

Tropical orchard soil microbiomes shaped by crop management harbor azole-resistant *Candida tropicalis* clade 4 as an integrated community member

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Abstract

Soil microbial communities represent one of the planet's most diverse ecosystems, performing essential ecological functions including nutrient cycling, plant growth promotion, and biological disease suppression. Agricultural practices reshape these communities, yet their interactions with agrochemical residues and opportunistic pathogens remain poorly characterized. We investigated how orchard management, azole fungicide residues, and *Candida tropicalis* presence influence soil microbial composition and functional profiles across 55 orchard soils (papaya, grape, wax apple) in Taiwan using 16S rRNA and ITS metabarcoding. Orchard management was the primary determinant of microbial community structure, explaining 10–11% of compositional variance and significantly structuring both bacterial and fungal functional guilds. Predictive functional annotation via FAPROTAX and FUNGuild revealed orchard-specific metabolic profiles, mostly enriched for anaerobic fermentation, iron respiration, and methanol oxidation. Neither *C. tropicalis* presence nor azole residues significantly altered overall community composition. However, cumulative azole burden selectively suppressed fungal but not bacterial diversity. Cross-kingdom co-occurrence analysis revealed that *C. tropicalis* clade 4 showed exclusively positive co-occurrence with 8 microbial taxa. These findings demonstrate that agricultural management overwhelms pathogenic and chemical drivers in structuring soil microbiomes, while total azole concentration exposure drives shifts in fungal diversity, and the *C. tropicalis* clade 4 may act as integrated community members.

Introduction

Soils constitute one of the most biologically diverse environments on the planet, with a single gram containing approximately 10^{10} bacterial cells and 10^6 fungal propagules^{1,2}. Beyond taxonomic richness, this vast microbial diversity underpins important ecosystem functions, including cycling biochemical nutrients, sustaining plant vitality, and suppressing disease³. The collective enzyme activities of soil microbial communities determine nutrient cycling rates that act as primary constraints on productivity across all terrestrial ecosystems⁴. However, soil microbial communities are not stochastically assembled; rather, they are shaped by environmental filters and biotic interactions that exert long-term selective pressure⁵. Elucidating the primary factors constraining microbial community assembly is a prerequisite for predicting ecosystem function under anthropogenic pressures such as agricultural intensification, fungicide residue persistence, and climate change⁶.

Agricultural intensification, associated with anthropogenic practice interventions including tillage, nutrient management, and rotation cycles, has fundamentally reshaped soil microbial diversity and composition^{7,8}. These anthropogenic interventions generate strong selective forces that can supersede the effects of inherent edaphic gradients on microbial community structure. For instance, the distinction between organic and conventional farming practices explains 12–20% of bacterial community variance, exceeding the influence of soil pH, historically considered the primary driver of microbial structure^{7–9}.

In parallel with agricultural management, the application of synthetic fungicides exerts direct chemical selection pressure on soil microbial communities. Among these, azole compounds accounted for approximately 2 million tons sold in 2020, representing approximately 16% of the global fungicide market volume, with sales on a continuing upward trajectory^{10,11}. Despite being designed for targeted disease control—specifically to inhibit fungal ergosterol biosynthesis—azoles exhibit broad-spectrum activity against the non-target soil mycobiome. Azole residues can persist in soil for up to three years, depending on the specific compound and soil conditions^{11,12}.

In addition to reducing fungal diversity, azole fungicides selectively enrich for azole-resistant opportunistic yeasts, including *Candida* species¹³. *Candida tropicalis*, a globally distributed yeast and an emerging azole-resistant human pathogen, has been detected and inhabits in agricultural soils, particularly in environments under intensive fungicide treatment^{14,15}. The enrichment of drug-resistant *C. tropicalis* in farming systems represents a pivotal One Health concern, as this reservoir can drive cross-transmission among humans, animals, and crops¹⁵.

In Southeast Asia and South America, *C. tropicalis* is the most prevalent non-albicans *Candida* species causing candidemia, with pooled prevalence of 29.2% in Southeast Asia¹⁶, alongside all-cause mortality from invasive infections reaching 55–60%¹⁷. Beyond the clinical setting, the environmental origins of the azole resistance have become a growing concern, particularly in agricultural ecosystems where azole fungicides are widely used. In Taiwan, azole exposure in agriculture is increasingly implicated in resistance emergence through environmental selection, as fluconazole resistance in *C. tropicalis* was detected ranging from 11.1% to 50% of environmental isolates from orchards^{15,18}. Yet *Candida* species are also integral members of soil fungal communities, fulfilling diverse ecological roles within these ecosystems^{19,20}; for instance, soil yeast in this genus has been reported to inhibit the growth of the plant pathogen *Moniliophthora perniciosa* in organic farm soil²¹; *C. tropicalis* also exhibits inhibitory activity against phytopathogen *Fusarium culmorum* and *F. graminearum*²². Moreover, *C. tropicalis* outcompetes bacterial populations in soil amended with n-alkanes, likely due to soil acidification resulting from yeast metabolism²³. As the resident *C. tropicalis* found in Taiwanese agricultural soils¹⁵, a critical knowledge gap remains: whether these soil-resident *C. tropicalis* functionally integrate into the indigenous soil microbial network, and whether azole fungicide exposure disrupts these ecological roles while selecting for those resistant to clinical azole medicines.

Papaya (*Carica papaya* L.), wax apple (*Syzygium samarangense* Merr.& Perry), and grape (*Vitis* spp.) are recognized as high-value prioritized export fruits in Taiwan²⁴. To meet stringent international quality standards, these crops are managed under a standardized and intensive cultivation regime²⁵ that involves strategic fungicide applications to ensure compliance with international residue standards²⁶. Critically, continuous crop exposure across orchard types creates differential selection pressure on the soil microbiome, providing an ecologically relevant gradient for examining how orchard management, azole residue pressure, and the presence of *C. tropicalis* drive shifts in soil microbial community structure and composition. Accordingly, this study assessed microbial variation across these three

orchard types, determined whether azole residues correlated with shifts in community composition, and investigated the association between *C. tropicalis* presence and specific microbial taxa within these agricultural soils.

Results

Soil orchard microbiome

The 55 soil sampling sites were distributed in central and southern areas where most orchards in Taiwan are located in. This mapping sample site was designed to capture a comprehensive overview of soil microbiome environments supporting three important crop cultivations (papaya, grape, and wax apple) in Taiwan. Targeting the 0–10 cm topsoil layer, which represents the primary interface for agricultural management, effectively captured the microbiome and chemical profile within orchard soils.

Candida tropicalis isolation and clade 4 detection

We also evaluated the presence of *C. tropicalis* and found it in 35 soil samples, with resistance lineage clade 4 accounting for up to 80% (n = 28) of *Candida*-positive sites. Although grape orchard displayed the highest prevalence at 78.9%, the clade 4 genotype was equally distributed (n = 12) in both grape and papaya soils. The ubiquitous presence of *Candida* clade 4 indicates that agricultural environments serve as a reservoir for this species.

