



Research paper

Changes in soil microbial communities associated to winter cover crop introduction and soil management in irrigated maize monocropping system

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ARTICLE INFO

Keywords:

Metagenomics
Soil microbiome
Diversity
Tillage
Sustainability

ABSTRACT

Cover cropping is a practice commonly associated with regenerative agriculture that has been implemented with greater or lesser intensity for approximately a century. Cover crops do not only provide a series of benefits and ecosystem services to the cash crops, but also contribute to the regeneration of the microbial component of soils and contribute to overcoming their microbiological exhaustion when they are subjected to very intensive cultural practices. This work analyzes certain soil biophysical properties and the composition and structure of microbial communities associated with the introduction of cover crops based on three different leguminous species (*Vicia villosa*, *Vicia sativa* and *Pisum sativum*) in a 2-ha field cropped with maize during the last four years under two different soil management systems for the cover crop sowing, namely conventional tillage and reduced tillage, combining for eight treatments. No significant differences were detected between management strategies and cover crops in terms of soil organic carbon and nitrogen contents, and microbial biomass in the topsoil. Concerning the microbiome, characterized through Next Generation Sequencing methods, this study showed that soil management was the main factor defining the structure of fungal and bacterial populations. The shaping effect of the leguminous species was smaller, although differences were observed in the composition and frequency of appearance of the different OTUs characterized, depending on the plant species employed.

1. Introduction

The employment and benefits of cover crops as a management strategy has been studied for over a century, although in recent decades, their interest has increased as a sustainable alternative to conventional agricultural systems that rely on tillage (Wittwer and van der Heijden, 2020). Some of the positive impacts of establishing cover crops have been largely recognized in most agricultural systems (Kim et al., 2020b). Cover crops can reduce nitrate leaching, enhance soil organic carbon sequestration (Li et al., 2018), increase available nitrogen (Erana, 2021), modulate nitrous oxide emissions (Tonitto et al., 2006; Kaye and Quemada, 2017), minimize wind or water erosion risks, and/or exert weed control (Wittwer and van der Heijden, 2020). For instance, the use of

various leguminous species as a cover crop usually increases crop yields, while the introduction and management of grasses can have disparate effects (Van Eerd et al., 2023).

Moreover, the maintenance and conservation of soil health is a driving factor in agricultural sustainability that directly affects productivity. Nonetheless, soils, and especially those from agricultural areas, are threatened globally mainly due to unsustainable practices (Dubey et al., 2019). In this context, the study of the roles played by microbial communities in agricultural soils have gained attention in the last decades, being considered as a key driver to maintain and restore soil health under a changing climate scenario (Purohit et al., 2024). In this context, high-throughput sequencing methods applied to a huge range of agronomically important crops (Goel et al., 2017) has revealed

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<https://doi.org/10.1016/j.apsoil.2025.106483>

Received 24 June 2025; Received in revised form 17 September 2025; Accepted 17 September 2025

Available online 22 September 2025

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the significant role of microbial communities, particularly those found in the root zone and surrounding soil (rhizosphere). These communities can vary notably depending not only on agroecological and management conditions but also on the genetic profile of each plant species. Plant-microbe selective interactions can enhance the presence of microorganisms that provide specific adaptive benefits, enabling plants to better withstand biotic stresses, such as pathogens and pests, as well as abiotic challenges, including drought, salinity, or nutrient limitations (Bordenstein and Theis, 2015). In this context, a new paradigm considers plant species not only as an individual genotype, but also as a larger biological entity that also includes its associated microorganisms (microgenome), introducing the concept of “holobiont” (Rosenberg and Zilber-Rosenberg, 2016).

Focusing on the soil system and regarding the relationship between agricultural management based on cover crops and the soil microbiome, numerous studies have revealed positive effects of these practices, such as the increase in microbial biomass, enzymatic activities, and the evenness of relative abundances of both bacterial and fungal taxa (Kim et al., 2020b). In addition, the impact of practices such as direct sowing and no-till on microbial communities have been investigated for many decades, and clear differences have been observed between tilled and non-tilled soils (Mirás-Avalos et al., 2011; Li et al., 2020). In this sense, many studies have suggested that, when comparing soil microbiomes between conventionally tilled and no-till managements, the taxonomic composition of both prokaryotic and eukaryotic microbes changes even when there is no observable difference in microbial community richness (Frøsvlev et al., 2022). In the case of certain cereal species such as maize (Vendruscolo et al., 2022) or barley (Bziuk et al., 2021), the adoption of certain long-term soil management practices, including no-till or the introduction of cover crops, have shown significant shifts in the microbiota of the rhizosphere. However, the interaction between tillage and cover crops has been rarely assessed in previous studies.

In addition, no-till can also induce a reduction in the soil structural porosity due to the absence of mechanical fragmentation of the topsoil (Guérif, 1994), increasing soil water content and promoting anaerobic activities such as denitrification (Pekrun et al., 2003). In contrast, tillage may alter the composition of soil microbial communities because of the modification in the environmental conditions for these microbes, such as aeration, temperature and permeability, as well as the mechanical disruption of fungal mycelia (Young and Ritz, 2000). Nevertheless, when changing from one strategy to another, a transition period is expected to occur for three years and, then, a new equilibrium is reached (Aon et al., 2001). Therefore, the assessment of microbial communities must be carried out after the equilibrium has been reached. In this context, the objective of this work was to assess the effects that leguminous winter cover cropping and its soil management at sowing (conventional tillage versus direct sowing) have on the diversity and structure of soil bacterial and fungal communities in an irrigated maize field under semiarid conditions after four years of establishing these practices. We compared the combination of three cover crop species (common vetch, hairy vetch and pea) and two soil management systems at cover crop sowing (conventional tillage and direct sowing) after four years of their establishment. A control treatment with no cover crop was also considered under both soil management systems. The main novelty of this work is the assessment of the interactive effects of soil management and cover crop species on the bacterial and fungal communities of a soil devoted to maize.

2. Materials and methods

2.1. Study site and experimental design

The study was performed sampling soil from an experiment aimed at assessing the effects (including crop yields, nitrogen fixation, soil physical and chemical properties, microbial diversity and biomass, etc.) of the introduction of winter cover crops combined with no-till on a

maize crop that was carried out from October 2018 to October 2022 at the experimental field ‘SotoLezcano’ (Zaragoza, Spain) under irrigated semiarid Mediterranean-continental conditions. The mean annual air temperature for the site is 14.6 °C and the mean annual rainfall and reference evapotranspiration (ET₀) amount to 318 mm and 1243 mm, respectively (period 2004–2019). The trial was established on a deep (>120 cm) Typic Xerofluvent soil (Soil Survey Staff, 2014), with a clay loam texture and pH greater than 8. The field had been cultivated continuously with maize since 2015 and it was irrigated with a solid-set irrigation system. Reference ET₀ was calculated using the FAO Penman-Monteith method (Allen et al., 1998) with data collected by a nearby weather station. The crop evapotranspiration (ET_c) was calculated multiplying the daily ET₀ by the estimated single crop-coefficient (k_c). Maize irrigation requirements were determined weekly as the difference between the ET_c and the effective precipitation. Maize was irrigated with 702, 627, 583, and 643 mm year⁻¹ in the 2019, 2020, 2021 and 2022 growing seasons, respectively. The cover crops were slightly irrigated (26, 4, 16, and 14 mm year⁻¹ in the 2019, 2020, 2021, and 2022 seasons, respectively) to facilitate crop establishment.

