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# Shift in soil microbial communities along ~160 years of natural vegetation restoration on the Loess Plateau of China

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ABSTRACT

Natural restoration of vegetation has been widely implemented as an effective strategy for recovery of degraded ecosystems. However, how soil microbial communities vary with natural restoration of vegetation and associated drivers remains unclear. Here, we investigated the changes in soil microbial communities at 0–60 cm soil depths along ~160 years of natural restoration of vegetation from farmland to pioneer weeds, to herbs, to shrublands and early forests, and finally to climax forests on the Loess Plateau of China. Our phospholipid fatty acids (PLFA) analysis showed that natural restoration of vegetation largely enhanced the abundances of the total PLFA, the bacterial, fungal, Gram-positive ($G^+$) bacterial, Gram-negative ($G^-$) bacterial, arbuscular mycorrhizal fungal (AMF), actinomycete, monounsaturated, branched, and saturated straight-chain (SSC) PLFA in the surface soil layer (0–20 cm). However, its effect was negligible on the deep soil layer (20–60 cm). The biomass of these microbial communities declined sharply along with soil depth at each of the restoration stage. Natural restoration of vegetation significantly changed composition proportion of soil microbial communities. In particular, the ratio of $G^+$: $G^-$, proportions of $G^+$ bacterial PLFA, and AMF PLFA in the total PLFA gradually decreased, while the proportions of bacterial PLFA and $G^-$ bacterial PLFA in the total PLFA gradually increased in the surface soil layer along vegetation restoration stages. Our finding suggested that natural restoration of vegetation altered the biomass and composition proportion of soil microbial communities, which was strongly driven by variations in soil nutrient substrates and physiochemical properties (e.g., soil moisture and pH), as well as litter and root biomass along the long gradient of vegetation. This study revealed that natural restoration of vegetation was an effective strategy for reestablishment of soil microbial communities. Additionally, the impacts of natural restoration of vegetation on soil microbial communities were primarily concentrated in the surface soil layer rather than deep soil layer, and soil microbial biomass reached maximum in the climax forests.

## 1. Introduction

Incessant deforestation has resulted in significant land degradation worldwide, and greatly threatens forest ecosystem service, as well as the survival of humanity (Smith et al., 2016; Guo et al., 2018). Natural restoration of vegetation in conjunction with continued deforestation has led to a vast increase in secondary forests (Chai et al., 2019). Natural restoration of vegetation has been extensively implemented as an effective strategy for recovery of degraded ecosystems through reestablishment of eco-environmental conditions, enhancing net ecosystem productivity, and prevention of desertification (Lozano et al., 2014; Baker and Eckerberg, 2016). Vegetation restoration has been documented to alter the quantity and quality of litter, root biomass and architecture (Schedlbauer and Kavanagh, 2008), soil physicochemical properties (Xiao et al., 2017), and ecosystem carbon (C) and nitrogen (N) sequestration through varying composition and coverage of plant species (Deng et al., 2014; Zhong et al., 2021). As the primary decomposers of organic matter in terrestrial ecosystems (Yang et al., 2016), soil microbes play a key role in biogeochemical cycles at a global scale (Palomo et al., 2016), particularly in ecosystem C and N cycling

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(Zhong et al., 2018) and the formation and decomposition of soil organic matter (SOM) (Delgado-Baquerizo et al., 2016). Thus, understanding impacts of natural vegetation restoration on soil microbial communities contributes to an improved elucidation of the mechanisms that drive ecosystem C and N cycling.

The impacts of natural restoration of vegetation on soil microbial communities in terrestrial ecosystems have garnered increasing attention (Kuramae et al., 2010; Jangid et al., 2011; Strickland et al., 2017; Hu et al., 2020). Previous studies have indicated that vegetation restoration improved the abundance and diversity of microbial communities by increasing the inputs of plant residues, and soil organic C and N (Hu et al., 2020). Conversely, other studies have reported that vegetation restoration leads to decreased biomass and/or richness of soil microbial communities (Kuramae et al., 2010), or no obvious changes (Jangid et al., 2011; Strickland et al., 2017). For instance, Kuramae et al. (2010) demonstrated a decrease in microbial richness after restoration in chalk grasslands. Strickland et al. (2017) observed time lags of changes in the composition and function of soil microbial communities behind changes in aboveground vegetation following 16 years of vegetation restoration. Cui et al. (2018) revealed that although there was an improvement in soil nutrients, the structure of soil bacterial communities was not altered at three vegetation restoration stages. These inconsistent results may be associated with multiple factors, including the heterogeneity of ecosystems, previous land use history, restoration types and durations, and variations in the soil substrates (Bardgett and van der Putten, 2014; Hu et al., 2020). Therefore, more studies are necessary to fully understand effects of natural vegetation restoration on soil microbial communities.

Soil microbial communities are primarily driven by biotic (e.g., plant properties) (Prescott and Grayston, 2013; Steinauer et al., 2016) and abiotic factors, such as climate (Kang et al., 2021), soil substrate quality and quantity (Heitkötter et al., 2017), soil pH (Fernández-Calviño et al., 2011), soil temperature and moisture (Ahmed et al., 2019), and oxygen concentration (Fierer et al., 2003). Generally, high levels of plant biomass and diversity can enhance soil microbial diversity as the result of abundantly available resources from litter and root exudates (Steinauer et al., 2016). Apart from the quantity of plant residues (i.e., litter and root), soil microbial communities are affected by the chemical properties of plant residues (e.g., C:N ratio) (Prescott and Grayston, 2013). Furthermore, soil properties are considered to be vital factors that drive soil microbial communities (Kang et al., 2021). For instance, changes in the quantity and quality of soil nutrient substrates can significantly alter the composition of soil microbial communities. This is due to different microbial groups that exhibit different strategies for the utilization of substrates (Heitkötter et al., 2017; Zheng et al., 2021). Soil pH has been widely recognized as predominant factor that drives soil microbial communities (Fernández-Calviño et al., 2011). Soil moisture can modify soil microbial communities by physically altering the environment for microorganisms (Suseela et al., 2011), and improving the microbes' access to nutrients, while mediating the osmotic potential and substrate diffusion (Schimel, 2018; Ullah et al., 2021). Variations in the oxygen concentration with soil depth may also influence the composition and activities of microbial communities (Fierer et al., 2003). Numerous studies have shown that natural restoration of vegetation greatly altered the accumulation of soil organic C and N (Gao et al., 2020; Zhong et al., 2021; Zhu et al., 2021), and physicochemical properties (e.g., soil moisture and bulk density) (Xiao et al., 2017). The identification of biotic and abiotic factors in regulating variations in soil microbial communities is conducive for better understanding the effects of natural restoration of vegetation on soil microbial communities.

The Loess Plateau, which is situated at the interface of arid and semiarid regions in China, encompasses a total area of $6.4 \times 10^5$ $km^2$ and is recognized as one of the most severely eroded areas worldwide, owing to its naturally erodible soil and frequent anthropogenic disturbances (Fu et al., 2011). A series of ecological development strategies have been implemented by the Chinese government since the1950's, with the aim of reducing soil erosion and restoring fragile ecosystems. For example, the "Grain for Green" program (1999) endeavored to convert abandoned croplands (slopes of >15°) to forests, shrubs, and grasslands through natural restoration of vegetation devoid of anthropogenic disturbance (Deng et al., 2014; Guo et al., 2018). These ecological development strategies have played a positive role in decreasing soil erosion (Zhang et al., 2017), promoting net primary productivity, expanding C sink capacities (Lu et al., 2018), and augmenting soil quality in fragile ecosystems. Numerous studies have documented that natural restoration of vegetation altered the aboveground vegetation, soil organic C and N contents (Deng et al., 2014; Gao et al., 2020; Zhu et al., 2021), and soil physicochemical properties (Xiao et al., 2017) on the Loess Plateau of China. Previous studies have focused primarily on the responses of soil microbial communities to managed vegetation restoration (i.e., afforestation) (Wang et al., 2019; Hu et al., 2020), and/or natural restoration of vegetation in the short-term (Xiao et al., 2017). However, the long-term effects of natural restoration of vegetation on the biomass and composition proportion of soil microbial communities along different restoration stages remain uncertain. We hypothesized that long-term natural restoration of vegetation alters the biomass and composition proportion of soil microbial communities by modifying the properties of plant residues, soil nutrient substrates, and physicochemical characteristics. To test this hypothesis, we examined the soil microbial biomass and composition proportion through phospholipid fatty acids (PLFA) analysis, which encompassed plant and soil properties, including soil moisture, bulk density, pH, soil organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), available phosphorus (AP), water-soluble organic carbon (WSOC), ammonium nitrogen ($NH_4^+$-N), and nitrate nitrogen ($NO_3^-$-N) at 0–20, 20–40 and 40–60 cm soil depths. The substitution of space for time method was used in a long-term natural restoration of vegetation, spanning a ~160-year period from farmland, to pioneer weeds, to herbs to shrublands and early forests, and finally to climax forests on the Loess Plateau of China.