Chemical azole residue

Among 55 collected soil samples, azole residues were detected in 52 of 55 orchard soil samples (94.5%). This contamination created distinct selective environments across the three orchard types. Grape soils contained a diverse mixture of fungicidal compounds, averaging 6 distinct azoles per sample. Wax apple soils exhibited the highest cumulative chemical loads, dominated by tebuconazole and difenoconazole. Meanwhile, papaya soils maintained an average total azole concentration below 0.01 mg/kg due to cultivation habits and expansion of predominant azole-resistant *C. tropicalis* genotype from 2012 to 2018; evidence from orchard environments in Taiwan¹⁸

Molecular work and downstream analyses

High-quality genomic DNA and PCR amplicons were successfully generated for all 55 orchard soil samples. After quality filtering and denoising using NanoCLUST, the final dataset contained 4,466,137 bacterial 16S rRNA sequences with 7,172 clusters and 8,202,480 fungal ITS sequences with 4,664 clusters. The rarefaction curves reached a clear saturation plateau for both markers in every sample (Supplementary Fig. 1.). This depth was further validated by high coverage, which reached 100% for both bacterial and fungal datasets.

Diversity metric of microbial community structure

We evaluated the impact of four previously defined variables on species diversity of bacteria and fungi (Shannon index) across 55 soil samples. None of the variables significantly influenced soil microbial species diversity (all $P > 0.05$). The diversity of fungal species remained stable across all comparisons, while bacterial species diversity showed a marginal trend by orchard type ($P = 0.054$). Pairwise comparisons indicated that this trend was driven primarily by differences between grape and wax apple orchards, with grape orchards harboring a more diverse bacterial species pool than wax apple orchards (Fig. 1).

Microbial community structure and composition

To assess whether soil microbial community composition differed across four categorical variables, we calculated Bray-Curtis dissimilarity matrices for bacterial (16S rRNA) and fungal (ITS) communities across 55 orchard soil samples. Among the four tested variables, NMDS ordination showed that orchard management type was a significant determinant of both bacterial and fungal community composition (PERMANOVA, $P = 0.001$; Fig. 2a). This factor explained 10–11% of the total compositional variance for both kingdoms. Additionally, significant differences in multivariate dispersion between orchard types (betadisper: bacteria $P = 0.039$; fungi $P = 0.004$; Supplementary Table 1) indicate that these communities differ not only in centroid position but also in within-group heterogeneity. In contrast, neither *C. tropicalis* presence, resistance clade 4 membership, nor azole residue detection significantly affected bacterial or fungal community structure.

Azole residue correlation with microbial diversity

Aggregate azole burden showed no significant correlation with bacterial diversity (16S: Spearman $\rho = -0.095$, $P = 0.491$; Fig. 3a) but was significantly negatively correlated with fungal diversity (ITS: Spearman $\rho = -0.295$, $P = 0.029$; Fig. 3B). This finding indicates that cumulative azole exposure primarily suppresses fungal community diversity while leaving bacterial communities unaffected.

Individual compound analysis focused on the three most frequently detected compounds ($\geq 50\%$ detected across samples): difenoconazole (72.7% of samples), tebuconazole (69.1% of samples), and hexaconazole (56.4% of samples). None showed significant associations with bacterial or fungal diversity after FDR correction (Fig. 4c–e), although difenoconazole approached significance for bacterial diversity (FDR-adjusted $P = 0.083$). These results suggest that the cumulative burden of multiple azole compounds, rather than any single compound, drives the observed reduction in fungal diversity.

Microbial functional annotation among the three orchard soils

FAPROTAX and FUNGuild were used to predict the potential ecological roles of bacterial and fungal communities across different habitats in three orchard soils. Based on the taxonomic annotation of 16S

rRNA and ITS sequences, 14 bacterial functional groups and 19 fungal ecological guilds were inferred (Fig. 4a–b). Aerobic chemoheterotrophy was the dominant predicted role across all sites for the bacterial communities, reflecting a shared baseline for organic matter decomposition in these agricultural management systems. Moreover, LEfSe analysis identified orchard-specific enrichment patterns (LDA > 2.0, $P < 0.05$; Supplementary Table 2), though with notable variation (Fig. 4a). The grape orchard displayed enrichment of anaerobic and respiratory processes, including fermentation and iron respiration. Meanwhile, papaya orchards showed greater abundance of methanol-oxidizing bacteria, and wax apple orchards showed no significant enrichment for these bacterial functions.

For fungal communities, nine fungal guilds were significantly represented across samples (Supplementary Table 2). Among all orchards, saprotrophic activity constituted the functional baseline. This activity represents the primary energy acquisition for the majority of the fungal community. Despite the shared baseline, LefSe analysis revealed orchard-specific enrichment patterns, though patterns varied among individual samples (Fig. 5b). Wax apple orchards tended to enrich multi-trophic assemblages of animal pathogen-endophyte-plant pathogen guilds. In contrast, grape and papaya soils showed trends toward specialized saprotrophic and endophytic roles. This result suggests that wax apple lacks a bacterial predicted functional profile but shows variation in fungal communities.

Co-occurrences between *C. tropicalis* clade 4 and indigenous soil microorganisms and correlation with azole fungicide residues.

Candida tropicalis clade 4 was detected in 28 of 55 orchard soils, and to assess ecological integration, we identified the 8 microbial taxa that strongly correlated with *C. tropicalis* clade 4 across 28 positive samples. These partners comprised 6 fungi and 2 bacteria spanning Ascomycota, Basidiomycota, Actinomycetota, and Acidobacteriota (Fig. 5a). All of these 8 partners have significant positive associations ($r = 0.44$ – 0.85 and $P < 0.05$), whereas negative associations were weak to moderate ($r = -0.33$) and none were statistically significant.

To determine whether azole residues could account for these co-occurrence patterns, we examined correlations between the eight co-occurring taxa and the 13 azole compounds detected in *C. tropicalis* clade 4–positive samples. None were statistically significant (Fig. 5b). Most correlations were negative, with the strongest observed between *Occallatibacter savanae* and hexaconazole ($\rho = -0.906$). At the same time, *T. yunnanense* and *T. crassum* showed positive associations with multiple azoles.

Discussion

This study examined the relationships among agricultural management, azole fungicide residues, and the clinically relevant fungus *C. tropicalis* clade 4 in orchard soils of central and southern Taiwan. Our results show that orchard type drove taxonomic turnover without reducing species richness, cumulative azole residues selectively suppressed fungal but not bacterial diversity, and *C. tropicalis* clade 4 integrated into local soil communities independently of residual azole levels. Together, these findings

reveal that agricultural management, chemical inputs, and pathogen presence each leave distinct signatures on the soil microbiome.

