalties (Brown et al., 2014). We then assessed whether the diversity and relative abundances of plant family-specific ASVs increased in cover crop mixtures as compared to monocultures.

We analyzed the qPCR data of specific soil-borne pathogens in the same way as the biomass data, but in this case omitted the cross-sites assessment with linear-mixed-effects models because site-specific analyses revealed no significant effects of cover crop treatments, and wide variability in the detection of species across sites.

3. Results

3.1. Biomass production of cover crop monocultures and mixtures

The overall biomass production of the cover crop monocultures ranged from 3004 ± 598 (sd) kg ha^{-1} mean dry weight produced by *Vicia sativa* at the DSV location, to 11,182 ± 1343 kg ha^{-1} produced by *Avena strigosa* at the Joordens location. The geographic location affected the overall productivity ($F_2 = 17.4$, $P < 0.001$), with lowest productivity

at DSV (mean across treatments, excluding fallow: 6248 ± 1774 kg ha^{-1}) and the highest at Vandinter (7679 ± 1796 kg ha^{-1}), as well as the productivity of different cover crop treatments (location × cover crop interaction: $F_{20} = 3.5$, $P < 0.001$). However, the patterns in productivity among the cover crops were similar at all three locations, with *Trifolium alexandrinum*, *V. sativa* and *Linum usitatissimum* producing the least biomass everywhere, and *Raphanus sativus* ssp. *oleiferus* and *A. strigosa* the most (Fig. 2a; Fig. S1).

The linear mixed-effects models confirmed the different productivity of cover crops across locations ($X^2 = 500.8$, df = 11, $P < 0.001$; Fig. 2), as location explained 18.2 % of total variance, whereas the experimental blocks within each location only explained 2.5 %. They also showed that both cover crop mixtures (SM5 and SM8) produced significantly more biomass than the least productive crops, *T. alexandrinum*, *V. sativa* and *L. usitatissimum*, and only produced significantly less biomass than the most productive crop, *A. strigosa* (Fig. 2a). We found a similar trend, with small variations in pairwise differences between treatments, when we used models to compare above-ground ($X^2 = 501.2$, df = 11, $P < 0.001$; Fig. 2b) and below-ground productivity separately ($X^2 = 134.6$, df = 11, $P < 0.001$; Fig. 2c).

