Alfalfa with Forage Crop Rotation Alleviates Continuous Alfalfa Obstacles through Regulating Soil Enzymes and Bacterial Community Structures

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Abstract: Alfalfa is a perennial herbaceous forage legume that is significantly and adversely affected by monocropping. Crop rotation is the most effective measure to overcome continuous cropping obstacles. However, the mechanisms of how bacterial communities are affected and the potential links between these effects and cropping systems remain poorly understood. Based on a long-term field experiments with continuous alfalfa and forage crops with alfalfa rotation in the black soil region of the western Songnen Plain in Northeast China, the alterations in soil bacterial community structure using high-throughput sequencing of the 16S rRNA gene and soil chemical properties and enzyme activities were analyzed. The alfalfa–forage oats–silage maize–alfalfa and alfalfa–silage maize–forage oats–alfalfa system significantly increase the levels of total phosphorus and available phosphorus, and promote the activities of acid phosphatase, β-glucosidase, leucine aminopeptidase, and N-acetyl-β-glucosaminidase in comparison to continuous alfalfa. While alfalfa crop rotation did not affect the α-diversity of soil bacteria, it significantly altered the bacterial community composition and structure. Some key taxa are significantly enriched in the crop rotation system soils, including *Bacillus*, *Sphingobium*, *Paenibacillus*, *Hydrogenispora*, *Rubrobacter*, *Haliangium*, and *Rubellimicrobium*. Additionally, crop rotation with alfalfa increased the stability and complexity of the soil bacterial co-occurrence network. Based on our findings, we recommend promoting the alfalfa–forage oats–silage maize–alfalfa and alfalfa–silage maize–forage oats–alfalfa rotation systems as ideal practices for overcoming the challenges associated with continuous cropping of alfalfa. These systems not only enhance soil nutrient content and enzyme activities but also foster a beneficial microbial community, ultimately improving soil functionality and crop performance.

Keywords: alfalfa crop rotation; continuous cropping obstacles; soil chemical properties; enzyme activities; bacterial community structure

1. Introduction

The practice of growing the same crop on the same land year after year without interruption is a common farming practice due to economic benefit or natural agroecological conditions [1]. However, this approach usually leads to severe continuous cropping obstacles (CCOs), with poor crop growth, increased incidence of pests and diseases, and
a decrease in both yield and quality [2,3]. Continuous cropping obstacles affect several crops, including medicinal plants [4], watermelon [5], cotton [6], and alfalfa [7]. Numerous studies have demonstrated that the development of CCOs for crops is highly complex, involving factors such as damaged soil property, reduced soil enzyme activity, imbalance in soil microbial community structure and allelopathic-autotoxic effects [8]. Decades of continuous sugarcane cropping resulted in soil acidification, a reduction in the carbon–nitrogen ratio, an increase in alkali hydrolyzable nitrogen, a decline in organic matter as well as total sulfur content when compared to newly planted fields [9]. Additionally, the accumulation of self-toxic substances, such as phenolic acids, in soils from continuous cropping inhibits the growth of crops such as cucumbers, strawberries, and tobacco [10–12]. Many studies have also shown that continuous cropping significantly increases the relative abundance of fungal pathogens, leading to severe diseases in corn, soybeans, and melons [13–15].

Alfalfa (Medicago sativa L.) is a perennial leguminous plant renowned as the “Queen of forages” for its high yield, rich protein content, excellent palatability and wide adaptability [16,17]. Furthermore, alfalfa exhibits a high ability of root nodulation with N2-fixing Rhizobia and produces numerous fibrous roots, which enhances soil organic matter and improves soil aggregate structure [18]. The Songnen Plain in northeast China serves as a crucial region for the development of alfalfa industry, is also an important commodity grain base and livestock husbandry development base. This region plays an important role in safeguarding national food security and regional ecological stability [19,20]. However, in order to meet the demand for animal feed, the widespread cultivation of alfalfa in the region has led to an increasingly common phenomenon of continuous cropping, consequently resulting in a gradual decline in production capacity year by year [21]. The reduced production capacity is demonstrated by a number of factors, such as declined root activity, decreased seed germination rates, increased incidences of root rot, and challenges in resowing or replanting [22]. This phenomenon is a typical CCOs of alfalfa, which is accompanied by a decline in soil quality. This, in turn, is transforming the black soil area in the western part of the Songnen Plain from a “functional ecological zone” to a “vulnerable ecological zone”. Therefore, urgent measures are needed to address the adverse effects of CCOs on alfalfa yield and ecosystem health.

Crop rotation is an effective approach to alleviate the CCOs. Rational crop rotation not only enhances ecological and economic benefits but also effectively improves soil physical and chemical properties, thereby achieving increased yields and income [23,24]. For example, the implementation of a spring wheat–alfalfa crop rotation can obviously reduce the loss of soil nutrients in semi-arid areas, thereby promoting the sustainability of agroecosystems [25]. Alfalfa crop rotation compared with continuous corn is a means to greatly reduce the leaching of NO3–N to the aquifer and improve the surface soil, with the potential to increase soil organic carbon sequestration [26]. Previous studies have pointed out that, compared with continuous cropping, crop rotation could increase soil microbial biomass, alter microbial diversity, and mediate community composition [27–29]. The incorporation of alfalfa into a tomato–maize rotation has been demonstrated to result in the recruitment of a diverse array of bacterial and fungal taxa, including arbuscular mycorrhizal fungi, and taxa associated with plant growth promoting and biocontrol abilities [30]. However, most research available primarily focuses on the rotational patterns of alfalfa with crops such as corn, soybeans, and wheat, whereas studies on the rotational benefits of alfalfa with other forage crops are relatively limited. Furthermore, investigations into the formation process of soil bacterial communities with what key bacteria are recruited by alfalfa under different rotational systems remain unexplored.

