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Streptomyces Application Triggers Reassembly and Optimization of the Rhizosphere Microbiome of Cucumber

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Abstract: *Streptomyces partum* Act12 and *Streptomyces roche* D74 are biocontrol strains that can promote plant growth and enhance stress resistance in different crops. However, their effects on the rhizosphere microbiome and the role of the reassembled microbiome in plant growth promotion and stress resistance enhancement remain unclear. This study investigated the variation in the rhizosphere microbiome induced by *Streptomyces* application through a cucumber (*Cucumis sativus* L. cv. "Youliang") pot experiment. The bacterial and fungal communities of rhizosphere soils inoculated with and without *Streptomyces* were, respectively, compared based on 16S rRNA and internal transcribed spacer rRNA gene sequences. Following *Streptomyces* application, the bacterial alpha diversity increased significantly, while the fungal alpha diversity exhibited the opposite trend. The bacterial and fungal communities' compositions clearly shifted in the inoculated soil. Compared with the uninoculated control, the relative abundance of the genus *Streptomyces* increased by 68.3%, and the bacterial co-occurrence network in the rhizosphere soil was enriched significantly. The relative abundance of bacteria associated with nitrogen fixation was increased by 7.5% following *Streptomyces* application. Based on the results of this study, we conclude that the application of *Streptomyces* Act12 and D74 can be used to reassemble and optimize the rhizosphere microbiome of cucumber, which is conducive to plant survival.

Keywords: *Streptomyces* biocontrol agent; cucumber; rhizosphere microbiome; microbial community composition; microbial function



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1. Introduction

Microbiomes are considered functional drivers in their eukaryotic hosts. Plant microbiomes can expand the genomic and metabolic capabilities of their hosts, providing or facilitating a range of essential life-support functions, including nutrient acquisition, immunity modulation, and stress tolerance [1]. In addition, the composition and functions of plant microbiomes influence their beneficial effects on plant growth and health.

The rhizosphere is the soil zone near the plant roots, where microbial activity is affected by the availability of plant-derived nutrients and oxygen. It is not a defined zone with a specific size or shape, but rather a zone with gradients of physical, chemical, and biological properties, which change along the radial and vertical directions of the roots [2]. According to Edward et al. [3], the root-associated microbiome extends from the rhizosphere to the rhizoplane (root surface) and to the endosphere (root interior) of rice. The soil can be considered a "seed bank" for root microbiota, the rhizosphere a "selective growth chamber," the rhizoplane a specific habitat or a transitional boundary,

and the endosphere a restricted area [4]. The rhizosphere microbiome plays a key role in the formation of the plant microbiome and influences plant growth and health [5].

In recent studies, distinct rhizosphere microbiomes have been observed between healthy and diseased plants [6,7]. In addition, the potential occurrence of *Fusarium* wilt can be predicted using key biological indicators and features common to diseased soil microbiomes [8]. Moreover, *Arabidopsis thaliana* plants have been reported to recruit beneficial microbes to resist the *Pseudomonas syringae* pv. *tomato* pathogen through root exudates [9], which function in the subsequent generation as legacy in the soil [10]. Therefore, regulation of the rhizosphere microbiome could facilitate the control of plant diseases and contribute to sustainable agriculture.

Actinobacteria are widespread in soil, produce diverse antibiotics, and have beneficial effects in the form of plant disease control and growth promotion [11]. Under continuous potato cropping, Chen et al. [12] observed that healthy plants were associated with higher Actinobacteria abundance in the rhizosphere soil compared to diseased plants, and the Actinobacteria associated with healthy plants showed greater antagonistic potential. *Streptomyces*, which exhibits easy cultivation, high yield, and long survival characteristics, is the most diverse and most widely distributed Actinobacteria group. In recent years, some *Streptomyces* strains (e.g., *S. partum* Act12 and *S. roche* D74) have been reported to have the capacity to (i) facilitate wheat [13] and corn growth [14] and to (ii) enhance plant resistance to viruses [15], drought [16], heavy metal toxicity [17], allelopathic inhibitors [18], and parasitic weeds [19].

The benefits of microbes (including *Streptomyces*) to plants as biocontrol agents are mainly attributed to (i) the release of growth hormones, (ii) the provision of plant nutrients, (iii) the direct inhibition of pathogens by the production of antimicrobial compounds [20], and (iv) the indirect inhibition of pathogens by stimulating plant immune systems—otherwise known as induced systemic resistance [21–24]. However, these mechanisms are generally attributed to interactions between beneficial microbes and their host plants, while the diversity and involvement of the rhizosphere microbiome are largely ignored. Therefore, the currently reported mechanisms by which beneficial microbes enhance plant performance cannot account for the overall enhancement observed following *Streptomyces* inoculation.

