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Shifts in Soil Microbial Communities Under Cover Cropping in Citrus Orchards

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Abstract

Cover crops are increasingly used by growers in the United States as an effective approach to enhance soil health. The interactions between cover crops and soil microbiomes have been studied extensively; however, research evaluating the impact of cover crops on rhizosphere microbial communities in tree crop systems, particularly under nutrient-limited field soils in Florida, remains limited. To address these knowledge gaps, a three-year field study was conducted to evaluate the effects of cover crop mixtures on soil bacterial and fungal communities in orchards of different citrus species and cultivars across seasons. The trial included 'Star Ruby' grapefruit trees grafted on 'US-942', 'Bearss' lemon trees grafted on 'Sour Orange', and 'OLL-8' sweet orange trees grafted on 'US-942' rootstock in a randomized complete block design comparing conventional management (no cover crop-control) with cover crop treatments. Bacterial community composition showed consistent taxonomic patterns across cultivars and treatments, with *Proteobacteria* consistently the most abundant phylum across grapefruit, lemon, and orange orchards, followed by *Actinobacteriota*, *Acidobacteriota*, and *Firmicutes*. Despite this overall stability, cultivar was the strongest determinant of bacterial community structure. Additionally, functional analysis revealed significant treatment-associated shifts in predicted guilds related to nitrogen transformations, carbon metabolism, sulfur oxidation, and manganese oxidation. In contrast, fungal community composition and predicted functional profiles were primarily structured by season, with limited effects of cultivar and cover cropping. Fungal communities were consistently dominated by *Ascomycota* across orchards, sampling dates, and treatments.

Keywords: cover crops; citrus; soil microbiome; functional analysis; nitrogen transformation

Academic Editor: Claudio Ciavatta

Received: 19 August 2026
Revised: 7 October 2026
Accepted: 20 October 2026
Published: 4 November 2026

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1. Introduction

Florida flatwood soils are sandy (>90%), poorly drained, and low in organic matter (less than 1%) [1], especially in the citrus-growing regions. Most citrus orchards in Florida are grown as monocultures or with weedy inter-rows coupled with large-scale fertilizer application to meet the nutrient requirements and enhance yield. However, growers' interest in the use of sustainable agricultural practices to enhance nutrient retention in orchards is increasing. Various organic amendments, such as mulching [2–5], manure [6,7], compost [8,9], and biochar applications [10–12] have been reported to increase soil fertility. However, cover cropping is another emerging practice that has been reported to effectively enhance soil organic matter, nutrient availability and microbial diversity [13–15]. Cover cropping has been widely adopted throughout the United States cropland systems, increasing by 50% between 2012–2017 [16], followed by an additional 17% rise from 2017-2022 [17]. The potential benefits of cover crops, especially related to soil fertility, are of great interest to citrus growers in Florida. Brewer et al. [18] found that inter-row cover crops in citrus increased NO_3^--N concentrations (31%) and soil organic matter [SOM (17%)]. Additionally, cover crops significantly enhanced SOM and nitrogen in citrus orchards after just one year. These improvements were driven by shifts in microbial activity and community composition [19].

The rhizosphere represents the area adjacent to plant roots that contains diverse microbial populations, including the bacteria and fungi that can establish symbiotic relationships with the host plant [20]. The activity of these microorganisms plays a key role in regulating soil and nutrient dynamics, as well as plant growth [21]. These microbial populations can impact plant health by regulating nutrient cycling through decomposition, nitrogen fixation, and phosphate solubilization, thereby increasing nutrient availability to plants [22,23]. Additionally, soil microbes may enhance plant growth through the production of phytohormones that protect plants by releasing antibiotic substances that inhibit pathogens [24]. Rhizosphere bacteria that promote plant growth are known as plant growth-promoting rhizobacteria (PGPR) [25].

Several studies have indicated that cover cropping enhances microbial diversity. For example, cover crops altered the bacterial and fungal communities and significantly increased the alpha diversity and relative abundance of bacterial taxa in citrus orchards [26]. Additionally, in a greenhouse study, the mixture of three cover crops (alfalfa—brassica-fescue) showed that increasing the cover crop density enhanced the PGPR, including *Stentotrophomonas* spp., *Azospirillum* sp., and *Achromobacter xylosoxidans* [27]. However, these previous studies have not examined the effects of season-based cover cropping on microbial diversity and community composition in citrus orchards.

As citrus growers in southeast Florida increasingly adopt cover cropping, it is important to understand how these management practices influence rhizosphere microbial communities over time, particularly in the region's sandy, poorly drained soils. Understanding these temporal microbial dynamics is critical because repeated cover crop establishment may alter soil resource availability and plant–microbe interactions, potentially shaping the long-term development and stability of rhizosphere microbial communities. To address this knowledge gap, this study evaluated the effects of repeated warm- and cool-season cover crops on rhizosphere microbial community composition across different citrus orchards over a three-year period. We hypothesized that cover crop management would alter microbial succession over time.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

A three-year field study (January 2022–July 2024) was conducted in Fort Pierce, Florida, across three citrus groves. The first grove (27°26′31″ N, 80°38′28″ W) consisted of 1.5-year-old 'Star Ruby' grapefruit (*Citrus* × *paradisi*) grafted on 'US-942' rootstock and was located on soils classified as Basinger sand, Pople sand, and Riviera fine sand. Basinger sand is classified as siliceous, hyperthermic Spodic Psammaquents, while Pople sand and Riviera fine sand are classified as loamy, siliceous, hyperthermic Arenic Glossaqualfs. The second grove (27°28′03″ N, 80°32′18″ W) consisted of three-year-old 'Bearss' lemon (*Citrus* × *limon*) grafted on sour orange (*Citrus* × *aurantium*) rootstock and was located on Riviera fine sand. The third grove (27°27′27″ N, 80°35′38″ W) consisted of three-year-old 'OLL-8' sweet orange (*Citrus* × *sinensis*) grafted on 'US-942' rootstock and was located on Winder loamy sand and Arents organic substratum. Winder loamy sand is classified as fine-loamy, siliceous, hyperthermic Typic Glossaqualfs. Baseline soil properties differed among groves, with cation exchange capacity values of 11.50, 11.95, and 4.80 meq 100 g^{-1}, soil pH values of 7.38, 7.33, and 5.25, and organic matter contents of 0.88, 0.62, and 0.87% for the grapefruit, lemon, and sweet orange groves, respectively. Due to the poorly drained soils in the region, all groves used a raised bed system with two tree rows per bed. The climate is characterized as a humid subtropical climate [28]. A collaborating grower managed all groves using standard industry insecticide and nutrient management practices [29]. A randomized complete block design was used, with orchard included as a blocking factor in the PERMANOVA to account for variation among orchards. Three orchards served as blocks, each containing six conventional and six treatment plots. Winter and summer cover crop mixtures were purchased from Hancock Seed (Dade City, FL, USA) and planted annually by the grower, as described in Table 1.

Table 1. Cover crop mixtures planted in the cool and warm seasons from 2022–2024. All mixtures were planted at a rate of 50 lbs $acre^{-1}$.