Soil microbial diversity serves as a critical bioindicator of agricultural ecosystem health, as high diversity sustains essential services such as nutrient cycling and pathogen suppression even under anthropogenic pressure²⁷. Our results revealed a decoupling between microbial diversity and community composition. Both bacterial and fungal richness remained stable across orchard types, yet community composition underwent significant turnover. This pattern indicates that crop identity does not reduce soil carrying capacity but instead replaces one set of species with another. Stable richness coupled with high turnover suggests that host identity determines which taxa inhabit the rhizosphere without altering overall niche saturation²⁸. Orchard type explained 10–11% of the total variance in species structure, a deterministic contribution that aligns with the lower range of values (12–20%) reported for management system-driven community shifts in long-term studies⁷. These findings suggest that while the host identity acts as a clear deterministic driver, the majority of community assembly in this open-field ecosystem remains governed by stochastic processes²⁹.

In contrast to orchard management, neither the presence of azole residual in soil nor the enrichment of *C. tropicalis* clade 4 was associated with changes in diversity and community composition. The negligible effect of azole residues on overall richness is likely a compensatory dynamic in which sensitive taxa are replaced by tolerant opportunistic guilds, preserving species counts³⁰. Similarly, the insignificant influence of *C. tropicalis* clade 4 suggests that this organism functions as an integrated community member occupying minor niches within a healthy soil matrix rather than acting as a disruptive agent²³.

Residual azole fungicides in orchard soils elicited differential responses between fungal and bacterial communities. Fungal diversity declined in response to cumulative total azole concentration rather than to any single fungicide (Fig. 3c–d). This pattern is mechanistically aligned with the mode of action of azole fungicides, which inhibit the cytochrome P450 enzyme (CYP51) essential for fungal ergosterol biosynthesis³¹. A recent global meta-analysis similarly reported that fungicide application significantly reduces fungal abundance and diversity, with fungal populations declining by 14.5–33.8% depending on the application concentration³².

In contrast, bacterial communities exhibited resilience in the overall diversity ($P = 0.49$). This differential response reflects the fundamental biochemical distinction that bacteria lack ergosterol and instead utilize hopanoids for membrane integrity³³. The observed bacterial resilience to azole exposure is consistent with findings that bacterial communities undergo minimal changes under triazole fungicide treatments³⁴ and with vineyard soil studies showing significant fungal loss without corresponding bacterial decline³⁵.

By using FAPROTAX and FUNGuild, we predict functional profiles of bacterial and fungal communities across orchard soils. Functional composition differed significantly between agricultural systems for both

bacteria ($P = 0.016$) and fungi ($P = 0.008$), corroborating the taxonomic turnover observed above. This concordance confirms that orchard management acts as a strong trend shaping both community composition and metabolic potential³⁶. Aerobic chemoheterotrophy was the major predicted bacterial function across all orchard types, reflecting a shared baseline of organic matter decomposition. The most abundant taxa supported this role, including *Chujaibacter soli* (mean 6.18%, present in 64% of samples), an aerobic bacterium associated with plant growth promotion and nutrient cycling under nutrient-rich conditions^{37,38}, *Gaiella occulta* (mean 5.3%, present in 98% of samples), a specialist in soil organic matter decomposition³⁹, and *Pseudolabrys taiwanensis* (mean 2.86%, present in 85% of samples). For fungi, saprotrophic activity constituted the functional baseline. The most abundant fungal taxa — *Mortierella globalpina* (mean 8.76%, 55% of samples), *Pseudogeomyces hebridensis* (mean 7.5%, 65% of samples), and *Trichoderma yunnanense* (mean 4.41%, 56% of samples) — were all classified as saprotrophs or decomposers. Notably, three *Mortierella* species ranked among the top ten fungal taxa (combined abundance > 12%), consistent with the genus being among the most abundant and diverse saprotrophic fungi across a wide soil pH range⁴⁰. *Trichoderma yunnanense* is known for prolific production of cellulases and hemicelluloses required to decompose woody plant biomass⁴¹.

Beyond this shared baseline, functional divergence among orchards was driven by the metabolic requirements of crop-specific dominant taxa. In grape orchards, bacterial functions were enriched for fermentation and iron respiration (Fig. 4c), consistent with the abundance of *Poalibacter uvarum* (1.62% mean abundance), a taxon specialized in degrading complex polymers⁴². In papaya orchards, methanol oxidation was the distinguishing bacterial function (LDA = 2.43), supported by the high abundance of *Methylobacterium palustris* (3.62% mean abundance), which utilizes methanol released from pectin demethylation in growing roots as its sole carbon source⁴³. For fungal communities, endophyte guilds complemented the saprotrophic baseline across orchards. For example, *M. rishikeshia* (5.91% abundance) is widely recognized as a plant growth-promoting fungus that colonizes the root endosphere to enhance nutrient uptake⁴⁴. Together, these results demonstrate that while bacterial chemoheterotrophy and fungal saprotrophy provide a shared ecological foundation, agricultural management and crop-specific inputs drive differentiation at the level of specialized guilds and functions.

The positive association correlations between *C. tropicalis* clade 4 partners align with the global observation that soil microbial communities are dominated by positive associations (54.3–99.2% across 20 regions), which supports microbial cooperation and ecosystem functioning⁴⁵. This pattern aligns with evidence that *Candida* species frequently participate in synergistic multispecies biofilms and microbial consortia⁴⁶. Although no prior evidence documents direct interaction between *Candida* and the specific taxa identified here, the predominantly positive co-occurrence pattern observed is consistent with shared environmental or functional niche preferences, as positively associated soil microbes tend to cluster around similar habitat conditions through environmental filtering^{47,48}. Notably, none of the correlations between the eight co-occurring taxa and 13 detected azole compounds were statistically significant, indicating that partnership composition is independent of azole residue levels. This independence is

consistent with studies showing that while chemical stress reshapes co-occurrence networks and selects for resistance in particular lineages, many microbial associations persist regardless of chemical gradients^{49,50}.

In conclusion, this study demonstrates that agricultural management is one of the dominant deterministic forces structuring soil microbial communities in tropical orchards, overriding the effects of azole fungicide residues and the presence of *C. tropicalis*. Orchard type explained 10–11% of compositional variance and drove taxonomic turnover while maintaining species richness through replacement rather than loss. Cumulative azole burden exerted kingdom-specific suppression of fungal diversity, whereas bacterial communities remained resilient, consistent with their distinct biochemical insensitivity to ergosterol-targeting fungicides. *Candida tropicalis* clade 4 formed positive associations with cross-kingdom microbial partners, suggesting ecological integration into soil microbial communities independent of azole residue levels. These findings carry One Health implications, i.e., agricultural soils simultaneously harbor clinically relevant azole-resistant *C. tropicalis* lineages while sustaining functionally diverse microbial communities shaped primarily by crop management rather than chemical contamination.

