forests

MDPI

Article

Changes in the Soil Bacterial Community in a Chronosequence of Temperate Walnut-Based Intercropping Systems

Pengxiang Gao [1], Xiaofeng Zheng [1], Lai Wang [1], Bin Liu [1] and Shuoxin Zhang [1,2,*]

[1] College of Forestry, Northwest A&F University, Yangling 712100, China; pengxiang.gao@nwafu.edu.cn (P.G.); evermyamelia@163.com (X.Z.); wanglaia_724@163.com (L.W.); lbpt@nwafu.edu.cn (B.L.)

[2] Qinling National Forest Ecosystem Research Station, Yangling 712100, China

* Correspondence: sxzhang@nwafu.edu.cn; Tel.: +86-29-8708-2993

Received: 4 February 2019; Accepted: 27 March 2019; Published: 31 March 2019

Abstract: Agroforestry (tree-based intercropping) is regarded as a promising practice in sustainable agricultural management. However, the impacts of converting cropland to an agroforestry system on microbial communities remain poorly understood. In this study, we assessed the soil bacterial communities in conventional wheat monoculture systems and a chronosequence (5–14 years) walnut-wheat agroforestry system through the high-throughput sequencing of 16S rRNA genes to investigate the effect of agroforestry age on soil bacterial communities and the correlation between soil properties and bacterial communities in the agroecosystem. Our results demonstrate that establishing and developing walnut tree-based agroforestry increased soil bacterial diversity and changed bacterial community structure. *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Acidobacteria* were the dominant soil bacterial phyla and *Bacillus* was the dominant genus. Crop monoculture systems were characterized by the *Bacillus* (*Firmicutes*)-dominated microbial community. The relative abundance of *Bacillus* decreased with agroforestry age; however, subgroups of *Proteobacteria* and *Actinobacteria* increased. Of the selected soil physicochemical properties, soil pH and bulk density were significantly correlated with bacterial alpha diversity, and soil pH and organic carbon were the principal drivers in shaping the soil microbial structure as revealed by redundancy analysis (RDA).

Keywords: afforestation; soil properties; 16S rRNA high-throughput sequencing; the Loess Plateau; tree-based intercropping

1. Introduction

Agroforestry is an intentional management of shade trees with agricultural crops [1], which is regarded as a promising approach for the sustainable development of agriculture. Agroforestry is a land-use type especially suitable for marginal land, which maximizes the provision of ecosystem goods and services [2]. The Loess Plateau region in China is one of the most eroded hilly areas in the world. In response to the erosion and land degradation, Grain-for-Green (GFG) and other afforest programs were carried out to replace the marginal cropland in this region [3]. Walnut tree (*Juglans regia* L.) is a widely planted afforestation species. The incorporation of walnut into the former cropland (walnut-based intercropping systems) is popular in this hilly area to balance the benefits of ecology and economy. Both the ecological and economic benefits of walnut-based intercropping systems have been demonstrated in previous research, for example, increasing light-use and land-use efficiency [4,5], improving soil infiltration [6], maintaining soil carbon sequestration [7] and promoting farmers' incomes [4,5].

Soil microorganisms play pivotal roles in some soil ecological processes, including the decomposition of organic matter and formation of soil aggregates [8], which directly impact on

not only soil fertility but also a series of ecosystem services [9]. Consequently, soil microbial diversity and richness are regarded as key indicators of soil health [10] and long-term soil productivity [11]. In agroforestry systems, trees can change the microclimate and supply abundant and diverse energy sources to the agroecosystems [12,13], which could be of benefit to microbial diversity. Bardhan [11] argued that, compared with crop agriculture, agroforestry systems would promote habitat diversity which results in higher species diversity. However, studies on soil microbial diversity are limited and current studies explore the spatial differences inside agroforestry systems. Bardhan [11] compared the bacterial communities between tree rows and crop alleys in the silver maple-based intercropping systems, and no significant difference in bacterial diversity was observed. Banerjee [14] analyzed great samples in Canadian agroforestry systems, and indicated that tree plots promote bacterial abundance and, to some extent, species richness. In a study of the microbial beta-diversity in Québec and Ontario, Lacombe [15] regarded the tree-based intercropping as integration. Based on the measure of dispersion among phospholipid fatty acid (PLFA) profiles, significantly higher diversity in the agroforestry system than in the crop monoculture system was only found in Québec [15]. Microbial species composition in an ecosystem is also important. Some keynote microbial groups have long been recognized for critical roles in the overall structure and function. Vallejo [16] indicated that conventional monoculture pastures promoted the dominance of Gram-negative bacteria, while the tree-based silvopastoral system favored actinomycetes and fungal biomass. On the contrary, Unger [17] suggested that agroforestry soil was characterized by a greater proportion of total bacteria, Gram-negative bacteria, anaerobic bacteria and mycorrhizal fungi than the cropland soil. Compared with crop monoculture, agroforestry is more complex in architecture. Shade trees live longer than herbaceous plants and the associated agroforestry management could have a lasting effect on the soil microbes. Seldom has the dynamic of microbial communities been studied in agroforestry systems, and the effect of agroforestry age remains poorly understood.

The soil is the harbor of soil microorganisms and physicochemical properties are directly correlated with the soil microbial communities. Soil pH, organic carbon, nitrogen, phosphorus and other soil physicochemical properties have been widely studied and correlated with soil bacterial communities in agroecosystems and forest ecosystems [14,18–20]. There are only a few studies that focus on the relationship of soil physicochemical properties and soil microbial communities in the agroforestry system. Clarifying these relations could help us better understand the impact of agroforestry on the microbial community. In addition, soil physicochemical properties are more accessible for us to manage soil biodiversity, thus enabling us to maintain agricultural land use [21].

In this study, we assessed the soil physicochemical properties and bacterial communities in walnut-wheat agroforestry systems converted from cropland, and the adjacent conventional crop monoculture systems. To address the temporal dynamics of soil bacterial communities with walnut succession in agroforestry systems, a chronosequence of 5–14-year-old walnut-based agroforestry systems was assessed. We hypothesized that, (i) walnut-based intercropping would increase soil bacterial diversity compared with crop monoculture, which would be strengthened with the agroforestry age; (ii) soil physicochemical properties would affect the structure and diversity of bacterial communities in the agroecosystems.

2. Materials and Methods

2.1. Study Site

The field trial of the study was located in Qishan (107°43′47″ E, 34°21′30″ N, 590–650 m a.s.l.), Shaanxi Province, China, which is located in the southern part of the Loess Plateau. This site is a semi-arid habitat, characterized by a temperate continental climate with hot, rainy summers and cold, dry winters. The mean annual temperature is 12.0 °C and the mean annual precipitation is 615 mm; ca. 70% falls between June and September. The soil in the study site is silt loam (27% sand, 49% silt and

24% clay) derived from loess and it is categorized as Calcaric Cambisols base on IUSS Working Group WRB [22]. The water table is over 30 m from the soil surface.

