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Preliminary evidence suggests that interrow cover crops may enrich potentially beneficial bacterial groups that confer soil-suppressive capacity against the olive pathogen *Verticillium dahliae*

Sandra Tienda [a,b], Mariia Kolodeznaia [a,b], Víctor J. Carrión [a,b], Ana Lia Gayan-Quijano [c], Belén Delgado-Martín [a,b], Ben O. Oyserman [c], Francisco Javier Peris-Felipo [c], Jose Antonio Gutiérrez-Barranquero [a,b], Francisco M. Cazorla [a,b,*]

[a] Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos, Avda. Louis Pasteur 31, 29071, Málaga, Spain
[b] Grupo de Biología y Control de Enfermedades de Plantas, Área de Protección de Cultivos, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", IHSM-UMA-CSIC, Avda. Louis Pasteur 49, 29010, Málaga, Spain
[c] Syngenta Crop Protection AG, Rosentalstrasse 67, 4058, Basel, Switzerland

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ABSTRACT

Interrow cover crop implementation is considered a promising and sustainable agronomic practice for enhancing crop performance and soil health in agroecosystems, mediated by improvement of the soil microbiome. This study assessed how interrow cover crop implementation affected the microbial community in the soil, and may enhance its suppressiveness against *Verticillium dahliae* in a commercial olive orchard. The experiments were performed in a commercial olive orchard divided into two different management zones: conventional (trees without interrow cover crops treatment) and the LivinGro® protocol (trees with interrow cover crops treatment). Soil samples were collected focusing in 2 sampling times (September 2021 and January 2022). Soil DNA was extracted, and 16S rRNA genes and ITS regions sequences analyzed to profile soil microbial communities. Cover crop implementation does not increase microbial richness and alpha diversity values. However, we observed that the use of cover crops influences the composition of both fungal and bacterial soil microbial communities. Thus, cover crop implementation in the soil significantly increased the relative abundance of some putative beneficial bacterial groups, such as *Bacillaceae*, *Blastocatellaceae* and *Koribacteraceae*. In addition, compared with conventional soil, the soil treated with cover crops displayed increased suppressiveness against the olive soil-borne pathogen *Verticillium dahliae*.

1. Introduction

Olive (*Olea europaea* L. subsp. *europaea* var. *europaea*) is mainly cultivated in Mediterranean-type climate regions between latitudes 30° and 45° of both hemispheres. The olive tree is the most emblematic tree of the Mediterranean area, with enormous ecological, social and economic importance. In addition, olive cultivation has multiple uses as a source of food, cattle fodder and wood (Montes-Osuna and Mercado-Blanco, 2020). Spain is currently considered the main producer of olive oil and table olives, and in the last decades, modern systems and intensification of olive cultivation have improved production and mechanization. However, these advances can lead to several problems. Specifically, conventional soil management techniques, such as vegetation control using herbicides or tillage in olive orchards in the Mediterranean area (Spain), can produce soil losses systematically exceeding more than 20 Ton ha^{-1} yr^{-1} in sloping areas (Gómez et al., 2009; Vanwalleghem et al., 2011). Erosion caused by run-off in tillage areas, mainly carries away the finest soil particles, from the clay fraction, which rapidly reduces the natural fertility of the soil and its productive ability in olive orchards (Gómez et al., 2009; Guimaraes et al., 2021). On the other hand, the development of super-high density hedgerow orchards and the high inputs of fertilisers or fungicides can also have consequences, such as a reduction in the genetic diversity of the olive tree or harmful effects on the soil microbiota

* Corresponding author. Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos, Avda. Louis Pasteur 31, 29071. Málaga, Spain.
E-mail address: cazorla@uma.es (F.M. Cazorla).

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(Fernández-González et al., 2019, 2020; Mercado-Blanco et al., 2018). These disturbances could help to enhance problems related to increased incidence and severity of specific olive pests and soil-borne diseases.

Various pathogens, including bacteria and fungi, can cause diseases to olive trees. Currently, one of the most devastating olive diseases and a major limiting factor for olive oil production is Verticillium wilt of olive (VWO) disease, caused by soil-borne fungus *Verticillium dahliae*. VWO is characterized by wilting, leaf rolling, chlorosis, defoliation, and dead brown leaves remaining attached to the branches (Montes-Osuna and Mercado-Blanco, 2020). The fungus can endure adverse conditions through stable microsclerotia structures that allow it to persist in the soil for several months to years (Klimes et al., 2008). In addition to causing economic losses due to decreased fruit production and tree mortality, recently it was shown that olive trees infected by *V. dahliae*. showed a negative effect on the commercial value of virgin olive oil due to the poor organoleptic properties of fruits (Landa et al., 2019; Montes-Osuna and Mercado-Blanco, 2020). Therefore, it is necessary to optimize environmental sustainability to reduce the impact of intensive management on olive orchard ecosystems, without reducing productivity.

The management and control of VWO is complex. Available fungicides are ineffective once the fungus has reached the plant vascular system, and together with the current restrictive policy to prevent chemical application to the field, makes difficult their use (Colla et al., 2012). Thus, environmentally friendly approaches are promoted as additional treatments in order to reach an integrate VWO management. Among these strategies, are included the development of resistant cultivars that have being pursued for long time to mitigate the impact of the pathogen (Montes-Osuna and Mercado-Blanco, 2020), the soil solarization during the early stages of the disease (López-Escudero and Blanco-López, 2001), or the development of biological control alternatives using beneficial microbes that need to be also adapted to field treatments (Castro et al., 2020; Sallami et al., 2023). As a complementary alternative, the use of cover crops has emerged as a suitable practice for soil and water conservation in olive (Gómez et al., 2009). This approach may protect olive soils against erosion, improve soil structure and increase organic matter contents. However, non-cropped weeds should be avoided since they can be used by *V. dahliae* as alternative host to survive and multiply. By the same way, inert covers made of pruning debris from diseased olive trees are completely discouraged, since they constitute a primary source of pathogen inoculum spreading (López-Escudero et al., 2008).

