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Microbial Community Response to Alpine Meadow Degradation and Its Impact on Soil Nutrient Cycling

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Abstract: The degradation of alpine meadows on the Qinghai–Tibet Plateau is a major issue affecting both the ecology and the economy. Microorganisms play an important role in soil nutrient cycling and the regulation of ecosystem function. This study aimed to investigate the species composition and diversity of microbial communities and understand the response of microbial communities to changes in physicochemical properties resulting from meadow degradation. In this study, the soil bacterial and fungal communities' composition and diversity of alpine meadows of degradation gradient were sequenced by high-throughput sequencing. During the process of grassland degradation, there were 59 bacterial taxa and 29 fungal taxa showing significant differences. The relative abundance of meadow pathogenic fungi significantly increased ($p < 0.05$). PICRUSt2 analysis showed a decrease in synthesis-related functional gene abundance and an increase in metabolism-related functional gene abundance. FUNGuild analysis showed that symbiotic and saprophytic nutrient fungi decreased significantly ($p < 0.05$). The soil nutrient cycling was mainly influenced by the beta diversity of microbial communities. Grassland degradation affects soil structure, thereby affecting the diversity of soil microbial composition and functional soil nutrient content. This work reveals the response of microbial communities to the degradation of alpine meadows and their impact on nutrient cycling, providing theoretical support for the protection and sustainable development of alpine meadows.

Keywords: biomarker; degraded alpine meadows; functional prediction; nutrient cycling; soil microorganisms

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1. Introduction

Alpine meadow is the most dominant grassland type in the Qilian Mountains and plays a key role in regional sustainable development, biodiversity and water conservation [1]. They constitute an irreplaceable ecological barrier [2], providing a wide range of ecosystem services for humans [3]. Animal husbandry is a local pillar industry, and meadows are seriously overloaded. With the continuous export of grass and livestock products, there is serious nutrient loss and soil fertility decrease, plant growth hinderance and a yearly increase in degraded grassland area. This has led to various ecological and environmental problems [4,5], such as climate change [6], recharge of rivers, sandstorm and soil erosion [7,8] and loss of biodiversity [9]. Therefore, the degradation of alpine meadow is a serious impediment to the conservation and sustainable development of local ecosystems [10].

Microorganisms play an important role in soil nutrient mineralization and cycling, energy flow and the regulation of ecosystem function [11]. And, they are important regulators of plant productivity [12]. The structure and function of soil microbial communities

are considered to be one of the key indicators of grassland degradation, but the response to grassland degradation has not been adequately studied. Previous studies have shown that vegetation degradation in alpine meadow significantly affected soil microbial diversity and structure [13]. For example, the distribution of carbon- and nitrogen-cycling bacterial genera is correlated with soil properties, the carbon/nitrogen ratio has a major influence on the structure of bacterial communities and their diversity in alkaline soils, while soil moisture is mainly responsible for building fungal communities [14]. Previous studies have found that the number of pathogenic bacteria in degraded soil increases, leading to limitations in plant growth. Due to grassland degradation, their living environment is altered [15]. Functional properties of microbial communities have also changed during the degradation process [16]. PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states) is a computational approach to predict the functional composition of a metagenome using marker gene data and a database of reference genomes [17]. It is possible to understand the microecological activities of bacterial communities in soils of degraded alpine meadows. However, the differences in soil microbial community structure, diversity and function in alpine meadow of degradation gradient remain to be explored. The aim of this study was to explore the response of microbial communities to grassland degradation and their driving forces on nutrient cycling by analyzing the relationship between soil microbial communities and soil physicochemical factors in the degradation gradient of alpine meadows.

Understanding how meadow degradation affects changes in soil microbial communities is critical for predicting the response of soil ecosystems to global climate change and anthropogenic activities [18,19]. This study aimed to answer the following questions: (1) How does soil microbial composition, diversity and function respond to different grassland degradation gradients? (2) What microorganisms can serve as indicators of degraded alpine meadows and the species differences between alpine meadows with different degrees of degradation? (3) What is the relationship between soil nutrient cycling and changes in microbial communities?

To answer the above questions, the experiment investigated the vegetation cover of degraded alpine meadows, measured the soil physicochemical properties, determined the structural composition of soil microbial communities by 16S and ITS rRNA high-throughput sequencing of soil bacteria and fungi, and predicted the functions of soil bacterial and fungal communities of degraded alpine meadows by PICRUSt2 and FUNGuild.

2. Materials and Methods

2.1. Natural Profile of the Study Area

The study site is located in the eastern foothills of the Qilian Mountains (Table S1). The climate is continental, cool and humid. Average annual precipitation and evaporation are 400 mm and 1164.3 mm, respectively. The average annual temperature is below 4 °C and the average annual solar radiation is 5722.5–6110.2 MJ m^{-2} (data are from the Chinese meteorological data website http://data.cma.cn/ accessed on 15 October 2022). The soil is alpine meadow soil, as Cambisols in FAO/UNESCO taxonomy. According to national standard of "parameters for degradation, sandification and salification of rangelands" (GB19377-2003) [20], we selected three degraded meadows, including lightly degraded (LD), moderately degraded (MD), severely degraded (SD) and non-degraded (ND) alpine meadow. The dominant plant species were *Kobresia pygmaea* C. B. Clarke and *Poa annua* L. in the ND meadow, *Stipa purpurea* Griseb., *Saussurea superba* Anth. in the LD meadow, *Carex alatauensis* S. R. Zhang in the MD meadow and *Polygonum sibiricum* (Laxm.) Tzvelev in the SD meadow.

2.2. Sample Collection and Measurement

A total of 12 sampling sites were selected for soil sample collection from three gorges. Three samples were collected from each site at ND, LD, MD, and SD, respectively. Three large plots (10 m × 10 m) were set up for each site, with a total of three replicates. The

minimum spacing between plots is 10 m. Each of the quadrats (10 m × 10 m) was divided into 4 quadrats (5 m × 5 m). We randomly selected 3 of 4 smaller quadrats for sampling (Figure S1). Using random sampling method, one sample square (1 m × 1 m) was respectively set in 3 selected smaller squares to determine coverage (CD) and aboveground biomass (AGB). Determination of CD within the sample square was conducted using the needle punching method. We cut 1 square meter of grassland plants on the ground, and then weighed and calculated AGB. And, 3 samples for soil total porosity (STP) and bulk density (BD) were collected in each smaller quadrats by the core cutter. A total of 5 drill soils (5 cm internal diameter) at 0–20 cm were randomly collected from each selected quadrat, and total of 15 drill soils were mixed from the 3 small quadrats. A total of 36 soil samples were collected. Plant roots and stones were removed from the samples, and each sample was fully mixed using sterile gloves and sterile self-sealing bags to avoid contamination during the sampling process. Approximately 2 g of soil was taken from the well-mixed samples into 1.5 mL lyophilized tubes, immediately stored in ice boxes for sequencing of community DNA fragments. Other samples were stored in self-sealing bags and brought back to the laboratory for nutritional analysis.