This study conducted field experiments on alfalfa continuous cropping and different alfalfa crop rotation systems in the black soil region of the western Songnen Plain, China. The changes in soil physicochemical properties and soil enzyme activities were analyzed, and the effects of different alfalfa crop rotation systems on soil bacterial com-
Community structure were investigated by using high-throughput sequencing technology. The objective of this study is to delve into the interrelationship between different alfalfa crop rotation systems and soil microbial community structure. The findings will provide crucial insights for the design of strategies to enrich microbial communities, and thus alleviating the obstacles of alfalfa monocropping.

2. Materials and Methods

2.1. Experimental Design

This study established a continuous cropping/crop rotation platform of alfalfa in Fulaerji district, Qiqihar city, Heilongjiang province, China (47°15′ N, 123°41′ E). The area belongs to the black soil region of the western Songnen Plain, with an average annual temperature of 3 °C and an average annual precipitation of 450 mm. The soil studied is aeolian sandy soil in a semiarid region, with an average pH value above 7.0 and salinity of approximately 0.24%.

The experimental plots were established in 2011, and a continuous cropping of alfalfa was conducted for eight years until 2019. Then, in 2019, the land was ploughed and a crop rotation system was implemented. By 2023, the first crop rotation cycle was completed. The experiment consisted of four crop rotation treatments: alfalfa–silage maize–silage maize–alfalfa (ACCA); alfalfa–silage maize–forage oats–alfalfa (ACOA); alfalfa–forage oats–forage oats–alfalfa (AOOA); and alfalfa–forage oats–silage maize–alfalfa (AOCA). Continuous alfalfa cropping served as the control, denoted as 8 years of alfalfa (AAAA). There was a total of 5 treatments, each covering an area of 300 m². Six replicates were set up for each treatment, with a total of 30 plots. Before the start of the experiment, alfalfa seeds were sown at a density of 18 kg·ha⁻¹, forage oats at 180 kg·ha⁻¹, and silage maize at 22.5 kg·ha⁻¹. The field layout and crop rotation schedule are shown in Table S1.

2.2. Soil Sample Collection and Soil Parameter Determination

In the final year of the rotation cycle, soil sampling was collected from each treatment during the alfalfa flowering stage. Five replicate samples were collected in a five-point method and then homogenized to provide one composite sample per replicated plot. Alfalfa roots were randomly uprooted and clipped from crown. Using the soil shaking method, soil adhering to the alfalfa roots was gently shaken off, with soil within 2 mm of the roots considered rhizosphere soil [31]. The bulk soil was collected simultaneously, and the bulk soil collection depth was 20 cm. After mixing each type of soil, they were sieved through a 2 mm mesh. Part of the soil was air-dried for subsequent analysis of soil physicochemical properties, while part was stored at −80 °C for soil DNA extraction.

The soil pH was determined using a pH meter in a soil water suspension (1:2.5 w/v). The soil total carbon (TC) and total nitrogen (TN) contents were measured using an elemental analyzer (VarioEL III, Elementar, Hanau, Germany) [32]. Soil total phosphorus (TP) digested with H₂SO₄-HClO₄, available phosphorus (AP) extracted with 0.5 M NaHCO₃, and ammonium nitrogen (NH₄⁺-N) and nitrate nitrogen (NO₃⁻-N) extracted with 2.0 M KCl were assayed using a continuous flow analytical system (SAN, SKALAR, Breda, The Netherlands) [33,34]. Available potassium (AK) extracted with 1.0M CH₃COONH₄ was quantified using inductively coupled plasma-atomic emission spectrometry (ICPS-7500, Shimadzu, Kyoto, Japan) [35].

Four soil enzymes examined were acid phosphatase (ACP), β-glucosidase (βGC), leucine aminopeptidase (LAP), and N-acetyl-β-D-glucosaminidase (NAG). These enzymes primarily participate in the cycling of elements such as carbon, nitrogen, and phosphorus, and could be used to assess the health and biological activity of soil ecosystems. ACP activity was determined by the p-nitrophenol method [36]. βGC activity was assessed through incubation of the enzyme with 5 mM para-nitrophenyl-d-glucopyranoside (pNPG) in 50 mM citrate buffer (pH 6.0) within a 0.55 mL reaction mixture, maintained at 50 °C for 15 min [37]. The activities of LAP and NAG were deter-
mined using a microplate fluorescence method, with L-leucine-7-amido-4-methylcoumarin (L-leucine-AMC) and 4-methylumbelliferyl-N-acetyl-β-d-glucosaminide as fluorogenic substrates, respectively [38,39]. The four enzyme activities were all expressed in µmol day⁻¹ g⁻¹.

