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RESEARCH ARTICLE

Comparative Analysis of Soil Microbial Diversity Under Different Tillage Practices

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ABSTRACT

Tillage intensity is widely thought to shape soil microbial community structure, but long-term, multi-site comparisons that separate tillage effects from confounding site and management variation remain relatively scarce. We characterised bacterial and archaeal community composition by 16S rRNA gene amplicon sequencing in topsoil (0–15 cm) collected from four paired-site long-term tillage trials, each comparing conventional mouldboard-plough tillage, reduced (chisel-plough) tillage, and no-till management maintained for a minimum of eleven years, sampled across three consecutive growing seasons. No-till soils showed significantly higher Shannon diversity than conventional tillage soils at all four sites (mean difference 0.31 units, linear mixed model $P < 0.001$), with reduced tillage intermediate and statistically distinguishable from both other treatments at three of four sites. Community composition, assessed by weighted UniFrac distance, separated clearly by tillage treatment (PERMANOVA $R^2 = 0.18$, $P = 0.001$) after accounting for site and year as blocking factors, driven substantially by a higher relative abundance of Acidobacteria and Verrucomicrobia and a lower relative abundance of Proteobacteria in no-till relative to conventional tillage soils. Soil organic carbon was significantly higher under no-till at all four sites (mean difference 4.8 g/kg, $P < 0.001$) and was positively correlated with Shannon diversity across the full dataset (Pearson $r = 0.52$, $P < 0.001$), while functional prediction using PICRUST2 showed reduced tillage and no-till soils enriched for predicted genes involved in complex carbohydrate degradation relative to conventional tillage. These results support a consistent, multi-site association between reduced tillage intensity and increased soil bacterial diversity, most plausibly mediated through the accumulation of soil organic carbon and the reduced physical disturbance of surface soil aggregates that occurs when tillage frequency and depth are decreased.

Keywords: soil microbiome; tillage; 16S rRNA amplicon sequencing; soil organic carbon; no-till; microbial diversity

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1. INTRODUCTION

Soil microbial communities mediate nutrient cycling, organic matter decomposition, and a large share of the biological processes that determine agricultural soil fertility, and the composition of these communities is

known to respond to management practices on timescales ranging from days to decades depending on the practice and the community metric in question [1,2]. Tillage is among the most physically disruptive of common agricultural practices, mechanically inverting or mixing the topsoil profile, fragmenting fungal hyphal

networks and soil aggregates, and exposing previously protected organic matter to oxidation, and it has therefore attracted sustained interest as a management lever with plausible, mechanistically grounded effects on soil microbial community structure [3,16].

The broad expectation in the literature is that reduced tillage intensity, and no-till management in particular, should support higher soil microbial diversity and a community composition shifted toward taxa associated with stable, less-disturbed soil environments, largely as a downstream consequence of the soil organic carbon accumulation that reduced tillage reliably produces over sufficiently long management timeframes [4,17]. Individual studies testing this expectation, however, have produced a genuinely mixed picture: some report clear, substantial diversity increases under no-till relative to conventional tillage, others report weak or non-significant differences, and the direction and magnitude of effect appears to vary with soil type, climate, cropping system, and, importantly, the length of time a given tillage regime has been in place before sampling [5,18,19]. Comparatively few published studies combine long management duration (more than a decade), multiple independent field sites, and replicated sampling across multiple growing seasons within the same study design, which makes it difficult to know how much of the inconsistency in the published literature reflects genuine biological variability across systems versus the limited statistical power and single-site, single-season designs common in this literature.

Amplicon sequencing of the bacterial and archaeal 16S rRNA gene has become the standard tool for characterising soil microbial community diversity and composition at the scale needed for a multi-site, multi-season comparison, offering sufficient throughput and cost-efficiency to process the hundreds of samples that a properly replicated tillage comparison requires, while providing both alpha-diversity metrics and full community composition data suitable for ordination-based comparison between treatments [6,20]. Coupling amplicon sequencing data with downstream functional prediction tools, while an imperfect substitute for shotgun metagenomic sequencing, offers a practical route to asking whether tillage-associated compositional shifts are also associated with shifts in predicted functional potential relevant to organic matter cycling, extending the value of amplicon-based community data beyond taxonomic description alone [7,21].