Year 1 (2022)

Cool Season			Warm Season		
Scientific Name	**Common Name**	**Percent of Mixture**	**Scientific Name**	**Common Name**	**Percent of Mixture**
Secale cereale	Cereal Rye	16%	*Urochloa ramosa*	Brown Top Millet	45%
Avena sativa	Coker Oats	48%	*Indigofera hirsuta*	Hairy Indigo	10%
Vicia villosa	Hairy Vetch	16%	*Helianthus annuus*	Peredovik sunflower	15%
Brassica rapa	Purple Top Turnips	2%	*Crotalaria juncea*	Sunn hemp	30%

Year 2 (2023)

Cool Season			Warm Season		
Scientific Name	**Common Name**	**Percent of Mixture**	**Scientific Name**	**Common Name**	**Percent of Mixture**
Urochloa ramosa	Brown Top Millet	30%	*Vigna unguiculata*	Cowpea	36%
Raphanus sativus	Daikon Radish	10%	*Indigofera hirsuta*	Hairy Indigo	6%
Indigofera hirsuta	Hairy Indigo	15%	*Abelmoschus esculentus*	Okra	8%
Vigna unguiculata	Iron Clay Cowpeas	20%	*Crotalaria juncea*	Sunn hemp	20%
Sorghum bicolor	Sterile Sorghum	25%	*Sorghum* × *drummondii*	Sorghum Sudangrass	30%

Year 3 (2024)

Cool Season			Warm Season		
Scientific Name	**Common Name**	**Percent of Mixture**	**Scientific Name**	**Common Name**	**Percent of Mixture**
Urochloa ramosa	Brown Top Millet	30%	*Vigna unguiculata*	Cowpea	26%

Raphanus sativus	Daikon Radish	10%	*Crotalaria juncea*	Sunn hemp	21%
Indigofera hirsuta	Hairy Indigo	15%	*Pennisetum glaucum*	Pearl Millett	15%
Vigna unguiculata	Iron Clay Cowpeas	20%	*Setaria italica*	Foxtail Millett	18%
Sorghum bicolor	Sterile Sorghum	25%	*Fagopyrum esculentum*	Buckwheat	20%

2.2. Treatments

Each grove was divided into two sections: conventional (perennial turfgrass grove floor) and cover crops (experimental treatment). The conventional grove floor management consisted of a perennial turfgrass species, St. Augustine (*Stenotaphrum secundatum*) and bahiagrass (*Paspalum notatum*). Grass was mowed as needed throughout the year. Cover crops were planted twice per year in June (warm season) and October (cool season). Cover crop mixtures changed between seasons and years; however, all experimental treatments in each grove received the same cover crop mixture and management (Tables 1 and 2). Cover crops were planted at a rate of 56 kg ha^{-1} using either a broadcast seed spreader (3-point 386 kg) (Behlen Country, Columbus, NE, USA) or a seed drill (Genisis 8, RTP, Winona, MN, USA) at a depth of 3.81 cm. At the end of the season, roughly six months after planting, cover crops were terminated and reincorporated via tillage and/or mowing (Table 2).

Table 2. Cover crop planting date, planting method, termination date, and termination method from 2022–2024.

Year	Season	Planting Month	Planting Method	Termination Month	Termination Method
2022	Cool season	October	Broadcast seed	June	Tillage
	Warm season	June	Broadcast seed	October	Tillage
2023	Cool season	October	Seed Drill	June	Mowing
	Warm season	June	Seed Drill	October	Mowing
2024	Cool season	October	Seed Drill	June	Mowing
	Warm season	July	Seed Drill	October	Mowing

2.3. Sampling Time and DNA Extraction

Soil samples were collected from the center of the raised bed at 5 different time points (summer 2022, winter and summer 2023, winter and summer 2024). The sampling location was selected because the cover crops are planted in the center of the raised bed, away from the dripline of the citrus trees to reduce potential competition. Additionally, most nutrient applications (fertigation and granular) were applied directly around the dripline of the citrus trees; thus, sampling in the center of the raised beds reduced possible influences from fertilizers. Soil DNA was extracted from 0.25 g of soil using the DNeasy PowerSoil Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's instructions.

2.4. DNA Amplification and 16S and ITS Sequencing

Genomic DNA was prepared for sequencing on a PacBio Revio (Pacific Biosciences, Menlo Park, CA, USA) sequencer using a two-stage PCR protocol, similar to that described for Illumina amplicon library preparation [30]. In the first stage of PCR, DNA was PCR amplified with primers 27F and 1492R (TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG and ACTTGCCTGTCGCTCTATCTTCRGYTACCTTGTTACGACTT, respectively) using domain-level bacterial primers as described by PacBio. The underlined regions, however, represent Oxford Nanopore 'Universal sequences for tailing PCR primers' linker sequences. For fungal amplicons, the

following primers were used for the first PCR step: ONTLinkF_PacBioITS1F: TTTCTGTTGGTGCTGATATTGC ACCWGCGGARGGATCATTA and ONTLinkR_PacBioITS4R: ACTTGCCTGTCGCTCTATCTTC CCTSCSCTTANTDATATGC.

In the second stage of PCR, amplicons from the first stage were used as templates and amplified with primers that contained PacBio Kinnex adapter sequences, combinatorial dual indices, and the ONT linker sequences at the 3′ ends of the primers (e.g., Kinnex_Fwd: **CTACACGACGCTCTTCCGATCT**NNNNNNNNNNTTTCTGTTGGTGCTGATATTGC and Kinnex_Rev: **AAGCAGTGGTATCAACGCAGAG**NNNNNNNNNNACTTGCCTGTCGCTCTATCTTC). Kinnex adapters are bolded, combinatorial indices are indicated with "NNNNNNNNNN", and ONT linkers are underlined. PCR products from the second stage of amplification were pooled, purified and subject to Kinnex library preparation and loading on the Revio instrument. Two-stage library preparation was performed at the Genomics and Microbiome Core Facility (GMCF) at Rush University (Chicago, IL, USA) and Kinnex library preparation, and PacBio sequencing was performed at the DNA Services Facility at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign, IL, USA.

First-stage PCR amplifications were performed in 10-microliter reactions in 96-well plates, using repliQa HiFi ToughMix (Quantabio, Beverly, MA, USA). Genomic DNA input was 4 μL per reaction. PCR conditions were 98 °C for 2 min, followed by 24 cycles of 98 °C for 10 s, 50 °C for 2 s and 68 °C for 3 s. The second PCR amplification was performed in 10-microliter reactions in 96-well plates using repliQa HiFi ToughMix. Each well received a separate primer pair containing unique combinatorial indices. One microliter of PCR product from the first-stage amplification was used as template for the second stage, without cleanup. Two microliters of primer were used per reaction. Cycling conditions were 98 °C for 2 min, followed by 8 cycles of 98 °C for 10 s, 60 °C for 1 s and 68 °C for 1 s. PCR products were pooled and cleaned using a AMPure XP beads (Beckman Coulter, Brea, CA, USA).

2.5. Sequencing Data Analysis

Full-length 16S and ITS microbiome bioinformatics analysis was performed with QIIME 2 (version 2023.5; QIIME 2 development team, Flagstaff, AZ, USA) [31]. Raw sequence data were checked for quality using seqkit. 27F-1492R for 16S and ITS1F-ITS4R for ITS; primer sequences were removed using the cutadapt algorithm [32]. The trimmed sequences were then imported into the QIIME2 pipeline and denoised using the DADA2 [33] (via q2-dada2-ccs). Alpha-diversity metrics [34], Shannon Index [35], and beta diversity metrics were calculated using q2-diversity after samples were rarefied (10,750 sequences/sample for 16S and 200 sequences/sample for ITS). The ITS rarefaction depth was selected to retain the maximum number of samples while accounting for differences in sequencing depth among samples, thus minimizing the loss of samples at higher thresholds. Specifically, rarefaction to 200 sequences per sample retained 174 of the 182 samples (95.6%). Taxonomy was assigned to ASVs against the SILVA-138-99-nb reference sequence database [36] for 16S and the unite_ver9_99_s_all_25.07.2023 reference database for ITS. The contaminant removal software, decontam [37], was employed to detect any contaminants based on the prevalence of the ASVs in the reagent-negative blank controls using default parameters.

Bacterial functional guild prediction was done using FAPROTAX [38]. Relative abundances were computed at the phylum and genus levels (taxonomic) and at the functional guild level (FAPROTAX). Taxa/guilds with mean relative abundance ≥1% were retained; the remainder were pooled as "Other".

For both bacterial and fungal communities, dissimilarities were quantified with Bray–Curtis distances on relative-abundance data. Differences among groups were tested

by PERMANOVA (vegan:adonis 2, 9999 permutations) using the model distance ~ Cultivar × Treatment × Season. Within-season analyses (summer, winter) were run separately as stratified, but only at the descriptive level, because the design is not independent experiments; the same plots were applied with different cover crops each season and measured over time. Beta-diversity was visualized with Principal Coordinates Analysis (PCoA) on the same Bray–Curtis distances.

To identify guilds responding to treatment, each guild's relative abundance was modeled with a linear mixed model (lmerTest). Treatment *p*-values were corrected across guilds by Benjamini–Hochberg FDR. Similarly to PERMANOVA above, we split the analysis by season to compare treatment effects with each season descriptively.