Both culture-dependent [25] and culture-independent [15,26] studies have shown that the rhizosphere microbiomes of different plants (i.e., ginseng and monkshood) shift following *Streptomyces partum* Act12 and *Streptomyces roche* D74 application. In addition, the introduction of *S. partum* Act12 promotes wheat growth by stimulating the growth of beneficial bacteria such as *Pseudomonas koreensis* populations [27]. Moreover, *S. partum* Act12 and *S. roche* D74 participate in the regulation of the rhizosphere microbiomes of tomato and monkshood, which are associated with disease resistance [15,26]. Therefore, in the present study, we hypothesized that *Streptomyces* application improves plant function largely via the reassembly and optimization of the rhizosphere microbiome. Our specific objectives were to investigate (i) how *Streptomyces* spp. influence the rhizosphere microbiome and (ii) whether the reassembled rhizosphere microbiome participates in plant growth promotion or disease resistance, and the underlying mechanisms.

To address the questions above, a cucumber pot experiment was carried out in the present study. To address the questions above, cucumber was selected as the experimental plant to set up a pot experiment. Cucumber is an important vegetable with highly economic value and market demand; it is mainly planted in the greenhouse and generally constrained by soil sickness [28]. According to previous studies, the significant growth promotion effect and microbiome vitiation (by the culture-dependent method) in the rhizosphere of *Streptomyces* on cucumber has been proved [29].

The composition and functions of the rhizosphere microbiome were compared between soils with and without *Streptomyces* application using high-throughput sequencing analysis of the bacterial 16S rRNA gene and fungal internal transcribed spacer (ITS), regions. The aim of the present study was to unravel the variation in the rhizosphere microbiome induced

by *Streptomyces* application, and the effect of this variation on cucumber productivity based on biomass.

2. Materials and Methods

2.1. Pot Experiment

Experimental design: The pot experiment was conducted from 28 February to 30 May 2020 in a solar greenhouse at Northwest A&F University in Yangling, Shaanxi Province, China (34.26° N, 108.07° E). A two-group experimental design was used, in which one group of pots contained soil without *Streptomyces* application (control) and the other group of pots contained soil with *Streptomyces* application (treatment). Each group had nine pots.

Streptomyces agent preparation: The *Streptomyces* agent consisted of *S. partum* Act12 (accession number: MH542148) and *S. rochei* D74 (accession number: KJ145878). Solid fermentation powder from strains Act12 and D74 was mixed at a 1:1 (*w/w*) ratio to formulate the *Streptomyces* agent with a total viable count of 8×10^9 colony-forming units g^{-1} , counted by the spread plate method. *Streptomyces* has been shown to have long-term viability in field experiments and biocontrol strain colonization tests [26].

Soil preparation: The experimental soil was a silty loam obtained from the topsoil (0–20 cm) in the farmland at the experimental site in Yangling. The soil was derived from loess material and classified as Eum-Orthic Anthrosol (Cumulic Haplustalf in the USDA system). The collected soil was air-dried, ground, passed through a 2 mm sieve, and mixed with an organic fertilizer (125 $g\ kg^{-1}$; Dadi Agricultural Organic Fertilizer Company, Baoji, China). Subsequently, the soil was divided into two portions for the preparation of treatment and control, respectively.

Inoculation of the treatment soil: Before the pot experiment, the *Streptomyces* agent was inoculated by mixing it thoroughly with the reserved soil (2 $g\ kg^{-1}$ dry soil). There was no inoculation during the pot experiment.

Pot preparation: Each pot was filled with 5 kg of the prepared soil according to the experimental design.

Cucumber seeding: Cucumber seeds (*Cucumis sativus* L. cv. “Youliang”) were soaked with 1% sodium hypochlorite solution for 15 min and then washed using distilled water. Germination was carried out at 25 °C over soaked cotton cloth in disinfected Petri dishes. The most robust germinated seeds were selected and transplanted into pots (three seeds per pot). During the experimental period, all the pots were placed randomly in the greenhouse and transposed every day. The field capacity of the soil was maintained at 20% by watering every two days.

2.2. Sampling and Biomass Estimation

Three-month-old cucumber plants were dug out using a sampling shovel to obtain the complete root system along with the root-zone soil. The loosely attached soil was shaken off, and only the tightly attached soil from the roots was collected. The roots from each plant were placed in a sterile Ziplock bag, kept on ice, and immediately transferred to the laboratory. The root-adhering soil was gently removed using fine forceps. The soil retained on the root surface was considered the rhizosphere soil. The three roots from the same pot were washed with sterile water to form one composite slurry sample directly. The slurry was centrifuged (at $1.1 \times 10^4 \times g$), the supernatant was removed, and the precipitate was stored as rhizosphere soil at –80 °C for further study. Each fresh plant was washed and dried with absorbent paper. Plant biomass was measured in terms of fresh shoot weight, fresh fruit weight, and dry fruit weight (after oven-drying at 60 °C).