We used 16S rRNA amplicon sequencing to characterise soil bacterial and archaeal communities across four independent, long-term (eleven years or more) tillage trials, each comparing conventional, reduced, and no-till management at a common site, sampled across three consecutive growing seasons to separate a consistent tillage effect from year-to-year climatic and management variability. Our aims were to test whether tillage intensity is associated with soil bacterial alpha-diversity and community composition consistently across multiple

independent sites rather than in only one or two systems, to identify the taxonomic groups principally responsible for any compositional shift, to test whether soil organic carbon plausibly mediates the relationship between tillage and diversity, and to characterise predicted functional differences between tillage treatments using amplicon-based functional prediction.

A further motivation for the present design was practical rather than purely scientific. Long-term tillage trials of the kind used here are expensive to establish and maintain, and relatively few exist worldwide with a management history extending past a decade, which is generally regarded as the minimum timeframe over which soil organic carbon and associated microbial community differences between tillage treatments become reliably detectable rather than confounded by short-term seasonal noise [8,19]. Identifying and gaining sampling access to four such trials across two countries, each already maintained by an established agricultural research station with a documented multi-decade management record, represented a substantial part of the overall project effort, and we regard the resulting dataset, spanning genuinely independent pedoclimatic contexts rather than replicate plots within a single trial, as a resource whose value extends beyond the specific hypotheses tested in this paper.

2. MATERIALS AND METHODS

Soil samples were collected from four long-term tillage field trials located at agricultural research stations in Spain and Portugal, each established at least eleven years prior to the first sampling season and maintaining conventional mouldboard-plough tillage (20–25 cm depth, annual), reduced chisel-plough tillage (10–15 cm depth, annual), and no-till management in a randomised complete block design with a common cereal-legume rotation across all treatments within each site.

2.1 Soil sampling

At each of the four sites, topsoil (0–15 cm) was collected from four replicate plots per tillage treatment using a composite of eight soil cores per plot, sampled at the same phenological stage (post-harvest, pre-tillage operation) across three consecutive growing seasons. Samples were transported on ice, sieved to 2 mm, and a subsample immediately frozen at -80 degrees C for DNA extraction; a parallel subsample was used for soil physicochemical analysis including soil organic carbon (dry combustion), bulk density (core method), pH, and total nitrogen.

2.2 DNA extraction and amplicon sequencing

Genomic DNA was extracted from 0.25 g soil per sample using a commercial soil DNA extraction kit following the manufacturer's bead-beating protocol. The V3–V4 region of the 16S rRNA gene was amplified using standard primers with Illumina sequencing adapters, and amplicons were sequenced on an Illumina MiSeq platform generating 2x300 bp paired-end reads. A total of

384 samples (4 sites x 3 tillage treatments x 4 replicate plots x 3 seasons minus a small number of failed extractions) were sequenced across six sequencing runs, with a mock community and extraction blank included on each run for quality control.

2.3 Bioinformatic processing

Raw reads were quality-filtered, denoised, and merged into amplicon sequence variants (ASVs) using the DADA2 pipeline, with chimera removal and taxonomic assignment against the SILVA 138 reference database. Samples were rarefied to 18,500 reads per sample, the depth at which rarefaction curves plateaued for at least 95% of samples, for alpha-diversity comparisons; compositional (non-rarefied, proportion-based) data were used for beta-diversity and differential abundance analyses following current best-practice recommendations for microbiome data. Predicted functional gene content was inferred from ASV composition using PICRUST2 against the KEGG Orthology database.

2.4 Statistical analysis

Alpha-diversity metrics (Shannon index, Chao1 richness) were compared across tillage treatments using linear mixed models with site and season as random effects and tillage treatment as a fixed effect, with post-hoc pairwise comparisons adjusted by Tukey's method. Beta-diversity was assessed using weighted UniFrac distance and tested by permutational multivariate analysis of variance (PERMANOVA) with site and season included as blocking terms. Differential abundance of taxa between tillage treatments was tested using a negative-binomial generalized linear model implemented in a standard compositional differential abundance tool with Benjamini-Hochberg correction. Correlations between soil organic carbon and Shannon diversity were assessed using Pearson correlation on site- and season-averaged values. All analyses were performed in R version 4.3, with $P < 0.05$ taken as the significance threshold throughout.