For the fungal community, the FUNGuild database was used to classify ASVs into the following putative functional guilds: saprotroph, pathotroph, symbiotroph. Because of the uncertainties surrounding ecological functional guilds of many soil microbes, many ASVs were assigned to the following combined categories: pathotroph–saprotroph, saprotroph–symbiotroph, pathotroph–symbiotroph, and pathotroph–saprotroph–symbiotroph [39]. Following trophic mode assignment of represented fungal taxa, data were then filtered to include only trophic assignments at confidence levels of 'highly probable' or 'probable'. This filtering resulted in a total of 205 fungal ASVs (saprotroph = 98, pathotroph–saprotroph = 54, pathotroph–saprotroph–symbiotroph = 20, pathotroph = 17, saprotroph–symbiotroph = 10, symbiotroph = 4, pathotroph–symbiotroph = 2).

3. Results

3.1. Relative Abundance of Bacterial Communities

Across the sampling dates, bacterial community composition revealed a consistent taxonomic pattern across cultivars and treatments. At the phylum level, *Proteobacteria* was consistently the most abundant phylum across grapefruit, lemon, and orange orchards, followed by *Actinobacteriota*, *Acidobacteriota*, and *Firmicutes*. Other consistently detected phyla included *Planctomycetota*, *Bacteroidota*, *Myxococcota*, *Gemmatimonadota*, *Chloroflexi*, *Nitrospirota*, and *Verrucomicrobiota*, although their relative abundances varied among sampling dates. At the genus level, *Bacillus*, *67-14*, *RB41*, *Microvirga*, *Sphingomonas*, *Gaiella*, and several uncultured or unclassified taxa were among the more abundant genera. The proportion of taxa represented by the "Other" category remained substantial, indicating that the bacterial community comprised a diverse assemblage of lower-abundance genera. Differences between control and cover crop treatments were generally modest and appeared primarily as shifts in the relative abundance of individual genera across sampling dates rather than major changes in the overall community composition (Figure 1).

3.2. Functional Guild Relative Abundance

FAPROTAX analysis identified significant differences in several predicted bacterial functional guilds between control and cover crop treatments, with responses varying by cultivar and season (Figure 2). Significant treatment effects were observed for guilds associated with nitrogen transformations, carbon metabolism, sulfur oxidation, and manganese oxidation, including denitrification, nitrate and nitrite denitrification, nitrogen fixation, methylotrophy, methanol oxidation, and anoxygenic photoautotrophy. Several nitrogen- and sulfur-related functions generally showed higher relative abundances in control plots, whereas manganese oxidation, methanol oxidation, methylotrophy, and photoheterotrophy were frequently higher under cover crop conditions. Treatment differences were most evident in grapefruit, whereas fewer differences were observed in both orange and lemon (Figure 2).

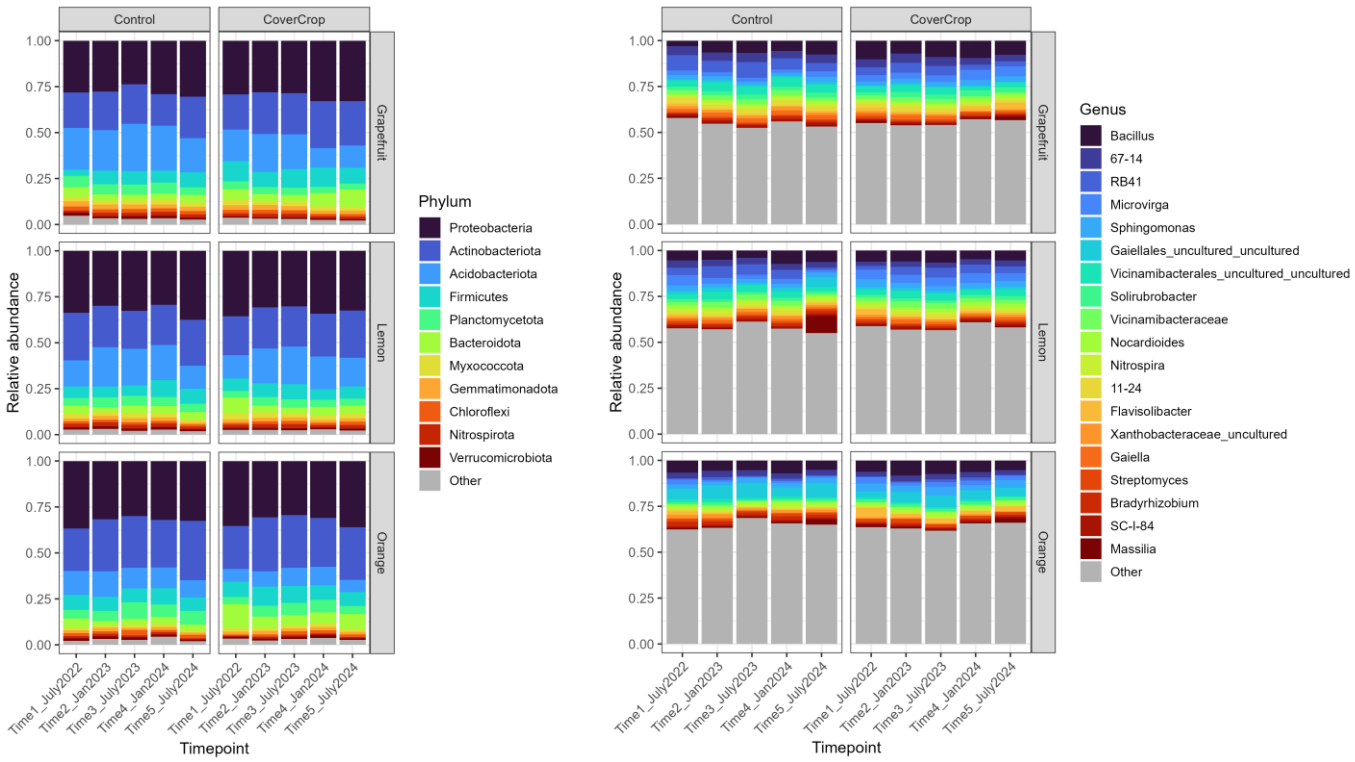
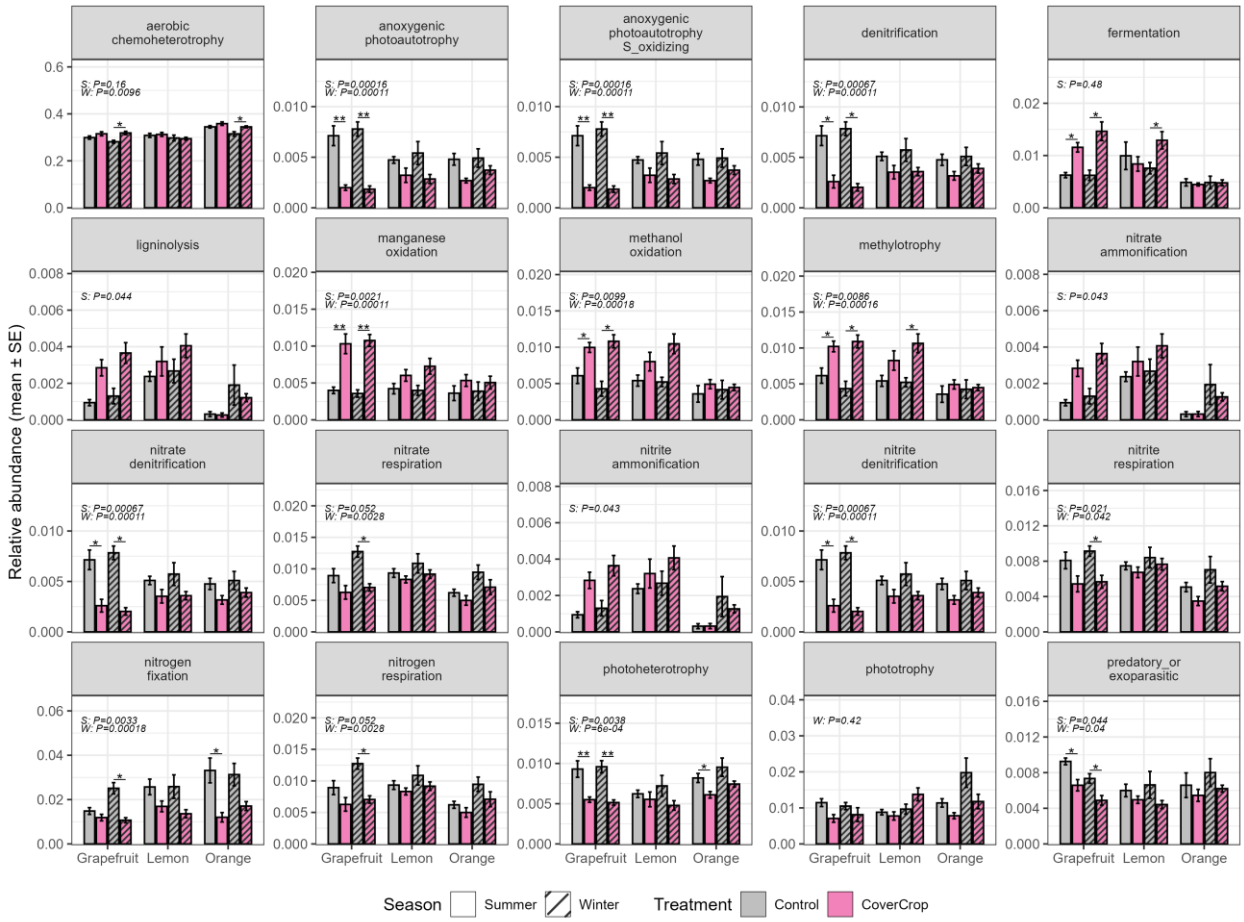