2.3. DNA Extraction

According to the manufacturer's instructions, total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA) and stored at −20 °C for further analysis. NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis were used to measure the quantity and quality of DNA extracted.

2.4. 16. S and ITS rRNA Gene Amplicon Sequencing

PCR amplification of the bacterial 16S rRNA genes V3-V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR amplification of the fungal ITS rRNA genes V1 region was performed using the forward primer 1737F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and the reverse primer 2043R (5′-GCTGCGTTCTTCATCGATGC-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μL of buffer (5×), 0.25 μL of Fast pfu DNA Polymerase (5 U·μL^{-1}), 2 μL (2.5 mM) of dNTPs, 1 μL (10 uM) of each Forward and Reverse primer, 1 μL of DNA Template, and 14.75 μL of ddH_2O. Thermal cycling consisted of initial denaturation at 98 °C for 5 min, followed by 25 cycles consisting of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 45 s, with a final extension of 5 min at 72 °C. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2250 bp sequencing was performed using the Illlumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

2.5. Sequence Analysis

Microbiome bioinformatics were performed with QIIME2 2019.4 [21] with slight modification according to the official tutorials (https://docs.qiime2.org/2019.4/tutorials/ accessed on 2 November 2022). Briefly, raw sequence data were demultiplexed using the Demux plugin following by primers cutting with Cutadapt plugin [22]. Sequences were then quality filtered, denoised, merged and chimera was removed using the DADA2 plugin [23]. Non-singleton amplicon sequence variants (ASVs) were aligned with Mafft [24] and used to construct a phylogeny with fasttree2 [25]. Alpha-diversity metrics [26], beta diversity metrics (Bray–Curtis dissimilarity) were estimated using the diversity plugin with samples, each of which were rarefied to 164744 sequences. Taxonomy was assigned

to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in feature–classifier plugin [27] against the SILVA Release 132/UNITE Release 8.0 Database [28].

2.6. Bioinformatics and Statistical Analysis

Sequence data analysis was mainly conducted using QIIME2 and R packets (v3.2.0). ASV-level alpha diversity indices, such as the Chao1 richness estimator, were calculated using the ASV table in QIIME2 and visualized as box plots. ASV-level ranked abundance curves were generated to compare the richness and evenness of ASVs among samples. Venn diagram was generated to visualize the shared and unique ASVs among samples or groups using R package "Venn Diagram", based on the occurrence of ASVs across groups regardless of their relative abundance [29]. LEfSe (Linear Discriminant Analysis Effect Size) was used to detect clusters with rich differences between groups using default parameters [30]. Random forest analysis was applied to discriminating the samples from different groups using QIIME2 with default settings [31,32]. Microbial functions were predicted by PICRUSt2 (Phylogenetic investigation of communities by reconstruction of unobserved states) [33] upon MetaCyc (https://metacyc.org/ accessed on 23 November 2022) and KEGG (https://www.kegg.jp/ accessed on 23 November 2022) databases. Fungal and bacterial community function was predicted by the FUNGuild and tax4fun upon Majorbio Cloud tool (https://cloud.majorbio.com/page/tools/ accessed on 23 November 2022).

2.7. Determination of Soil Physical and Chemical Properties

The remaining soils after taking microbial samples were taken to the lab, air-dried and passed through a 2 mm sieve to determine the physicochemical properties. Soil organic matter (SOM) was determined using the potassium dichromate-sulphuric acid oxidation method. The content of the soil total nitrogen (TN) was determined by the semi-micro Kjeldahl method. Soil total phosphorus (TP) was determined via spectrophotometer. Soil total potassium (TK) and soil available potassium (AK) were determined by flame photometer. Soil available nitrogen (AN) was determined by alkali-hydrolyzed reduction diffusing method. Soil available phosphorus (AP) was determined by Mo-Sb colorimetric method. Soil pH was determined in a 1:2.5 (*v*:*v*) soil/water suspension. Determination of soil water content (SWC) was conducted by drying method. STP and BD were determined using the core cutter method. The procedure was based on the methods of soil analysis [34]. The vegetation and soil indicators of the degraded gradient alpine meadow sample sites were averaged as the results.

2.8. Sequence Processing Analysis and Diversity Indices

A total of 36 mixed soil samples were analyzed by Illumina MiSeq high-throughput sequencing to systematically analyze the bacterial and fungal communities in alpine meadows of degradation gradient. An average of 97,719 effective bacterial sequences and 74,318 high quality sequences were obtained from the soil. An average of 72,461 sequences were obtained by removing amplicon sequence variants (ASVs) with an abundance of 1 from all samples. A total of 2,608,599 ASVs were obtained.

An average of 81,498 effective sequences of fungi were obtained from alpine meadows of degradation gradient, and 80,389 high-quality sequences were obtained from the soil. An average of 80,389 sequences of ASVs were obtained by removing with an abundance of 1 from all samples. The total number of ASVs obtained was 2,894,022. After removing the questionable sequences and low-quality sequences, the number of high-quality sequences in each sample was counted. After the quality control, the lengths of high-quality sequences in all samples were concentrated around 404–433 bp for bacteria and 185–305 bp for fungi.

3. Results

3.1. Basic Physicochemical Properties in Alpine Meadows of Degradation Gradient

The data and statistical results of vegetation and soil physical and chemical properties in alpine meadows of degradation gradient were shown in Table 1. It can be seen that with

the increase in degradation, CD and AGB had a significant decrease with the degradation gradient of alpine meadows ($p < 0.05$). The pH ranged from 7.01 to 7.31, which was weakly acidic and fell within the normal pH range for alpine meadow soils. BD significantly increased ($p < 0.05$). AP, AK, AN, TP, TK, TN, SOM, SWC and SOM of the degraded alpine meadows of degradation gradient (including LD, MD and SD) decreased significantly ($p < 0.05$).

Table 1. Soil physicochemical properties and vegetation properties in alpine meadows of degradation gradient (mean ± SEM). ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded; CD: cover degree; AGB: Aboveground biomass; BD: Bulk density; SWC: Soil water content, AN: Available nitrogen; AP: Available phosphorus; AK: Available potassium; TN: Total nitrogen; TP: Total phosphorus; TK: Total potassium; SOM: Soil organic matter; STP: Soil total porosity.

