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Impact of a Single Lignite Humic Acid Application on Soil Properties and Microbial Dynamics in Aeolian Sandy Soils: A Fourth-Year Study in Semi-Arid Inner Mongolia

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Abstract: Humic acid (HA) is considered a promising soil amendment for improving soil fertility. However, the effects of HA application on the microbial community, especially in aeolian sandy soils of semi-arid regions, remain insufficiently elucidated. To address this gap, a field experiment was conducted to investigate the changes in soil properties, bacterial and fungal diversity, and community structure in a buckwheat field in the fourth year after a single application of lignite humic acid (L-HA) at 0 (L-HA0), 2 (L-HA1), 4 (L-HA2), and 6 (L-HA3) ton·ha^{-1} in an aeolian sandy soil in Inner Mongolia, China. The results demonstrated that four years after L-HA application, there was a significant ($p < 0.05$) decrease in soil pH, accompanied by an increase in soil water content and nutrient levels, including organic matter and total N, available P, and K. Additionally, the application of L-HA enhanced microbial biomass C and N and stimulated enzyme activities, such as urease and invertase, with these effects being more pronounced at higher application rates (L-HA2 and L-HA3). However, HA addition did not significantly ($p < 0.05$) affect soil microbial biomass P or alkaline phosphatase activity. The L-HA amendment enhanced the α-diversity indices of soil bacteria but did not significantly ($p < 0.05$) affect soil fungal diversity. The addition of L-HA induced significant changes in the composition of the soil microbial community at both the phylum and genus levels, with significant variability in microbial responses observed across the different L-HA application rates. The incorporation of L-HA notably enriched the composition of bacterial and fungal communities at the phylum level, particularly those involved in carbon cycling, including the bacterial phyla *Proteobacteria* and *Actinobacteriota* and the fungal phyla Ascomycota and Rozellomycota. At the genus level, higher L-HA application rates, specifically L-HA2 and L-HA3, exerted statistically significant ($p < 0.05$) effects on most bacterial and fungal genera. Specifically, these treatments increased the abundance of bacterial genera, such as *Rokubacterium* and fungal genera, including *Plectosphaerella*, *Tausonia*, *Talaromyces*, and *Clonostachys*. Conversely, the relative abundance of the bacterial genera *Vicinamibacter* and *Subgroup_7*, as well as the fungal genus *Niesslia*, was significantly reduced. Redundancy analysis (RDA) indicated that bacterial community compositions were closely associated with soil parameters, such as available P (AP), microbial biomass carbon (SMC), microbial biomass nitrogen (SMN), microbial biomass phosphorus (SMP), and invertase, while all tested soil parameters, except for alkaline phosphatase, significantly influenced the fungal community structure. Given that the changes in these soil parameters were highly correlated with the amounts of L-HA addition, this suggests that the impacts of long-term L-HA amendment on the soil bacterial and fungal communities were linked to alterations in soil physicochemical and biological properties.

Keywords: lignite humic acid; soil microorganisms; buckwheat field; aeolian sandy soil

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1. Introduction

It is proposed that the global population will increase by 3.7 billion, reaching 9.7 billion by around 2050. This substantial population growth is expected to lead to a 70% increase in food demand from the current level, which will exert pressure to expand agricultural land [1]. Moreover, climate and environmental changes, coupled with the shrinking area of cultivated land, underscore the urgent need to improve the utilization of marginal soils, such as sandy soils, to address the shortage of cultivable land [2]. Globally, sandy soils cover approximately 4.99×10^9 ha, accounting for 31% of the total land area [3]. In China alone, sandy soils cover approximately 1.7×10^8 ha [4]. Therefore, effectively utilizing sandy soils represents a promising approach to expand cultivable land globally.

Due to the increasing population and climate change, there is great pressure to increase food production in China. Sandy soil areas in China are crucial for both food production and food security. The Horqin sandy land is one of the four largest sandy lands in China, occupying 5.06×10^4 km^2 in northeast China. It lies in the semi-arid agropastoral transitional zone of Inner Mongolia [5]. In the process of improving sandy soils, the addition of organic substances is a sustainable approach for enhancing their properties and productivity [6].

Humic acid (HA) is a complex organic substance derived from the microbial decomposition and transformation of plant and animal residues under both aerobic and anaerobic conditions, as well as through various geochemical processes [7,8]. As an important constituent of soil organic matter (SOM), HA, with its rich functional groups and complex chemical structures, is recognized as a crucial component of healthy and fertile soil [9]. HA influences soil through its physical, chemical, and biological effects, which contribute to soil aggregate formation, enhance cation exchange capacity, regulate soil pH, and improve soil water retention, nutrient retention, aeration capacity, and microbial activity [7,10]. Despite the significant roles of HA in soil remediation and improvement [11,12], there is limited research on soil microbial communities in aeolian sandy soils, particularly in arid–semiarid regions.

Soil is a complex and dynamic ecosystem in which biochemical processes are carried out with the participation of soil microorganisms. Soil microorganisms represent vital components of soil ecosystems, serving as key indicators for assessing soil quality and fertility [13]. Soil bacteria are indispensable to ecosystem functioning, playing key roles in organic matter transformation, nutrient cycling, and energy flow. These processes are critical for maintaining soil health and sustainability, supporting plant growth, and ensuring ecosystem resilience and productivity [14]. Soil fungi are a crucial component of soil microbial communities and serve as key indicators of ecosystem health due to their roles in nutrient cycling, organic matter decomposition, and symbiotic relationships with plants. Many soil fungi, particularly mycorrhizal fungi, depend on vegetation for their carbon (C) supply, obtained through symbiotic relationships with plant roots [15]. Soil management practices, such as the use of organic materials, directly influence the abundance, diversity, and composition of soil microbial communities [16]. Organic substances' addition could directly alter the physicochemical and microbiological characteristics of soil, thereby influencing soil microorganisms [9,17]. HA addition in a continuous cropping peanut field decreased the abundance of bacteria while increasing the abundance of fungi [18]. Bacterial and fungal communities are significantly influenced by various environmental changes, such as temperature fluctuations, moisture levels, and soil pH. These microbial communities are crucial indicators of soil health and ecosystem stability [19]. However, due to the complexity of microbes, the use of HA on soil bacterial and fungal community composition remains unclear, especially in an aeolian sandy soil.

Although numerous studies have extensively evaluated the impact of HA and HA-related fertilizer addition on soil properties, plant growth, and crop yields, such as those conducted by Rekaby et al. [20], Al-Fraihat et al. [21], and Li et al. [18], there is a gap in the understanding of the effects of HA on soil microbial community, especially in aeolian sandy soil in arid–semi-arid regions in China. Buckwheat (*Fagopyrum esculentum* Moench) is a common cultivated crop with a long history of cultivation in China, and Inner Mongolia

is a major buckwheat production area, particularly in its northeastern region [22]. Due to its tolerance of low-fertility soils and significant health benefits, it is widely cultivated in semi-arid regions, and it has been in high demand in China in recent years [23]. As a result of overcultivation, excessive fertilizer application, and intensified farming practices, the soil in regions where common buckwheat is grown has degraded. Therefore, it is essential to find and implement environmentally sustainable methods to boost soil fertility and productivity, thereby maintaining and improving soil health. In this study, a four-year field experiment was established to evaluate the impact of varying lignite humic acid (L-HA) application rates (0, 2, 4, and 6 ton·ha^{-1}) on soil physicochemical and biological properties, as well as on bacterial and fungal communities within a buckwheat field. We hypothesized that L-HA application would improve the properties of aeolian sandy soil and modify microbial α-diversity and community composition.