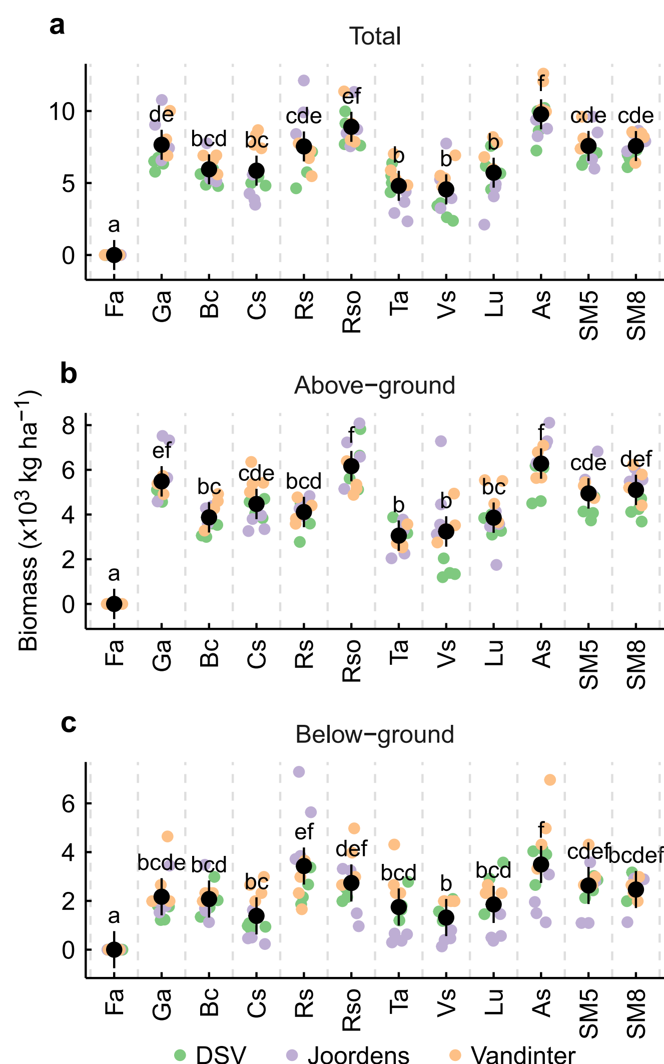

Fig. 2. Dry biomass of nine cover crop monocultures and two species mixtures across the three locations DSV, Joordens, and Vandinter. The plots show the fitted values (black dots) with 95 % confidence intervals (error bars) of the dry total biomass (a), above-ground biomass (b), and below-ground biomass (c) produced by each treatment, according to linear-mixed-effects analysis. The colored points show the raw biomass values for each test plot. The letters above the points indicate significant differences at $P \leq 0.05$, based on Tukey's HSD post hoc tests. See Table 1 for cover crop treatment abbreviations.

3.2. Effect of cover crops on the soil microbiome

The profiling of bulk soil microbial communities using Illumina MiSeq sequencing for the three microbial groups resulted in 6,130,754 quality-filtered reads representing 32,853 bacterial ASVs (median: 42,657.5 reads and 1261 ASVs per sample), 4,533,104 reads representing 4134 fungal ASVs (30,823.5 reads, 134.5 ASVs per sample), and 993,815 reads representing 831 ASVs of AMF (12,705 reads, 28 ASVs per sample). In the AMF dataset, two samples of plots of the Brassicaceae *R. sativus* (Joordens) and *R. sativus* ssp. *oleiferus* (Vandinter) did not yield any ASV. The diversity of the three organismal groups, measured by the Shannon's index, varied differently across locations and the cover crop treatment within each location (Fig. S2a). However, there were no consistent effects of cover crop treatment on microbial diversity across locations, including the cover crop mixtures, based on linear mixed-effects models in which location and experimental block were considered as random effects (Fig. S2b).

The location also was the strongest determinant of community differentiation in bacteria and fungi (Fig. 3). Bacterial communities were unaffected by cover crop treatment, while cover crop treatment explained a significant amount of variation of the fungal and AMF communities (Fig. 3b,c). Differences in AMF communities among cover crop treatments were more evident, as differences among sites were less pronounced (Fig. 3c).

Because of the large variation in microbial communities among the three locations, we ran separate analyses of community structure and composition to analyze the effects of cover crop treatment and experimental block at each site (Fig. 4; Table 2). We found that fungi were the most responsive group of microbes to both cover crop treatment and location, since their communities differed by cover crop treatment at Joordens and Vandinter (Table 2). Contrastingly, the bacterial community differed only among cover crop treatments in Vandinter, while AMF assembly did not significantly vary in response to any factor at each of the three locations (Table 2). At neither location did cover crop mixtures assemble microbial communities different from those of the monocultures (Fig. 4a,b,c).

As with community structure, the taxonomic composition of the microbial communities varied largely with the location but was relatively similar across cover crop treatments at each site (Fig. 4b). Overall, bacterial communities (including 5 % of ASVs classified within the domain Archaea) were dominated by members of the classes Gamma- (16 % of all bacterial reads) and Alphaproteobacteria (14 %), followed by Bacteroidia (11 %). This pattern was mirrored at DSV and Joordens, but at Vandinter there were significantly more Acidobacteria and Verrucomicrobiae, surpassing the abundance of Bacteroidia (Fig. S3). Similarly, at genus level there was a clear differentiation in the taxa dominating at each site, with members of the bacterial Subgroup_6_ge (Acidobacteria), an unidentified Nitrososphaeraceae (Archaea), and RB41 (Pyrinomonadaceae) being abundant at DSV, Subgroup_6_ge at Joordens, and *Candidatus Udaeobacter* and Subgroup_2_ge (Acidobacteriia) at Vandinter (Fig. 4b).