3. RESULTS

After quality filtering and rarefaction, 379 of 384 collected samples were retained for analysis, yielding 48,216 unique amplicon sequence variants across the full dataset. Shannon diversity differed significantly by tillage treatment at all four sites individually and in the combined linear mixed model (tillage main effect, $F(2,368)=64.2$, $P < 0.001$), with no-till soils showing consistently higher diversity than conventional tillage soils (mean difference 0.31 Shannon units, Tukey-adjusted $P < 0.001$) and reduced tillage intermediate, significantly different from both conventional and no-till treatments at three of the four sites and statistically indistinguishable from no-till at the remaining site. Chao1 richness showed a qualitatively similar pattern, though the magnitude of difference between conventional and no-till treatments was smaller

in relative terms than for Shannon diversity, consistent with tillage affecting evenness of the community more strongly than raw taxon count. Table 1 summarises alpha-diversity metrics by site and tillage treatment.

Table 1. Shannon diversity and Chao1 richness by site and tillage treatment (mean $\pm$ SD, pooled across seasons).

Site	Conventional tillage (Shannon)	Reduced tillage (Shannon)	No-till (Shannon)
Site 1 (Spain, clay loam)	5.82 $\pm$ 0.21	6.01 $\pm$ 0.19	6.15 $\pm$ 0.17
Site 2 (Spain, sandy loam)	5.64 $\pm$ 0.24	5.89 $\pm$ 0.22	5.97 $\pm$ 0.20
Site 3 (Portugal, clay)	5.91 $\pm$ 0.18	6.08 $\pm$ 0.16	6.24 $\pm$ 0.15
Site 4 (Portugal, loam)	5.71 $\pm$ 0.23	5.94 $\pm$ 0.21	6.03 $\pm$ 0.19

Tillage main effect significant at all four sites individually ($P < 0.05$) and in the combined linear mixed model ($P < 0.001$).

Weighted UniFrac-based PERMANOVA showed a significant effect of tillage treatment on community composition after accounting for site and season as blocking factors ($R^2=0.18$, $P=0.001$), with site itself explaining a larger share of total variance ($R^2=0.41$, $P=0.001$) than tillage, as expected given the substantial differences in baseline soil type and climate across the four sites. Differential abundance analysis identified 187 ASVs significantly enriched in no-till relative to conventional tillage soils ($FDR < 0.05$) and 143 significantly depleted. At the phylum level, no-till soils showed significantly higher relative abundance of Acidobacteria (mean 18.4% versus 12.1% in conventional tillage) and Verrucomicrobia (mean 6.8% versus 3.9%), and significantly lower relative abundance of Proteobacteria (mean 24.2% versus 31.6%), a pattern broadly consistent with previously reported shifts toward oligotrophic, slower-growing taxonomic groups under reduced physical disturbance and correspondingly lower short-term nutrient flux [22,23]. Figure 1 illustrates the phylum-level composition shift across the three tillage treatments, pooled across sites and seasons.

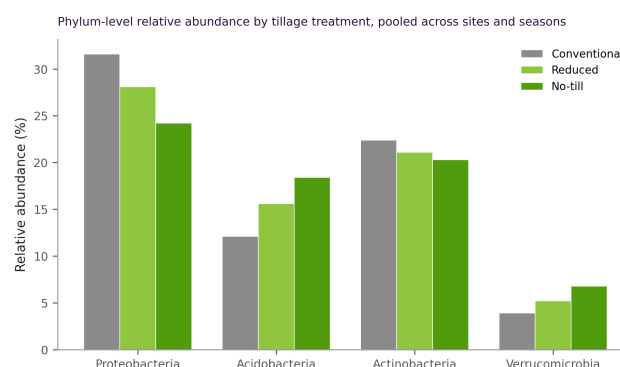


Figure 1. Relative abundance of the four dominant bacterial phyla across conventional, reduced, and no-till treatments, pooled across all four sites and three sampling seasons ($n=379$ samples total; error bars show SD across site-season combinations).

