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Effects of Microbial Inoculants Combined with Chemical Fertilizer on Growth and Soil Nutrient Dynamics of Timothy (*Phleum pratense* L.)

Shanmu He [1,2], Ying Zhang [1,2], Xiaolei Yang [1,2], Qi Li [1,2], Changning Li [1,2] and Tuo Yao [1,2,*]

[1] College of Grassland Science, Gansu Agricultural University, Lanzhou 730070, China; hesm@st.gsau.edu.cn (S.H.); zhangying@st.gsau.edu.cn (Y.Z.); zhangxl@st.gsau.edu.cn (X.Y.); liqi@st.gsau.edu.cn (Q.L.); licn@gsau.edu.cn (C.L.)
[2] Key Laboratory of Grass Ecosystem, Ministry of Education, Gansu Agricultural University, Lanzhou 730070, China
* Correspondence: yaotuo@gsau.edu.cn

Abstract: Microbial inoculants derived from plant growth-promoting rhizobacteria (PGPR) offer eco-friendly alternatives to traditional chemical fertilizers, maintaining microbiota balance in agricultural systems. However, limited research has explored the combined effects of microbial inoculants and chemical fertilizers on crop growth and soil properties. In this study, we investigated seven fertilizer combinations, ranging from no fertilizer to various proportions of chemical fertilizers with microbial inoculants, on timothy (*Phleum pratense* L.) growth, chlorophyll content, soil properties, enzyme activities, and soil microbial communities. A randomized block design was employed to analyze these effects. The results indicate that the combination of 85% chemical fertilizer with microbial inoculants significantly increased timothy yield and chlorophyll content. In addition, a reduction to 55% chemical fertilizer in conjunction with microbial inoculants resulted in comparable yield to that of 100% fertilizer with no inoculants. The microbial inoculants treatments notably elevated soil catalase, urease, acid phosphatase, and invertase activities, along with soil fast-acting nutrient content. The sequencing results show that the abundance of beneficial bacteria increased, while that of fungi decreased in the soil rhizosphere after the application of microbial inoculants. This study underscored the potential of microbial inoculants combined with reductions in chemical fertilizers to enhance soil microbiology, nutrient content, and beneficial microbial abundance while suppressing pathogenic fungi, thereby promoting timothy growth and yield. These findings provide a theoretical basis for the use of microbial inoculants in sustainable agricultural practices, providing valuable insights for optimizing microbial inoculants and chemical fertilizer formulations to mitigate the sustainability challenges posed by conventional fertilizers.

Keywords: soil microbiology; chemical fertilizers; microbial inoculants; timothy; soil physicochemical properties

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Citation: He, S.; Zhang, Y.; Yang, X.; Li, Q.; Li, C.; Yao, T. Effects of Microbial Inoculants Combined with Chemical Fertilizer on Growth and Soil Nutrient Dynamics of Timothy (*Phleum pratense* L.). *Agronomy* **2024**, *14*, 1016. https://doi.org/10.3390/agronomy14051016

Academic Editors: Robert P. Larkin and Chengsheng Zhang

Received: 14 March 2024
Revised: 3 May 2024
Accepted: 9 May 2024
Published: 10 May 2024

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1. Introduction

Timothy (*Phleum pratense* L.), also known as *Phleum pratense*, is a perennial grass forage recognized as part of the standard hay mixture, providing high-quality nutrition for racehorses and serving as a staple food for domesticated pets such as rabbits, guinea pigs, and chinchillas [1]. Additionally, the entire grass plant holds medicinal properties, offering benefits such as antipyretic, antitussive hypothermic, and hemostatic effects. It serves as the primary cash crop in Minxian County, Gansu Province, China [2]. The prevalence of timothy cultivation in Minxian County is closely linked to soil nutrients, climatic conditions, planting practices, and economic considerations. However, the pursuit of high-yield and high-quality timothy cultivation faces several obstacles, as previous studies have highlighted factors such as soil physicochemical property degradation, decreased soil enzyme

activity, and alterations in soil microbial community structure as significant influences on yield and quality [3]. To mitigate these challenges, growers have explored various strategies, including the application of different fertilizer types, combinations of organic and metal-chelated fertilizers, and the use of farmyard manure, often in combination [4]. Combinations of different fertilizers are widely used due to their convenience, effectiveness, and cost efficiency [5]. Nevertheless, reliance on chemical fertilizers has led to reduced soil fertility, environmental pollution, and reduced soil microbial diversity. Taswar et al. [6] showed that the combination of compound fertilizer and microbial inoculants increased the enzymatic activity and bacterial abundance in rhizosphere soil. Microbial inoculants contain numerous beneficial microorganisms, which are known as plant root-promoting bacteria (PGPR), as most of them originate from the inter-root zone of plants and exert growth-promoting effects. PGPR can improve soil structure and microbiota, induce systemic resistance in plants, and secrete indole-3-acetic acid (IAA), gibberellin (GA), and cytokinin (CTK) that directly promote plant growth [7]. In recent years, microbial inoculants have been primarily focused on promoting the growth of cash crops, crop disease control, and other applications. For example, Li et al. [8] applied PGPR microbial inoculants to oats, alfalfa, and cucumber, demonstrating significant improvements in oat growth indices, as well as soil urease (SURE), invertase (SIN), alkaline phosphatase (SAKP), peroxidase (SCAT) activities, and fast-acting nutrients. Similarly, Hema et al. [9] pointed out that PGPR provide an environmentally friendly green alternative to synthetic pesticides and conventional farming practices for achieving sustainable agriculture through improved plant growth and stress resistance. PGPR exist in the soil rhizosphere and positively interact with plant roots. Furthermore, Shehzad et al. [10] showed that inoculation with indigenous microbial inoculants contributed to food security by biocontrolling important potato diseases (*Fusarium* dry rot and wilts). Although more PGPR mycorrhizal agents have been developed, fewer broad-spectrum adaptable agents have emerged. This may be attributed to the complex environments in which PGPR in microbial inoculants live and their sensitivity to external factors, such as climatic conditions, soil moisture, soil temperature, soil physicochemical properties, and plant cultivation practices [11]. At the same time, the research sites and relative abundances of microbial inoculants and chemical fertilizers are relatively small, which is insufficient to provide a theoretical basis for reducing the application of chemical fertilizers and microbial inoculants.

The development of microbial inoculants and application modes of chemical fertilizers is particularly important. Compared with the single application of microbial inoculants, this new mode of application can effectively provide the nutrients needed for plant growth and meet the requirements of modern agricultural development. Meng et al. [12] stated that the application of microbial inoculants promotes an increase in the nutrient content of the soil and enhances soil enzyme activities. Soil enzyme activity and microbial diversity have been identified as potential indicators of fertility. Soil enzymes are the metabolic powerhouses of soil organisms and play an important role in the material cycle and energy transformation of soil ecosystems.

The effects of microbial inoculants and chemical fertilizer pairings on soil enzyme activity and soil microbial diversity have not yet been fully explored in the context of timothy cultivation history. In this study, a field trial was conducted using a specialized forage microbial inoculant prepared earlier by our research team with different rates of chemical fertilizers to assess the effects of seven different fertilization treatments on the growth of timothy. In addition, ensuring the high yield and quality of timothy under the given conditions, investigating the impact of the addition of microbial inoculants on soil bacterial and fungal diversity in plant roots, as well as the detailed exploration of changes in soil enzyme activity and microbial diversity caused by different fertilization options may help develop fertilization strategies to manage soil microbial diversity and provide a comprehensive evaluation of the effectiveness of various fertilization methods. This will offer a theoretical basis and basic data for the application of microbial inoculants to ensure the efficient and stable production of timothy.

2. Materials and Methods

2.1. Research Site

Field experiments were carried out from April 2022 to November 2022 at Kaishengyuan Agricultural Professional Cooperative, Minxian County, Dingxi City, Gansu Province, China (103°48′25″ E, 34°29′20″ N, elevation 2603.15 m). The research site is located in the transition zone from a temperate semi-humid climate to an alpine humid climate, with an average annual temperature of 5.5 °C. The annual precipitation is 635 mm, the annual precipitation is 635 mm, and the average sunshine duration is 2214.9 h. The soil type is black soil with a pH of 7.97, alkaline-dissolved nitrogen of 94.52 mg·kg^{-1}, 21.80 mg·kg^{-1} quick phosphorus, 41.11 mg·kg^{-1} quick potassium, and 24.11 g·kg^{-1} organic matter [13].

2.2. Experimental Design

The experimental materials were selected from a local timothy (*Phleum pratense* L.) species (Minshan) and planted in strips (row spacing 20 cm, sowing rate 15 kg·ha^{-1}). A single-factor random block design was implemented, and experiments were repeated three times. The experiment included seven fertilization modes: CK (no fertilization), chemical fertilizers, microbial inoculants, and microbial inoculants with chemical fertilizers (100%, 85%, 70%, 55%). The specific fertilizer application rates are shown in Table 1.

Table 1. Application rates of different types of fertilizer.

