

# Rhizosphere biochemical and microbial community patterns across documented penicillin application histories: a blocked citrus-orchard study

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## Research Article

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# Abstract

## Background

Antibiotic use in citrus production may alter non-target rhizosphere processes, but replicated field evidence remains limited. We evaluated whether documented penicillin application histories were associated with coordinated soil biochemical and bacterial and fungal community patterns in a commercial Wogan mandarin orchard.

## Results

A blocked retrospective comparison included three spatial orchard blocks and four documented application-history groups (no recorded application or application recorded in one, two, or three years), with one plot per group in each block. Rhizosphere soil from three focal trees per plot was pooled, yielding 12 plot-level samples from 36 trees. Nine soil biochemical variables were integrated with near-full-length bacterial 16S rRNA gene and fungal ITS amplicon profiles. All nine reported soil variables differed among groups after block adjustment. Observed bacterial richness and Shannon diversity showed strong non-linear group-associated patterns, whereas fungal alpha-diversity evidence was less robust across parametric and exact Friedman sensitivity analyses. Block-restricted PERMANOVA detected bacterial and fungal Bray-Curtis separation, but multivariate dispersion also differed in both domains; centroid and dispersion effects were therefore interpreted jointly. Taxonomic and environmental associations were exploratory, and no FAPROTAX-predicted function remained significant after false-discovery-rate correction.

## Conclusions

Documented penicillin application history was associated with coordinated, non-linear rhizosphere biochemical and microbial patterns. Because the histories were retrospective and residue concentrations and several soil and disease covariates were unavailable, the findings support field associations and testable hypotheses rather than a direct cumulative penicillin effect.

## Background

The citrus rhizosphere is a biologically active interface in which root-derived resources, soil physicochemical conditions, and microbial interactions jointly influence nutrient acquisition and plant health. Global surveys show recurrent bacterial lineages across citrus rhizospheres, while local soil properties, host status, disease, and management shape community composition <sup>[1]</sup>. Huanglongbing (HLB) can alter the enrichment and functional potential of root-associated microbiota, making disease status an important covariate in citrus microbiome studies <sup>[2–4]</sup>.

Antibiotics used in plant agriculture can affect non-target microorganisms and select for altered community structure or resistance-related traits. Their environmental effects depend on the active ingredient, dose, delivery route, sorption, transformation, repeated exposure, and soil conditions <sup>[5–7]</sup>. Experimental soil exposure to penicillin has altered microbial genotype composition and activity, although responses vary among soils and exposure regimes <sup>[8]</sup>.

This issue is relevant to citrus because trunk-injected antibiotics may affect microbial associates beyond the intended pathogen niche. Penicillin injection has been investigated experimentally for HLB management, with reported changes in tree-associated bacterial communities and possible phytotoxic or other side effects <sup>[9, 10]</sup>. These studies do not establish that trunk-injected penicillin reaches rhizosphere soil at an ecologically active concentration; residue measurements in trunks, roots, rhizosphere soil, and bulk soil are required to link application history with belowground exposure.

Soil extracellular enzymes provide a complementary view of rhizosphere state. Acid phosphatase, urease, sucrase, and catalase assays index potential phosphorus acquisition, nitrogen mineralization, labile-carbon transformation, and oxidative processes, respectively. These activities integrate microbial biomass, enzyme production, substrate availability, stabilization, and soil chemistry and are not direct measures of in situ process rates. Enzyme potential is strongly related to organic matter, pH, and microbial nutrient demand <sup>[11]</sup>.

We compared soil biochemical profiles, bacterial and fungal alpha diversity, community configuration, dominant taxa, and cross-domain concordance among four documented penicillin application-history groups. The groups were compared within three spatial orchard blocks to account for broad position-related heterogeneity. We tested whether the history groups were associated with distinct biochemical and microbial profiles and whether the patterns were linear or non-linear across the ordered history categories.

## Methods

### Study site and blocked retrospective design

Rhizosphere samples were collected during a spring 2025 survey of a commercial Wogan mandarin orchard in Maosang Village, Xiangxian Town, Shanglin County, Nanning, Guangxi, China (23.2863° N, 108.6395° E; 160.1 m above sea level). Orchard records indicated that the sampled trees were 7–8 years old and planted at approximately 3 m within rows and 3–4 m between rows. The study was a cross-sectional, blocked retrospective comparison with three spatial orchard blocks. Each block contained four approximately 5 × 5 m plots, one representing each documented penicillin application-history group, and each plot was centered on three focal trees.

The four history categories were no recorded penicillin application (K-Y-0) and penicillin application recorded in one (K-Y-1), two (K-Y-2), or three years (K-Y-3). The K-Y labels are category codes and do not

denote repeated sampling time points. Within each block, one plot represented each category, giving three plots per category and 12 plots in total. Rhizosphere soil from the three focal trees within a plot was pooled as one plot-level composite sample. Thus, 36 trees yielded 12 plot-level samples; the plot, not the individual tree or soil subsample, was the sampling and analytical unit.

Penicillin histories were reconstructed from available orchard records for applications made before this study and were not assigned by the investigators. The groups therefore represent documented duration and frequency of use rather than randomized treatments or calculated cumulative exposure. The same cultivar, tree-age class, and routine orchard management were used across the study area; product manufacturer, concentration, per-tree injection volume, exact application dates, rootstock, complete co-management history, and HLB status were unavailable.

## Rhizosphere sampling and soil biochemical measurements

At each focal tree, fine roots were exposed approximately 50 cm from the trunk at a soil depth of about 20 cm. Approximately 1 kg of root-zone soil containing fine roots was collected. Loosely adhering soil was shaken off, and soil remaining attached to fine-root surfaces was brushed into sterile bags without phosphate-buffered-saline washing or centrifugation. Approximately equal contributions from the three focal trees in each plot were mixed to form one composite sample, and molecular and biochemical measurements used the same composite-sample identifiers.

All composite samples were kept at 4°C before laboratory processing, and the same handling was applied to all samples. Soil pH, moisture, texture, total organic carbon, total nitrogen, and penicillin residues were not measured.

Nine soil biochemical variables were measured: acid phosphatase activity (ACP), catalase activity (CAT), urease activity (URE), sucrase activity (SUC), hydrolysable nitrogen (HN), available phosphorus (AP), available potassium (AK), humic acid (HA), and cation-exchange capacity (CEC). For biochemical analysis, subsamples were air-dried at room temperature, ground, and sieved; enzyme-assay soil was passed through a 30-50-mesh sieve, and soil for nutrient and exchange-property assays was passed through a 2-mm sieve. Results were reported on an air-dried soil basis.

ACP, CAT, URE, and SUC were assayed with commercial soil-enzyme kits (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China; ACP, BC0140; CAT, BC0100; URE, BC0120; SUC, BC0240) following the manufacturer's spectrophotometric protocols. Briefly, ACP used phenyl disodium phosphate substrate and 4-aminoantipyrine colorimetry at 660 nm after 37°C incubation; CAT was calculated from H<sub>2</sub>O<sub>2</sub> decomposition at 240 nm; URE used urea substrate and indophenol-blue colorimetry at 630 nm after 37°C incubation; and SUC used sucrose substrate and 3,5-dinitrosalicylic-acid colorimetry at 540 nm after 37°C incubation. Enzyme activities were expressed as U g<sup>-1</sup> soil according to kit definitions.

