Assessment of Soil Fungal Diversity under Reduced Tillage Systems Using High-Throughput Sequencing

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ABSTRACT

Background: Soil fungi are central regulators of nutrient cycling, organic matter decomposition, and aggregate stability, making fungal community diversity a key indicator of agroecosystem sustainability. Intensive conventional tillage disrupts fungal hyphal networks and homogenizes microbial habitats, whereas reduced tillage is widely promoted as a conservation practice that preserves soil structure and biological activity.

Objective: This study assessed the influence of reduced tillage on soil fungal community diversity, composition, and structure relative to conventional tillage using high-throughput amplicon sequencing.

Methods: Soil samples (0–15 cm depth) were collected from a two-year field experiment arranged in a randomized complete block design comparing conventional tillage and reduced tillage plots, each replicated four times. Genomic DNA was extracted, and the fungal ITS2 region was amplified and sequenced on the Illumina MiSeq platform. Sequences were processed using DADA2 within QIIME2, taxonomically classified against the UNITE database, and analyzed for alpha diversity (Shannon, Simpson, Chao1, ACE), beta diversity (Bray–Curtis, PCoA, PERMANOVA), and correlation with soil physicochemical properties.

Results: Reduced tillage plots exhibited significantly higher fungal richness and evenness, with Shannon index values averaging 5.42 compared with 4.68 under conventional tillage ($p < 0.05$). *Ascomycota* dominated both treatments, but *Basidiomycota* and *Mortierellomycota* were proportionally enriched under reduced tillage, while beta diversity analysis revealed distinct community clustering by tillage regime (PERMANOVA, $R^2 = 0.31$, $p = 0.002$). Fungal diversity correlated positively with soil organic carbon and moisture content.

Conclusion: Reduced tillage promotes greater fungal diversity and a community composition associated with saprotrophic and mycorrhizal functions beneficial to soil health. High-throughput sequencing provides a robust framework for monitoring tillage-induced shifts in soil fungal communities, supporting its integration into conservation agriculture and precision soil management strategies.

Keywords: Reduced tillage; Soil fungal diversity; ITS2 amplicon sequencing; Illumina MiSeq; QIIME2; UNITE database; Conservation agriculture

1. Introduction

Soil biodiversity underpins the ecological processes that sustain agricultural productivity, including nutrient cycling, organic matter turnover, water retention, and disease suppression [1]. Among the diverse soil biota, fungi occupy a disproportionately influential ecological niche: their extensive hyphal networks bind soil particles into stable aggregates, decompose recalcitrant plant residues, and mediate symbiotic nutrient exchange with plant roots through mycorrhizal associations [2, 3]. Fungal community composition is therefore widely regarded as a sensitive bioindicator of soil health and land management intensity.

Conventional tillage, characterized by repeated mechanical soil inversion, physically disrupts fungal hyphae, accelerates organic matter oxidation, and destabilizes soil aggregates, collectively reducing fungal biomass and diversity [4, 5]. In response, reduced tillage and no-till systems have been promoted within conservation agriculture frameworks as practices that minimize soil disturbance, preserve residue cover, and enhance biological activity [6]. However, the magnitude and consistency of reduced tillage effects on fungal community structure remain variable across soil types, climates, and cropping systems, warranting site-specific molecular investigation [7].

Advances in next-generation sequencing have transformed soil microbial ecology by enabling culture-independent, high-resolution characterization of complex fungal assemblages [8]. High-throughput sequencing of fungal barcode regions, particularly the internal transcribed spacer (ITS), permits simultaneous taxonomic profiling of thousands of operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) per sample, far exceeding the resolution of traditional culture-based or morphological approaches [9, 10].

Coupled with robust bioinformatics pipelines and curated reference databases such as UNITE; these methods allow rigorous quantification of alpha and beta diversity in relation to management practices [11].