Treatment	Processing Method	Chemical Fertilizers	Microbial Inoculants
CK	Unprocessed	0 kg·ha^{-1}	0 L·ha^{-1}
CM	Microbial inoculants	0 kg·ha^{-1}	75 L·ha^{-1}
CF	100% Diammonium phosphate	375 kg·ha^{-1}	0 L·ha^{-1}
0.85 CF+CM	85% Diammonium phosphate + microbial inoculants	320 kg·ha^{-1}	75 L·ha^{-1}
0.70 CF+CM	75% Diammonium phosphate + microbial inoculants	263 kg·ha^{-1}	75 L·ha^{-1}
0.55 CF+CM	55% Diammonium phosphate + microbial inoculants	206 kg·ha^{-1}	75 L·ha^{-1}
CF+CM	Diammonium phosphate + microbial inoculants	375 kg·ha^{-1}	75 L·ha^{-1}

Microbial inoculants were produced by Gansu Lanzhou Hong Yuan Biotechnology Co., Ltd. The effective number of live bacteria was $\geq 10^8$ CFU/g, and the bacterial composition contained highly active strains of *Bacillus mycoides*, *Bacillus subtilis*, and *Pseudomonas synxantha* [14]. The chemical fertilizer (diammonium phosphate) was a commercial product of Mason Agro Co., Ltd. (Beijing, China), containing 14% N and 21% P_2O_5. All fertilizers were applied once to the soil before planting as base fertilizers. Chemical fertilizer application was conducted at the percentage required for each treatment, while microbial inoculants were applied near the surface with an electric sprayer. Weeds were initially removed after seed emergence and after 20 days. Artificial water replenishment was carried out once after grass planting, and no artificial water replenishment was carried out afterward. No additional fertilizer and chemical pesticides were applied after planting to the mowing period, and other conventional field management methods were adopted.

2.3. Plant Measuring

Plant growth was determined 210 days after sowing, and plant height (natural state) was determined by randomly sampling 30 plants in each treatment. Leaf weight and stem weight were determined separately, and the stem–leaf ratio was calculated. A 1 m × 1 m sample plot was randomly selected within each plot, mowed to a uniform height, and weighed to obtain the fresh weight. The dry weight was determined using the drying method [15]. Leaf chlorophyll content was determined using an ECA-051 portable chlorophyll meter (Beijing Yi Kang Nong Science and Technology Development Co., Ltd.). Leaf area was determined using an LI-3000C portable leaf area meter (Qingdao Ju Chuang Jia Heng Analytical Instrument). The root system was scanned using a desktop scanner, and

the root system was imaged with Win-RHIZO root analysis system software to obtain relevant indicators.

2.4. Soil Sampling

Soil near plant roots was used for subsequent analyses. Five samples were randomly sampled from each replicate treatment. Next, soil samples were taken from 0 to 30 cm using a shovel, debris was removed, and soil samples attached to roots were collected by brushing and sweeping with a brush; soil samples from the same replicate treatment were evenly mixed and placed in enzyme-free and sterile self-sealing bags. All samples were divided into two parts: one part was preserved with dry ice and stored at −80 °C and sent to Shanghai Ling En Biologicals for high-throughput sequencing within one week; the other part was air-dried at room temperature and passed through a 60-mesh sieve with an aperture of approximately 0.25 mm for the determination of soil physicochemical properties and enzyme activity.

2.5. Soil Physicochemical Properties

Soil pH was determined using a pH meter (soil:H_2O ratio 1:2.5); soil electrical conductivity (EC, soil:H_2O ratio 1:5) was assessed using a conductivity meter; the total N (TN) was determined with the Kjeldahl method; total potassium (TK) was determined using the sodium hydroxide-flame photometry; total phosphorus (TP) was quantified with the molybdenum antimony colorimetric method. Organic matter (SOM) was determined using the potassium dichromate volumetric method—dilution heat method; alkaline nitrogen (AN) was assessed with the alkaline diffusion method; available phosphorus (AP) content was measured using the molybdenum blue method; available potassium (AK) was quantified using the sodium tetraphenyl–boron turbidimetric method [16,17].

2.6. Soil Enzyme Activities

The activities of soil catalase (CAT) and urease (SURE) were determined by titration with $KMnO_4$ and the indophenol blue colorimetric method, respectively. Soil acid phosphatase (ACP) activity was analyzed using the colorimetric method with benzene disodium phosphate. Soil invertase (SIN) activity was determined using the 3,5,dinitrosalicylic acid method [18,19].

2.7. DNA Extraction, PCR, and Sequencing

A thoroughly mixed soil sample (0.5 g) was taken from each treatment replicate, and sample DNA was extracted using EZNA®Soil DNA Kit (OMEGA; Beijing Jiehui Bogao Biotechnology Co., Ltd.). DNA concentration and purity were detected using a NanoDrop 2000. Illumina PE250 high-throughput sequencing was used to sequence the bacterial 16S rDNA region (341F-ITS2R) with the following primer sequences: 5′-CCTAYGGGRBGCASCAG-3′ and 5′-GGACTACNNGGG-TATCTAAT-3′. The fungal ITS rDNA region (ITS1F-ITS2R) was sequenced using the following primers: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and 5′-GCTGCGTTCTTCATCG-ATGC-3′ [20]. PCR reaction conditions: pre-denaturation at 98 °C for 2 min; denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min for 28 cycles [21]. PCR products were quantified using a QuantusTM Fluorometer. The corresponding proportion of mixing was carried out according to the sequencing volume requirements. The PacBio library was constructed by the repair and formation of dumbbell-like structures of joints, with the subsequent removal of unconnected joint fragments using an exonuclease. Subsequently, bipartite sequencing was conducted using the PacBio triple sequencing platform (Pacific Biosciences of California, USA), and consistent circular consensus sequencing (CCS) sequences were obtained using SMRTLINK (v9), with CCS sequences achieving QV20 (97% accuracy) levels of accuracy. Soil sequencing was conducted on an Illumina HiSeq 2500 platform (Shanghai Ling En Biozeron Co., Ltd., Shanghai, China).

2.8. Statistical Analysis

Excel 2019 was used to collate the experimental data. Statistical analysis was performed using SPSS 21.0 statistical software (IBM SPSS, Sommers, New York, NY, USA). SPSS 21.0 statistical software was used to conduct one-way ANOVA and Tukey's multiple comparison test to analyze the statistical significance of differences among different fertilization schemes ($p < 0.05$); SAS 9.4 software was used for principal component analysis. The effects of microbial agent and fertilizer combination on soil microbial community composition (redundancy analysis RDA) and Spearman correlation were analyzed using the Lingen Biocloud platform. Origin 2021 was used to visualize the data.

3. Results

3.1. Changes in the Growth of Timothy (Phleum pratense L.) under Different Fertilizer Combinations

Compared with no fertilization, different proportions of chemical fertilizers combined with microbial inoculants significantly affected agronomic traits, yield, leaf area, and chlorophyll content in field experiments (Figure 1). Overall, reducing chemical fertilizer usage with microbial inoculants significantly improved the yield components of timothy, with the combination of 85% chemical fertilizers with microbial inoculants (0.85 CF+CM) resulting in the highest timothy plant height, total root length, stem-to-leaf ratio, and fresh grass yield. The 0.70 CF+CM also increased plant height, root length, stem–leaf ratio, and grass yield relative to the remaining treatments, and the 0.55 CF+CM treatment resulted in comparable plant height, root length, and grass yield as both of the fully fertilized treatments (CF, CF+CM). Overall, significant differences in plant height, total root length, stem-to-leaf ratio, and fresh herbage yield were observed among the four combinations of microbial inoculants and chemical fertilizers, while the differences in timothy agronomic traits and yield between the CF and CM treatments were not significant. Compared to the control, plant height, total root length, stem-to-leaf ratio, and fresh grass yield, under the 0.85 CF+CM treatment, increased by 46.24%, 138.99%, 27.95%, and 49.26%, respectively. These results suggest that reducing chemical fertilizer usage combined with microbial inoculation significantly enhances the agronomic characteristics and yield of timothy. The leaf area and chlorophyll content of timothy plants in the different treatments followed the same trend as the agronomic traits. The leaf area of timothy under the 0.85 CF+CM treatment increased by 72.66% compared to CK, while the chlorophyll content of timothy under all treatments of microbial inoculants with chemical fertilizers (CF), except CF+CM, increased by more than 10% compared to CK, which was a significant difference ($p < 0.05$).

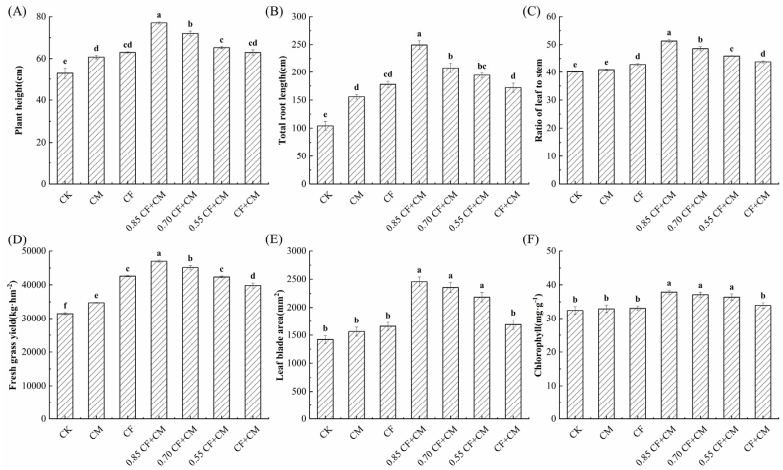

Figure 1. Effect of different fertilizer treatments on the height, total root length, stem-to-leaf ratio, fresh grass yield, leaf area, and chlorophyll content of timothy (*Phleum pratense* L.). (**A**) Plant height; (**B**) Total root length; (**C**) Stem–leaf ratio; (**D**) Fresh grass yield; (**E**) Leaf blade area; (**F**) Chlorophyll. Note: Different lowercase letters indicate significant differences ($p < 0.05$).