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

Total genomic DNA was extracted from the 18 soil precipitates using the hexadecyl trimethyl ammonium bromide (CTAB) method. The extracted DNA was checked on 1% (*w/v*) agarose gel, quantified using a UV–VIS spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA), and diluted to 1 $ng\ \mu L^{-1}$. The univer-

sal primer pairs 341F/806R (forward: 5'-GTGCCAGCMGCCGCGGTAA-3'/reverse: 5'-GGACTACHVGGGTWTCTAAT-3') and ITS-1737F/ITS-2043R (forward: 5'-TCCGTAGGTG AACCTGCGG-3'/reverse: 5'-GCTGCGTTC-TTCATCGATGC-3') were used to amplify the V3–V4 region of the bacterial 16S rRNA gene and the ITS region of the fungal 18S rRNA gene, respectively [30]. DNA fragments of the target genes were amplified from template DNA with Phusion Hot Start Polymerase (New England Biolabs, Ipswich, MA, USA) and Phusion® High-Fidelity PCR Master Mix with GC buffer according to the manufacturer's instructions. The final amplicon libraries were subjected to the Illumina NovaSeq6000 platform (Illumina Inc., San Diego, CA, USA) for sequencing at Novogene Bioinformatics Technology Co. Ltd (Beijing, China). All the resulting sequences were deposited in the NCBI Sequence Read Archive database (<https://www.ncbi.nlm.nih.gov/sra> (accessed on 29 January 2021)) under BioProject number PRJNA695869 for bacteria and PRJNA698281 for fungi.

2.4. Bioinformatics Analysis

Raw reads of the 16S rRNA gene and ITS region were processed using self-R scripts and USEARCH v.10.0 as follows [31]: assemble paired-end Illumina reads; relabel sequencing names (-fastq_mergepairs); remove barcodes and primers (-fastx_truncate); filter of low-quality reads (expected error per base greater than 1% were discarded, and sequence records with abundance less than 0.0001% of total amplicons were filtered out); find non-redundancy reads (-fastx_uniques); denoise using the command “unoise3” in USEARCH; obtain amplicon sequence variants (ASVs); remove plastid and non-bacterial sequences (for bacteria); and generate the feature table by USEARCH.

All samples were rarefied to the same number of reads as the sample with the lowest number of reads. For each representative ASV, the SILVA 138.1 (for bacteria) and RDP ITS (WARCUP Training Set V2, Jun-2016, for fungi) [32] databases were used to annotate the taxonomic information. Alpha-diversity analysis was carried out using the richness index and the Shannon index [33]. Functional annotation of fungal taxa was carried out using FUNGuild (<https://github.com/UMNFuN/FUNGuild> (accessed on 17 December 2020), update by zewei Song) [34].

2.5. Data Analysis and Statistics

Biomass analysis: As plant growth differences were observed within one pot, cucumber plants within one pot were divided into three groups based on plant height (high, medium, and low), and then the biomass data were analyzed by grouping, according to the model of a Randomized Block Design.

One-way analysis of variance was performed to determine differences in taxa abundance, and microbial alpha-diversity indices between the control and *Streptomyces* treatments. Statistically significant differences were defined at the level of $p < 0.05$. Principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity algorithm was used to assess variation in microbial community beta diversity between the control and *Streptomyces* treatments.

Co-occurrence network analysis was performed to assess the connections within bacterial and fungal communities at the genus level. The bacterial and fungal genera with average relative abundances higher than 0.1% were selected. The Spearman correlation matrix between the relative abundance of two genera was calculated. Both the correlation matrix (r matrix) and the significance matrix (P matrix) were estimated using the “Hmisc” package in R, v.4.0.3 (<http://www.r-project.org/> (accessed on 10 Oct 2020)). Correlation data were filtered with a cut-off of $|r| = 0.6–0.93$ and significant correlation at $p < 0.05$. The data were loaded into Gephi software version 0.9.2 (<https://gephi.org/> (accessed on 26 Sep 2017)) to construct co-occurrence networks and visualized using the Fruchterman–Reingold layout algorithm [7]. The following topological features were calculated: average connectivity, average path length, average clustering coefficient, and modularity [15]. The STAMP software v2.1.3 (<http://kiwi.cs.dal.ca/Software/STAMP> (accessed on 26 Jun

2015)) was used to identify and visualize the phyla and genera with significant differences in relative abundance between the control and the *Streptomyces* treatments (*t*-test, $p < 0.05$).

3. Results

3.1. Biomass of Cucumber Plants

The weights of the cucumber shoots and fruits are shown in Table 1. Regardless of the size of the cucumber plant, the fresh shoot weight of cucumber had no significant differences between control and *Streptomyces*-treated plants. *Streptomyces* application resulted in weight increases of 58% in the fresh fruit and 65% in the dry fruit for the high group ($p < 0.05$); however, these weights were not affected by *Streptomyces* application in the middle group. The potted plants in the low group did not bear cucumber fruit.

Table 1. Average biomass of cucumber plants in each pot inoculated with or without *Streptomyces*.