Basic Physicochemical Properties	ND	LD	MD	SD
CD	92 ± 1.05 a	82 ± 0.53 b	74.44 ± 0.47 c	54 ± 0.85 d
AGB ($g \cdot m^{-2}$)	283.45 ± 11.95 a	139.33 ± 3.9 b	88.3 ± 1.57 c	50.33 ± 2.33 d
pH	7.31 ± 0.04 a	7.22 ± 0.04 a	7.02 ± 0.06 b	7.01 ± 0.05 b
BD ($g \cdot cm^{-3}$)	0.93 ± 0.00 d	1.05 ± 0.04 c	1.24 ± 0.02 b	1.31 ± 0.02 a
SWC (%)	39.16 ± 0.31 a	28.11 ± 1.32 b	27.35 ± 0.53 b	22.56 ± 0.57 c
AN ($mg \cdot kg^{-1}$)	30.22 ± 0.87 a	27.91 ± 1.13 b	24.09 ± 0.6 c	21 ± 0.38 d
AP ($mg \cdot kg^{-1}$)	9.53 ± 0.08 a	8.48 ± 0.09 b	3.68 ± 0.13 c	3 ± 0.14 d
AK ($mg \cdot kg^{-1}$)	130.9 ± 6.5 a	136.47 ± 7.59 a	65.56 ± 2.88 b	67.89 ± 1.27 b
TN ($g \cdot kg^{-1}$)	4.49 ± 0.09 a	4.17 ± 0.26 a	3.07 ± 0.06 b	3.2 ± 0.06 b
TP ($g \cdot kg^{-1}$)	0.65 ± 0.01 a	0.64 ± 0.00 b	0.62 ± 0.00 c	0.61 ± 0.00 d
TK ($g \cdot kg^{-1}$)	19.34 ± 0.06 a	18.09 ± 0.19 b	17.91 ± 0.16 b	17.34 ± 0.04 c
SOM ($g \cdot kg^{-1}$)	132.55 ± 5.68 a	82.38 ± 0.48 b	61.04 ± 0.51 c	61.92 ± 0.73 c
STP (%)	65.08 ± 0.15 a	60.32 ± 1.43 b	53.18 ± 0.82 c	50.39 ± 0.61 d

Note: Values presented with different lowercase letters are significantly different at $p < 0.05$.

An average of 16 phyla, 94 classes, 215 orders, 659 families, 3256 genera and 84 species of bacteria were obtained at different taxonomic levels for the degradation gradient alpine meadow soils (Figure S2a). An average of 23 phyla, 23 classes, 43 orders, 41 families, 89 genera and 111 species of fungi were obtained at different taxonomic levels (Figure S2b).

With increasing sequencing depth, the bacterial and fungal ASVs richness dilution curves of the degraded alpine meadow soils reached the saturation stage, revealing that the amount of data from sequencing reads was sufficient to detect most sequence types (Figure S2c,d). The total number of bacterial ASVs for the four different alpine meadow soils was 64,787 with 2362 (3.64%) shared ASVs (Figure S2e), and the total number of fungal ASVs was 8351 with 270 (3.23%) shared ASVs (Figure S2f).

The abundance of bacteria showed a trend of initially increasing and then decreasing during the degradation of alpine meadows. The Chao1 index of LD was lower than SD ($p < 0.01$) (Figure 1a). The alpha-diversity index of the fungal community was different from that of the bacterial community, with fungal richness decreasing as the degradation level increased, and significantly lower in SD than in ND ($p < 0.01$), LD ($p < 0.001$) and MD ($p < 0.01$) (Figure 1b).

The patterns of bacterial and fungal community structure were visualized with non-metric multidimensional scaling (NMDS) based on Bray–Curtis distances. NMDS analysis showed that bacterial and fungal communities at different stages of degradation were not clearly separated, suggesting that no significant difference in bacterial and fungal community structure among the gradient alpine meadows (Figure 1c,d).

From Figure S3, fungal community richness was negatively correlated with BD ($p < 0.001$) and positively correlated with pH, SWC, STP, AN, TN, TP, SOM ($ps < 0.001$), AP, AN ($ps < 0.01$) and TK ($p < 0.05$). Richness with no significant correlation between diversity and soil physicochemical properties.

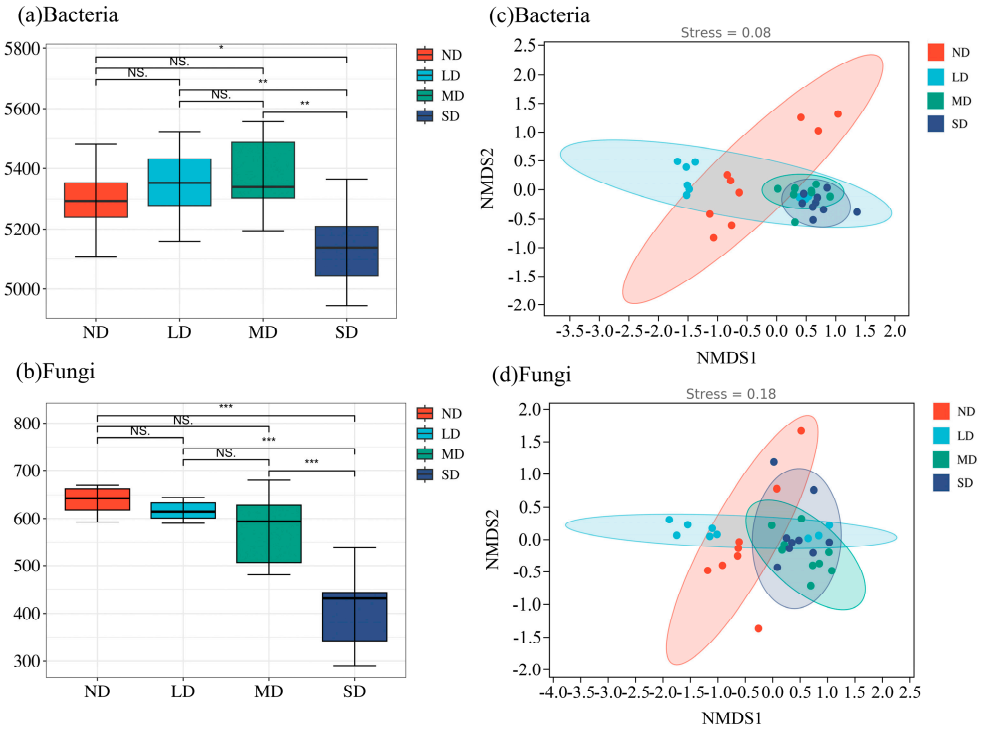

Figure 1. Degradation gradient of alpine meadow soil alpha diversity index for soil bacteria (**a**) and fungi (**b**) and beta diversity index for soil bacteria (**c**) and fungi (**d**). NMDS showed the structure of microbial community for soil, 95% confidence ellipses were shown around the samples grouped based on the degradation gradient alpine meadow. NS represents no significant difference, asterisk represents significant level, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded.