Despite growing interest in conservation tillage, few studies directly compare fungal diversity under reduced versus conventional tillage within a controlled, replicated experiment while linking community structure to soil physicochemical properties. This study addresses that gap by (i) characterizing fungal diversity and composition under reduced and conventional tillage using Illumina MiSeq ITS2 sequencing, (ii) evaluating alpha and beta diversity differences, and (iii) identifying relationships between fungal communities and soil properties, thereby informing sustainable tillage management.

2. Related Work

Soil fungal ecology has received increasing attention as molecular tools have matured. Early culture-based surveys substantially underestimated fungal diversity because most soil fungi are unculturable under standard laboratory conditions [12]. The advent of ITS metabarcoding resolved this limitation, establishing ITS1 and ITS2 as the universal fungal barcode regions recommended by the international fungal barcoding consortium [9]. Subsequent studies using Illumina MiSeq and related platforms have generated extensive datasets characterizing fungal communities across agricultural, forest, and grassland soils [13, 14].

Within conservation agriculture research, several investigations have reported that reduced or no-till systems increase fungal biomass and hyphal length relative to conventional tillage, attributing this pattern to preserved residue inputs and undisturbed hyphal networks [15, 16]. Other studies, however, report inconsistent or non-significant diversity differences, suggesting that soil texture, climate, and cropping history modulate tillage effects [17, 18]. Comparative meta-analyses indicate that arbuscular mycorrhizal fungal abundance is particularly responsive to tillage intensity, given the vulnerability of their extraradical hyphae to physical disruption [19].

Bioinformatics pipelines such as QIIME2 and DADA2 have standardized fungal amplicon processing, enabling denoising of sequencing errors and generation of ASVs with single-nucleotide resolution, an improvement over earlier OTU-clustering approaches that inflated diversity estimates [20, 21]. Diversity analyses typically combine alpha indices (Shannon, Simpson, Chao1, ACE) with beta diversity ordination (PCoA, NMDS) and PERMANOVA to partition community variance attributable to treatment [22].

Collectively, prior work establishes that (a) fungal communities are highly sensitive to soil disturbance, (b) high-throughput sequencing is the current gold standard for fungal diversity assessment, and (c) tillage effects are context-dependent. A critical limitation across much of the existing literature is the frequent absence of paired physicochemical data analyzed alongside diversity metrics, which restricts mechanistic interpretation of why fungal communities shift under different tillage regimes. This study integrates soil physicochemical characterization with amplicon sequencing to address this limitation directly. Table 1 summarizes representative prior studies referenced above.

Table 1: Summary of Representative Studies on Tillage Effects on Soil Fungal Communities

Study / Reference	Methodological Approach	Key Reported Finding
Kabir [15]	Field survey of mycorrhizal colonization under tillage	No-till increased arbuscular mycorrhizal fungal colonization relative to conventional tillage
Helgason *et al*. [16]	Molecular survey of hyphal networks	Ploughing disrupted extraradical mycorrhizal hyphal networks
Hontoria *et al*. [17]	Comparative fungal diversity survey across managements	Diversity differences were inconsistent across soil texture and cropping history
Wang *et al*. [18]	Illumina sequencing of topsoil fungal communities	Tillage effects on diversity were context-dependent and non-uniform
Sharma-Poudyal *et al*. [4]	Long-term no-till dryland wheat system survey	No-till was a major driver shaping fungal community structure
Kabir *et al*. [19]	Vertical-distribution sampling of AMF under maize	Extraradical AMF hyphae were highly sensitive to physical soil disruption
Bolyen *et al*. [20] / Callahan *et al*. [21]	QIIME2 / DADA2 bioinformatics pipelines	ASV-based denoising improved resolution over OTU clustering, standardizing amplicon processing

3. Study Area and Experimental Design

The field experiment was conducted over two consecutive cropping seasons at a research farm situated within a semi-arid subtropical agroecological zone characterized by a mean annual temperature of 24.3°C and mean annual precipitation of 780 mm, concentrated primarily during the monsoon period. The predominant soil type was classified as a sandy clay loam (*Inceptisol*) with a pre-experiment pH of 7.2 and organic carbon content of 0.61%. The cropping system followed a maize–wheat rotation representative of regional agricultural practice.