2. Materials and Methods

2.1. Study Sites

The field experiment was initiated in July 2020 at the Xiliaohe village, Tongliao, Inner Mongolia, China (43°44′ N, 122°24′ E). The region has a temperate continental monsoon climate characterized by a mean annual temperature of 6.4 °C, a mean annual precipitation of 400 mm, and a frost-free period lasting approximately 150 days. The soil is classified as aeolian sandy soil. The basic properties of the soil (0–20 cm) were as follows: pH 8.23, soil organic matter 7.46 g·kg^{-1}, ammonium N (NH_4^+-N) 13.25 mg·kg^{-1}, nitrate N (NO_3^--N) 78.31 mg·kg^{-1}, available P 7.12 mg·kg^{-1}, and available K 81.44 mg·kg^{-1}. The local daily mean temperature and daily precipitation during the growing seasons of 2020, 2021, 2022, and 2023 were recorded and are presented in Figure S1.

2.2. Lignite Humic Acid and Field Experiment

Lignite humic acid (L-HA) was purchased from Songmo Technology Co., Ltd., Langfang, China. The raw material is weathered coals, a black powder with a humic acid content of ≥70%, a fulvic acid content of 15% to 20%, an ash content of 10%, and a pH of 6.5. The lignite humic acid contains 628.74 g·kg^{-1} organic carbon, 12.3 g·kg^{-1} total N, 1.14 g·kg^{-1} total P, and 113.58 g·kg^{-1} total K.

The experiment was carried out in a completely randomized design with four L-HA treatments, namely, L-HA0 (0 ton·ha^{-1}), L-HA1 (2 ton·ha^{-1}), L-HA2 (4 ton·ha^{-1}), and L-HA3 (6 ton·ha^{-1}). The experiment began in July 2020, and L-HA was applied uniformly to the top 15 cm of soil at one time and then thoroughly mixed into the soil using a rototiller over five passes to achieve uniform integration of the amendments. Each treatment had three replicates (3 m × 10 m), and plots were separated by a ridge (30 cm wide and 10 cm high) to prevent water and nutrient runoff. All of the field plots were annually planted with common buckwheat (cv. Tongqiao No. 5) at a rate of 45 kg·hm^{-2} in July and harvested in September from 2020 to 2023. Each year, all of the experimental plots received 36 kg·ha^{-1} N, 40 kg·ha^{-1} P_2O_5, and 81 kg·ha^{-1} K_2O as the base fertilizers. These were applied once before buckwheat sowing per year. All other aspects of field management, including weeding and irrigation, were applied consistently across all treatments in accordance with local cultivation practices. After harvest, the buckwheat straw was uniformly removed from the field in all treatments.

2.3. Soil Sampling and Analysis

On 13 August 2023 (buckwheat flowering stage), soils were sampled from buckwheat rhizosphere and bulk soil at a depth of 0–20 cm after removing surface litter. Rhizosphere soil samples were collected by gently shaking off loosely attached soil from the roots and then removing the soil attached to the roots with a soft sterile brush [24]. Any visible roots and debris were removed and subsequently passed through a 2 mm sieve to combine the replicate samples into a composite sample. A total of four replicate samples were selected for each treatment, resulting in 16 samples for the rhizosphere and 16 samples for the

bulk soil. For the bulk soil, the soil sample was divided into two parts: one part was stored at 4 °C for measurements of soil microbial biomass carbon (SMC), nitrogen (SMN), phosphorus (SMP), and enzyme activity, and the remaining portion was air-dried in the shade at room temperature for two weeks to assess soil physicochemical properties. For the rhizosphere soil, the soil sample was promptly frozen in dry ice and transferred to −80 °C for DNA analysis.

Soil physicochemical properties were assessed following established methodologies. Briefly, the soil pH was determined using a pH meter at a soil/water ratio of 1:2.5 (*w*/*v*) [25]. The soil water content (SWC) was measured by oven-drying at 105 °C for 24 h to a constant weight [5]. The soil organic carbon content (SOC) was quantified using a potassium dichromate oxidation method [26]. The total nitrogen content (TN) was analyzed using the Kjeldahl method [26]. The soil available phosphorus content (AP) was assessed using the Olsen method [27]. The available potassium content (AK) was measured via flame photometer following extraction with ammonium acetate [28]. The soil microbial biomass carbon (SMC), soil microbial biomass nitrogen (SMN), and soil microbial biomass phosphorus (SMP) were measured using the fumigation extraction method [29–31]. Three soil extracellular enzymes—specifically, soil invertase (EC 3.2.1.26), urease (EC 3.5.1.5), and alkaline phosphatase (EC 3.1.3.1)—were assayed using the following methods: urease activity was assessed using the sodium phenate–sodium hypochlorite colorimetric method, invertase activity was determined via the 3, 5-dinitrosalicylic acid colorimetric assay, and alkaline phosphatase activity was measured using disodium phenyl phosphate colorimetry, as outlined by Zhou et al. [32] and Guan et al. [33].

2.4. DNA Extraction and Sequencing

Soil DNA was extracted from 0.25 g of soil using the OMEGA Soil DNA Kit (M5636-02) from Omega Bio-Tek, Norcross, GA, USA, following the manufacturer's instructions, and subsequently stored at −20 °C for future use. The quantity and quality of the extracted DNA samples were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. Bacterial and fungal communities were assessed using high-throughput sequencing of the 16S rRNA gene and an internal transcribed spacer (ITS), respectively. PCR amplification targeting the V3–V4 region of soil bacterial 16S rRNA genes was conducted using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [34], while the soil fungal ITS region was amplified using ITS1F with specific primers (ITS5-1737F: 5′-GGAAGTAAAAGTCGTAACAAGG-3′; ITS2-2043R: 5′-GCTGCGTTCTTCATCGATGC-3′) [35]. Subsequently, the amplicons underwent purification, quantification, equimolar pooling, and sequencing on the Illumina NovaSeq platform with the NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The raw sequencing data were deposited into the NCBI database (Accession Number: PRJNA1134377).

2.5. Bioinformatic Analysis

Sequence data analyses were primarily conducted using QIIME2, with minor adaptations according to the official tutorials (https://docs.qiime2.org/2021.8/tutorials/, accessed on 15 December 2023). The cutadapt plugin was used to remove the primers, and the DADA2 plugin was applied for quality filtering, denoising, merging, and removal of chimeric sequences from pre-processed data [36,37]. Sequences exhibiting 100% similarity were subsequently clustered into amplicon sequence variants (ASVs) [38]. Taxonomic assignments for bacteria and fungi utilized SILVA Release 132 and UNITE Release 8.0, respectively [39].