3.2. Changes in Soil Physicochemical Properties under Different Fertilizer Combinations

The effects on soil physicochemical properties were compared with the application of different rates of fertilizer with microbial inoculants (Table 2). Interestingly, the 0.85 CF+CM treatment showed a decreasing trend in soil pH and conductivity, whereas organic matter, quick nutrients (nitrogen, phosphorus, and potassium), and total nutrients (nitrogen, phosphorus, and potassium) were elevated compared to CK, and the differences were significant. CM significantly reduced soil pH and conductivity compared to the CF treatment, indicating that microbial inoculant addition can affect soil salinity. Compared to a single application of fertilizer (CF), the soil alkali–hydrolytic nitrogen, available phosphorus, and available potassium contents were significantly increased by the application of microbial inoculants combined with chemical fertilizer. There were no significant differences in total nitrogen, potassium, or phosphorus among the four combinations of microbial inoculants and chemical fertilizers. Soil alkaline-dissolved nitrogen, quick-acting potassium, and quick-acting phosphorus contents increased by 22.40%, 67.25%, and 26.14%, respectively, in the 0.85 CF+CM treatment compared with the CM treatment. The results show that microbial inoculants combined with chemical fertilizers significantly increased soil fertility and improved soil quality.

Table 2. Effect of different fertilizer combinations on soil physicochemical properties.

Treatment	pH	Electrical Conductance ($us \cdot cm^{-1}$)	Organic Matter ($g \cdot kg^{-1}$)	Available Nitrogen ($mg \cdot kg^{-1}$)	Available Phosphorus ($mg \cdot kg^{-1}$)	Available Potassium ($mg \cdot kg^{-1}$)	Total Nitrogen ($g \cdot kg^{-1}$)	Total Phosphorus ($g \cdot kg^{-1}$)	Total Potassium ($g \cdot kg^{-1}$)
CK	7.97 ± 0.009 a	211.64 ± 1.79 a	24.26 ± 0.54 f	94.92 ± 2.00 e	21.70 ± 0.55 f	41.22 ± 0.56 e	1.68 ± 0.006 e	0.70 ± 0.006 e	17.16 ± 0.01 e
CM	7.82 ± 0.012 b	135.83 ± 1.13 d	27.78 ± 0.17 e	135.75 ± 2.58 b	24.23 ± 0.56 e	55.47 ± 0.41 d	1.70 ± 0.003 e	0.72 ± 0.006 e	17.22 ± 0.01 e
CF	7.93 ± 0.007 b	185.95 ± 2.77 b	32.97 ± 0.78 d	116.23 ± 2.69 d	27.89 ± 0.13 d	58.78 ± 1.40 d	2.42 ± 0.006 a	1.00 ± 0.009 a	18.66 ± 0.01 a
0.85 CF+CM	7.82 ± 0.015 d	146.31 ± 3.06 c	38.23 ± 0.19 a	142.26 ± 1.68 ab	35.18 ± 0.53 a	98.31 ± 2.82 a	2.19 ± 0.006 b	0.93 ± 0.017 b	18.51 ± 0.02 b
0.70 CF+CM	7.80 ± 0.003 c	131.33 ± 5.25 d	36.12 ± 0.21 b	140.13 ± 2.39 ab	32.60 ± 0.69 b	82.47 ± 0.58 b	2.09 ± 0.006 c	0.86 ± 0.006 c	18.17 ± 0.02 c
0.55 CF+CM	7.77 ± 0.006 c	118.84 ± 4.20 e	34.26 ± 0.75 c	128.91 ± 2.26 c	30.28 ± 0.27 c	75.27 ± 0.64 c	1.97 ± 0.012 d	0.81 ± 0.006 d	17.84 ± 0.02 d
CF+CM	7.87 ± 0.012 c	187.95 ± 3.58 b	35.73 ± 0.60 bc	146.19 ± 2.35 a	33.6 ± 0.62 bc	84.25 ± 0.54 b	2.43 ± 0.023 a	1.02 ± 0.030 a	18.63 ± 0.02 a

Note: Date are mean ± SE. Different letters in the same column indicate significant differences at $p < 0.05$ level.

3.3. Changes in Soil Enzyme Activities under Different Fertilizer Combinations

Soil enzyme activities serve as potential indicators of soil microbial activity and productivity, significantly influencing soil fertility. In treatments with microbial inoculants, soil enzyme activity increased in the vicinity of plant roots. All microbial inoculant treatments (CM, CF+CM, 0.85 CF+CM, 0.70 CF+CM and 0.55 CF+CM treatments) demonstrated significant increases in urease and invertase activities compared to the non-inoculated treatments (CK and CF treatments) (Figure 2). Soil catalase and acid phosphatase activities were also increased in some treatments with microbial inoculants. Furthermore, soil enzyme activity exhibited an increase with microbial inoculant application and a decrease with chemical fertilizer application. In comparison to the control, the activities of urease, invertase, catalase, and acid phosphatase in soil treated with 0.55 CF+CM increased by 91.10%, 28.57%, 57.68%, and 13.91%, respectively. The combined application of microbial inoculants and chemical fertilizers significantly increased urease, invertase, catalase, and acid phosphatase activities.

3.4. Economic and Principal Components Analyses of Fertilization and Microbial Inoculant Treatments

Timothy harvest was completed in November 2022. Based on the 2022 timothy (fresh grass) purchase price of USD 0.58 kg^{-1}, average chemical fertilizer market price of USD 0.50 kg^{-1}, and average microbial inoculants market price of USD 1.14 kg^{-1}, timothy yields varied considerably under different fertilizer combinations. The ladder pasture yield for each fertilizer treatment was highest at 0.85 CF+CM and second-highest at 0.70 CF+CM (Table 3). The yield of timothy under 0.85 CF+CM (85% chemical fertilizers + microbial inoculants) treatment was 46,979.33 $kg \cdot ha^{-1}$ with a net return of USD 24,161.5 per hectare, which was USD 2534 higher than that of CF (chemical fertilizers alone), with a high output ratio of 9.003 as compared to only 6.210 in CK. Even the reduced fertilizer treatment of 0.55 CF+CM produced returns comparable to those of full fertilization but without the microbial inoculant (CF).

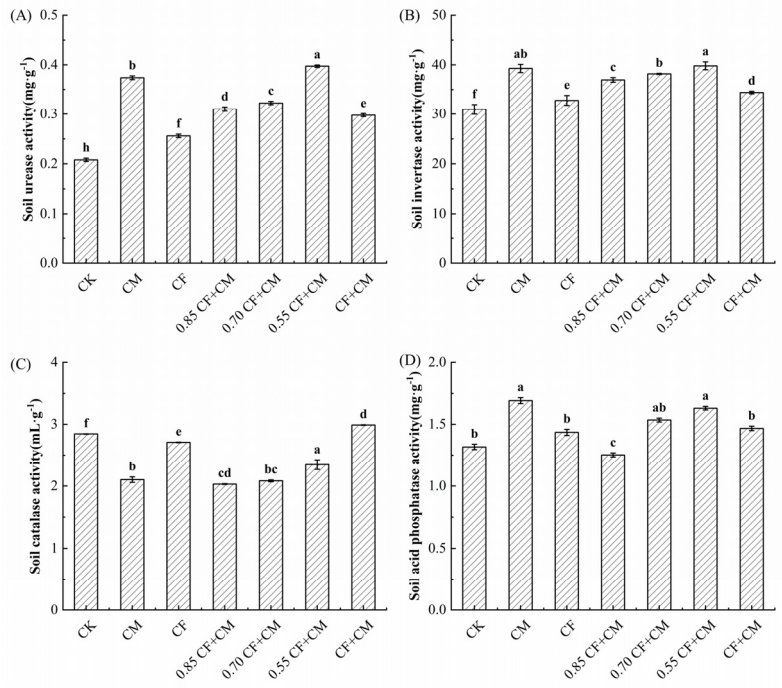

Figure 2. Effects of different fertilizer treatments on soil enzyme activities. (**A**) Soil urease activity; (**B**) Soil invertase activity; (**C**) Soil CAT activity; (**D**) Soil phosphatase activity. Note: Different lowercase letters indicate significant differences ($p < 0.05$).

Table 3. Evaluation of the economic efficiency of timothy under different fertilizer combinations.

Treatment	Gross Pasture Income	Chemical Fertilizer	Microbial Inoculant	Labor Cost	Cost of Fertilization	Land Rent	Pure Pasture Income	Output Ratios
CK	125,896	0	0	2462	0	15,000	108,434	6.210
CM	138,960	0	1200	1812	100	15,000	120,848	6.672
CF	170,326.7	1350	0	2086	500	15,000	151,390.7	7.995
0.85 CF+CM	187,917.3	1152	1200	1035	400	15,000	169,130.3	9.003
0.70 CF+CM	180,573.3	946.8	1200	1285	350	15,000	161,791.5	8.614
0.55 CF+CM	169,422.7	741.6	1200	1581	270	15,000	150,630.1	8.015
CF+CM	159,238.7	1350	1200	2230	600	15,000	138,858.7	6.813

According to the integrated principal component function model (where b is the contribution rate, m is the number of principal components, and Z is the main component), the integrated principal component values of each treatment were calculated, ranked, and a comprehensive evaluation was performed on the agronomic traits and soil nutrient levels of timothy (plant height, total root length, stem–leaf ratio and fresh grass yield; soil alkalolytic nitrogen, available potassium, available phosphorus, total nitrogen, total phosphorus, total potassium, urease, invertase, catalase, and acid phosphatase) under treatments with different proportions of chemical fertilizers and microbial inoculants. The results show that 0.85 CF+CM treatment had the highest composite score, followed by 0.85 CF+CM, 0.70 CF+CM, CF, 0.55 CF+CM, and CM treatments in decreasing order, with scores of 0.487, 0.359, 0.264, −0.007, and −0.784, respectively. The CK score was only −1.170. The composite scores of the indicators under different ratios of chemical fertilizers and microbial inoculants showed a significant increase with decreasing chemical fertilizer application (Figure 3).