## 2. Materials and methods

### 2.1. Study area

The study area was located on the northwest side of the Ziwuling Mountains, in the middle region of the Loess Plateau (36°00′14″–36°01′09″N and 109°00′56″–109°01′52″E), Fu County, Shaanxi Province, China (Fig. 1a, b). This region is home to a temperate semi-arid to sub-humid transition zone, with a continental monsoon climate (Zhang et al., 2018). The mean annual precipitation ranges from 500 mm to 620 mm, which is mostly concentrated during the summer. The mean annual temperature ranges between 7 °C and 8 °C, and the elevation is from 1300 to 1700 m (Zhang et al., 2018). Soil type in this study area is cinnamon soil and classified as Ustalfs (FAO Soil Taxonomy) (Yan et al., 2020). The exclusive secondary forest region that remains in the Ziwuling Mountains, on the Loess Plateau of China, covers an area of 23,000 $km^2$ (Zhang et al., 2018).

Based on previous research in the study area, natural restoration of vegetation has naturally regenerated on abandoned farmland. The *Zea mays* L. is the only rotation crop prior to restoration in this study area. The farmland was abandoned at different times along the emigration of local inhabitants from this area due to war, famine, and other disasters that occurred since 1860. Consequently, various stages of restoration, from grasslands to shrublands and climax forests (*Quercus liaotungensis* Koidz) have been observed in this area over the last ~160 years (Zhong et al., 2018). Previous investigations reported that *Populus davidiana* Dode comprised 70% of the vegetative cover in this area in the 1950's, after ~100 years of vegetation restoration (Chen, 1954). The ages of pioneer weeds, herbs, and shrublands communities were estimated through consultations with local elders, and by considering land contracts between farmers and the government. The investigation of natural restoration of vegetation for this study took place in 2019. We selected six vegetation restoration stages for this study: (FL) farmland stage

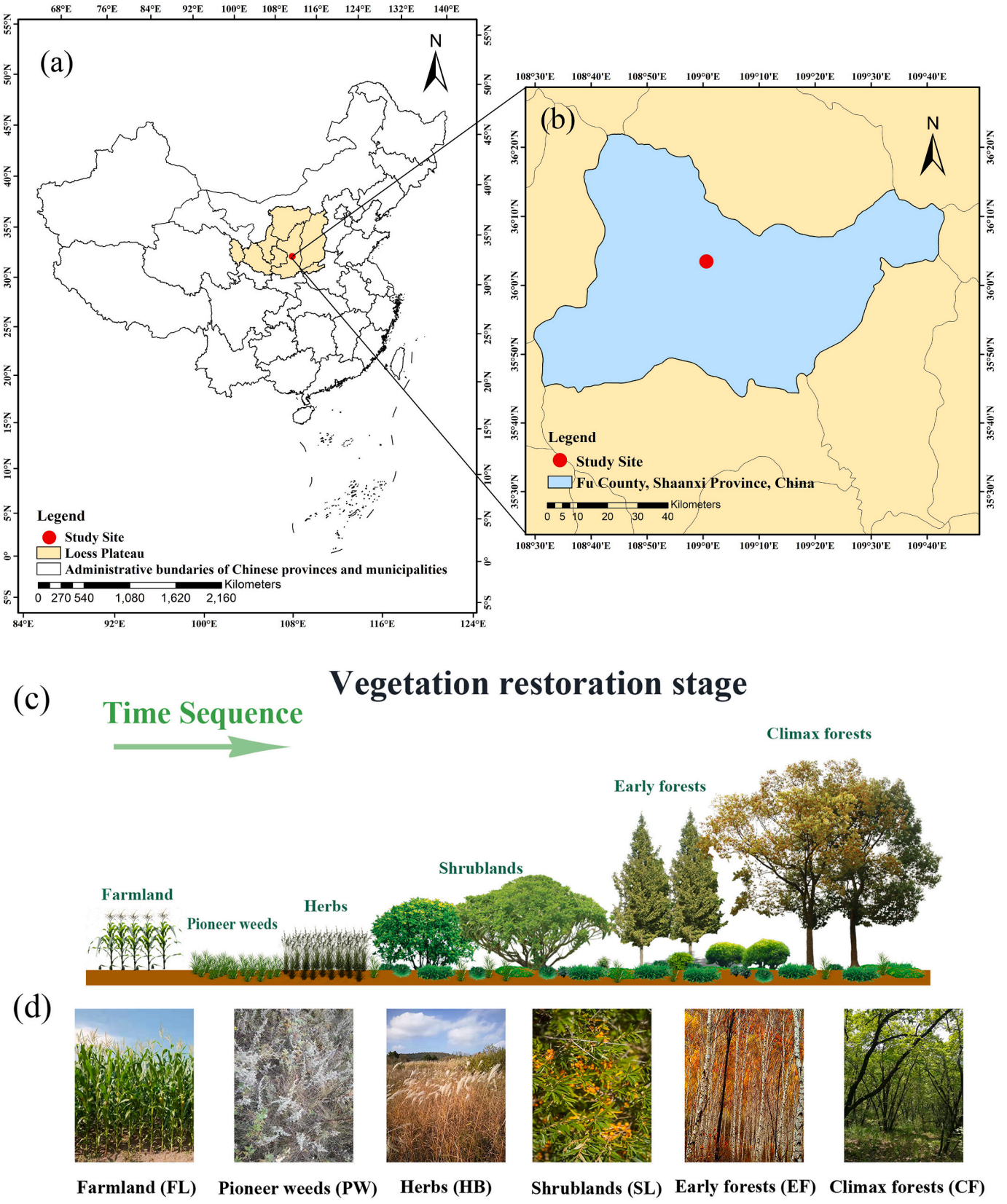


**Fig. 1.** (a) Geographical location of the Loess Plateau, China, (b) study site in Fu County, Shanxi Province, China, (c) conceptual diagram of the main stages during the natural vegetation restoration process, (d) photographs of the study site at each vegetation restoration stage.

during which *Zea mays* L. was the only rotation crop prior to restoration in this study area, thus farmland was selected as the control (0 year); (PW) pioneer weeds stage (~15 years) dominated by *Artemisia lavandulaefolia* DC. and *Stipa bungeana* Trin.; (HB) herbs stage (~30 years), during which *Triarrhena sacchariflora* (Maxim.) Nakai was the main herbaceous species; (SL) shrublands stage (~50 years) dominated by *Hippophae rhamnoides Linn.*; (EF) early forests stage (~110 years) dominated by *P. davidiana*; and (CF) climax forests stage (~160 years) dominated by *Q. liaotungensis* communities (Fig. 1c, d). The other vegetation and geographical features were shown in Table 1.

### 2.2. Plant and soil sampling

By employing the substitution of space for time, 24 plots (6 restoration stages × 4 replications) were established in October 2019. The dimensions of the sample plots were 20 m × 20 m for the forests, 5 m × 5 m for the shrublands, and 2 m × 2 m for the herbs, pioneer weeds, and farmland. The distance between four independent replicates was not less than 200 m but not more than 2 km for each restoration stage, and the altitude was lower than 120 m to ensure consistent environmental and climatic conditions. All plots for each restoration stage had similar slope aspects, slope gradients, and altitudes. Each plot of each restoration stage was at least 5 m away from vegetation community boundary at each restoration stage to avoid being affected by edge effects. All surveyed soils were developed from the same parent materials (i.e., loess parent material) and soil type (i.e., cinnamon soil). Prior to soil sampling, geographical data and the aboveground vegetation communities of each restoration stage were investigated (Table 1). Nine soil cores (5 cm diameter × 20 cm deep) were randomly collected from the 0–20 cm soil layer (the humus layer was removed at the forest sites), 20–40 cm, and 40–60 cm soil layer, respectively, using the S-shaped sampling method in each plot. The soil cores from each soil layer of each plot were thoroughly mixed to create single composite samples. A total of 72 soil samples were collected (24 plots × 3 soil depths). Three 1 × 1 m quadrats were randomly selected from each plot to collect the aboveground litter samples. Three root sampling blocks (10 cm diameter × 20 cm deep) were randomly collected using root drill at three soil depths (0–20, 20–40, and 40–60 cm, respectively) to obtain root samples from each plot. A total of 72 litter samples and 216 root samples were collected. To determine the soil bulk density of each plot, a ring cutter was used in the 0–20, 20–40, and 40–60 cm depth ranges in the soil profile. All the samples were sifted through a 2 mm sieve to remove roots and other debris. A portion of each soil sample was frozen, placed in a dry-ice box and transferred to the laboratory. The tubes were stored at −80 °C pending the determination of the PLFA. Once the remaining fresh soil was transported to the laboratory, it was divided into four subsamples after thorough mixing. The first soil subsample was used to determine soil moisture, whereas the second soil subsample was air-dried and sifted through a 1 mm sieve to determine the soil pH and SOC. The third subsample was air-dried and passed through 0.15 mm sieve for the determination of the soil TN, TP, AP, $NH_4^+$-N, and $NO_3^-$-N concentrations. The fourth subsample was sifted through a 2 mm sieve and stored at 4 °C for the determination of the soil WSOC.