2.6. Statistical Analysis

Data analysis was conducted using R version 4.2.2 (https://cran.dcc.uchile.cl/, accessed on 1 June 2024) and visualized using both R version 4.2.2 and Origin Pro 2021

software (Origin Lab, Northampton, MA, USA). A Venn diagram was created using the R package 'VennDiagram' (version 1.7.3) to visualize the shared and unique ASVs across samples or groups [40]. Diversity indices at the ASV level, including the Chao1 richness estimator [41], observed species, Shannon diversity index [42], and Simpson index [43], were computed using the ASV table in QIIME2 and presented as box plots. Differences in soil properties, α-diversity, and microbial community composition were assessed using one-way ANOVA. β-diversity, comprising permutational multivariate analysis of variance (PERMANOVA) analysis [44] and principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarities [45] at the ASV level, were calculated using the 'vegan' package (version 2.6-8). Redundancy analysis (RDA) [46] was employed to examine the relationship between microbial community composition and relevant environmental factors using R with the 'vegan' package.

3. Results

3.1. Soil Properties

The application of L-HA significantly ($p < 0.05$) altered the properties of aeolian sandy soil, except for the SMP content and alkaline phosphatase activity (Table 1). Notably, regarding the pH and SWC, only the L-HA3 treatment induced significant changes, resulting in a reduction of 0.15 units in pH and an increase of 33.9% in SWC compared to non-L-HA-amended soils. L-HA2 and L-HA3 treatments significantly elevated the soil SOC content by 7.48% and 12.70%, respectively, compared to L-HA0. The TN content exhibited a significant increase under the L-HA2 treatment, whereas other treatments showed only minor alterations in response to L-HA addition. For available nutrients, higher rates of L-HA application significantly enhanced AP and AK contents, with increases up to 17.48% and 8.78%, respectively, compared to the control. The SMC content significantly ($p < 0.05$) rose with increasing L-HA addition rates, ranging from 8.91% to 45.77% over the control. A similar upward trend was observed for SMN and invertase activity across various L-HA treatments, although no significant differences were detected between L-HA2 and L-HA3 treatments. Soil urease activity was 6.17% to 23.42% higher in L-HA-treated soils relative to untreated soils.

3.2. Soil Bacterial and Fungal Community Diversity

Based on the Venn diagram (Figure 1a), there were 2197 core ASVs among the four treatments for bacterial ASVs. A gradual increase in the number of unique ASVs was observed with increasing rates of L-HA addition, with 8624, 9790, 9869, and 10,526 unique ASVs in L-HA0, L-HA1, L-HA2, and L-HA3, respectively. Compared to L-HA0, the addition of L-HA significantly elevated the Chao1 and observed species indices; however, no significant differences were detected among the L-HA1, L-HA2, and L-HA3 treatments. The Simpson index remained unaffected by L-HA application. Notably, L-HA1 and L-HA3 treatments resulted in a significant increase in the Shannon index (Figure 2a).

The PCoA using Bray–Curtis dissimilarity was employed to evaluate the similarities and differences in bacterial β-diversity across all treatments (Figure 3a). PCoA1 and PCoA2 explained 10.4% and 8.4%, respectively, of the total variation among the treatments. All treatments were clearly separate from each other, indicating differences in the bacterial community among the treatments. Furthermore, PERMANOVA analysis revealed a significant difference in bacterial community structure among the treatments ($p < 0.05$) (Table 2).

Table 1. Effects of different amounts of L-HA addition on soil physicochemical and microbiological properties.

Treatment	pH	SWC (%)	SOC ($g \cdot kg^{-1}$)	TN ($g \cdot kg^{-1}$)	AP ($mg \cdot kg^{-1}$)	AK ($mg \cdot kg^{-1}$)	SMC ($mg \cdot kg^{-1}$)	SMN ($mg \cdot kg^{-1}$)	SMP ($mg \cdot kg^{-1}$)	Urease ($mg \cdot g^{-1} \cdot 24\ h^{-1}$)	Invertase ($mg \cdot g^{-1} \cdot 24\ h^{-1}$)	Alkaline Phosphatase ($mg \cdot g^{-1} \cdot 24\ h^{-1}$)
L-HA0	8.20 ± 0.08 a	8.14 ± 0.62 b	4.41 ± 0.07 c	0.43 ± 0.01 b	8.29 ± 0.15 b	84.45 ± 1.09 b	118.43 ± 4.80 d	6.88 ± 0.14 b	4.15 ± 0.06 a	150.22 ± 4.37 d	1.46 ± 0.03 c	8.30 ± 0.14 ab
L-HA1	8.18 ± 0.04 a	8.29 ± 0.43 b	4.52 ± 0.07 c	0.45 ± 0.02 b	8.33 ± 0.16 b	83.56 ± 2.30 b	128.98 ± 3.55 c	6.87 ± 0.27 b	4.25 ± 0.03 a	170.55 ± 6.20 b	1.52 ± 0.03 b	8.20 ± 0.18 b
L-HA2	8.13 ± 0.02 ab	8.91 ± 0.26 b	4.74 ± 0.07 b	0.48 ± 0.01 a	8.97 ± 0.14 a	86.14 ± 0.85 b	155.17 ± 4.09 b	8.05 ± 0.09 a	4.18 ± 0.03 a	159.49 ± 2.63 c	1.61 ± 0.04 a	8.46 ± 0.09 ab
L-HA3	8.05 ± 0.02 b	10.9 ± 0.72 a	4.97 ± 0.04 a	0.45 ± 0.01 b	8.75 ± 0.11 a	91.87 ± 1.31 a	172.64 ± 3.33 a	8.18 ± 0.04 a	4.28 ± 0.13 a	185.40 ± 4.26 a	1.62 ± 0.01 a	8.53 ± 0.27 a

Mean (SE) with ANOVA results ($n = 4$). Different letters in each column represent significant differences between different treatments ($p < 0.05$). L-HA0, L-HA1, L-HA2, and L-HA3 represent L-HA application rates at 0, 2, 4, and 6 $ton \cdot ha^{-1}$, respectively. SWC: soil water content; SOC: soil organic carbon; TN: total nitrogen; AP: available phosphorus; AK: available potassium; SMC, SMN, and SMP represent soil microbial biomass carbon, nitrogen, and phosphorus, respectively.