Several limitations should be noted. The modest sample size of *C. tropicalis* clade 4–positive soils ($n = 28$) constrained statistical power to detect individual azole–taxon associations, and functional annotations derived from FAPROTAX and FUN-Guild represent taxonomic inference rather than measured metabolic activity. Future research should experimentally test whether the eight co-occurring taxa provide metabolic or niche facilitation to *C. tropicalis* clade 4, and quantify how cumulative azole exposure affects fungal diversity under repeated, long-term agricultural management regimes.

Materials and methods

Sample collection

Five samples from the center, east, west, south, and north of each orchard were collected. A total of 55 soil samples were collected from three different orchard types, consisting of papaya ($n = 18$), grape ($n = 19$), and wax apple ($n = 18$) in 2023 across central and southern Taiwan. The soil was collected at 0–10 cm depth beneath the soil surface, pooled into a sterile plastic bag, mixed gently, and transferred into a 50 ml sterile Falcon tube. The samples were subsequently stored at -20°C for further analysis. Sample collection was conducted as part of the orchard survey titled “Species distribution and drug susceptibility of yeast pathogens in farmers and fields”, which was approved by the Human Experiment and Ethics Committee of the National Health Research Institutes (NHRI), Taiwan (approval number EC1070117). Informed consent was obtained from all participating farmers prior to sampling.

Candida tropicalis isolation and clade 4 detection

The isolation of *C. tropicalis* was performed according to the previously established protocol¹⁵. Briefly, approximately 1 g of soil was transferred into a 50 ml tube containing 10 ml of Yeast Extract Peptone Dextrose (YPD) medium and incubated at 25⁰C for three days. The cell suspension will be streaked onto CHROMagar Candida (BBL, Becton Dickinson, Cockeysville, MD, USA) containing chloramphenicol at room temperature for 2–3 days. *Candida tropicalis* isolates were identified by rDNA sequencing of the internal transcribed spacer (ITS) and/or D1/D2 regions¹⁵, while clade 4 genotype were detected using MLST primers^{51,52}. The primers used in this study are listed in Supplementary Table 3. One isolate per sample type per orchard was further analyzed.

Chemical azole residue measurement

We followed the method established previously⁵³. Five soil samples from each orchard were combined and air-dried naturally in a shaded area for approximately 5 to 7 days, then passed through a 2-mm standard sieve. The sieved samples were then stored in high-density polyethylene (HDPE) bottles at room temperature. A 10-g portion of the soil was extracted with 120 mL of an acetone and dichloromethane mixture (1:1, v/v) using an automated Soxhlet extraction system (Soxtec Extraction System Model 2050, Foss, Sweden). The extract was concentrated to near dryness and reconstituted with 2 mL of an acetone and n-hexane mixture (1:1, v/v). A 1-mL aliquot of this solution was filtered through a 0.2-µm PTFE filter and analyzed by gas chromatography-tandem mass spectrometry (GC-MS/MS). The remaining 1-mL aliquot was evaporated to near dryness under a stream of nitrogen, reconstituted with 1 mL of methanol, filtered through a 0.2-µm PTFE filter, and analyzed by ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The 15 pesticide compounds of bitertanol, bromuconazole, cyproconazole, difenoconazole, paclobutrazol, epoxiconazole, fenbuconazole, imibenconazole, myclobutanil, penconazole, propiconazole, tebuconazole, tetraconazole, triadimefon, and triadimenol were analyzed by GC-MS/MS, and 5 pesticide compounds of fluconazole, hexaconazole, amisulbrom, metconazole, and prochloraz were analyzed by UPLC-MS/MS, respectively.

Molecular work and downstream analyses

Soil DNA extraction

Genomic DNA from the soil sample was extracted using the Super Soil DNA Extraction Protocol⁵⁴. The quantity and quality of eluted DNA were measured using the Qubit dsDNA HS Assay kit and the Qubit Flex Fluorometer 4.0 (Thermo Fisher Scientific) according to the manufacturer's instructions.

16S rRNA and Fungal ITS amplification

Amplification was performed using a two-step barcoding approach as described by⁵⁵. The first-step PCR was performed in 25 µl reaction mixture containing of 1 µl of template DNA, 0.5 µl of each forward and reverse primer 12.5 µl of Kappa HIFI HotStart PCR ready mix, and 10.5 µl of nuclease-free water (ddH₂O). Bacterial 16S rRNA gene were amplified using universal primers 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1429R (5'TACGGYTACCTTGTTACGACTT-3'). Fungal Internal Transcribe Spacer (ITS) were targeted

using the primer ITS1-F_KYO2 (5'-TAGAGGAAGTAAAAGTCGTAA-3') and ITS4ngsUni (5'-CCTSCSCTTANTDATATGC-3'). The first PCR cycling conditions consisted of an initial denaturation at 95°C for 3 minutes, followed by 25 cycles of denaturation at 98°C for 20 seconds, an annealing step at 66°C (16S rRNA) and 60°C (ITS) for 15 seconds, and an extension at 72°C for 30 seconds. Meanwhile, the second-step PCR reaction mixture was 15 µL and consisted of 0.75 µL of the first-step DNA product as template, 0.6 µL pre-mixed barcoded primers, 7.5 µL of 2X Zeju Fast PCR master mix, and 6.15 µL of ddH₂O. The thermal cycling conditions for the second PCR started with an initial denaturation at 95°C for 3 minutes, followed by 15 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 15 seconds, and extension at 72°C for 20 seconds. PCR products were subsequently verified via 1% agarose gel electrophoresis. The barcoded amplification products were purified using Ampure XP beads with 0.4X (16S) and 0.6X (ITS) product-to-bead ratios, both procedures following the manufacturer's protocol.

Amplicon sequencing, quality control, and microbial profiling

Bacterial (16S rRNA) and fungal (ITS) amplicon libraries (2nd PCR product) were pooled and sequenced using Oxford Nanopore Technology (ONT). We loaded a 75 ul DNA library onto an R10.4.1 flow cell and sequenced on a MinION device using MinKNOW software v23.07.5. Raw POD5 files were basecalled using Dorado v0.5.2 (github.com/nanoporetech/dorado) with the Super Accuracy (SUP) model to generate high- quality FASTQ files. Following basecalling, reads underwent quality filtering using NanoACT pipeline (github.com/Raingel/nanoACT), retaining fragments between 500–2000 bp for (16S) and 800–1200 bp for (ITS) with a minimum Q-score of 10. The filtered sequences underwent a specialized header standardization process (`fastq_header_clean`) to remove redundant metadata and ensure data integrity.