### 2.3. Laboratory analysis

Each root sampling block was repeatedly flushed with water through a 0.15 mm sieve, and the roots that finally remained were collected. All litter and root materials were carefully cleaned and oven-dried at 65 °C to a constant weight, for measuring the litter and root biomass, respectively. The fresh soil was dried at 105 °C to constant weight for the determination of the soil moisture content. The intact soil cores that were obtained by the ring cutter were dried to constant weight for bulk density measurements. The pH value of soil was quantified using a pH meter in a 1:2 (soil/water) suspension. The WSOC was measured using a Liqui TOCII analyzer (Elementar Analysensystem GmbH, Germany) via the method described by Yang et al. (2016). The SOC was measured with a CN elemental analyzer (Vario PYRO cube elemental analyzer, Germany). Prior to determination, the dried soil samples were treated at room temperature with 1 M HCl for 24 h to remove the total inorganic C. The TN was measured using an AA3 continuous flow analyzer (Auto-Analyzer 3-HR Continuous-Flow Analyzer, Germany) after digestion with $H_2SO_4$ and extraction with 1 M KCl (Bao, 2018). The $NH_4^+$-N and $NO_3^-$-N were measured using an AA3 continuous flow analyzer after the samples were extracted with 1 M KCl (Bao, 2018). The TP and AP were determined using the molybdophosphate method that digested the soil with $HClO_4$ and $H_2SO_4$ before measuring with an AA3 continuous flow analyzer (Parkinson and Allen, 1975).

**Table 1**
Geographical features and vegetation of the different restoration stages in the Ziwuling forest region of the Loess Plateau, China.

| Vegetation restoration stage | Longitude (E) | Latitude (N) | Altitude (m) | Aspect (°) | Slope (°) | Canopy density (%) | Mean height (m) | Mean DBH (cm) | Mean tree density (tree/ha) | Dominant species | Minor plant species |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Farmland (FL) | 109°01′52″ | 36°00′39″ | 1245.13 | NE73 | 2–7 | – | 2.1 | – | – | *Zea mays* L. | – |
| Pioneer weeds (PW) | 109°01′52″ | 36°00′41″ | 1238.42 | NE45 | 8–15 | – | 0.5 | – | – | *A. lavandulaefolia*, *S. bungeana* | *A. gmelinii*, *L. barystachys*, *P. discolor*, *V. sepium* |
| Herbs (HB) | 109°01′44″ | 36°00′14″ | 1119.10 | NW30 | 8–18 | – | 2.3 | – | – | *T. sacchariflora* | *A. pilosa*, *H. altaicus*, *C. lanceolata*, *R. cordifolia*, *P. discolor* |
| Shrublands (SL) | 109°01′00″ | 36°00′24″ | 1037.98 | NE20 | 9–20 | – | 3.2 | – | – | *H. rhamnoides* | *C. lanceolata*, *S. bungeana*, *C. indicum*, *P. humile* |
| Early forests (EF) | 109°00′56″ | 36°00′20″ | 1074.21 | NE60 | 9–23 | 40–60 | 11.0 | 11.28 | 956 | *P. davidiana* | *R. xanthina*, *V. schensianum*, *L. ferdinandii*, *H. rhamnoides*, *L. maackii*, *H. rhamnoides* |
| Climax forests (CF) | 109°01′47″ | 36°01′09″ | 1201.30 | NE56 | 10–21 | 55–70 | 12.0 | 17.15 | 600 | *Q. liaotungensis* | *V. schensianum*, *S. pubescens*, *L. maackii*, *L. ferdinandii*, *S. pekinensis*, *R. xanthina* |

### 2.4. Soil microbial PLFA analysis

The soil microbial biomass was determined via PLFA analysis. The detailed procedures for PLFA analysis in this study were in line with the method described by Bossio and Scow (1998), and Yang et al. (2016). The content of each PLFA (ng $g^{-1}$ dry soil) was calculated from the 19:0 internal standard (5 μg $mL^{-1}$). The abundance of the PLFA in each sample were expressed as ng PLFA $g^{-1}$ dry soil and were used to estimate soil microbial biomass. The techniques for classifying the fatty acids into designated soil microbial communities referred to the following standard: The bacteria consisted of the PLFA of i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, 14:1ω5c, 15:1ω6c, 16:1ω7c, 18:1ω5c, 18:1ω7c, 15:0, and 17:0 (Frostegård and Bååth, 1996; Bossio and Scow, 1998; Bååth and Anderson, 2003; Cao et al., 2010; Zhao et al., 2012, 2015). The fungi were indicated by the sum of the PLFA of 18:1ω9c, 18:2ω6c, and 20:1ω9c (Kourtev et al., 2002; Bååth and Anderson, 2003; Swallow et al., 2009; Yang et al., 2016). The Gram-positive ($G^+$) bacteria were comprised of the PLFA of i13:0, i14:0, i15:0, a15:0, i16:0, a16:0, i17:0, and a17:0 (Yang et al., 2016). The Gram-negative ($G^-$) bacteria were composed of the PLFA of 14:1ω5c, 15:1ω6c, 16:1ω9c, 16:1ω7c, 17:1ω8c, 18:1ω5c, and 18:1ω7c (Kourtev et al., 2002, 2003; Wilkinson et al., 2002; Sampedro et al., 2006; Cao et al., 2010; Yang et al., 2016). The arbuscular mycorrhizal fungal (AMF) was represented by the PLFA of 16:1ω5c (Olsson, 1999; Kourtev et al., 2002; Yang et al., 2016). The PLFA of 16:0 10-methyl, 17:0 10-methyl, and 18:0 10-methyl represented the content of actinomycetes (Bossio et al., 2006; Zhao et al., 2015). The saturated straight-chain (SSC) PLFA were quantified by the sum of the PLFA of 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, and 24:0 (Bossio and Scow, 1998; Bossio et al., 2006; Yang et al., 2016). The monounsaturated PLFA were identified by the PLFA of 14:1ω5c, 15:1ω6c, 16:1ω5c, 16:1ω7c, 16:1ω9c, 17:1ω8c, 18:1ω5c, 18:1ω7c, 18:1ω9c, and 20:1ω9c (Kourtev et al., 2003; Bossio et al., 2006; Cao et al., 2010; Yang et al., 2016). The branched PLFA were identified by the PLFA of i13:0, i14:0, i15:0, a15:0, i16:0, a16:0, i17:0, a17:0, 10me 16:0, 10me 17:0, and 10me 18:0 (Bossio and Scow, 1998; Bossio et al., 2006; Cao et al., 2010; Yang et al., 2016). The total PLFA of the soil microorganisms included: $G^+$ bacterial PLFA, $G^-$ bacterial PLFA, fungal PLFA, AMF PLFA, actinomycete PLFA, and SSC PLFA (Yang et al., 2016). The corresponding PLFA were utilized to calculate the ratios of fungal PLFA: bacterial PLFA (F:B), $G^+$ bacterial PLFA: $G^-$ bacterial PLFA ($G^+$: $G^-$), and monounsaturated PLFA: branched PLFA (monounsaturated: branched), respectively.

### 2.5. Statistical analyses

The statistical analysis of the data was performed using SPSS Statistics 24.0 software. One-way ANOVA was performed to determine the effects of vegetation restoration and soil depth, respectively, on plant-related (i.e., litter and root biomass), and soil properties (i.e., moisture, bulk density, pH, SOC, TN, TP, AP, WSOC, $NH_4^+$-N, and $NO_3^-$-N) and various types of PLFA. Two-way ANOVA of variance was used to determine the impacts of vegetation restoration, soil depth, and their interactions on plant and soil properties, and various types of PLFA. Pearson correlations analysis was performed among litter and root biomass, soil properties, and each microbial type (i.e., each of PLFA). The relationships between soil microbial communities (all types of PLFA), and plant and soil properties were conducted using redundancy analysis (RDA) in CANOCO 4.5 software. The Monte Carlo permutation test (499 permutations) was employed to test the statistical significance of RDA, which was determined at $P < 0.05$.

## 3. Results

### 3.1. Plant and soil characteristics

The litter biomass increased significantly along vegetation restoration stages, and reached 955.88 g $m^{-2}$ in the climax forests (Fig. S1). The root biomass in the farmland, early forests, and climax forests in the 0–20 cm soil layer was significantly higher than that in the other restoration stages (Table 2). However, the root biomass in the 20–60 cm soil layer was not altered significantly among the vegetation restoration stages (Table 2). Vegetation restoration markedly affected the root biomass, soil moisture, pH, SOC, TN, AP, WSOC, and $NO_3^-$-N (Table 2; Fig. S2). Soil pH (0–20 cm) was highest in the early restoration stages (S0-S2), and lowest in the later restoration stages (S5; Table 2). Soil bulk density (0–20 cm) was highest in the farmland among the vegetation restoration stages (Table 2). Soil moisture, SOC, and TN concentrations in the 0–20 cm soil layer was highest in the climax forests, followed by the early forests, shrublands, and pioneer weeds stages compared with the farmland and herbs stages (Table 2). Soil AP concentration (0–60 cm) was highest in the farmland (Table 2). The WSOC concentration in the 0–20 cm soil layer gradually increased along vegetation restoration stages (Fig. S2a). Soil $NH_4^+$-N concentration (0–20 cm) was highest in the climax forests and lowest in the farmland (Fig. S2b). Soil $NO_3^-$-N concentration (0–20 cm) in the farmland and shrublands stages were significantly higher than that in the other restoration stages (Fig. S2c). However, soil bulk density (20–60 cm), SOC (40–60 cm), and TP (0–60 cm) were not significantly different among the vegetation restoration stages (Table 2). Soil properties were significantly affected by soil depth (Table 3). The concentrations of SOC, TN, WSOC, $NO_3^-$-N, and root biomass in the 0–20 cm soil layer were significantly higher than those in the 20–60 cm soil layer for each restoration stage (Table 2; Fig. S2). The pH in the 0–20 cm soil layer was significantly lower than that in the 20–60 cm soil layer for each restoration stage (Table 2). Pearson correlation analysis revealed that the SOC and TN had significantly positive correlations with the litter and root biomass, soil moisture, TP, WSOC, $NH_4^+$-N, and $NO_3^-$-N, whereas they showed significantly negative correlations with the soil bulk density and pH (Table 4). Soil WSOC, $NH_4^+$-N, and $NO_3^-$-N were closely related to the soil moisture, SOC, TN and root biomass (Table 4).