Group	Treatment	Fresh Shoot Weight Mean	Fresh Fruit Weight Mean	Dry Fruit Weight Mean
High	Control	87.15 ± 5.20 a	149.44 ± 2.96 b	5.79 ± 0.56 b
	With <i>Streptomyces</i>	93.42 ± 9.51 a	237.03 ± 16.52 a	9.57 ± 1.46 a
Middle	Control	101.97 ± 4.51 a	56.26 ± 39.43 a	2.62 ± 1.49 a
	With <i>Streptomyces</i>	82.62 ± 17.48 a	100.02 ± 8.84 a	4.19 ± 0.77 a
Low	Control	78.78 ± 3.30 a	0	0
	With <i>Streptomyces</i>	79.05 ± 14.47 a	69.23 ± 19.15	3.16 ± 0.63

The duplicates within one pot were divided into three groups with high, middle, and low plant height, respectively. The control was treated without *Streptomyces*. Values are means ± standard deviation ($n = 3$). Different letters indicate statistically significant difference between the control and *Streptomyces* treatments, at the level of $p < 0.05$.

3.2. Distinctive Microbial Community Diversity

The bacterial alpha diversity (richness index and Shannon index) of rhizosphere soil treated with *Streptomyces* was significantly higher than that of the control, while the alpha diversity of the fungal community exhibited the opposite trend (Figure 1). Rarefaction curves of all the samples showed high quality (Supplementary Figure S1). After *Streptomyces* application, the richness index and the Shannon index of bacteria increased from 2761 to 2906 (Figure 1c) and from 6.5 to 6.8 (Figure 1e), respectively ($p < 0.05$); in contrast, the fungal richness index and Shannon index decreased from 338 to 271 (Figure 1d) and from 3.4 to 2.4 (Figure 1f), respectively ($p < 0.05$). The PCoA results showed clear separation of the bacterial (Figure 2a) and fungal (Figure 2b) communities between the rhizosphere soils with and without *Streptomyces* inoculation.