3.2. Differences in Microbial Community Composition

There were differences in the relative abundance of soil microorganisms at the phylum level in degraded gradient alpine meadows. At the bacterial phylum level, Proteobacteria, Actinobacteria, Acidobacteria, and Chloroflexi were the four phyla with the highest relative abundance, accounting for over 80% of all bacteria. The abundance of Proteobacteria and Acidobacteria showed an initially decreasing and then increasing trend, with the lowest abundance of LD. The abundance of Actinobacteria showed a trend of increasing first and then decreasing, with the highest abundance of LD, while that of Chloroflexi showing an increasing trend (Figure 2a).

At the fungal phylum level, Ascomycota, Basidiomycota and Mortierellomycota were the 3 dominant phyla in relative abundance in all samples. The abundance of Ascomycota showed a trend of initially increasing and then decreasing, with the highest abundance in MD. The abundance of the phylum Basidiomycota showed a trend of decreasing. Mortierellomycota has the highest abundance in SD (Figure 2b). In addition, at the genus level, the relative abundance of phytopathogenic fungi Nectria, Aspergillus and Didymella showed a trend of increasing (Figure 2c).

To understand the relationship between soil physicochemical factors and dominant bacterial and fungal taxa, the top 10 abundant bacterial and fungal phyla were selected for Pearson correlation analysis with soil physicochemical factors, as shown in Figure S4. The relative abundance of bacterial and fungal phyla responded differently to environmental variables. Most bacterial phyla abundance was influenced by environmental factors, while fungal phyla were less affected. Soil pH was negatively correlated with most bacterial phyla. SWC was negatively correlated ($p < 0.05$) with an abundance of five bacterial phyla ($p < 0.05$). These results suggest that meadow degradation and the resulting changes in soil properties have had different effects on the composition of different soil bacterial and

fungal communities. Therefore, we have explained how the composition and diversity of soil microbial communities respond to grassland degradation.

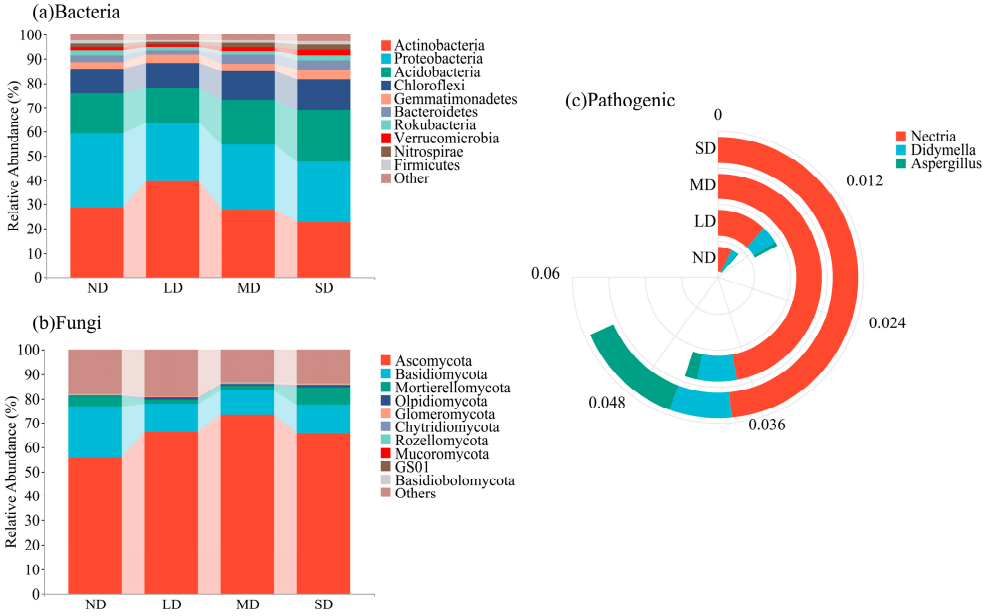

Figure 2. The bacteria (**a**) and fungi (**b**) community in alpine meadows of degradation gradient at the phylum levels relative abundance of soil pathogens in alpine meadows of degradation gradient. Abundance of pathogenic bacteria at the fungal genus level (**c**). ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded.

3.3. Prediction of Soil Microbial Community Functions in Alpine Meadows of Degradation Gradient

The functional potential of soil microorganisms in alpine meadows was explored using high-throughput sequencing and MetaCyc database [35] comparisons. The analysis revealed that soil bacteria from alpine meadows of degradation gradient contained seven types of metabolic pathways at the primary functional level, namely biosynthesis, degradation/utilization/assimilation, detoxification, generation of precursor metabolite and energy, glycan pathways, and macromolecule modification. During the degradation of alpine meadows, the abundance of genes associated with degradation/utilization/assimilation, detoxification, generation of precursor metabolite and energy functional pathways decreased, while the abundance of genes associated with all other functional pathways tended to increase. A total of 60 metabolic pathways were analyzed for their secondary pathway abundance. The main pathways with higher abundance were amino acid biosynthesis, cofactor, prosthetic group, electron carrier, and vitamin biosynthesis, and nucleoside and nucleotide biosynthesis, which belong to biosynthesis. SD mainly enriches amino acid biosynthesis and nucleoside nucleotide biosynthesis (Table S2).

There were three inter-group differential pathways in grassland soil samples with different degrees of degradation. There was a significant difference in the PWY-6572 pathway in ND and MD, a significant difference in the PWY-3081 pathway in ND and SD, and a significant difference in the PWY-7398 pathway in LD and MD.

There were 5 primary metabolic pathways analyzed in fungal communities in alpine meadows of degradation gradient (Table S3). The lowest abundance was of metabolic clusters, generation of precursor metabolite and energy, glycan pathways, and functional pathways in MD. The highest abundance of biosynthesis was in SD. A total of 29 metabolic pathways were analyzed for their secondary pathway abundance. The main pathways with higher abundance were nucleoside and nucleotide biosynthesis, as well as electron transfer respiration, which belong to the biosynthesis and generation of precursor metabolites and

energy. There is no inter-group difference pathway in soil samples of grasslands with different degrees of degradation.