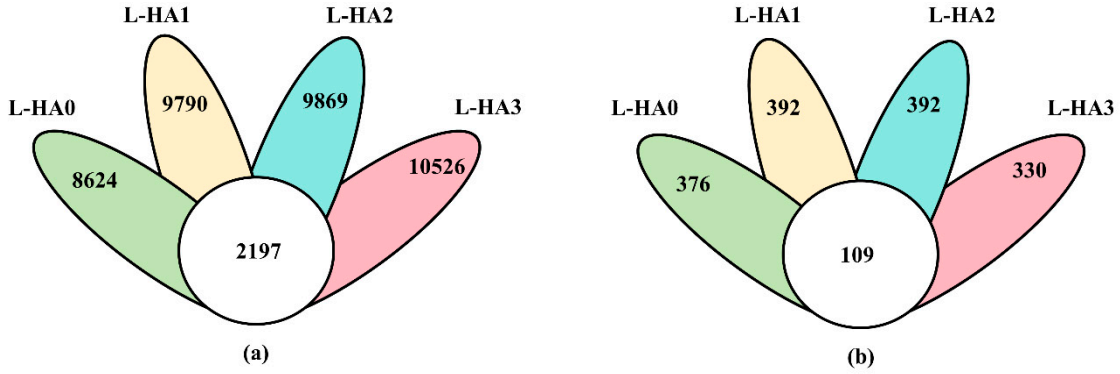

Figure 1. Venn diagram illustrating unique and shred amplicon sequence variations (ASVs) of bacterial (**a**) and fungal (**b**) communities across the different L-HA treatments. L-HA0, L-HA1, L-HA2, and L-HA3 represent L-HA application rates at 0, 2, 4, and 6 ton·ha^{-1}, respectively.

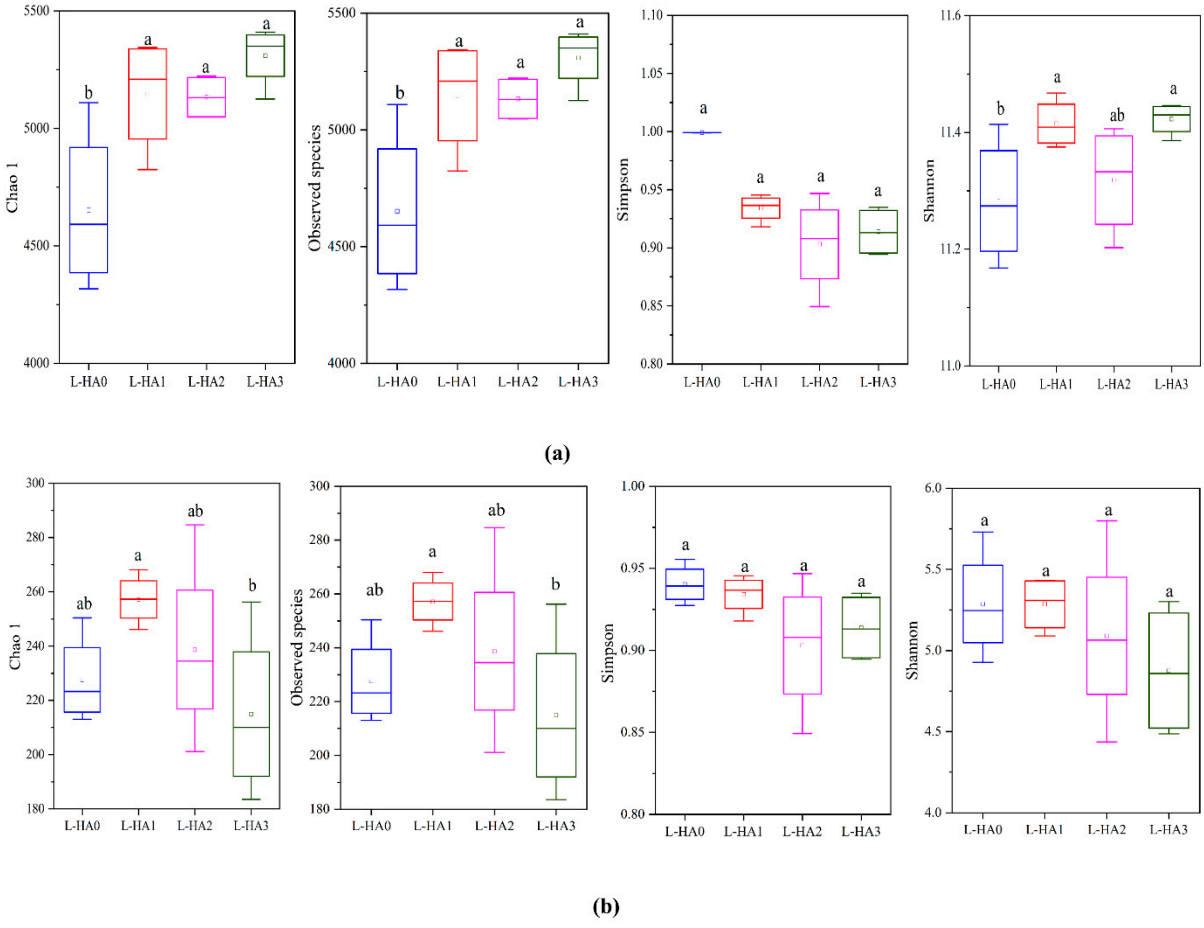

Figure 2. Alpha diversity (α-diversity) of soil bacterial (**a**) and fungal (**b**) communities under different L-HA treatments. Data are means ± SE (n = 4). In each plot, different lowercase letters show statistically significant differences ($p < 0.05$). L-HA0, L-HA1, L-HA2, and L-HA3 represent L-HA application rates at 0, 2, 4, and 6 ton·ha^{-1}, respectively.

For fungal ASVs (Figure 1b), the four treatments shared 109 ASVs. The highest number of unique ASVs was 392 in both the L-HA1 and L-HA2 treatments, while the L-HA3 treatment exhibited the lowest unique number of ASVs, with 330. Compared to L-HA0, the addition of L-HA did not affect the α-diversity (i.e., Chao1, observed species,

Simpson index, and Shannon index) of fungi. However, the L-HA3 treatment demonstrated lower Chao1 and observed species compared to L-HA1 (Figure 2b).

PCoA1 (Axis1) and PCoA2 (Axis2) explained 17.4% and 13.7% of the total variance, respectively. All four treatments exhibited statistically significant differences from each other (Figure 3b, Table 2), indicating that the fungal community structure significantly differed among the L-HA treatments ($p < 0.05$).