The experiment was arranged in a randomized complete block design (RCBD) with two tillage treatments—conventional tillage (CT), involving disc harrowing and moldboard plowing to a depth of 20–25 cm, and reduced tillage (RT), limited to shallow chisel tillage at 8–10 cm with 30% residue retention—each replicated four times across eight plots measuring 6 m × 8 m. Full experimental parameters, including block arrangement and treatment codes, are summarized in Table 2.

Table 2: Experimental Design, Tillage Treatments, Soil Characteristics, and Sampling Details

Parameter	Details
Location / Climate	Semi-arid subtropical zone; mean annual temp. 24.3°C; mean annual rainfall 780 mm
Soil type	Sandy clay loam (*Inceptisol*)
Cropping system	Maize–wheat rotation
Experimental design	Randomized Complete Block Design (RCBD)
Treatments	Conventional Tillage (CT) vs. Reduced Tillage (RT)
Tillage depth	CT: 20–25 cm (disc harrow + moldboard plow); RT: 8–10 cm (chisel tillage, 30% residue retention)
Plot size	6 m × 8 m
Replications	4 per treatment (8 plots total)
Sampling depth	0–15 cm topsoil
Sampling period	Physiological maturity of wheat crop
Sample preservation	Air-dried (physicochemical); −80°C (molecular analysis)

Composite soil samples were collected at the physiological maturity stage of the wheat crop from the 0–15 cm topsoil layer, corresponding to the zone of maximum root and microbial activity. Within each plot, five soil cores were collected in a zigzag pattern and homogenized to form a single composite sample, yielding 32 samples (2 treatments × 4 replicates × 4 sampling sub-cores pooled per plot, analyzed as biological replicates). Samples were immediately placed on ice, transported to the laboratory within four hours, sieved through a 2 mm mesh, and subdivided for physicochemical analysis (air-dried) and molecular analysis (stored at −80°C until DNA extraction). The overall workflow, from field sampling through bioinformatic analysis, is illustrated in Figure 1.

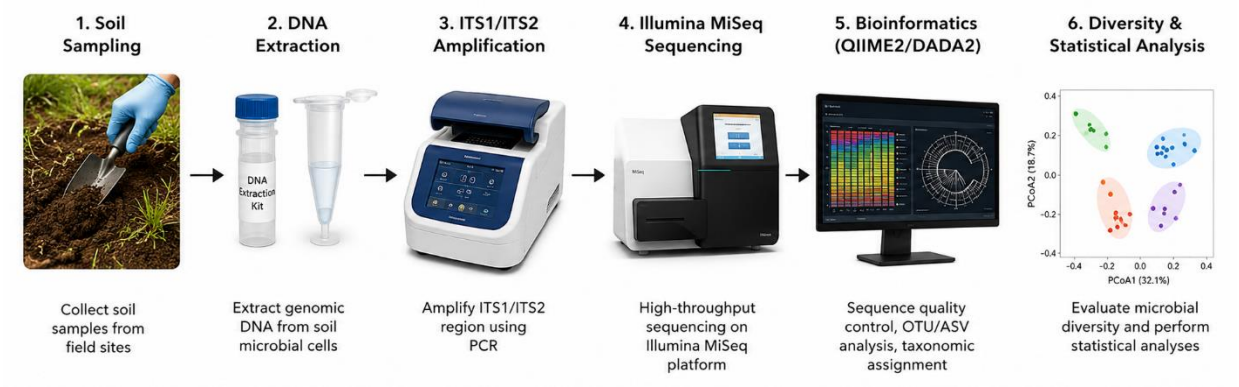

Fig 1: Experimental Workflow for Soil Sampling, DNA Extraction, ITS Amplification, High-Throughput Sequencing, and Bioinformatics Analysis

4. Materials and Methods

4.1. Soil Sampling

Composite sampling followed a standardized zigzag transect within each plot as described above. Five sub-samples per plot were pooled, homogenized, and passed through a 2 mm sieve to remove roots and debris. Each composite sample was divided into three aliquots for physicochemical, molecular, and archival analysis, with molecular aliquots flash-frozen and stored at −80°C prior to DNA extraction.