We established agroforestry fields with the Weiweiyuan Walnut Professional Farmer Cooperative in the level terrace. Before the agroforestry systems were established, crops had been planted for over thirty years. The main cropping system was wheat–fallow, and occasionally rotated with soybean/maize. One-year-old walnut (*Juglans regia* L.) seedlings were planted in the cropland in 2002, 2007 and 2011, respectively, in different fields at an interval of 4 m along the row and 6 m between rows; so the agroforestry age we referred to in this study is equal to the tree age. The tree rows were in a north–south orientation. Wheat (*Triticum aestivum* L.) was annually grown among the tree rows in the 1–7-year-old agroforestry systems and reduced to being biennially grown in agroforestry systems older than 7 years. The 1.6-m wide tree rows were left untilled. Soybean was rotated in 2013. Wheat was sowed (2.1×10^6 ha^{-1}) in early October and harvested in June of the next year between the tree rows as well as the adjacent crop monoculture system. The field left fallow for the rest of the year. Basic fertilizers of ca. 160 kg nitrogen ha^{-1} were equally applied and additional fertilizer was applied as needed. Cropland and the crop rows in the agroforestry system were tilled to 20 cm before wheat sowing. There was no irrigation.

2.2. Soil Sampling

This study was carried out in September 2015 before soil tillage and wheat sowing, following a completely randomized block design. Three spatially separated blocks were chosen including continuous conventional wheat monoculture system (C), 5-year-old agroforestry system (AF_5, established in 2011), 9-year-old agroforestry system (AF_9) and 14-year-old agroforestry system (AF_{14}) plots (Table 1). Each plot is approximately 50 min length and 15 m in width.

Table 1. Plots characteristics.

Plot	Altitudem	Slope Aspect	Tree Age Year	Tree Diameter * cm	Tree Canopy Diameter m	Tree Height m
Block 1						
1	648	N	NA	NA	NA	NA
2	635	N	5	7.08	0.92	3.18
3	620	N	9	14.20	2.20	5.70
4	648	N	14	16.02	2.43	7.20
Block 2						
1	594	S	NA	NA	NA	NA
2	600	S	5	8.10	1.23	3.73
3	594	S	9	13.53	2.02	5.43
4	600	S	14	15.60	2.23	6.77
Block 3						
1	589	S	NA	NA	NA	NA
2	594	S	5	7.88	1.17	3.33
3	600	S	9	14.10	1.89	4.88
4	620	N	14	14.48	2.17	6.06

* Tree diameter was measured at 80 cm in height.

In each agroforestry plot, six sample grids of 1×1 m^2 were randomly selected between the tree rows. In wheat monoculture plots, six sample grids of the same size were randomly selected. Soil samples were collected from 0 to 10 cm depth of the sample grids, and then mixed with samples from the same plot. So eventually, there was a composite sample from each crop monoculture plot and agroforestry plot. The composite samples were transported in an ice box to the laboratory. After removal of the litters and stones, the samples were sieved through a 2-mm mesh and the homogenized soils were divided into three portions. One portion was stored at −80 °C until DNA extraction. One portion was stored at 4 °C prior to assessing the soil water content (Wc), dissolved organic carbon (DOC), ammonium nitrogen (N-NH4$^+$) and nitrate nitrogen (N-NO3$^-$). This left a

portion that was air-dried for approximately a week for the measurement of soil organic carbon (SOC), total nitrogen (TN), pH and available phosphorus (AP). In addition, an undisturbed 5-cm-diameter core was collected at each grid of the plots for measuring the soil bulk density (BD). The average BD value of the grids stood for the BD value of the plots.

2.3. Soil Physicochemical Property Measurement

Wc was determined by weighing 25 g fresh soil oven-dried at 105 °C to constant weight. BD was calculated using the ring cylinder method. Soil pH was measured in 1:2.5 soil water solutions with a digital pH meter at 25 °C. SOC was analyzed by titration after wet oxidation in concentrated H_2SO_4 and 2 mol L^{-1} $K_2Cr_2O_7$. TN was measured with the Kjeldahl method using a Foss Kjeltec 8400 analyzer (Foss Tecator AB, Hoganas, Sweden). The concentrations of N-NH_4^+ and N-NO_3^- were measured with Continuous-Flow AutoAnalyzer III (Bran and Luebbe, Norderstedt, Germany) after fresh soil extracted by 2 M KCl. DOC content was determined using liquid TOCII analyzer (Elementar, Langenselbold, Germany) with the fresh soil solution of 0.5 M K_2SO_4. AP was analyzed using the colorimetric method with 0.5 M $NaHCO_3$ extraction.

2.4. Soil DNA Extractions and Sequencing of Bacterial 16S rRNA

Total soil DNA from different samples was extracted using the E.Z.N.A.^{TM} Soil DNA Kit (Omega Bio-Tec, Inc., Norcross, GA, USA) according to the manufacturer's directions. Purified DNA concentration was determined and purity was confirmed with NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Soil DNAs were stored at −80 °C until measurement in the PCR by LC-Bio Technology Co., Ltd, Hangzhou, Zhejiang, China.

The V3–V4 region of the prokaryotic 16S rRNA gene was amplified with the primers 319F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The 5′ ends of the primers were tagged with specific barcodes per sample and sequencing universal primers. PCR amplification was performed in a total volume of 25 µL reaction mixture containing 25 ng of template DNA, 12.5 µL of Phusion Master Mix (2×, NEB, Ipswich, MA, USA), 2.5 µL of each primer at 0.1 µM. The PCR reaction followed the procedure: an initial denaturation at 98 °C for 30 seconds; 35 cycles of denaturation at 98 °C for 10 seconds, annealing at 54 °C for 30 seconds, and extension at 72 °C for 45 seconds; and then final extension at 72 °C for 10 min. The PCR products were confirmed with 2% agarose gel electrophoresis. A negative control, with ultrapure water instead of a sample solution, was used to exclude the possibility of false-positive PCR results throughout the DNA extraction process. The PCR products were purified by AMPPure XP beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, Carlsbad, CA, USA). The amplicon pools were prepared for sequencing and the size and quantity of the amplicon library were assessed on Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. PhiX Control library (v3) (Illumina) was combined with the amplicon library (expected at 30%). The libraries were then sequenced on 300 PE MiSeq runs. The amplicons sequences were submitted to the National Center for Biotechnology Information (NCBI) genbank (SAMN10240058-63,67-69,73-75) under the bioproject PRJNA496378.