The experiment was laid out in a split-plot design with complete blocks with 3 replications (3 m wide × 60 m long) and eight treatments combining two factors: soil management of the cover crop (conventional till vs reduced till) as a main factor (whole plots) and cover crop (CC) species (control and three leguminous species) as second factor. To facilitate the tillage operations with standard machinery, the reduced tilled plots (first factor) were located at one half of the field and the tilled plots at the other side. The reduced till treatment consisted in no-till and direct sowing of the cover crop with a commercial no-till seed drill and the conventional till included a shredder/cultivator/roller operation and then sowing the cover crop. For the first factor (specie of cover crop) the following treatments were considered:

- Control: bare soil from maize harvest until the next sowing.
- CC-pea: cover crop (pea, *Pisum sativum* L.) sown in October (200 kg of seeds per ha, approximately).
- CC-sativa: cover crop (common vetch, *Vicia sativa* L.) sown in October (100 kg of seeds per ha, approximately).
- CC-villosa: cover crop (hairy vetch, *Vicia villosa* Roth) sown in October (50 kg of seeds per ha, approximately).

The seed rates used for sowing each cover crop were caused by the great difference in seed weight between species, as they were sown using the seed density commonly used when establishing them as the sole crops in a field. Nevertheless, the average number of plants was similar for the three species (71, 83 and 79 plants m⁻², respectively for pea, common vetch and hairy vetch). The cover crops were established either with direct sowing (reduced till treatment) or with conventional tillage (conventional till treatment). Thus, a total of 8 different treatments (4 cover crops × 2 soil managements) with 3 replicates were established. No fertilizer was applied to the cover crops. All the maize residues were incorporated into the soil. The seeds of the cover crops were not inoculated since these species were grown historically in the field and their rhizobia populations are already present in the soil. In tilled plots, the residues were chopped and incorporated before CC planting in autumn, whereas in the no-tilled plots both the maize and CC residues were incorporated together before sowing maize in spring. In the spring, all the plots were tilled before maize sowing using sequentially a shredder, disk harrow and cultivator with a maximum depth of 0.25 m. Then, a long-season maize (*Zea mays*, var. ‘P0933Y’, FAO 500), at a sowing rate of 89,000 seeds ha⁻¹ was sown at a row spacing of 0.75 m. The maize growing season was from April–May to September–October and maize was harvested with a commercial combined and only the grain was removed from the field. The aboveground biomass of the cover crops and the companion weeds were manually sampled before their incorporation at the end of March, harvesting two samples of 0.5 m² in each plot. The samples of cover crop and weeds were oven dried at 65 °C and

then weighed to estimate the total biomass per hectare. Maize was fertilized through the sprinkler irrigation system by applying 150 kg N/ha in three split applications: 50 kg before sowing (along with 100 kg/ha of $P_2O_5-K_2O$), 50 kg at the V6 stage (June) and 50 kg at the V12 stage (July).

2.2. Soil samples

Soil samples for subsequent metagenomic analyses were collected in October 2022 from each field replication. In total, after harvesting the main crop (maize), 24 composite soil samples were taken between plant rows, collecting 10 cores in each of them at about 15 cm depth with an auger and subsequently mixing them to provide a pooled sample in each plot. After this, the soil samples were stored at $-85^{\circ}C$ until further processing (Lane et al., 2022).

Soil microbial biomass was measured by the chloroform fumigation-extraction method (Vance et al., 1987) on fresh soil samples. The organic carbon and total nitrogen contents were determined by the dry combustion method (TruSpec CN, LECO, St. Joseph, MI).

2.3. DNA extraction and sequencing

Prior to performing total genomic DNA extraction, pooled soil samples were spread out on the surface of a laminar flow cabinet to be defrosted and sifted through a sterile metal strainer with the help of a spatula. Then, approximately 400 mg of sifted soil per sample was used to extract total genomic DNA with the DNeasy Power Soil PRO-kit® (Qiagen, Germany), following the manufacturer's instructions. The concentration of extracted DNA was measured using a NanoDrop® ND-1000 spectrophotometer device (Thermo Scientific, MA, USA). The integrity of DNA was checked through 1.5 % agarose gel electrophoresis. Then, samples were sent to Stab Vida, Lda. (Caparica, Portugal) for NGS analysis. The ITS (ITS2 fragment) region was selected as the target amplicon for the metagenomic study of the fungal community, while V3 and V4 variable regions of ribosomal 16S fragment were chosen as target amplicon for bacterial taxa. The Illumina metagenomic sequencing library preparation protocol was used to construct the library, and the resulting DNA fragments were sequenced on the Illumina MiSeq platform using 300 bp paired-end reads.

After sequencing, the generated raw data (for 16S 2,900,132 reads in total, from 58,918 to 209,040 raw sequence reads per sample; for ITS 4,488,362 reads in total, from 83,978 to 324,394 raw sequence reads per sample) were downloaded and processed through a galaxy work-flow using DADA2 (Galaxy Version 1.20 + galaxy0) (Callahan et al., 2016). The sequences were trimmed and quality filtered, the error rate was denoised, and bimeras were removed. The reads were then grouped into OTUs, allowing a maximum of 3 mismatches for sequences to be considered the same OTU, and classified by taxon using the DADA2 modules *assignTaxonomy* and *addSpecies*. For fungal taxonomy, the 8.3 version of the general fasta release of the UNITE database was used (Abarenkov et al., 2021). For bacterial taxonomy, Silva NR99 v138.1 was used (Yilmaz et al., 2014).

2.4. Statistical analysis

Organic carbon, total nitrogen, microbial biomass, and the 4-year average (from 2019 to 2022) cover crop biomass (plus weeds) data were submitted to a two-way ANOVA, using tillage (factor 1) and cover crop species (factor 2), after checking the normality and homoscedasticity assumptions using the Shapiro-Wilks and Bartlett tests, respectively. Differences among treatments were considered significant when the *p*-value was less than 0.05.

Statistical analysis of the NGS data was performed in Rstudio using the *phyloseq* package (McMurdie and Holmes, 2013). Alpha-diversity was determined using four of the most common indexes (Chao, Shannon, Fisher, and Simpson) (Whittaker, 1972). For beta-diversity analysis

purposes, principal coordinate analysis (PCoA) and ordination plots of OTU data were constructed using the *phyloseq* package for R. The *DESeq2* package was used to detect differentially abundant OTU between types of hypotheses (soil management and cover species) and with default parameters (Wald test). To analyze the effects of alternate mycobiota communities in the different plots, FUNGuild annotation tools were utilized for functional predictions (Nguyen et al., 2016). Additionally, Picrust2 (Douglas et al., 2020) was run to identify functional variation but no KEGG pathway showed variability between conditions.