Among fungi, members of the orders Filobasidiales (20 %) and Mortierellales (16 %) consistently dominated at all locations, but the Thelebolales, and to a lesser extent the Tremellales, were particularly abundant at Vandinter (Fig. S4). These abundances matched those across all three locations, with *Solicoccozyma* (Piskurozymaceae) and *Mortierella* (Mortierellaceae) dominating at all three locations, with higher abundances of members of *Thelebolus* (Thelebolaceae), *Saitozyma* (Trimorphomycetaceae), and *Pseudogymnoascus* (Pseudeurotiaceae) in Vandinter (Fig. 4d). As expected, AMF communities were strongly dominated by members of the order Glomerales (98 %; Fig. S5), with the genus *Glomus* almost entirely dominating in the soils at Joordens and Vandinter and being less abundant at DSV due to higher abundance of

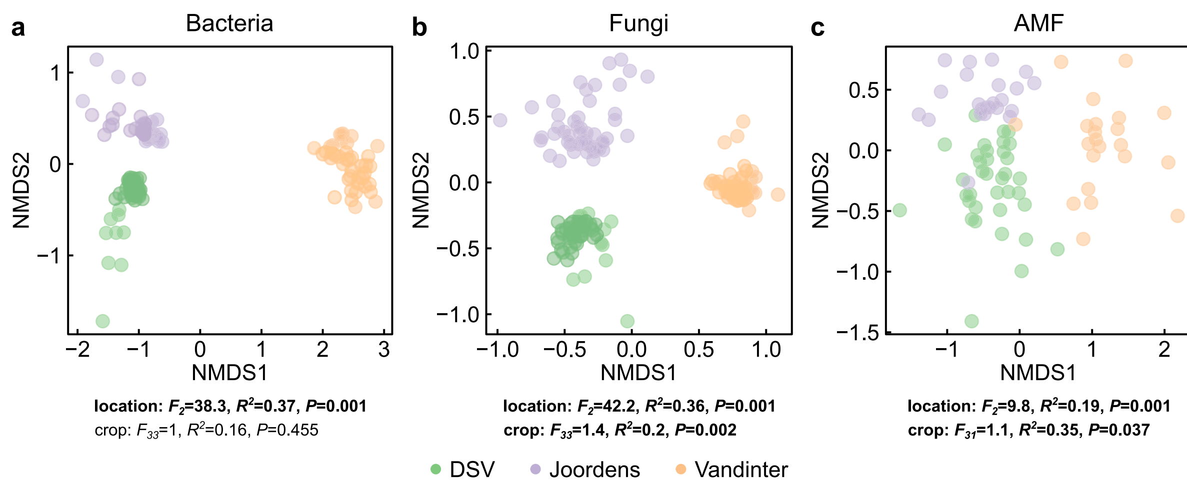

Fig. 3. Structure of bulk soil microbial communities associated with 12 cover crop treatments across the three locations DSV, Joordens, and Vandinter. The plots show non-metric multidimensional scaling (NMDS) ordinations based on Bray-Curtis dissimilarities of communities of (a) bacteria (stress = 0.07), (b) fungi (stress = 0.16), and (c) arbuscular mycorrhizal fungi (AMF; stress = 0.13). The points represent communities of individual test plots and are colored according to the geographical location. Below each plot, the results of permutational analysis of variance (PERMANOVA) testing the effects of cover crop treatments nested in geographical location are shown. Bold: effects with $P \leq 0.05$.

ASVs from another genus of the Glomeraceae, *Rhizophagus* (Fig. 4f).

3.3. Recruitment of host plant-specific microbes by cover crop mixtures

Using multispecies GLMs, we identified bacterial and fungal ASVs at all locations exhibiting putative specificity toward all the cover crop families included in the experiment (Table S3), based on significant positive associations of their abundances with cover crop monocultures. In AMF, we could only detect plant family-specific ASVs at DSV, but not at the other two locations (Table S3). The Poaceae, represented by the species *A. strigosa*, followed by the Asteraceae, represented by *Guizolia abyssinica*, consistently showed the largest number of specific ASVs in all organismal groups across all sites (Table S3).