2.3. Soil DNA Extraction, PCR Amplification and High-Throughput Sequencing

Soil DNA was extracted from the frozen soil samples (0.5 g wet weight) using a E.Z.N.A Soil DNA Kit (OMEGA, Norcross, GA, USA) according to the manufacturer’s instructions [40]. The extracted DNA was stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The quality and concentration of DNA were determined using a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The bacterial 16S rRNA gene of the V4-V5 hypervariable regions was amplified using 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) [41]. Each 25 µL amplification system contains 10 µL of SYBR Premix Ex TaqTM (TaKaRa, Da-lian, China), 0.5 µL of forward and reverse primers at a concentration of 10 µM, 2 µL of DNA sample (1–10 ng/µL) as the template, and 12 µL of sterile water to make up the total volume to 25 µL. PCR amplification cycling was initial denaturation at 95 °C for 5 min, followed by 30 cycles (95 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min) with a final extension at 72 °C for 5 min.

Using the QIAEX Agarose Gel Extraction Kit (II) (QIAGEN, Shanghai, China), PCR products were purified. The purified products were quantified using the Quantus™ Fluorometer (Promega, Madison, WI, USA). Each PCR product was purified and pooled in equimolar amounts, and then prepared for sequencing using an Illumina MiSeq platform (PE 300).

2.4. Processing of Bacterial 16S rRNA Sequencing Data

The raw sequences were processed using QIIME (v 1.9.1) (http://qiime.org/tutorials/tutorial.html) (accessed on 15 May 2023) [42]. FLASH software (http://www.cbcb.umd.edu/software/flash) (accessed on 15 May 2023) was employed for merging paired reads [43]. Then, the deblur algorithm was applied to eliminate low-quality sequences, and subsequent quality control involved the removal of chimeric sequences using the Uchime algorithm [44,45]. High-quality sequences were clustered into operational taxonomic units (OTUs) based on a 97% similarity threshold [46]. Taxonomic assignments for bacterial OTU representative sequences were conducted using the RDP naïve Bayesian classifier, following the SILVA (v 128) database [47,48]. The raw sequence data were deposited into the Sequence Read Archive (SRA) database at the National Center for Biotechnology Information (NCBI) under accession number [PRJNA1085785].

2.5. Statistical Analysis

The Shannon diversity index, representing alpha diversity, was computed using the QIIME Pipeline (v 1.9.1). For the beta diversity, principal coordinate analysis (PCoA) based on the Bray–Curtis distance using the “vegan” package in R (v 4.1.0) was conducted to compare the differences in bacterial community structures among different treatments [49,50]. Differential analysis at the OTU level between the continuous cropping and alfalfa crop rotation was conducted using “DESeq2” package in R (v 4.1.0) by fitting a generalized linear model with a negative binomial distribution [51]. OTUs with p < 0.05 and |log2(fold change)| ≥ 1 were considered a significant difference. Venn diagrams for differential enriched OTUs in different treatments were constructed using tool Venny (v 2.1.0). Co-occurrence networks were utilized to assess the relationships between each bacterial OTU with a relative abundance greater than 0.1%. The Spearman coefficient between OTUs was determined using the “Hmisc” and “igraph” packages in R (v 4.1.0) [52]. When the Spearman coefficient between OTUs was greater than 0.8 or less than −0.8, with a significance level of p < 0.05, the OTU was included in the network analysis.
The networks were explored and visualized using Gephi (v 0.8.2) [54]. Redundancy analysis (RDA) and Pearson correlation analysis were utilized to determine which soil parameter was significantly correlated with the bacterial community structure [55]. The statistical significance of the soil-related indicators, bacterial α-diversity, and bacterial relative abundance at various taxonomic levels were tested using an analysis of variance (ANOVA) and pairwise comparisons with Tukey's HDS.

3. Results

3.1. Soil Chemical Properties and Soil Enzyme Activities

Soil TP and AP contents from different alfalfa crop rotation systems were significantly higher than AAAA (Table 1). The value of alfalfa crop rotations for TP was ACOA > AOOA > AOCA > ACCA > AAAA, while that of alfalfa crop rotations for AP was AOOA > ACCA > AOCA > ACOA > AAAA. On the contrary, the alfalfa crop rotation treatments showed average reductions of 5.58%, 7.11%, and 24.2% in TC, TN, and NO₃⁻-N, respectively, compared to AAAA. Nevertheless, the soil pH, NH₄⁺-N, and AK contents in the alfalfa crop rotation treatments were similar to those in the AAAA.