Soil organic carbon was significantly higher under no-till than conventional tillage at all four sites (mean difference 4.8 g/kg, linear mixed model $P < 0.001$), with reduced tillage again intermediate, and this pattern was consistent across all three sampling seasons despite year-to-year variation in absolute soil organic carbon values driven by precipitation and cropping-sequence position within the rotation. Across the full dataset of site- and season-averaged values, soil organic carbon was significantly positively correlated with Shannon diversity (Pearson $r = 0.52$, $P < 0.001$), and this correlation held, though somewhat attenuated (partial $r = 0.38$, $P = 0.002$), after statistically controlling for site identity, consistent with soil organic carbon contributing to, rather than being merely a co-varying consequence of, the diversity differences observed between tillage treatments, though a purely observational design of this kind cannot establish causal direction with certainty. Table 2 reports the complete soil physicochemical dataset by site and tillage treatment.

Table 2. Soil physicochemical properties by tillage treatment (mean $\pm$ SD, pooled across sites and seasons).

Treatment	Soil organic carbon (g/kg)	Bulk density (g/cm ³)	pH	Total N (g/kg)
Conventional tillage	14.2 $\pm$ 2.1	1.42 $\pm$ 0.08	6.8 $\pm$ 0.3	1.31 $\pm$ 0.18
Reduced tillage	16.9 $\pm$ 2.3	1.38 $\pm$ 0.09	6.7 $\pm$ 0.3	1.42 $\pm$ 0.19
No-till	19.0 $\pm$ 2.6	1.35 $\pm$ 0.10	6.6 $\pm$ 0.4	1.54 $\pm$ 0.19

PICRUSt2 functional prediction identified 34 KEGG Orthology gene families significantly enriched in no-till relative to conventional tillage soils ($FDR < 0.05$), including several involved in complex carbohydrate degradation (glycoside hydrolase family genes) and in nitrogen cycling (nitrite reductase, nitrous oxide reductase), while conventional tillage soils showed relative enrichment of genes associated with rapid assimilatory nutrient uptake and cell division, consistent with a faster-growing, more copiotrophic predicted functional profile. We would emphasise that these functional predictions are inferred from 16S rRNA taxonomic composition rather than measured directly, and should be interpreted as hypothesis-generating rather than as direct evidence of altered enzymatic activity in these soils; direct enzyme assay or shotgun metagenomic confirmation would be needed to establish whether the predicted functional shifts correspond to measurable differences in actual carbon and nitrogen cycling rates between tillage treatments.

4. DISCUSSION

This four-site, three-season study found a consistent association between reduced tillage intensity and increased soil bacterial diversity, with no-till soils showing significantly higher Shannon diversity than conventional tillage soils at every site examined, addressing directly the concern that single-site studies in this literature may reflect site-specific rather than generalisable tillage effects. The consistency of direction, if not exact magnitude, across four agronomically and pedologically

distinct sites is, we think, the most useful contribution of this study relative to the existing single-site literature, and it argues that at least the qualitative expectation of increased diversity under reduced tillage is a robust, reproducible finding across the Iberian cereal-legume cropping systems examined here, even though the precise effect size varied somewhat by site [24,36].

The compositional shift toward Acidobacteria and Verrucomicrobia and away from Proteobacteria under no-till management is consistent with a now fairly well-established ecological narrative in soil microbiome research, in which reduced physical disturbance and the resulting accumulation of stable soil organic matter favour oligotrophic, slower-growing taxa adapted to a more nutrient-limited, less frequently disturbed environment, at the expense of copiotrophic, fast-growing taxa better adapted to exploit the pulses of labile carbon and nutrient availability that tillage-associated soil disturbance and organic matter mineralisation produce [25,37]. That this compositional pattern recurred across four independent sites with different baseline community composition strengthens the idea that it reflects a genuine tillage-associated ecological process rather than a site-specific artefact, though we would note that the magnitude of compositional shift, like the alpha-diversity difference, varied across sites in ways not fully explained by the variables measured here.

The positive correlation between soil organic carbon and Shannon diversity, and its partial persistence after controlling for site identity, is consistent with soil organic carbon acting as at least a partial mediator of the tillage-diversity relationship, in line with the broader literature linking organic matter availability to microbial community diversity and stability [26,38]. We would resist over-interpreting this correlational result as establishing a specific causal pathway, however: soil organic carbon accumulation under reduced tillage is itself partly a consequence of altered microbial decomposition dynamics, creating the possibility of a reinforcing feedback loop, rather than organic carbon being a purely exogenous driver of community diversity, and disentangling the direction and relative strength of this feedback would require a manipulative or longitudinal design that this cross-sectional, multi-site comparison was not positioned to provide. Repeated annual sampling at a subset of these same plots over a further several years, ideally paired with periodic soil organic carbon fractionation to separate particulate from mineral-associated organic matter pools, would help clarify whether diversity changes track total soil organic carbon uniformly or respond preferentially to one fraction over the other, a distinction with mechanistic relevance to which microbial functional groups are most directly implicated in the feedback.