Due to the limited reliability of the PICRUSt's analysis results, we used FUNGuild [36] for supplementary analysis. The saprophytic trophic phenotype was the dominant trophic pattern in the degradation of alpine meadows. Compared to ND, there were no significant differences in pathotroph and saprotroph fungi between degraded meadows, while symbiotroph fungal abundance showed a significant decrease during degradation ($p < 0.05$). The abundance of saprotroph–symbiotroph fungi decreased significantly with increasing degradation ($p < 0.05$). The abundance of pathotroph–saprotroph–symbiotroph fungi significantly increased with the degradation gradient (Figure 3).

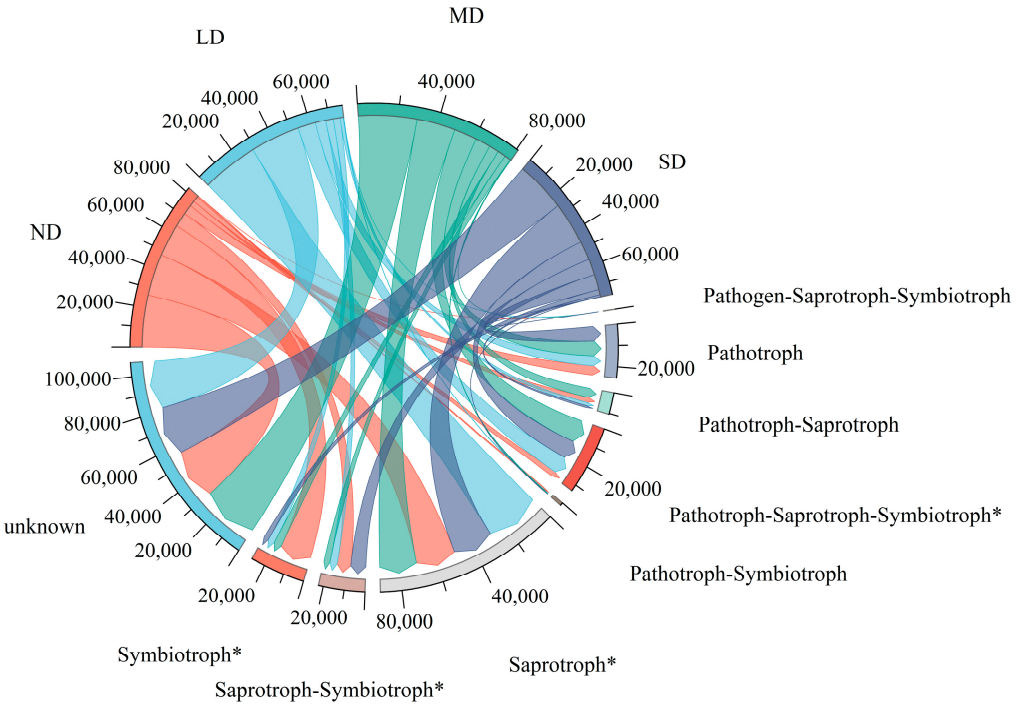

Figure 3. Different fungal trophic types and their relative abundance in alpine meadows of degradation gradient. *: $p < 0.05$. ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded.

Correlation analysis showed a highly significant negative correlation between AK and pathotroph–saprotroph–symbiotroph ($p < 0.01$) and a significant positive correlation with saprotroph ($p < 0.01$). SWC was significantly negatively correlated with pathotroph–saprotroph–symbiotroph ($p < 0.01$) and positively correlated with symbiotroph (Figure S5).

3.4. Analysis of Inter-Group Variation in Microbial Communities

This study used Lefse to estimate the degree to which the abundance of each species in degraded alpine meadows affects the differential effects of soil microbial communities. The results showed that there were five phyla, eleven classes, thirteen orders, eighteen families and twelve genera of soil bacterial species that differed between degraded alpine meadows (Figure 4a). Soil fungal communities differed in three phyla, three classes, five orders, eight families and ten genera. This indicates an important taxonomic shift in the overall microbial community (Figure 4b).