HN was determined by alkaline hydrolysis-diffusion (LY/T 1228–2015); AP by 0.50 mol L<sup>-1</sup> NaHCO<sub>3</sub> extraction (pH 8.5) and molybdenum-antimony spectrophotometry at 880 nm (HJ 704–2014); AK by

neutral 1 mol L<sup>-1</sup> ammonium acetate extraction and flame photometry (NY/T 889–2004); HA by sodium pyrophosphate/NaOH extraction and potassium-dichromate oxidation with external heating (LY/T 1238–1999); and CEC by 1 mol L<sup>-1</sup> ammonium-acetate exchange at pH 7.0, ethanol washing, steam distillation of exchanged NH<sub>4</sub><sup>+</sup>, and acid titration (LY/T 1243–1999). HN, AP, and AK were expressed as mg kg<sup>-1</sup>; HA as percentage; and CEC as cmol(+) kg<sup>-1</sup>. HA values in the source workbook were decimal mass fractions and were multiplied by 100 for presentation.

## DNA amplification, PacBio sequencing, and sequence processing

Total soil DNA was extracted using an E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA; catalog no. D5625-01) according to the manufacturer's instructions. Amplicon preparation, library construction, and PacBio sequencing were performed by Shanghai LingEn Biotechnology Co., Ltd. (Shanghai, China). Near-full-length bacterial 16S rRNA genes were amplified with 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), and fungal ITS regions with ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')<sup>[12–14]</sup>. The archived provider protocol recorded triplicate 20-μL FastPfu reactions, initial denaturation at 95°C for 5 min, cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s, and final extension at 72°C for 10 min. Reaction composition and cycle number were not retained. Triplicate products were pooled before library construction.

SMRTbell libraries were prepared using kit version 3.0 and sequenced on a PacBio Sequel IIe platform. Circular consensus sequences were generated in SMRT Link version 11 with at least three passes and a minimum predicted accuracy of 0.99, then demultiplexed and primer-trimmed. UPARSE version 10 was used for chimera filtering and operational taxonomic unit (OTU) clustering at 98.65% identity<sup>[15, 16]</sup>. The threshold was applied to both domains as an operational criterion rather than a universal species boundary. Taxonomy was assigned with a UCLUST confidence threshold of 0.80 against SILVA SSU release 138.1 for bacterial reads and UNITE release 8.2 for fungal reads<sup>[17, 18]</sup>.

## Statistical analyses

All analyses used plot-level composite samples (n = 12; three plots per history category). When descriptive summaries are reported as mean ± SD, the SD was calculated among the three independent plot-level composites in each category and does not represent variation among the three pooled trees or technical replicates. Soil variables and alpha-diversity indices were analyzed with linear models containing application-history group and spatial block as explanatory terms. Partial η<sup>2</sup> quantified the group effect. Exact Friedman tests enumerating all within-block group permutations provided distribution-free sensitivity analyses, and orthogonal linear, quadratic, and cubic contrasts evaluated pattern shape across the ordered history categories. Tukey-adjusted group contrasts were calculated from the block-adjusted model, and Benjamini-Hochberg false-discovery-rate adjustment was applied

within each test family <sup>[19]</sup>. Analyses used Python 3.13.5, NumPy 2.3.5, SciPy 1.17.0, and statsmodels 0.14.6 with seed 20260714.

For beta diversity, bacterial and fungal OTU tables were rarefied to 22,517 and 28,058 reads per sample, respectively, and Bray-Curtis dissimilarities were ordinated by principal coordinates analysis. Group effects were tested by PERMANOVA with block entered before group and permutations restricted within blocks <sup>[20]</sup>. Multivariate dispersion was evaluated by PERMDISP and interpreted jointly with PERMANOVA <sup>[21, 22]</sup>. Pairwise PERMANOVA was omitted because a two-group comparison across three blocks permits only eight within-block label swaps. Normalization sensitivity was assessed by comparing Bray-Curtis matrices from rarefied counts and full counts converted to within-sample proportions using Mantel correlation as a descriptive diagnostic.

Taxonomic composition was summarized at phylum and genus levels as relative read proportions. Genus-level centered-log-ratio (CLR) one-way ANOVA and Welch contrasts against the no-record category (K-Y-0) were used only as hypothesis-generating screens because  $n = 3$  plots per category; these analyses did not include block and are not ANCOM-BC or ANCOM-BC2 results <sup>[23]</sup>. Environmental fitting, genus-environment correlations, and cross-domain Mantel analysis were likewise treated as exploratory because nine soil variables were evaluated in only 12 samples and both soil and microbial variables covaried strongly with application-history group. FAPROTAX outputs were interpreted as taxonomic functional predictions rather than direct functional measurements <sup>[24]</sup>.

## Results

### Soil biochemical properties differed among application-history groups

All nine reported soil biochemical variables differed among history categories in block-adjusted models ( $F_{3,6} = 11.92\text{--}12,946.52$ ; BH  $q \leq 0.0061$ ; partial  $\eta^2 = 0.856\text{--}0.9998$ ; Fig. 1; Additional file 1: Supplementary Table S1). Exact Friedman sensitivity tests also supported category differences for all variables after false-discovery-rate correction ( $q \leq 0.033$ ). The clearest biochemical signal was a non-linear group profile: the two-year-history group had the lowest means for many enzyme and nutrient measurements, whereas the three-year-history group had higher means. The sample-level standardized soil profile is shown in Additional file 2: Supplementary Figure S1.

Representative observed values (mean  $\pm$  SD;  $n = 3$  plot-level composites per group) were as follows. ACP was  $32,304.9 \pm 1,451.7$ ,  $13,987.4 \pm 368.7$ , and  $43,835.8 \pm 3,317.7$  U g<sup>-1</sup> in the no-record, two-year-history, and three-year-history groups, respectively. URE was  $299.4 \pm 9.4$ ,  $180.2 \pm 4.2$ ,  $109.0 \pm 3.3$ , and  $211.3 \pm 2.4$  U g<sup>-1</sup> in the no-record, one-year-history, two-year-history, and three-year-history groups, respectively. AP was  $184.4 \pm 3.1$ ,  $22.3 \pm 0.3$ , and  $82.8 \pm 0.3$  mg kg<sup>-1</sup> in the no-record, two-year-history, and three-year-history groups, respectively. The highest group means for HN and HA occurred in the three-year-history

group, the highest AK mean in the one-year-history group, and the lowest CEC mean in the two-year-history group. Complete summaries and individual plot values are provided in Fig. 1 and Additional file 1: Supplementary Table S1.