We examined whether cover crop mixtures accumulated more host-specific ASVs than monocultures, consistent with the hypothesis that mixtures would accumulate microorganisms that preferentially associate with each plant species represented therein. However, we did not observe a surge in microbial diversity in cover crop mixtures, when considering subsets of communities containing only host-specific ASVs (Fig. S6).

3.4. Response of soil-borne pathogens to cover crop treatments

We quantified the abundance of the plant pathogens *R. solani* AG2–1, *R. solani* AG3, and *V. dahliae* at all three study locations using pathogen-specific qPCR assays. A fourth pathogen we targeted, *R. solani* AG2–2IIIB, was not found in any of the soil samples we analyzed. The abundance of the detected pathogens significantly differed across the three sites (AG2–1, $F_2 = 20.1$, $P < 0.001$; AG3, $F_2 = 7.1$, $P = 0.001$; *V. dahliae*, $F_2 = 129.3$, $P < 0.001$), but it was barely affected by cover crop treatment at each location, including both cover crop mixtures ($P > 0.05$; Fig. 5). Only *V. dahliae* showed a significant variation at Joordens ($F_{11} = 2.9$, $P < 0.017$), with a surge in the treatment with *L. usitatissimum* respect to fallow, but the effect was lost in both mixtures even though they included this cover crop species (Fig. 5).

4. Discussion

Our findings indicate that the use of cover crop mixtures does not have significant effects on overall cover crop biomass production, nor on the microbial community composition of the bulk soil as compared to monocultures of the constituent species. These results were consistent across three locations in the Netherlands exhibiting different soil conditions. Overall, the mixed stands had an average productivity as compared to all monocultures, producing significantly more than low-yielding monocultures but less than the most productive species. While different cover crop treatments assembled specific bulk soil microbiota and recruited specific microbial taxa, the cover crop mixtures did not increase the taxonomic and functional diversity, nor did they reduce or increase pathogen pressure. However, these results should be interpreted in light of the short duration of our experiment (three months), as it may take longer to induce significant changes in the bulk soil microbiota. Cover crop termination after three months of growth is a common practice in North-Western Europe, to provide enough time for nutrient release into the soil before the cash crop cycle (Cicek et al., 2015). It is well established that plant diversity consistently enhances ecosystem productivity, but the build-up of this effect following establishment of a plant community can last several years (Cardinale et al., 2007; Eisenhauer et al., 2012). The duration of our experiment aligns with common practices, particularly in rotation cycles between cash crops, rather than in other scenarios such as intercropping with perennial cover crops (Nevins et al., 2018; Walker et al., 2022). Therefore, as compared to monocultures, no added value, but also no disadvantage of cover crop mixtures was observed under the studied settings. This does not exclude the possibility that ecosystem services beyond the ones evaluated in this study (yield and effects on the soil microbiome) are enhanced by cover crop mixtures.

4.1. Biodiversity effects on cover crop biomass

The observed patterns in biomass production between cover crop mixtures and monocultures align well with other studies with similar or different sets of species and at different locations (Elhakeem et al., 2021, 2019; Finney et al., 2016; Florence et al., 2019). Similar to our findings, these studies also reported that yields of cover crop mixtures are comparable to those of the most productive monocultures. These data therefore contradict our first hypothesis that mixing cover crop species may increase biomass productivity as compared to monocultures. The relatively high yields observed for the mixtures were primarily attributable to the low productivity of some monocultures and not niche complementarity, and thus resource use efficiency (Elhakeem et al., 2021; Florence et al., 2019). It appears thus that the relative high productivity of cover crop mixtures is driven by species sorting rather than other mechanisms.