Figure 1. Mean relative abundance of bacterial communities across time points, faceted by cultivar (rows) and treatment (columns). Features with mean relative abundance ≥1% are shown; the remainder are pooled as "Other" (grey).



https://doi.org/10.3390/agronomy16212206

Figure 2. Functional guild relative abundance bars (bacterial community): Relative abundance of FAPROTAX functional guilds differing significantly between Control and Cover Crop treatments, shown by cultivar and season. Within each cultivar, bars are grouped by season (summer = solid, winter = diagonal stripes) and colored by treatment (grey = Control, pink = Cover Crop). Bars are means ± standard error across plots (n = 6 plots per cultivar × treatment). Two significance layers are shown: italicized values in the upper-left of each panel give the overall Treatment main-effect p-value from per-season, per-guild linear mixed models, FDR-corrected across guilds (S: summer; W: winter); asterisks above each cultivar × season bar pair denote Control vs. Cover Crop contrasts (Wilcoxon rank-sum on plot means, FDR-corrected across cultivars within each guild; * adjusted p < 0.05, ** < 0.01).

3.3. Relative Abundance of Fungal Communities

At the phylum level, fungal community composition was consistently dominated by Ascomycota across orchards, sampling dates, and treatments, while Basidiomycota and Rozellomycota occurred at comparatively low relative abundances (Figure 3). The relative abundance of Ascomycota varied temporally, particularly in the lemon orchard, but its overall dominance was maintained under both control and cover-crop treatments. At the genus level, the communities were more heterogeneous, with *Fusarium*, *Marasmius*, *Dothideomycetes_gen_Incertae_sedis*, *Cladosporium*, *Trichoderma*, *Curvularia*, and *Penicillium* among the predominant identified taxa. The relative abundance of these genera varied among orchards and sampling dates, and a substantial proportion of sequences remained classified as "Other" (Figure 3).

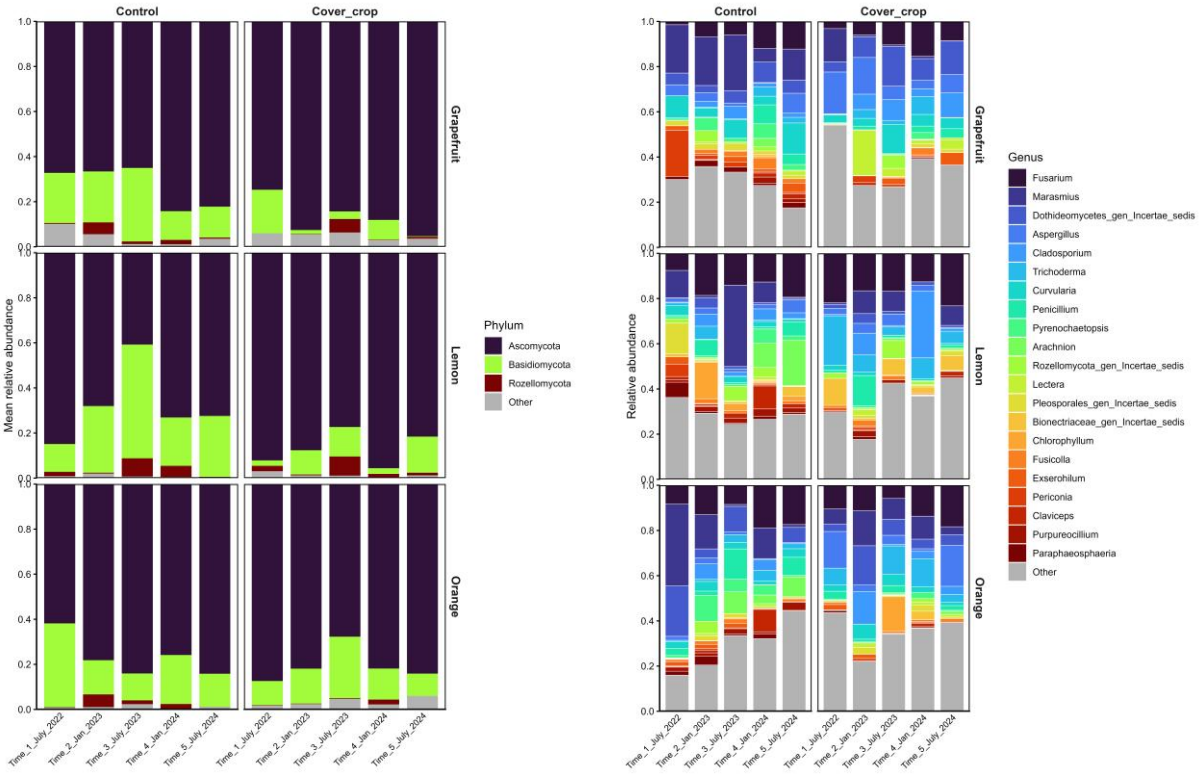


Figure 3. Mean relative abundance of fungal community (phylum and genus level) across time points, faceted by cultivar (rows) and treatment (columns). Features with mean relative abundance ≥1% are shown; the remainder are pooled as "Other" (grey).

3.4. Alpha Diversity Analysis (Bacterial and Fungal Communities)

Bacterial Shannon diversity varied across orchards, sampling time points, and treatments (Table 3). In the grapefruit orchard, the control treatment exhibited significantly

higher Shannon diversity than the cover crop treatment in July 2022 and July 2023. In the orange orchard, bacterial Shannon diversity did not differ significantly between treatments at most sampling time points. However, in January 2024, the cover crop treatment had significantly greater Shannon diversity than the control. Although slightly higher values were observed under the cover crop treatment during January 2023, July 2023, and July 2024, these differences were not statistically significant. However, pairwise comparisons showed significant differences between grapefruit and orange and between lemon and orange orchards for the bacterial community, whereas no significant differences in fungal Shannon diversity were detected among the three orchard types (Tables S1–S8).

Table 3. Shannon entropy of bacterial and fungal communities in grapefruit, orange, and lemon orchards under control and cover crop treatments across sampling time points. Values are presented as mean ± standard deviation. Within each orchard and sampling timepoint, means followed by different lowercase letters are significantly different according to Tukey's honestly significant difference (HSD) test ($p < 0.05$).