Table 1. Effects of continuous cropping/crop rotation of alfalfa on soil chemical properties.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>pH</th>
<th>TC</th>
<th>TN</th>
<th>NH₄⁺-N</th>
<th>NO₃⁻-N</th>
<th>AP</th>
<th>AK</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAAA</td>
<td>7.49 ± 0.017 a</td>
<td>19.9 ± 0.372 a</td>
<td>1.39 ± 0.031 a</td>
<td>0.461 ± 0.023 c</td>
<td>105 ± 2.72 ab</td>
<td>6.50 ± 0.116 a</td>
<td>22.2 ± 1.41 b</td>
</tr>
<tr>
<td>ACCA</td>
<td>7.53 ± 0.014 a</td>
<td>18.6 ± 0.198 b</td>
<td>1.27 ± 0.007 b</td>
<td>0.599 ± 0.041 b</td>
<td>109 ± 3.37 ab</td>
<td>4.87 ± 0.362 b</td>
<td>29.4 ± 1.99 a</td>
</tr>
<tr>
<td>ACOA</td>
<td>7.51 ± 0.025 a</td>
<td>19.0 ± 0.189 b</td>
<td>1.32 ± 0.009 ab</td>
<td>0.695 ± 0.018 a</td>
<td>99.0 ± 2.67 b</td>
<td>4.78 ± 0.095 b</td>
<td>28.0 ± 1.04 a</td>
</tr>
<tr>
<td>AOCA</td>
<td>7.53 ± 0.011 a</td>
<td>18.8 ± 0.300 b</td>
<td>1.31 ± 0.034 ab</td>
<td>0.617 ± 0.009 b</td>
<td>104 ± 3.18 ab</td>
<td>5.05 ± 0.223 b</td>
<td>29.4 ± 1.93 a</td>
</tr>
<tr>
<td>AOOA</td>
<td>7.52 ± 0.018 a</td>
<td>18.7 ± 0.349 b</td>
<td>1.26 ± 0.040 b</td>
<td>0.622 ± 0.009 b</td>
<td>112 ± 4.15 a</td>
<td>5.00 ± 0.522 b</td>
<td>29.5 ± 0.915 a</td>
</tr>
</tbody>
</table>

P 0.466 0.032* 0.023* 0.0001*** 0.101 0.003** 0.0092** 0.2780

Note: TC: total carbon; TN: total nitrogen; TP: total phosphorus; NH₄⁺-N: ammonium nitrogen; NO₃⁻-N: nitrate nitrogen; AP: available phosphorus; AK: available potassium. Between different lowercase letters are significantly different at p<0.05. Significance levels are as follows: * p<0.05; ** p<0.01; *** p<0.001.

Different alfalfa crop rotation systems exhibited a significant effect on the activities of four enzymes (Table 2). In detail, all alfalfa crop rotation systems resulted in an augmentation of ACP, βGC, LAP, and NAG activities, with ACOA and AOCA showing the most pronounced enhancements. ACOA demonstrated a notable increase in the activities of ACP, βGC, LAP, and NAG by 30.0%, 56.3%, 136%, and 94.9%, respectively, compared to AAAA. Similarly, AOCA exhibited a significant increase in the activities of ACP, βGC, LAP, and NAG by 21.4%, 54.0%, 126%, and 96.0%, respectively, compared to AAAA.

Table 2. Effects of continuous cropping/crop rotation of alfalfa on soil enzyme activities.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>ACP</th>
<th>βGC</th>
<th>LAP</th>
<th>NAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAAA</td>
<td>6.43 ± 0.103 b</td>
<td>27.2 ± 1.22 c</td>
<td>2.79 ± 0.136 d</td>
<td>2.70 ± 0.353 c</td>
</tr>
<tr>
<td>AOCA</td>
<td>7.80 ± 0.326 a</td>
<td>41.9 ± 1.72 a</td>
<td>6.31 ± 0.184 a</td>
<td>5.28 ± 0.258 a</td>
</tr>
<tr>
<td>ACCA</td>
<td>6.65 ± 0.153 b</td>
<td>33.2 ± 2.71 b</td>
<td>4.56 ± 0.326 b</td>
<td>3.86 ± 0.202 b</td>
</tr>
<tr>
<td>AOOA</td>
<td>6.74 ± 0.182 b</td>
<td>29.1 ± 1.08 bc</td>
<td>3.65 ± 0.226 c</td>
<td>3.57 ± 0.247 b</td>
</tr>
<tr>
<td>AOOA</td>
<td>8.36 ± 0.626 a</td>
<td>42.5 ± 2.17 a</td>
<td>6.59 ± 0.437 a</td>
<td>5.25 ± 0.181 a</td>
</tr>
</tbody>
</table>

P 0.0013 0.0001 0.0001 0.0001

Note: ACP: acid phosphatase; βGC: β-glucosidase; LAP: leucine aminopeptidase; NAG: N-acetyl-β-D-glucosaminidase. Between different lowercase letters are significantly different at p<0.05.

3.2. Soil Bacterial Diversity and Structure

A total of 3,954,125 high-quality 16S rRNA sequences were generated in this study and identified as 13,530 bacterial OTUs. There was no significant difference in bacterial Shannon index among all treatments, regardless of bulk soil or rhizosphere soil (Figure 1a,b). Principal coordinate analysis (PCoA) was employed to analyze the β-diversity of
bacterial communities (Figure 1c,d), and showed a significant discrimination between alfalfa crop rotation samples and the continuous cropping samples. This indicates that rotation practices have a notable effect on both bulk soil ($p = 0.0001$) and rhizosphere soil ($p = 0.029$) bacterial communities.