2.5. Bioinformatics Analyses

The achieved paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (1.2.8) [23]. Quality filtering on the raw reads was performed by filtering 1) reads containing undetermined nucleotides ratio greater than 5%; 2) reads having the base number of Q (Sequencing quality) ≤10 more than 20%. The specific information of the read number before and after quality filtering is listed in Table 2. Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs) by Usearch. Representative sequences were chosen for each OTU, and

taxonomic data were then assigned to each representative sequence using the RDP (Ribosomal Database Project) classifier with an 80% confidence threshold [24].

Table 2. Sequence number before and after quality filtering.

Plot	Raw Read Number	Clean Read Number
Block-1		
C	41,842	3,2196
AF_5	36,725	25,531
AF_9	18,552	14,845
AF_{14}	24,830	17,550
Block-2		
C	29,841	22,638
AF_5	35,911	28,070
AF_9	19,026	14,847
AF_{14}	25,688	20,885
Block-3		
C	28,844	21,768
AF_5	23,592	18,187
AF_9	29,201	23,483
AF_{14}	29,803	24,056

C = crop monoculture; AF5 = 5-year-old walnut-based agroforestry; AF9 = 9-year-old walnut-based agroforestry; AF14 = 14-year-old walnut-based agroforestry.

2.6. Statistical Analyses

To assess bacterial diversity among samples in a comparable manner, OTUs abundance information' was normalized using a standard of sequence number corresponding to the sample with the least sequences. Alpha diversity was applied in analyzing the complexity of species diversity (at OTU level) for a sample through four indices, namely, Observed species (N_{obs}), Chao1, Shannon and Simpson; the indices were calculated with QIIME 1.8.0 [25] as follows:

Chao1 index [26],

$$\text{Chao1} = N\text{obs} + \frac{{F_1}^2}{2F_2} \quad (1)$$

where F_1 and F_2 are the count of singletons and doubletons, respectively.

Shannon index,

$$H' = -\sum_{i=1}^{S} p_i \log_2 p_i \quad (2)$$

Simpson index,

$$1 - \lambda = 1 - \sum_{i=1}^{S} {p_i}^2; \quad (3)$$

where p_i is the proportion of the ith species (OTU); S is the richness of the species (OTUs).

Soil physicochemical properties, bacterial alpha diversity indices and bacterial taxa were analyzed by two-way analysis of variance (ANOVA) with the agroforestry age and block as fixed factors in IBM SPSS Statistics (IBM, Chicago, IL, USA). The normality and the homoscedasticity of the variances were checked. Data was transformed into its square root when either of the requirements was not satisfied (as for available phosphorus values). Beta diversity analysis was used to evaluate the differences of samples in species complexity. Principal coordinate analysis (PCoA) of unweighted UniFrac distances between taxonomic units (at OTUs level) was used to screen the compositional differences among bacterial communities in the agroforesty systems and crop monoculture systems, based on the 'pcoa' function of the 'vegan' package in R, version 3.4.1 (R Development Core Team, Auckland, New Zealand). Two-way permutational multivariate analysis of variance (PERMANOVA) [27] was used to

test the effect of agroforestry age and block on the bacterial community variances through 'adonis' function, with 999 random permutations.

Correlation tests were performed to identify potential correlations of tree and soil properties to bacterial diversity indices and the abundant genera. Pearson correlation coefficients were calculated between the variables. Redundancy analysis (RDA) was applied to relate the variation of bacterial community (at the genus level) to specific soil physicochemical properties. To test for a significant effect of soil physicochemical properties on bacterial community, a Monte-Carlo permutation test (permutation = 999) was performed.

3. Result

3.1. Soil Physicochemical Properties

Soil pH, BD and DOC were significantly affected by the agroforestry age (Table 3). Soil DOC was significantly higher in wheat monoculture (C) than that in the intercropping crops. Meanwhile, soil Wc was much lower in the crop monocultures. Soil pH, Wc, BD and N-NH_4^+ generally increased with agroforestry age (Table 3). SOC, TN and N-NO_3^- decreased at the earlier stage of agroforestry treatment (AF_5 and AF_9), but increased in the older agroforestry treatment.

Table 3. Soil physicochemical properties in crop monoculture and agroforestry of different age classes measured at 0–10 cm depth.

Age	pH	Wc %	BD g·cm^{-3}	SOC g·kg^{-1}	DOC mg·kg^{-1}	TN g·kg^{-1}	N-NH_4^+ mg·kg^{-1}	N-NO_3^- mg·kg^{-1}	AP mg·kg^{-1}
C	7.58(0.05)	15.13(0.77)	1.23(0.03)	11.09(0.41)	125.6(10.92)	1.49(0.05)	1.72(0.24)	10.91(0.63)	9.21(2.3)
AF_5	7.78(0.01)	18.72(0.87)	1.24(0.02)	9.91(0.21)	75.2(4.82)	1.30(0.10)	2.16(0.28)	9.32(0.46)	10.32(4.48)
AF_9	7.95(0.12)	21.50(0.94)	1.34(0.03)	8.94(0.19)	87.2(4.09)	1.20(0.05)	2.07(0.04)	7.89(0.25)	7.55(1.22)
AF_{14}	8.17(0.04)	21.16(0.92)	1.32(0.02)	10.90(0.60)	77.6(1.60)	1.48(0.14)	2.89(0.32)	14.32(0.97)	15.28(7.85)
P values from two-way analysis of variance									
Age	**0.013**	0.103	**0.034**	0.412	**0.014**	0.167	0.132	0.159	0.852
Block	0.957	0.591	0.426	0.483	0.944	0.606	0.424	0.282	0.743

C = crop monoculture; AF_5 = 5-year-old walnut-based agroforestry; AF_9 = 9-year-old walnut-based agroforestry; AF_{14} = 14-year-old walnut-based agroforestry. Wc, water content; BD, bulk density; SOC, soil organic carbon; DOC, dissolved organic carbon; TN, total nitrogen; AP, available phosphorus. Values shown are the mean of treatment replicates (n = 3) with standard error. Values in bold mean the main effect is significant at $p < 0.05$.

3.2. Soil Bacterial Community Structure and Diversity

The obtained sequences from the 16S rRNA gene sequencing of all the soil samples were clustered in 6504 OTUs, identified to 36 bacterial phyla.

The bacterial alpha diversity varied with agroforestry age and block (Table 4). Bacterial alpha diversity indices increased with agroforestry age.

Table 4. Bacterial alpha diversity indices of crop monoculture and agroforestry of different age class.