## Sequencing depth and alpha-diversity patterns

The bacterial dataset contained 385,182 clean reads across 12 samples, with 30,446 – 34,200 reads per sample and a mean read length of 1,448.0 bp. The fungal dataset contained 467,163 clean reads, with 31,924 – 44,119 reads per sample and a mean read length of 631.7 bp (Additional file 1: Supplementary Table S2; Additional file 3: Supplementary Figure S2). All samples exceeded the domain-specific rarefaction depths.

Observed bacterial richness (mean  $\pm$  SD) was  $4,779.3 \pm 177.7$ ,  $3,290.0 \pm 31.4$ ,  $3,344.3 \pm 31.0$ , and  $4,336.3 \pm 97.9$  OTUs in the no-record, one-year-history, two-year-history, and three-year-history groups, respectively. The block-adjusted group effect was strong ( $F_{3,6} = 142.93$ ,  $q = 1.47 \times 10^{-5}$ , partial  $\eta^2 = 0.986$ ), and all three recorded-history groups differed from the no-record group in Tukey-adjusted comparisons. Bacterial Shannon diversity showed the same non-linear category pattern ( $F_{3,6} = 147.95$ ,  $q = 1.47 \times 10^{-5}$ , partial  $\eta^2 = 0.987$ ; Fig. 2A,B; Additional file 1: Supplementary Tables S3 and S4). Exact Friedman tests supported both endpoints ( $q = 0.017$ ).

The highest observed fungal richness mean occurred in the three-year-history group and the lowest in the no-record group ( $F_{3,6} = 4.87$ ,  $q = 0.0476$ , partial  $\eta^2 = 0.709$ ). Fungal Shannon diversity had its lowest group mean in the two-year-history group ( $F_{3,6} = 6.74$ ,  $q = 0.0418$ , partial  $\eta^2 = 0.771$ ). However, exact Friedman sensitivity results were not significant after multiplicity correction for observed richness ( $q = 0.207$ ) or Shannon diversity ( $q = 0.061$ ). Fungal alpha-diversity evidence was therefore considered suggestive rather than robust (Fig. 2C,D).

## Bacterial and fungal community configurations differed among application-history groups

Bray-Curtis ordination separated application-history groups in both microbial domains (Table 1; Fig. 3; Additional file 1: Supplementary Table S6). For bacteria, PCoA1 and PCoA2 explained 46.23% and 26.60% of variation; for fungi, the first two axes explained 49.90% and 22.89%. Block-restricted PERMANOVA detected group-associated separation for bacteria ( $R^2 = 0.853$ ,  $P = 0.0017$ ) and fungi ( $R^2 = 0.806$ ,  $P = 0.0017$ ).

Multivariate dispersion also differed for bacteria ( $F = 40.88$ ,  $P = 0.0039$ ) and fungi ( $F = 39.62$ ,  $P = 0.0061$ ; Fig. 3C,D; Additional file 1: Supplementary Table S7). Consequently, the ordination and PERMANOVA patterns reflect differences in both group centroids and within-group spread, not a pure location shift.

Rarefied and proportional Bray-Curtis matrices were nearly identical (bacteria  $\rho = 0.9991$ ; fungi  $\rho = 0.9995$ ), indicating that the principal distance pattern was insensitive to the two normalization approaches (Additional file 1: Supplementary Table S8).

Table 1  
Beta-diversity, multivariate-dispersion, and normalization-sensitivity results

| Domain   | Rarefaction depth | PCoA1 / PCoA2 (%) | Block-restricted PERMANOVA   | Block-restricted PERMDISP  | Normalization sensitivity |
|----------|-------------------|-------------------|------------------------------|----------------------------|---------------------------|
| Bacteria | 22517             | 46.23 / 26.60     | $R^2 = 0.853$ ; $P = 0.0017$ | $F = 40.88$ ; $P = 0.0039$ | Mantel $\rho = 0.9991$    |
| Fungi    | 28058             | 49.90 / 22.89     | $R^2 = 0.806$ ; $P = 0.0017$ | $F = 39.62$ ; $P = 0.0061$ | Mantel $\rho = 0.9995$    |

PERMANOVA and PERMDISP used block-restricted permutations. Significant PERMDISP indicates that group-associated separation reflects differences in both group location and within-group dispersion. PCoA, principal coordinates analysis; PERMANOVA, permutational multivariate analysis of variance; PERMDISP, permutational analysis of multivariate dispersion. The normalization sensitivity statistic compares Bray-Curtis matrices derived from rarefied counts and within-sample proportions.

## Dominant taxa showed application-history-associated compositional profiles

Proteobacteria and Acidobacteria dominated the bacterial communities. Group-mean Proteobacteria proportions were 39.3%, 39.9%, 37.1%, and 32.7% in the no-record, one-year-history, two-year-history, and three-year-history groups, respectively; the corresponding Acidobacteria proportions were 23.3%, 31.3%, 32.2%, and 40.0% (Fig. 4; Additional file 1: Supplementary Table S5). Chloroflexi had its highest group mean in the three-year-history group, Bacteroidetes was less abundant in the recorded-history groups than in the no-record group, and Firmicutes had its highest mean in the two-year-history group. These relative read proportions do not measure absolute microbial abundance.

At genus level, the group-mean relative proportion of *Candidatus Koribacter* was 6.5% in the no-record group and 25.5% in the three-year-history group. *Rhodanobacter* and *Occallatibacter* had their highest means in the two-year-history and one-year-history groups, respectively, whereas *Metallibacterium* had its lowest mean (0.2%) in the three-year-history group. Fungal communities were dominated by Ascomycota. *Trichoderma* comprised 57.6% and 66.1% in the one-year-history and two-year-history groups, respectively. The *Fusarium* mean was lower in both intermediate-history groups than in the no-record group and was 9.1% in the three-year-history group. *Torula* and *Mortierella* were more prominent in the three-year-history group, whereas *Endogone* was concentrated in the no-record group.

Because  $n = 3$  plots per category and the genus-level screens did not include block, these taxonomic contrasts are hypothesis-generating (Additional file 4: Supplementary Figure S3; Additional file 1:

Supplementary Tables S13 and S14).

## Exploratory environmental and functional associations

Exploratory marginal analyses aligned soil sucrase activity, available phosphorus, hydrolysable nitrogen, and catalase activity with microbial community structure. Variance-inflation-screened db-RDA retained sucrase activity, available phosphorus, and hydrolysable nitrogen for bacteria and sucrase activity, available phosphorus, and catalase activity for fungi (Fig. 5A,B; Additional file 5: Supplementary Figure S4; Additional file 1: Supplementary Table S9). These fitted variables summarize the observed biochemical-community gradients rather than independent effects.

Bacterial and fungal Bray-Curtis matrices were strongly concordant in an exploratory Mantel analysis ( $p = 0.926$ ). Genus-environment heatmaps identified multiple false-discovery-rate-controlled correlations, but both taxa and soil variables covaried with application-history group; these associations therefore do not demonstrate direct taxon-level effects or independent causal drivers (Fig. 5C,D; Additional file 6: Supplementary Figure S5; Additional file 1: Supplementary Table S10).