In general, cover crop management may provide a wide range of benefits in biological properties of soil (Castellano-Hinojosa et al., 2020; Li et al., 2024). Previous studies showed that cover crop in olive orchards contributed to soil enrichment in biological properties, especially on the microbial community structure (Bechara et al., 2018; Sofo et al., 2014), increasing some specific microbial groups and microbial functional activities (Arias-Giraldo et al., 2021). In other woody crops, similar studies have shown that cover crops drive different effects on soil microbial diversity indices, either increasing it (Kim et al., 2020; Vukicevich et al., 2016), reducing it (Cazzaniga et al., 2023a, 2023b), or even showing no effects in the diversity indices values. Cover cropping can selectively reduce soil microbial diversity (Banerjee et al., 2019), making the link between alpha diversity and soil health context-dependent (Shade, 2017). Additionally, the use of cover crops has been shown to influence the composition of microbial communities (Chen et al., 2022; Zheng et al., 2018a, 2018b). In some cases, increase of arbuscular mycorrhizal fungi (*Acaulospora morrowiae* and *Scutellospora calospora*) and rhizospheric bacteria (*Pseudomonas* and *Bacillus* spp.) were reported. These microbial groups have been previously described to have a beneficial effect on the soil cycle of different nutrients, and also may show biological control and plant growth promotion activities (Bever et al., 2015; Hamel et al., 2005, Mazzola et al., 2012). Because this increase of beneficial soil microorganisms, cover crops can also promote suppressiveness of soil-borne pests and diseases (McNeill et al., 2012; Van Elsas et al., 2002). This has been previously proved in different pathosystems, for example reducing *Fusarium* pathogens in tomato (Wang et al., 2018) or reducing the index of bacterial wilt disease (caused by the pathogen *Ralstonia solanacearum*) in a tobacco field (Qi et al., 2020).

The evaluation of cover crops in perennial systems, particularly for disease suppression potential is novel and is timely as many commercial operations seek to reduce agrochemical inputs (Bergtold et al., 2019). While the benefits of cover cropping are increasingly recognized in annual agriculture, their effects in established perennial systems, characterized by potentially distinct soil zones influenced by permanent plant rows, remain comparatively underexplored (Quintarelli et al., 2022). Our research specifically highlights the novelty of evaluating these localized impacts, acknowledging that soil microbial communities may respond differently in interrow areas compared to the immediate vicinity of the perennial crop. For this purpose, the implementation of cover crops in olive conventional cropping systems could be a good strategy to improve the main problems currently faced by the olive cultivar, such as the control of fungal diseases, specifically *V. dahliae*, and in general to improve the health of the olive agricultural system. In this work, the international LivinGro® protocol was applied in an olive tree orchard. This protocol focuses on the study of the effects of the implementation of a specific composition of interrow cover crops on agricultural ecosystem, with the aim of improving soil microbial communities and soil health. The different plants contained in the cover crop mix have been selected for their ability to attract pollinators, to improve soil structure and for its ability to control pests, among them fungicidal activity (Aguado et al., 2015). More specifically, we focus on the effects of this sustainable management on the soil microbiome. Metabarcoding analysis and an investigation into whether a cover crop could improve soil suppressiveness against Verticillium wilt of olive (VWO) have been carried out.

2. Materials and methods

2.1. Experimental design and sample collection

The experiments were conducted on a commercial olive orchard of 12-years-old trees of *Olea europaea* (cv. Picual), growing in lines of approximately 200 m length (every line containing approximately 40–45 dip-irrigated trees per line), with a separation of 5 m between tree lines. The experimental plots (Fig. 1A and Supplementary Fig. S1) were located in Pozaldez (Valladolid, Spain; 41°21′47.0″N - 4°47′58.8″W), and trees are growing in sandy loam soil. This field was selected because it has been previously challenged by two occasional mild outbreaks of Verticillium wilt of olive (VWO) in the last six years (personal communication). This olive orchard was maintained without cover plants or weeds following conventional agricultural management (early-season residual herbicide application and/or mechanical weed maintenance during the season to keep the field free of spontaneous vegetation; Fig. 1B), while three experimental consecutive rows in the centre of the commercial olive field were used for cover crops (Fig. 1C). Interrow cover crops (LivinGro® protocol) were introduced in September 2020. Temperature and precipitation values were recorded during the assay (Supplementary Fig. S2).

After one year of management, soil samples were taken in September 2021 and January 2022 to assess the impact of interrow cover crops on soil microbial diversity. In the same field orchard, we tested two treatments (LivinGro or conventional) in tree lines of about 200 trees. Two experimental lines (treated and untreated with the cover crops), separated approximately 100 m each other, were selected and further sampled. We selected two different zones under the same treatment (tree row and line). Three independent sampling points were established per zone, approximately allocated in the centre of the line, with 20 m of separation from each other sampling point. To study the overall effect of the LivinGro® treatment, independent soil samples were taken in each sampling point from both under the tree line and in the centre of the

Fig. 1. Experimental design of the LivinGro® project in a commercial olive field. A) Aspects of LivinGro® and conventional treatment described above. B) Picture showing the tree line and interrow line in LivinGro® C) and the conventional treatment.

interrow line. To obtain each soil sample, 20-cm-deep soil core samples were taken via a 15-cm-diameter manual soil auger, and approximately 1 kg of soil (with organic debris previously removed from the surface) was collected per sample. In this study, a total of 24 soil samples were collected to study the effects of interrow cover crops on soil microbial biodiversity. Twelve samples (6 LivinGro® and 6 conventional soil samples) were taken at each sampling time (September 2021 and January 2022).

The collected soil samples were placed in cold storage (4 °C), transported to the laboratory, and sieved through a 2 mm pore-size sieve to remove visible residues, such as organic debris, rocks and roots. Sieved fresh soil was used to test soil suppressiveness and to obtain a microbial suspension to test microbial antagonism against the olive pathogen *V. dahliae* V150I (D pathotype, isolated from olive and belonging to the vegetative compatibility group (VCG) 1A; Collins et al., 2005). The remaining soil was preserved frozen at −80 °C, and then thawed to extract DNA for microbial community analyses (Li et al., 2023).

2.2. Cover crop mixture plant selection

The LivinGro® protocol consists of the application of a cover crop using an herbaceous mixture (in percentage by weight of seeds) composed of *Chrysanthemum* spp. (3 %), *Coriandrum sativus* L. (10 %), *Eruca vesicaria* L. Cav. (5 %), *Melilotus officinalis* L. Pall. (8 %), *Onobrychis viciaefolia* Scop. (22 %), *Salvia pratensis* L. (10 %), *Trifolium vesiculosum* Savi. (4 %) and *Vicia sativa* L. (30 %).

This seed mixture was sown manually or by an electric drill with air distribution. The soil was prepared beforehand by a flail mower and subsequently covered with a drag. The sowing dose used was 15 kg seed mixture/ha. The seasonal management of mowing and replanting the cover crop in autumn was implemented to optimize soil health. Mowing the existing biomass in autumn facilitates its decomposition over winter, releasing nutrients back into the soil. Replanting with a new cover crop in autumn provides continued soil cover, preventing nutrient leaching and erosion, while also establishing a root system that will further improve soil structure for the following spring.

2.3. Soil DNA extraction, PCR amplification and Illumina sequencing

Total genomic DNA was extracted from 0.25 g of each soil sample using the PowerSoil® DNA Isolation Kit (Qiagen Iberia S.L., Madrid, Spain) following the manufacturer's instructions. The DNA extraction quantity and quality (total amount ≥200 ng and A260/A280: 1.8–2.0) were evaluated via a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, United States). Additionally, DNA quality was analyzed by agarose gel electrophoresis and RedSafeTM staining (Labotaq, Seville, Spain).