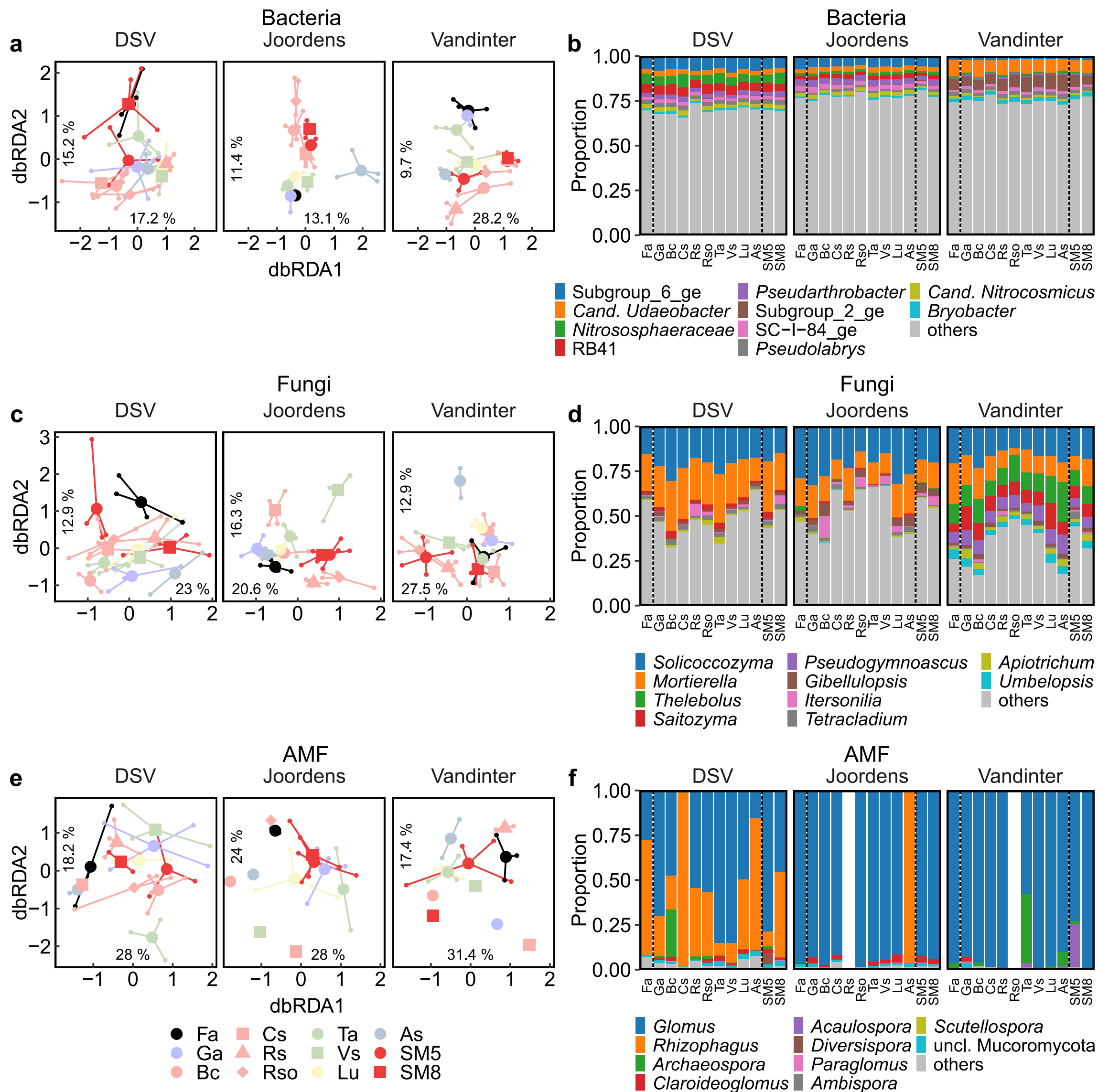

Fig. 4. Bulk soil microbial community structure and composition associated with 12 cover crop treatments within three test locations. Plots a, c, and e show distance-based redundancy analysis (dbRDA) ordinations of (a) bacterial, (c) fungal, and (e) arbuscular mycorrhizal fungal (AMF) communities in response to the cover crop treatments at each location. The large points indicate the centroids for each treatment replicated in four blocks, with lines connecting to smaller points representing individual test plots within treatments. Point colors and shapes differentiate individual samples, with shapes coding for plant species and colors for plant families, or different categories for the fallow and mixed cover crops treatments. Percentage values indicate the proportion of variance represented by the adjacent dbRDA axis. Bar plots b, d, and f represent the relative proportion of the 10 most abundant genera of (b) bacteria, (d) fungi, and (f) AMF. The dashed lines within the plots delimit the monoculture treatments from the fallow (left) and cover crop mixture (right) treatments. Empty bars in f correspond to samples from the Brassicaceae *Raphanus sativus* (Joordens) and *R. sativus* ssp. *oleiferus* (Vandinter), where no AMF were found. See Table 1 for cover crop treatment abbreviations.

Table 2
Results of permutational analysis of variance (PERMANOVA) of the effects of cover crop treatment and experimental block on the structure of bulk soil microbial communities at three locations.