None of the 81 FAPROTAX-predicted bacterial functions remained significant at BH  $q < 0.05$  (Additional file 7: Supplementary Figure S6; Additional file 1: Supplementary Tables S11 and S12).

## Discussion

### Documented penicillin application history was associated with a non-linear rhizosphere pattern

Across the three spatial orchard blocks, the four application-history groups differed consistently in soil biochemical profiles, bacterial alpha diversity, and bacterial and fungal community configuration. The dominant profile was non-linear: the one-year-history and two-year-history groups generally had lower enzyme activities and bacterial diversity than the no-record group, whereas the three-year-history group had higher values than the two-year-history group and a distinct community state. Because the categories encode documented years of use rather than measured cumulative exposure, this is an association and not a conventional dose-response relationship.

Recent citrus studies provide direct evidence that systemic antibiotic use can perturb belowground microbial communities. In healthy *Citrus reticulata*, streptomycin and oxytetracycline reduced culturable rhizosphere bacteria, altered the relative abundance of *Pseudomonas*, *Agrobacterium*, and *Streptomyces*, changed rhizosphere metabolite profiles, and reduced photosynthetic performance<sup>[25]</sup>. In a commercial huanglongbing-affected orchard, oxytetracycline trunk injection was followed by detectable residues in leaves, bark, and roots and by short-term reductions in the abundance and diversity of active prokaryotes in the bark and rhizosphere<sup>[26]</sup>. Earlier work also showed that penicillin trunk injection changed citrus-associated bacterial community structure<sup>[10]</sup>.

These studies support the plausibility of an antibiotic-associated rhizosphere pattern, but they cannot establish direct penicillin causation in the present orchard. Penicillin residues and complete product, dose, and timing information were unavailable. The higher values in the three-year-history group could reflect community reassembly, enrichment of tolerant populations, plant-mediated changes, disease status, or unmeasured edaphic variation; because different plots represented the categories and were sampled once, they are not evidence of temporal recovery.

## **Soil biochemical responses indicate altered nutrient-processing potential**

The group-associated changes in ACP, CAT, URE, SUC, AP, HN, HA, AK, and CEC indicate markedly different rhizosphere biochemical states. Extracellular enzyme assays measure potential activity under standardized conditions rather than in situ nutrient-transformation rates. The low means for several enzyme activities and nutrient variables in the two-year-history group are therefore best interpreted as a coordinated biochemical profile rather than direct inhibition of specific carbon, nitrogen, or phosphorus processes.

The broader soil literature supports both antibiotic sensitivity and strong context dependence. A 2024 hierarchical meta-analysis based on 2,564 observations from 60 publications found that antibiotics reduced soil microbial biomass, alpha diversity, and enzyme activity on average, while antibiotic class, soil texture, temperature, and exposure configuration modified the magnitude of the response <sup>[27]</sup>. In controlled agricultural-soil incubations, tetracycline antibiotics altered microbial community structure and produced enzyme-specific responses: urease was inhibited more consistently, whereas phosphomonoesterase and beta-glucosidase responses varied with soil and concentration <sup>[28]</sup>. Thus, the present enzyme patterns are compatible with a history of microbial disturbance, but they cannot identify penicillin as the sole driver.

Nutrient status and the citrus rhizobiome are also shaped by orchard management and host traits. A field study showed that citrus rootstock and compost interacted to alter active rhizosphere bacterial diversity, community composition, predicted functions, and relationships with root nutrient concentrations <sup>[29]</sup>. Rootstock and major soil properties were not measured or fully documented here, limiting separation of application-history associations from edaphic and host effects. Sample handling was uniform across groups, so the biochemical measurements are most appropriately interpreted as within-study comparisons.

## **Bacterial diversity and taxonomic turnover reflect community reorganization**

Observed bacterial richness and Shannon diversity provided the most statistically robust biological signals. Group means were lower in the one-year-history and two-year-history groups than in the no-record group; the three-year-history group was higher than both intermediate-history groups but remained below the no-record group. Bray-Curtis separation further showed that the groups differed in

overall bacterial composition. These findings are consistent with citrus antibiotic experiments that detected changes in rhizosphere bacterial abundance or composition after streptomycin or oxytetracycline exposure <sup>[25, 26]</sup>.

Nevertheless, modern citrus microbiome studies demonstrate that community composition is jointly structured by geography, orchard environment, plant compartment, host genotype, rootstock, and management. A global survey across 28 citrus groves in nine countries identified reproducible core root and rhizosphere members but also substantial location- and host-associated variation <sup>[30]</sup>. Within a California orchard, microbial diversity and assembly differed among citrus tissues and ecological compartments <sup>[31]</sup>. Host genetic traits also contributed to citrus microbiome composition and assembly <sup>[32]</sup>, while rootstock-by-compost interactions altered the active rhizobiome under field conditions <sup>[29]</sup>.

Accordingly, the group-associated profiles of *Acidobacteria*, *Candidatus Koribacter*, *Rhodanobacter*, *Occallatibacter*, and *Metallibacterium* are best regarded as biomarkers of different plot-level rhizosphere states rather than confirmed penicillin-responsive or functionally diagnostic taxa. Phylum- and genus-level assignments contain substantial ecological heterogeneity, and relative abundance does not distinguish population growth from declines in other taxa. Establishing antibiotic tolerance or a specific function would require absolute quantification, isolate-level characterization, resistance measurements, and direct residue data.

## **Fungal turnover was stronger for community configuration than alpha diversity**

Fungal alpha-diversity evidence was less consistent than the bacterial evidence because the block-adjusted models were significant whereas the exact Friedman sensitivity tests did not remain significant after multiplicity correction. By contrast, fungal Bray-Curtis configuration differed strongly among groups. Fungal community composition and dispersion varied among histories, whereas evidence for group effects on fungal richness or evenness remained suggestive.

The large relative-abundance changes in *Trichoderma*, *Fusarium*, *Torula*, *Mortierella*, and *Endogone* should not be translated directly into disease-suppression or pathogenicity claims. A recent global analysis identified *Fusarium* and *Mortierella* among recurrent core fungal genera of citrus roots and rhizospheres across diverse production regions <sup>[30]</sup>, demonstrating that their presence is not itself a functional diagnosis. Similarly, a 2025 comparison of historically undisturbed and conventionally managed citrus groves linked long-term orchard context with rhizosphere composition and apparent HLB susceptibility <sup>[33]</sup>, reinforcing the possibility that management history and disease background can covary with fungal and bacterial profiles.

The reciprocal proportions of *Trichoderma* and *Fusarium* in some groups may partly reflect the closed-sum nature of amplicon data: expansion of one dominant taxon necessarily reduces the reported proportion of others even if their absolute abundance is unchanged <sup>[23]</sup>. Species and strains within both

genera vary widely in ecology. Demonstrating antagonism, pathogenicity, or biocontrol would require species-resolved identification, cultivation, absolute quantitative PCR or spike-in normalization, metabolite or transcript measurements, and plant or pathogen bioassays.