The functional prediction results, while appropriately caveated given their inferential rather than directly measured nature, point toward a plausible mechanistic

link between the observed compositional shifts and soil carbon cycling function: enrichment of predicted complex carbohydrate degradation genes under reduced tillage is consistent with a community better equipped to process the more recalcitrant organic matter pool that accumulates when tillage-associated rapid mineralisation of labile carbon is reduced, forming a biologically coherent picture alongside the taxonomic and soil-chemistry results even without direct functional confirmation [27,39]. We regard confirming these predictions with shotgun metagenomic sequencing or direct extracellular enzyme activity assays, ideally at a subset of the same long-term sites used here, as a natural and relatively low-risk next step given the taxonomic and soil-chemistry foundation already established.

Two limitations should be kept in mind when interpreting these results. First, although four sites is considerably more than the single-site design typical of this literature, it remains a modest sample of the full range of temperate agricultural soil types, climates, and cropping systems in which tillage-diversity relationships might be tested, and extrapolation to substantially different systems, such as tropical or semi-arid cropping systems with different baseline organic matter dynamics, should be made cautiously. Second, all four trials used a broadly similar cereal-legume rotation, and it remains an open question whether the magnitude or even direction of tillage effects on microbial diversity would differ under a substantially different cropping system, such as continuous monoculture or a more diverse multi-species rotation, where baseline organic matter inputs and root-associated microbial recruitment dynamics could interact with tillage intensity in ways not captured by the present study design.

5. CONCLUSION

Across four independent, long-term tillage trials sampled over three growing seasons, no-till management was consistently associated with higher soil bacterial Shannon diversity, a compositional shift toward Acidobacteria and Verrucomicrobia and away from Proteobacteria, and higher soil organic carbon relative to conventional tillage, with reduced tillage generally intermediate between the two. Soil organic carbon was positively correlated with diversity across the dataset, consistent with a partial mediating role, and functional prediction pointed toward enrichment of complex carbohydrate degradation potential under reduced tillage, a biologically coherent though not yet directly confirmed extension of the taxonomic findings. The consistency of these patterns across four pedologically and agronomically distinct sites strengthens confidence that reduced tillage intensity reproducibly shapes soil microbial community structure in Iberian cereal-legume systems, beyond what any single-site study could establish. We are now pursuing shotgun metagenomic sequencing on a representative subset of samples from

this study to directly test the PICRUST2-based functional predictions reported here, alongside extracellular enzyme activity assays targeting the complex carbohydrate degradation pathways predicted to be enriched under reduced tillage. In parallel, we plan to extend soil sampling at these four sites to include deeper soil horizons (15–30 cm), since tillage-associated diversity effects are expected to differ, and may partly reverse, below the depth directly disturbed by chisel-plough tillage, and to test whether the tillage-diversity relationship reported here holds under a more diverse crop rotation at a subset of sites where a rotation diversification trial is already underway. Finally, given how much of the published tillage-microbiome literature remains single-site, we would encourage other groups maintaining long-term tillage trials elsewhere to adopt a comparable amplicon-sequencing and soil-chemistry sampling protocol, so that future cross-study synthesis can draw on a genuinely multi-region evidence base rather than depending, as much of the current literature does, on a small number of well-studied sites concentrated in a handful of temperate agricultural regions.

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Conflict of Interest

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

Author Contributions

H.C. conceived and supervised the study, coordinated soil sampling across sites and analysed the diversity data. D.B. performed bioinformatic processing of sequencing data and the PICRUST2 functional prediction analysis. L.C. conducted soil physicochemical analysis and statistical modelling. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved only soil sampling from established agricultural research trials and laboratory-based molecular analysis; it did not involve human participants, human data, or animals, and therefore did not require institutional ethics committee review. Sampling was conducted with permission from the participating agricultural research stations.

Data Availability

Raw 16S rRNA amplicon sequencing data have been deposited in the European Nucleotide Archive under a project accession available from the corresponding author on reasonable request. Soil physicochemical data and R analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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