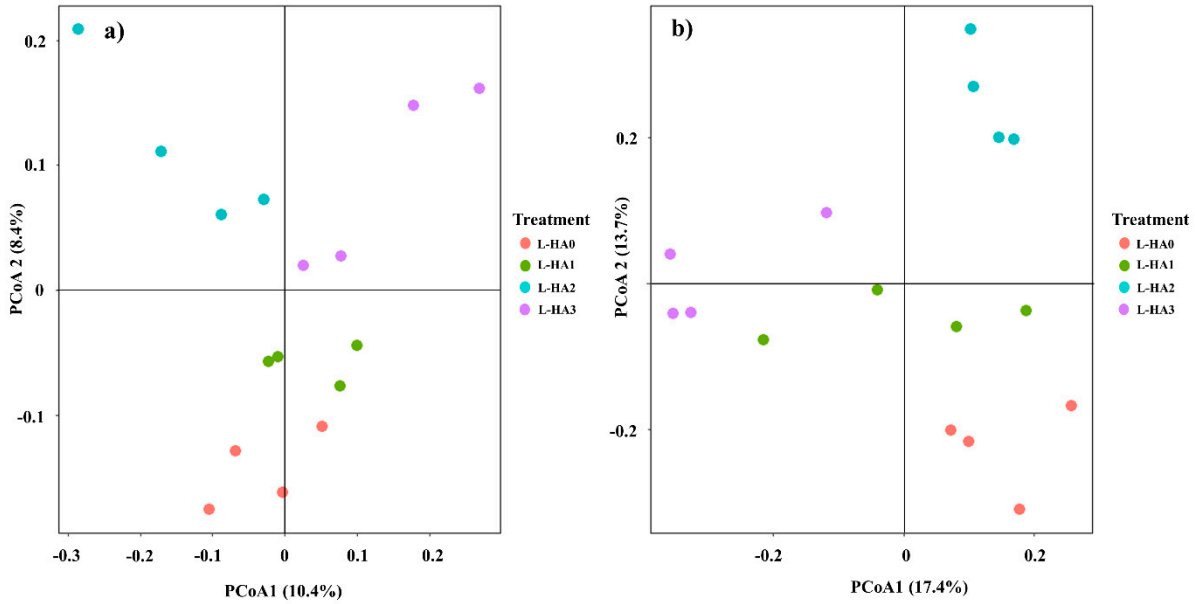

Figure 3. Principal coordinate analysis (PCoA) of bacterial (**a**) and fungal (**b**) communities in different L-HA treatments based on the Bray–Curtis distances. L-HA0, L-HA1, L-HA2, and L-HA3 represent L-HA application rates at 0, 2, 4, and 6 ton·ha^{-1}, respectively.

Table 2. PERMANOVA analysis for the relative bacterial abundance of bacteria and fungi.

PERMANOVA	*p*-Value	
	Bacteria	Fungi
L-HA0 vs. L-HA1	0.025	0.033
L-HA0 vs. L-HA2	0.028	0.037
L-HA0 vs. L-HA3	0.029	0.028
L-HA1 vs. L-HA2	0.032	0.031
L-HA1 vs. L-HA3	0.032	0.022
L-HA2 vs. L-HA3	0.021	0.024

3.3. Bacterial and Fungal Community Composition

L-HA application induced significant changes in the relative abundance of soil bacterial and fungal taxa at both the phylum and genus levels (Figure 4). The dominant bacterial phyla, including *Proteobacteria* (23.95–32.47%), *Acidobacteriota* (15.03–22.33%), *Actinobacteriota* (13.25–17.08%), *Gemmatimonadota* (9.73–13.62%), *Chloroflexi* (6.67–8.86%), and *Bacteroidota* (3.92–7.34%), accounted, in total, for over 85% of the bacterial sequences across all treatments (Figure 4a). Compared to L-HA0, the L-HA2 treatment significantly increased the abundance of *Actinobacteriota* while significantly decreasing the abundance of *Acidobacteriota* (Figure S2a). *Proteobacteria* and *Bacteroidota* were least abundant in L-HA3, with significantly lower values than in L-HA2 (Figure S2a). Stable alterations were observed in the community composition of fungi at the phylum level across the four treatments (Figure 4b). The fungal community was dominated by four main phyla: Ascomycota (49.92–80.64%), Basidiomycota (13.9–35.42%), Rozellomycota (1.69–21.26%), and Mortierellomycota (0.92–6.80%) (Figure 4b). The L-HA1 treatment exhibited the highest relative abundance of Ascomycota (74.04%), whereas the L-HA3 treatment showed the lowest

(54.85%). Additionally, L-HA3 significantly increased the relative abundance of Rozellomycota while concomitantly decreasing the relative abundance of Mortierellomycota (Figure S3a).

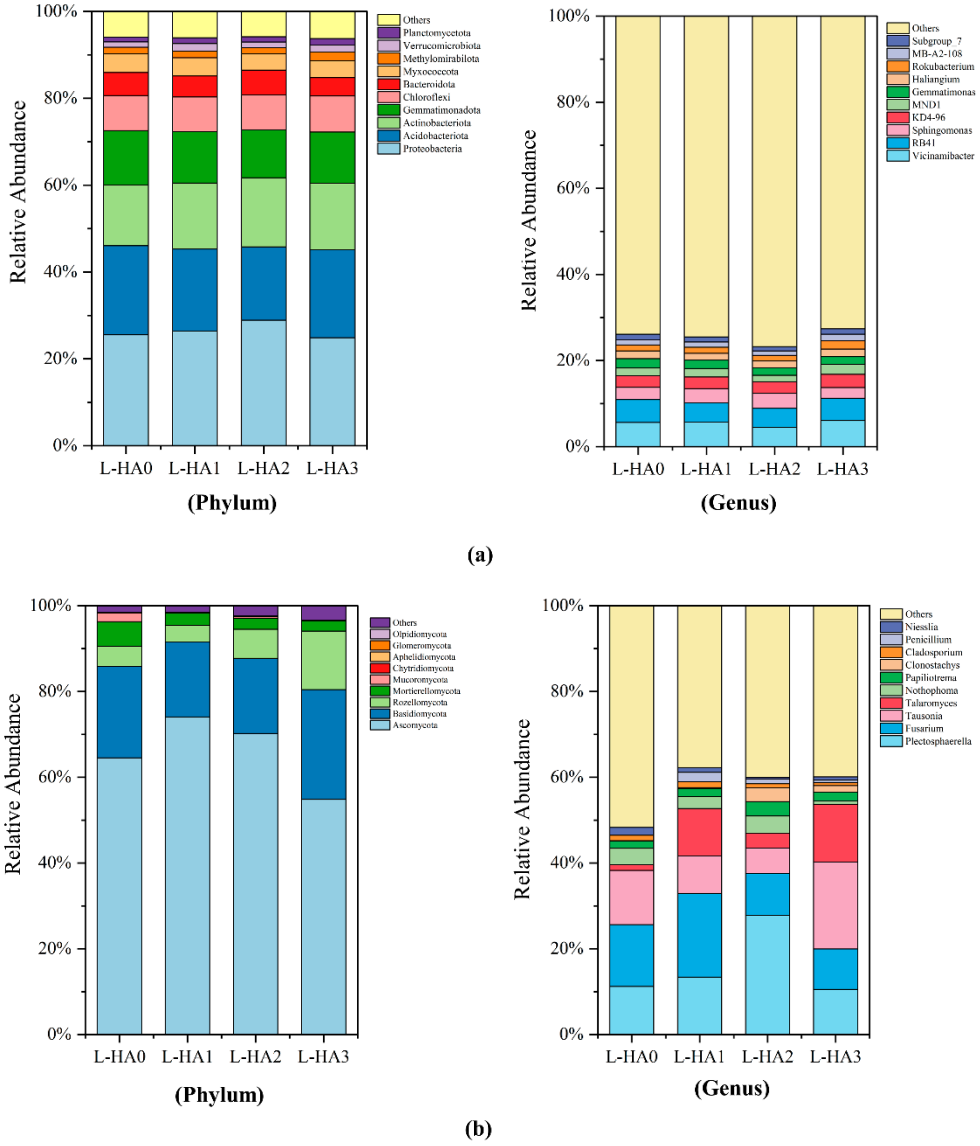

Figure 4. Bacterial (**a**) and fungal (**b**) relative abundance at the phylum and genus levels. L-HA0, L-HA1, L-HA2, and L-HA3 represent L-HA application rates at 0, 2, 4, and 6 ton·ha^{-1}, respectively.