Extracted DNA samples were sent to Novogene Company (Tianjin, China) to obtain the DNA sequences for the bacterial 16S rRNA gene and fungal internal transcribed spacer (ITS) amplicon. For 16S rRNA, the V3-V4 region was amplified via the PCR primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT), resulting in a 470 bp fragment. For the ITS region, the ITS1-1F region was amplified using the PCR primers ITS1-1F-F (CTTGGTCATTTA-GAGGAAGTAA) and ITS1-1F-R (GCTGCGTTCTTCATCGATGC), resulting in a 200–400 bp product. The PCR products were sequenced via Illumina NovaSeq 6000 technology (Illumina, USA). PCR amplification was performed using Phusion® High-Fidelity PCR Master Mix, following Novogene's standardized protocols. Amplified products were purified with AMPure XP beads, and dual-index barcodes were added using the Nextera XT Index Kit. Libraries were purified, quantified, and assessed for fragment size, then pooled in equimolar amounts. Sequencing was conducted on an Illumina NovaSeq 6000 platform, following Novogene's protocols for amplicon-based profiling (Caporaso et al., 2012; Toju et al., 2012) with a sequencing depth of 100,000 reads per sample.

2.4. Illumina data processing

The raw reads were processed via Cutadapt (v3.4, Martin, 2011) to remove amplicon primers and fastp (v0.23.4, Chen et al., 2018) to perform quality controls and filtering based on quality scores and length. Amplicon sequence variants (ASVs) were inferred using the DADA2 pipeline (v1.26.0, Callahan et al., 2016). Taxonomic assignment was performed via the SILVA v138.1 database for bacteria (Quast et al., 2013) and UNITE v7.2 database for fungi (Kõljalg et al., 2013). ASVs below 0.01 % of total reads were excluded by converting read counts to relative abundances, retaining only higher-abundance ASVs to reduce noise. Data were normalized by applying cumulative sum scaling (CSS) using the metagenomeSeq package (Paulson et al., 2023). Alpha and beta diversity estimates were calculated using the phyloseq R package (v1.42.0; McMurdie et al., 2013). Alpha diversity and richness were quantified using the Shannon and Chao1 indices, respectively. A a two-tailed *t*-test was performed to assess the statistical significance of differences between groups (LivinGro® and Conventional). To assess beta diversity, data were transformed to proportions and analyzed using principal coordinate analysis (PCoA) based on the Bray-Curtis distance. The significance of the effects of sampling time and treatment on this beta diversity was subsequently evaluated using a two-way nonparametric test (PERMANOVA; Anderson, 2001). Additionally, one-way PERMANOVA was applied when analysing each sample individually. For taxonomic composition analysis, ASVs with an abundance of less than 10 counts in 10 % of the samples were excluded, and counts were normalized using total sum scaling method. To determine which microbial groups showed significantly different abundance in the samples where LivinGro® cover crops were applied, marker analysis was performed at the family level. This analysis utilized the linear discriminant analysis (LDA) effect size (LEfSe) method implemented in the microbiomeMarker R package (v1.6.0, Cao et al., 2022). The statistical thresholds for this analysis were a Kruskal–Wallis (KW) test cut-off <0.01 and an LDA score cut-off >2. Furthermore, the ecological guild traits were determined using the FungalTraits database (Põlme et al., 2020), after aggregating ASVs by their primary lifestyle based on genus classification. Statistical comparisons between treatments were

conducted using Wilcoxon rank-sum tests with Benjamini-Hochberg false discovery rate (FDR) correction for multiple comparisons. All findings were deemed statistically significant at $P < 0.05$.

2.5. Suppressiveness of LivinGro® soil against the pathogen Verticillium dahliae

A soil suppressiveness assay against the olive pathogen *V. dahliae* V150I was performed to determine the response of LivinGro® treatment, using soil samples treated or untreated with a cover crop from the last sampling time (January 2022). Before starting the assay, 300 g of soil stored at 4 °C from each treatment was incubated at 25 °C for 3 d in the dark to allow the microbial activity to stabilize it, as previously described by Li et al. (2023).

The soil suppressiveness was evaluated by a sandwich plate assay described previously (Li et al., 2023) with some modifications. Briefly, for each replicate, 20 g of this soil was evenly spread on the bottom of a Petri dish. The lid of the Petri dish contained 15 mL of sterile PDA medium, and a fungal disk of *V. dahliae* V150I (0.6 cm diameter) that was grown on potato dextrose agar (PDA) was placed in the centre. A piece of sterilized 0.22-μm microporous membrane was then placed on top of the soil to prevent contamination in the upper compartment and hermetically sealed with parafilm so that the fungus was exposed to the volatile compounds produced by the soil and the containing microorganisms. The plates were subsequently incubated at 25 °C for 5 days. Control of fungal growth (without soil) was included in the assay. After incubation, the area of fungal growth was determined, and the growth was compared between the treatments. Three independent biological experiments were performed, and from each one, 8 technical replicates per treatment were obtained. The data were analyzed via one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test with Bonferroni correction ($P = 0.05$) comparing the values obtained between LivinGro® (with cover crop), conventional (without cover crop) and control (without soil). All data analyses were performed via GraphPad Prism (version 5.0; GraphPad Software Inc., La Jolla, CA).

2.6. Antagonism of bacterial community of LivinGro® soil against pathogen Verticillium dahliae

To further evaluate whether the antagonistic effect against *Verticillium* observed by the LivinGro® soil is due to its prokaryotic community, an antagonistic test against this pathogen was performed by preparing bacterial suspensions at the last sampling time (January 2022), as previously described (Li et al., 2023) with some modifications. First, 3 g of soil were added to 6 ml of phosphate buffer (KH_2PO_4, 1 g/L, pH = 6.5) and mixed on a shaker at 150 rpm for 1.5 h at 4 °C. The soil suspension was filtered through a 5-μm membrane to remove a high portion of fungal hyphae. A piece of sterilized cellulose filter (3 cm × 1 cm) was then placed on the PDA plate, and 50 μL of the suspension was inoculated onto it. A PDA disk with *V. dahliae* V150I hyphae (0.6 cm diameter) was placed on the centre of the PDA media plate and the plates were incubated at 25 °C for 10 days. The distance of fungal growth was subsequently measured from the centre of the fungal disk to the outer edge closest to the paper inoculated with the bacterial suspension. As a control, a piece of sterilized cellulose filter impregnated with sterile deionized water were used. Three independent biological experiments, with 12 technical replicates per each one, were performed. The data were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test with Bonferroni correction ($P = 0.05$) comparing the values obtained between LivinGro® (bacterial suspension of soil with cover crop), conventional (bacterial suspension of soil without cover crop) and control (sterile deionized water). All data analyses were performed via GraphPad Prism (version 5.0; GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Alpha diversity and richness

To analyse the microbiota composition of this olive orchard, ASVs were assigned for taxonomy. For 16S data, 49945 ASVs were obtained. Both sampling times were filtered by abundance together, which resulted in 14158 ASVs. For ITS data, 6891 ASVs were obtained, and filtering resulted in 3158 ASVs. To investigate how bacterial and fungal alpha diversity and richness was affected by the cover crop, the Shannon and Chao1 indices were calculated for each sampling time separately.