3. Results and discussion

3.1. Soil analyses

Independently of the species in the cover crop, soil organic carbon content was similar between till (1.158 %) and reduced-till (1.147 %) plots. However, total nitrogen was slightly higher in reduced-till plots (0.135 % vs. 0.122 %), although this difference was marginally significant (*p*-value = 0.051, Table 1). Soil microbial biomass was similar between tillage practices (436 and 406 mg C-biomass/kg soil for till and reduced-till plots, respectively). No significant differences among soil management practices (till and reduced-till) and cover crops were detected for organic carbon content and microbial biomass (Table 1). However, total nitrogen contents were affected significantly by the cover crop species, while the effect of soil management was marginally significant (*p*-value around 0.05) (Table 1). Soil total nitrogen content was lower in plots from the conventional tillage management with *V. sativa* as cover crop when compared with those from the reduced tillage management with *V. villosa* and control plots (Table 1). The total biomass of the cover crop and the weeds incorporated before the maize was not affected by the soil management, but the control plots (without cover crop) tended to present lower biomass than the plots with cover crop, specially under conventional tillage. However, in reduced-till plots, the differences in biomass were not significant due to the considerable growing of the weeds in the control treatment (Table 1). The main weeds found in the field before maize sowing were *Sinapis arvensis* L., *Poa annua* L., and *Diplotaxis eruroides* DC.

Previous studies in monocropping maize systems reported that replacing winter fallow with a legume increased significantly soil organic carbon levels (Kong et al., 2005; Fernández-Ortega et al., 2025); however, this could not be confirmed in the current work, likely due to the fact that maize straw was also incorporated to the soil and that the control plots allowed to grow a significant amount of weeds during the intercrop period (October to March) in monocropping maize systems, which was comparable in biomass to the cover crop treatments (Table 1). The observed differences among treatments in soil total nitrogen, although minimal, align with previous studies in which cover crops reduced the levels of nitrogen in the soil before winter (Kramberger et al., 2009), but translated into increases in maize grain and nitrogen contents (Zugasti-López et al., 2024).

3.2. DNA sequencing

The processed OTU tables were composed of 1,313,157 reads for fungi and 466,514 reads for bacteria. In the case of fungal amplicons, a total of 2386 OTUs were obtained, while 8330 OTUs were resolved for the procaryotic taxa. According to these data, species diversity was significantly higher for bacterial 16S fragment amplifications compared to fungal taxa. However, among all these resolved OTUs, 652 corresponded with fungal taxonomic binomials, and 291 represented procaryotic taxa determined at the species level. So, the level of taxonomic resolution for bacteria was considerably lower than that recorded for fungi. Focusing on the genus level, a total of 2944 generic taxa were determined for bacteria compared to 1161 OTUs for fungi, which provided a more accurate view of the magnitudes of taxonomic diversity

Table 1
Organic carbon and total nitrogen contents, and microbial biomass at the end of the experiment (October 2022) as a function of soil management and winter cover crop in a maize system. The four-year average of the total cover crop biomass (including weeds) in each treatment is also presented. Data are averages $\pm$ standard errors.

Soil management	Cover crop	Organic C	Total N	Microbial biomass	¹ Cover crop biomass
		%		mg C-MB/kg soil	kg/ha
Conventional tillage	Control	1.105 $\pm$ 0.076	0.124 $\pm$ 0.008 ab	400.9 $\pm$ 167.8	1634 $\pm$ 245 a
	Pea	1.078 $\pm$ 0.033	0.125 $\pm$ 0.008 ab	436.0 $\pm$ 73.0	2918 $\pm$ 342 bc
	<i>V. sativa</i>	1.205 $\pm$ 0.061	0.103 $\pm$ 0.006 a	464.1 $\pm$ 116.5	3025 $\pm$ 351 bc
	<i>V. villosa</i>	1.242 $\pm$ 0.079	0.136 $\pm$ 0.012 ab	431.4 $\pm$ 109.7	2232 $\pm$ 240 ab
Reduced till	Control	1.147 $\pm$ 0.087	0.152 $\pm$ 0.012 b	269.0 $\pm$ 128.4	2622 $\pm$ 530 abc
	Pea	1.182 $\pm$ 0.090	0.129 $\pm$ 0.006 ab	438.3 $\pm$ 147.9	3439 $\pm$ 383 c
	<i>V. sativa</i>	1.115 $\pm$ 0.049	0.116 $\pm$ 0.007 ab	377.4 $\pm$ 76.4	2835 $\pm$ 272 abc
	<i>V. villosa</i>	1.142 $\pm$ 0.081	0.142 $\pm$ 0.009 b	492.6 $\pm$ 88.3	2417 $\pm$ 301 ab
Statistical analysis (p-values)					
Sources of variation		Organic C	Total N	Microbial biomass	Cover crop biomass
Tillage		0.834	0.051	0.689	0.1852
Cover crop species		0.782	0.014	0.717	0.0417
Tillage $\times$ Cover crop species		0.435	0.563	0.808	0.4505

¹ The cover crop biomass of the control treatment is the weed biomass. Different letters in the column indicate significant differences between cover crop species.

existing between both groups of microorganisms. These results principally reflect limitations in current bacterial reference databases or challenges in resolving bacterial species from 16S rRNA sequences. They also highlight the complexity of microbial community profiling and the importance of considering both diversity and resolution when interpreting amplicon sequencing data.

3.3. Alpha-diversity analyses

Results corresponding to the diversity analysis within each type of plot (alpha diversity), and for the four richness indexes proposed, showed (as expected) that, in general, the bacterial populations were more diverse and complex compared to fungal populations, generally obtaining higher values in all the indexes analyzed (Fig. 1). Thus, for the ACE and Chao1 indexes, two non-parametric abundance-based species richness estimators, bacterial diversity reached values in the range of 750–1500 for both indexes, while in the case of fungal populations they were in the range of 350–500 for both estimators, clearly below those obtained for prokaryotic microbial communities. A comparable pattern was observed when analyzing the Shannon diversity index (H') across both microbial groups obtaining H' between 6 and 7 for bacteria compared to 3.8–4.5 for fungal populations. Bacterial

H' values are in concordance with what is usually reported for prokaryotes in bare soil, and can be considered as high in species diversity for this group of microorganisms. In the case of fungi, diversity values were slightly above those usually reported for this taxonomic group in metagenomic surveys in soils (Labouyrie et al., 2023; Djemiel et al., 2024). Finally, the values of the Simpson index in bacterial communities were higher than in fungal populations, and, interestingly, these diversity values were similar (for both groups of microorganisms) in plots with the same type of cover crop or soil management modality, suggesting that no treatment decreased intra-plot microbial diversity. Previous studies have reported that bacterial microbiomes tend to be more complex than fungal communities in agricultural soils (Wagg et al., 2019; Zhang et al., 2024). Thus, soil bacterial communities are often found to be more diverse compared to fungal groups, and the number of unique fungal OTUs can only rival or exceed the number of unique bacterial OTUs in certain agroecosystems with very specific conditions (Fierer et al., 2007). For instance, prokaryotic diversity is often more sensitive to environmental changes and agricultural practices, which can lead to a higher variety of bacterial taxa in different soil conditions (Wang et al., 2015). It is commonly assumed that bacterial communities tend to show higher richness and evenness than fungal microbiomes (Simon et al., 2024). As in the present work, previous studies (Zhang