## **Environmental fitting and cross-domain concordance are hypothesis-generating**

The alignment of bacterial and fungal community structure with SUC, AP, HN, and CAT indicates that microbial and biochemical variation formed coherent plot-level gradients. This is compatible with the close links between active citrus rhizosphere bacteria and nutrient status reported under rootstock and compost treatments <sup>[29]</sup>. However, the present db-RDA models evaluated nine strongly intercorrelated soil variables in only 12 samples, and both datasets varied with application-history group. The selected variables should therefore be interpreted as axes summarizing the observed gradient, not as independent causal drivers.

The strong bacterial-fungal Mantel correlation likewise indicates that samples similar in one domain tended to be similar in the other. It does not demonstrate direct bacterial-fungal interactions, because both domains may have responded to the same application-history group, host, disease, or soil conditions. Network or interaction claims would require substantially greater replication, temporal information, absolute abundance data, and experimental validation.

No FAPROTAX-predicted function remained significant after false-discovery-rate correction. Taxonomic functional prediction maps functions documented in cultured representatives onto marker-gene profiles and cannot resolve strain-specific gene content, gene expression, or process rates <sup>[24]</sup>. The functional data therefore do not support claims of altered denitrification, carbon degradation, or other specific pathways. Shotgun metagenomics, metatranscriptomics, targeted functional-gene assays, or direct process measurements would be needed to test such mechanisms.

## **Statistical interpretation, strengths, limitations, and future directions**

The blocked sampling structure is a strength because each history group was represented once in each spatial block and analyses used plot-level units. Block adjustment and restricted permutations accounted for broad position-related heterogeneity. However, applications predated the study and were not assigned by the investigators, so this remains an observational comparison rather than a randomized experiment. PERMDISP was significant for both bacteria and fungi; PERMANOVA separation therefore represents a combination of centroid displacement and unequal within-group dispersion rather than a pure group-centroid effect <sup>[22]</sup>. The near identity of rarefied and proportional Bray-Curtis matrices indicates that the main ordination pattern was not created by the selected normalization approach, although rarefaction should not be treated as universally optimal <sup>[34]</sup>.

The principal limitations are the single-orchard setting, small number of spatial blocks, one-time cross-sectional sampling, absence of pretreatment measurements, non-random historical antibiotic application, incomplete product and application metadata, and the lack of penicillin residues, HLB status and titer, rootstock information, soil pH, moisture, texture, total organic carbon, and total nitrogen. The 16S and ITS data also represent relative marker-gene profiles rather than absolute microbial biomass or activity. The observations were replicated across blocks, but these limitations constrain generalizability and preclude causal attribution.

A definitive follow-up should prospectively randomize a known penicillin product and dose across more blocks, collect pretreatment samples, and repeatedly sample after injection. Penicillin and major transformation products should be quantified in trunk, root, rhizosphere, and bulk-soil compartments, together with HLB titer, rootstock, plant physiological traits, soil pH, moisture, texture, organic carbon, and total nitrogen. Absolute bacterial and fungal loads should accompany compositional sequencing, and function should be evaluated with shotgun or transcript-based methods and direct enzyme or nutrient-flux measurements. Such a design would distinguish direct exposure effects from plant-mediated responses, transient suppression, community reassembly, and background orchard heterogeneity.

Documented penicillin-use history was associated with coordinated, non-linear rhizosphere biochemical and microbial states across the spatial blocks. The principal contribution is the replicated field evidence for this coordinated pattern, which now warrants residue-informed longitudinal testing.

## Conclusions

Documented penicillin application history was associated with coordinated, non-linear differences in reported soil biochemical properties, bacterial alpha diversity, and bacterial and fungal community configuration across three spatial orchard blocks. The blocked comparison supports plot-level group contrasts but does not demonstrate temporal recovery or direct antibiotic causation. Mechanistic interpretation remains limited by the single-orchard setting, small replication, non-random historical exposure, incomplete product and application metadata, and unmeasured soil, residue, rootstock, and disease covariates. The study therefore provides field associations and testable hypotheses for longitudinal, residue-informed validation.

## Abbreviations

ACP

acid phosphatase activity

AK

available potassium

ANCOM-BC

analysis of compositions of microbiomes with bias correction

AP  
available phosphorus  
BH  
Benjamini-Hochberg  
CAT  
catalase activity  
CEC  
cation-exchange capacity  
CLR  
centered log-ratio  
db-RDA  
distance-based redundancy analysis  
FAPROTAX  
Functional Annotation of Prokaryotic Taxa  
FASTQ  
sequence read file format with quality scores  
HA  
humic acid  
HLB  
huanglongbing  
HN  
hydrolysable nitrogen  
ITS  
internal transcribed spacer  
OTU  
operational taxonomic unit  
PCoA  
principal coordinates analysis  
PERMANOVA  
permutational multivariate analysis of variance  
PERMDISP  
permutational analysis of multivariate dispersion  
rRNA  
ribosomal RNA  
SRA  
Sequence Read Archive  
SUC  
sucrase activity  
URE  
urease activity.

# Declarations

## Ethics approval and consent to participate

Not applicable. This study did not involve humans, human data or tissue, or animals.

## Consent for publication

Not applicable.

# Competing interests

The authors declare that they have no competing interests.

# Authors' information

Not applicable.

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# Author Contribution

LL and BS conceived the study. LL, YZ, YL, QL, SC and YQ developed the methodology. LL, YZ, YL, QL, SC, YQ and WY performed the formal analyses. YL and QQ conducted the investigation. LL curated the data. YL prepared the visualizations. LL drafted the manuscript. SC and BS reviewed and edited the manuscript. BS supervised the work. All authors read and approved the final manuscript.

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## Availability of data and materials

The bacterial 16S rRNA gene and fungal ITS amplicon reads have been deposited in the NCBI Sequence Read Archive under BioProject PRJNA1498691 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1498691>); the associated BioSample accessions are SAMN61854641-SAMN61854652. Supplementary analysis tables and selected source data are included within the article and its Additional file 1.