### 3.2. The biomass and composition proportion of soil microbial communities

The total microbial biomass, which was estimated as the total PLFA ranged from 2592 to 10,310 ng $g^{-1}$, 1155 to 2057 ng $g^{-1}$, and 572 to 1735 ng $g^{-1}$ in the 0–20, 20–40 and 40–60 cm soil layers, respectively, among the vegetation restoration stages (Fig. 2). The total PLFA, and bacterial, fungal, $G^+$ bacterial, $G^-$ bacterial, AMF, actinomycete, SSC, monounsaturated, and branched PLFA was significantly affected by soil depth (Table 3), which in the 0–20 cm soil layer was significantly higher than that in the 20–40 and 40–60 cm soil layers for each restoration stage (Figs. 3, 4). The PLFA of the total, as well as the vast majority of microbial communities for each restoration stage, displayed no significant differences between the 20–40 and 40–60 cm soil depths (Figs. 3, 4). The abundances of the total PLFA, and bacterial, fungal, $G^+$ bacterial, $G^-$ bacterial, AMF, actinomycete, monounsaturated, branched, and SSC PLFA in the 0–20 cm soil layer were lowest in the farmland, which gradually increased along vegetation restoration stages, and reached maximum in the climax forest (Figs. 2–4). Soil microbial biomass in the 20–60 cm soil layer showed no obvious increasing trend along vegetation restoration stages (Figs. 2–4).

The F:B ratio in the 0–20 and 20–40 cm soil layers were not significantly different among the vegetation restoration stages (Fig. 3c). The F:B ratio in the 40–60 cm soil layer of climax forests was significantly higher than that of shrublands, herbs, pioneer weeds, and farmland (Fig. 3c). The $G^+$: $G^-$ ratio in the 20–40 and 40–60 cm soil layers were significantly higher than that in the 0–20 cm soil layer for each restoration stage, except for shrublands (Fig. 3f). The $G^+$: $G^-$ ratio in the 0–20 cm soil layer decreased with vegetation restoration stages (Fig. 3f). The monounsaturated:branched ratio in the 0–20 cm soil layer showed no significant difference among the vegetation restoration stages

**Table 2**
Plant and soil properties (mean ± SE) of different vegetation restoration stages in the Ziwuling forest region on the Loess Plateau, China.

| Vegetation restoration stage | Soil depth (cm) | Moisture (%) | BD (g $cm^{-3}$) | pH | SOC (g $kg^{-1}$) | TN (g $kg^{-1}$) | TP (g $kg^{-1}$) | AP (mg $kg^{-1}$) | RB (g $m^{-2}$) |
|---|---|---|---|---|---|---|---|---|---|
| Farmland (FL) | 0–20 | 20.48 ± 0.22$^{Da}$ | 1.16 ± 0.04$^{Aa}$ | 8.38 ± 0.05$^{Ab}$ | 9.74 ± 0.64$^{CDa}$ | 2.430 ± 0.028$^{CDa}$ | 0.306 ± 0.031$^{Aa}$ | 70.00 ± 5.15$^{Aa}$ | 2905 ± 189$^{Aa}$ |
| | 20–40 | 22.28 ± 3.16$^{Aa}$ | 1.21 ± 0.06$^{Aa}$ | 8.57 ± 0.03$^{Aa}$ | 5.16 ± 0.47$^{ABb}$ | 2.077 ± 0.018$^{Ab}$ | 0.175 ± 0.022$^{Ab}$ | 34.85 ± 7.25$^{Ab}$ | 766 ± 456$^{Ab}$ |
| | 40–60 | 19.44 ± 0.07$^{Ba}$ | 1.22 ± 0.03$^{Aa}$ | 8.59 ± 0.03$^{ABa}$ | 3.27 ± 0.33$^{Ac}$ | 1.980 ± 0.011$^{Bc}$ | 0.152 ± 0.025$^{Ab}$ | 39.48 ± 9.26$^{Ab}$ | 478 ± 258$^{Ab}$ |
| Pioneer weeds (PW) | 0–20 | 20.87 ± 0.14$^{CDa}$ | 1.12 ± 0.04$^{ABa}$ | 8.36 ± 0.03$^{Ab}$ | 12.03 ± 0.56$^{Ca}$ | 2.523 ± 0.022$^{BCa}$ | 0.291 ± 0.052$^{Aa}$ | 13.50 ± 1.41$^{Ba}$ | 1593 ± 231$^{Ba}$ |
| | 20–40 | 20.95 ± 0.48$^{Aa}$ | 1.16 ± 0.03$^{Aa}$ | 8.59 ± 0.01$^{Aa}$ | 4.98 ± 0.44$^{ABb}$ | 2.143 ± 0.022$^{Ab}$ | 0.148 ± 0.028$^{Ab}$ | 9.57 ± 0.82$^{Bb}$ | 990 ± 266$^{Aab}$ |
| | 40–60 | 20.85 ± 0.24$^{Aa}$ | 1.23 ± 0.08$^{Aa}$ | 8.62 ± 0.04$^{Aa}$ | 3.66 ± 0.35$^{Ab}$ | 2.041 ± 0.020$^{ABc}$ | 0.157 ± 0.014$^{Ab}$ | 10.77 ± 1.22$^{Bab}$ | 620 ± 163$^{Ab}$ |
| Herbs (HB) | 0–20 | 20.21 ± 0.62$^{Da}$ | 1.14 ± 0.03$^{ABa}$ | 8.40 ± 0.04$^{Ab}$ | 8.15 ± 0.32$^{Da}$ | 2.303 ± 0.019$^{Da}$ | 0.241 ± 0.024$^{Aa}$ | 6.88 ± 0.67$^{Bab}$ | 1111 ± 142$^{Ba}$ |
| | 20–40 | 20.80 ± 0.29$^{Aa}$ | 1.16 ± 0.06$^{Aa}$ | 8.56 ± 0.05$^{Aa}$ | 4.07 ± 0.29$^{Bb}$ | 2.068 ± 0.024$^{Ab}$ | 0.239 ± 0.009$^{Aa}$ | 4.66 ± 0.83$^{Bb}$ | 719 ± 20$^{Ab}$ |
| | 40–60 | 21.44 ± 0.16$^{Aa}$ | 1.22 ± 0.01$^{Aa}$ | 8.56 ± 0.03$^{ABa}$ | 3.44 ± 0.12$^{Ab}$ | 2.008 ± 0.012$^{ABb}$ | 0.239 ± 0.019$^{Aa}$ | 7.54 ± 1.00$^{Ba}$ | 549 ± 113$^{Ab}$ |
| Shrublands (SL) | 0–20 | 23.03 ± 0.30$^{Ca}$ | 1.02 ± 0.04$^{Ba}$ | 8.17 ± 0.02$^{BCc}$ | 11.94 ± 1.00$^{Ca}$ | 2.690 ± 0.109$^{ABa}$ | 0.212 ± 0.025$^{Aa}$ | 8.47 ± 1.00$^{Ba}$ | 1491 ± 136$^{Ba}$ |
| | 20–40 | 20.97 ± 0.66$^{Aa}$ | 1.08 ± 0.03$^{Aa}$ | 8.38 ± 0.01$^{Cb}$ | 5.10 ± 0.65$^{ABb}$ | 2.179 ± 0.046$^{Ab}$ | 0.170 ± 0.044$^{Aa}$ | 9.83 ± 1.86$^{Ba}$ | 959 ± 252$^{Aab}$ |
| | 40–60 | 21.05 ± 0.94$^{Aa}$ | 1.26 ± 0.13$^{Aa}$ | 8.44 ± 0.01$^{Ca}$ | 3.90 ± 0.76$^{Ab}$ | 2.063 ± 0.027$^{Ab}$ | 0.158 ± 0.031$^{Aa}$ | 13.42 ± 3.08$^{Ba}$ | 560 ± 131$^{Ab}$ |
| Early forests (EF) | 0–20 | 26.15 ± 1.48$^{Ba}$ | 1.05 ± 0.03$^{ABa}$ | 8.29 ± 0.05$^{ABb}$ | 16.77 ± 1.07$^{Ba}$ | 2.724 ± 0.053$^{Aa}$ | 0.177 ± 0.014$^{Aa}$ | 6.70 ± 0.48$^{Ba}$ | 3099 ± 413$^{Aa}$ |
| | 20–40 | 21.32 ± 0.07$^{Ab}$ | 1.09 ± 0.10$^{Aa}$ | 8.51 ± 0.04$^{ABa}$ | 4.43 ± 0.33$^{ABb}$ | 2.161 ± 0.029$^{Ab}$ | 0.168 ± 0.024$^{Aa}$ | 4.43 ± 0.27$^{Bb}$ | 1587 ± 412$^{Ab}$ |
| | 40–60 | 21.43 ± 0.19$^{Ab}$ | 1.15 ± 0.06$^{Aa}$ | 8.54 ± 0.02$^{ABa}$ | 3.24 ± 0.41$^{Ab}$ | 2.020 ± 0.011$^{ABc}$ | 0.147 ± 0.023$^{Aa}$ | 5.27 ± 0.57$^{Bab}$ | 567 ± 104$^{Ab}$ |
| Climax forests (CF) | 0–20 | 29.63 ± 0.86$^{Aa}$ | 1.06 ± 0.02$^{ABb}$ | 8.07 ± 0.05$^{Cb}$ | 21.76 ± 1.26$^{Aa}$ | 2.874 ± 0.078$^{Aa}$ | 0.218 ± 0.015$^{Aa}$ | 8.54 ± 0.63$^{Ba}$ | 3360 ± 152$^{Aa}$ |
| | 20–40 | 21.80 ± 0.24$^{Ab}$ | 1.19 ± 0.02$^{Aa}$ | 8.46 ± 0.01$^{BCa}$ | 5.94 ± 0.51$^{Ab}$ | 2.167 ± 0.030$^{Ab}$ | 0.188 ± 0.016$^{Aa}$ | 3.76 ± 0.05$^{Bb}$ | 1165 ± 222$^{Ab}$ |
| | 40–60 | 21.34 ± 0.35$^{Ab}$ | 1.15 ± 0.02$^{Aab}$ | 8.52 ± 0.02$^{Ba}$ | 4.52 ± 0.06$^{Ab}$ | 2.079 ± 0.016$^{Ab}$ | 0.238 ± 0.013$^{Aa}$ | 3.55 ± 0.05$^{Bb}$ | 906 ± 112$^{Ab}$ |