4.2. Soil Physicochemical Analysis

Soil pH and electrical conductivity were measured in a 1:2.5 soil–water suspension using a calibrated pH/EC meter. Organic carbon was determined by the Walkley–Black wet oxidation method, with organic matter estimated using the conventional conversion factor. Total nitrogen was quantified by the Kjeldahl method, available phosphorus by the Olsen method, and available potassium by flame photometry following ammonium acetate extraction. Soil moisture was determined gravimetrically, and bulk density was measured using the core method.

4.3. DNA Extraction

Genomic DNA was extracted from 0.25 g of homogenized soil using a commercial soil DNA extraction kit employing bead-beating lysis and silica-membrane purification. DNA quality was assessed via agarose gel electrophoresis and A260/280 absorbance ratios, while concentration was quantified fluorometrically. Extracts with ratios between 1.8 and 2.0 were retained for amplification.

4.4. PCR Amplification

The fungal ITS2 region was amplified using primer pair ITS3–ITS4 with Illumina-compatible adapters. PCR was performed in triplicate under: initial denaturation 95°C/3 min; 30 cycles of 95°C/30 s, 55°C/30 s, 72°C/45 s; final extension 72°C/5 min. Amplicons were verified by gel electrophoresis, pooled, and purified using magnetic bead-based purification.

4.5. High-Throughput Sequencing

Purified libraries were indexed with dual-barcoded adapters, quantified, normalized, and pooled equimolarly. Paired-end sequencing (2 × 300 bp) was performed on the Illumina MiSeq platform. Raw reads underwent quality filtering to remove low-quality bases, adapter contamination, and reads shorter than 200 bp.

4.6. Bioinformatics Analysis

Demultiplexed reads were processed in QIIME2 using the DADA2 plugin for quality filtering, denoising, chimera removal, and inference of amplicon sequence variants (ASVs), which offer higher taxonomic resolution than traditional OTU clustering. Taxonomic assignment was performed using a naive Bayes classifier trained against the UNITE reference database

for fungal ITS sequences. Relative abundance tables were generated at phylum, class, order, family, genus, and species levels for comparative analysis between treatments. Table 3 summarizes the key methodological parameters applied across the extraction, amplification, sequencing, and bioinformatics stages, and Figure 2 illustrates the sequential bioinformatics data-processing pipeline.

Table 3: Summary of Molecular and Sequencing Methodology Parameters

Methodological Step	Parameters / Conditions
DNA extraction	0.25 g homogenized soil; commercial kit with bead-beating lysis and silica-membrane purification; A260/280 retained between 1.8–2.0
Target region	Fungal ITS2 region; primer pair ITS3–ITS4 with Illumina-compatible adapters
PCR cycling	95°C/3 min initial denaturation; 30 cycles of 95°C/30 s, 55°C/30 s, 72°C/45 s; final extension 72°C/5 min
Library preparation	Dual-barcoded indexing, fluorometric quantification, equimolar normalization and pooling
Sequencing platform	Illumina MiSeq, paired-end 2 × 300 bp
Quality filtering	Removal of low-quality bases, adapter contamination, and reads < 200 bp
Bioinformatics pipeline	QIIME2 with DADA2 plugin for denoising, chimera removal, and ASV inference; taxonomy via naive Bayes classifier trained on UNITE
Statistical software	R (vegan, phyloseq) and SPSS for statistics; QIIME2, DADA2, USEARCH for sequence processing