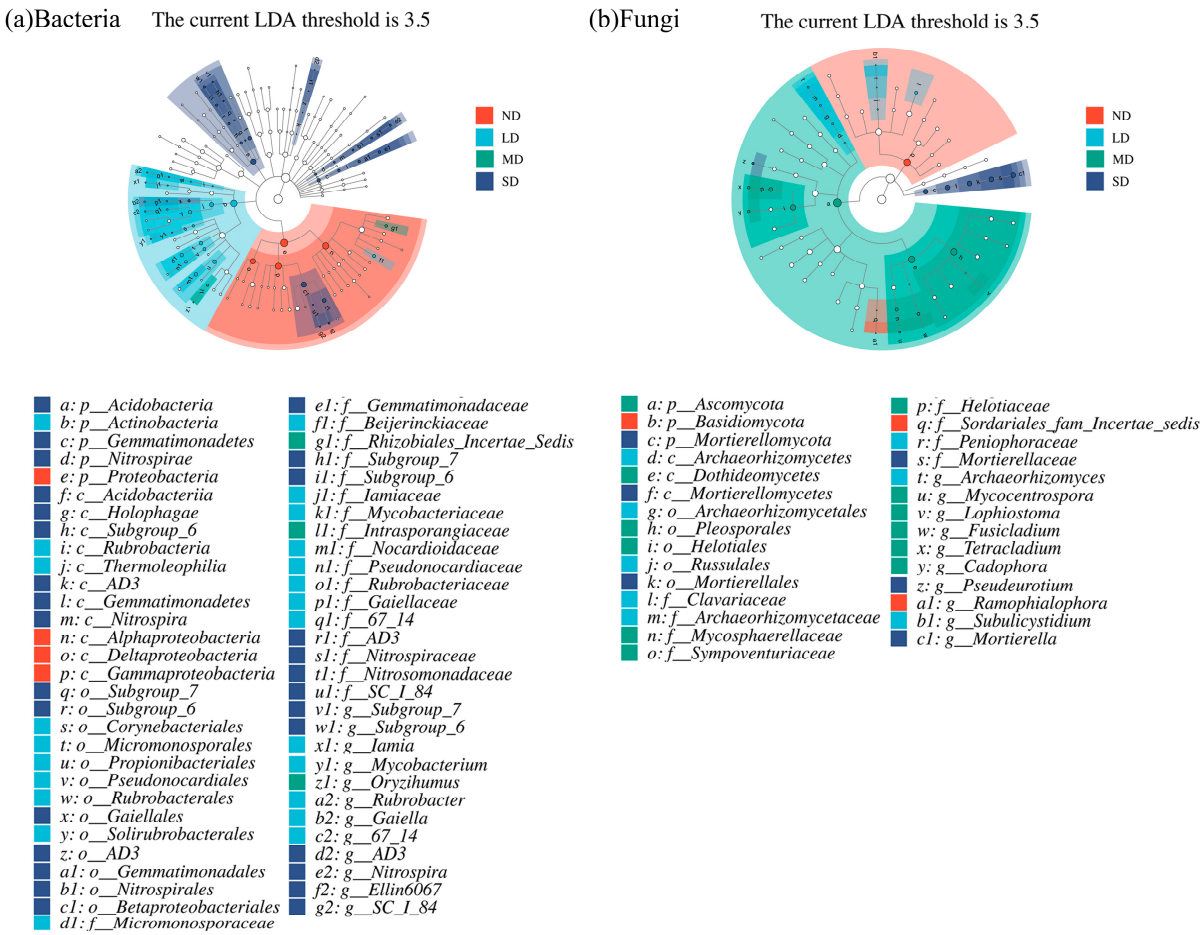

Figure 4. Cladogram showing the phylogenetic distribution of the bacterial (**a**) and fungal (**b**) lineages in alpine meadows of degradation gradient. Different-colored regions represent different treatments. Circles indicate phylogenetic levels from phylum to genus. The diameter of each circle is proportional to the abundance of the group. p: phylum, o: order, c: class, f: family, g: genus. ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded.

Random forest analysis showed the microbial populations that contributed significantly to the differences in soil microbial community construction in alpine meadows of degradation gradient (Figure 5). The differences in soil bacterial community construction at the degradation gradient were mainly influenced by populations of *mle1-7*, *Ellin6067*, *AKYH767*, *Subgroup-2*, which were significantly enriched in SD soils. *0319-7L14* was significantly enriched in LD soils, while *Hyphomicrobium* was significantly enriched in MD soils (Figure 5a). The differences in the construction of fungal communities at the degradation gradient were mainly influenced by *Pseudeurotium*, *Mortierella*, *Ophiosphaerella*, which were significantly enriched in SD soils. The abundance of *Tetracladium*, *Cadophora*, was significantly enriched in MD soils. The abundance of *Archaeorhizomyces*, *Microdochium* was significantly enriched in LD soils. This suggests that alpine meadow degradation affects microbial abundance and the community structure (Figure 5b). The above results explain our second question, which involves 59 bacterial marker species and 29 fungal marker species in the process of grassland degradation. At the genus level, *mle1-7* in bacteria and *Pseudeurotium* in fungi are the most important species, both of which have the highest abundance in SD.

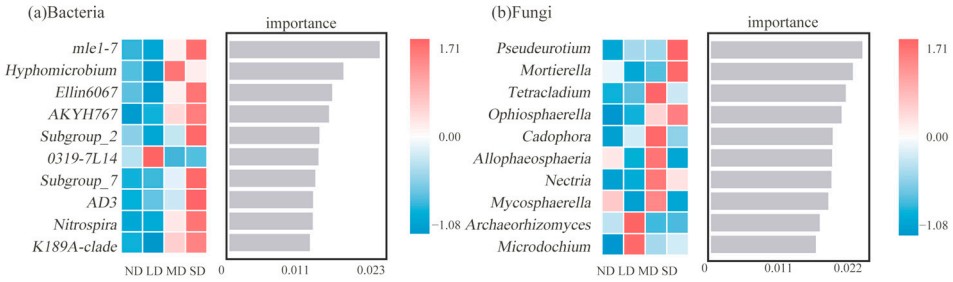

Figure 5. Heat maps of the top 10 most important bacterial (**a**) and fungal (**b**) genera, the blue mark indicates higher relative abundance and red mark indicates lower relative abundance. ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded.

3.5. Microbial Community Changes Drive Nutrient Cycling

To disentangle the potential main drivers of soil nutrient cycling in degradation gradient alpine meadows, we identified the main microbial predictors for the soil multi-nutrient cycling index by random forest (RF) analysis (Figure 6a). Bacterial beta-diversity was found to be the most important variable for predicting the soil multi-nutrient cycling index throughout the degradation gradient, followed by fungal beta-diversity.

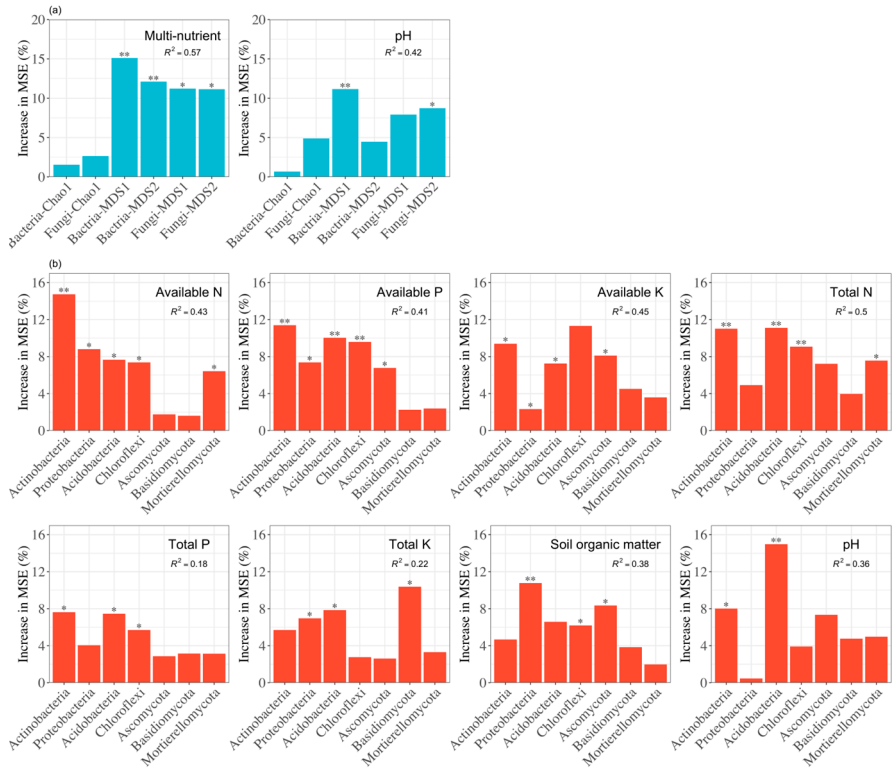

Figure 6. Potential driving factors for nutrient cycling changes in gradient degraded alpine meadow soil. Random Forest (RF) mean predictor importance of microbial alpha- and beta-diversity indices as drivers for the soil multi-nutrient cycling separately (**a**). RF mean predictor importance (percentage of increase of mean square error) of dominant phyla (>5% of total community) as drivers for soil properties (**b**). *: $p < 0.05$. **: $p < 0.01$. MSE: mean squared error.