At the bacterial level, the results revealed significant differences between LivinGro® and conventional samples, with higher values in the conventional treatment group in January 2022, according to the Chao1 index (P value = 0.007; Fig. 2B). In September 2021, no significant differences between soil management were observed (Fig. 2A and B) using two-tailed t-test to assess the statistical significance.

At the fungal level, the Shannon index revealed that there were no significant differences between the LivinGro® and conventional treatments (Fig. 2C). However, with the Chao1 index, we did observe significantly higher Chao1 index values in the conventional soil samples in September 2021 with P value = 0.007 (Fig. 2D) using the same test as for bacterial level.

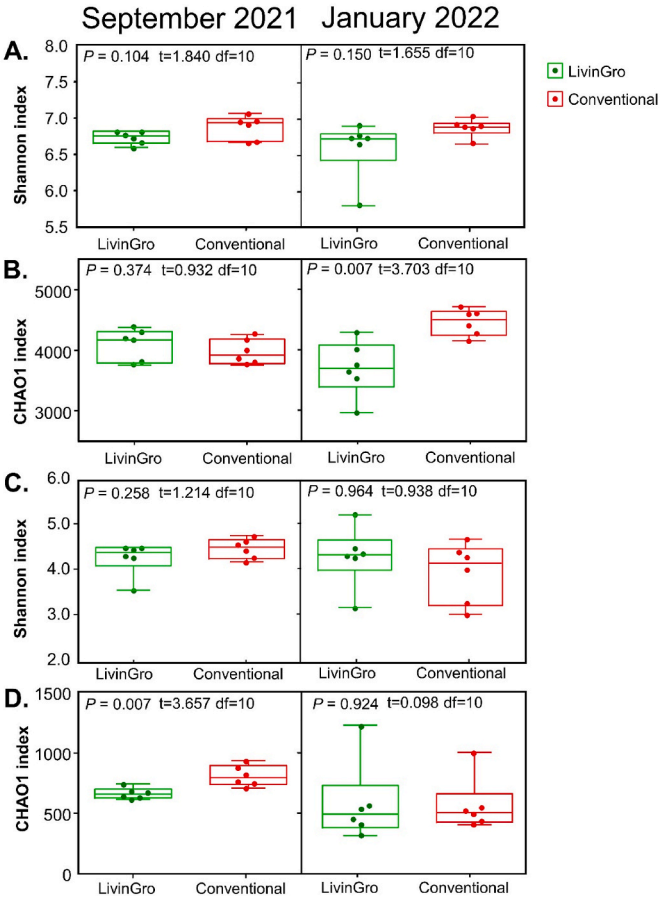

Fig. 2. Alpha diversity and richness analysis at the bacterial level with A) Shannon and B) Chao1 indices and at the fungal level with C) Shannon and D) Chao1 indices; the results are separated by sampling time (September 2021 and January 2022). The LivinGro® and conventional samples are reported in green and red, respectively. Significant differences between treatments were indicated with $P \leq 0.05$ (unpaired Student's ttest).

3.2. Changes in soil bacterial and fungal communities in relation to cover crop treatment

To statistically assess the impact of vegetation cover and sampling time on the soil microbial community composition, a Permutational Multivariate Analysis of Variance (PERMANOVA) was performed. Principal Coordinates Analysis (PCoA) ordinations showed that bacterial communities from soil samples taken at different times formed distinct clusters (Fig. 3A, PERMANOVA, Time $P = 0.001$). Similar results were obtained for fungal beta diversity analysis, where we observed that the sampling time clearly influenced the fungal community structure (Fig. 3B, PERMANOVA, Time $P = 0.001$). In addition, the two-way nonparametric test revealed that time had an effect on treatment at both the bacterial (Fig. 3A, PERMANOVA, Interaction $P = 0.001$) and fungal community (Fig. 3B, PERMANOVA, Interaction $P = 0.024$) levels. Analysing each sampling time independently, we observed that at the first sampling time, in September 2021, bacterial (Fig. 3C, PERMANOVA, $P = 0.007$) and fungal communities (Fig. 3D, PERMANOVA, $P = 0.003$) were separated by treatment, with LivinGro® and conventional samples grouped into separate groups. The similar results were obtained in January 2022, when both bacteria (Fig. 3E, PERMANOVA, $P = 0.003$) and fungi (Fig. 3F, PERMANOVA, $P = 0.020$).

To analyse the composition of the microbiota of this olive tree field, ASVs were assigned to taxonomy, and each treatment at each sampling time was analyzed (at genera level, those with relative abundance>1 %). At the bacterial level in September 2021, the genera with the highest relative abundance were *Sphingomonas, Pseudarthrobacter*, *Bacillus*, *Nocardioides* and *Microvirga* (Fig. 4A). In January 2022, *Bacillus*, *Blastococcus*, *Pseudarthrobacter*, *Nocardoides*, *Microvirga* and *Rubrobacter* had the highest relative abundances (Fig. 4B). At the fungal level, in September 2021, we observed relatively high relative abundances of the *Talaromyces, Fusarium* and *Aspergillus* genera (Fig. 4C). Similar results were obtained in January 2022 (Fig. 4D), with high abundances of the genera *Fusarium, Aspergillus* and *Talaromyces*. However, we observed that between September and January, fungal population abundance fluctuated more than bacterial relative abundance at genera level, with those more than 1 % abundance. At the fungal level, a higher relative abundance of *Aspergillus* and *Penicillium* was observed in September 2021 than in January 2022. In contrast, a greater abundance of *Fusarium* and *Cladosporium* was observed in January 2022 than in September 2021. Additionally, some genera appeared at a given sampling time and disappeared at other sampling times, such as those observed with the *Rhizoctonia* genus.