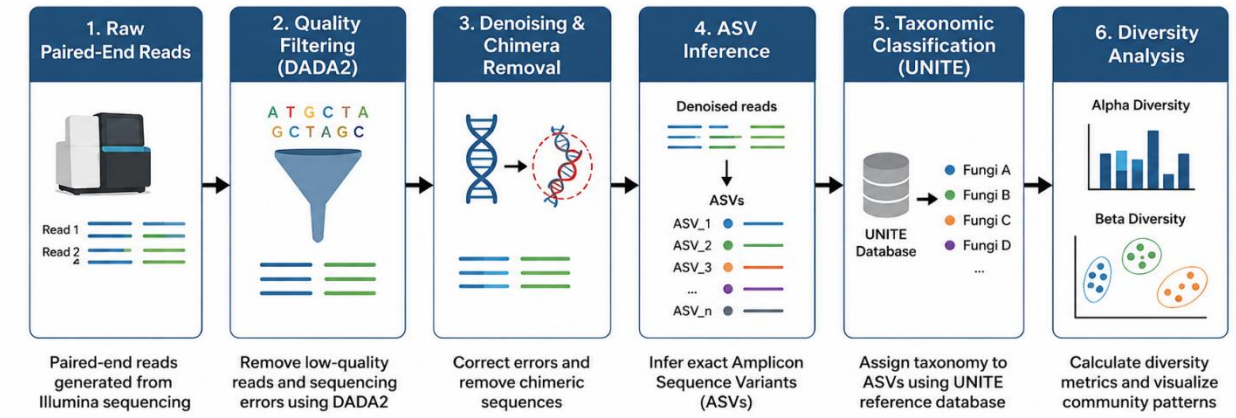

Fig 2: Bioinformatics Data-Processing Pipeline from Raw Reads to Diversity Analysis

4.7. Diversity Analysis

Alpha diversity was assessed using the Shannon diversity index, Simpson index, Chao1 richness estimator, and abundance-based coverage estimator (ACE), calculated on rarefied ASV tables to normalize sequencing depth across samples. Beta diversity was evaluated using Bray–Curtis dissimilarity, visualized through Principal Coordinate Analysis (PCoA) and non-metric multidimensional scaling (NMDS), to assess compositional differences between conventional and reduced tillage communities.

4.8. Statistical Analysis

Differences in physicochemical properties and alpha diversity between treatments were evaluated using one-way ANOVA with Tukey's HSD post-hoc test at $p < 0.05$. Beta diversity was tested using PERMANOVA (999 permutations). Pearson correlation examined relationships between soil properties and diversity indices, while PCA explored multivariate associations among physicochemical variables. Analyses were performed in R (vegan, phyloseq) and SPSS, with sequence processing in QIIME2, DADA2, and USEARCH.

5. Results

5.1. Soil Physicochemical Characteristics and Diversity Indices

Reduced tillage significantly increased soil organic carbon, organic matter, total nitrogen, and moisture content relative to conventional tillage ($p < 0.05$), while bulk density was significantly lower under reduced tillage, reflecting improved soil structure. Soil pH and electrical conductivity did not differ significantly between treatments, indicating that observed fungal community shifts were more plausibly driven by physical and organic matter dynamics than by changes in soil chemistry. Alpha diversity indices were consistently higher under reduced tillage: the Shannon index averaged 5.42 ± 0.21 versus 4.68 ± 0.24 under conventional tillage ($p = 0.006$), while Chao1 richness averaged 612 ± 34 versus 487 ± 29, respectively ($p = 0.011$). These physicochemical and diversity results are summarized together in Table 4.