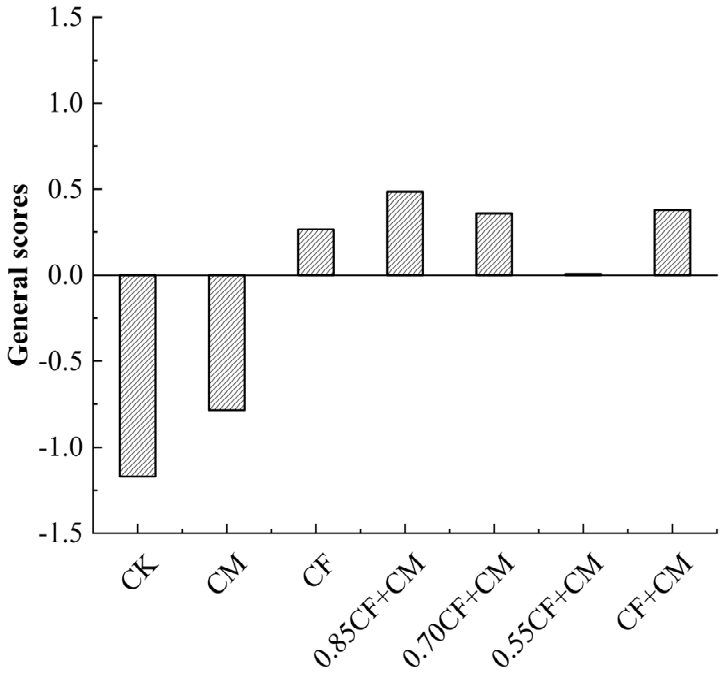

Figure 3. Combined scores of agronomic traits and soil nutrients of timothy under different fertilization treatments (the *Y*-axis value is the comprehensive evaluation score (F). The contribution rate corresponding to each principal component is the weight factor, and the comprehensive evaluation function can be obtained $F = 0.615F_1 + 0.285F_2$. Based on the principal component function model, the comprehensive scores of 7 different fertilizer treatments were calculated).

3.5. Changes in Soil Microbial Community Structure under Different Fertilizer Combinations

Based on the comparison of the four treatments after the addition of microbial inoculants and the single microbial inoculants, the 0.85 CF+CM treatment resulted in the highest yield and the highest content of soil available nutrients. Principal component analysis and economic benefit evaluation showed that 0.85 CF+CM treatment had the most potent growth-promoting effect on timothy production. Under the premise of ensuring the high yield of crops, the changes in soil microbial community structure induced by microbial inoculants were further evaluated. Therefore, three treatments, namely CK (no fertilizer applied), CF (100% CF), and 0.85 CF+CM (85% chemical fertilizers + microbial inoculants), were selected for comparison.

3.5.1. Soil Microbial Community Composition

Following sequencing of the rhizosphere soil samples, 609,283 bacterial 16S rRNA and 71,815 ITS sequences were obtained for further analysis. The high-quality bacterial community sequences ranged from 46,594 to 66,025 (mean ± SD, 57,723 ± 6805). Similarly, high-quality fungal community sequences ranged from 59,208 to 76,162 (mean ± SD, 64,615 ± 6731), with a dominant length of 201~250. All high-quality sequences were taxonomically classified from phylum to genus using QIIME's default settings, employing a 97% sequence similarity threshold. Notably, soil OTUs and structures varied significantly across different fertilizer treatments, as shown in the Venn diagrams (Figure 4A,B). A total of 22,177 bacterial and 4920 fungal OTUs were detected in the 12 inter-root soil samples, with bacterial and fungal counts per sample ranging from 1429 to 2223 and from 213 to 503, respectively, and with means of 1824 and 352, respectively. Figure 4A illustrates that the number of bacteria shared among CK, CF, and 0.85 CF+CM was 1382, while the number of OTUs exclusively found in CK, CF, and 0.85 CF+CM was 306, 285, and 294, respectively. In contrast, the CK, CF, and 0.85 CF+CM treatments had 173 fungal OTUs, whereas 49, 32, and 30 OTUs were unique to the CK, CF, and 0.85 CF+CM treatments, respectively (Figure 4B).

Figure 4. Number of different and shared bacterial (**A**) and fungal (**B**) OTUs between CK, CF, and 0.85 CF+CM.

3.5.2. Effects of Adding Microbial Inoculants on Microbial Diversity

Given the sensitivity of α-diversity to fertilizer application, we evaluated the impact of chemical fertilizers and microbial inoculants on bacterial and fungal diversity indices. The Chao1 and Shannon indices were determined at a 97% similarity level. An increasing trend in the number of bacteria and fungi was observed with the addition of microbial inoculants compared to the control (CK; Figure 5). Specifically, the bacterial Chao 1 index was significantly affected by the 0.85 CF+CM treatment, showing a 19.28% increase compared to the control ($p < 0.05$; Figure 5A). Conversely, the application of CF alone resulted in an 11.33% decrease in the bacterial Chao 1 index compared to the control ($p < 0.05$). The Chao1 value of bacteria treated with 0.85 CF+CM increased by 34.14% compared with that of the CF treatment ($p < 0.05$). Similarly, the fungal Chao 1 index increased by 19.28% ($p < 0.05$) in the 0.85 CF+CM treatment. However, there were no significant differences in the Shannon indices of bacteria and fungi between the CK and CF groups (Figure 5C,D).

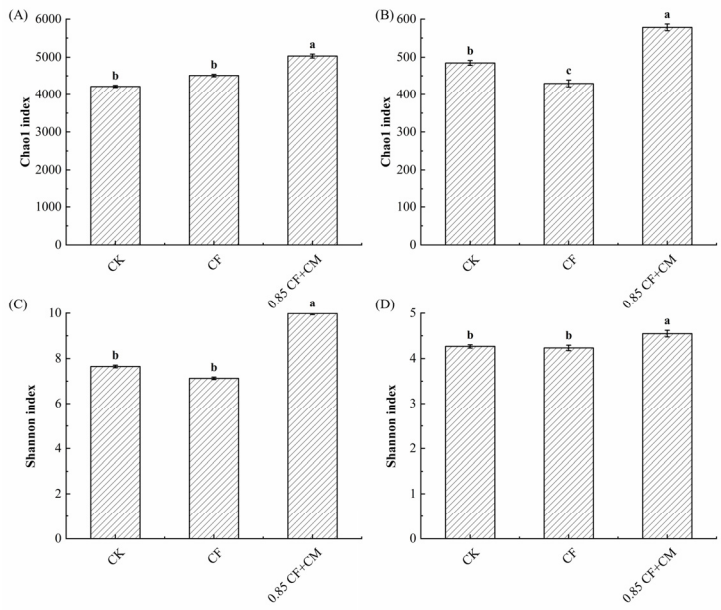

Figure 5. Bacterial and fungal diversity in plant inter-root soils under various fertilization treatments. (**A**,**B**) depict Chao 1 values for bacterial and fungal communities, respectively, while (**C**,**D**) illustrate Shannon values for bacterial and fungal communities, respectively. Note: Different lowercase letters indicate significant differences ($p < 0.05$).

3.5.3. Microbial Community Structure Analysis

The relative abundance and community composition of bacterial and fungal phyla were somewhat altered in the different fertilization treatments, as shown in Figure 6A. The dominant bacterial phyla in different treatments were *Proteobacteria* (25.96–29.32%), *Actinobacteria* (18.23–24.87%), *Actinobacteria* (8.33–13.54%), *Bacteroidetes* (5.36–12.87%), *Chloroflexi* (2.77–3.74%), *Bacillus* (2.43–4.98%), and *Verrucomicrobia* (1.39–3.18%), with mean relative abundances of 27.81%, 21.68%, 11.35%, 9.15%, 3.55%, 3.35%, and 2.85%, respectively. In addition, significant changes in the relative abundance of the dominant phyla were observed with the addition of microbial inoculants. The application of CF reduced the relative abundance of Bacillus by 29.87% ($p < 0.05$). Conversely, compared with the control, the 0.85 CF+CM application significantly increased the relative abundance of *Bacillus* by 39.41% and also significantly elevated the relative abundance of *Chloroflexi* by 25.14% ($p < 0.05$).

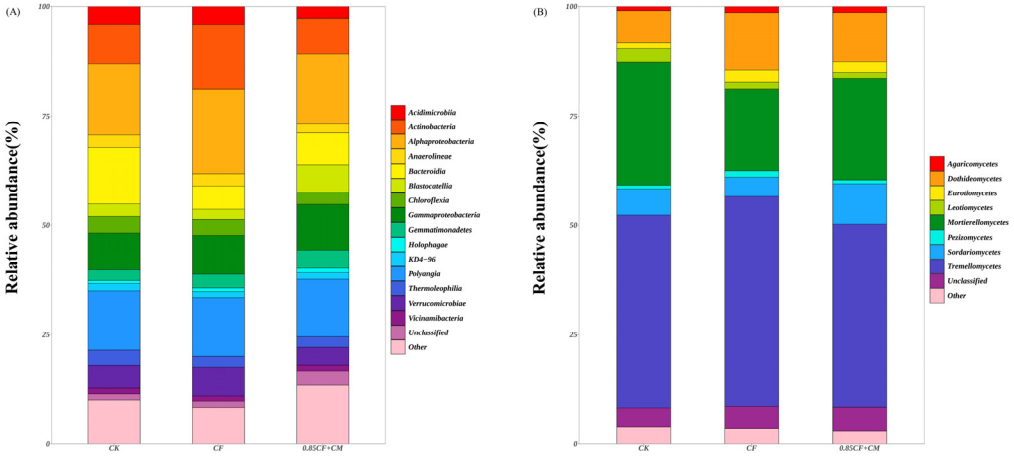

Figure 6. Classification of bacterial (**A**) phyla and fungal (**B**) orders between different fertilizer treatments and CK. T-columns labeled "Other" indicate that the relative abundance of all phyla or orders is not listed.