To investigate the composition of the microbiota and explore which bacterial and fungal taxa were influenced by the application of the cover crop (LivinGro® treatment), LEfSe package analysis was performed. It was observed that statistically significant differential biomarkers appeared with cover crop implementation. In September 2021, at the bacterial level, LEfSe revealed seventy-one bacterial families whose relative abundance significantly differed between LivinGro® and conventional treatments (Supplementary Fig. S3). There are 44 biomarkers for LivinGro® and 27 for conventional methods. The families enriched with the highest LDA values in the LivinGro® treatment were *Oxalobacteraceae, Coleofasciculaceae*, *Bacillaceae*, *Comamonadaceae*,

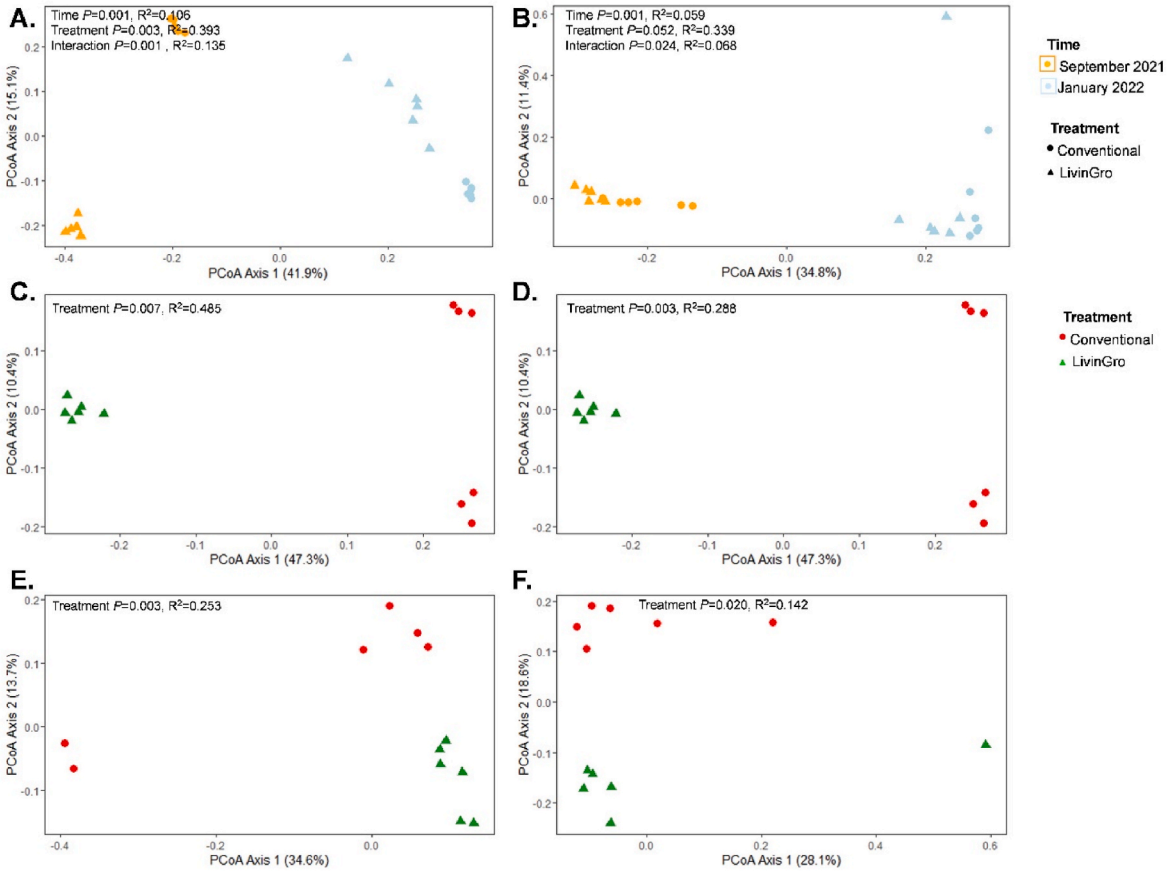

Fig. 3. Principal coordinate analysis (PCoA) of Bray–Curtis distances in soil microbial communities for samples from two sampling times are included together at the A) bacterial and B) fungal levels. Yellow shapes represent samples collected in September 2021, and blue shapes represent samples collected in January 2022. △: represent samples with cover crop, ◯: represent samples with the conventional treatment. Each sampling time are plotted separately, at bacterial level for C) September 2021 and D) January 2022 and at fungal level for E) September 2021 and F) January 2022. The LivinGro® and conventional samples are represented by green triangles and red, respectively. Permutational multivariate analysis of variance (PERMANOVA) was applied to test the significance of differences for bacterial and fungal communities.