Orchard	Timepoint	Treatment	Shannon Entropy of Bacterial Community	Shannon Entropy of Fungal Community
Grapefruit	July 2022	Control	11.36 ± 0.17 [a]	3.44 ± 1.22
		Cover crops	10.87 ± 0.24 [b]	3.31 ± 1.16
	Jan 2023	Control	11.15 ± 0.19	4.46 ± 1.04
		Cover crops	11.04 ± 0.34	4.06 ± 0.52
	July 2023	Control	11.13 ± 0.17 [a]	4.13 ± 0.47
		Cover crop	10.91 ± 0.08 [b]	4.28 ± 0.24
	Jan 2024	Control	11.03 ± 0.31	4.76 ± 0.29
		Cover crop	10.89 ± 0.22	4.64 ± 0.15
	July 2024	Control	11.12 ± 0.19	4.21 ± 0.96
		Cover crop	11.04 ± 0.16	4.54 ± 0.32
Orange	July 2022	Control	10.73 ± 0.29	3.60 ± 1.53
		Cover crops	10.77 ± 0.25	4.09 ± 0.59
	Jan 2023	Control	10.84 ± 0.38	4.25 ± 0.98
		Cover crops	11.10 ± 0.20	3.51 ± 1.27
	July 2023	Control	10.84 ± 0.29	4.40 ± 0.43
		Cover crop	11.05 ± 0.26	3.51 ± 1.73
	Jan 2024	Control	10.83 ± 0.18 [b]	3.94 ± 0.85
		Cover crop	11.11 ± 0.17 [a]	4.26 ± 0.66
	July 2024	Control	10.79 ± 0.28	4.40 ± 0.40
		Cover crop	11.01 ± 0.10	3.86 ± 1.37
Lemon	July 2022	Control	10.98 ± 0.18	4.17 ± 0.42
		Cover crops	11.02 ± 0.23	3.63 ± 0.81
	Jan 2023	Control	11.09 ± 0.36 [b]	3.75 ± 1.82
		Cover crops	11.46 ± 0.16 [a]	4.49 ± 0.26
	July 2023	Control	11.23 ± 0.18	3.68 ± 1.29
		Cover crop	11.09 ± 0.17	4.29 ± 0.33
	Jan 2024	Control	10.90 ± 0.25 [b]	4.26 ± 0.60
		Cover crop	11.28 ± 0.09 [a]	3.71 ± 0.62
	July 2024	Control	10.61 ± 0.56 [b]	3.76 ± 1.09
		Cover crop	11.44 ± 0.05 [a]	4.12 ± 0.62

In the lemon orchard, the cover crop treatment significantly increased bacterial Shannon diversity compared with the control in January 2023, January 2024 and July 2024. However, no significant differences were recorded for fungal Shannon diversity (Table 3).

3.5. Beta Diversity (Bacterial Community)

Bray–Curtis PCoA revealed distinct differences in bacterial community composition among citrus cultivars, with PCoA1 and PCoA2 explaining 33.5% and 13.8% of the variation, respectively. PERMANOVA identified cultivar as the primary factor explaining community variation (R^2 = 0.276; p < 0.001), followed by treatment (R^2 = 0.047; p = 0.005) and season (R^2 = 0.009; p < 0.001) (Figure 4B). The cultivar × treatment interaction was not significant (R^2 = 0.023; p = 0.273), whereas interactions involving season were significant. Within-season analyses confirmed the strong effect of cultivar in both summer (R^2 = 0.277; p < 0.001) and winter (R^2 = 0.302; p < 0.001). Treatment also significantly influenced bacterial community composition in summer (R^2 = 0.045; p = 0.007) and winter (R^2 = 0.064; p = 0.003), while cultivar × treatment interactions were not significant in either season (Figure 4).

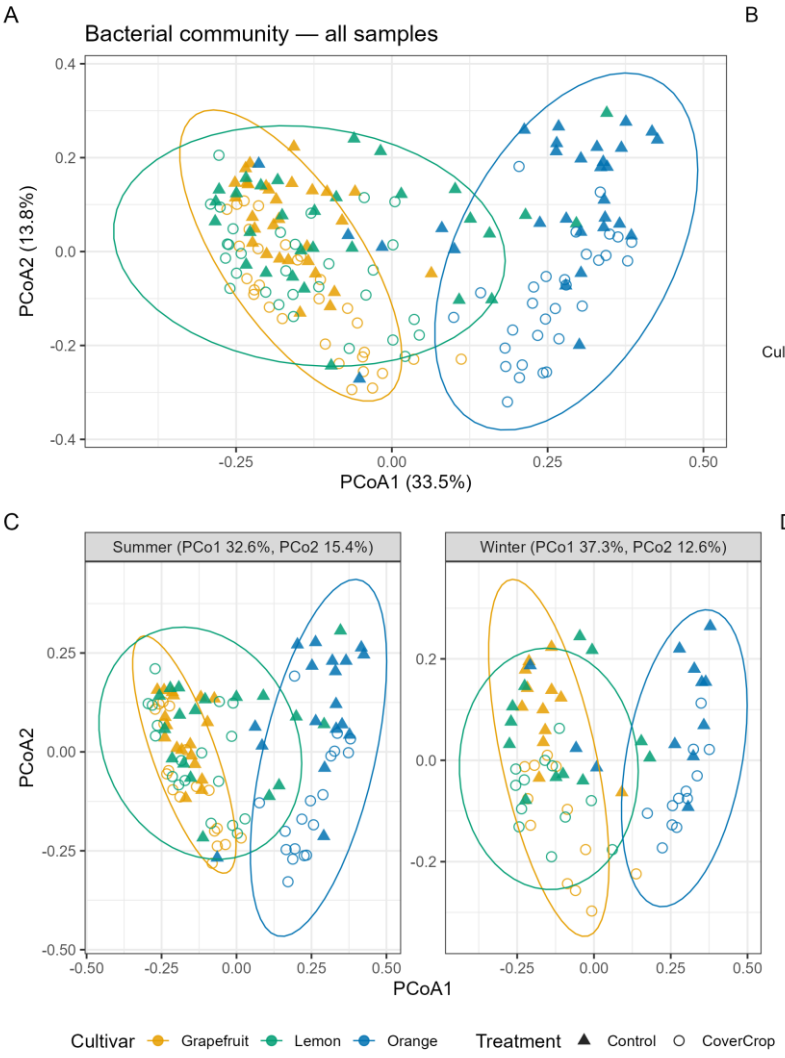


Figure 4. Taxonomic beta-diversity (bacterial community): PCoA on Bray–Curtis distances of rarefied 16S data. (**A**) All samples, ellipses by cultivar; (**B**) combined PERMANOVA (Cultivar × Treatment × Season, 9999 permutations restricted to whole plots); (**C**) within-season PCoA (summer, winter); (**D**) within-season PERMANOVA (Cultivar × Treatment, 9999 permutations). Points colored by cultivar; shapes denote treatment (Cover Crop = open circle, Control = filled triangle). † Season and its interactions are within-plot factors; *p*-values under the whole-plot permutation scheme are approximate and should be interpreted with caution. R^2 = proportion of variation explained; Df = degrees of freedom; F = pseudo-F statistic.

Functional beta-diversity of bacterial communities differed significantly among cultivars and treatments (Figure 5). The overall PCoA explained 46.9% of the variation across the first two axes (PCoA1 = 29.4%, PCoA2 = 17.5%). PERMANOVA identified cultivar as the strongest determinant of functional composition (R^2 = 0.202; p < 0.001), followed by treatment (R^2 = 0.065; p < 0.001) and season (R^2 = 0.020; p < 0.001). The cultivar × season and cultivar × treatment × season interactions were also significant (p = 0.007 and 0.008, respectively), whereas the cultivar × treatment interaction was not significant (p = 0.133). Within-season analyses confirmed significant cultivar and treatment effects in both summer and winter. Cultivar explained 21.1% and 22.6% of the variation in summer and winter, respectively (both p < 0.001), while treatment explained 5.6% in summer (p = 0.002) and 9.3% in winter (p < 0.001). The cultivar × treatment interaction was significant only in winter (R^2 = 0.059; p = 0.029), indicating a season-dependent response of bacterial functional profiles to treatment (Figure 5).