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# Figures

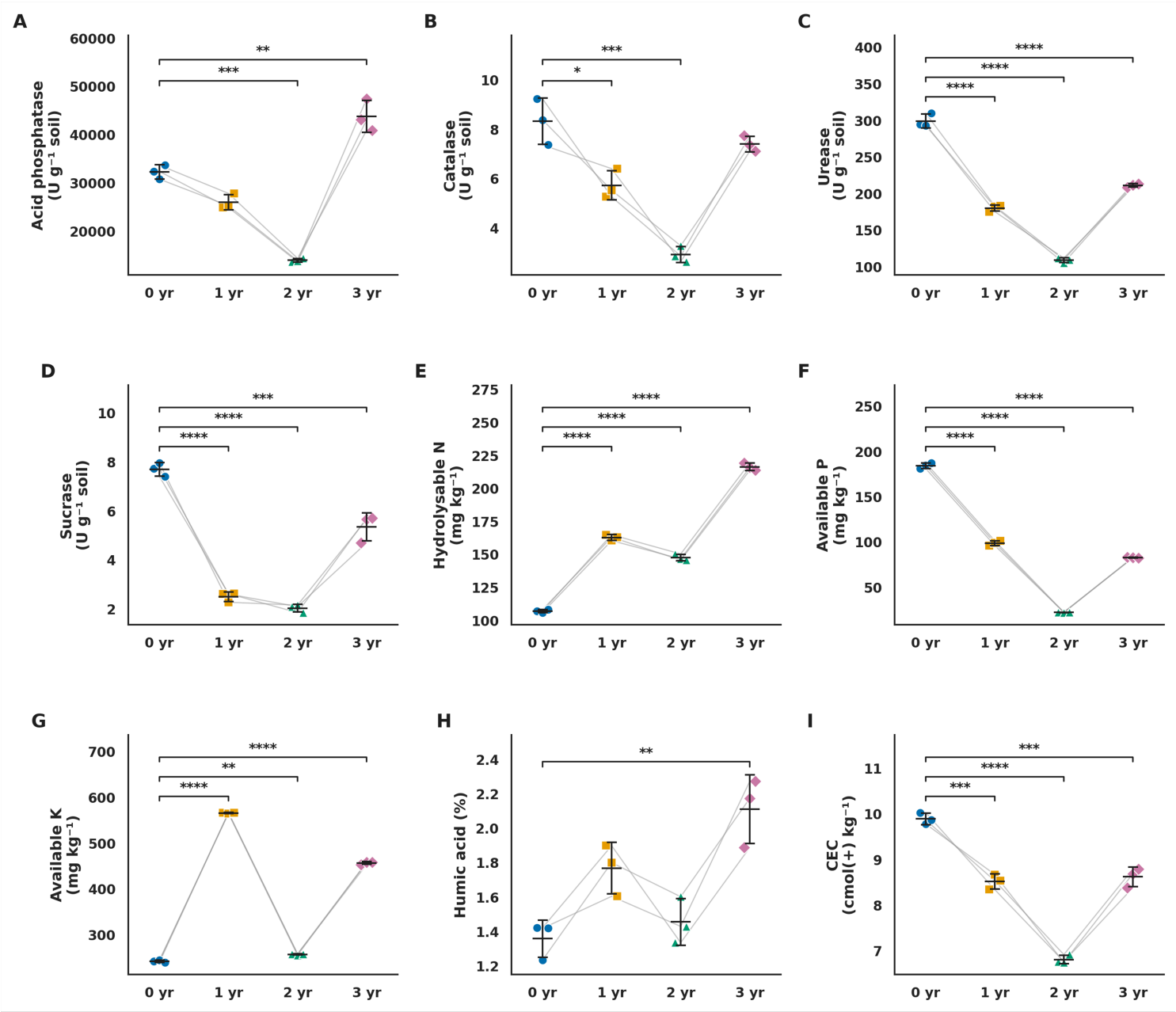


Figure 1

## Soil biochemical properties across documented penicillin application-history groups.

Group codes denote no recorded application (K-Y-0) or application recorded in one (K-Y-1), two (K-Y-2), or three years (K-Y-3). Points show the three plot-level samples per group, gray lines connect categories within the same spatial block, and black bars show mean  $\pm$  SD across the three plot-level composites. Brackets show Tukey-adjusted comparisons with K-Y-0 from the block-adjusted model; only adjusted  $P < 0.05$  comparisons are displayed (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ ). ACP, CAT, URE, and SUC are expressed as U g<sup>-1</sup> soil; HN, AP, and AK as mg kg<sup>-1</sup>; HA as %, and CEC as cmol(+) kg<sup>-1</sup>.

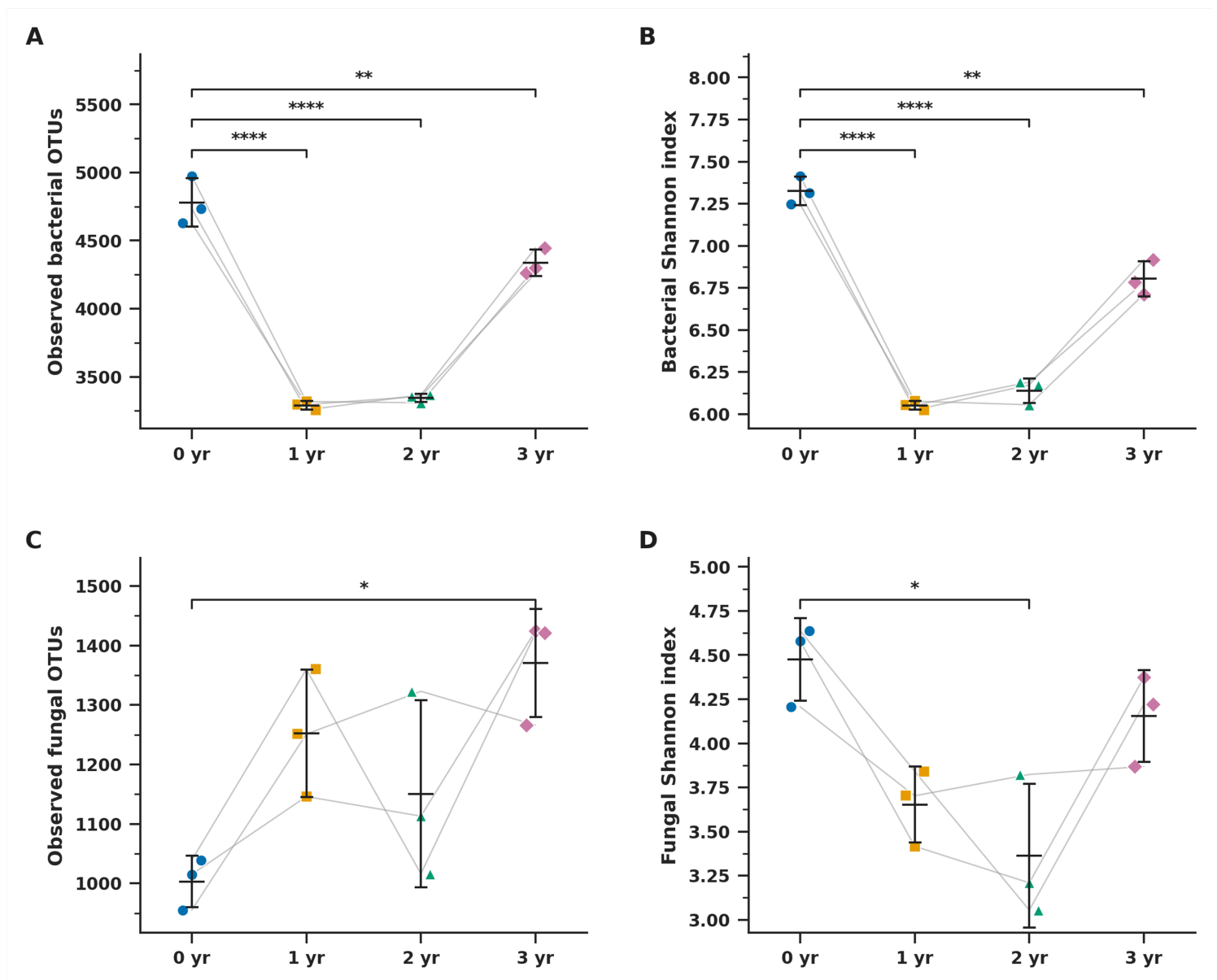


Figure 2

### Bacterial and fungal alpha diversity across documented penicillin application-history groups.

Group codes denote no recorded application (K-Y-0) or application recorded in one (K-Y-1), two (K-Y-2), or three years (K-Y-3). Panels A and B show bacterial observed OTUs and Shannon diversity; panels C and D show the corresponding fungal indices. Points show plot-level samples, gray lines connect categories within the same spatial block, and black bars show mean  $\pm$  SD across the three plot-level composites. Brackets show Tukey-adjusted contrasts with K-Y-0 from block-adjusted models.