![Figure 1.](image)

**Figure 1.** Effects of continuous cropping/crop rotation of alfalfa on soil bacterial diversity. Bacterial Shannon diversity in bulk soil (a) and rhizosphere soil (b). Principal coordinate analysis (PCoA) based on Bray–Curtis distance for differences in the composition of bacterial communities in bulk soil (c) and rhizosphere soil (d). * $p < 0.05$; ** $p < 0.001$; NS: not significant.

Across all bulk soil samples, the dominant bacterial phyla (relative abundance higher than 5%) were Proteobacteria (27.3–31.6%), Actinobacteria (21.7–25.2%), Acidobacteria (11.1–13.3%), and Chloroflexi (6.07–7.05%). Additionally, relative abundance at phyla level in alfalfa crop rotation was improved compared to continuous cropping (Figure 2a). For example, compared to continuous cropping, alfalfa crop rotation resulted in higher richness of Acidobacteriota, Chloroflexi, Gemmatimonadota, and Firmicutes. Conversely, the relative abundance of Proteobacteria and Bacteroidota decreased significantly in the alfalfa crop rotation treatment. In terms of all rhizosphere soil samples, it was found that Proteobacteria had the highest relative abundance (17.8–21.2%), which was significantly lower in ACOA than in AAAA (Figure 2b). Furthermore, compared to AAAA, an increase in the relative abundance of Firmicutes and Myxococccota was found among different alfalfa crop rotation treatments, although the differences were not significant.
Figure 2. The relative abundance of soil bacterial communities at phylum level. Changes in the relative abundance of bacterial phylum in bulk soil (a) and rhizosphere soil (b) under different alfalfa continuous cropping/crop rotation treatments. Significance levels are as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The relative abundance exceeding 1% was considered to be dominant bacterial genera (Figure 3). With regards to bulk soil bacterial communities, the alfalfa crop rotation treatment exhibited higher relative abundance of *norank_Vicinamibacteraceae*, *norank_Gemmatimonadaceae*, *Novosphingobium*, *RB41*, and *Nitrospira*, but lower relative abundance of *Microvirga* and *Ramlibacter*. For rhizosphere bacterial communities, it was noted that ACOA significantly reduced the relative abundance of *Arthrobacter* and *Microvirga* in comparison with AAAA.

Figure 3. The relative abundance of soil bacterial communities at genus level. Changes in the relative abundance of dominant genera in bulk soil (a) and rhizosphere soil (b) under different alfalfa continuous cropping/crop rotation treatments. Significance levels are as follows: * $p < 0.05$; ** $p < 0.01$.

The differences in bacterial species between alfalfa continuous cropping and alfalfa crop rotation at the OTU level were investigated (Table S2). In bulk soil, compared to AAAA, both ACCA and ACOA showed a significantly higher number of depleted OTUs than enriched OTUs, with enriched/depleted OTU numbers being 210/231 and 186/208, respectively (Figure 4a–d). For AOCA and AOOA, the numbers of significantly enriched OTUs were close to the numbers of depleted OTUs, with enriched/depleted OTU numbers being 185/184 and 191/190, respectively. In rhizosphere soil, compared to in AAAA, AOCA exhibited a significantly higher number of enriched OTUs than depleted OTUs, with enriched/depleted OTU numbers being 85/75. Conversely, ACOA showed the opposite pattern, with enriched/depleted OTU numbers being 58/80. Furthermore, both
ACCA (80/78) and AOOA (70/70) demonstrated a similar number of significantly enriched and depleted OTUs (Figure 4f–i).

To further explore the relationships among differential OTUs under different planting systems, the unique and shared enriched OTUs between different alfalfa crop rotation and continuous cropping treatments were presented. The Venn diagrams revealed an overlap of 20 OTUs among different rotation treatments in bulk soil (Figure 4e). These OTUs were affiliated with taxa such as *Pontibacter*, *Sphingobium*, *Bacillus*, *Parasegetibacter* and *norank_Vicinamibacteraceae* (Table S3), suggesting their potential importance in plant growth under various crop rotation treatments. Additionally, a considerable number of unique OTUs were observed in AOCA, ACCA, AOOA, and ACOA treatments, numbering 119, 99, 96, and 79, respectively. However, the enriched OTUs in rhizosphere soil had no overlap among different crop rotation treatments, indicating a lower likelihood of mutual colonization among these OTUs (Figure 4j). Furthermore, in AOCA treatment, the proportion of unique OTUs was higher, reaching 23.5%. These OTUs belonged to taxa such as *Paenibacillus*, *norank_Ardenticatenales*, *Sporosarcina*, *Rubrobacter* and *Rubellimicrobium*, indicating the prevalence of these taxa in response to the AOCA treatment. Meanwhile, specific OTUs such as *Pirellula*, *Phenylobacterium*, *norank_Roseiflexaceae*, *Pajaroellobacter*, *Hydrogenispora*, *Bacillus*, and *Segetibacter* were significantly enriched in other crop rotation treatments (Table S3).