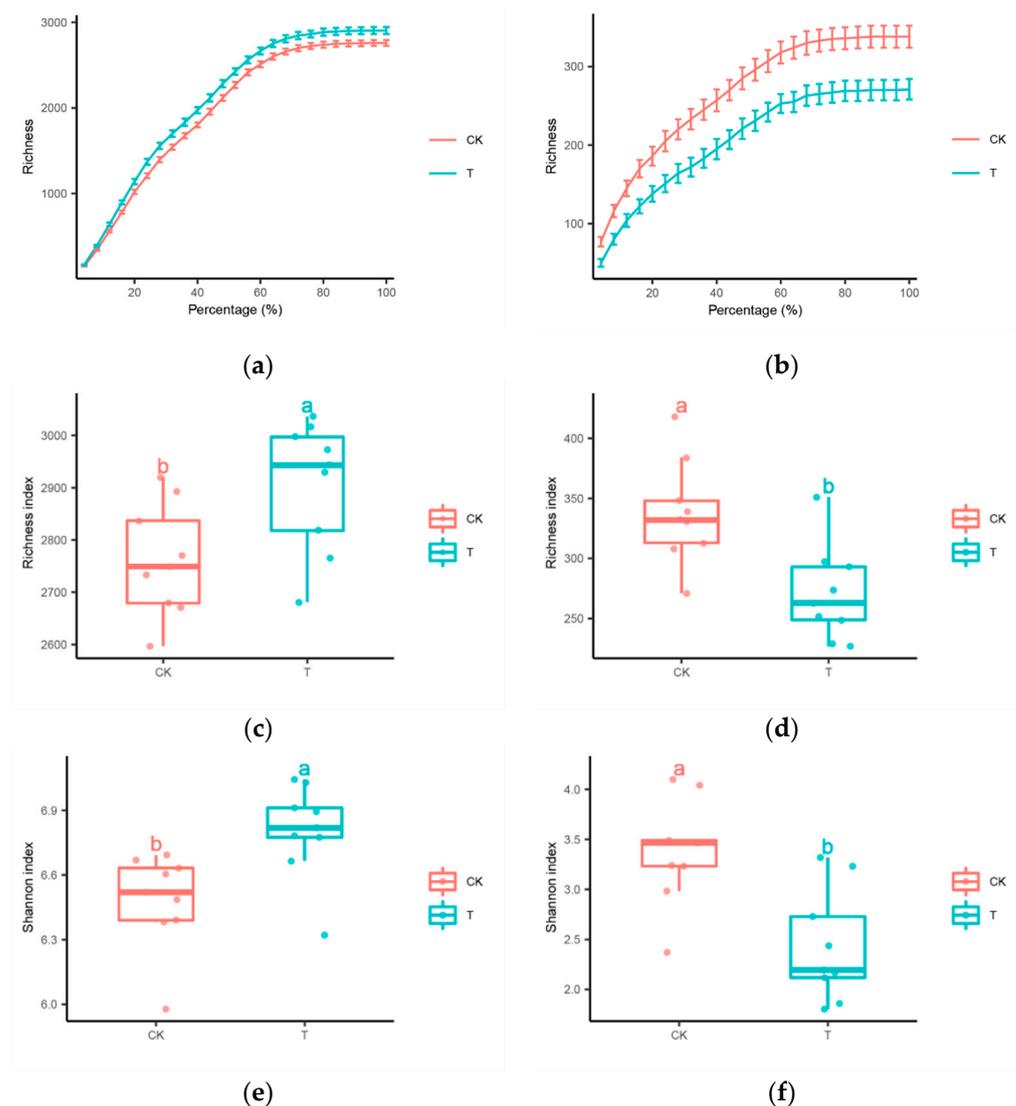


Figure 1. Alpha diversity of the rhizosphere microbiome associated with cucumber plants in *Streptomyces*-inoculated soil (T) and uninoculated control soil (CK). (a) Rarefaction curves of bacteria; (b) rarefaction curves of fungi; (c) richness index of bacteria; (d) richness index of fungi; (e) Shannon index of bacteria; (f) Shannon index of fungi. The horizontal bars within boxes represent the medians. The tops and bottoms of boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers extend to data no more than $1.5 \times$ the interquartile range from the upper edge and lower edge of the box, respectively. Different letters above boxes indicate significant differences at $p < 0.05$.

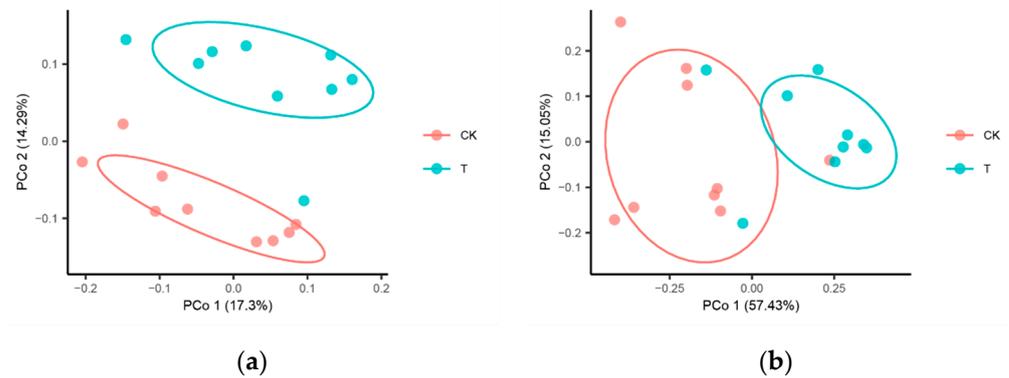


Figure 2. Principal coordinate analysis (PCoA) of bacterial (a) and fungal (b) beta diversity based on the Bray–Curtis distance between all rhizosphere soil samples inoculated with *Streptomyces* (T) and without *Streptomyces* inoculation (CK).

3.3. Shifted Microbial Community Composition

The rhizosphere bacteria were classified into 32 phyla and 366 genera based on the 16S rRNA gene sequences. The five most abundant bacterial phyla in the control treatment were Proteobacteria (48.7% of total bacterial sequences), Bacteroidetes (14.1%), Acidobacteria (9.3%), Gemmatimonadetes (5.9%), and Actinobacteria (4.6%); their relative abundances varied by −6.0%, −3.0%, −2.8%, 15.2%, and 50.4%, respectively, in *Streptomyces*-treated soil (Figure 3a).

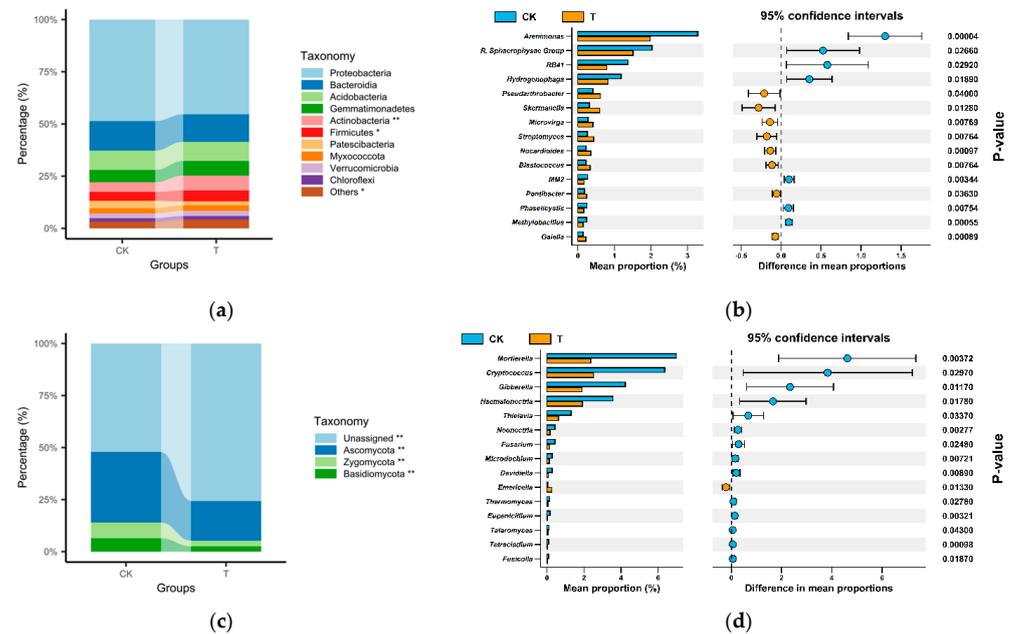


Figure 3. The relative abundances of major bacterial (a) and fungal (c) phyla, and the top 15 genera of bacteria (b) and fungi (d) with significant differences in relative abundance, in the rhizosphere soils of cucumber inoculated with *Streptomyces* (T) and without *Streptomyces* inoculation (CK). * $p < 0.05$; ** $p < 0.01$.