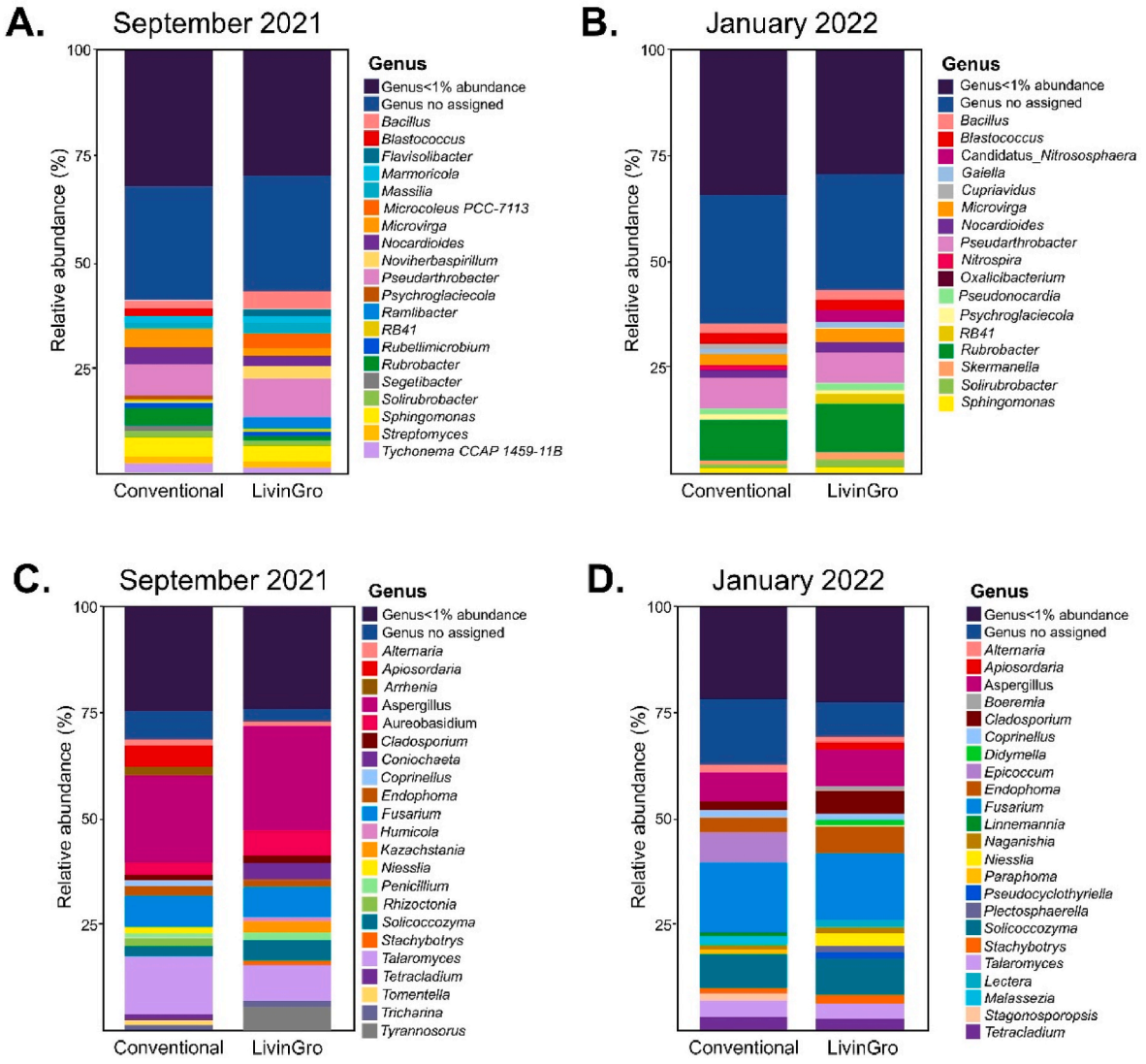

Fig. 4. The composition of the microbial communities at different sampling times (May 2020, September 2021 and January 2022). The samples were grouped by treatment (LivinGro® or conventional) for each sampling time. The stacked column graph shows the relative abundances of A) bacterial genera and B) fungal genera. Each column represents the mean of the corresponding groups.

Acidobacteriales, *Solibacteraceae* and *Koribacteraceae* (Fig. 5A). In January 2022, 52 bacterial families were identified (Supplementary Fig. S4), 5 of which were enriched in LivinGro® and 47 of which were enriched in conventional treatment. The 5 families enriched in LivinGro® with the highest levels of LDA were *Blastocatellaceae*, *Chthoniobacteraceae*, *Xanthobacteraceae*, *Koribacteraceae* and *Weeksellaceae* (Fig. 5B). Interestingly, the *Koribacteraceae* family was enriched by the LivinGro® treatment at both sampling times. While that *Longimicrobiaceae* family was enriched by the conventional treatment at both sampling times.

At the fungal level, LEfSe analysis presented fourteen and nine biomarker families in September 2021 (Fig. 5C) and January 2022 (Fig. 5D), respectively. In September 2021, the biomarkers in LivinGro® soil samples were *Venturiaceae*, *Coniochaetaceae* and *Saccharomyceae*. In January 2022, the families enriched in LivinGro® soil samples were *Plectosphaerellaceae*, *Leptosphaeriaceae* and *Basidiomycota incertae sedis*.

Additionally, an analysis of ASVs from conventional and Livingro samples using FungalTraits (Põlme et al., 2020) to predict their primary lifestyle showed that in September 2021 (Supplementary Figure S5A), the Livingro treatment had a significantly higher relative abundance in the dung_saprotroph, mycoparasite, and pollen_saprotroph categories compared to the conventional treatment. However, in January 2022 (Supplementary Figure S5B), Livingro showed a significantly lower relative abundance in the dung_saprotroph and pollen_saprotroph categories compared to the conventional treatment.

3.3. Soil managed with a cover crop improve suppressiveness against *Verticillium dahliae*

In vitro experiments were conducted to decipher the putative implications of the change in microbial communities in soil treated with the cover crop (LivinGro® treatment) by examining the suppressive effects of volatiles produced in the soil against the pathogen *V. dahliae* V150I. For this, soil volatile diffusion tests were conducted by sandwich plate tests. The results revealed that volatile metabolites produced by both soil samples (managed with LivinGro® and under conventional management) inhibit *V. dahliae* growth when compared with the control without any soil sample. Volatile metabolites produced by LivinGro® soil samples showed an average fungal growth area of 36.12 mm^2, and the conventional treatment showed an average fungal growth area of 60.30 mm^2. The control growth of *V. dahliae* without any soil samples showed an average growth area of 257.46 mm^2 (Fig. 6A). These results showed a significantly lower area of fungal growth (Student's t-test, $P < 0.05$) from LivinGro® soil samples. LivinGro® soil samples exhibited

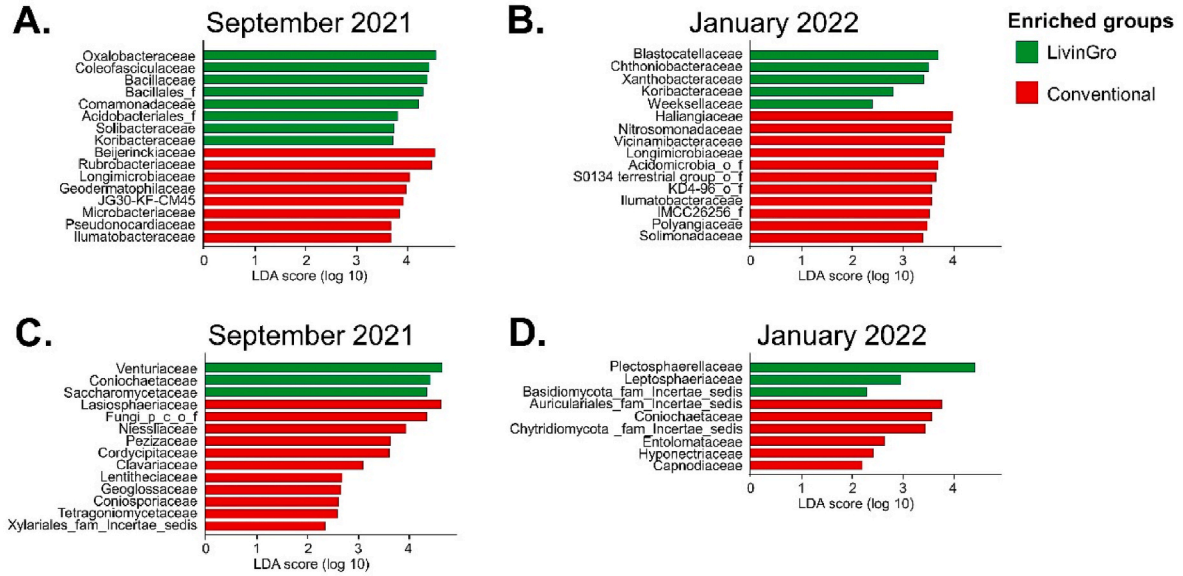

Fig. 5. Families whose abundance significantly differed according to LEfSe analysis. At the bacterial level A) in September 2021 and B) in January 2022. At the fungal level, C) in September 2021 and D) in January 2022. The samples are grouped by treatment, and the enriched families in LivinGro® are green and red under conventional conditions. For bacterial analysis, only the families with the highest LDA values are shown.