The taxonomic information at the fungal class level is shown in Figure 6B. Fungal communities in the different treatment groups mainly comprised 29 classes. Among them, *Sordariomycetes* (43.71–59.53%), *Zygomycetes* (18.04–28.20%), *Dothideomycetes* (6.22–15.46%), *Pezizomycetes* (4.43–12.07%), *Tremella* (4.20–6.25%), *Eurotiales* (1.24–3.94%), and *Agaricomycetes* (1.08–2.43%) had average relative abundances of 51.88%, 23.21%, 10.84%, 8.25%, 5.25%, 2.55%, 2.35%, and 1.75%, respectively, with the dominant classes mostly belonging to *Ascomycota* and *Basidiomycota*. Similar to the results for bacteria, the relative abundance of fungal groups in the dominant phyla varied significantly with different fertilizer treatments. The relative abundance of CF *Sordariomycetes* increased by 10.87% with the application of CF compared to the control, whereas the relative abundance of *Sordariomycetes* was significantly reduced by 14.67% with the application of 0.85 CF+CM treatment compared to the control ($p < 0.05$). In addition, the 0.85 CF+CM treatment significantly reduced the relative abundance of *Mortierella* perforatum by 19.79%, but the relative abundance of *Pezizomycetes discoideum* was significantly higher than that of the other treatments, being 4.12 times higher than that of the control ($p < 0.05$). The 0.85 CF+CM treatment had a significant effect and influenced the rate of decrease in the RA of *Sordariomycetes*.

3.5.4. Exploring Biomarkers in Microbial Communities

Differences in microbial communities between treatments were assessed using linear discriminant analysis effect size (LEfSe) analysis at a linear discriminant analysis (LDA) threshold of 3 (Figure 7). Significant differences in the relative abundance of bacteria across the bacterial community (LDA $\geq$ 3.0) were observed in three phyla and nine orders. More

bacterial phyla and orders were significantly enriched in the 0.85 CF+CM soil compared to the CK and CF soils (Figure 7A). Among them, *Flavobacterium*, *Bacteroidetes*, and *Chloroflexi* were significantly enriched in the CK soil ($p < 0.05$), with LDA values of 4.3, 4.1, and 4.1, respectively. *Proteobacteria*, *Actinobacteria*, and *Gemmatimonadetes* were significantly enriched in the 0.85 CF+CM soil, with LDA values ranging from 4.1 to 4.3. In contrast, CF soil was enriched in *Actinobacteria* and *Proteobacteria* with LDAs of 3.8 and 3.2, respectively. At the class level, nine bacterial classes, including *Alphaproteobacteria*, *Actinomycetes*, *Gemmatimonadetes*, and *Acidimicrobidae*, were enriched in the 0.85 CF+CM soil; *Actinobacteria*, *Bacteroides*, and *Flavobacteria* were significantly enriched in CK soils; six bacterial phyla, including *Bacteroidetes*, *Actinobacteria*, *Rubrobacteridae*, and *Chloroflexi*, were enriched in CF soils.

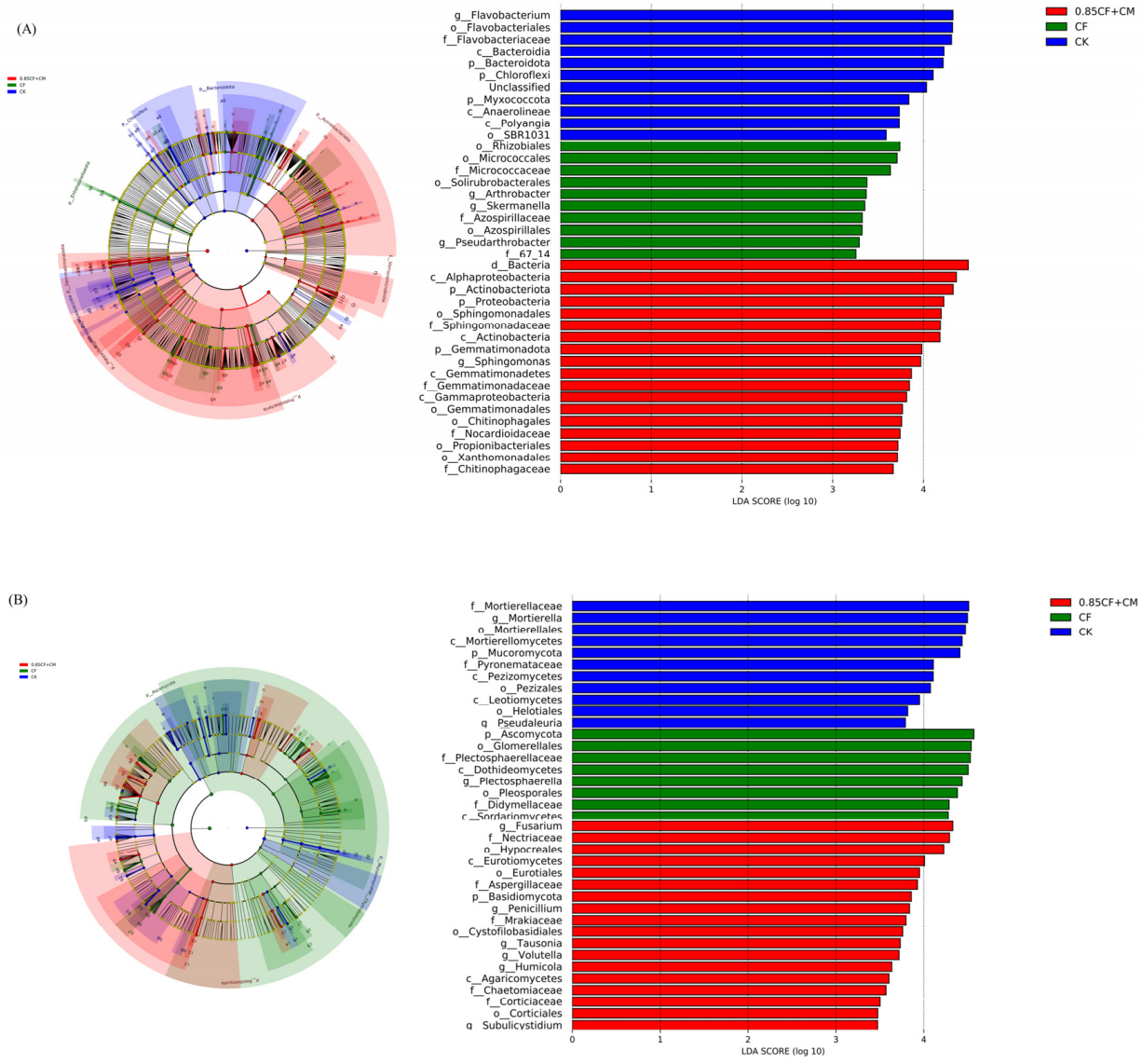

Figure 7. Phylogenetic distribution of soil bacterial spectrum (**A**) and fungal spectrum (**B**) under different fertilization treatments. The right panel shows histograms of linear discriminant analysis (LDA) scores indicating differences in the abundance of soil bacterial and fungal communities across fertilization treatments, with a threshold of 3.0. Branching plots derived from LEfSe analyses depict distinct enrichment changes in bacterial (**A**) and fungal (**B**) communities at various taxonomic levels. Circles radiate from the center to the periphery of the graph, representing taxonomic levels from phylum to genus. Different colored nodes on the branches indicate microbial communities that play significant roles in each group. Species names in letters are provided in the annex.

Similarly, LEfSe analysis was used to detect fungal communities that showed significant differences in relative abundance between the addition of microbial inoculants and conventional fertilization (Figure 7B). Across the fungal community, the most abundant genera were *Ascomycota* and *Basidiomycete*, with species differences observed among the three treatments. The CF treatment was significantly more enriched ($p < 0.05$, LDA > 3.0) than the other treatments in *Sphaeriales*, *Fusarium*, *Mycosphaerella*, *Ulocladium,* and *Beauveria*, with RA being 4.14-, 21.02-, 3.27-, 1.35, and 5.22 times higher than in CK, respectively. RA was significantly enriched ($p < 0.05$, LDA > 3.0) in *Glomus mosseae*, *Ascomycetes*, and *Phoma* after the application of 0.85 CF+CM treatment, with RA being 1.25-, 1.64, and 3.08 times higher than that of CK, respectively.

3.6. Correlation of Plant Inter-Root Soil Microbial Community Composition with Soil Environmental Factors

Microbial composition and diversity of timothy (*Phleum pratense* L.) roots under different treatments were influenced by environmental factors. RDA was used to analyze the correlation between microbial communities and environmental factors, further elucidating the impact of strongly correlated variables (Figure 8). Analysis of the top 10 dominant bacterial populations and environmental factors in the timothy rhizosphere soil revealed significant effects of environmental factors on bacterial communities across the three fertilization modes. Collectively, RDA1 and RDA2 accounted for 91.48% of the total variance, with contributions of 58.06% and 33.42%, respectively. Soil physical and chemical factors were divided into two groups (Figure 8A). The first category comprised soil organic matter (TOM), available potassium (AK), AP, TP, and total nitrogen (TN), exhibiting similar effects on bacterial communities. In contrast, other environmental factors, apart from AN, constituted a separate group, indicating different effects on bacterial community outcomes. Among these, EC emerged as the most significant factor, followed by AN, pH, TOM, AP, AK, TP, and TN. These rankings underscored the importance of each environmental factor in shaping bacterial community structure. At the bacterial genus level, EC exerted the greatest influence on bacterial community structure. The negative correlation between soil EC and the abundance of dominant bacterial species suggests that reduced soil EC may favor bacterial growth, thereby enhancing our understanding of the relationship between soil environmental factors and bacterial community diversity, with implications for farmland fertilization pattern establishment and management.