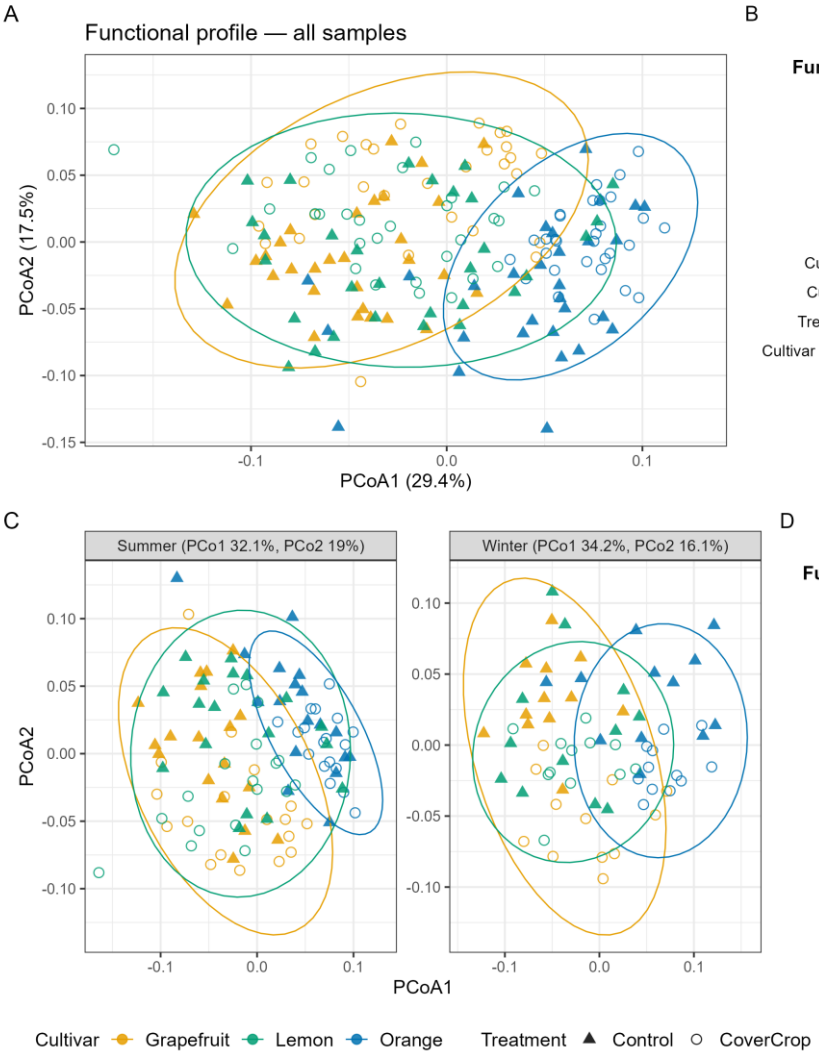
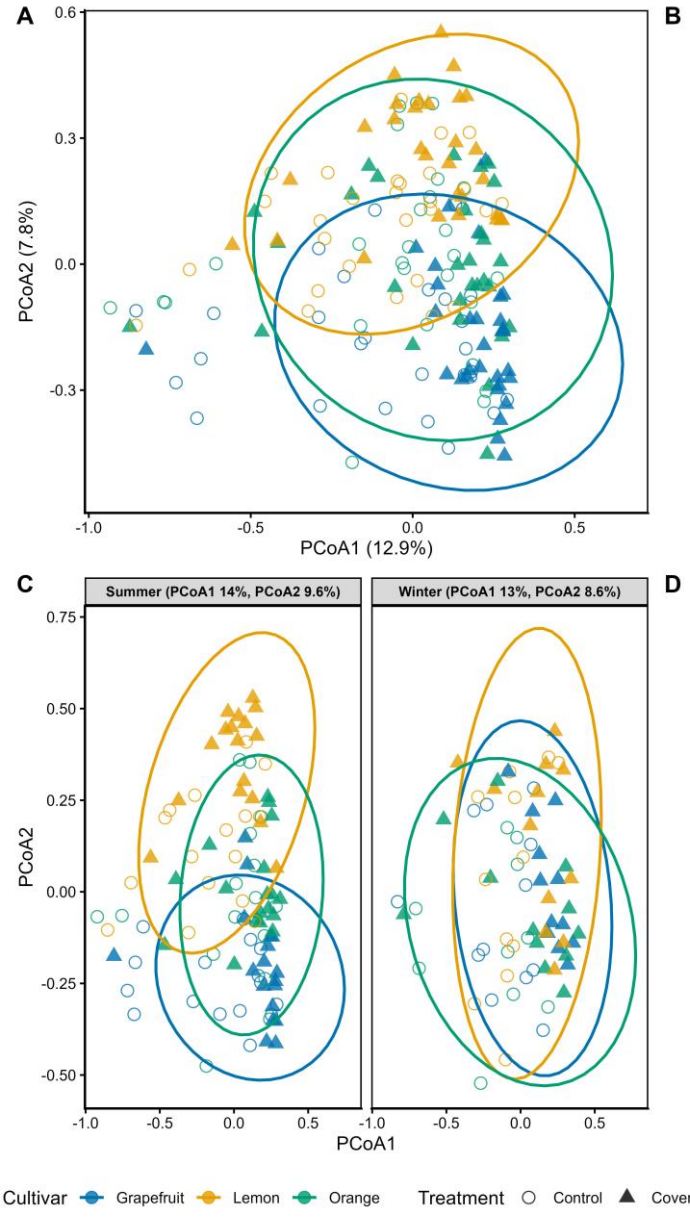


Figure 5. Functional beta-diversity (Bacterial community). PCoA on Bray–Curtis distances of predicted functional guilds (FAPROTAX). (**A**) All samples, ellipses by cultivar; (**B**) combined PERMANOVA (Cultivar × Treatment × Season, 9999 permutations restricted to whole plots); (**C**) within-season PCoA (summer, winter); (**D**) within-season PERMANOVA (Cultivar × Treatment, 9999 permutations). Points colored by cultivar; shapes denote treatment (Cover Crop = open circle, Control = filled triangle). † Season and its interactions are within-plot factors; *p*-values under the whole-plot permutation scheme are approximate and should be interpreted with caution. R^2 = proportion of variation explained; Df = degrees of freedom; F = pseudo-F statistic.

https://doi.org/10.3390/agronomy16212206

3.6. Beta Diversity (Fungal Community)

Weather, cultivar and treatment significantly affected taxonomic beta-diversity of fungal communities (Figure 6A). The overall PCoA explained 20.7% of the variation across the first two axes (PCoA1 = 12.9%, PCoA2 = 7.8%). PERMANOVA indicated that all main effects, as well as all interactions, significantly affected soil fungal community dissimilarities, with a strong three-way interaction between cultivar, treatment and weather (Figure 6B). Within-season analyses revealed that these main effects were persistent across seasons (Figure 6C,D). Notably, the two-way interaction between cultivar and treatment was significant in summer months, but not in winter sampling periods (Figure 6D).