**Figure 4.** Certain OTUs are enriched and depressed under alfalfa continuous cropping/crop rotation. Volcano plot was used to describe OTU differences in AOCA (a), ACCA (b), AOOA (c), and ACOA (d) in bulk soil was expressed as BAAAA, BAOCA, BACCA, BAOOA, and BACOA, respectively. The OTU differences in AOCA (f), ACCA (g), AOOA (h), and ACOA (i) in rhizosphere soil was expressed as RAAAA, RAOCa, RACCA, RAOOA and RACOA, respectively. Each point represents an individual OTU, and the position along the y axis represents the abundance fold.
change compared with the abundance fold change in AAAA. Number of significantly enriched differential OTUs between continuous cropping and rotation treatments in bulk soil (e) and rhizosphere soil (j).

3.3. Soil Bacterial Co-Occurrence Network

The interrelated characteristics of bacteria in both bulk soil and rhizosphere soil were investigated through the utilization of co-occurrence network analysis at the OTU level (with a relevance abundance threshold of more than 0.1%), then the network topological features of these networks for each treatment were calculated (Figure 5; Table 3). Compared to the BAAAA network, the alfalfa crop rotation network exhibited significantly higher values in average path length, average clustering coefficient, average weighted degree, and modularity, while the number of nodes, edges, and average degree was lower. These results suggest that the alfalfa crop rotation network in the bulk soil is more complex (Figure 5a–e; Table 3). Among each network, BAOCA, BACCA, and BAOOA had similar numbers of nodes, edges, graph density, and average path length. BAOOA showed the highest modularity and average weighted degree.

Moreover, there were discernible variations in the response of rhizospheric bacterial community networks to different alfalfa crop rotation treatments (Figure 5f–j; Table 3). Specifically, compared to the RAAAA network, both RAOCA and RACOA had similar node numbers but possessed more edges, positive/negative correlations, average degree, graph density and average weighted degree. They also showed lower average path length, average clustering coefficient, and modularity. In contrast, RACCA displayed opposite characteristics with fewer nodes and edges, lower negative correlations, as well as higher average clustering coefficient and modularity. Additionally, RAAAA was similar to RAOOA with slight differences in the number of nodes and edges, but in terms of positive and negative correlations, RAOOA had more positive correlations. Overall, alfalfa crop rotation led to an increased complexity in the bacterial network structure in the rhizosphere soils, while RACOA and RAOCA exhibited even more intricate network structures.

In order to evaluate the possible key role of taxa in the network, closeness centrality and betweenness centrality were used to describe the topological role of bacterial OTUs in the network (Table S4). It was found that most of these key taxa belonged to Proteobacteria, Actinobacteriota, and Acidobacteriota. In bulk soil, key taxa from different alfalfa crop rotation treatments included OTU1123 (Lysobacter), OTU7993 (Terrimonas), OTU2302 (Mycetocola), OTU2114 (norank_Gaiellales), and OTU2189 (Acidibacter). In rhizosphere soil, key taxa from different alfalfa crop rotation treatments were OTU2294 (Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium), OTU1426 (norank_Gemmatinimonadaceae), OTU1142 (Nocardioides), OTU1042 (Pedomicrobium), and OTU784 (norank_Xanthobacteraceae).
Figure 5. Effects of continuous cropping/crop rotation of alfalfa on soil bacterial co-occurrence network. The bacterial co-occurrence network of AAAA (a), AOCA (b), ACCA (c), AOOA (d) and
ACOA (e) in bulk soil was expressed as BAAAA, BAOCA, BACCA, BAOOA, and BACOA, respectively. The bacterial co-occurrence network of AAAA (f), AOCA (g), ACCA (h), AOOA (i), and ACOA (j) in rhizosphere soil was expressed as RAAAA, RAOCA, RACCA, RAOOA, and RACOA, respectively. The nodes are colored according to bacterial phylum. Node size indicates the degree of connection. Edge color represents positive (red) and negative (green) correlations. The key taxa are annotated on the network.