Location	Variable	Bacteria				Fungi				Arbuscular Mycorrhizal Fungi			
		Df	*F*	R^2	*P*	Df	*F*	R^2	*P*	Df	*F*	R^2	*P*
DSV	Cover crop	11	1.0	0.24	0.326	11	1.1	0.25	0.217	11	1.1	0.34	0.165
	Block	3	1.2	0.07	0.092	3	1.0	0.06	0.446	3	1.0	0.08	0.418
Joordens	Cover crop	11	1.1	0.23	0.087	**11**	**1.9**	**0.33**	**0.001**	10	1.1	0.49	0.278
	Block	**3**	**3.9**	**0.22**	**0.001**	**3**	**2.8**	**0.14**	**0.001**	3	0.9	0.12	0.652
Vandinter	Cover crop	**11**	**1.2**	**0.30**	**0.036**	**11**	**1.7**	**0.35**	**0.001**	10	1.1	0.50	0.315
	Block	3	1.3	0.09	0.054	**3**	**1.5**	**0.08**	**0.004**	3	0.9	0.13	0.574

PERMANOVA test statistics include the degrees of freedom (Df), the pseudo-*F* statistic (*F*), the partial R^2 value of residual variance, and the *P*-value (*P*). Significant effects ($P \leq 0.05$) are indicated in bold-face.

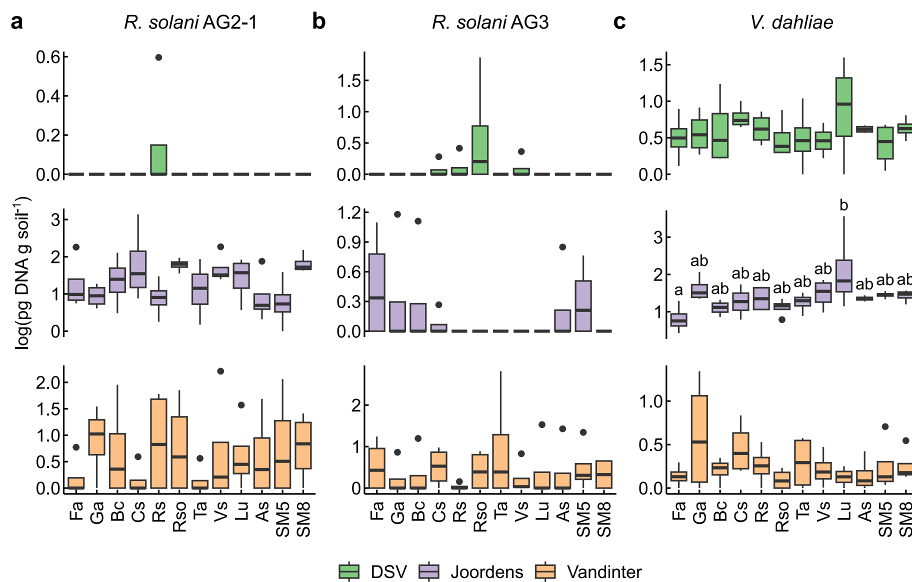

Fig. 5. Quantification of selected soil-borne fungal pathogens in bulk soil associated with 12 cover crop treatments. The box-and-whisker plots show the abundance of (a) *Rhizoctonia solani* AG2-1, (b) *R. solani* AG3, and (c) *Verticillium dahliae* in bulk soil associated with each cover crop treatment at the three study locations, quantified using pathogen-specific TaqMan quantitative PCR assays. Results for a fourth pathogen, *R. solani* AG2-2, are not shown because it was not detected at these locations. Different letters above box plots indicate significant differences at $P \leq 0.05$, based on Tukey's HSD post hoc tests. See Table 1 for cover crop treatment abbreviations.