Regarding the bacterial community composition at the genus level (Figure 4a), approximately 75% of the genera were identified, with *Vicinamibacter*, *RB41*, *Sphingomonas*, and *KD4-96* being the dominant taxa. There were no significant differences among the four treatments, except for *Vicinamibacter*, which exhibited lower abundance in the L-HA2 treatment compared to the others (Figure S2b). Generally, the L-HA3 treatment demonstrated the highest relative abundances, notably for genera like *MND1*, *Rokubacterium*, and *MB-A2-108* (Figure S2b). Significant alterations in the fungal community composition at the genus level were observed following L-HA addition (Figure 4b). The L-HA2 treatment showed the highest relative abundances of *Plectosphaerella* and *Clonostachys* compared to other treatments (Figure S3b). Additionally, the L-HA3 treatment had the highest relative abundances of *Tausonia* and *Talaromyces* compared to the others (Figure S3b).

3.4. The Linkage Between Soil Microbial Community Structure and Environmental Factors

Redundancy analysis was conducted to assess the influence of soil properties on the composition and structure of bacterial (Figure 5a) and fungal (Figure 5b) communities at the genus level. The first and second sorting axes explained 71.94% and 53.65% of the total variation in bacterial and fungal composition, respectively. Soil properties, including AP, SMC, SMN, SMP, and invertase, were identified as significant factors influencing bacterial community structure ($p < 0.05$). All tested soil parameters significantly influenced fungal community structure, except for alkaline phosphatase activity ($p < 0.05$).

In Figure 5a, bacterial genera, such as *Gemmatimonas*, *Haliangium*, and *Subgroup_7*, were closely associated with L-HA0 samples, primarily positively influenced by soil pH. Conversely, *Sphingomonas* exhibited a strong association with L-HA3 samples, predominantly positively affected by TN and AP. Figure 5b shows that fungal genera like *Cladosporium*, *Fusarium*, *Niesslia*, and *Nothophoma* were closely associated with L-HA0 and L-HA1 samples, primarily positively influenced by soil pH. In contrast, *Plectosphaerella* and *Papiliotrema* were closely associated with L-HA1 samples, mainly influenced by TN. *Tausonia* and *Talaromyces* showed strong correlations with SMP, urease, AK, SWC, SOM, and SMC in L-HA3 samples.

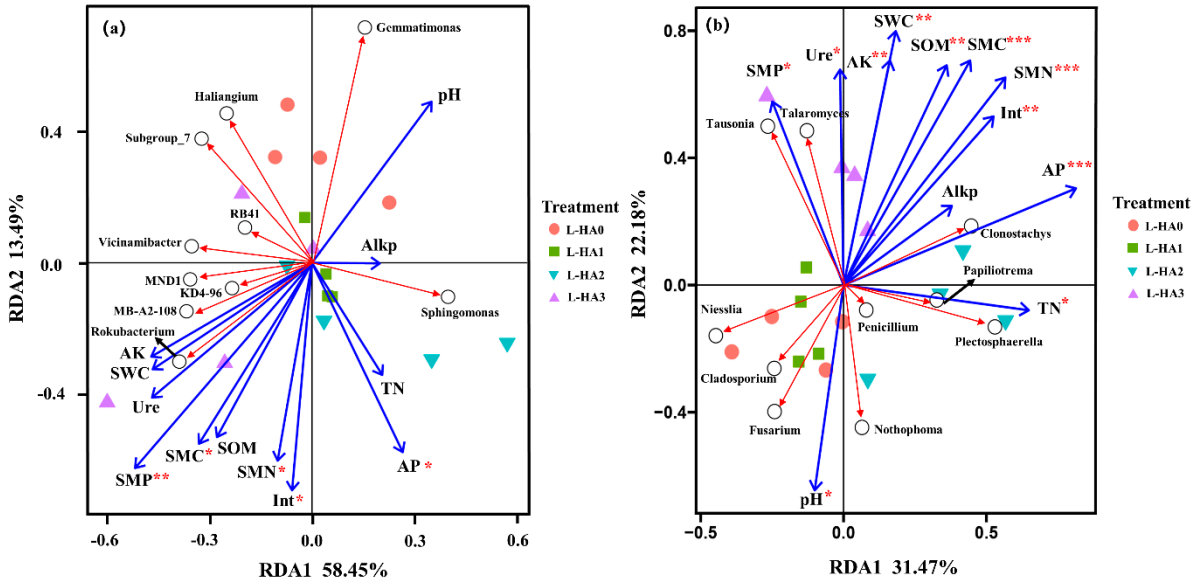

Figure 5. Redundancy analyses (RDAs) of the correlations between soil properties (blue arrows) and the composition of bacterial (**a**) and fungal (**b**) communities at the genus level (red arrows). *, **, and *** indicate significant differences at 0.05, 0.01, and 0.001 probability levels, respectively. L-HA0, L-HA1, L-HA2, and L-HA3 represent L-HA application rates at 0, 2, 4, and 6 ton·ha^{-1}, respectively.

4. Discussion

4.1. Changes in Soil Properties with L-HA Addition

In this study, the initial soil pH was alkaline, and only the L-HA3 treatment (6 ton·ha^{-1}) significantly decreased the pH, which is consistent with Xu's findings [47]. This observation could be attributed to HA enhancing the soil's ability to release and exchange H^+ ions [48]. However, the lower L-HA addition rates used in this study (L-HA1 and L-HA2) did not have a noticeable effect on soil pH. This may be related to the L-HA addition rate, as lower L-HA resulted in a lower exchange capacity for H^+. Additionally, the inherently stronger buffering capacity of the soil tested in this study could also contribute to this finding.

HA is a primary fraction of humic substances and one of the most active components in soil and compost organic matter [49]. In the current study, the addition of L-HA increased the SOC content by 2.49% to 12.70% (Table 1). Previous studies have confirmed that the SOC content was enhanced following HA addition due to the organic matter present in HA. Furthermore, the presence of non-degradable carbon in HA reduces SOC mineralization by

replenishing the SOC pool [50]. As expected, L-HA addition also increased the nutrient content of aeolian sandy soil, including TN, AP, and AK. HA serves as an essential component of organic matter in soil ecosystems, providing substrates for decomposing microbes and enhancing the soil structure and the water-holding capacity [51]. The results indicate that the L-HA amendment increased the soil nitrogen pool and nutrient availability. Soil organic matter is the primary natural source of soil-available nutrients, acting as a reservoir of essential nutrients, such as nitrogen, phosphorus, and sulfur. These nutrients are gradually mineralized and made available to plants through microbial activity, ensuring a sustained release of nutrients and thereby enhancing the soil's nutrient supply capacity [20]. Our findings suggest that L-HA addition increased soil nutrient levels, which was consistent with previous studies [18,52]. These results confirmed the long-term effect of L-HA on improving soil nutrient status based on four years of experimental data.