Different superscript upper case letters indicate statistically significant differences at the α = 0.05 level among the vegetation restoration stages at the same soil depth. Different superscript lower case letters indicate statistically significant differences at the α = 0.05 level among the soil depths in the same vegetation restoration stage. BD: bulk density; SOC: soil organic carbon; TN: total nitrogen; TP: total phosphorus; AP: available phosphorus; RB: root biomass. See Table 1 for abbreviations.

(Fig. 4f). The monounsaturated:branched ratio in the 0–20 cm soil layer was significantly higher than that in the 20–40 and 40–60 cm soil layers for each restoration stage (Fig. 4f).

The proportions of bacterial PLFA and $G^-$ bacterial PLFA in the total PLFA of the 0–20 cm soil layer gradually increased along vegetation restoration stages, respectively (Fig. S3). However, the proportion of $G^+$ bacterial PLFA in the total PLFA of the 0–20 cm soil layer revealed a decreasing trend along vegetation restoration stages (Fig. S3). The fungal PLFA, AMF PLFA, and actinomycete PLFA accounted for 11.20–15.03%, 3.05–6.53%, and 9.09–14.59% of the total PLFA in the 0–60 cm soil layer among the vegetation restoration stages, respectively (Fig. S3). The proportion of fungal PLFA in the total PLFA of the 40–60 cm soil layer markedly increased along vegetation restoration stages (Fig. S3). The proportion of actinomycete PLFAs in the total PLFA of the 0–20 cm soil layer was higher in the early restoration stages (S0-S3) but lower in the later restoration stages (S4–S5; Fig. S3).

### 3.3. Linking soil microbial communities to environmental variables

Twelve variables of plant and soil properties (i.e., litter biomass, root biomass, soil moisture, bulk density, pH, SOC, TN, TP, AP, WSOC, $NH_4^+$-N, and $NO_3^-$-N) explained 96.9% of the total variability of the PLFA in the 0–20 cm soil layer, 77.8% of the total variability of the PLFA in the 20–40 cm soil layer, and 76.4% of the total variability of the PLFA in the 40–60 cm soil layer (Fig. S4). Variations in the PLFA were strongly correlated with soil moisture ($F = 63.48$, $P = 0.0020$), AP ($F = 28.66$, $P = 0.0020$), TP ($F = 10.90$, $P = 0.0040$), and SOC ($F = 4.72$, $P = 0.0490$) in the 0–20 cm soil layer (Fig. S4a); AP ($F = 16.82$, $P = 0.0020$), and $NO_3^-$-N ($F = 10.85$, $P = 0.0060$) in the 20–40 cm soil layer (Fig. S4b); and the soil pH ($F = 16.90$, $P = 0.0020$), and SOC ($F = 8.92$, $P = 0.0120$) in the 40–60 cm soil layer (Fig. S4c). In the 0–20 cm soil layer, Axis 1 explained 95.0% of the total variations of the PLFA, and Axis 2 explained 1.5% (Fig. S4a). In the 20–40 cm soil layer, Axis 1 explained 74.8% of the total variations in the PLFA and Axis 2 explained 1.7% (Fig. S4b). In the 40–60 cm soil layer, Axis 1 explained 70.1% of the total variations in the PLFA and Axis 2 explained 4.1% (Fig. S4c). Pearson correlation analyses indicated that the total PLFA, bacterial, fungal, $G^+$ bacterial, $G^-$ bacterial, AMF, actinomycete, SSC, monounsaturated, and branched PLFA were significantly positively correlated with the soil moisture, SOC, TN, WSOC, $NH_4^+$-N, $NO_3^-$-N, litter, and root biomass, while they were significantly negatively associated with the soil bulk density and pH (Table 4). The F:B ratio was significantly negatively correlated with SOC, TN, AP and $NO_3^-$-N (Table 4). The $G^+$: $G^-$ ratio was significantly negatively associated with soil moisture, SOC, TN, TP, WSOC, $NH_4^+$-N, $NO_3^-$-N, and root biomass (Table 4). The monounsaturated:branched ratio was significantly positively correlated with soil moisture, SOC, TN, TP, WSOC, $NH_4^+$-N, $NO_3^-$-N, and root biomass, and was significantly negatively correlated with the soil bulk density and pH (Table 4).

## 4. Discussion

Natural restoration of vegetation greatly altered the soil microbial biomass in the surface soil layer (0–20 cm) on the Loess Plateau of China (Figs. 2–4). The abundances of total PLFA, and bacterial, fungal, $G^+$

**Table 3**
Statistical significance (F value $^{P\ value}$) of the effects of the vegetation restoration stage, soil depth and their interactions on soil properties and microbial communities based on two-way ANOVA.

| Source of variation | Moisture | BD | pH | SOC | TN | TP | AP | WSOC | $NH_4^+$-N | $NO_3^-$-N | RB | Total PLFA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vegetation restoration | 7.190*** | 1.473 | 18.510*** | 29.501*** | 16.321*** | 3.169* | 80.078*** | 5.013*** | 1.826 | 20.813*** | 9.112*** | 32.755*** |
| Soil depth | 12.176*** | 6.101** | 108.276*** | 441.649*** | 323.836*** | 10.276*** | 9.440*** | 33.745*** | 30.360*** | 87.737*** | 76.778*** | 357.235*** |
| Vegetation restoration × soil depth | 5.276*** | 0.608 | 2.338* | 19.309*** | 6.378*** | 2.336* | 5.550*** | 2.853** | 1.820 | 4.360*** | 4.702*** | 22.491*** |

| Source of variation | Bacterial PLFA (B) | Fungal PLFA (F) | F: B ratio | $G^+$ bacterial PLFA | $G^-$ bacterial PLFA | $G^+$: $G^-$ ratio | AMF PLFA | Actinomycete PLFA | SSC PLFA | Monoun-saturated PLFA (Mo) | Branched PLFA (Br) | Mo: Br ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vegetation restoration | 34.245*** | 35.343*** | 3.922** | 26.291*** | 36.691*** | 6.032*** | 13.234*** | 26.236*** | 34.786*** | 31.494*** | 23.172*** | 5.751*** |
| Soil depth | 359.777*** | 331.807*** | 6.968** | 254.147*** | 418.522*** | 109.887*** | 320.616*** | 326.913*** | 316.309*** | 420.674*** | 244.284*** | 129.863*** |
| Vegetation restoration × soil depth | 25.216*** | 21.466*** | 2.713** | 16.208*** | 29.815*** | 2.684*** | 8.265*** | 14.927*** | 24.311*** | 25.292*** | 14.273*** | 1.843 |

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. WSOC: soil water-soluble organic carbon; $NH_4^+$-N: ammonium nitrogen; $NO_3^-$-N: nitrate nitrogen; PLFA: phospholipid fatty acids; $G^+$: Gram-positive; $G^-$: Gram-negative; AMF: arbuscular mycorrhizal fungal; SSC: saturated straight-chain; See Table 2 for abbreviations.