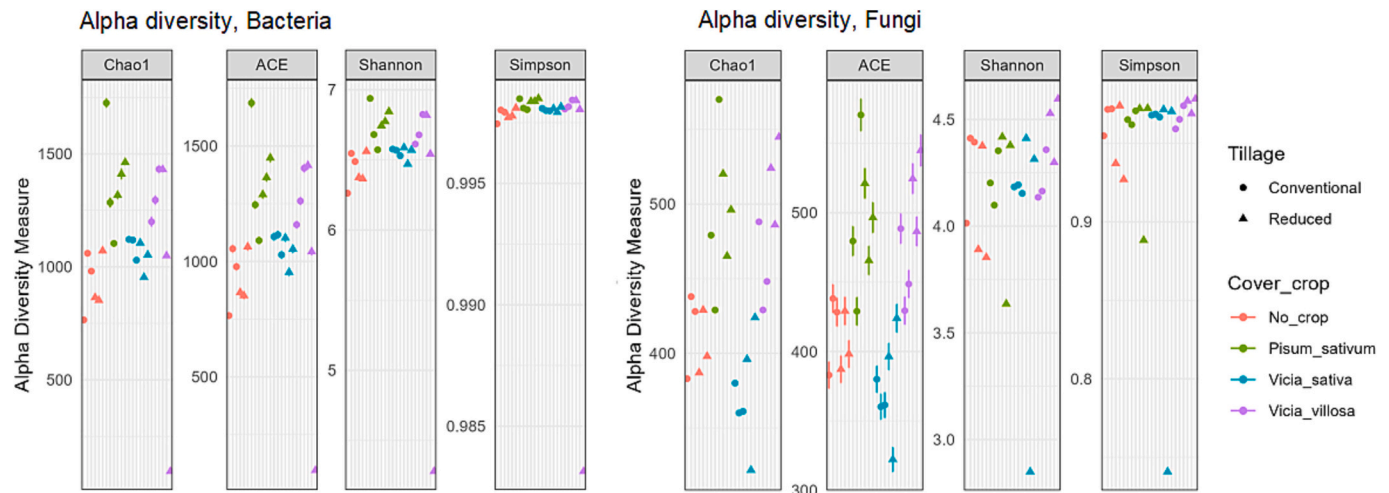


Fig. 1. Alpha diversity indexes of bacterial (left) and fungal (right) communities associated with bare soil in a maize monocropping system under different cover crops and types of management.

et al., 2019) showed that regardless of the type of management, the prokaryotic microbiome seems to have a greater degree of complexity and taxonomic diversity compared to fungal populations. However, in our study, alpha diversity indexes did not show significant differences between soil management types or cover crop species, although differential trends were observed in the values of alpha diversity indices according to the type of vegetation cover species used. In this sense, slightly higher diversity values were obtained from soil samples coming from plots with *P. sativum* as cover crop, both in the case of fungi and bacteria and regardless the till management regime considered. A similar, although less marked, trend was also observed in the case of samples with *V. villosa* as cover legume, while alpha diversity values similar to those of the control soil were recorded in the case of *V. sativa*.

3.4. Beta-diversity analyses

Comparative diversity analyses (β -diversity) showed that the composition and distribution of taxa (prokaryotes and eukaryotes) was more clearly differentiated according to soil management than in relation to the cover crop species considered. Non-metric multidimensional scaling (NMDS) analyses were used to compare microbial community composition under different management types. For both fungi and bacteria, the results showed clear differences in community structure depending on the tillage regime applied (Fig. 2) which is relevant considering that the differences in tillage occurred only for the cover crop sowing, while the tillage for the maize sowing was the same in both treatments. Thus, management type (particularly tillage versus reduced-till) had a strong influence on fungal community composition, as it clearly separated the populations associated with each soil management practice, regardless of the cover crop species used. Numerous previous studies have studied the effect of tillage on the response of soil microbial diversity, especially with regard to bacterial communities. In the case of fungi, there are less experimental data on their diversity and composition in response to this type of management. Li et al. (2020) reviewed a series of previous works on this topic and concluded that, in general, the absence of tillage increased bacterial microbial diversity, but it did not seem to have the same effect on fungal populations, contrary to what was observed in the present study. It is interesting to note that bacteria are often considered (Dong et al., 2017) to be more sensitive to changes in soil properties, considering fungi as more generalist microorganisms, and somewhat more resilient to this type of changes. Additionally, previous studies about reducing tillage on soil fungal communities indicated that mycorrhizal fungi are negatively affected by tillage, as

this practice disrupts their mycelia (Mirás-Avalos et al., 2011). Our study seems to indicate that the type of management is capable (in a short period of time) of altering and differentially shaping the type of fungal community associated with different tillage regimes in the agroecosystem under study. Regarding bacterial communities, a separation between microbial populations was also observed according to the type of soil management employed, suggesting, as previously reported (Srouf et al., 2020), that tillage is the main factor driving beta-diversity in the detected bacterial soil microbial communities. Furthermore, in the case of prokaryotic populations associated with reduced-till plots, a tendency toward the differentiation of microbiomes according to the type of cover crop species was also observed. In this sense, Ouversen et al. (2022) reported that the presence and composition of cover crops affect the evenness and community composition of soil bacterial communities.

Concerning cover crop species, previous studies have analyzed the effect of introducing several cover crops like hairy vetch and rye (Nevins et al., 2018), grass mixtures (Momesso et al., 2022), or legumes (Dörr de Quadros et al., 2019; Sui et al., 2025). In all these cases, an enrichment and the increase in the complexity of microbial populations was recorded as a consequence of the increase in carbon and nitrogen levels in soils due to the incorporation of cover crop biomass, generally observing increases in microbial biomass and enzymatic activities. Specifically, it has been shown that the inclusion of legume cover crops, enhance soil fertility and microbial richness and diversity, potentially reducing the need for chemical fertilizers, although in the present work the values associated with different diversity indices in soils under cover crops were not significantly higher than those found for control plots.

In the case of the fungal species, it was observed that the type of leguminous species employed could shape the composition and structure of the populations, this driving effect being more evident in the case of soils from reduced-till plots (Fig. 3). In the case of bacterial diversity, it was observed that the composition of the bacterial communities under reduced till management tended to be more homogeneous between replicates with the same condition, in at least two of the cover species and the control plots (an exception for this was found for *V. villosa* samples). Thus, the composition of the prokaryotic communities associated with the three legume species used was more similar in reduced tillage, even than those resolved from samples from the control plots.

Numerous studies (Smith et al., 2016; Sun et al., 2016; Zhang et al., 2022) have reported that no-till practices, particularly when combined with cover crops (Nivelle et al., 2016), have a positive impact on soil microbial communities in maize crops. These practices have been