Soil microorganisms play an essential role in nutrient cycling and energy flow, significantly contributing to plant nutrient acquisition. These processes are vital for ensuring the long-term sustainability of agricultural systems [32]. Soil enzyme activities and microbial biomass are sensitive indicators that provide immediate and accurate information about small disturbances in soils [53]. In the present study, SMC and SMN content, as well as urease and invertase activity, were significantly improved in L-HA treatments compared to untreated soils (Table 1). This result indicates that the addition of L-HA promoted microbial growth and accelerated enzyme secretion. Notably, the L-HA3 treatment exhibited the most significant enhancements in SMC, SMN content, urease, and invertase activity, indicating a positive, dose-dependent response in soil microbial activity and nutrient cycling. These improvements have the potential to enhance soil fertility, increase nutrient availability, and improve overall soil health and related key factors, which are key factors for promoting sustainable agriculture and ecosystem stability. However, no significant difference was observed in SMP and alkaline phosphatase activity among the four L-HA treatments. Conversely, Liu et al. [54] revealed that HA addition significantly increased soil acid phosphatase activity, and Ni et al. [50] reported that the activity of soil alkaline phosphatase increased after the addition of HA compound fertilizer in red soil. These inconsistent findings may be due to competition among microorganisms for phosphorus resources. In our test field, where phosphorus availability is limited, microorganisms may prioritize phosphorus uptake for their own growth and metabolism, leading to a reduced proportion of phosphorus being incorporated into microbial biomass. The lack of a significant effect on alkaline phosphatase activity could be attributed to various factors. It is possible that the specific microorganisms responsible for alkaline phosphatase production were not notably affected by the application of L-HA. Additionally, other factors, such as soil pH, temperature, and moisture levels, can impact alkaline phosphatase activity and may have obscured any potential effects of L-HA. In summary, while the application of L-HA can improve phosphorus availability and stimulate microbial biomass carbon and nitrogen content, the limited effect on microbial biomass phosphorus and alkaline phosphatase activity indicates the involvement of other factors or mechanisms. This emphasizes the complexity of interactions among the soil, microorganisms, and organic matter.

4.2. Response of Soil Bacterial Community Composition to L-HA Addition

Soil bacterial richness and community structure are influenced by alterations in soil ecological factors, including temperature, oxygen availability, pH, and available organic carbon [55]. HA, a component of organic matter, plays a crucial role in modifying soil structure while introducing organic substances and essential nutrients that affect soil microbial populations. The Chao index is a robust estimator of species richness within microbial communities encompassing both bacteria and fungi, with higher values indicative of greater species diversity [56]. Our findings demonstrated that treatments incorporating L-HA resulted in an increase in the Chao1 index by 13.34% to 14.12% compared to treatment without L-HA. This enhancement can be attributed to the addition of L-HA, which ameliorates soil physicochemical and biological properties, thereby creating a more conducive

environment for microbial proliferation [57]. The Shannon and Simpson indices are critical metrics for assessing species richness and evenness within a community. Our analysis revealed that compared with the non-L-HA treatment, the Simpson index significantly increased by an average of 0.75% in L-HA treatments (except for L-HA2), while the Shannon index remained largely unaffected (Figure 2a). These findings are consistent with those of Cole et al. [58], who observed that the addition of organic substances, such as biochar, had a negligible impact on α-diversity indices (Shannon and Simpson) three years post-application. Overall, our results demonstrated that long-term addition of L-HA exerts a positive effect on soil α-bacterial diversity, suggesting that four years later, L-HA addition supports a more stable, resilient, and productive soil ecosystem.

In this study, the bacterial communities across the four treatments exhibited similar compositions, with the main phyla being *Proteobacteria* (23.95–32.47%), *Acidobacteriota* (15.03–22.33%), and *Actinobacteriota* (13.25–17.08%), which is consistent with findings from previous research [59,60]. The lack of discernible trends in the relative abundances of bacteria at the phylum level, despite differing rates of L-HA application compared to the control, underscores the complex nature of microbial responses to soil amendments.

In intensive agricultural systems, characterized by various physical disturbances and large fluctuations in organic carbon availability, the soil microbiome often exhibits a high abundance of fast-growing opportunistic bacteria, such as *Proteobacteria*. Conversely, oligotrophic bacteria like *Acidobacteria* are typically depleted in these environments [61]. *Proteobacteria* are known to utilize easily degradable substances derived from HA; however, their abundance tends to decrease once these substances are depleted. In this study, L-HA-amended treatments exhibited no significant effect on the relative abundance of *Proteobacteria* when compared to the non-L-HA-amended soil four years post-application. This indicates that the readily degradable substances from L-HA, which *Proteobacteria* decompose, may have been depleted. Less readily degradable forms of humic acid-C, such as fused aromatic rings, may be decomposed by other microorganisms, such as fungi [62]. *Actinobacteriota* is recognized for its significant role in the decomposition of organic materials, such as cellulose and chitin, thereby contributing substantially to organic matter turnover and carbon cycling [63]. Four years after the incorporation of L-HA, a higher abundance of *Actinobacteriota* was observed compared to the control, suggesting an increased degradation rate of soil organic matter and a subsequent rise in organic carbon turnover. Within the phylum of *Actinobacteria*, the relative abundances of several abundant genera, including *Rokubacterium* and *MB-A2-108*, were markedly increased under L-HA amendment at a rate of 6 ton·ha^{-1} (Figure S2b), thereby contributing significantly to the observed changes in the relative abundance of *Actinobacteriota*. Notably, in this study, the varying rates of L-HA exhibited differential impacts on bacterial community composition at both the phylum and genus levels, suggesting that the influence of L-HA on bacterial abundance does not necessarily follow a linear relationship with dosage. For example, *Acidobacteria*, typically enriched in soils with low resource availability, are often associated with lower rates of SOC mineralization [64]. Only L-HA2 treatment resulted in a significant difference in *Acidobacteria*, which was 17.66% lower compared to the control. This decrease suggests that the moderate application of L-HA (4 ton·ha^{-1}) altered soil conditions sufficiently to decrease the competitive advantage of *Acidobacteria*, which are typically adapted to low-nutrient environments. In contrast, both the lower application rate (L-HA1, 2 ton·ha^{-1}) and the higher application rate (L-HA3, 6 ton·ha^{-1}) did not show a significant difference in *Acidobacteria* abundance compared to the control. This lack of significant difference suggests that there might be a threshold effect where a certain concentration or application rate of L-HA (in this case, L-HA2 at 4 ton·ha^{-1}) is necessary to induce a noticeable change in *Acidobacteria* populations. Furthermore, the differential responses of *Acidobacteria* to varying L-HA application rates underscore the complexity of soil microbial ecology and the need for further research to unravel the mechanisms driving these changes.