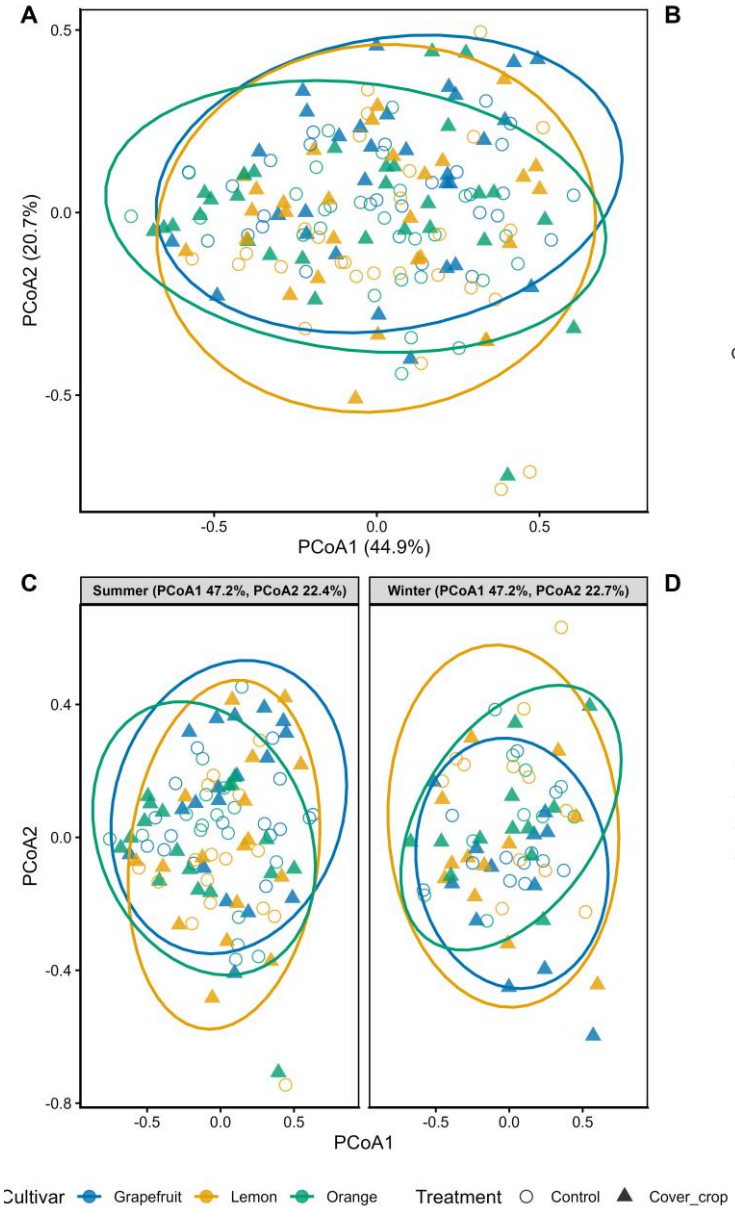
Effect	Df	R2	F	p
Cultivar	2	0.04840	4.7220	<0.001
Treatment	1	0.04018	7.8408	<0.001
Weather	1	0.02020	3.9417	<0.001
Cultivar × Treatment	2	0.01676	1.6350	0.0058
Cultivar × Weather	2	0.03051	2.9763	<0.001
Treatment × Weather	1	0.00796	1.5542	0.0408
Cultivar × Treatment × Weather	2	0.01604	1.5645	0.0090
Residual	161	0.81996	NA	NA

Season	Effect	Df	R2	F	p
Summer	Cultivar	2	0.09608	5.5945	<0.001
Summer	Treatment	1	0.04599	5.3564	<0.001
Summer	Cultivar × Treatment	2	0.03362	1.9577	0.0006
Summer	Residual	96	0.82431	NA	NA
Winter	Cultivar	2	0.05510	2.0565	<0.001
Winter	Treatment	1	0.05485	4.0949	<0.001
Winter	Cultivar × Treatment	2	0.03271	1.2208	0.1245
Winter	Residual	64	0.85734	NA	NA

Figure 6. Taxonomic beta-diversity (fungal community): PCoA on Bray–Curtis distances of rarefied 16S data. (**A**) All samples, ellipses by cultivar; (**B**) combined PERMANOVA (Cultivar × Treatment × Season, 9999 permutations restricted to whole plots); (**C**) within-season PCoA (summer, winter); (**D**) within-season PERMANOVA (Cultivar × Treatment, 9999 permutations). Points colored by cultivar; shapes denote treatment (Cover Crop = open circle, Control = filled triangle). Season and its interactions are within-plot factors; *p*-values under the whole-plot permutation scheme are

approximate and should be interpreted with caution. NA = not applicable; R^2 = proportion of variation explained; Df = degrees of freedom; F = pseudo-F statistic.

Bray–Curtis PCoA of predicted fungal functional profiles showed that the first two axes explained 65.6% of the total variation (PCoA1 = 44.9%, PCoA2 = 20.7%) (Figure 7A). PERMANOVA identified a significant effect of season ($R^2 = 0.032$; $p = 0.001$), while cultivar showed a marginal effect ($R^2 = 0.022$; $p = 0.051$) and treatment was not significant ($p = 0.107$). No significant cultivar × treatment, cultivar × season, treatment × season, or three-way interaction effects were detected (all $p > 0.17$). Within-season analyses showed a significant cultivar effect during summer ($R^2 = 0.042$; $p = 0.038$), whereas treatment and the cultivar × treatment interaction were not significant. In winter, neither cultivar, treatment, nor their interaction significantly influenced functional community composition (all $p \geq 0.096$) (Figure 7).



Effect	Df	R2	F	p
Cultivar	2	0.022	2.017	0.051
Treatment	1	0.010	1.885	0.107
Weather	1	0.032	5.733	0.001
Cultivar × Treatment	2	0.003	0.331	0.961
Cultivar × Weather	2	0.016	1.428	0.172
Treatment × Weather	1	0.003	0.607	0.637
Cultivar × Treatment × Weather	2	0.014	1.303	0.229
Residual	160	0.896	NA	NA

Season	Effect	Df	R2	F	p
Summer	Cultivar	2	0.042	2.189	0.038
Summer	Treatment	1	0.005	0.570	0.668
Summer	Cultivar × Treatment	2	0.015	0.811	0.575
Summer	Residual	96	0.935	NA	NA
Winter	Cultivar	2	0.033	1.183	0.299
Winter	Treatment	1	0.028	2.008	0.096
Winter	Cultivar × Treatment	2	0.023	0.836	0.544
Winter	Residual	64	0.913	NA	NA

Figure 7. Functional beta-diversity (fungal community). PCoA on Bray–Curtis distances of predicted functional guilds (FAPROTAX). (**A**) All samples, ellipses by cultivar; (**B**) combined PERMANOVA (Cultivar × Treatment × Season, 9999 permutations restricted to whole plots); (**C**) within-

season PCoA (summer, winter); (**D**) within-season PERMANOVA (Cultivar × Treatment, 9999 permutations). Points colored by cultivar; shapes denote treatment (Cover Crop = open circle, Control = filled triangle). Season and its interactions are within-plot factors; *p*-values under the whole-plot permutation scheme are approximate and should be interpreted with caution. NA = not applicable; R^2 = proportion of variation explained; Df = degrees of freedom; F = pseudo-F statistic.

Following the filtering of taxa representing low confidence in trophic mode assignments, PERMANOVA results indicate independent significant effects of weather (R^2 = 0.03; p = 0.001) and cultivar (R^2 = 0.02; p = 0.05) on composition of fungal functional guilds (Figure 7A,B). Alternatively, cover cropping had no significant effect on composition of fungal functional guilds (R^2 = 0.01; p = 0.10). Within-season analyses revealed a significant effect of cultivar, but only in summer months (R^2 = 0.04; p = 0.038). When assessing proportional relative abundances of trophic modes, fungal pathotrophs constituted a larger proportion of the fungal community in winter months, compared to summer months (Figure S1). When considering citrus cultivar, overall differences in trophic mode assemblages appeared to be largely driven by low relative abundance of pathotrophs in orange groves, relative to grapefruit and lemon groves, coupled with greater relative abundance of saprotrophs in orange and lemon groves, compared to grapefruit groves (Figure S1).

4. Discussion

The bacterial community exhibited a relatively stable overall taxonomic structure across control and cover crop treatments, with *Proteobacteria*, *Actinobacteriota*, *Acidobacteriota*, and *Firmicutes* consistently representing the dominant phyla across cultivars and seasons. The high abundance of these groups suggests that these bacteria may contribute to ecosystem functioning through their reported roles in carbon and nitrogen cycling, organic matter decomposition, nutrient transformation and their potential to promote plant growth [40]. The observed temporal shifts in the relative abundance of specific genera, including *Bacillus*, *RB41*, *Microvirga*, *Sphingomonas*, and *Gaiella*, may reflect changes in seasonal conditions, substrate availability, and rhizosphere inputs rather than a uniform response to cover cropping. The predicted functional profiles provided additional evidence that cover cropping influenced selected components of the bacterial community**.** Differences in functions associated with nitrogen transformations, carbon metabolism, sulfur oxidation, and manganese oxidation varied among cultivars and seasons, indicating that the response was context dependent rather than uniform across the orchard system. The greater relative abundance of several carbon- and manganese-associated functional groups under cover crop treatment may be consistent with increased availability of plant-derived substrates and residue inputs. Conversely, greater representation of some denitrification-related and anoxygenic photoautotrophic functions in control plots for specific cultivar–season combinations suggests that microbial functional responses may depend on both host and environmental conditions. The fungal communities shared a core mycobiome although their relative abundance varied among orchards, which suggests stability that results by forming extensive hyphal networks that allow them to access nutrients over large areas and participate in decomposition and organic turnover. This aligns with the previous findings that perennial agroecosystems often maintain a core microbiome despite spatial variability [41]. The consistent occurrence of *Fusarium*, *Cladosporium*, *Trichoderma*, and *Penicillium* across all orchards suggests these taxa function as decomposers with broad ecological tolerance and roles in nutrient cycling.