In addition to absolute productivity, other studies have shown that mixtures, compared to monocultures, provide improved yield resilience under variable environmental conditions and additional ecosystem services, such as increased rates of mineralization of organic matter or retention of nutrients (Couëdel et al., 2018a, 2018b; Elhakeem et al., 2021). While our study was well-suited for evaluating the stability of cover crops productivity across locations with varying environmental conditions, it did not reveal differences in biomass yield among cover crop treatments across the three study locations. In summary, our results reinforce the findings of numerous previous reports of good and stable yields of cover crop mixtures compared to monocultures.

4.2. Effects of geographic location and cover crop treatments on soil microbiota

The soil-borne microbiome, encompassing bacterial, fungal, and AMF communities, differed primarily by geographical location. This aligns with well-established findings from numerous studies on microbial biogeography, which highlight that climate, soil, and dispersal limitation are the primary determinants of soil microbial community composition and structure, overriding the influence of vegetation (Fierer and Jackson, 2006; Hazard et al., 2013; Thiergart et al., 2020). This is reflected, for example, in the larger dissimilarity between the bacterial and fungal communities at Vandinter and those at the other two locations. Vandinter is geographically further apart from the other locations. It also showed more dissimilar soil conditions, including substantially higher organic matter and nitrogen contents, and a lower pH and clay content (Fig. S7; Table S1), which likely explains the local abundances of Acidobacteria and of the fungal genus *Thelebolus*, known to be linked with those conditions (De Hoog et al., 2004; Ivanova et al., 2020). The differences in the taxonomic composition of the AMF communities at DSV compared to other locations could be explained by the markedly lower bio-available P levels of the soil (Egerton-Warburton and Allen, 2000). Therefore, there seems to be no characteristic core soil microbiome for specific cover crop species across locations.

Nonetheless, recent studies investigating the soil microbiome associated with various cover crop species have revealed significant cover crop effects on multiple groups of soil microbes (Cazzaniga et al., 2023a, 2023b; Liu et al., 2021). Notably, brassicaceous species stand out by their ability to suppress several microbial taxa, even in bulk soil after the cash crop that followed the cover crops was harvested (Cazzaniga et al., 2023b). This effect is likely attributable to secretion of antimicrobial compounds, such as glucosinolates, into the rhizosphere (Bressan et al., 2009; Schlaeppi et al., 2021; Schreiner and Koide, 1993), which has led some researchers to propose specific brassicaceous species as eco-friendly biofumigants to control soil-borne plant diseases (Tagele et al., 2021; Walker et al., 2022). However, the impact of cover crops on the bulk soil microbiome appears to be weaker than previously reported. This arguably is attributed to the fact that previous studies have focused on the rhizosphere soil, highly influenced by root exudation and with higher microbial richness and activity (Cazzaniga et al., 2023a), or on bulk soil after incorporation of aboveground cover crops biomass (Cazzaniga et al., 2023b).

4.3. Effect of cover crop mixtures on soil microbial communities

Contrary to our second hypothesis, we did not find a clearly distinct soil microbiota in cover crop mixtures as compared to monoculture or fallow treatments, neither in terms of microbial diversity nor in composition. This may be attributable to relatively small differences among the microbial communities of the different cover crop species not leading to a conspicuous merging of host-specific soil microbiomes when different plant species were grown together. Plant diversity has frequently been postulated to enhance soil microbial diversity by creating a more diverse resource pool, including exudates and litter materials, as well as by increasing overall plant biomass able to sustain a larger microbial richness and abundance (De Deyn et al., 2011; Milcu et al., 2006; Sauheitl et al., 2010). However, the support for such an effect has been mixed and sometimes absent across studies, even in perennial systems (Dassen et al., 2017; Hannula et al., 2020; Mommer et al., 2018). These conflicting outcomes can be attributed, at least partially, to the age of plant communities, which might require years to gradually build-up a specific soil microbial community with specific and increasing feedback on plant growth and health (Eisenhauer et al., 2012; Maciá-Vicente et al., 2023). Plausibly, an effective strategy to increase the impact of plant diversity on soil microbiota is the incorporation of