In the present study, the application of L-HA significantly ($p < 0.05$) influenced the composition of the rhizosphere bacterial community (β-diversity) compared to the control (Table 2). Several studies have reported that the addition of organic substances shifted the microbial community structures by altering soil properties [57,62]. The high nano-porosity and large surface area of HA can enhance soil aeration and water retention, thereby creating a more favorable habitat for beneficial soil bacteria [50,57]. Additionally, chemical properties may also explain the alteration of the bacterial community. For instance, the addition of L-HA increased nutrient contents and decreased the soil pH (Table 1). However, in our study, the L-HA2 and L-HA3 treatments exhibited similar levels of β-diversity, indicating that increasing L-HA beyond a certain concentration does not significantly alter the soil bacterial community composition. This suggests a threshold effect, where additional L-HA applications beyond a certain point do not further change the bacterial community composition. This finding underscores the potential stability and resilience of these microbial populations under varying levels of organic amendments.

Variations in the compositions of the bacterial community are closely correlated with soil parameters [65]. In this study, the addition of L-HA significantly affected soil properties, with RDA results revealing that soil AP, SMC, SMN, SMP, and invertase were the main factors influencing bacterial communities. Additionally, these soil parameters were closely correlated with L-HA application (Figure 5a). These findings suggests that the L-HA amendment indirectly influenced bacterial community composition by altering these specific soil properties. Furthermore, the dominant bacterial genera exhibited correlations with environmental factors. *Vicinamibacter*, *RB41*, *Sphingomonas*, and *KD4-96* showed positive correlations with SMC, SMN, SMP, and invertase but negative correlations with AP. This indicates that specific soil properties influenced by L-HA application can selectively promote or suppress particular bacterial taxa, ultimately shaping the overall microbial community structure.

4.3. Response of Soil Fungal Community Composition to L-HA Addition

Soil fungi play crucial roles in decomposing complex organic matter, cycling essential nutrients like nitrogen and phosphorus, improving soil fertility through organic matter breakdown, and directly influencing crop growth and development [66]. In our study, no significant differences were found in α-diversity indices (e.g., Shannon diversity, Simpson diversity) among the four L-HA treatments. This suggests that the overall richness and evenness of the microbial community remained stable across different treatments, indicating a high level of microbial community resilience. The fungal communities across the four treatments exhibited similar compositions dominated by the phyla Ascomycota (54.85–74.04%), Basidiomycota (17.53–25.57%), and Rozellomycota (4.68–13.67%) (Figure 4b). These findings align with previous studies conducted in agricultural soils [63,67]. In contrast to the magnitude of change observed in the bacterial community structure, the fungal community structure exhibited significant alterations after four years of L-HA addition. Compared to L-HA0, the relative abundance of Ascomycota increased by 14.76% and 8.78% under L-HA1 and L-HA2, respectively, but it was 14.97% lower in L-HA3. Ascomycota represent the most diverse group of saprotrophic fungi, characterized by a wide range of ecological roles and metabolic capabilities [68]. They are key decomposers in agricultural soils, thriving due to the increased availability of organic carbon provided by organic substances [69]. Contrary to expectations, the relative abundance of Ascomycota decreased with increasing L-HA rates, reaching its lowest value in L-HA3 compared to L-HA0. This decline may be attributed to the suppressive effect of excessively high nutrient content, which overshadowed the positive effects of organic substances on Ascomycota growth [70]. Another reason could be the competitive relationships within the fungal community, particularly in response to the highest levels of L-HA. As the L-HA addition rate increased, the relative abundance of Rozellomycota and Mortierellomycota exhibited opposite trends. Specifically, L-HA3 resulted in the highest increase in the relative abundance of Rozellomycota, while the relative abundance of Mortierellomycota decreased to the lowest observed value

(Figure S3a). Most Rozellomycota species prefer to consume organic substances [71]. The increase in Rozellomycota suggests that the addition of L-HA provides additional organic materials, thereby promoting their growth. However, the decrease in Mortierellomycota could be attributed to the competition for carbon sources among dominant fungal phyla in the soil, potentially reducing the abundance of Mortierellomycota.

The composition of fungal communities exhibited variable changes in response to different rates of L-HA addition at the genus level compared to the phylum level. For example, L-HA2 treatment showed the highest relative abundance of *Plectosphaerella* and *Clonostachys*, while L-HA3 favored *Tausonia* and *Talaromyces*. This indicates that L-HA addition promoted the growth of these genera, with the magnitude of increase correlating to the application rate.

Fungal community composition varies primarily due to several factors, including soil chemical properties (such as pH, nutrient availability, and soil texture) and organic compounds released by plant roots, known as root exudates. The impact of L-HA addition on fungal community composition was confirmed through PCoA analysis, which illustrated distinct differences between fungal communities in L-HA-amended and non-amended soils (Figure 3b, Table 2). Furthermore, the rate of L-HA addition significantly influenced the fungal community composition (Figures 4b and S3). These observations likely result from changes in soil physicochemical properties, as evidenced by RDA results showing significant separation among the four treatments based on measured soil parameters, which correlated with the relative abundance of fungal genera.

5. Conclusions

In the aeolian sandy soil region of Inner Mongolia, the application of L-HA has been observed to enhance soil fertility and induce notable changes in both bacterial and fungal community structures after four years of amendment. In particular, higher rates of L-HA amendments exert more pronounced effects under typical conditions. The fungal community exhibited greater sensitivity to L-HA application compared with soil bacteria, specially at the genus level. Furthermore, L-HA application shifted bacterial community composition, particularly at the genus level, leading to an increase in the relative abundances of genera proficient in organic matter decomposition. In contrast, the impact of L-HA amendment on fungal community composition was more pronounced and marked by substantial increases in the relative abundances of the Rozellomycota and Ascomycota phyla and decreases in the Mortierellomycota phyla. Changes in bacterial community composition were mainly affected by AP, SMC, SMN, SMP, and invertase, while all tested soil parameters, except for alkaline phosphatase, significantly influenced the fungal community structure. Our study underscores the significant role of L-HA in influencing microbial biomass and altering community compositions four years post-amendment, with potential implications for soil C cycling. However, as a soil amendment, further research is necessary to explore the long-term impacts of L-HA on soil quality, microbial community composition, and crop growth characteristics. Specifically, studies should focus on carbon and nitrogen-cycle-related gene communities and diversities in aeolian sandy soil within arid and semi-arid regions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14112581/s1. Figure S1. Daily mean temperature and precipitation during the four buckwheat growing seasons in 2020–2023. Figure S2. Relative abundance of the 10 most abundant phyla (a) and genera (b) of bacteria (>1%) across all treatments. Vertical bars are standard errors of the mean (n = 4). Pairs of bars with the same letter between treatments within the same phylum and genus are not significantly different at $p \leq 0.05$. L-HA0, L-HA1, L-HA2, and L-HA3 represent L-HA application rates at 0, 2, 4, and 6 ton·ha^{-1}, respectively. Figure S3. Relative abundance of the 4 most abundant phyla (a) and the 10 most abundant genera (b) of fungi (>1%) across all treatments. Vertical bars are standard errors of the mean (n = 4). Pairs of bars with the same letter between treatments within the same phylum and genus are not significantly

different at $p \leq 0.05$. L-HA0, L-HA1, L-HA2, and L-HA3 represent L-HA application rates at 0, 2, 4, and 6 ton·ha^{-1}, respectively.

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