In addition, the relative abundances of bacterial genera *Arenimonas*, *R. sphaerophysae*, RB41, and *Hydrogenophaga* decreased by 39.7%, 25.7%, 42.2%, and 30.1%, respectively, following *Streptomyces* application ($p < 0.05$) compared to the control. In contrast, the relative abundances of all 11 of the other bacterial genera increased following *Streptomyces* application ($p < 0.05$; Figure 3b).

The rhizosphere fungi were classified into 4 phyla and 102 genera based on ITS gene sequences in total. The three most abundant fungal phyla were Ascomycota (33.9% of

total fungal sequences), Zygomycota (7.6%), and Basidiomycota (6.4%). Their relative abundances decreased by 44.7%, 64.9%, and 60.4%, respectively, in *Streptomyces*-treated soil ($p < 0.05$; Figure 3c). The relative abundances of the fungal genus *Emericella* increased following *Streptomyces* application ($p < 0.05$), while the abundance of the 14 other fungal genera decreased ($p < 0.05$; Figure 3d).

3.4. Increased *Streptomyces* Abundance and Enriched Bacterial Networks

After *Streptomyces* application, the relative abundances of the phylum Actinobacteria and the genus *Streptomyces* increased considerably, by 50.4% and 69.9%, respectively, in the rhizosphere soil of cucumber (t -test, $p < 0.05$; Figure 4). The microbial co-occurrence networks of bacterial and fungal genera were compared between the rhizosphere soils inoculated with *Streptomyces* and without inoculation (Figure 5; Table S1). The bacterial network of the control soil had 296 nodes and 2342 links (Table S1). In the *Streptomyces*-treated soil (Figure 5b) there were 313 nodes in the bacterial network, which was similar to the number in the bacterial network of the control (Figure 5a). However, the number of links in the bacterial network of the *Streptomyces*-treated soil increased markedly to 3536, which was 51.0% higher than the number in the control treatment. In particular, the links connected with *Streptomyces* increased from 20 to 41, which was 105.0% higher than that in the control. The fungal network in the control soil (Figure 5c) had 62 nodes and 405 links, which changed slightly to 69 nodes and 399 links in the fungal network in the *Streptomyces*-treated soil (Figure 5d).

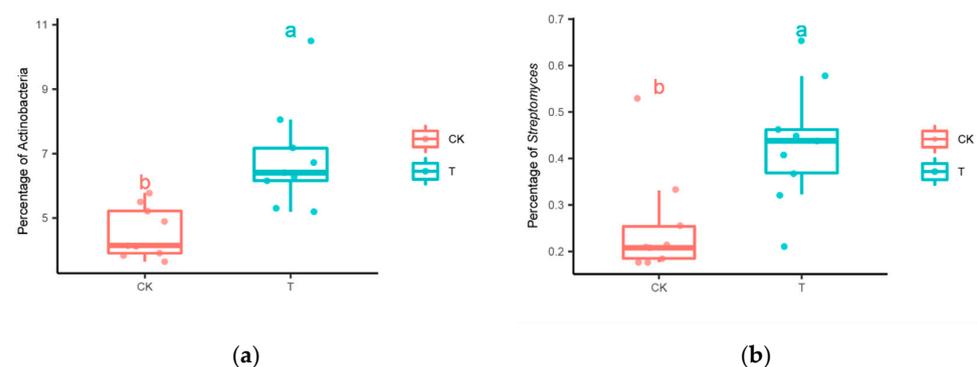


Figure 4. Variation in the relative abundance of the phylum Actinobacteria (a) and genus *Streptomyces* (b) in the rhizosphere soil of cucumber inoculated with *Streptomyces* (T) and without *Streptomyces* inoculation (CK). The horizontal bars within boxes represent the medians. The tops and bottoms of boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers extend to data no more than 1.5× the interquartile range from the upper edge and lower edge of the box, respectively. Different letters above boxes indicate significant differences at $p < 0.05$.