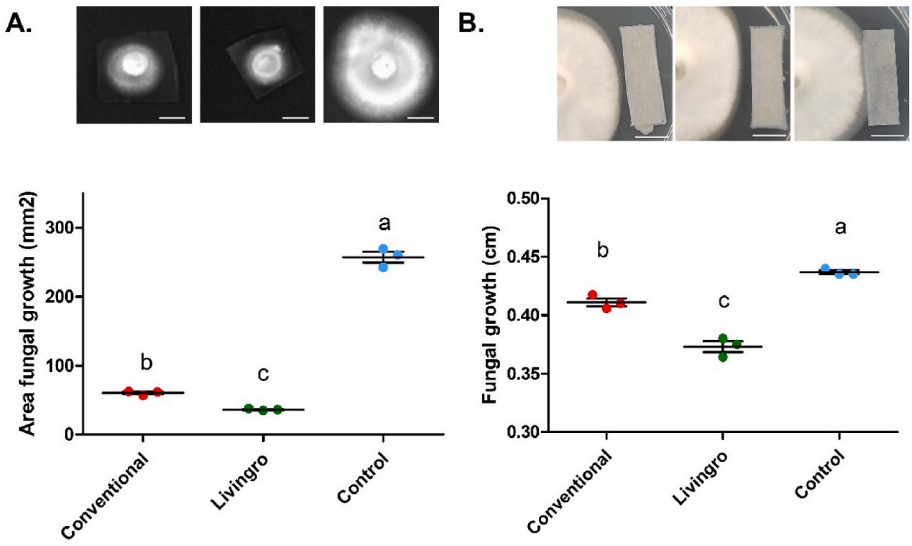

Fig. 6. Pathogen-suppressive ability of soil samples and bacterial communities extracted from treated and untreated soils with interrow cover crops (LivinGro® and conventional treatments). A) Results of the response of the mycelial growth of the pathogenic fungus *V. dahliae* V150I to soil volatiles; the area of fungal growth in mm2 is shown. The size of the bars is 0.06 cm. B) Results of the response of the growth of the pathogenic fungus *V. dahliae* V150I to the soil bacterial suspension; the radius of fungal growth in mm was measured. The size of the bars is 1 cm. The conventional, LivinGro® and control treatments are shown in red, green and blue, respectively. Statistical differences between treatments were assessed by one-way ANOVA followed by Bonferroni post-hoc correction, considering results statistically significant at $P < 0.005$.

40.16 % more fungal growth inhibition compared to soil samples under conventional treatment.

3.4. Bacterial suspensions from soil samples treated with the cover crops confer antagonism against Verticillium dahliae

To analyse the possible implications of the change in bacterial communities in the soil to which the cover crop was applied (LivinGro® treatment), antagonism assays with soil bacterial suspensions against the pathogen *V. dahliae* V150I were performed to examine the effects on the inhibition of fungal pathogen growth. The results revealed that the bacterial microbial communities from the LivinGro® soils (average growth radius of 0.37 cm) inhibited *V. dahliae* V150I growth more strongly than did the conventional soil communities (average growth radius of 0.41 cm). The untreated control without the bacterial suspension showed an average fungal growth radius of 0.44 cm (Fig. 6B). These results showed that LivinGro® microbial suspensions can inhibit the fungal pathogen growth by 10 % more when compared with the microbial suspensions obtained from soil samples of conventional treatments.

4. Discussion

The implementation of interrow cover crops is considered a

promising ecological measure for sustainable agriculture, as it has been shown to influence soil organic matter dynamics, improve soil structure, prevent soil erosion, increase fertility, and improve pest control and soil general biodiversity (Giese et al., 2014; Muscas et al., 2017; Garcia et al., 2018; Hall et al., 2020; Chen et al., 2022). More specifically, the implementation of interrow cover crops can be an effective way to increase soil microbial diversity in some soils (Garbeva et al., 2004).

In this study, a soil management using vegetation covers was implemented one year before sampling in order to provide sufficient time to influence the soil microbial communities. In addition, samples were collected in September and January, as these are two key moments in the phenological stage of the olive crop in the Mediterranean area (Sanz-Cortés et al., 2005). In September, olive fruit development begins and additionally, this is a hard stage for the tree, as it faces a lack of water after the increase in temperatures during the summer season (August). In January, the ripening of the olive fruit takes place, where the oil accumulates and the harvesting of the olives begins, and in the soil, the growth of the root ends. The study highlights how microbial communities are influenced not only by the type of cover crop but also by environmental conditions such as temperature, precipitation, and crop phenological stage. Samples were collected at two key times (September and January), capturing changes in microbial dynamics during the growing season. This suggests that microbial community composition can fluctuate significantly over time due to various environmental factors (Kivlin and Hawkes, 2020; Averill et al., 2019).

Based on the Shannon and Chao1 indices, no increase in alpha diversity and richness values were observed in the soil samples from interrow cover crop implementation in this olive orchard. Interestingly, we observed a decrease in some richness values when the cover crop was applied. Observed patterns may reflect selective microbial enrichment rather than functional loss (Hartmann et al., 2015), suggesting that reduced alpha diversity can sometimes indicate beneficial functional specialization for soil health (Philippot et al., 2013), thus requiring careful interpretation in agroecosystems. The introduction of interrow cover crops is recognized for its potential to enhance soil organic matter, soil structure, and contribute to better pest control and biodiversity. Although cover crops have shown increased microbial diversity in some soils, this study did not observe a significant change in alpha diversity or microbial richness, contrasting with previous studies that reported positive impacts (Kim et al., 2020; Vukicevich et al., 2016).

The most abundant bacterial and fungal genera observed in the olive orchard studied, have been also reported in previous studies on the olive microbiome, being *Bacillus*, *Rubrobacter* and *Sphingomonas* the most abundant ones (Wentzien et al., 2023; Fernández-González et al., 2020). At the fungal level, *Aspergillus* also appears reported as one of the most abundant genera in others olive orchards (Fernández-González et al., 2019). On the other hand, we observed that the composition of fungal communities was highly dynamic, influenced by sampling time. Thus, some putative soil-borne pathogens, such as *Aspergillus, Fusarium*, *Cladosporium* and *Rhizoctonia*, which are widely described as having the potential to cause plant disease, fluctuated in abundance with the sampling time (Arie, 2019; Bensch et al., 2012; Nji et al., 2023; Senapati et al., 2022). This further highlight that microbial communities are not static and are subject to interactive factors, including soil type, plant genotype, and management practices (Jumpponen et al., 2010; Fernández-González et al., 2020).