Analysis of the diversity and composition of the rhizosphere microbial community also provides valuable insights into soil and plant health, because these communities play an important role in nutrient cycling, organic matter decomposition, and plant-microbe interactions [42,43]. Bacterial diversity in citrus orchards has been associated with

improved growth and enhanced disease suppression via key microbial processes [44]. Similarly, Shu et al. [45] found that cover crops enhanced the microbial biomass and altered the composition of microbial communities. Differences in bacterial composition across citrus orchards, particularly in orange orchards, suggest that soil type plays a major role in shaping distinct microbial communities, consistent with previous findings in tree crops [46,47]. These changes likely result from multiple biotic and physicochemical interactions, including nutrient cycling alterations, root biomass, and exudate diversity [48]. In the present study, beta-diversity analyses and functional analyses revealed distinct drivers of bacterial and fungal community structure in the citrus rhizosphere. Bacterial communities were primarily structured by cultivar, which suggests that host-associated factors may exert a greater influence on bacterial community assembly than cover-crop management. Differences among cultivars may reflect variation in root characteristics, nutrient acquisition, and the quantity and composition of root-derived compounds, which can create distinct rhizosphere niches for microbial communities. Conversely, fungal communities were primarily structured by season, with limited effects of cultivar and no significant influence of cover-crop treatment on overall functional composition. This contrasting pattern suggests that fungal community assembly may be more strongly influenced by temporal changes in environmental conditions than by host genotype or cover-crop management. Seasonal variation in temperature, soil moisture, substrate availability, and plant physiological activity may therefore represent stronger determinants of fungal community composition in the citrus rhizosphere [49]. Similarly, previous work has indicated that dominant bacterial genera can be seasonally stable but can vary based on the soil depth [50]. Moreover, the comparison of control and cover crop groups showed differentiation in soil microbial communities, suggesting that application of cover crops enhanced the functional activities of microbiomes [26]. Similarly, Rodriguez-Ramos et al. [51] reported that cover crops enhanced the bacterial composition in pecan (*Carya illinoinensis*) orchards in middle row soils. The variation in bacterial Shannon diversity further suggests that the effects of cover cropping were orchard-dependent rather than consistent across sampling periods. Significant differences between grapefruit and orange and between lemon and orange orchards further support the influence of orchard-specific factors on bacterial diversity. In contrast, fungal alpha diversity did not differ significantly among citrus orchards, treatments, or sampling timepoints, suggesting that fungal alpha diversity was relatively stable despite variations in host species, cover cropping, and temporal conditions. Similar patterns have been reported in agroecosystems, where cover cropping alters community composition without necessarily affecting overall diversity metrics [52].

After filtering for only fungal taxa that could be assigned to trophic modes with moderate to high confidence, assembly of trophic modes differed by orchard type and season, but not cover cropping. These results suggest that shifts in fungal community composition, whether through identity or relative abundance of key taxa, does not necessarily alter the broader ecological roles occupied by the local fungal community. Several taxa represented in our data were assigned to trophic modes spanning two, or even all three, of the possible broad categories (pathotroph, saprotroph, or symbiotroph). This could be due to uncertainties regarding the functional roles of individual taxa. Alternatively, some fungal taxa are known to occupy two or more of these trophic modes, depending on resource availability and other edaphic factors. Lastly, the sequencing depth in the present study was relatively low, which may have resulted in filtering of rare taxa, which may limit inferences of detailed community composition. However, previous studies using similar rarefaction thresholds have indicated that these sampling depths are adequate for detecting broad differences in community composition across treatments [53,54].

The bacterial and fungal community responses observed in the present study can be considered alongside the soil fertility responses reported from the same three-year field

experiment. Hallman et al. [55] reported that cover cropping influenced soil moisture, temperature, organic matter, and nutrient dynamics, including increases in soil nitrate and phosphorus, although responses varied among orchards and sampling periods. These changes in soil resource availability and physicochemical conditions provide a plausible ecological context for the orchard- and time-dependent shifts in bacterial diversity and community composition observed in the present study, as microbial communities are closely associated with changes in nutrient availability and organic matter inputs. In contrast, fungal alpha diversity remained relatively stable across orchards, treatments, and sampling periods, suggesting that bacterial and fungal communities may respond differently to changes associated with cover cropping. The additional tree-growth study further reported variable responses in leaf nutrient concentrations, canopy growth, trunk diameter, and other tree performance indicators under cover cropping [56].

While bacteria and fungi represent the dominant and most extensively studied components of the soil microbiome, other microbial groups such as archaea, protists, and viruses also play important roles in regulating soil ecosystem processes, including nutrient cycling and microbial interactions. However, due to their well-established ecological roles as primary drivers of decomposition, nutrient mineralization, and plant–microbe interactions, this study focused on bacterial and fungal communities.

5. Conclusions

In conclusion, this three-year study revealed that cover crops produced modest shifts in bacterial community composition, particularly among taxa associated with nutrient cycling, although the responses were orchard dependent, However, the fungal microbiome exhibited a stable, resilient structure across orchards, treatments, and time points, suggesting a conserved core community. Overall, our findings demonstrate that cover cropping can modify the bacterial component of soil microbiomes across citrus orchards while maintaining relatively stable fungal diversity. However, future studies should explore functional gene analysis to further clarify mechanisms and guide more targeted cover crop recommendations for citrus systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy16212206/s1, Table S1: Pairwise comparison of bacterial alpha diversity (Shannon) across orchard types, cover crop treatments, and within-season time points; Table S2: Pairwise comparison of fungal alpha diversity (Shannon) across orchard types, cover crop treatments, and within-season timepoints; Table S3: Pairwise comparison for bacterial Evenness across orchard types, cover crop treatments, and within-season time points; Table S4: Pairwise comparisons for bacterial Observed Features across orchard types, cover crop treatments, and within-season time points; Table S5: Pairwise comparisons for bacterial Simpson Diversity Index across orchard types, cover crop treatments, and within-season time points; Table S6: Pairwise comparison for fungal Evenness across orchard types, cover crop treatments, and within-season time points; Table S7: Pairwise comparisons for bacterial Observed Features across orchard types, cover crop treatments, and within-season time points; Table S8: Pairwise comparisons for fungal Simpson Diversity Index across orchard types, cover crop treatments, and within-season time points; Figure S1: Proportional abundances of fungal trophic modes summarized by (A) citrus cultivar, (B) season, and (C) cover crop-ping treatment.

Author Contributions: Conceptualization, K.H., L.M.H. and L.R.; methodology, K.H., L.M.H., J.-P.F. and L.R.; formal analysis, Y.X., E.B.D., Y.W. and K.H.; resources, M.A.R. and D.M.K.; writing—original draft preparation, K.H.; writing—review and editing, K.H., L.M.H., J.-P.F., Y.W., E.B.D., Y.X., M.A.R., D.M.K. and L.R. All authors have read and agreed to the published version of the manuscript.

https://doi.org/10.3390/agronomy16212206

Funding: This research was funded by the UF/IFAS Citrus Initiative and the U.S. Department of Agriculture (USDA) HATCH grant #FLA-IRC-005743 and #TX0-811 and Multistate HATCH #TEX0-7052 (S1070). D.K. acknowledge the support of USDA HATCH grant #006185. Y.X. and Y.W. acknowledge the support of USDA HATCH grant #TEX0-8008, Texas A&M University and AgriLife Research. E.B.D acknowledge the support of USDA HATCH grant #TEX0-008158, Texas A&M University and AgriLife Research.

Data Availability Statement: The original sequencing data presented in this study are openly available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number PRJNA1526745 at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1526745 (accessed on 5 October 2026).

Acknowledgments: The authors express their gratitude to Daniel Scott from Scott Citrus Management in Fort Pierce, FL, USA, for granting access to his commercial grove for the entire duration of the study.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

SOM	Soil organic matter
PERMANOVA	Permutational multivariate analysis of variance
PGPR	Plant growth promoting rhizobacteria

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