To generate polished consensus sequences from noisy long-reads, we performed clustering analysis using NanoCLUST (github.com/genomicsITER/NanoCLUST)⁵⁶. Reads were clustered using UMAP and HDBSCAN for density-based clustering, and clusters containing fewer than 5 reads were excluded to minimize noise and avoid losing rare but important taxa. Representative sequences from each cluster were subsequently polished using Medaka (model: DNA_R10.4.1_E8.2_400bps_SUP). Final consensus amplicons were taxonomically assigned using BLAST against curated NCBI 16S RefSeq and NCBI ITS RefSeq databases. Classification for both markers was conducted using a maximum E- value of 1×10^{-5} and minimum identity threshold of 97% for species identification.

Alpha diversity

To evaluate the factors influencing microbial diversity and community structure, four primary variables were defined in this study as follows: 1. Orchard type; 2. *Candida tropicalis* presence/absence, determined by culture-dependent methods; 3. *Candida tropicalis* clade 4 presence/absence, identified by molecular typing of those culture-dependent isolates, and 4. Azole residues quantified by GC-

MS/MS/UPLC-MS/MS. Microbial (16S and ITS) alpha diversity was assessed using Shannon diversity at the species level in samples for four categorical variables (orchard type, *C. tropicalis* presence/absence, azole residue detection, and *C. tropicalis* clade 4 presence/absence). To assess differences among categorical groups, the Kruskal-Wallis test was applied for comparisons among more than two groups (e.g., orchard types), with post-hoc pairwise comparisons using Dunn's test and multiple testing correction (Benjamini-Hochberg; FDR at $\alpha = 0.05$). Meanwhile, the Wilcoxon rank-sum test was employed for two-group comparisons (e.g., *Candida* and azole presence).

Beta diversity

Beta diversity was carried out at the species level with Bray-Curtis dissimilarity index computed using the `vegdist()` function in `vegan` R package. Non-Metric Multidimensional Scaling (NMDS) ordination was used to visualize community compositional differences. Statistical differences in community composition among groups were tested using Permutational Multivariate Analysis of Variance (PERMANOVA) implemented in the `adonis2()` function in the R package with 999 permutations⁵⁷. Additionally, we used the `betadisper()` function, followed by a permutation test, to test homogeneity of group dispersion.

Azole fungicide microbiome correlation

To assess the relationship between azole fungicide residues and the soil microbiome, we first examined the aggregate effect of total azole concentration, followed by examining individual compound contributions across 55 orchard soil samples, of which 52 (94.5%) contained detectable azole residues. Spearman's rank correlation was used to examine associations between total azole concentration and Shannon diversity index for both bacterial and fungal communities. Among the 20 pesticide compounds screened, 13 azole compounds were detected in at least one sample, and we continued with individual compound analysis. The 13 detected compounds varied widely in detection frequency (1.8–72.8%), making a uniform statistical approach inappropriate. We therefore screened for individual azoles with > 50% detection frequency for further analysis⁵⁸. False Discovery Rate (FDR) was corrected using the Benjamini-Hochberg method to account for multiple comparisons, and statistical significance was declared at $\text{FDR} < 0.05$.

Bacterial function and Fungal Guild annotation

To understand the potential ecological roles of soil microorganisms, bacterial predicted function and fungal guild were analyzed using FAPROTAX^{59,60} and FUNGuild⁶¹, respectively. Both tools provide functional prediction based on taxonomic assignment with > 1% filter abundance was applied to the taxonomic data prior to functional mapping preventing rare taxa loss. The difference in overall predicted functional guild composition among variable groups was tested using PERMANOVA. To identify bacterial function and guild biomarkers that distinguish orchard types, we first used Linear Discriminant Analysis of Effect Sizes (LEfSe) with the Kruskal-Wallis rank-sum test across all groups ($\alpha = 0.05$) to identify bacterial functions and fungal guilds with significantly different abundances. Secondly, biologically

meaningful biomarkers are defined as those with a Linear Discriminant Score (LDA) score of 2.0 ($\log_{10}$ scale) or higher.

Microbial co-occurrence network and azole concentration correlation

A microbial co-occurrence network was constructed using the center log-ratio (CLR) transformation to mitigate compositional artifacts followed by Pearson correlation on the transformed data⁶². Pearson correlation coefficients were calculated between CLR-transformed *C. tropicalis* presence and all bacterial and fungal clusters across 28 orchard soil samples. Meanwhile, Spearman's rank correlation coefficients (ρ) were calculated between the relative abundance of *C. tropicalis* partners and the concentrations of 15 detected azole compounds. Moreover, multiple testing correction was performed using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) at $\alpha = 0.05$ across all partner azoles. Network visualization was performed using igraph package in R and all statistical analyses were conducted in R version 4.5.1.

Declarations

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Ethics declarations

This study was conducted as part of the orchard survey, "Species distribution and drug susceptibility of yeast pathogens in farmers and fields," which received ethical approval from the Human Experiment and Ethics Committee of the National Health Research Institutes (NHRI), Taiwan (EC1070117). All procedures complied with the relevant guidelines and regulations. Written informed consent was obtained from all participating farmers before sampling.

Competing interest

The authors declare no competing interests.

Author Contribution

S.Y.R. contributed to conceptualization, methodology, investigation, formal analysis, manuscript drafting, manuscript visualization, and editing H.J.L. contributed to conceptualization, methodology, and resource K.Y.T. contributed to project administration and methodology P.Y.J. contributed to the

investigation R.N.L. contributed to supervision and manuscript review Y.T.H. contributed to conceptualization, methodology, investigation, manuscript review, data curation, resource, supervision

Data Availability

The raw sequencing data generated in this study (16S and ITS) have been deposited in the NCBI Sequence Read Archive (SRA) database under BioProject PRJNA1432446 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1432446>) and will be made publicly available upon publication. The following confidential reviewer link is provided for the editor and peer reviewers to access the dataset during review process (<https://dataview.ncbi.nlm.nih.gov/object/PRJNA1432446?reviewer=v4cl7saob3nc39jr2qfrifjs53>).