Age	Richness		Diversity	
	N_{obs}	Chao1	Shannon	Simpson
C	1484	3244	6.30	0.76
AF_5	1622	3525	7.06	0.84
AF_9	1724	3753	7.63	0.86
AF_{14}	2162	4417	9.60	0.97
P values from two-way analysis of variance				
Age	**0.019**	**0.016**	**0.013**	0.055
Block	**0.006**	0.063	**0.002**	**0.005**

C = crop monoculture; AF_5 = 5-year-old walnut-based agroforestry; AF_9 = 9-year-old walnut-based agroforestry; AF_{14} = 14-year-old walnut-based agroforestry. Values in bold mean the main effect is significant at $p < 0.05$.

The PCoA plot shows that the bacterial community was specific to the agroforestry age (Figure 1). AF_9 and AF_{14} were well separated from C and AF_5. The two-way PERMANOVA result (F_{Age} = 1.142, P_{Age} = 0.042; F_{Block} = 1.159, P_{Block} = 0.046) reinforced that the soil bacterial community composition depended on the agroforestry age. Of the identified phyla, *Firmicutes* (40.34%), *Proteobacteria* (21.30%), *Actinobacteria* (14.82%) and *Acidobacteria* (12.18%) were dominant in soil microbial communities in the studied plots (Figure 2). The relative abundance of *Firmicutes* and *Proteobacteria* was significantly affected by the agroforestry age (respectively, $p = 0.012$, $p < 0.001$). The relative abundance of *Firmicutes* decreased with agroforestry age, whereas *Proteobacteria* increased with agroforestry age (Figure 2a). At the genus level, *Bacillus* (31.10%) was dominant (Figure 2b). The relative abundance of *Bacillus* decreased with agroforestry age ($p = 0.012$).

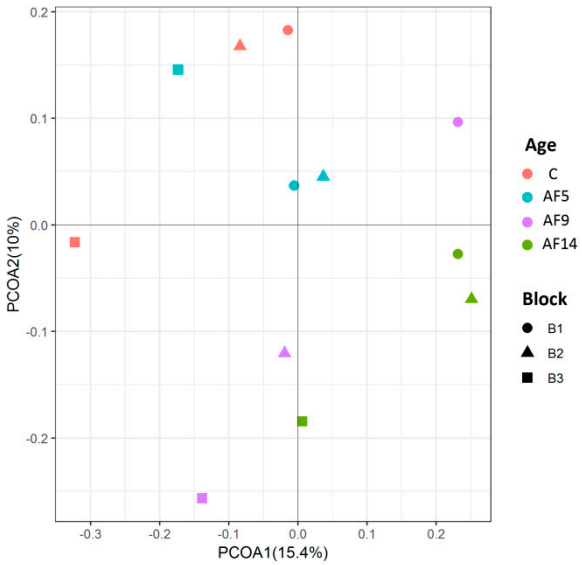

Figure 1. Principal coordinate analysis (PCoA) plot showing patterns of β-diversity in bacterial communities (OTU, operational taxonomic units level) of different agroforestry ages. C = crop monoculture; AF_5 = 5-year-old walnut-based agroforestry; AF_9 = 9-year-old walnut-based agroforestry; AF_{14} = 14-year-old walnut-based agroforestry.

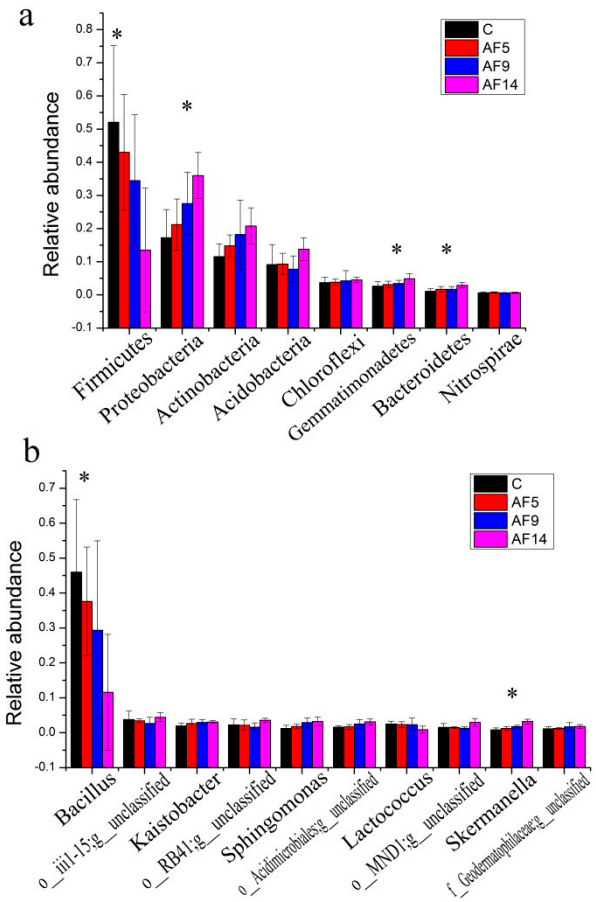

Figure 2. The relative abundance of bacterial phyla (**a**) and genera (**b**) of different agroforestry ages. C = crop monoculture; AF_5 = 5-year-old walnut-based agroforestry; AF_9 = 9-year-old walnut-based agroforestry; AF_{14} = 14-year-old walnut-based agroforestry. Asterisks indicate that the effect of agroforestry age is significant through ANOVA at $p = 0.05$.

3.3. Influence of Soil Physicochemical Properties on Bacterial Community

Soil BD, pH and Wc had a positive correlation with soil bacterial alpha diversity and some genera belonged to *Proteobacteria* and *Actinobacteria* analyzed by a correlation test (Figure 3). However, they had a negative correlation with *Bacillus* and *Lactococcus* which belongs to *Firmicutes*. On the contrary, TN was negatively correlated with abundant genera in *Actinobacteria* and *Proteobacteria*.

In RDA, the selected soil physicochemical properties explained 90.80% (adjusted $R^2 = 0.494$) of the total variation of bacterial communities at the genus level, and the first two axes explained 67% of the variation (Figure 4). SOC ($p = 0.015$) and pH ($p = 0.050$) were revealed to be factors significantly affecting the bacterial community through the permutation test (Table S1).