RDA analyses of the top 10 populations with soil environmental factors showed significant changes in the timothy community structure in the inter-root soils of under different treatments. RDA1 and RDA2 accounted for 39.47% and 29.86% of the total variance, respectively, highlighting major environmental factors affecting fungal communities. As depicted in Figure 8B, soil physicochemical property factors were broadly divided into two groups. The first group included TN, TP, AP, AK, and TOM, suggesting potential interactions or effects on the fungal community. Conversely, EC and pH constituted the second group, implying differential impacts on dominant fungal populations. The foremost influences on the timothy community in inter-root soil stemmed from soil physicochemical properties, followed by AN, pH, AK, TP, TOM, AP, and TN. This ranking delineated the varying degrees of influence of different environmental factors on fungal community structure in each environment. TP and TN emerged as the primary environmental factors affecting fungal communities. TP showed significant positive correlations with fungal communities, such as the abundance of *Pseudombrophila hepatica*, suggesting that increased soil levels of total phosphorus may promote the growth of these genera. These findings shed light on the multifaceted and dynamic roles of environmental factors in soil and fungal communities.

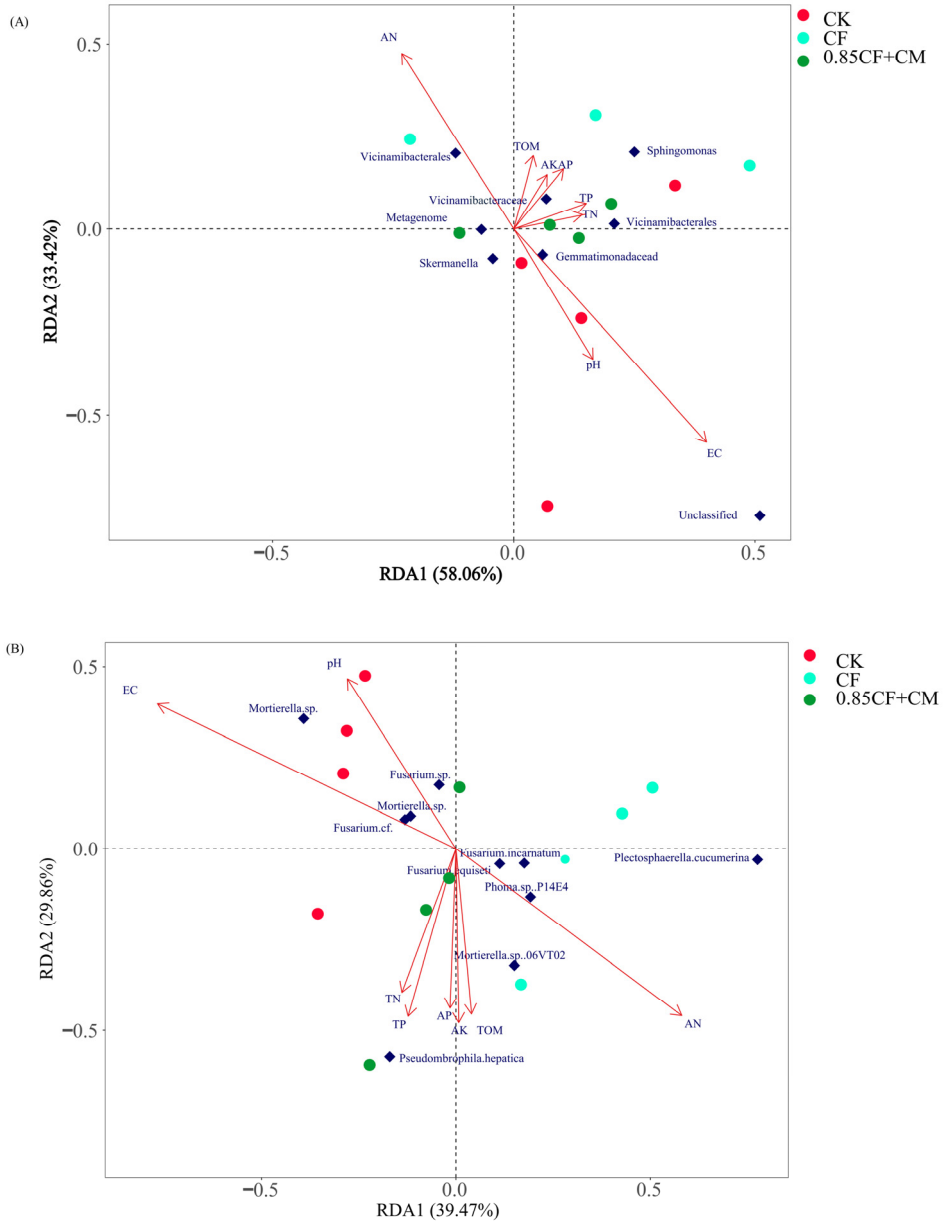

Figure 8. RDA analysis plot depicting the relationship between soil bacterial and fungal communities and soil properties under different fertilizer treatments. Soil physicochemical properties are indicated by red lines, while dominant bacterial and fungal populations are represented in dark blue (**A**,**B**). EC: electrical conductivity, AN: alkali-dissolved nitrogen, AK: quick-acting potassium, AP: quick-acting phosphorus, TN: total nitrogen, TP: total phosphorus, TK: total potassium, and TOM: total organic matter.

3.7. Functional Prediction of Rhizosphere Soil Bacterial Communities

Figure 9 illustrates spatial variations in the functional groups of bacteria across different fertilization modes. The functional predictions in the figure indicate involvement in the entire chemical cycling process by nitrogen (two groups), nitrate (seven groups), and manganese (one group). Classification of soil bacterial functions under the three fertilization treatments, based on the metabolic functions of the third level of inter-root bacteria, revealed similarities between the bacterial functions of CF and 0.85 CF+CM, which clustered together. In contrast, the bacterial functions of CK (unfertilized) were distinctly different from the other two treatments. Dominant bacterial functions included nitrate denitrification and nitrite respiration in CK, nitrate respiration, nitrogen respiration, and

degradation of aromatic compounds in CF, and nitrogen fixation, manganese oxidation, and oxygen-producing photosynthesis autotrophs in 0.85 CF+CM, which were significantly higher than those observed in the other treatments.

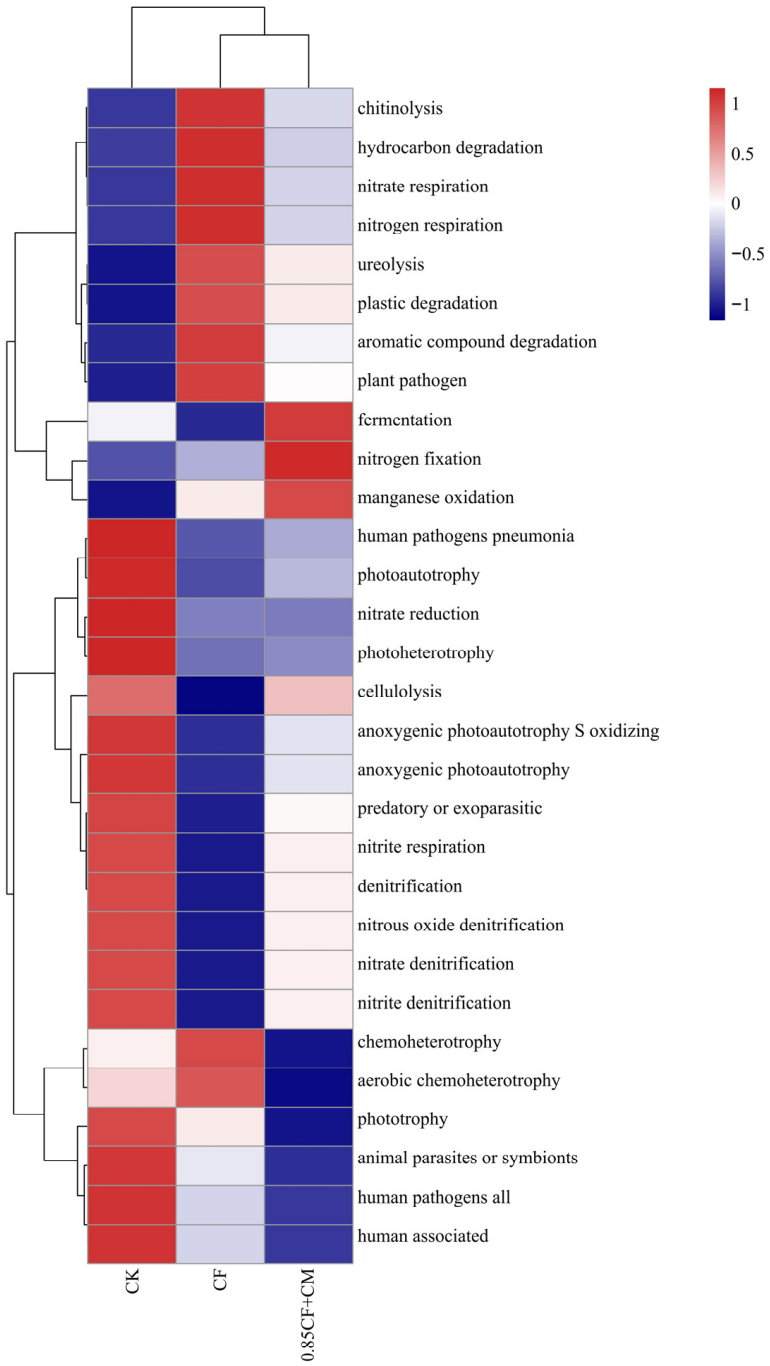

Figure 9. Functional heatmap of predicted microbial communities at the OTU level based on the Functional Annotation of Taxonomic Groups (FAPROTAX1.1) database.