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Figures

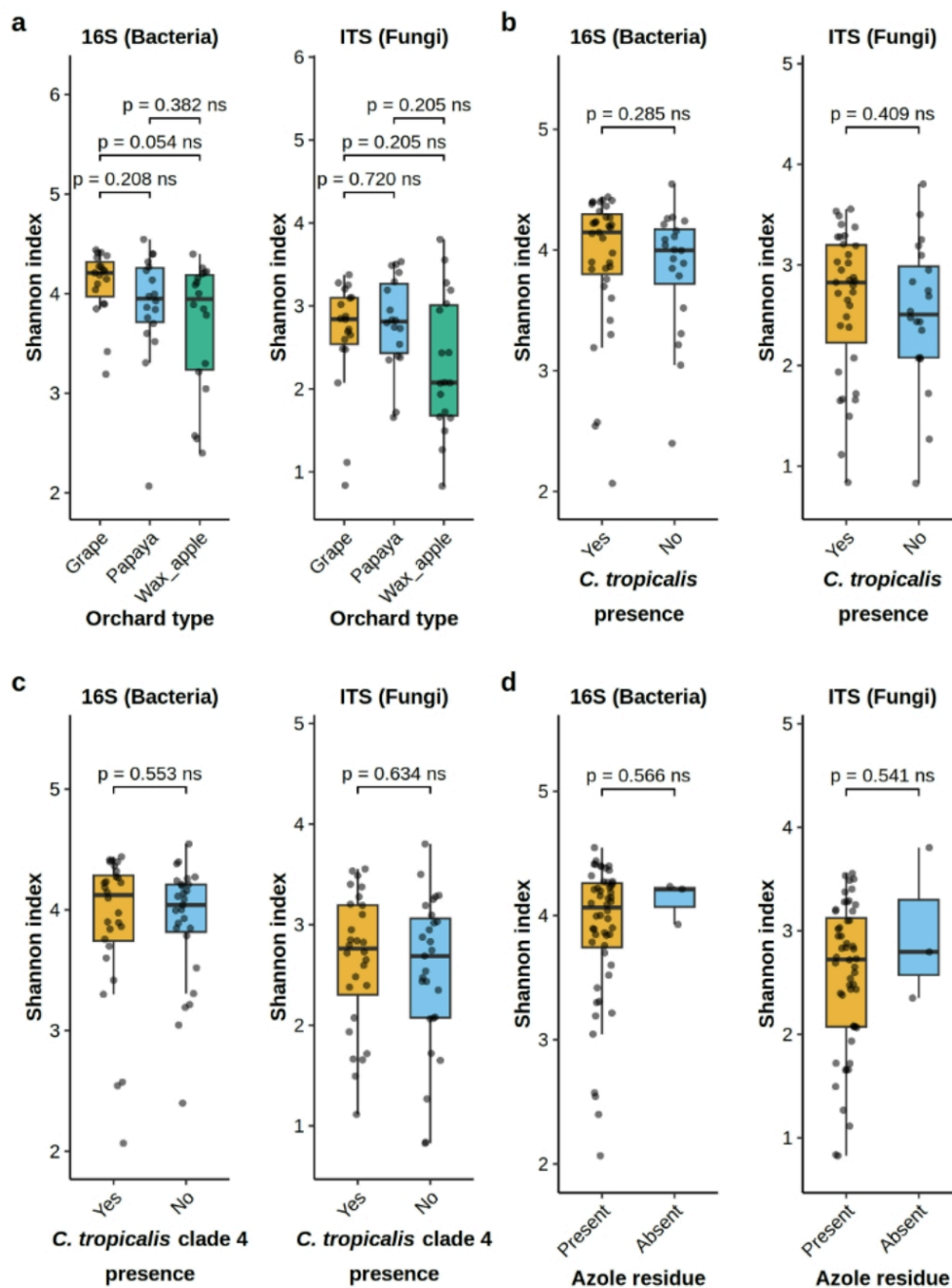


Figure 1

Comparative alpha diversity of soil microbial communities. Shannon diversity indices for bacterial (16S) and fungal (ITS) communities under four conditions: (a) orchard types, (b) *C. tropicalis* presence/absence, (c) *C. tropicalis* clade 4 presence/absence, and (d) azole residue detection status. Both bacterial and fungal communities showed no statistically significant differences across any factor.

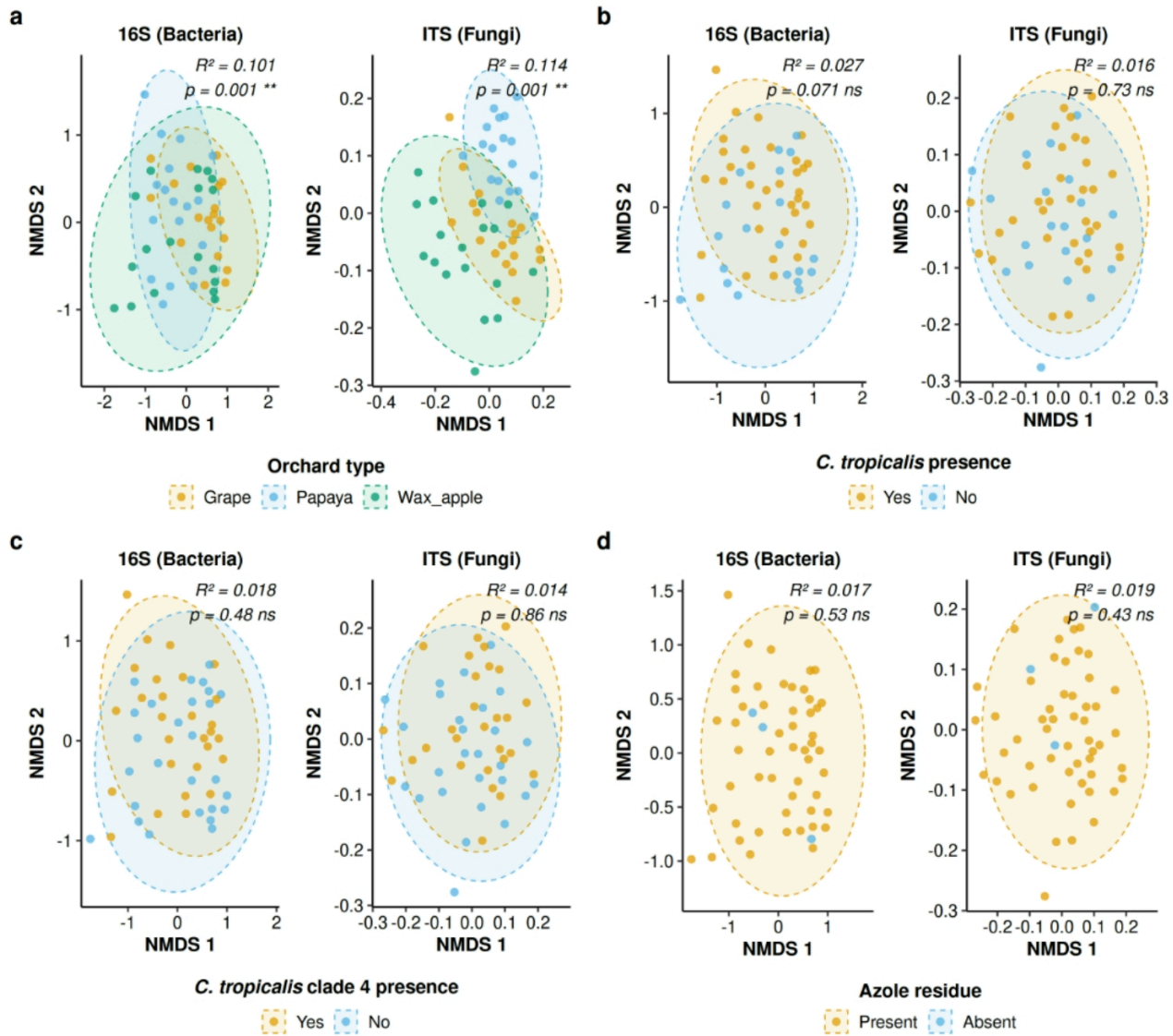


Figure 2

Non-metric multidimensional scaling ordination of soil microbial beta diversity. Plots are based on Bray-Curtis dissimilarities for bacterial (16S) and fungal (ITS) communities under four conditions: (a) orchard types, (b) *C. tropicalis* presence/absence, (c) *C. tropicalis* clade 4 presence/absence, and (d) azole residue detection status. Only the orchard type significantly influenced community composition for both bacterial and fungal communities.