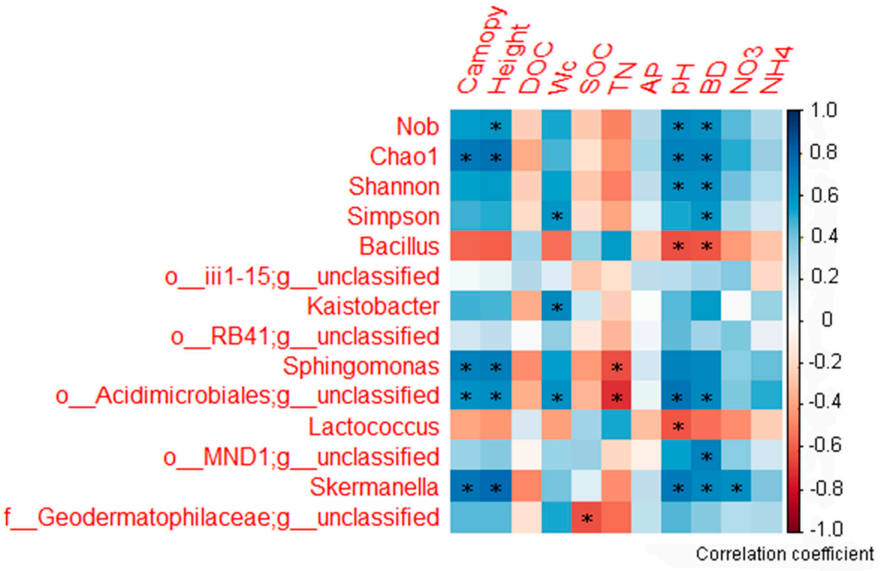

Figure 3. Relation of tree and soil physicochemical properties with bacterial alpha diversity and abundant genera. Canopy, average tree canopy diameter in a plot; Height, average tree canopy diameter in a plot; DOC, dissolved organic carbon; Wc, water content; SOC, soil organic carbon; TN, total nitrogen; AP, available phosphorus; BD, soil bulk density; NO_3, nitrate nitrogen; NH_4, ammonium nitrogen; Nob, observed species number. Asterisks indicate significant correlation at $p < 0.05$.

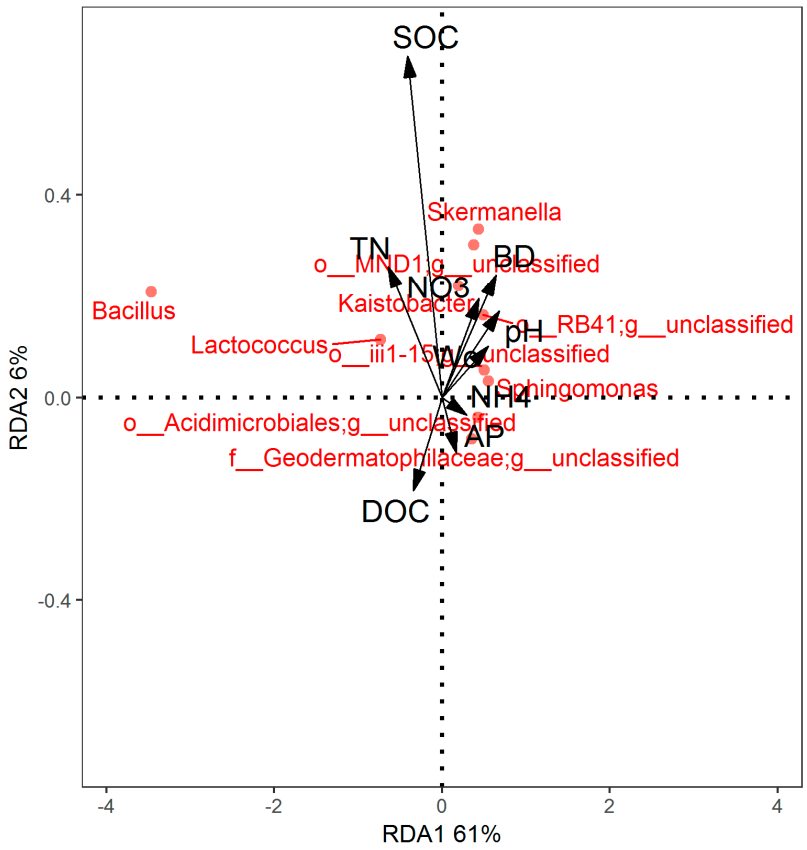

Figure 4. Redundancy analysis (RDA) ordination diagram of bacterial genera patterns with soil physicochemical properties (represented by arrows). DOC, dissolved organic carbon; Wc, water content; SOC, soil organic carbon; TN, total nitrogen; AP, available phosphorus; BD, soil bulk density; NO_3, nitrate nitrogen; NH_4, ammonium nitrogen. The ten most abundant bacterial genera are shown in the diagram.

4. Discussion

4.1. Bacterial Communities along Agroforestry Chronosequence

Our results revealed that the application of agroforestry systems increased soil bacterial diversity and changed soil bacterial community structures. Agroforestry is regarded as an effective afforestation approach in the Loess Plateau region. However, the effects of agroforestry on soil microorganisms have seldom been studied. Compared with the cropland, agroforestry increased the aboveground vegetation diversity. Diversified plant species and the resultant rhizosphere chemistry and litter quality could affect the soil microbial community [28]. Some previous studies in the same region found that microbial diversity increased with afforestation [29–31], and the microbial diversity associated with the aboveground vegetation. Zhang [32] observed that soil bacterial diversity decreased at the early stage of restoration on farmland along with the aboveground vegetation decrease. Compared with the undisturbed restoration, the constitution of aboveground vegetation in agroforestry was more stable during its building process. Zak [33] indicated that the soil microbial diversity was decided by the plant diversity-associated production change, rather than plant diversity. This could be an explanation of the bacterial diversity increase along agroforestry age, where the aboveground vegetation diversity is limited by agricultural management. In the correlation test, we found positive relations between tree biomass (represented by tree canopy and height) and bacterial diversity. This could partly clarify the important role of aboveground biomass in the soil bacterial diversity.

The bacterial community structure changed with agroforestry age (Figures 1 and 2). The most abundant bacterial phyla in the present study, including *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Acidobacteria*, were also observed in some former studies in the same region [34,35]. They make up over 85% of the total sequences. The soil bacterial community changed from being *Firmicutes* dominated to a more evenly distributed community along the agroforestry age. Guo [28] found a higher abundance of *Firmicutes* and lower abundance of *Proteobacteria* and *Actinobacteria* in cropland than in the restoration site, which is in accordance with our result. However, the higher frequency of *Firmicutes* in the studied site was in sharp contrast to the relatively lower abundance reported in other studies [29–31]; this is mainly due to the high abundance of *Bacillus*. *Firmicutes*, typically *Bacillus* and its derived genera, are a group of aerobic endospore-forming bacteria abundant in soils [36]. In Tian et al.'s study, the abundance of *Firmicutes* was much higher in the bare fallow site than in the vegetation-covered site [37]. *Firmicutes* were reported to be enriched in the soil with lower moisture content [38] and they could survive under stressful conditions, such as warming and desiccation [39]. *Bacillus cereus* (*Fermicutes*) was the most abundant bacterial species in our study. It could secrete poisonous toxins [40]. A reduction of *Bacillus cereus* abundance would be beneficial to agricultural production. *Proteobacteria* always receive great attention due to their role in soil nitrogen cycling [41]. Besides *Rhizobiales*, which belong to *Alphaproteobacteria*, some other subgroups in *Proteobacteria* have been discovered in nodules [41]. *Actinobacteria* have a critical role in the decomposition of soil organic materials, such as cellulose and chitin [42]. The increase of subgroups in *Proteobacteria* and *Actinobacteria* may improve nutrient cycling in agroforestry systems.