We also evaluated the biological contributions of dominant microbial phyla to soil properties via a RF analysis (Figure 6b). Evidently, not all microbial phyla contributed alike to the various edaphic variables. For example, Acidobacteria was the most important variable for predicting soil properties, including AP, TN, pH (*ps* < 0.01), indicating its importance in soil nutrient cycling during reforestation. Other important variables for

predicting soil properties were the Actinobacteria, for pH and AN, AP, TN, ($ps < 0.01$), the proteobacteria, for SOM ($p < 0.01$), the Chloroflexi, for AP and TN ($ps < 0.01$)

4. Discussion

4.1. Soil Microbial Community Composition, Diversity and Function in Response to Meadow Degradation

The results of this study confirmed that the community composition of microorganisms remained largely stable during meadow degradation, but there were significant differences in the relative abundance of bacterial communities. The bacterial community was dominated by phyla Actinobacteria, Proteobacteria, Acidobacteria and Chloroflexi, which were common in soils around the world [37]. Our results were consistent with those of alpine swamp meadows, alpine meadows and alpine deserts on the Qinghai–Tibet Plateau [38]. We found that the relative abundance of Proteobacteria gradually decreased during the degradation of alpine meadows, but increased in SD. The phylum Actinobacteria showed a trend of initially increasing and then decreasing with the degradation gradient. These results may be explained with the different life strategies of bacterial taxa. The phylum Proteobacteria is able to produce extracellular polysaccharides that contribute to biocrust formation [39] and soil stability [40]. Also, it is associated with phenolic compounds and aromatic groups in the soil [41]. The degradation of meadows is characterized by a gradual reduction in vegetation cover and apomictic material, which affects species composition and diversity during species succession and is negatively correlated with biological crust cover [42]. This explains the changing trends of the Proteobacteria phylum during meadow degradation. Actinobacteria plays an important role in the organic matter cycle [43]. It inhibits the growth of plant inter-rooted phytopathogens and decomposing dead plants, and contributes significantly to improve nutrient and mineral availability and stabilizing humus formation, thereby improving the soil carbon cycle [44]. The overall trend of decreasing relative abundance of the above bacterial phyla indicates a decrease in substrate degradation capacity during degradation and a change in the trophic structure of the microbial community [45].

The major phylum in the fungal community included Ascomycota and Basidiomycota, which is in agreement with previous studies in alpine meadows [46]. Species in both phyla participate in the carbon cycle by degrading organic matter [47,48]. Some fungal groups within the phylum Basidiomycota form symbiotic relationships with algae and mosses [49]. In general, members of the Ascomycota are often found in harsh environments, while members of the Basidiomycota prefer environments with abundant resources and high plant richness [50]. This explains the opposing trends in the relative abundance of the Ascomycota and Basidiomycota at our study sites. It has been suggested that both alpine meadow desertification and highland sage-grouse activity may increase the risk of infectious diseases, particularly fungal diseases, in alpine ecosystems [51]. This is consistent with the finding of a significant increase in plant pathogens during the degradation of alpine meadows in this study, suggesting that the degradation process is detrimental to the health of the meadow system [52]. An increase was found in the relative abundance of the plant pathogenic fungi Nectria, Aspergillus and Didymella. This may be due to a reduction in above- and below-ground biomass during meadow degradation. Transformation of dominant species from herbaceous plants is favored by livestock to shrubs [53]. Phytopathogenic fungi usually feed on plant roots and compete with mycorrhizal fungi [54]. In the early stages of degradation, soil conditions begin to deteriorate and become unsuitable for the survival of clump-forming mycorrhizal fungi. Pathogenic fungi dominate the competitive process, and their relative abundance increases. And, a reduction in root biomass and apoplastic inputs may be responsible for the decrease in pathogenic fungi.

PICRUSt2 was used to predict the function of soil microbial communities in degraded alpine meadows, and secondary functional gene analysis was performed in MetaCyc database. Amino acid biosynthesis, cofactor, prosthetic group, electron carrier, and vitamin biosynthesis, nucleoside and nucleotide biosynthesis had the highest abundance. Amino

acid metabolism mainly produces a-ketoacids, amines, and carbon dioxide through deamination, transamination, combined deamination, or decarboxylation [55]. Amino acids are involved in cellular reactions, and therefore, they influence a number of physiological processes such as plant growth and development, intracellular pH control, generation of metabolic energy or redox power [56]. This indicates that the microbial community in the alpine meadow actively participates in the synthesis process.

Using FUNGuild to predict the function of fungal communities, the three trophic modes of symbiotic trophic, pathogenic symbiotic trophic and saprophytic symbiotic trophic were significantly different in the non-degraded, compared with the degraded meadows. Symbiotroph obtain nutrients by exchanging resources with host cells [36] and are beneficial to plant health and nutrition. The significant decrease in symbiotic trophic fungi during the degradation of the meadow may be due to a reduction in below-ground biomass. Pathotrophs obtain their nutrients mainly by damaging host cells and can have a negative impact on other components of the fungal community [57]. During meadow degradation, pathotrophic fungi showed a trend of increasing. The increase may be due to a decrease in the stability of the meadow system of degradation [58], leading to a decrease in host cell resistance and an increase in pathogenic fungal content because of a large number of pathogenic bacteria attacking host cells [13].

4.2. Biomarkers of Degraded Gradient Alpine Meadows

The results of the diversity analysis, Lefse analysis and random forest analysis showed that the diversity and composition within the community have changed, but the community structure has not undergone complete changes during the degradation of alpine meadows. This is because during degradation, above- and below-ground biomass is reduced [59,60], which reduces inputs of apoplastic matter and root secretions [61,62]. Then, soil microbial populations and microbial activity are suppressed due to soil nutrient depletion [63]. Degradation leads to soil compaction [64] and low soil oxygen and nutrient content [65,66], resulting in a decrease in microbial diversity [67]. At the same time, soil microorganisms also indirectly influence the C cycle by improving soil aggregation, thereby protecting SOM [68,69]. Changes in soil microbial diversity and composition during degradation can affect vegetation development [70], resulting in worse meadow degradation and a vicious cycle.

In addition, the LDA analysis results indicated that most biomarker bacteria belonged to Acidobacteria, and fungi belonged to Ascomycota. Their relative abundance increased with the degradation gradient, possibly due to their ability to decompose organic matter. Some biomarkers are involved in carbon and nitrogen metabolism [71,72]. The increase in these species indicates that the relative abundance of microorganisms related to material degradation in grasslands will increase during the degradation process. At the genus level, 12 marker species were found in the bacterial community, and 10 marker species were found in the fungal community to predict the degradation of alpine meadows.