**Table 4**
Correlation analysis (Pearson correlation coefficient $^{P\ value}$) of plant and soil properties and microbial communities at 0–20, 20–40 and 40–60 cm soil depths among different vegetation restoration stages in the Ziwuling forest region on the Loess Plateau, China.

| | Moisture | BD | pH | SOC | TN | TP | AP | WSOC | $NH_4^+$-N | $NO_3^-$-N | LB | RB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SOC | 0.717** | −0.412** | −0.850** | 1 | 0.958** | 0.236* | −0.014 | 0.785** | 0.648** | 0.561** | 0.647** | 0.773** |
| TN | 0.623** | −0.458** | −0.864** | 0.958** | 1 | 0.299* | 0.007 | 0.712** | 0.642** | 0.632** | 0.544** | 0.756** |
| TP | −0.089 | −0.133 | −0.195 | 0.236* | 0.299* | 1 | 0.184 | 0.114 | 0.087 | 0.287* | −0.052 | 0.286* |
| AP | −0.167 | 0.124 | 0.048 | −0.014 | −0.007 | 0.184 | 1 | −0.087 | −0.007 | 0.526** | −0.494* | 0.237** |
| WSOC | 0.542** | −0.280* | −0.675** | 0.785** | 0.785** | 0.114 | −0.087 | 1 | 0.518** | 0.315** | 0.363 | 0.669** |
| $NH_4^+$-N | 0.363** | −0.269* | −0.589** | 0.648** | 0.642** | 0.087 | −0.007 | 0.518** | 1 | 0.387** | 0.142 | 0.523** |
| $NO_3^-$-N | 0.256* | −0.248* | −0.583** | 0.561** | 0.561** | 0.287* | 0.526** | 0.315** | 0.387** | 1 | −0.203 | 0.519** |
| Total PLFA | 0.726** | −0.426** | −0.842** | 0.944** | 0.919** | 0.115 | −0.147 | 0.779** | 0.660** | 0.482** | 0.747** | 0.684** |
| Bacterial PLFA (B) | 0.734** | −0.419** | −0.840** | 0.945** | 0.915** | 0.110 | −0.142 | 0.783** | 0.658** | 0.478** | 0.754** | 0.687** |
| Fungal PLFA (F) | 0.731** | −0.424** | −0.832** | 0.945** | 0.912** | 0.110 | −0.159 | 0.794** | 0.661** | 0.455** | 0.736** | 0.689** |
| F: B ratio | −0.106 | −0.018 | 0.215 | −0.246* | −0.254* | −0.099 | −0.255* | −0.045 | −0.153 | −0.343** | 0.343 | −0.169 |
| $G^+$ bacterial PLFA | 0.713** | −0.426** | −0.839** | 0.928** | 0.905** | 0.100 | −0.162 | 0.765** | 0.635** | 0.482** | 0.758** | 0.665** |
| $G^-$ bacterial PLFA | 0.741** | −0.415** | −0.841** | 0.951** | 0.919** | 0.118 | −0.130 | 0.786** | 0.668** | 0.481** | 0.743** | 0.695** |
| $G^+$: $G^-$ ratio | −0.451** | 0.320* | 0.729** | −0.751** | −0.786** | −0.347** | −0.006 | −0.521** | −0.635** | −0.549** | −0.182 | −0.577** |
| AMF PLFA | 0.576** | −0.420** | −0.812** | 0.870** | 0.884** | 0.174 | −0.143 | 0.680** | 0.649** | 0.498** | 0.554** | 0.594** |
| Actinomycete PLFA | 0.692** | −0.447** | −0.858** | 0.938** | 0.929** | 0.151 | −0.134 | 0.753** | 0.656** | 0.534** | 0.749** | 0.674** |
| SSC PLFA | 0.745** | −0.422** | −0.824** | 0.941** | 0.907** | 0.089 | −0.154 | 0.788** | 0.656** | 0.449** | 0.726** | 0.688** |
| Monounsaturated PLFA (Mo) | 0.722** | −0.421** | −0.845** | 0.949** | 0.924** | 0.130 | −0.130 | 0.779** | 0.673** | 0.491** | 0.739** | 0.687** |
| Branched PLFA (Br) | 0.713** | −0.417** | −0.836** | 0.929** | 0.898** | 0.116 | −0.148 | 0.764** | 0.639** | 0.483** | 0.759** | 0.671** |
| Mo:Br ratio | 0.459** | −0.373** | −0.752** | 0.797** | 0.851** | 0.291* | 0.011 | 0.584** | 0.687** | 0.556** | 0.075 | 0.606** |

* $P < 0.05$; ** $P < 0.01$. LB: litter biomass. See Tables 2 and 3 for abbreviations.

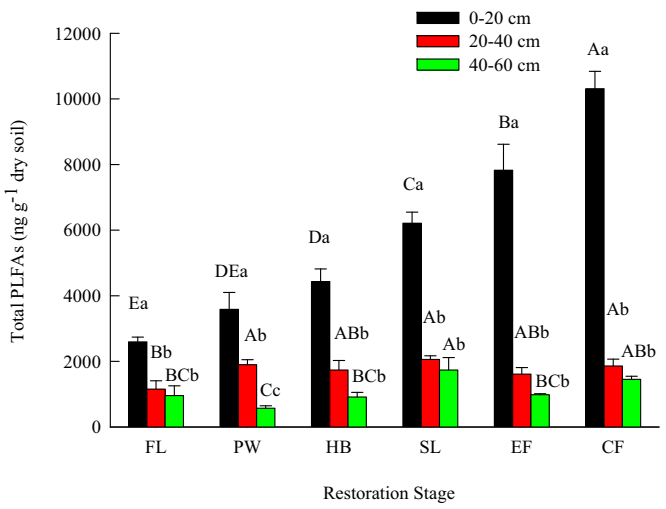


**Fig. 2.** Soil total phospholipid fatty acids (PLFA) (mean ± SE) of different vegetation restoration stages in the Ziwuling forest region on the Loess Plateau, China. Different superscript upper case letters indicate statistically significant differences at the $\alpha = 0.05$ level among the vegetation restoration stages at the same soil depth. Different superscript lower case letters indicate statistically significant differences at the $\alpha = 0.05$ level among the soil depths at the same vegetation restoration stage. FL: farmland; PW: Pioneer weeds; HB: Herbs; SL: Shrublands; EF: Early forests; and CF: Climax forests.

bacterial, $G^-$ bacterial, AMF, actinomycete, SSC, monounsaturated, and branched PLFA in the surface soil layer (0–20 cm) gradually increased along vegetation restoration stages, and attained their maximum in the climax *Q. liaotungensis* forests (Figs. 2–4). We also found that natural restoration of vegetation greatly promoted the accumulation of SOC, WSOC, and TN in the surface soil layer along restoration duration (Table 2, Fig. S2). It has been widely documented that soil C and N stocks are primarily determined by the quantity and quality of plant residues (i.e., litter and root) that enter the soil, and the loss of C and N via decomposition of SOM (Fissore et al., 2009; Assefa et al., 2017; Kohl et al., 2020). Root exudates can enrich soil nutrient substrates and provide easily assimilable C for soil microbes (Picariello et al., 2021). In this study, the litter and root biomass increased significantly along vegetation restoration stages (Table 2; Fig. S1), where SOC and TN were closely relevant to litter and root biomass in the surface soil layer (Table 4). It was inferred that greatly increased SOC and TN in the surface soil layer along vegetation restoration stages (Table 2; Fig. S2), were primarily attributed to a substantial quantity of litter residues and root exudates input into the soil (Fig. S1). In general, decreases in soil moisture and good aeration may stimulate the SOM decomposition and expedite loss of organic C and N in soils due to oxygen exposure (Mitra et al., 2005; Yang et al., 2016). In this study, soil moisture (0–20 cm) significantly increased in the later restoration stages (Table 2), probably owing to increasing shading that was caused by canopies of trees (Table 1), and increasing litter coverage (Fig. S1). Greatly increased soil moisture (0–20 cm) in the later restoration stages was conducive to avoid excessive exposure of SOM to soil atmosphere (Mitra et al., 2005), which can reduce loss of soil C and N (Table 2; Yang et al., 2016).

In this study, RDA analyses indicated that the variations in the soil microbial communities along vegetation restoration stages were strongly associated with soil moisture, SOC, AP, TP in the surface soil layer (Fig. S4a). It further demonstrated that soil moisture is a crucial factor that drove soil microbial communities (Brockett et al., 2012; Stefanowicz et al., 2021). Furthermore, SOC is widely considered to be a predominant driving factor for soil microbial communities (Orwin et al., 2016; Santonja et al., 2017), as it provides available nutrient substrates for soil microbes. Aside from soil moisture, SOC, AP, TP, $NO_3^-$-N, and pH, our Pearson correlation analyses clearly indicated that the total PLFA, bacterial, fungal, $G^+$ bacterial, $G^-$ bacterial, AMF, actinomycete, SSC, monounsaturated, and branched PLFA were intimately related to litter and root biomass, and soil nutrient substrates (i.e., SOC, TN, WSOC, $NH_4^+$-N, and $NO_3^-$-N) (Table 4). Thus, significantly increased soil (0–20 cm) microbial biomass along vegetation restoration stages were strongly driven by progressively increased litter and root biomass, soil nutrient substrates, and changes in soil properties, particularly soil moisture and pH with restoration time (Table 2; Figs. 2–4, S1, S2).