4.2. Drivers of Bacterial Community Variation

The microhabitat environment shaped by trees [43] is also important. In the present study, we focus on the effect of soil physicochemical properties, which are directly connected with the soil microbial community. Agroforestry is supposed to increase the soil organic carbon due to plenty of organic matter input in the form of litter fall and fine roots from trees [44]. However, in our study, SOC and TN even decreased in the agroforestry systems, especially in the 9-year-old agroforestry. Wang [45] found the same result during the long-term observation of agroforestry in this region. In the present study, we sampled from crop alleys, which may suffer less from tree litter or roots when the trees were small. With tree growth, the crop alleys would receive more organic matter from the trees. In addition, the high demand of nutrients for the younger trees to construct their biomass could lead to

a decrease in soil nutrients. As with the agroforestry management of Ketema [46], tillage frequency decreased with agroforestry age in the studied site, which minimized the human-disturbance effect on tree growth. This could be reflected through the BD increase with agroforestry age. Some studies pointed out that tree plantation would decrease soil water content [47,48] and this may be a threat of afforestation in the region. However, we sampled in September when surface evaporation was strong. Shade trees in the agroforestry systems alleviated the evaporation of remaining soil water.

Soil physicochemical properties are regarded as the driving force of soil microbial diversity [49–51]. Soil pH and SOC were identified as the most important factors shaping the bacterial community through RDA analysis. Soil pH is a universal predictor of bacterial composition and distribution [19,52,53]. Soil bacterial communities are always sensitive to pH change. Tight relationships between the abundant soil bacterial phyla, for example *Proteobacteria*, *Actinobacteria*, *Chloroflexi* and *Bacteroidetes*, and soil pH have been reported in different soils [19,54]. In our study, the pH positively correlated with bacterial alpha diversity and the abundant genera belonged to *Proteobacteria* and *Actinobacteria*. The negative correlation between soil pH and the dominant genus, *Bacillus*, was significant. SOC could significantly affect soil microbial communities [55,56]. Although the regression of SOC and the bacterial community was significant, the correlations between SOC and the abundant genera were not significant. SOC may affect the bacterial community through the control of other physicochemical properties. Soil BD and Wc were found to be positively correlated with the alpha diversity of soil bacteria and the abundant genera belonging to *Proteobacteria* and *Actinobacteria*, and negatively correlated with genera of *Firmicutes*. The positive correlations of soil bulk density with soil diversity could owe to the diminished use of tillage in the agroforestry system, especially in the older agroforestry systems. Some studies demonstrate that tillage would decrease soil bacterial communities [57,58]. Soil Wc increased with agroforestry age, which could alleviate the dominance of *Bacillus* and increase the bacterial diversity.

Besides the influence of soil physicochemical properties, the sample site (block) also significantly affected the soil bacterial community, which indicated that the distribution of the soil bacterial community even changed within small area.

5. Conclusions

Knowledge of soil biotic and abiotic factors in the agroforestry ecosystem could help us improve the management of sustainable land-use. In our study, establishing and developingwalnut tree-based agroforestry increased soil bacterial diversity and changed the bacterial community structure. *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Acidobacteria* were the dominant soil bacterial phyla and *Bacillus*, which belongs to *Firmicutes*, was the dominant soil bacterial genus. Crop monoculture systems were characterized by the *Bacillus* (*Firmicutes*)-dominant microbial community. The relative abundance of *Bacillus* decreased with agroforestry age; however, subgroups of *Proteobacteria* and *Actinobacteria* increased. Of the selected soil physicochemical properties, soil pH and bulk density were significantly correlated with bacterial diversity, and soil pH and organic carbon were the principal drivers in shaping the soil microbial structure, as revealed by RDA analysis.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/4/299/s1, Table S1: RDA result showing effect of soil properties on bacterial community.

Author Contributions: Conceptualization, P.G. and S.Z.; methodology, P.G.; software, X.Z.; validation, L.W., B.L. and S.Z.; formal analysis, P.G.; investigation, P.G. and X.Z.; writing—original draft preparation, P.G.; writing—review and editing, X.Z. and S.Z.; supervision, S.Z.; project administration, S.Z.; funding acquisition, S.Z.

Funding: This work was supported by the Chinese National Science and Technology Research Plan under Grant [2015BAD07B050202] and National Natural Science Foundation of China [31600493].