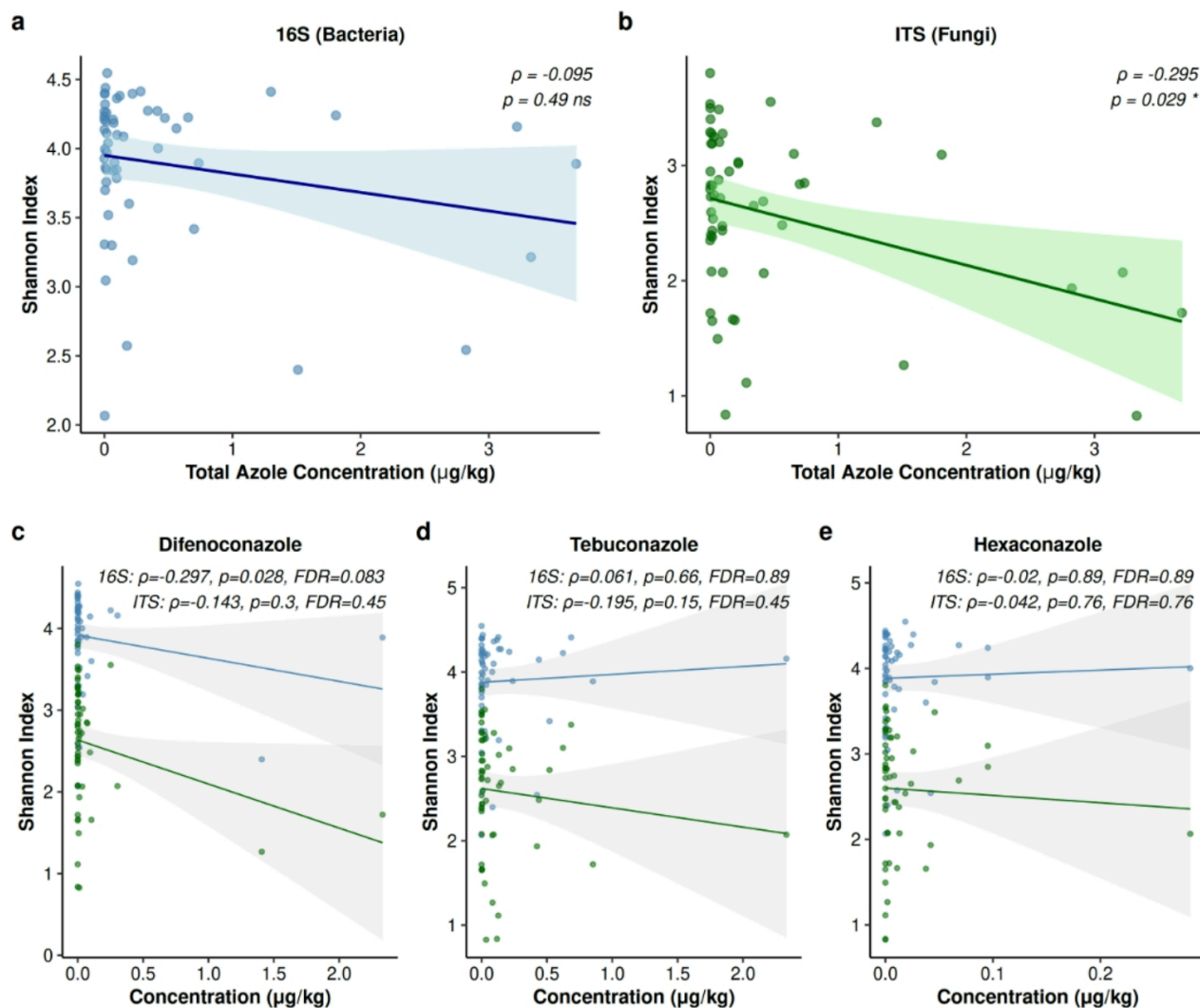


Figure 3

Azole concentration affects microbial diversity of orchard soils. Correlation between azole concentration and Shannon diversity indices for bacterial and fungal communities, (a) total azole concentration against bacterial diversity, (b) total azole concentration against fungal diversity, (c) difenconazole, (d) tebuconazole, and (E) hexaconazole. Only the total azole concentration showed a significant negative correlation with fungal diversity.