As anticipated, the soil microbial biomass sharply declined with soil depth for each restoration stage (Figs. 2–4). The total PLFA in the surface soil layer (0–20 cm) was five times higher than that of the deep soil layer (20–60 cm) in the climax *Q. liaotungensis* forests (Fig. 2). The soil microbial biomass in the deep soil layer did not markedly increase along vegetation restoration stages in comparison to the surface soil layer (Figs. 2–4). There were no obvious changes between the 20–40 cm and 40–60 cm soil layers for the majority of the restoration stages (Figs. 2–4). These results clearly showed that the impacts of vegetation restoration on soil microbial communities were primarily concentrated in the surface soil layer rather than deep soil layer on the Loess Plateau of China (Figs. 2–4). This was likely due to the fact that the litter was mainly trapped and incorporated into the SOM in the surface soil layer via microbial decomposition. This inference was supported by previous investigation that the effects of litter inputs on the microbial communities in the surface soil layer were greater than those of the deep soil layer (Liu et al., 2012). Moreover, the greatly reduced root biomass with soil depth translated to a significant decrease in root exudates which typically provide easily assimilable C for soil microbes (Table 2; Picariello et al., 2021). Meanwhile, the sharply decreased litter and root input into the soil induced lower SOC, TN, WSOC, $NO_3^-$-N for each restoration stage (Table 2, Figs. S1, S2). This ultimately resulted in the decline of the soil microbial communities in the deep soil layer due to the lack of an adequate supply of nutrient substrates (Table 2, Figs. 2–4, S2).

The F:B ratio indicates the responses of bacterial and fungal communities to environmental change (Spohn, 2016). Interestingly, the F:B ratio in the surface soil layer remained unchanged among the vegetation restoration stages (Fig. 3c). This was likely because bacterial and fungal communities exhibited similar increases in the surface soil layer along vegetation restoration stages (Fig. 3a, b; Hu et al., 2020). This was coincident with the finding of Liu et al. (2020) who reported that the bacterial and fungal abundance both showed a progressive increasing trend along secondary succession and tended to stabilize in the later successional stages. Our result was supported by Hu et al. (2020), who showed that the F:B ratio remained constant with natural and managed vegetation restoration in a subtropical karst region. In this study, the proportions of bacterial and fungal PLFA in the total PLFA of the surface soil layer accounted for 48.1% to 51.2%, and 11.4% to 12.6% among the vegetation restoration stages, respectively (Fig. S3a, b). It confirmed that the bacterial communities were dominant in terms of overall soil microbes (Fig. S3). The highest proportions of bacterial and fungal PLFA in the total PLFA of the surface soil layer were observed in the climax *Q. liaotungensis* forests, respectively (Fig. S3a, b). In general, soil fungal communities have been reported to exhibit a stronger capacity to degrade recalcitrant organic materials and have a greater competitiveness in nutrient-poor environments (Collins et al., 2018; Yang et al., 2019). Previous studies documented that soil nutrient substrates had a positive impact on soil fungal abundance (Peay et al., 2013; Yang et al., 2019), owing to the great majority of soil fungi being saprophytes (Zimudzi et al., 2018). This was supported by our finding that soil fungal PLFA was intimately associated with the SOC, TN, WSOC, $NH_4^+$-N, and $NO_3^-$-N (Table 4). Simultaneously, soil nutrient substrates are one of the overwhelming driving factors for soil bacterial communities due to bacteria favoring nutrient-rich conditions (Orwin et al., 2016; Santonja et al., 2017; Peguero et al., 2021). Thus, the climax *Q. liaotungensis* forests possessed the highest proportions of bacterial and fungal communities in the surface soil layer among the vegetation restoration stages (Fig. S3a, b), most likely due to its highest levels of SOC, TN,

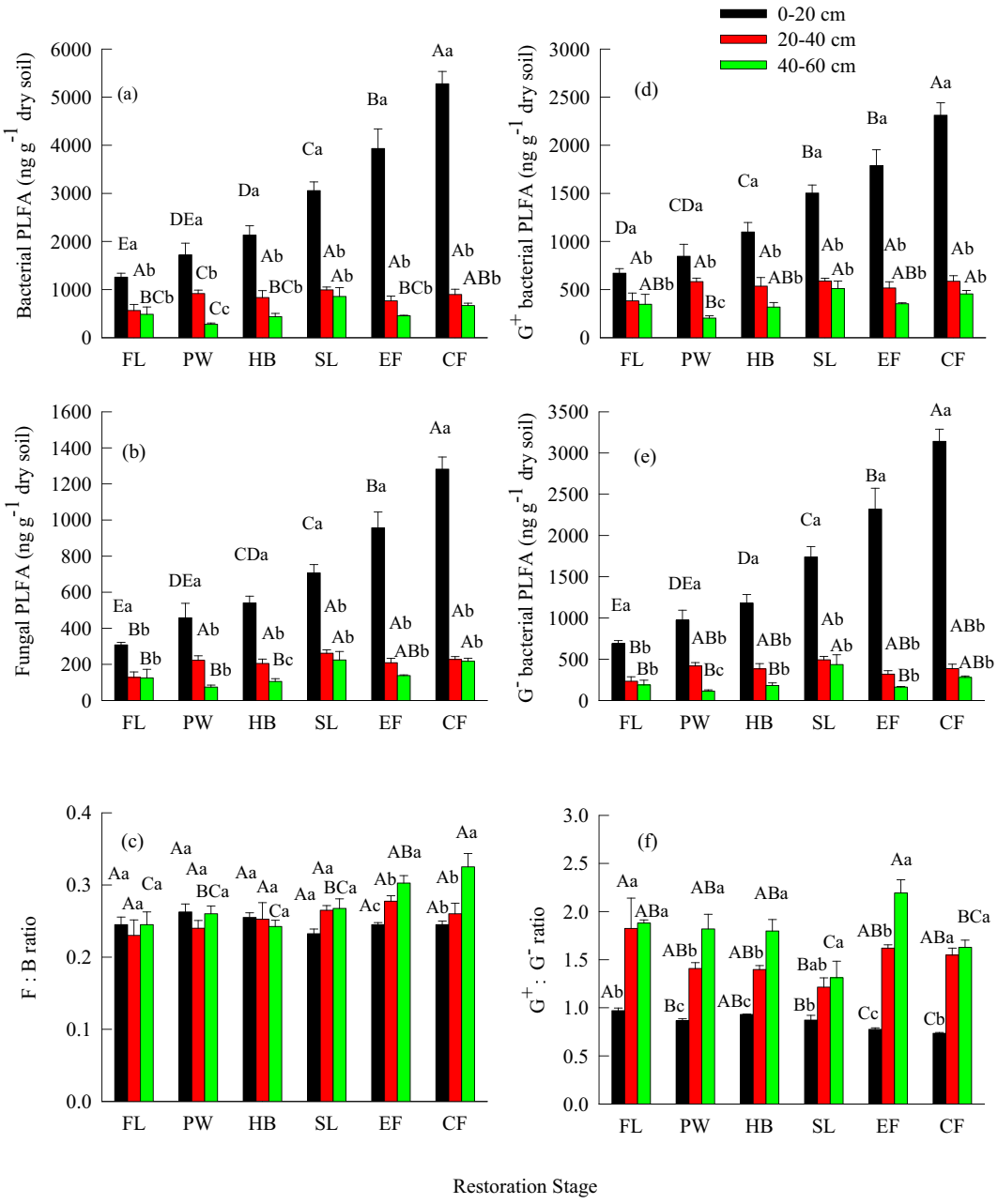


**Fig. 3.** (a) Soil bacterial phospholipid fatty acids (PLFA), (b) Fungal PLFA, (c) Fungal:Bacterial PLFA (F:B) ratio; (d) $G^+$ bacterial PLFA, (e) $G^-$ bacterial PLFA, (f) $G^+$: $G^-$ ratio (mean ± SE) of different vegetation restoration stages in the Ziwuling forest region, on the Loess Plateau, China. Different superscript upper case letters indicate statistically significant differences at the $\alpha = 0.05$ level among the vegetation restoration stages at the same soil depth. Different superscript lower case letters indicate statistically significant differences at the $\alpha = 0.05$ level among the soil depths at the same vegetation restoration stage. See Table 3 and Fig. 2 for abbreviations.

WSOC, $NH_4^+$-N, and $NO_3^-$-N. They provide enriched available nutrients to soil bacteria and fungi (Peay et al., 2013; Yu et al., 2019).