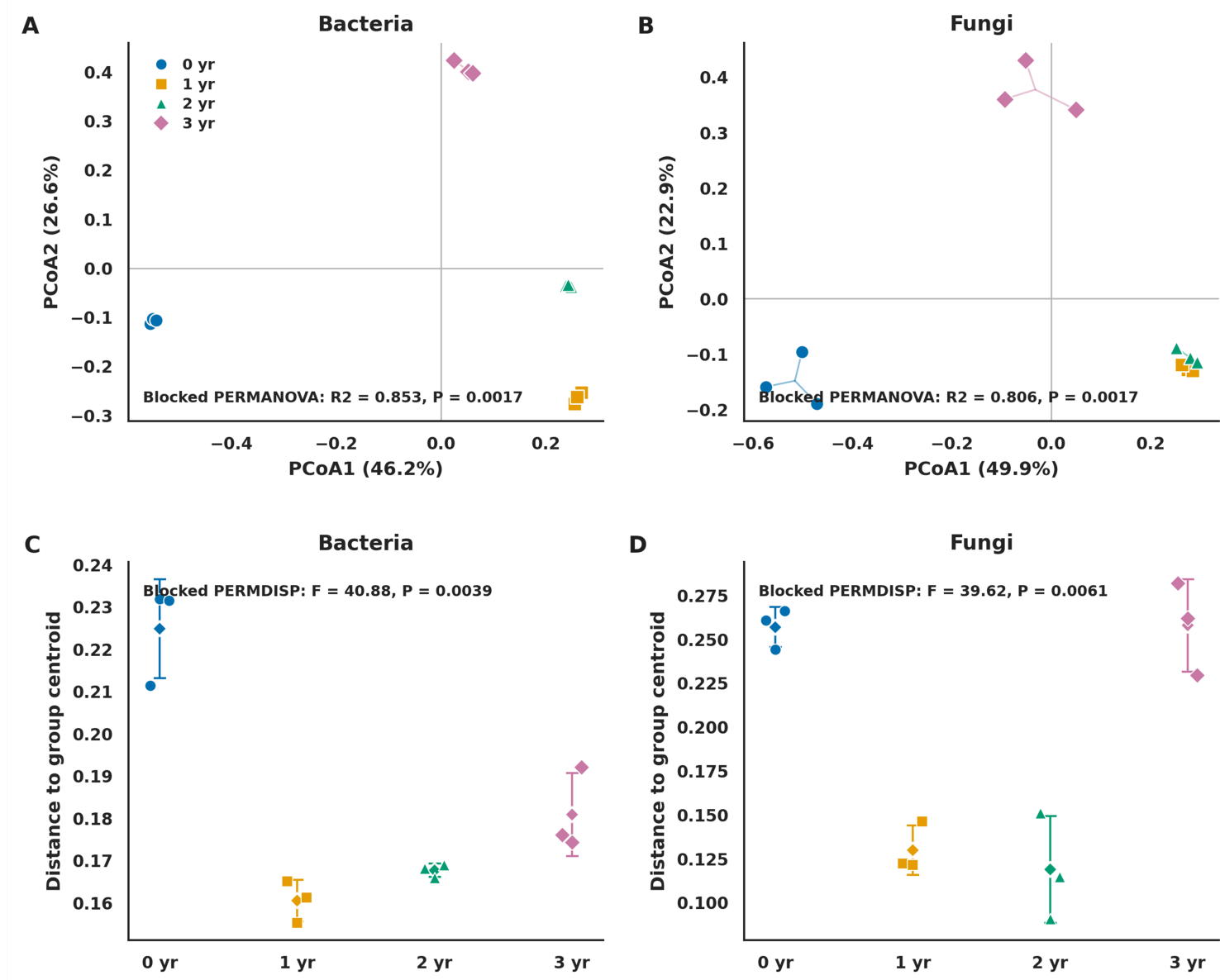


**Figure 3**

### Bray-Curtis beta diversity and multivariate dispersion.

Panels A and B show bacterial and fungal principal coordinates analyses; line segments connect samples to group centroids. Panels C and D show distances to the corresponding centroid with mean  $\pm$  SD across the three plot-level composites per group. Group P values were obtained by block-restricted PERMANOVA and PERMDISP. Because PERMDISP was significant in both domains, ordination separation reflects differences in both group location and within-group dispersion.