**Table 3.** The co-occurrence network characteristics in the alfalfa continuous cropping/crop rotation system.

<table>
<thead>
<tr>
<th>Network Indicator</th>
<th>AAAA</th>
<th>BAOCA</th>
<th>BACCA</th>
<th>BAOOA</th>
<th>BACOA</th>
<th>RAAAA</th>
<th>RAOCA</th>
<th>RACCA</th>
<th>RAOOA</th>
<th>RACOA</th>
</tr>
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<tbody>
<tr>
<td>Node</td>
<td>188</td>
<td>178</td>
<td>177</td>
<td>176</td>
<td>183</td>
<td>181</td>
<td>180</td>
<td>172</td>
<td>182</td>
<td>179</td>
</tr>
<tr>
<td>Edge</td>
<td>1699</td>
<td>1326</td>
<td>1372</td>
<td>1343</td>
<td>1538</td>
<td>1363</td>
<td>1354</td>
<td>1345</td>
<td>1580</td>
<td></td>
</tr>
<tr>
<td>Positive correlation</td>
<td>958</td>
<td>780</td>
<td>835</td>
<td>862</td>
<td>913</td>
<td>791</td>
<td>1194</td>
<td>796</td>
<td>893</td>
<td>1079</td>
</tr>
<tr>
<td>Negative correlation</td>
<td>741</td>
<td>546</td>
<td>537</td>
<td>481</td>
<td>625</td>
<td>572</td>
<td>660</td>
<td>539</td>
<td>452</td>
<td>501</td>
</tr>
<tr>
<td>Average degree</td>
<td>18.1</td>
<td>14.9</td>
<td>15.5</td>
<td>15.3</td>
<td>16.8</td>
<td>15.1</td>
<td>20.6</td>
<td>15.8</td>
<td>14.8</td>
<td>17.7</td>
</tr>
<tr>
<td>Average path length</td>
<td>2.92</td>
<td>3.05</td>
<td>3.01</td>
<td>3.00</td>
<td>2.96</td>
<td>3.01</td>
<td>2.89</td>
<td>3.00</td>
<td>3.07</td>
<td>2.88</td>
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<td>Graph density</td>
<td>0.097</td>
<td>0.084</td>
<td>0.088</td>
<td>0.087</td>
<td>0.092</td>
<td>0.084</td>
<td>0.115</td>
<td>0.092</td>
<td>0.082</td>
<td>0.099</td>
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<tr>
<td>Network diameter</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td></td>
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<tr>
<td>Average clustering coefficient</td>
<td>0.490</td>
<td>0.515</td>
<td>0.543</td>
<td>0.530</td>
<td>0.499</td>
<td>0.498</td>
<td>0.487</td>
<td>0.507</td>
<td>0.507</td>
<td>0.480</td>
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<tr>
<td>Average weighted degree</td>
<td>2.59</td>
<td>3.15</td>
<td>4.40</td>
<td>6.10</td>
<td>3.98</td>
<td>2.71</td>
<td>8.97</td>
<td>3.91</td>
<td>7.07</td>
<td>9.83</td>
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<td>1</td>
<td>1</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Modularity</td>
<td>0.423</td>
<td>0.467</td>
<td>0.468</td>
<td>0.493</td>
<td>0.455</td>
<td>0.437</td>
<td>0.335</td>
<td>0.446</td>
<td>0.473</td>
<td>0.421</td>
</tr>
</tbody>
</table>

Note: B represents the bulk soil samples. R represents the rhizosphere soil samples.

3.4. Correlations between Soil Parameters and Soil Bacterial Community Structure

The redundancy analysis (RDA) showed that the soil parameters (i.e., pH, TC, TN, AP, ACP, and LAP) had different effects on bacterial communities. Apart from ACP, pH, and AK, all other soil parameters significantly altered the structure of bacterial community in bulk soil (Figure 6a). In the rhizosphere soil, only AK was the vital factor that significantly affected the bacterial community structure ($p = 0.007$, Figure 6b).

**Figure 6.** Redundancy analysis (RDA) of bacterial OTUs and soil parameters. The relationship between bacterial OTUs and soil parameters in bulk soil (a) and rhizosphere soil (b). Significance levels are as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. 
The relationship between bacterial phyla and soil parameters was further analyzed (Table S5). For the bulk soil, most of the bacterial phyla were significantly associated with soil nutrients and enzyme activities, and responses to these parameters varied among phyla. For example, Proteobacteria and Bacteroidota were negatively correlated with TP but positively correlated with NO$_3^-$-N. In contrast, four other bacterial phyla including Chloroflexi were positively correlated with TP but negatively correlated with NO$_3^-$-N. Additionally, Proteobacteria and Bacteroidota also showed a positive response to AP, while Firmicutes and Acidobacteriota exhibited a negative response to AP. Bacteroidota, Chloroflexi, Gemmatimonadota, Firmicutes, and Acidobacteriota demonstrated significant associations with soil enzyme activities. Specifically, Chloroflexi was positively correlated with βGC activity. Bacteroidota was negatively correlated with LAP and NAG activity, while other phyla were positively correlated with these enzyme activities. Only Planctomycetota was negatively correlated with NH$_4^+$-N (Figure 7a).

In terms of the rhizosphere soil, the relative abundance of Chloroflexi was negatively correlated with TN and TC. Similarly, the relative abundance of Proteobacteria was also negatively correlated with βGC and LAP activity. However, Actinobacteriota and Firmicutes were positively correlated with AK and LAP activity, respectively. Additionally, Bacteroidota was negatively correlated with soil pH but positively correlated with TC (Figure 7b).

**Figure 7.** Heatmap showing the strength of correlations between soil parameters and soil bacterial communities at phylum level in bulk soil (a) and rhizosphere soil (b). Significance levels are as follows: * p < 0.05; ** p < 0.01. Color legend represents the values of correlation coefficients (r). Negative values indicate negative correlations, while positive values indicate positive correlations.

### 4. Discussion

Crop rotation, as an essential farming management practice, has been demonstrated to effectively enhance the soil microenvironment, increase microbial diversity, and promote the growth of beneficial microorganisms [56,57]. This study demonstrated that alfalfa crop rotation leads to the improvement of soil chemical properties, with significantly higher soil TP and AP than continuous cropping system (Table 1). These improvements in soil chemical properties contribute to the establishment of a more stable and healthier soil ecosystem, thereby reducing the occurrence of soil-borne diseases [58]. However, the present study found that the contents of TC, TN, and NO$_3^-$ are significantly lower in the alfalfa crop rotation system compared to the continuous cropping system. This indicates that alfalfa continuous cropping increases some soil nutrient indicators. This finding is consistent with the research by Chen et al. (2015), who observed that the soil TC, TN, AN, and AP was significantly increased after continuous cropping. This might be due to the long-term acclimatization where continuous cropping specifically enriched certain microbes that positively contributed to the soil nutrient status [59]. For
instance, Wang et al. (2017) discovered that continuous cropping significantly enriched certain microbial species capable of degrading cellulose [60].