4. Discussion

4.1. Effects of Different Fertilizer Combinations on Agronomic Traits and Photosynthesis of Timothy (Phleum pratense L.)

The long-term application of chemical fertilizers and pesticides, along with the environmental factors associated with continuous soil cultivation, leads to a decrease in soil nutrients, an increase in soil-borne diseases and pathogens, soil compaction, soil acidification, salinization, and environmental pollution [22]. The application of microbial inoculants

with chemical fertilizers not only improves soil enzyme activity but also accelerates the decomposition of soil nutrients, facilitating nutrient absorption by plants and promoting crop growth and development [23]. The results of this experiment demonstrate that the combination of microbial inoculants and varying proportions of chemical fertilizers significantly promotes the growth of timothy. Specifically, the combination of microbial inoculants and 85% chemical fertilizers had the most significant effect on growth, with plant height, total root length, stem–leaf ratio, and fresh grass yield surpassing those of CK and CF (100% CF). It is worth noting that the plant height, total root length, stem–leaf ratio and fresh grass yield of the timothy treated with the combination of microbial inoculants and 55% chemical fertilizers are equivalent to or even higher than that of the CF and CF+CM. These results are consistent with those reported by Kumar et al. [24], Gong et al. [25], and Marina et al. [26] in a study involving sesame, winter wheat, and cucumber, where the application of microbial inoculants combined with different proportions of fertilizers yielded similar results. Fan et al. [27], Xiao et al. [28], and Wang et al. [29] reported relatively similar results for rice, broccoli, and tea using organic rather than chemical fertilizers. The same conclusion was reached when the experimental data confirmed the growth-promoting effect of microbial inoculants, although the fertilizer substitution ratio was different. In the above studies, the fertilizer substitution ratio ranged from 15% to 50%, and most of them confirmed that the effect of a fertilizer substitution ratio between 15% and 50% is better; when the replacement ratio exceeds 50%, the effect is not as good as the full amount of fertilizer, which will cause a reduction in production. In this study, we found that, when the fertilizer ratio was not less than 55%, it did not cause a timothy population reduction. The reason for this is that the planting cycle of timothy in Minxian County is long, the growth period is about 155 days, and the nutrient demand time is long and balanced. The microbial inoculants used in this experiment contained three types of excellent rhizosphere growth-promoting bacteria, which can colonize the soil environment in this area, produce plant hormones, exert the ability of nitrogen fixation and phosphorus dissolution, and release and dissolve nutrient elements that are difficult to be absorbed and utilized by plants in soil and chemical fertilizers. The addition of microbial inoculants in this experiment can complement the quick release, short cycle, and low utilization efficiency of chemical fertilizers and extend the nutrient release cycle to coordinate with the needs of the plant growth period, which is conducive to the growth and quality improvement of timothy. Chlorophyll is the main pigment in plant photosynthesis and its content directly affects the photosynthetic capacity of plants [30]. Plant growth is closely related to the rate of photosynthesis, and an increase in chlorophyll content improves the absorption efficiency and utilization capacity of plant light energy and enhances photosynthesis. Attia et al. [31] found that the application of PGPR increased the contents of chlorophyll a, b, and total chlorophyll (a+b) in tomatoes. Similar results were observed in our experiments, where the chlorophyll content of timothy increased after the application of a microbial inoculant compared to that of CK and CF (Figure 1). This indicates that microbial inoculants can promote photosynthesis in grasses, which is consistent with the results reported by Lv et al. [32]. Microbial inoculants can increase crop vitality and resilience. Simultaneously, the active microorganisms in microbial inoculants can enter the rhizosphere soil to improve the soil structure and provide excellent living space for plants and beneficial microorganisms in plants. However, the application of microbial inoculants and the propagation of microorganisms should consider the effects of multiple factors, such as soil pH, environmental temperature, host plants, and changes in the soil indigenous microbial community structure [32]. Based on this, it is necessary to continue to conduct research on the combination of multi-species microbial inoculants and fertilizers and to select a reasonable fertilization plan according to local conditions to provide a scientific basis for promoting green food safety and reducing the application of fertilizers and pesticides.

4.2. Effects of Different Fertilizer Combinations on Soil Nutrients

The development of farmland ecosystems is closely related to dynamic changes in crop varieties, soil nutrients, and structure. Good crop quality and high yield can directly reflect high soil nutrient levels, with soil conditions serving as basic standards for evaluating crop growth [33]. In this experiment, the application of microbial inoculants with 85% chemical fertilizers not only promoted the growth of timothy but also improved the rhizosphere soil environment. Li et al. [8] investigated PGPR in oat, alfalfa, and cucumber seedlings, finding that soil enzyme activity and plant-available nutrient content increased 2–3 times. Olamide et al. [34] observed that the synergistic effect of straw and *Brevibacillus laterosporu* significantly increased soil organic carbon, TN, and soil microbial biomass compared to the uninoculated microbial control. In this study, catalase, urease, invertase, and acid phosphatase activities in the inter-root soil of timothy were significantly increased by the addition of microbial inoculants and increased with a decrease in the proportion of chemical fertilizers. The highest soil enzyme activities were observed when microbial inoculants were combined with the application of 55% of chemical fertilizers, with the second-highest observed when 0.70% of chemical fertilizers + microbial inoculants were applied. The addition of microorganisms increased the inter-root soil catalase, urease, invertase, and acid phosphatase activities of the timothy by 16.40% to 37.19%, 16.33% to 55.20%, 4.92% to 21.37%, and 2.24% to 17.88%, respectively, compared to CF. According to scholars and this paper, it has been found that active microorganisms in microbial inoculants can convert elements in chemical fertilizers into nutrients that can be absorbed and utilized by plants through nitrogen fixation and phosphorus solubilization capabilities [35]. In addition, microbial inoculants increase plant inter-root activity, organic acid secretion, and tolerance to abiotic stresses while decreasing soil pH and EC. The reduction in pH and salt content increases the effective nutrient concentration in the soil [35]. In contrast, the soil content of fast-acting nutrients is positively correlated with soil enzyme activity, which, in turn, is positively correlated with microbial inoculant application [36]. The addition of microbial inoculant accelerates soil organic matter decomposition and provides nutrients for soil enzyme activity, thereby creating an environment conducive to the proliferation of plant inter-root-promoting flora. Active microorganisms in microbial inoculants can also release volatile substances to regulate plant metabolism and induce plants to develop tolerance to nutrient deficiencies or fertilizer damage, thus indirectly promoting plant growth. Montaño et al. [37] showed that PGPR directly or indirectly influence plant metabolism, promote plant growth, and enhance root development. These abilities have been demonstrated across a wide range of plants (wheat, soybean, rice, maize, etc.) and are important for improving soil fertility and crop yields, thus mitigating the adverse impact of chemical fertilizers and pesticide application on the environment. These findings are consistent with those of the present study.

4.3. Effects of Different Fertilizer Combinations on Soil Microbial Communities

Soil microorganisms are pivotal in plant growth, constituting major components of the dynamic inter-root microenvironment [38]. The addition of beneficial microorganisms provides several benefits for sustainable soil management, serving as both plant growth enhancers and soil conditioners. The new fertilization approach not only fulfills plant growth requirements but also reduces environmental pollution stemming from chemical fertilizers and pesticides, thereby enhancing soil quality. Furthermore, the application of microbial inoculants to plant inter-root soil induces changes in microbial community structure, influencing root microecology, fostering plant growth, and facilitating the establishment of a green and sustainable agricultural system [39]. In this study, a notable observation was the significantly higher abundance of bacterial communities in treatments involving microbial inoculants combined with 85% fertilizer compared to conventional fertilization. This observation was corroborated by the Chao1 and ACE indices of bacteria. Conversely, the application of chemical fertilizers adversely affected soil microbial diversity and reduced bacterial abundance, as evident in the CF treatment. From the perspective

of microbial diversity and inter-root microecology, Simpson's and Shannon's indices of fungi showed more favorable outcomes for plant growth under the microbial inoculants with 85% chemical fertilizer treatment. The analysis presented in Figure 5 elucidates the differences in soil microbial communities across three scenarios: no fertilizer application, conventional fertilizer application (100% chemical fertilizer), and microbial inoculants with 85% chemical fertilizer. These findings bolster the scientific premise that various fertilizer types, including organic fertilizers, chemical fertilizers, and microbial inoculants, along with their application rates and mixing patterns, collectively influence soil microbial diversity. This assertion is supported by the results of Zhang et al. [40], Xu et al. [41], and Shu et al. [42], as they underscored the implications of fertilizer application strategies on soil microbial diversity. Thokchom et al. [43] demonstrated successful colonization of root tissues and soil by PGPR inoculation in broad-skinned citrus plants, inducing structural changes in root endophytes and inter-root bacteria. Similarly, in our study, PGPR may colonize the inter-roots of timothy, potentially enhancing fertilizer utilization efficiency in the presence of chemical fertilizer additions and contributing to the proliferation of inter-root microorganisms [44].