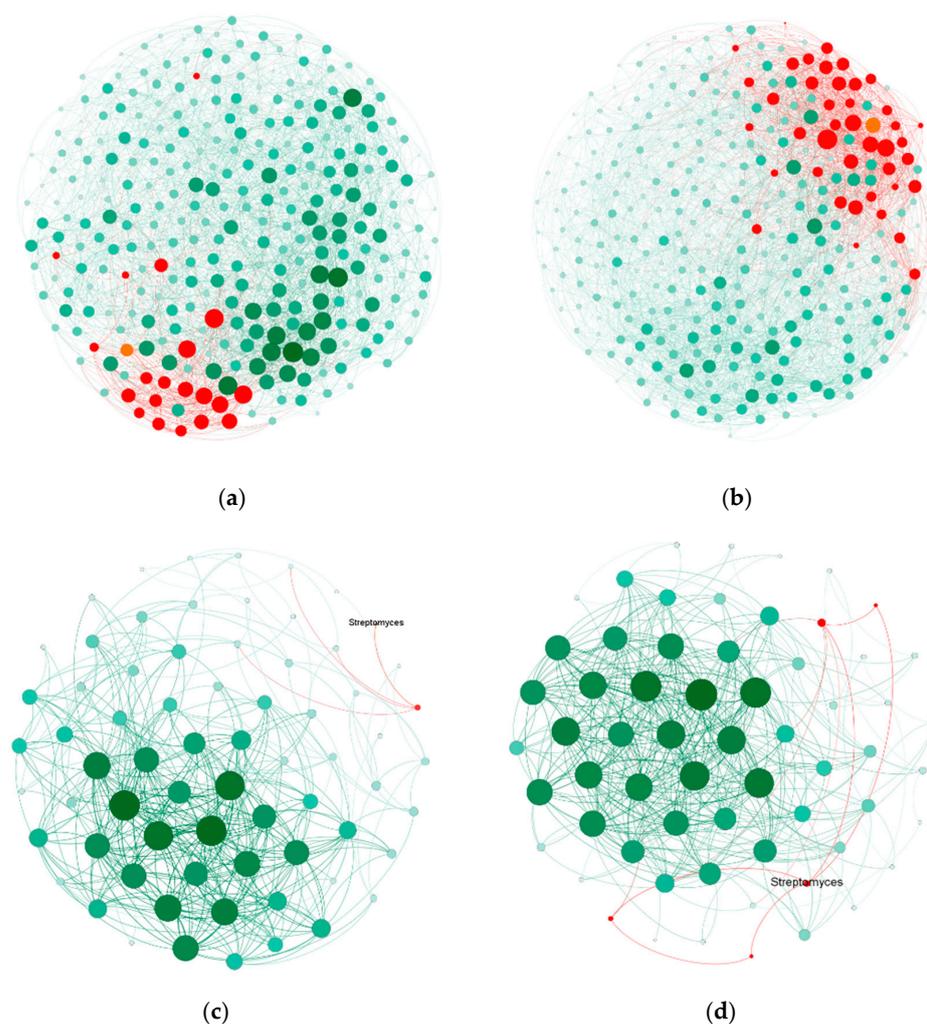


Figure 5. Co-occurrence networks of microbial genera in the rhizosphere soil of cucumber. (a) Bacterial network of the control soil; (b) bacterial network of *Streptomyces*-treated soil; (c) fungal network of the control soil; (d) fungal network of *Streptomyces*-treated soil. Orange spots refer to the genera of *Streptomyces*, red spots refer to the genera significantly correlated with *Streptomyces*, and green spots refer to other genera. Spot size indicates the strength of the connection to the genera.

3.5. Potential Functions of Rhizosphere Microbial Communities

Streptomyces application significantly altered the potential functions of the rhizosphere bacterial communities associated with cucumber plants (Figure 6). The relative abundances of bacteria associated with carbon cycling were higher in *Streptomyces*-treated soil than in the control soil (Figure 6). This was especially true for bacterial taxa associated with aromatic compound degradation, hydrocarbon degradation, and xylanolysis, which increased by 12.1%, 23.0%, and 85.1%, respectively (t -test, $p < 0.05$) after *Streptomyces* application. Furthermore, the relative abundances of bacteria associated with nitrogen fixation increased by 6.9% following *Streptomyces* application (t -test, $p < 0.05$). After *Streptomyces* application, the relative abundances of nitrification-associated bacteria increased by 39.2%, in contrast to a 2.3% reduction in the relative abundance of ammonification-associated bacteria.