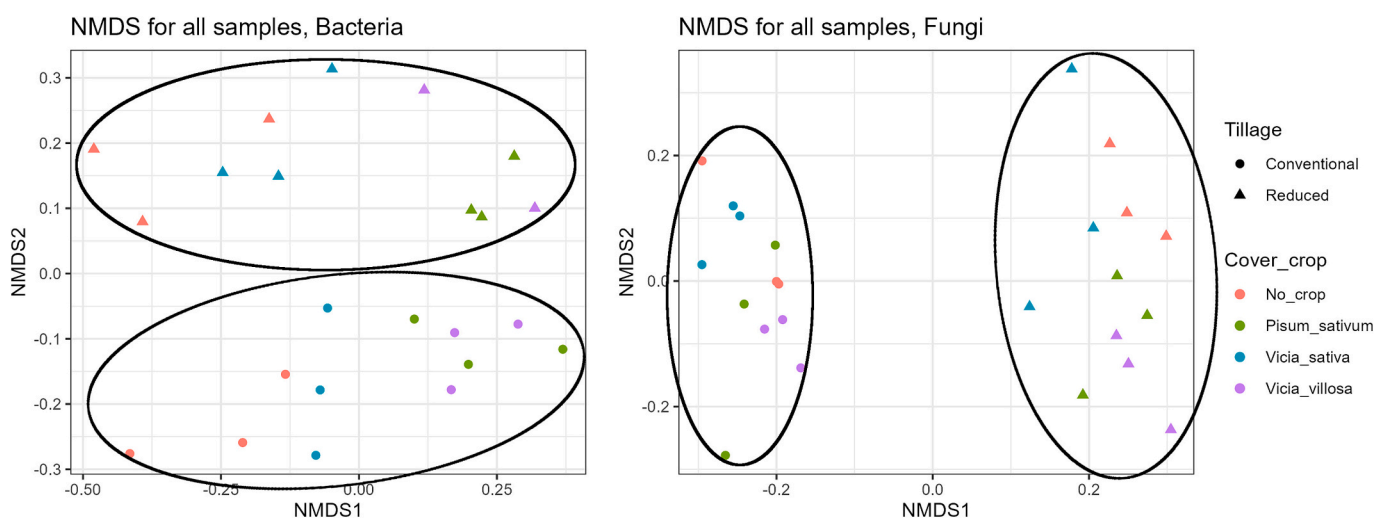


Fig. 2. Beta-diversity analysis of bacterial (left) and fungal (right) populations using non-metric multidimensional scaling (NMDS) comparing cover crop species and soil management practices. The third replicate of bacteria in reduced tillage has been removed from the analysis due to the extremely low alpha diversity values observed in it.

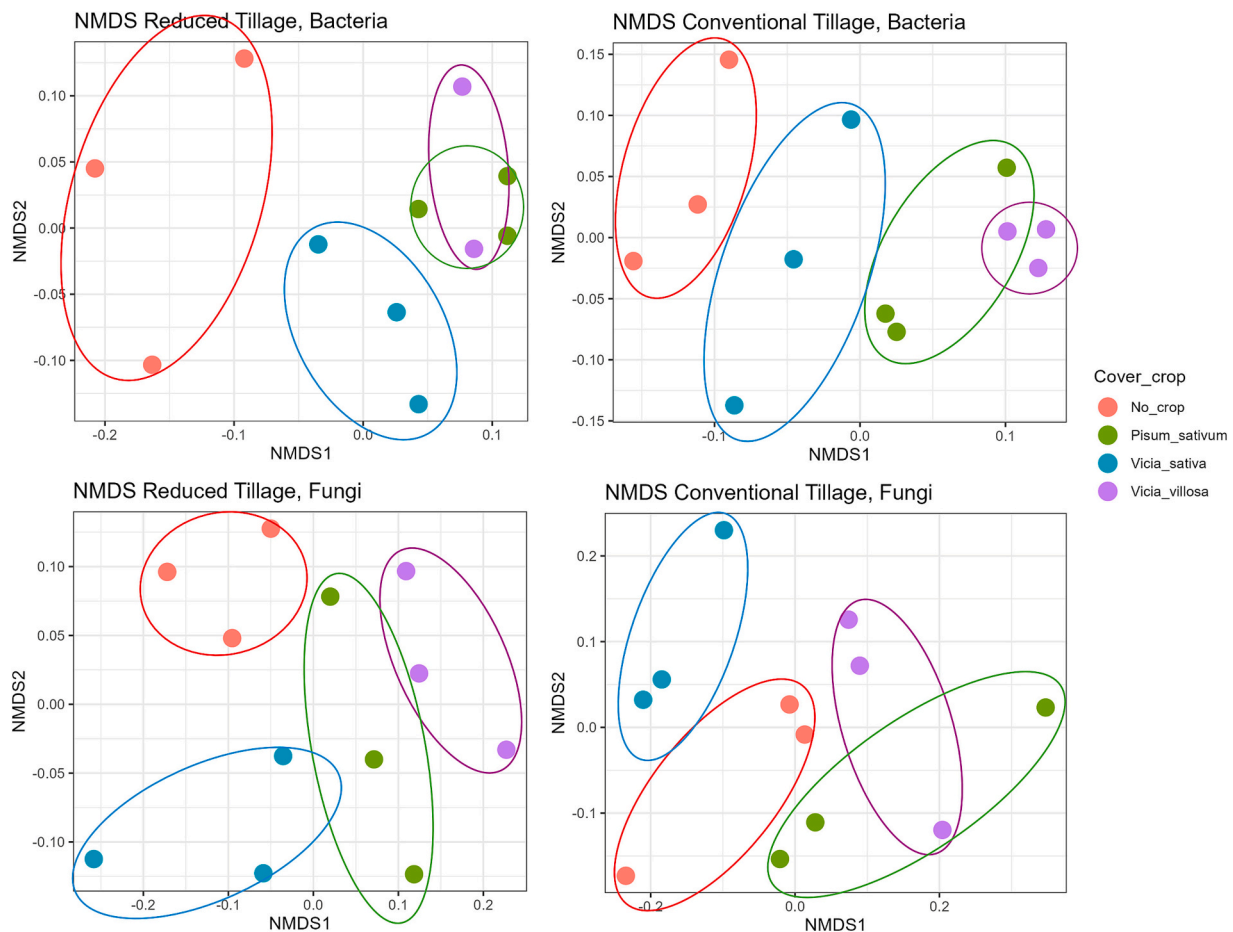


Fig. 3. Beta-diversity analysis of microbial populations using non-metric multidimensional scaling (NMDS) between crop cover plant species and soil management. Top row: Bacterial Reduced-till samples (left), Bacterial Tillage samples (right); Bottom row: Fungal Reduced-till samples (left), Fungal Tillage samples (right). The third replicate of bacteria in reduced tillage has been removed from the analysis due to the extremely low alpha diversity values observed in it.

associated with increased soil organic carbon (however, the results of the present study showed similar organic carbon rates under both till management conditions), enhanced microbial abundance and diversity, and improved soil biological quality and sustainability. Additionally, the inclusion of cover crops has shown to further enhance soil microbial metabolic activity and diversity, ultimately contributing to improved soil quality and crop yields. When performing a pairwise comparison of the fungal OTUs among reduced tillage and tilled plots, a series of taxa were clearly differentiated by the type of management used (Fig. 4). Thus, in plots with conventional management, the dominant fungal taxa belonged to soil-borne pathogenic genera such as *Fusarium*, *Neonectria*, *Plectosphaerella*, *Gibellulopsis*, *Thelonectria*, and *Paracremonium*, while genera such as *Aspergillus*, *Penicillium*, *Humicola*, and *Trichoderma*, the later more related with beneficial effects on the soils were more abundant in plots managed with conservation agriculture techniques.

Pathogenic genera, like the above mentioned *Gibellulopsis* (*Plectosphaerellaceae*), were always less abundant in plots with no crop vs those with any cover crop, both in till and reduced-till plots. In addition, the soil-borne genus *Dactylonectria* (*Nectriaceae*) was always less abundant in no cover crop plots vs *Vicia sativa* cover crop in both cultural management practices. Another soil pathogen, *Fusarium equiseti*, that was among the most abundant OTUs in the current study, was more abundant in control plots. These results seem to indicate that the absence of cover crops (regardless of tillage) maintains the presence in the soil of a contingent of species of generalist pathogenic soil-borne fungi adapted to survive long periods even in the absence of any plant host. Their low incidence in soils with cover crops could be due to a possible displacement by other species that include legumes in their host range.