The $G^+$: $G^-$ ratio has been proved to be a key indicator to reveal the variations in soil microbial community structure (Yang et al., 2021). $G^+$ and $G^-$ bacteria have different strategies for the utilization of substrates (Zheng et al., 2021). Previous studies documented that $G^-$ bacteria are copiotrophic r-strategists (Vangestel et al., 1993; Yang et al., 2021), which preferentially utilize readily degradable labile C sources from plant residues inputs (Creamer et al., 2016; Fanin et al., 2019; Kohl et al., 2020), and promoted the additional incorporation of residual C into the SOM (Bai et al., 2021). Conversely, $G^+$ bacteria are slow-growing (Yang et al., 2021), and favor the use of recalcitrant C compounds from aged organic residues or endogenous C components from SOM (Bai et al., 2021; Kang et al., 2021), which are regarded as oligotrophic K-strategists (Vangestel et al., 1993; Abbruzzese et al., 2021). Among soil nutrient substrates, WSOC was one of main components in the soil labile organic C pool (Yang et al., 2016), which served as a direct reservoir of readily available nutrients for microbial growth and metabolism (Yang et al., 2016). In this study, the decreased $G^+$: $G^-$ ratio in the surface soil layer of the climax *Q. liaotungensis* and early *P. davidiana* forests (Fig. 3f), was primarily due to the significantly increased soil labile C (i.e., WSOC) being favored by $G^-$ bacteria rather than $G^+$ bacteria (Fig. S2a; Kohl et al., 2020). On the other hand, soil microbial communities can change their composition in arid environments (Fuchslueger et al., 2016) who verified that $G^+$ bacteria was increased during a natural drought period. A higher $G^+$: $G^-$ ratio in the surface soil

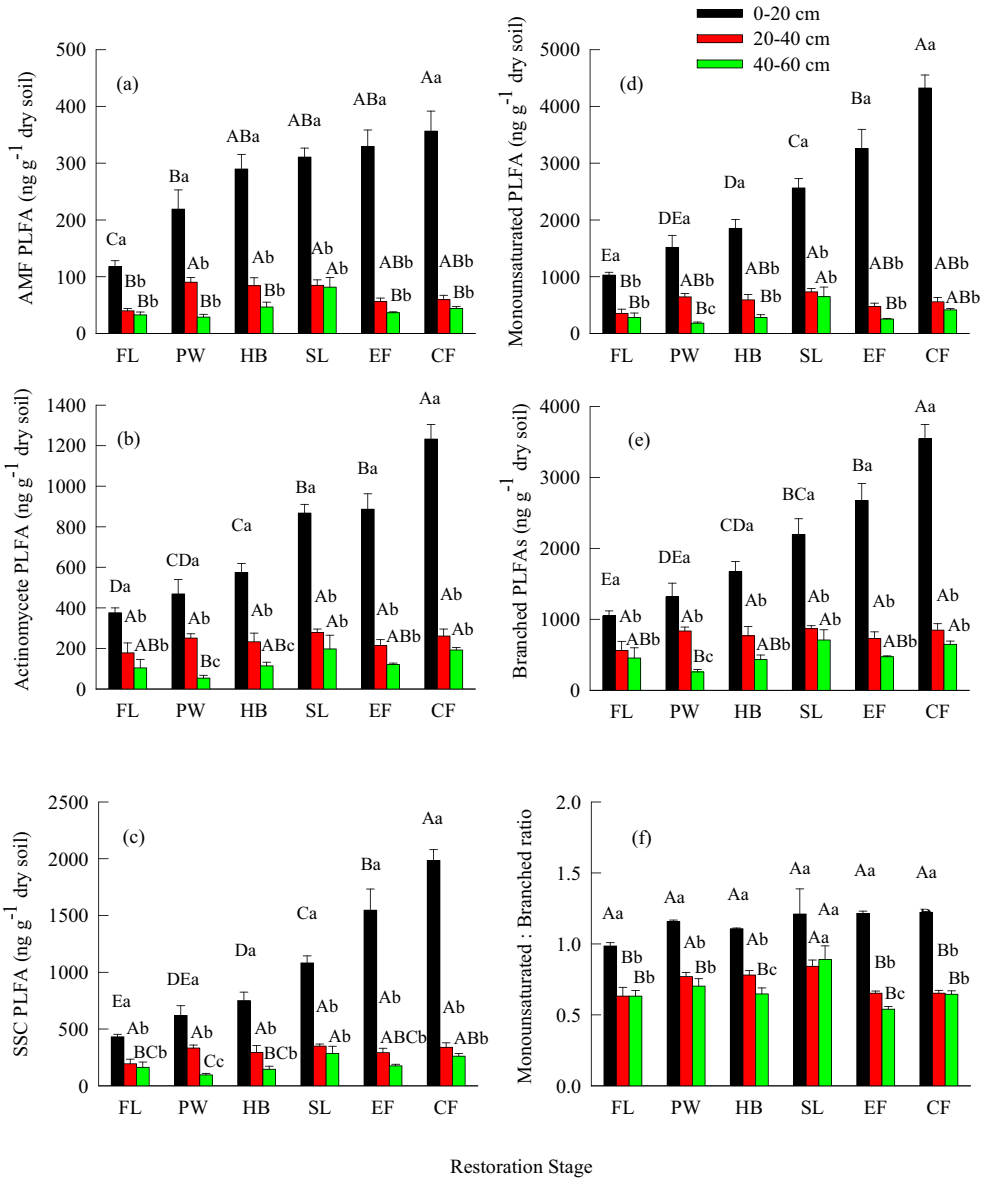


**Fig. 4.** (a) Soil arbuscular mycorrhizal fungal (AMF) PLFA, (b) Actinomycete PLFA, (c) Saturated straight-chain (SSC) PLFA, (d) Soil monounsaturated PLFA, (e) Branched PLFA, (f) Monounsaturated:branched ratio (mean ± SE) of different vegetation restoration stages in the Ziwuling forest region on the Loess Plateau, China. Different superscript upper case letters indicate statistically significant differences at the α = 0.05 level among the vegetation restoration stages at the same soil depth. Different superscript lower case letters indicate statistically significant differences at the α = 0.05 level among the soil depths at the same vegetation restoration stage. See Fig. 2 for abbreviations.

layer was found in the shrublands, herbs, pioneer weeds, and farmland stages (Fig. 3f), which may have partially resulted from lower soil moisture that facilitated the growth of $G^+$ bacteria in the early restoration stages (Table 2; Fuchslueger et al., 2016). Additionally, we inferred that an increased proportion of $G^-$ bacteria in the surface soil layer of the climax *Q. liaotungensis* and early *P. davidiana* forests boosted the incorporation of plant residues into the SOM (Fig. S3a; Bai et al., 2021), and enhanced the accumulation of soil C and N (Table 2). Meanwhile, the decreased proportion of $G^+$ bacteria in the surface soil layer of the climax *Q. liaotungensis* and early *P. davidiana* forests can impede the consumption of recalcitrant C (Fig. S3a; Bai et al., 2021; Kang et al., 2021), which can be instrumental for soil C and N sequestration (Table 2).

The soil monounsaturated:branched ratio can be widely applied to assess the relative ratio of aerobic to anaerobic microbes (Bossio et al., 2006; Yang et al., 2021). The monounsaturated:branched ratio ranged from 0.99 to 1.22 in the surface soil layer among the vegetation restoration stages (Fig. 4f), which suggested that the distribution of aerobic and anaerobic microbes was more balanced in the surface soil layer at different restoration stages. A significantly decreased monounsaturated:branched ratio was observed in the deep soil layer (Fig. 4f), which confirmed that anaerobic microbes dominated in the deep soil layer among the vegetation restoration stages. Ratke et al. (2020) reported that the soil oxygen content was reduced with soil depth owing to the decrease of soil pores. It was deduced that the decreased monounsaturated:branched ratio in the deep soil layer has likely caused by poor soil aeration relative to the surface soil layer among the vegetation restoration stages (Fig. 4f).

## 5. Conclusions

This study provides insights into how soil microbial communities shifted along long series of restoration stages of natural vegetation on the Loess Plateau of China over ~160 years. Our results revealed that natural vegetation restoration greatly promoted soil microbial biomass in the surface soil layer (0–20 cm) but had a negligible impact in the deep soil layer (20–60 cm). Greatly increased soil (0–20 cm) microbial biomass along the gradient of vegetation restoration was primarily attributed to gradually increased soil moisture and nutrient (i.e., SOC, TN, WSOC, and $NH_4^+$-N) availability, which resulted from progressively enhanced litter and root biomass entering the soil. Simultaneously, natural restoration of vegetation markedly altered composition

proportion of soil microbial communities. The ratio of $G^+$: $G^-$, the proportion of $G^-$ bacterial PLFA, $G^+$ bacterial PLFA, bacterial PLFA, and fungal PLFA in the total PLFA of the surface soil were changed along vegetation restoration stages, respectively. These alterations, particularly in the ratio of $G^+$: $G^-$ along vegetation restoration stages may regulate soil C and N sequestration and decomposition. This study comprehensively elucidate that long-term natural vegetation restoration can effectively restore the soil microbial biomass and alter composition proportion of soil microbial communities.

## Declaration of competing 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.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2022.104394.

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