Despite the lack of significant changes in alpha diversity, cover crops appear to affect microbial community composition, as indicated by beta diversity analysis. This suggests that while overall diversity remains unchanged, the structure and specific microbial groups present in the soil can be altered by cover crop implementation (Chen et al., 2022; Zheng et al., 2018a, 2018b). Additional benefits of cover crop implementation include the improvement of chemical, physical and biological characteristics: increasing soil organic matter, nitrogen, phosphorous, and water storage, helping prevention of soil erosion, reducing soil compaction, changing the composition of microbial communities, enhancing beneficial microorganisms, and helping to suppress soil-borne diseases and pests (Quintarelli et al., 2022; McNeill et al., 2012; Van Elsas et al., 2002). Additionally, beta diversity analysis of all samples using the two-way nonparametric test revealed that time had an effect on soil microbial communities. Many studies have shown that the structure of fungal and bacterial communities varies over time, indicating that the temporal dynamics are rather complex, where changes in microbial communities can be driven by a number of environmental factors, such as soil chemistry, nutrient availability and global factors, such as climate (Averill et al., 2019; Kivlin and Hawkes, 2020; Jumpponen et al., 2010; Lladó et al., 2017; Voříšková et al., 2014). Our results showed that both temperature and precipitation at each sampling time differed greatly. In September 2021, approximately, the average temperature was 20 °C and the total precipitation was 30 mm. While in January 2022 it was 10 °C and 10 mm. (Supplementary Fig. S2).

The study identified specific microbial biomarkers associated with cover crop treatments. Notably, the *Koribacteraceae* family, involved in carbon cycling, was enriched in cover crop soils. This suggests that cover crops may promote microbial communities that enhance organic matter decomposition, contributing to better long-term soil fertility (Ward et al., 2009). Carbon-rich soil organic matter provides an energy source for the soil food web, which in turn facilitates nutrient cycling essential for plant growth. Additionally, organic matter improves soil structure, creating a favourable environment for root development and function in crops. The other two biomarkers, *Longimicrobiaceae* and *Ilumatobacteraceae*, increased in abundance in the samples where vegetation cover was not implemented or decreased in abundance with vegetation cover. *Longimicrobiaceae* and *Ilumatobacteraceae* are commonly found in semi-arid soils with little organic matter (Korkar et al., 2022; Wu et al., 2022; Asem et al., 2018). Thus, cover crops could increase the number of bacterial groups involved in carbon degradation, possibly because the implementation of cover crops between rows results in the accumulation of soil organic matter, meaning that the amount of carbon available in the soil is greater than that in the soil without covers (Nyabami et al., 2024). The composition of fungal communities varies considerably over time, and no common fungal biomarker was identified between sampling points. This suggests that cover crops have a less pronounced effect on fungi compared to bacteria. This could be related to the specificities of fungi, which are influenced by factors other than soil organic matter, such as complex interactions with plant roots or microclimatic conditions.

Additionally, during interrow cover crop implementation, cover crops can enhance disease-suppressiveness in soils (Aiyer et al., 2022). The application of cover crops to watermelon has demonstrated the efficacy of cover crops in reducing *Fusarium* pathogens and disease incidence (Zhou and Everts, 2007). Additionally, the use of cover crops in grapevine cultivation has the potential to improve the control of black-foot disease caused by *Ilyonectria* spp. (Berlanas et al., 2018). Our results revealed that the interrow cover crop management in the olive field used, resulted in improved soil suppressiveness and higher fungal antagonism by *in vitro* assays against *V. dahliae* V150I, which is one of the most devastating fungi affecting the olive crop (Montes-Osuna and Mercado-Blanco, 2020).

Switching to a different management system (LivinGro® vs. conventional) could affect soil microbial composition in complex ways, impacting different functions. Some plant pathogens can be controlled by cover crop implementation, frequently associated with changes in the microbiome, and the increase of biological control agents in the soil communities (Benítez et al., 2007; Frasier et al., 2016; Romdhane et al., 2019). A key outcome of this study is the potential of cover crops to enhance disease suppression. Specifically, interrow cover crops in this olive orchard improved soil suppressiveness against the olive pathogen *Verticillium dahliae*, a devastating fungus. The increased presence of biocontrol agents such as *Bacillus* and *Pseudomonas* in cover crop-treated soils supports the idea that these crops can aid in managing soilborne pathogens (Hollister et al., 2013; Gaofu et al., 2020). Type of microbes

could differ during each sampling period, since over time, other factors influence the communities, such as temperature and humidity. In the first sampling time (September 2021), LEfSe analysis revealed an increase in the *Bacillales* and *Bacillaceae* groups in soils with cover crop implementation. These groups have potential use as biocontrol agents with protective activity against fungal plant pathogens and plant growth-promoting activities. (Almoneafy et al., 2014; Fira et al., 2018; Andrić et al., 2020). In January 2022, the *Blastocatellaceae* family of *Acidobacteria* was identified as a biomarker for cover crops and could be implicated in plant growth promotion and metal tolerance (Zhang et al., 2024). Furthermore, *Acidobacteria* genomes contain biosynthesis-related gene clusters that encode the synthesis of diverse secondary metabolites and other compounds, such as antibiotics, siderophores, and antinematodal and antifungal agents (Kalam et al., 2020; Crits-Christoph et al., 2018). Our results revealed that the LivinGro® implementation could suggest stimulated suppressiveness mediated by microorganisms that could be recruited by these cover crops.

In conclusion, the study demonstrates that interrow cover crops can significantly modify microbial communities in olive orchards, with variable impacts depending on sampling time and environmental conditions. The results also highlight the potential of cover crops to enhance disease suppression and improve soil health by promoting beneficial microbes. The suppressive effect against *Verticillium dahliae* is a promising result for the ecological management of diseases in olive cultivation, potentially reducing reliance on chemical treatments. In summary, while the study did not observe an overall increase in microbial diversity, it highlights the potential of interrow cover crops to influence microbial composition and disease suppression, which are crucial factors for sustainable agricultural practices. The dynamic nature of microbial communities and the temporal fluctuations observed underscore the complexity of soil management and its influence on crop health.

CRediT authorship contribution statement

Sandra Tienda: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Mariia Kolodeznaia:** Software, Formal analysis. **Víctor J. Carrión:** Software, Conceptualization. **Ana Lia Gayan-Quijano:** Resources. **Belén Delgado-Martín:** Software. **Ben O. Oyserman:** Methodology. **Francisco Javier Peris-Felipo:** Resources, Methodology, Funding acquisition. **Jose Antonio Gutiérrez-Barranquero:** Writing – review & editing, Methodology, Investigation. **Francisco M. Cazorla:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2025.101092.

Data availability

The 16S rRNA and ITS gene amplicon sequences associated with this study have been deposited in the NCBI SRA under accession number PRJNA1131742.

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