Table 4: Soil Physicochemical Properties and Fungal Alpha Diversity Indices under Different Tillage Systems (mean ± SE)

Property / Metric	Conventional Tillage	Reduced Tillage	p-value
pH (1:2.5)	7.24 ± 0.08	7.19 ± 0.06	0.412 (ns)
EC (dS/m)	0.34 ± 0.03	0.32 ± 0.02	0.351 (ns)
Organic carbon (%)	0.58 ± 0.04	0.79 ± 0.05	0.008
Total nitrogen (%)	0.062 ± 0.005	0.084 ± 0.006	0.014
Soil moisture (%)	16.8 ± 1.1	21.4 ± 1.4	0.019
Bulk density (g/cm³)	1.48 ± 0.04	1.32 ± 0.03	0.006
Shannon index	4.68 ± 0.24	5.42 ± 0.21	0.006
Chao1 richness	487 ± 29	612 ± 34	0.011
ACE index	502 ± 31	628 ± 30	0.009

5.2 Sequencing Output

High-throughput sequencing generated a total of 1,842,360 raw reads across 32 samples, of which 1,516,904 high-quality reads (82.3%) were retained after quality filtering and chimera removal, corresponding to an average of 47,403 reads per sample. A total of 3,286 fungal ASVs were identified across all samples.

5.3 Taxonomic Composition

Ascomycota was the dominant phylum in both treatments, accounting for 58.4% of relative abundance under conventional tillage and 46.2% under reduced tillage, while *Basidiomycota* and *Mortierellomycota* showed proportional enrichment under reduced tillage (27.8% and 14.6%, respectively) compared with conventional tillage (21.7% and 8.9%), as shown in Figure 3. At the genus level, saprotrophic taxa such as *Mortierella* and *Trichoderma*, along with putative beneficial genera including *Glomus*, were more abundant under reduced tillage, whereas potentially pathogenic *Fusarium*-affiliated taxa were relatively enriched under conventional tillage, consistent with reported associations between soil disturbance and opportunistic fungal proliferation.

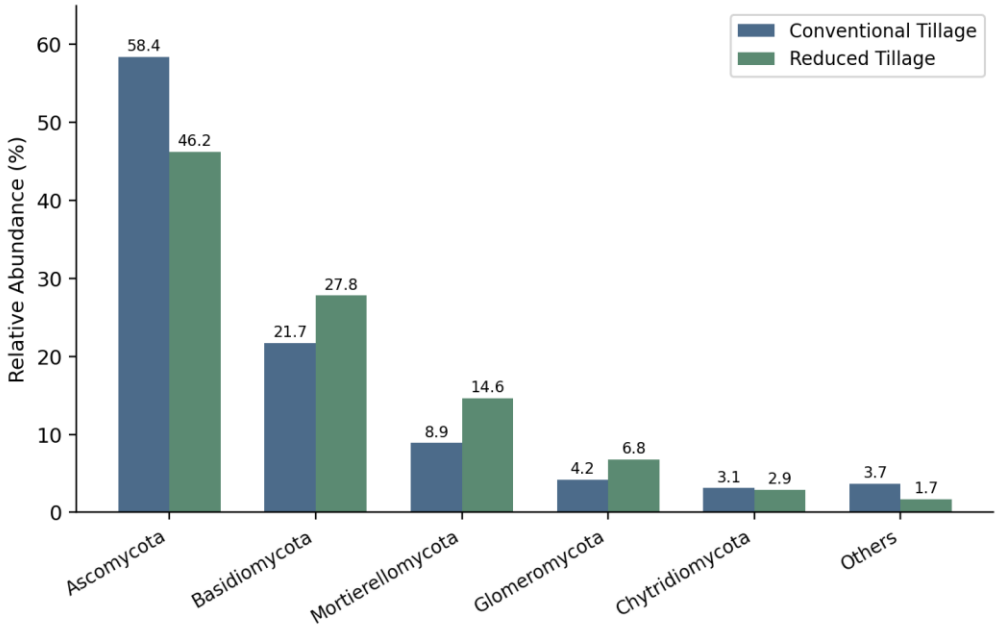

Fig 3: Relative Abundance of Major Soil Fungal Phyla under Conventional and Reduced Tillage Systems

5.4. Beta Diversity and Community Structure

Principal Coordinate Analysis based on Bray–Curtis dissimilarity revealed clear separation between conventional and reduced tillage fungal communities along the first ordination axis, which explained 34.7% of total community variance, with the second axis explaining an additional 18.2%. PERMANOVA confirmed that tillage treatment significantly influenced fungal community composition ($R^2 = 0.31$, $p = 0.002$), while within-treatment dispersion did not differ significantly, supporting a genuine compositional shift rather than an artifact of heterogeneous variance.