3.5. Fungal taxonomy

Soil management (conventional vs. reduced tillage) modeled the composition and specific weight of the different taxonomic groups resolved. Thus, at the family taxonomic level (Fig. 5), there was a higher incidence and abundance of taxa from the *Nectriaceae* and *Plectosphaerellaceae* families, in the plots with conventional management, and regardless of the type of cover crop used compared to all the plots managed with reduced tillage. These two taxonomic groups include species from *Fusarium*, *Neocosmospora*, *Cylindrocarpon*, *Plectosphaerella*, etc., pathogenic and ubiquitous soil-borne fungi with a wide range of potential hosts, including maize. Conversely, these families and genera of soil-borne pathogens were less abundant in the reduced-till plots. This contrasts with several studies on maize (Wang et al., 2020; Sommermann et al., 2018), which reported higher abundance of *Fusarium*-like fungi under no-till and conservation tillage systems, respectively. In those cases, residue retention was linked to an increased risk of diseases such as maize foot rot. In our study, the crop residues were kept in the soil in both management treatments and the maize planting was performed after tillage in all the plots, which means that all plots received at least a disk harrowing pass plus cultivator before maize planting. Furthermore, in the soil samples from most of the reduced-till plots, a differential abundance of representatives of the *Clavicipitaceae* family was recorded, especially species of the genus *Metarhizium* (more specifically the *M. brunneum* / *anisoplae* complex), an entomopathogenic species long known for its natural ability to regulate insect populations, and commonly used as a biological control agent (BCA) in integrated pest management programs.

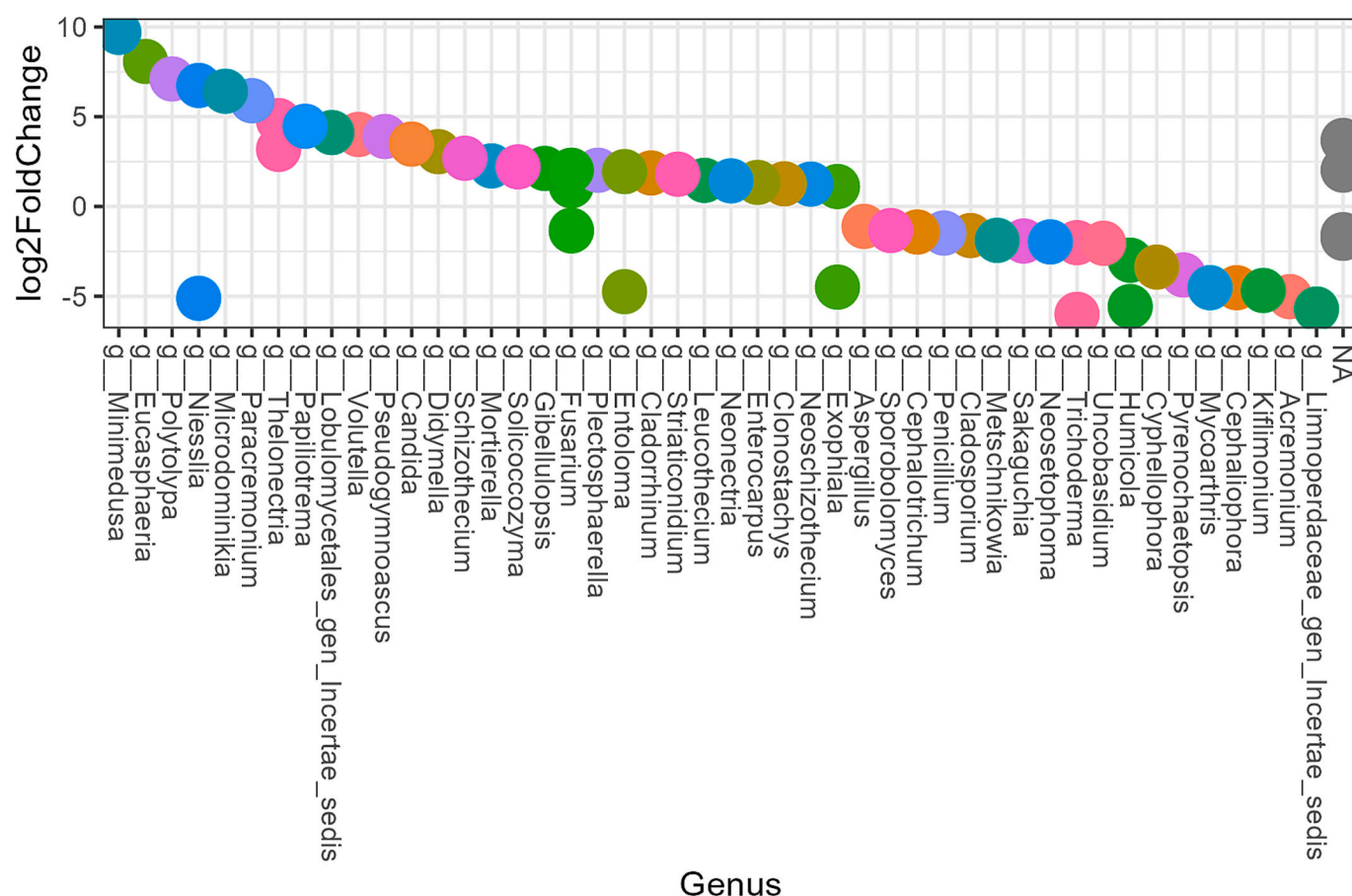


Fig. 4. Pairwise comparison. Differential abundant fungal OTUs grouped by genera ($p < 0.05$) in samples from tillage vs reduced-till management plots. Negative values (x-axis) indicate higher abundance in reduced-till plots and positive values indicate higher abundances in till-plots.

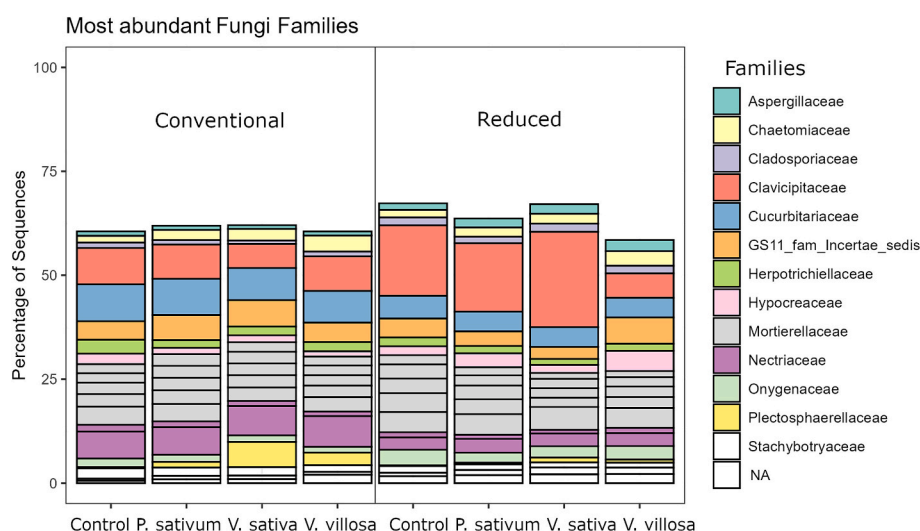


Fig. 5. Composite bar histogram showing the most abundant fungal families in the different cover treatments (Control; *V. sativa*; *V. villosa*; *P. sativum*) grouped by management regimes.