biomass into the topsoil following cover crop termination, a common agricultural practice. This approach has demonstrated its efficacy in modifying soil microbiota by altering both the quantity and quality of plant litter input to soil (Barel et al., 2018; Cazzaniga et al., 2023b; Liu et al., 2021), akin to the outcomes achieved through soil organic amendments (Clocchiatti et al., 2020). Hence, exploring how the incorporation of biomass of cover crop mixtures influences the taxonomic and functional composition of soil microbial communities stands as an important topic for future research.

4.4. Effect of cover crop mixtures on soil-borne pathogen populations

Beyond the influence on the entire microbial communities, cover crop mixtures had no effect on the population sizes of three agriculturally important soil-borne fungal pathogens present at all three study locations. This again is reminiscent of the generally stable pathogen abundances across monoculture and fallow treatments. We only found a significant increase of the pathogen *V. dahliae* in a *L. usitatissimum* monoculture compared to the fallow treatment, aligning with several reports suggesting a preference of the pathogen for this particular host plant (Fitt et al., 1991; Nuez et al., 2022). An increase in pathogen abundance with *L. usitatissimum* was, however, only found at the Joordens location and did not translate to the cover crop mixtures, containing the species as well. Our results thus do not support our third hypothesis that cover crop mixtures may dilute soil-borne pathogens, but neither suggest an increased risk of soil-borne diseases associated with mixing cover crop species. Nevertheless, we remain cautious about extending this finding to other scenarios, given the potential risks entailed for crop production. After all, we did not observe evident disease symptoms of the quantified pathogens across treatments. The pathogen dynamics could potentially vary in situations of higher pathogen pressure, or following incorporation of cover crop biomass into soil that could support pathogen multiplication and survival until seeding of a subsequent cash crop (Liu et al., 2021).

5. Conclusions

Overall, our findings indicate that combining cover crop species in mixtures does neither result in increased biomass production nor increased soil microbial diversity after one cropping cycle. However, we also find that multispecies cover crop stands do not increase the pathogen risk compared to monocultures, at least under the specific conditions of our experiment. When deciding whether to incorporate plant diversity in cover cropping, it is, however, important to consider additional potential ecosystem services beyond the ones we targeted, particularly in rotation systems between cash crops. We would like to emphasize that one round of cover cropping may not suffice to harness benefits from crop plant diversity (Cardinale et al., 2007). Therefore, further studies similar to ours but that include perennial cover crop species, examine the effect of incorporating cover crop biomass into the soil, and assess the long-term impact of mixed cover crops on the subsequent cash crop performance, are needed to fully grasp the potential benefits that biodiversity may bring to agriculture.

CRediT authorship contribution statement

Jose G. Maciá-Vicente: Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sara Cazzaniga:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Marie Duhamel:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Luc van den Beld:** Writing – review & editing, Methodology, Funding acquisition. **Carin Lombaers:** Writing – review & editing, Methodology, Investigation. **Johnny Visser:** Writing – review & editing, Investigation. **Geert Elzes:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Jos Deckers:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Peter Jan Jongenelen:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Leendert Molendijk:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Liesje Mommer:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Johannes Helder:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Joeke Postma:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Geert Elzes reports a relationship with Vandinter Semo that includes: employment. Jos Deckers reports a relationship with DSV Zaden that includes: employment. Peter Jan Jongenelen reports a relationship with Joordens Zaden that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The sequence data generated are available from the NCBI Sequence Read Archive under BioProject number PRJNA1144384

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2024.105573.

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