Acknowledgments: We appreciate Yulin Xu at Weiweiyuan Walnut Professional Farmer Cooperative, Qishan County, for his industrious work in governing the sample fields. We thank the editor and anonymous reviewers for their careful revisions and constructive comments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bhagwat, S.A.; Willis, K.J.; Birks, H.J.; Whittaker, R.J. Agroforestry: A refuge for tropical biodiversity? *Trends Ecol. Evol.* **2008**, *23*, 261–267. [CrossRef] [PubMed]
2. Tsonkova, P.; Quinkenstein, A.; Freese, D. Ecological benefits provided by alley cropping systems for production of woody biomass in the temperate region: A review. *Agrofor. Syst.* **2012**, *85*, 133–152. [CrossRef]
3. Gao, G.; Fu, B.; Wang, S.; Liang, W.; Jiang, X. Determining the hydrological responses to climate variability and land use/cover change in the Loess Plateau with the Budyko framework. *Sci. Total Environ.* **2016**, *557*, 331–342. [CrossRef] [PubMed]
4. Yun, L.; Bi, H.; Gao, L.; Zhu, Q.; Ma, W.; Cui, W.; Wilcox, B. Soil moisture and soil nutrient content in walnut-crop intercropping systems in the Loess Plateau of China. *Arid Soil Res. Rehabil.* **2012**, *26*, 285–296. [CrossRef]
5. Peng, X.; Zhang, Y.; Jing, C.; Jiang, Z.; Zhang, S. Photosynthesis, growth and yield of soybean and maize in a tree-based agroforestry intercropping system on the Loess Plateau. *Agrofor. Syst.* **2009**, *76*, 569–577. [CrossRef]
6. Wang, L.; Zhong, C.; Gao, P.; Xi, W.; Zhang, S. Soil infiltration characteristics in agroforestry systems and their relationships with the temporal distribution of rainfall on the Loess Plateau in China. *PLoS ONE* **2015**, *10*, e124767. [CrossRef] [PubMed]
7. Lu, S.; Meng, P.; Zhang, J.; Yin, C.; Sun, S. Changes in soil organic carbon and total nitrogen in croplands converted to walnut-based agroforestry systems and orchards in southeastern Loess Plateau of China. *Environ. Monit. Assess.* **2015**, *187*, 688. [CrossRef] [PubMed]
8. Eo, J.; Park, K.C. Long-term effects of imbalanced fertilization on the composition and diversity of soil bacterial community. *Agric. Ecosyst. Environ.* **2016**, *231*, 176–182. [CrossRef]
9. Lemanceau, P.; Maron, P.A.; Mazurier, S.; Mougel, C.; Pivato, B.; Plassart, P.; Ranjard, L.; Revellin, C.; Tardy, V.; Wipf, D. Understanding and managing soil biodiversity: A major challenge in agroecology. *Agron. Sustain. Dev.* **2015**, *35*, 67–81. [CrossRef]
10. Ashworth, A.J.; Debruyn, J.M.; Allen, F.L.; Radosevich, M.; Owens, P.R. Microbial community structure is affected by cropping sequences and poultry litter under long-term no-tillage. *Soil Biol. Biochem.* **2017**, *114*, 210–219. [CrossRef]
11. Bardhan, S.; Jose, S.; Udawatta, R.P.; Fritschi, F. Microbial community diversity in a 21-year-old temperate alley cropping system. *Agrofor. Syst.* **2013**, *87*, 1031–1041. [CrossRef]
12. Fischer, J.; Zerger, A.; Gibbons, P.; Stott, J.; Law, B.S. Tree decline and the future of Australian farmland biodiversity. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 19597–19602. [CrossRef] [PubMed]
13. Jose, S.; Gillespie, A.R.; Pallardy, S.G. Interspecific interactions in temperate agroforestry. *Agrofor. Syst.* **2004**, *61*, 237–255.
14. Banerjee, S.; Baah Acheamfour, M.; Carlyle, C.N.; Bissett, A.; Richardson, A.E.; Siddique, T.; Bork, E.W.; Chang, S.X. Determinants of bacterial communities in Canadian agroforestry systems. *Environ. Microbiol.* **2016**, *18*, 1805–1816. [CrossRef]
15. Lacombe, S.; Bradley, R.L.; Hamel, C.; Beaulieu, C. Do tree-based intercropping systems increase the diversity and stability of soil microbial communities? *Agric. Ecosyst. Environ.* **2009**, *131*, 25–31. [CrossRef]
16. Vallejo, V.E.; Arbeli, Z.; Terán, W.; Lorenz, N.; Dick, R.P.; Roldan, F. Effect of land management and *Prosopis juliflora* (Sw.) DC trees on soil microbial community and enzymatic activities in intensive silvopastoral systems of Colombia. *Agric. Ecosyst. Environ.* **2012**, *150*, 139–148. [CrossRef]
17. Unger, I.M.; Goyne, K.W.; Kremer, R.J.; Kennedy, A.C. Microbial community diversity in agroforestry and grass vegetative filter strips. *Agrofor. Syst.* **2013**, *87*, 395–402. [CrossRef]
18. Deng, Q.; Cheng, X.; Hui, D.; Zhang, Q.; Li, M.; Zhang, Q. Soil microbial community and its interaction with soil carbon and nitrogen dynamics following afforestation in central China. *Sci. Total Environ.* **2016**, *541*, 230–237. [CrossRef]
19. Lauber, C.L.; Hamady, M.; Knight, R.; Fierer, N. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl. Environ. Microb.* **2009**, *75*, 5111–5120. [CrossRef]

20. Nie, Y.; Wang, M.; Zhang, W.; Ni, Z.; Hashidoko, Y.; Shen, W. Ammonium nitrogen content is a dominant predictor of bacterial community composition in an acidic forest soil with exogenous nitrogen enrichment. *Sci. Total Environ.* **2017**, *624*, 407–415. [CrossRef]
21. Deakin, G.; Tilston, E.L.; Bennett, J.; Passey, T.; Harrison, N.; Fernández-Fernández, F.; Xu, X. Spatial structuring of soil microbial communities in commercial apple orchards. *Appl. Soil Ecol.* **2018**, *130*, 1–12. [CrossRef]
22. IUSS Working Group WRB. *World Reference Base for Soil Resources 2014, Update 2015. International Soil Classification System for Naming Soils and Creating Legends for Soil Maps*; World Soil Resources Reports No. 106; FAO: Rome, Italy, 2015.
23. Mago, T.; Salzberg, S.L. FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **2011**, *27*, 2957–2963. [CrossRef]
24. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microb.* **2007**, *73*, 5261–5267. [CrossRef] [PubMed]
25. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Pena, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef] [PubMed]
26. Chao, A. Non-parametric estimation of the number of classes in a population. *Scand. J. Stat.* **1984**, *11*, 265–270.
27. Anderson, M.J.; Walsh, D.C.I. Permanova, Anosim, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? *Ecol. Monogr.* **2013**, *83*, 557–574. [CrossRef]
28. Guo, Y.; Chen, X.; Wu, Y.; Zhang, L.; Cheng, J.; Wei, G.; Lin, Y. Natural revegetation of a semiarid habitat alters taxonomic and functional diversity of soil microbial communities. *Sci. Total Environ.* **2018**, *635*, 598–606. [CrossRef] [PubMed]
29. Xiao, L.; Liu, G.B.; Zhang, J.Y.; Xue, S. Long-term effects of vegetational restoration on soil microbial communities on the Loess Plateau of China. *Restor. Ecol.* **2016**, *24*, 794–804. [CrossRef]
30. Dong, L.; Huang, Y.; Sun, H.; An, S. The restoration age of Robinia pseudoacacia plantation impacts soil microbial biomass and microbial community structure in the Loess Plateau. *Catena* **2018**, *165*, 192–200.
31. Liu, J.; Yang, Z.; Peng, D.; Zhu, H.; Yang, G.; Ha, V.N.; Zhong, Z. Response of soil microbial community dynamics to *Robinia pseudoacacia* L. afforestation in the loess plateau: A chronosequence approach. *Plant Soil* **2018**, *423*, 327–338. [CrossRef]
32. Zhang, C.; Liu, G.; Xue, S.; Wang, G. Soil bacterial community dynamics reflect changes in plant community and soil properties during the secondary succession of abandoned farmland in the Loess Plateau. *Soil Biol. Biochem.* **2016**, *97*, 40–49. [CrossRef]
33. Zak, D.R.; Holmes, W.E.; White, D.C.; Peacock, A.D.; Tilman, D. Plant diversity, soil microbial communities, and ecosystem function: Are there any links? *Ecology* **2003**, *84*, 2042–2050. [CrossRef]
34. Zhong, Y.; Yan, W.; Shangguan, Z. Impact of long-term N additions upon coupling between soil microbial community structure and activity, and nutrient-use efficiencies. *Soil Biol. Biochem.* **2015**, *91*, 151–159. [CrossRef]
35. Hu, H.; Chen, X.; Hou, F.; Wu, Y.; Cheng, Y. Bacterial and fungal community structures in Loess Plateau grasslands with different grazing intensities. *Front. Microbiol.* **2017**, *8*, 606.
36. Subhash, Y.; Rajeev, K.; Saxena, A.K.; Arora, D.K. Diversity and phylogeny of plant growth-promoting bacilli from moderately acidic soil. *J. Basic Microb.* **2015**, *51*, 98–106.
37. Tian, H.; Wang, H.; Hui, X.; Wang, Z.; Drijber, R.A.; Liu, J. Changes in soil microbial communities after 10 years of winter wheat cultivation versus fallow in an organic-poor soil in the Loess Plateau of China. *PLoS ONE* **2017**, *12*, e0184223. [CrossRef]
38. Acosta-Martínez, V.; Dowd, S.E.; Sun, Y.; Wester, D.; Allen, V. Pyrosequencing analysis for characterization of soil bacterial populations as affected by an integrated livestockcotton production system. *Appl. Soil Ecol.* **2010**, *45*, 13–25. [CrossRef]
39. Battistuzzi, F.U.; Hedges, S.B. A major clade of prokaryotes with ancient adaptations to life on land. *Mol. Biol. Evol.* **2009**, *26*, 335–343. [CrossRef]
40. Arnesen, L.P.S.; Fagerlund, A.; Granum, P.E. From soil to gut: Bacillus cereus and its food poisoning toxins. *Fems Microbiol. Rev.* **2008**, *32*, 579–606. [CrossRef]