In addition, a differential abundance of OTUs belonging to the *Cucurbitariaceae* family (Pleosporales) was observed in the tilled, conventional plots, where genera such as *Cucurbitaria* or especially *Pyrenochaetopsis* were especially abundant. In this sense, [Chen et al. \(2022\)](#) have found that in multiple cropping systems based on maize, the diversity of soil fungal communities increased compared to spring maize

monocropping, and that this type of practice enriched beneficial fungi such as *Mortierella* and *Metarhizium*, in agreement with the findings of our study. Moreover, [Chen et al. \(2022\)](#) also found that potentially pathogenic fungi like *Pyrenochaetopsis* were less abundant in these systems compared to monocropping, as observed in the current study. Interestingly, a higher incidence of representatives of the *Aspergillaceae*

family, especially the genus *Penicillium*, was also observed in samples from reduced-till plots. Taxa of this large and well-known genus of fungi cause certain pathologies in maize crops, such as the so-called *Penicillium* ear rot, attributable to several species of the genus (Jackson-Ziems et al., 2012). These species are involved in the production of secondary metabolites (mycotoxins), which are toxic to animals and humans consuming the contaminated grain.

3.6. Trophic modes and guilds in fungal microbiome

In the last years, NGS sequencing studies have revealed significant insights into the diversity and distribution of fungal trophic modes across different environments and ecosystems. These approaches can help in understanding the trophic modes of fungal groups by providing insights into the taxonomic diversity and functional potential of fungal communities associated to certain agroecosystems (Nilsson et al., 2019). Concerning the trophic modes of the different fungal OTUs resolved in the present study, the prediction carried out showed differences in abundance and incidence by ecological role played according with the type of till treatment (Fig. 6). Thus, the trophic group representing those species with more generalist behavior (including taxa that can exhibit several lifestyles facultatively) was the most abundant regardless of the type of management considered. After this group of species, the contingents of strictly pathogenic, saprophytic, and facultative saprophytic taxa were represented with similar reading rates in the analysis, although all these groups were more abundant in the samples under reduced till.

Management practices in maize cropping, specifically tillage (CT) and no-till (NT), significantly influence the diversity and structure of fungal communities in the soil (Mirás-Avalos et al., 2011). These practices affect various fungal trophic groups, including arbuscular mycorrhizal fungi (AMF), filamentous fungi, and other beneficial or pathogenic fungi. In this sense, Schmidt et al. (2019) reported that different agricultural practices, such as no-till and cover cropping, shaped the structure and function of soil fungal communities. Cover cropping increased species diversity, while no-till shifted the symbiotroph / saprotroph ratio to favor symbiotrophs, indicating the ecological impact of these practices on soil fungal communities, as in the case of the present study, where the group of facultative symbiotroph taxa was more abundant under reduced tillage conditions. The significant presence of a large number of facultative symbiotrophic taxa in reduced till samples, suggests a “priming” effect of these non-conventional management

techniques, as previously reported. In this sense, some studies (Mirás-Avalos et al., 2011; Castelli et al., 2014) have shown that non-till systems tend to support higher densities and diversity of AMF compared to conventional systems. These authors propose that non-till management preserves the fungal mycelia from previous crops, allowing for a better colonization of maize roots. Regarding strict symbiotic species, previous studies like that of Castelli et al. (2014) identified in maize crops a significant abundance of common AMF genera, such as *Glomus* or *Gigaspora*, associated with non-till management. However, the number of reads and OTUs resolved in our study was similar for both types of crop management.

3.7. Bacterial taxonomy

It has been largely reported that the bacterial communities in maize crops are significantly influenced by tillage practices (Smith et al., 2016). Thus, reduced-till and conventional tillage methods create distinct environments that affect the composition, diversity, and functional roles of soil and rhizospheric bacteria. In the present study, we compared bacterial OTUs between the no cover-crop control and the three leguminous cover crop species under both tillage and reduced-till conditions (Fig. 7). Significant differences were observed at the phylum level. The soil management appeared to have a stronger influence on bacterial community composition than the cover crop species. This pattern is consistent with previous findings (Kim et al., 2020a). In this sense, the different legume species used as cover crops do not seem to drive microbial composition, and the three plant species employed shared certain bacterial groups with non-cover plots, regardless of the type of cultural management applied. However, in the case of plots with the three leguminous species employed as cover crops, OTUs representing the Planctomycetota were especially abundant under both soil management treatments, compared to control plots without leguminous cover crops. Moreover, representatives of the Firmicutes were differentially abundant in the plots with cover crops based on *P. sativum* and *V. sativa* only under conventional management with tillage. When focusing exclusively on the effect of tillage management on the taxonomic composition (Fig. 8), a significant divergence in bacterial relative abundance was observed at the high taxonomic ranks (phylum). Thus, phyla such as Actinobacteriota, Firmicutes, and Gemmatimonadota were more abundant under tillage regimes, whereas Bacteroidota and Verrucomicrobiota increased in relative abundance under reduced-till management, just as observed by Srouf et al. (2020), who compared

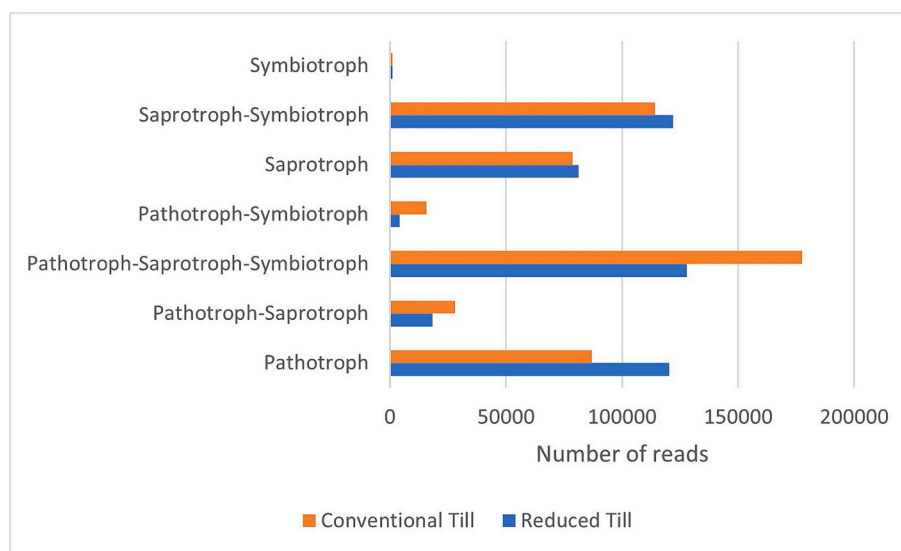


Fig. 6. Bar histogram showing the different trophic modes identified for fungal OTUs present in each treatment. Data were presented as total number of reads associated per OTU related to each trophic mode.