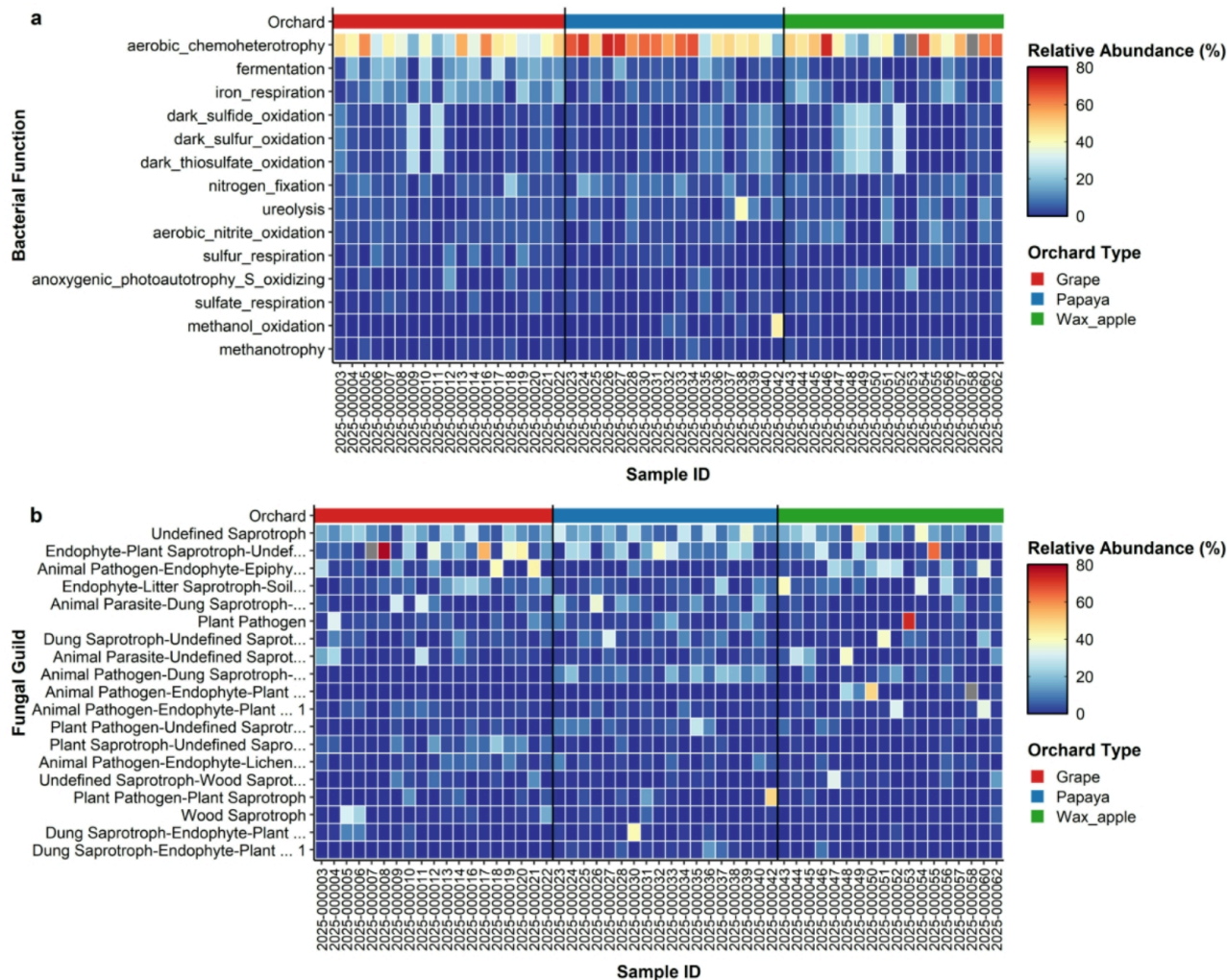


Figure 4

Divergence of microbial functional prediction analysis across 55 samples of orchard soils. (a) Heatmap profiling the relative functional abundance predictions of bacterial communities as predicted using FAPROTAX and (b) fungal ecological guild distribution based on FUNGuild.

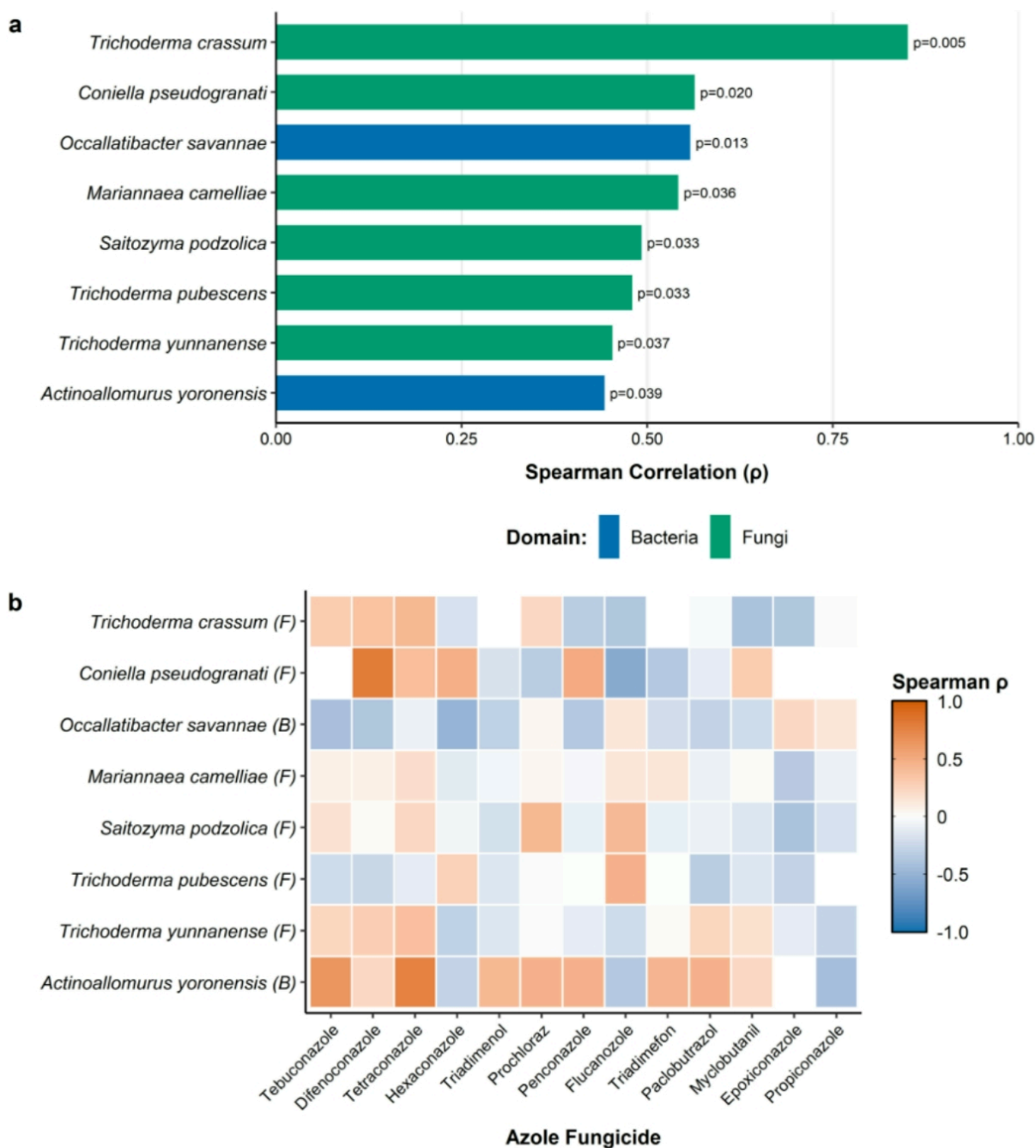


Figure 5

Microbial co-occurrence patterns of *C. tropicalis* and associations with azole fungicides. (a) Correlation coefficients of the top eight taxa co-occurring with *C. tropicalis* clade 4 identified by co-occurrence network analysis (6 fungi and 2 bacteria). (b) Correlation heatmap between the eight co-occurring taxa and 13 azole fungicide residues. Some associations showed strong correlations, but none were statistically significant (FDR < 0.05).

Supplementary Files

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- [OnlineNMDSCompositeSciReportsv3.png](#)
- [OnlineShannonCompositeAlphaSciReportsv2.png](#)