5.5. Correlation with Soil Properties

Pearson correlation indicated significant positive associations between the Shannon index and soil organic carbon ($r = 0.68$, $p < 0.01$), total nitrogen ($r = 0.59$, $p < 0.01$), and moisture ($r = 0.54$, $p < 0.05$), while bulk density was negatively correlated with richness ($r = -0.61$, $p < 0.01$). PCA of physicochemical variables showed organic carbon and bulk density loading strongly on the first component, aligning with the tillage-based separation in the fungal ordination, reinforcing the link between soil structure and fungal diversity.

6. Discussion

The results demonstrate that reduced tillage significantly enhances soil fungal diversity and reshapes community composition relative to conventional tillage, corroborating previous reports linking minimal soil disturbance to preserved hyphal networks and elevated fungal biomass [15, 16]. The proportional enrichment of *Basidiomycota* and *Mortierellomycota* under reduced tillage is ecologically significant, as taxa within these groups include efficient decomposers of complex organic substrates and contributors to

soil aggregate stabilization, processes central to long-term carbon sequestration [23].

The positive correlation between fungal diversity and soil organic carbon supports the hypothesis that reduced tillage fosters a self-reinforcing cycle in which preserved organic inputs sustain fungal decomposer communities, which in turn enhance humification and aggregate formation, further protecting organic matter from mineralization [24]. The negative association between bulk density and diversity indices similarly underscores the sensitivity of fungal hyphal networks to soil compaction and physical disruption, consistent with mechanistic explanations proposed in earlier tillage studies [17].

These findings extend previous meta-barcoding studies by explicitly linking beta diversity ordination patterns to measured physicochemical gradients, addressing a limitation noted in the related work section. The relative enrichment of *Fusarium*-affiliated taxa under conventional tillage warrants attention from a plant health perspective, as elevated abundance of potentially pathogenic genera may increase disease pressure, reinforcing the agronomic value of reduced tillage beyond diversity metrics alone [18].

Nonetheless, several limitations merit consideration. Amplicon sequencing captures relative rather than absolute abundance and cannot fully resolve functional activity, requiring complementary metatranscriptomic or enzyme-based approaches [25]. Classification accuracy remains constrained by incomplete reference-database representation of soil fungal taxa, particularly underexplored genera. Sampling was restricted to a single phenological stage across two seasons, limiting inference on seasonal variation. Future research should incorporate multi-seasonal sampling, metagenomic and metatranscriptomic profiling, and integration with precision agriculture platforms for real-time, site-specific biological monitoring supporting adaptive tillage management [26].

7. Conclusion

This study demonstrates that reduced tillage significantly enhances soil fungal diversity and favorably restructures fungal community composition relative to conventional tillage, as revealed through high-throughput ITS2 amplicon sequencing. Reduced tillage plots exhibited higher richness and evenness, proportional enrichment of decomposer and potentially beneficial fungal taxa, and distinct community clustering strongly associated with elevated soil organic carbon and reduced bulk density. These findings confirm that high-throughput sequencing offers a powerful, culture-independent framework for detecting management-induced shifts in soil fungal communities with a resolution unattainable through conventional methods. From an applied perspective, the results support the integration of reduced tillage into conservation agriculture programs as a means of enhancing soil biological health, nutrient cycling capacity, and long-term carbon storage potential. Soil health management strategies should incorporate fungal community monitoring as a biological indicator alongside conventional physicochemical assessments. Future work employing metagenomics, metatranscriptomics, and multi-year, multi-site field experiments will be essential to establish the temporal stability and broader generalizability of these findings, ultimately guiding evidence-based, sustainable soil management in diverse agroecological contexts.

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