41. Araujo, A.S.F.; Leite, L.F.C.; Iwata, B.D.F.; de Freitas Iwata, B.; de Andrade Lira, M.; Xavier, G.R. Microbiological process in agroforestry systems. A review. *Agron. Sustain. Dev.* **2012**, *32*, 215–226. [CrossRef]
42. Stevenson, A.; Hallsworth, J.E. Water and temperature relations of soil Actinobacteria. *Environ. Microbiol. Rep.* **2015**, *6*, 744–755. [CrossRef]
43. Lin, B.B. Agroforestry management as an adaptive strategy against potential microclimate extremes in coffee agriculture. *Agric. For. Meteorol.* **2007**, *144*, 85–94. [CrossRef]
44. Kaur, B.; Gupta, S.R.; Singh, G. Soil carbon microbial activity and nitrogen availability in agroforestry systems on moderately alkali sols in northern India. *Appl. Soil Ecol.* **2000**, *15*, 283–294. [CrossRef]
45. Wang, L. Effect Mechanism Underground on Productivity of Walnut-Wheat Intercropping System. Ph.D. Thesis, Northwest A&F University, Yangling, China, 2016.
46. Ketema, H.; Yimer, F. Soil property variation under agroforestry based conservation tillage and maize based conventional tillage in Southern Ethiopia. *Soil Tillage Res.* **2014**, *141*, 25–31. [CrossRef]
47. Chen, L.; Huang, Z.; Jie, G.; Fu, B.; Huang, Y. The effect of land cover/vegetation on soil water dynamic in the hilly area of the loess plateau, China. *Catena* **2007**, *70*, 200–208. [CrossRef]
48. Jia, X.; Shao, M.; Zhu, Y.; Luo, Y. Soil moisture decline due to afforestation across the Loess Plateau, China. *J. Hydrol.* **2017**, *546*, 113–122. [CrossRef]
49. Kerfahi, D.; Tripathi, B.M.; Dong, K.; Go, R.; Adams, J.M. Rainforest conversion to rubber plantation may not result in lower soil diversity of bacteria, fungi, and nematodes. *Microb. Ecol.* **2016**, *72*, 359–371. [CrossRef] [PubMed]
50. Thomson, B.C.; Tisserant, E.; Plassart, P.; Uroz, S.; Griffiths, R.I.; Hannula, S.E.; Buée, M.; Mougel, C.; Ranjard, L.; Veen, J.A.V. Soil conditions and land use intensification effects on soil microbial communities across a range of European field sites. *Soil Biol. Biochem.* **2015**, *88*, 403–413. [CrossRef]
51. Wang, J.; Ren, C.; Cheng, H.; Zou, Y.; Bughio, M.A.; Li, Q. Conversion of rainforest into agroforestry and monoculture plantation in China: Consequences for soil phosphorus forms and microbial community. *Sci. Total Environ.* **2017**, *595*, 769–778. [CrossRef] [PubMed]
52. Takeshi, T.; Seishi, I.; Chiaki, N.; Kiwamu, M.; Katsuhiko, A.; Satoshi, T.; Shigeaki, H. Molecular diversity of bacterial chitinases in arable soils and the effects of environmental factors on the chitinolytic bacterial community. *Soil Biol. Biochem.* **2009**, *41*, 473–480.
53. Landesman, W.J.; Nelson, D.M.; Fitzpatrick, M.C. Soil properties and tree species drive ß-diversity of soil bacterial communities. *Soil Biol. Biochem.* **2014**, *76*, 201–209. [CrossRef]
54. Chu, H.; Fierer, N.; Lauber, C.L.; Caporaso, J.G.; Knight, R.; Grogan, P. Soil bacterial diversity in the Arctic is not fundamentally different from that found in other biomes. *Environ. Microbiol.* **2010**, *12*, 2998–3006. [CrossRef] [PubMed]
55. Fierer, N.; Bradford, M.A.; Jackson, R.B. Toward an ecological classification of soil bacteria. *Ecology* **2007**, *88*, 1354–1364. [CrossRef]
56. Liu, J.; Sui, Y.; Yu, Z.; Shi, Y.; Chu, H.; Jin, J.; Liu, X.; Wang, G. High throughput sequencing analysis of biogeographical distribution of bacterial communities in the black soils of northeast China. *Soil Biol. Biochem.* **2014**, *70*, 113–122. [CrossRef]
57. Sengupta, A.; Dick, W.A. Bacterial community diversity in soil under two tillage practices as determined by pyrosequencing. *Microb. Ecol.* **2015**, *70*, 853–859. [CrossRef]
58. Helgason, B.L.; Walley, F.L.; Germida, J.J. No-till soil management increases microbial biomass and alters community profiles in soil aggregates. *Appl. Soil Ecol.* **2010**, *46*, 390–397. [CrossRef]