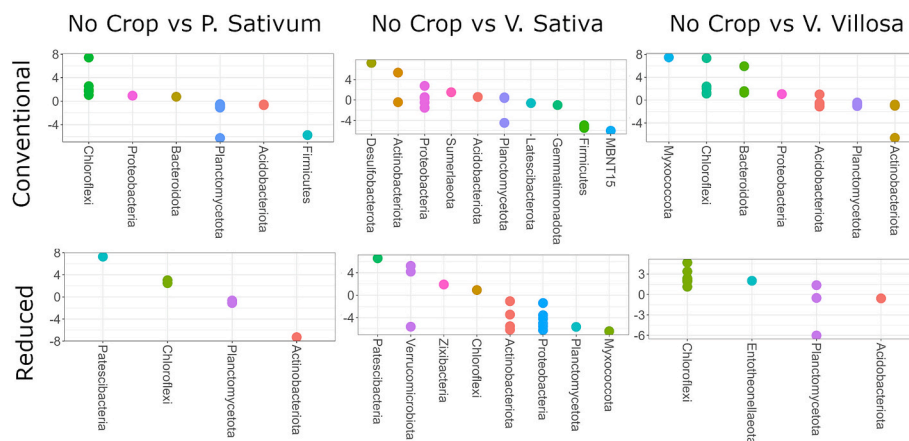


Fig. 7. Differential abundant bacterial OTUs grouped by phyla ($p < 0.05$) between no cover crop vs different cover crops under tillage vs reduced-till management. Negative “log2 Fold Change” values (x-axis) indicate for higher abundance in any of the three cover crop plots and positive values indicate higher abundances in no cover crop plots.

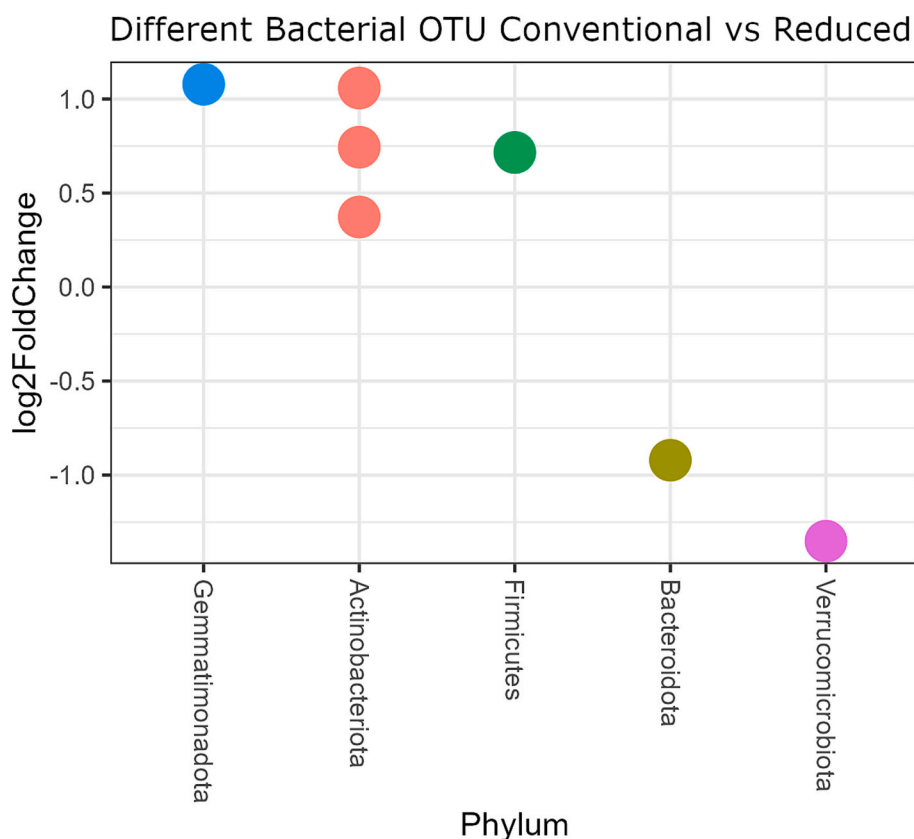


Fig. 8. Differential abundant bacterial OTUs grouped by phyla ($p < 0.05$) between tillage vs reduced-till management. Negative values (y-axis) indicate higher abundance in reduced-till plots and positive values indicate higher abundances in tillage plots.

the composition of microbial communities associated with long-term tillage and fertility treatments in maize crops. Navarro-Noya et al. (2013) reported that, for a maize system, the retention and incorporation into the soil of residues generated as a result of the introduction of cover crops, especially under no-till conditions, appears to have an effect on the composition and structure of bacterial populations, affecting the abundance of groups such as Bacteroidetes, Betaproteobacteria, Cyanobacteria, and Gemmatimonadetes. Just in the same way as observed in our analyses (Fig. 8), these authors stated that the number of OTUs belonging to the Bacteroidetes was significantly different in the treatments with crop residue retention than in those where it was removed,

while that of Gemmatimonadetes was found to be lower. Moreover, Khan et al. (2023) have reported that the retention and incorporation of plant debris from mulch into the soil under tillage increases the relative abundance of members of Proteobacteriota and Bacteroidota. In the present study, these differences were not found in the comparison between control plots and the three legume species used under tillage management. An increase in Proteobacteriota was only found in plots with a *V. sativa*-based cover with reduced-till.

4. Conclusions

In this study, the influence of tillage intensity and introduction of winter leguminous cover crops in monoculture maize on the composition and structure of soil-associated microbial populations was analyzed using high-throughput sequencing methods. After four years of cover crop introduction, no significant differences were detected in soil organic carbon and microbial biomass as a function of tillage intensity or cover crop species, but slight differences were observed in total nitrogen although not consistently related to soil tillage or cover crop introduction. For both the fungal and prokaryotic populations analyzed, soil management appeared to be the main factor shaping the differences in relative abundance and taxonomic composition, much more than the presence of any cover crop in the agroecosystem. Likewise, the type of leguminous species used as a cover crop, although it increased the abundance of nitrogen-fixing taxonomic groups in the case of bacteria, it did not appear to clearly define specific populations. Finally, the type of soil management also appears to shape the composition of associated fungal populations, where plots with conventional management tend to harbor a higher contingent of soil pathogens, especially those groups with a wide host range compared to reduced-till plots. This finding is in contradiction with what has been usually reported in previous studies, where conservation till or the lack of it usually increases the abundance of this type of fungal pathogens. Furthermore, conservation practices (reduced-till), on the one hand, tend to increase the relative abundance of arbuscular mycorrhizal fungi (AMF), together with the differential presence of potentially beneficial species, mainly biological control agents (BCAs), although it also favors the presence of certain groups of facultative or weak pathogens. In summary, a deeper understanding of the impact of management regimes on soil microbial communities in maize systems can guide plant-oriented strategies to improve productivity and agroecosystem sustainability.

CRedit authorship contribution statement

Vicente González-García: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **José Manuel Mirás-Avalos:** Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis. **Inés Zugasti-López:** Methodology, Investigation, Formal analysis. **Ramón Isla:** Writing – original draft, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Carmen Julián-Lagunas:** Methodology, Formal analysis. **Jérôme Grimplet:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis.

Funding

This study forms part of the AGROALNEXT programme and was supported by the Spanish Ministry of Science, Innovation and Universities with funding from European Union NextGenerationEU (PRTR-C17-I1 and the Spanish Ministry of Economy, Industry, and Competitiveness (AGL2017-84529-C3-2-R; PID2021-1263430B-C32). Inés Zugasti was granted with a predoctoral fellowship from Ministry of Science and Innovation (PRE2020-093635).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank to the field and laboratory personnel of the SAFMA Department of CITA and EEAD (CSIC).

Data availability

The sequencing data generated in this study have been deposited in the European Nucleotide Archive (ENA) under the project accession number PRJEB89644. All raw sequence reads can be publicly accessed at: <https://www.ebi.ac.uk/ena/browser/view/PRJEB89644>.

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