Cong et al. [45] and Zhu et al. [46] identified *Proteobacteria* as the most abundant and crucial bacterial phylum during pasture growth. Consistent with this, we observed a higher abundance of *Proteobacteria* in the rhizosphere microenvironment of timothy grass across all treatments. Moreover, the application of microbial inoculants with 85% chemical fertilizers led to increased abundances of *Proteobacteria*, *Actinobacteria*, *Acidobacteria,* and *Phylum Firmicutes*. *Actinobacteria* have been documented to mitigate the adverse effects of environmental pollution on plant growth and enhance enzyme activities in the plant root system [47]. The reason for the increase in *Proteobacteria* and *Actinobacteria* may be the successful colonization of active microorganisms in the soil treated with microbial inoculants, which contributed to a synergistic effect with the native microbial community. *Proteobacteria* and *Actinobacteria* play a pivotal role in the soil carbon cycle and the decomposition of organic matter. Therefore, when microbial inoculants and chemical fertilizers are applied, the abundance of *Proteobacteria* and *Actinobacteria* can increase in the soil microenvironment due to the growth-promoting effects of microorganisms. *Streptomyces*, a member of the *Actinobacteria* phylum, contributes to the decomposition of organic matter and serves as both antibiotics and herbicides for crop protection [48]. In addition to *Proteobacteria* and *Actinobacteria*, the addition of microbial inoculants also increased the abundance of *Phylum Firmicutes* phyla in the inter-root soil. Two of the three bacterial strains used in this study belonged to the test *Bacillus*. As an important branch of the *Phylum Firmicutes*, *Bacillus* is involved in many steps in soil nutrient dynamics. The use of microbial inoculants to improve the plant root microenvironment and microbial structure facilitates the formation of a sustainable soil nutrient dynamic change system. Microbial bacteria play a crucial role in enhancing enzyme activity and decomposing soil elements that are difficult to absorb, thus aiding in the breakdown of harmful substances in the soil. Ali et al. [49] reported that inoculation with Bacillus subtilis (FBL-10) can promote mineral uptake and energy metabolism in plants, significantly mitigating the toxicity of heavy metals in melons. This discovery holds significant potential for improving soil quality, as *Bacillus* exhibits biocontrol effects and contributes to the sustainable development of agricultural land due to its role as an excellent biotrophic bacterium. Additionally, Peng et al. [50] showed that *Bacillus* enhances nitrogen uptake and assimilation in plants by promoting soil nitrogen cycling. These bacteria also solubilize phosphorus, thus facilitating phosphorus cycling in plant inter-root soils. Moreover, under the application of microbial inoculants with 85% chemical fertilizer treatment, there was an observed tendency for Rhizobium to become the dominant genus. Although timothy is not classified as a legume, it is a perennial grass belonging to the ladder grass family. Our experiment revealed that mature timothy plants possess small roots with nodules, which facilitate improved nitrogen absorption by the crop. Of note, the three strains in the microbial inoculants selected in the experiment have nitrogen-fixing abilities, aiding in the formation of nodules in crop roots. Furthermore,

the results of this study indicate that the combined application of microbial inoculants and chemical fertilizers significantly alters the inter-root soil microenvironment, thereby modulating the structural composition and abundance of the bacterial community and promoting the emergence of beneficial bacterial genera as dominant genera [51].

Unlike bacteria, fungal diversity is closely related to changes in soil nutrient levels. This relationship is reflected by the fact that soil texture, pH, organic nitrogen, Cu, P, and other chemicals are important variables in the fungal OTU distribution model. Fungi act as important regulators of soil repair and biochemical processes [52]. In addition, fungi play a crucial role in the decomposition of organic matter and nutrient cycling in the cultivation system. Fungal communities form symbionts with most plants, which have an important regulatory role in soil carbon dynamics [53]. However, the soil fungal community is a highly sensitive index of changes in soil fertility, and most studies on changes in planting systems and fertilizer types have not fully revealed changes in the fungal community. In this study, we observed changes in fungal community structure and diversity under different fertilization regimes: full chemical fertilizers, microbial agent + fertilizer, and no fertilizer. The addition of microbial inoculants increased the biodiversity of fungal species. By adding different fertilizer combinations, we found that *Ascomycetes* and *Basidiomycetes* were prevalent and abundant in the fungal communities of the plant rhizosphere soil microbiome, consistent with previous soil studies on various cash crops such as wheat, maize, and rice [54]. Notably, the combination of microbial inoculants and chemical fertilizers resulted in a decrease in the relative abundance of some fungal phyla, including *Ascomycetes*, *Zygomycetes*, *Glomeromycota,* and *Basidiomycetes*. Among the three different fertilization schemes, *Ascomycetes* and *Basidiomycetes*, the important phyla of the fungal community in the soil microbiome of the timothy rhizosphere, contain a variety of plant pathogens, such as *Fusarium* and *Rhizoctonia solani*, which cause root rot of the timothy and can lead to plant leaf lesions, yellowing, and branch wilting, thereby affecting plant growth. Additionally, the economic benefits of timothy have decreased [55]. The combination of microbial inoculants and chemical fertilizers significantly reduced the relative abundance of *Ascomycetes* compared to the other treatments. This finding is similar to the results of previous studies showing that the addition of biofertilizers or biological agents can reduce *Fusarium* abundance and promote plant development [56]. Simultaneously, the abundance of beneficial fungi increases, and synergistic interactions between them and resident fungal populations are activated by the application of microbial inoculants [57].

4.4. Effects of Environmental Factors on Soil Microbial Communities

Changes in plant and soil environmental factors exert significant effects on soil microbial communities within alpine farmland ecosystems. Crop varieties, growth years, soil moisture, organic matter, nitrogen, phosphorus, and potassium directly correlate with soil fertility and influence both plant growth and soil microbial community structure [58]. In high-altitude and low-temperature farmlands, long-term environmental factors partially inhibit the propagation and impact of soil microorganisms [59]. In this study, redundancy analysis (RDA; Figure 8) showed that changes in environmental factors, such as soil pH, organic matter, AP, available potassium, and alkali-hydrolyzed nitrogen, predominantly influenced soil microbial communities. The application of microbial inoculants, chemical fertilizers, and environmental alterations can modify plant rhizophore flora. Following the application of microbial inoculants with chemical fertilizers, soil enzyme activity and nutrients increased, whereas microbial inoculants alone led to a reduction in soil pH and salt concentration [60]. The response of microbial communities to environmental factors can impact the abundance and composition of microorganisms related to plant growth, thereby affecting overall plant development. Previous studies have demonstrated that changes in carbon and nitrogen metabolism can alter soil microbial community structure. In this study, FAPROTAX function prediction was used to compare the effects of different fertilization approaches (no fertilization, sole application of chemical fertilizers, 85% chemical fertilizers, and microbial inoculants) on the bacterial community function of timothy

root flora. Functional prediction analysis based on FAPROTAX facilitates the identification of potential functional changes in microbial communities and offers insights into directions for soil improvement and restoration [61]. In this study, the bacterial community functions in plant root flora were primarily nitrogen fixation, oxygen-producing photoautotroph, and chemoheterotroph, which were attributed to the relatively high abundance of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes,* and *Gemmatimonadetes* in the plant root flora. These phyla are known for their roles in nitrogen fixation and chemoheterotrophy [62]. Among them, *Proteobacteria*, the main group of soil bacteria, also exhibited co-trophic behavior, consistent with the results of Li et al. [63]. Additionally, this study predicted higher nitrogen cycle-related functions, with microbial inoculants combined with chemical fertilizer treatments significantly increasing soil nitrogen and nitrate respiration, suggesting enhanced soil nitrogen cycling and reduced nitrogen loss [64]. *Bacillus* in microbial inoculants significantly increased nitrate denitrification compared to full chemical fertilizer treatment, with microorganisms involved in the nitrate reaction primarily belonging to the *Proteobacteria* phylum [65]. In contrast, full fertilizer application led to a significant reduction in the *Proteobacteria* phylum, resulting in decreased nitrate denitrification. The addition of microbial inoculants can improve soil fermentation. During the fermentation process and soil microbial activities, microbial inoculants interact with the soil to produce substances that promote plant growth hormones (gibberellins and cytokinins), stimulate plant growth, and regulate metabolism through the root microbial action pathway [66].

Firstly, this study only utilized one type of microbial inoculant, potentially limiting the generalizability of the findings. Different microbial inoculants may have varying effects on soil microbial communities and plant growth, warranting further investigation into a broader range of inoculant types. Secondly, this study's timeframe was limited to one year. Consequently, the long-term effects of microbial inoculant application on soil microbial communities and plant growth may not have been fully captured within this timeframe. Extending the duration of the study could provide valuable insights into the sustained impacts of microbial inoculants over time.

Lastly, while this study primarily focused on bacterial functions in plant root flora, the role of fungi and other microorganisms in soil processes and plant growth was not extensively explored. A more comprehensive analysis of microbial communities could offer a more holistic understanding of ecosystem dynamics and further enrich this study's findings.

5. Conclusions

The findings of this study address a knowledge gap in the field of soil remediation in alpine farmland systems and provide insight into the inter-relationships between soil, plants, and soil microbial community composition. The addition of microbial inoculants at various reduced fertilizer rates resulted in improved soil properties, plant growth, and yield. Previous studies have demonstrated that the combination of microbial inoculants and 85% chemical fertilizer represents the most effective fertilizer combination, but even a fertilizer reduction of 55% in conjunction with microbial inoculants resulted in comparable yield and growth characteristics as full fertilizer without microbial inoculant, plus improved soil properties. These treatments not only significantly enhanced soil nutrient content but also reduced soil pH and EC values. The introduction of microbial inoculants alters the structure of the fungal community in the plant's root zone, potentially diminishing the prevalence of pathogenic bacteria, thus fostering a favorable micro-ecological environment for the growth of timothy, ultimately leading to increased yield and soil enzyme activity. Changes in soil microbial community composition were primarily driven by changes in environmental factors. Notably, the addition of microbial inoculants led to an increase in the relative abundance of beneficial microorganisms, implying the successful effects of the microbial inoculants on soil remediation and enhancement. Nevertheless, it is worth noting that this study only employed one microbial inoculant over one year. Thus, further investigations are warranted to explore the long-term effects of microbial inoculant application on soil physicochemical properties and crop yield, facilitating a comprehensive understanding

of dynamic changes in microbial communities and alterations in functional genes within timothy-growing areas.

Author Contributions: S.H. and T.Y.; methodology, Y.Z.; software, S.H.; validation, S.H., X.Y., and C.L.; formal analysis, S.H.; investigation, Q.L.; resources, Y.Z.; data curation, S.H.; writing—original draft preparation, T.Y.; writing—review and editing, S.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Forage Industry Technology System Program, grant number CRAS-34.

Data Availability Statement: Data are contained within the article.

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

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