The correlation between bacterial community diversity and soil nutrient changes during grassland degradation is higher than that of fungal community [73]. This may be explained by the fact that bacteria dominate the initial stages of apoplastic decomposition, i.e., mineralization, with easily degradable organic matter (i.e., starch and pectin) as a source of nutrients, whereas fungi decompose more complex apoplastic compounds that rely more on stable organic matter (i.e., cellulose and lignin) [74,75]. The relative proportion of stable carbon inputs is likely to remain constant and the content of readily degradable organic matter is likely to decline.

4.3. Composition and Diversity of Soil Microbial Communities Driving Nutrient Cycling

The changes in soil microbial communities lead to changes in soil nutrient cycling [76]. In order to further study the impact of microbial community composition and diversity on nutrient cycling, correlation analysis and random forest analysis were conducted on key nutrient indicators and microbial composition and diversity. The degradation process of

alpine meadows affects the composition and diversity of soil microbial communities [77], thereby affecting soil nutrient cycling. Different microbial populations participate in the biogeochemical cycle of nutrients, thereby improving soil fertility and plant health [78] and drive nutrient cycling in environments with poor nutritional conditions through collaborative metabolism among bacterial communities during the degradation process of alpine meadows [79]. This explains the significant contribution of bacterial community composition and diversity to the cycling of multiple nutrients in soil. Many studies have reported the significant impact of pH on the structure of soil microbial communities [80,81]. Siciliano et al. [82] found that pH plays a greater role in determining the phylogenetic structure and composition of bacterial communities. The present study found that pH significantly influenced the composition of the soil microbial community, with different microbial populations responding differently to pH. The reasons why soil pH affects the structure of the microbial community may be: (1) the optimal pH value of bacterial community is limited by the pH value of the native environment, and a deviation of 1.7 pH units will reduce bacterial activity by 50% [83]. (2) pH, as a comprehensive index for evaluating soil conditions, can indirectly affect the activity of microorganisms and the correlation between microorganisms by adjusting the availability of soil nutrients [84]. Other studies suggest that climate, contents of nitrogen and phosphorus may be the main drivers of change in microbial communities [82,85–87]. Our research also supports the cycle of microbial composition primarily driving AP [88,89]. Phosphorus is an essential element for microbial growth and metabolism, and can change the structure of microbial communities [90]. Li et al. showed that the Acidobacteria was the main microbial phylum that determined soil phosphorus solubilization and immobilization turnover, explaining the correlation between the Acidobacteria and AP in this experiment.

5. Conclusions

The degradation of alpine meadows directly affects soil nutrient content, which in turn affects the diversity and function of soil microbial composition. The combined effect of soil nutrient reduction and microbial community changes reduces the stability of meadows. And, these changes may aggravate ecosystem degradation. This work reveals the response patterns and main environmental drivers of alpine meadow degradation in microbial communities. As alpine meadow degradation increases, the soil nutrient content decreased significantly ($p < 0.05$). It indirectly affects the composition, diversity and function of microbial communities. Among the dominant bacteria phyla, an abundance of Proteobacteria initially decreased and then increased, and that of Actinobacteria initially increased and then decreased. Among the fungi, an abundance of Ascomycota initially increased and then decreased, and that of Basidiomycota initially decreased and then increased. There were 59 bacterial taxa and 29 fungal taxa showing significant differences in four groups. The abundance of pathogenic fungi Nectria, Didymella and Aspergillus increases. The alpha-diversity index of bacteria showed a trend of initially increasing and then significantly decreasing during the degradation of alpine meadows ($p < 0.05$). But, the alpha-diversity index of the fungal community showed a decreasing trend during the degradation of alpine meadows. Among the potential functional groups of bacteria and fungi, there was a decrease in synthesis-related functional gene abundance and an increase in metabolism-related functional gene abundance. The symbiotic and saprophytic symbiotic nutrient fungi decreased significantly ($p < 0.05$). The above research results indicate that grassland degradation affects the composition and abundance of soil microbial communities, thereby altering the function of microorganisms. A total of 59 bacteria and 29 fungi from different levels can serve as marker species in the process of grassland degradation. The soil nutrient cycling is mainly influenced by the beta diversity of microbial communities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14010195/s1, Figure S1. Experimental design. ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded; Figure S2: Annotated diagram of species classification of soil bacteria (a) and fungi (b) in degraded gradient

alpine meadow. The abscissa of the alpine meadow soil bacteria (c) and fungi (d) sparse curves in the degradation gradient is the flattening depth, and the ordinate is the median and boxplot of the α-diversity index calculated 10 times. The flatness of the curve reflects the impact of sequencing depth on the diversity of observed samples. ASV Venn diagram of soil bacteria (e) and fungi (f) in degraded gradient alpine meadow ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded; Figure S3: Heat map of the correlation between soil bacterial and fungal alpha-diversity indices and vegetation and soil physicochemical factors in alpine meadows of degradation gradient. The red and blue mark indicates positive and negative correlation., while star marks indicate the significant level: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. BD: Bulk density; SWC: Soil water content, AN: Available nitrogen; AP: Available phosphorus; AK: Available potassium; TN: Total nitrogen; TP: Total phosphorus; TK: Total potassium; SOM: Soil organic matter; STP: Soil total porosity; Figure S4. Heat map of the correlation between the relative abundance of soil bacteria and fungi and soil physicochemical factors in alpine meadows of degradation gradient. The red and blue mark indicates positive and negative correlation, while star marks indicate the significant level: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. BD: Bulk density; SWC: Soil water content, AN: Available nitrogen; AP: Available phosphorus; AK: Available potassium; TN: Total nitrogen; TP: Total phosphorus; TK: Total potassium; SOM: Soil organic matter; STP: Soil total porosity; Figure S5. Heat map of correlation between fungal nutrient patterns and soil physicochemical factors. The red and blue mark indicates positive and negative correlation, while star marks indicate the significant level: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. BD: Bulk density; SWC: Soil water content, AN: Available nitrogen; AP: Available phosphorus; AK: Available potassium; TN: Total nitrogen; TP: Total phosphorus; TK: Total potassium; SOM: Soil organic matter; STP: Soil total porosity; Table S1: Distribution of sampling points ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded; Table S2: Function prediction of the bacterial communities in the degradation meadow (mean $\pm$ SD). ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded; Table S3: Function prediction of the fungal communities in the degradation meadow (mean $\pm$ SD). ND: Non-degraded; LD: Lightly degraded; MD: Moderately degraded; SD: Severely degraded.

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