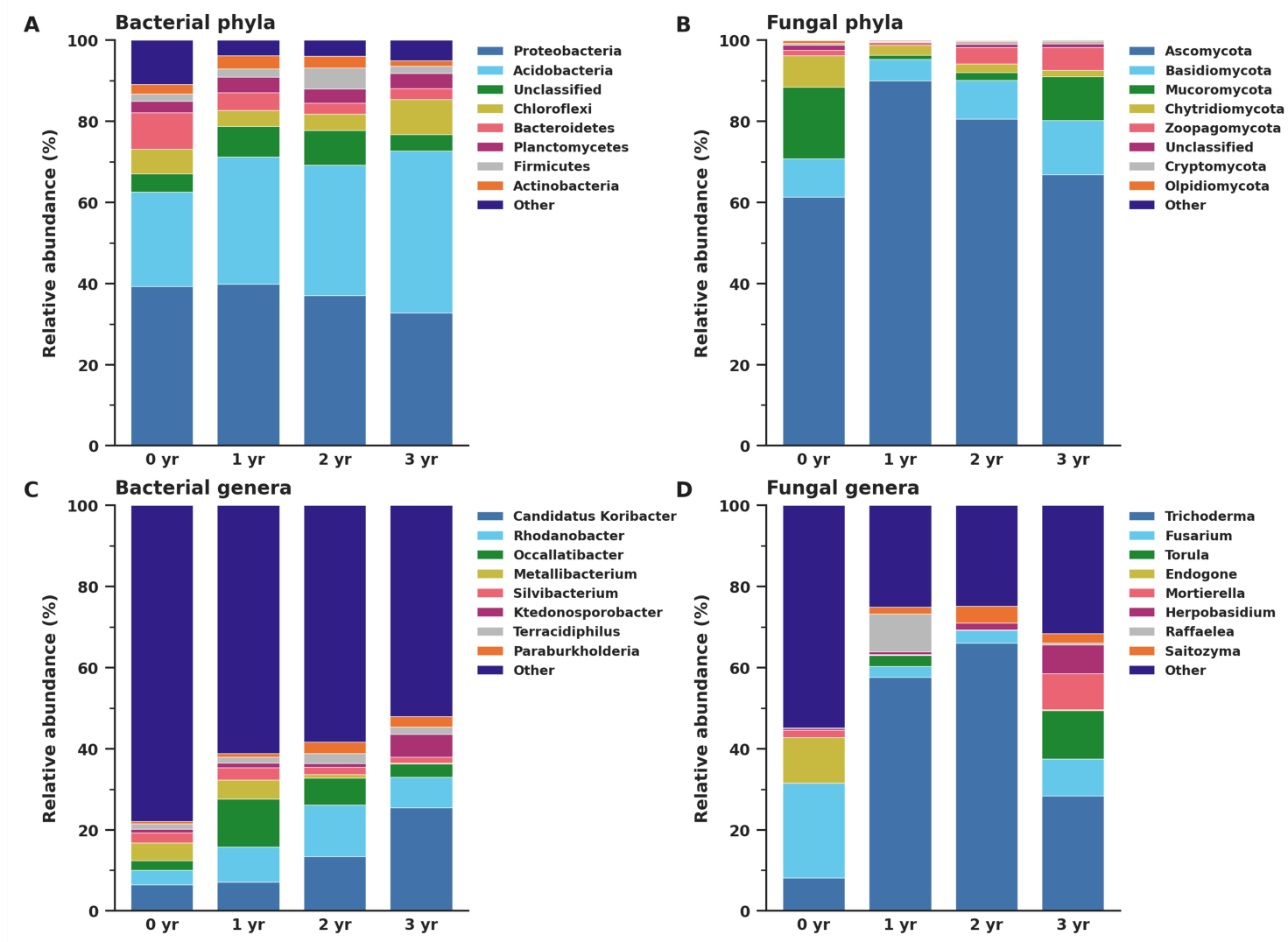


**Figure 4**

**Mean bacterial and fungal taxonomic composition across documented penicillin application-history groups at phylum and genus levels.**

Group codes denote no recorded application (K-Y-0) or application recorded in one (K-Y-1), two (K-Y-2), or three years (K-Y-3). Values are group-mean relative read proportions after domain-specific rarefaction; minor taxa are grouped as Other. Relative composition does not measure absolute microbial abundance.

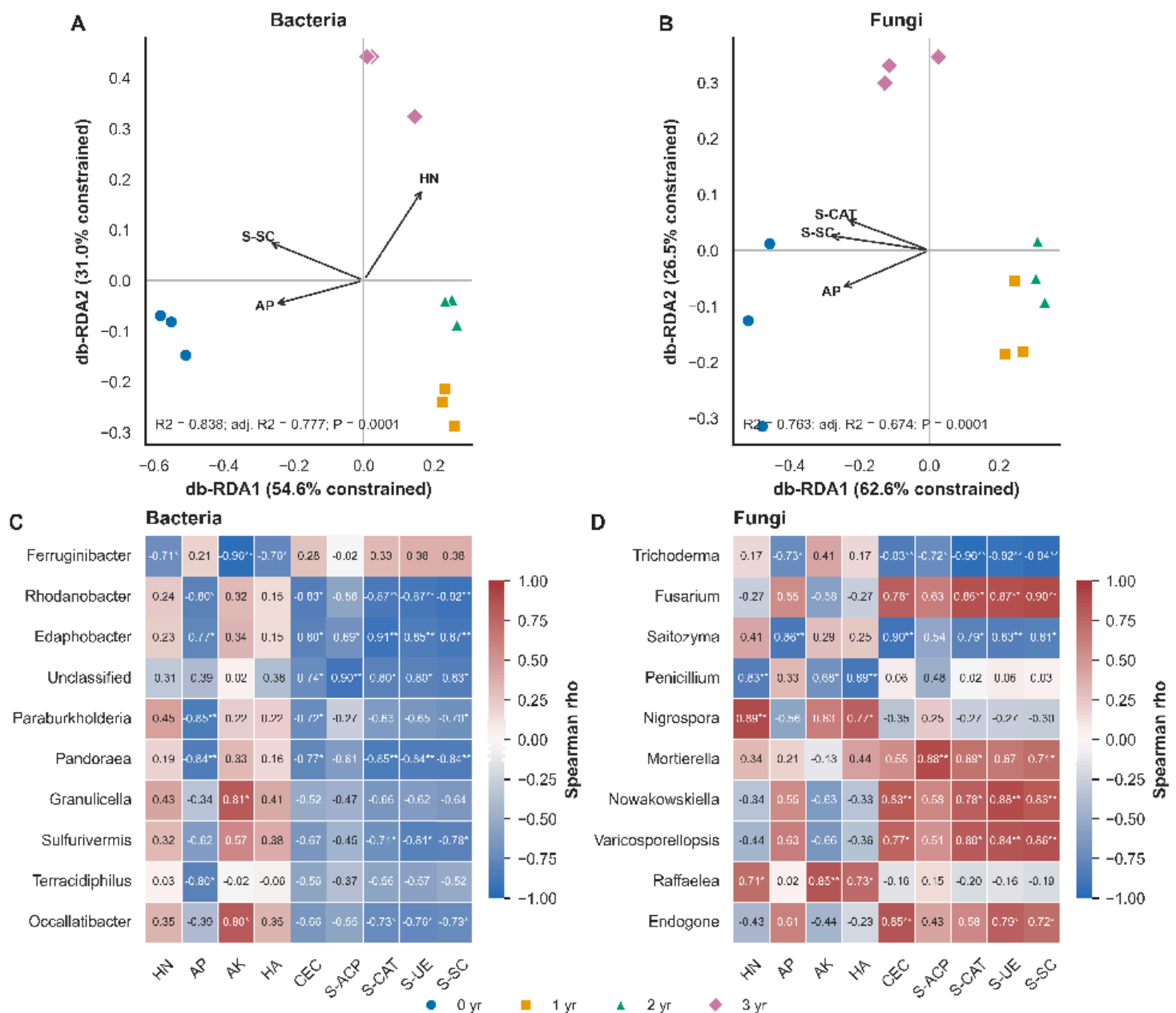


**Figure 5**

**Exploratory associations between microbial community structure and soil biochemical gradients.**

Panels A and B show bacterial and fungal distance-based redundancy analyses after marginal screening and variance-inflation control. Arrows denote selected standardized soil variables. Panels C and D show abundant genera with the strongest false-discovery-rate-controlled Spearman associations; \*  $q < 0.05$  and \*\*  $q < 0.01$ . S-SC, soil sucrase activity; S-CAT, soil catalase activity; AP, available phosphorus; HN, hydrolysable nitrogen. These patterns describe covariation within 12 samples and are not evidence of independent causal effects.

# Supplementary Files

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