Although alfalfa crop rotation significantly altered the microbial community structures in both bulk soils and rhizosphere soils (Figure 1), we found that continuous cropping has a greater impact on bulk soil than on rhizosphere soil, which is inconsistent with previous studies [61]. This discrepancy might be due to the differences in root exudates among various crops, the duration of continuous cropping and soil types, which could affect microbial community structures differently, leading to varying effects observed across different cropping systems [62]. The present study found that AP, TP, NAG, LAP, \(\beta\)GC, TN, TC, \(\text{NO}_3^-\)-N, and \(\text{NH}_4^+\)-N are the most significant factors influencing the bacterial community structure in different alfalfa crop rotation modes (Figure 6). This aligned with some findings from Lian et al. (2019) [63] and Liu et al. (2020) [61], who discovered that certain soil enzymes, along with TN and TC, played crucial roles in shaping microbial community structures.

In crop rotation systems, the role of complex interactions among soil microorganisms extends beyond maintaining soil health; it directly influences crop growth and resilience [57]. Our study underscores that alfalfa crop rotation fosters stronger intermicrobial relationships, culminating in intricate and resilient ecological networks (Figure 5). The higher numbers of nodes and edges of RAOCA and RACOA of bacterial network in the rhizospheric soils than those of RAAAA soils indicate that RAOCA and RACOA exhibit a greater network size and more microbial taxa involved in the potential microbial interactions than those in RAAAA treatment. These networks, by bolstering soil resilience, significantly contribute to its adaptability to environmental shifts. Similar results have been reported by Fan et al. (2018), that crop rotation promoted soil ecosystem stability and functionality [64].

The increased presence of beneficial soil microorganisms by alfalfa crop rotation, such as nitrogen-fixing bacteria and plant growth-promoting rhizobacteria has significant effects in mitigating the adverse effects of continuous cropping. In fact, *Bacillus* *niacin* is responsible for phosphate solubilization and siderophore production [65], while *Paenibacillus* has been reported to be associated with nitrogen fixation [66]. This not only could contribute to the reduction in the use of chemical pesticides but also naturally boost the health and yield of crops, thereby promoting the development of sustainable agriculture [67]. Thus, our results further highlight the indispensable role of microbial ecosystems in sustaining agricultural productivity and resilience through intricate woven fabric [68].

5. Conclusions

In conclusion, compared to continuous cropping, the alfalfa–forage oats–silage maize–alfalfa and alfalfa–silage maize–forage oats–alfalfa system could significantly increase the contents of TP, AP, ACP, and promote the \(\beta\)GC, LAP, and NAG activities, which is beneficial for enhancing soil nutrient supply and promoting crop growth and development. Although alfalfa crop rotation does not affect the \(\alpha\)-diversity of soil bacteria, it significantly alters the bacterial community structure, enriching beneficial bacterial taxa such as *Bacillus*, *Sphingobium*, *Paenibacillus*, *Hydrogenispora*, *Rubrobacter*, and *Rubellimicrobium*. Furthermore, the alfalfa crop rotation systems enhance the stability and complexity of soil bacterial co-occurrence networks. Therefore, it is recommended to promote the adoption of the alfalfa–forage oats–silage maize–alfalfa and alfalfa–silage maize–forage oats–alfalfa system as an ideal crop rotation system for alleviating alfalfa continuous cropping obstacles in the region. This study underscores the vital role of alfalfa crop rotation in mitigating the challenges of continuous cropping, enhancing soil nutrient content, promoting the diversity and abundance of beneficial bacteria, and thus improving soil functionality.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14071349/s1. Table S1: Field layout of alfalfa continuous cropping/crop rotation system. Note: A represents alfalfa, C represents silage maize, and O represents forage oats; Table S2: Bacterial OTUs that are significantly abundance in the bulk soil and rhizosphere soil (crop rotation vs continuous cropping, p < 0.05). Note: B represents the bulk soil samples. R represents the rhizosphere soil samples; Table S3: Common and unique bacterial OTUs in bulk soil and rhizosphere soil samples (crop rotation vs continuous cropping). Note: B represents the bulk soil samples. R represents the rhizosphere soil samples; Table S4: Topological characteristics of bacterial hubs observed in the bulk soil and rhizosphere soils across the alfalfa continuous cropping/crop rotation system. Note: B represents the bulk soil samples. R represents the rhizosphere soil samples; Table S5: Pearson correlation analysis of bacterial phylum and soil parameters in bulk soil and rhizosphere soil.

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Data Availability Statement: The original data presented in the study are openly available in the NCBI short-read archive under accession number PRJNA1085785.

Conflicts of Interest: We declare that we do not have any commercial or associative interests that represent conflicts of interest in connection with the work submitted here.

References


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