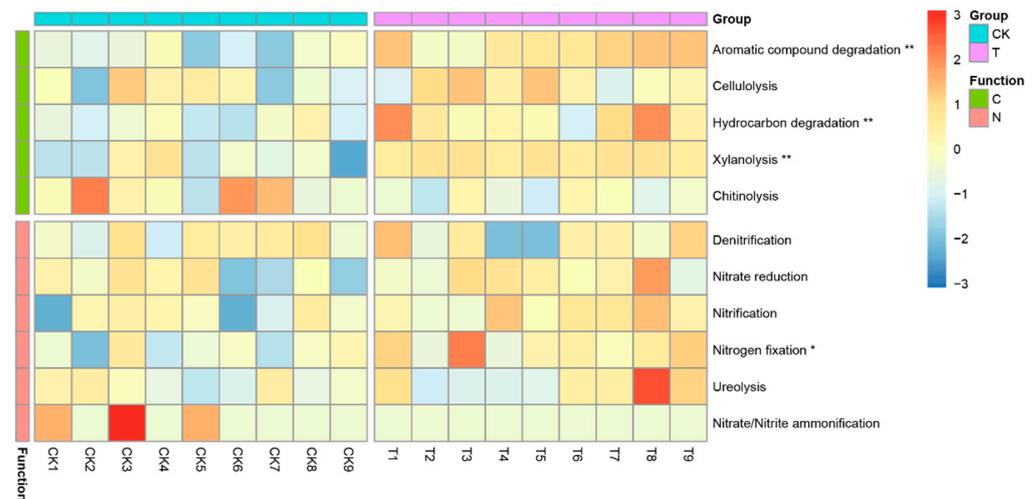


Figure 6. Heatmap of the potential functional groups of rhizosphere bacteria communities. T and the color purple represent *Streptomyces* treatment; CK and the color blue represent the control without *Streptomyces*. C and the color green represent bacteria abundance associated with the carbon cycling function; N and the color red represent bacteria abundance associated with the nitrogen cycling function. * $p < 0.05$; ** $p < 0.01$.

4. Discussion

The *Streptomyces* strains Act12 and D74 have been proved to have strong positive effects on plant growth and stress resistance in different crops, such as cucumber (Tables S2–S5), tomato [15], wheat [16], and corn [14]. Consistently, the biomass of cucumber plants was remarkably increased by the application of these two *Streptomyces* strains in the present study (Table 1). However, it remains unclear whether the effects of *Streptomyces* application on the rhizosphere microbiome contribute to plant growth. Therefore, we compared the structure and function of the rhizosphere microbiome of cucumber between soils inoculated with *Streptomyces* and those without inoculation.

4.1. Rhizosphere Microbiome Diversity and Structure Influenced by *Streptomyces* Application

In the present study, *Streptomyces* application increased the alpha diversity of the rhizosphere bacterial community but reduced the alpha diversity of the rhizosphere fungal community associated with cucumber plants. In the *Streptomyces*-treated rhizosphere soil, the increased richness index of bacteria and the reduced richness index of fungi are consistent with the findings of a previous culture-dependent study that concluded that the rhizosphere microbial community shifts from a fungal type to a bacterial type [33]. Furthermore, the beta diversity of both rhizosphere bacteria and fungi varied considerably between *Streptomyces* treatments and control treatments. The results are consistent with the findings of a previous study on monkshood [26], indicating that *Streptomyces* could considerably alter the diversity of rhizosphere microbial communities.

Among the top five bacterial phyla detected in the rhizosphere soil of cucumber, the relative abundances of phyla Proteobacteria, Bacteroidetes, and Acidobacteria decreased non-significantly following *Streptomyces* application. The relative abundances of Actinobacteria and Gemmatimonadetes increased following *Streptomyces* treatment, which could facilitate the healthy growth of plants. The phylum Actinobacteria comprises many plant-promoting rhizobacteria, including both *Streptomyces* and non-*Streptomyces* [35]. The phylum Gemmatimonadetes was reported to show an adaptation to drier soils [36], which may be correlated to plant drought resistance by *Streptomyces* application. On the other hand, Firmicutes also increased following *Streptomyces* treatment, which is consistent with a previous study that the enhanced Firmicutes was also observed when the same *Streptomyces* treatment was applied on tomato [15]. The phylum Firmicutes comprises the class Bacillus, and the function of growth promotion and disease resistance has been reported

in the genus *Bacillus aryabhatai* [37,38], *Bacillus subtilis* [39,40], and *Bacillus amyloliquefaciens* [41,42]. The enhancement of this phylum in the present study indicates that the stress resistance of the microbiota in the rhizosphere was enhanced by *Streptomyces*. Both Firmicutes and Actinobacteria comprise Gram-positive bacteria, which means that there was an increase in the abundance of Gram-positive bacteria in this study. This phenomenon may have been induced by the selection of antibiotics (unpublished results) secreted by the inoculated *Streptomyces*. All the above discussions were based on the relative abundance; the richness index of bacteria increased by 5.3% following *Streptomyces* application.

Compared with the annotation of bacteria, only 50% and 75% of fungi in the control and treatment groups were annotated at the phyla level, respectively. This may have been caused by the following reasons: (1) The fungal species are less abundant than bacterial species in the environment; usually, the true OUT or ASV is less than 500. Then, the same variation of absolute abundance would give higher variation in relative abundance in fungi than in bacteria. (2) The fungus database is not as comprehensive as the bacteria database. (3) In most pot experiments, the potted habitat is isolated with a fixed narrow space, compared with field experiments. The ecosystem stability is lower than that of field experiments, which is conducive to forming some species with high relative abundance. If the dominant species cannot be annotated, then the fungal annotation rates could be very low. Among the fungal phyla identified in the rhizosphere soil of cucumber, the relative abundances of Ascomycota, Zygomycota, and Basidiomycota decreased after *Streptomyces* application. Ascomycota includes plant-harming microorganisms such as *Myrothecium roridum*, *Monographella cucumerina*, *Humicola fuscoatra*, and *Verticillium dahlia* [26]. Such changes support the notion that plant pathogens were inhibited by *Streptomyces* application. All the above discussions were based on the relative abundance; the richness index of fungi decreased by 19.8% following *Streptomyces* application.

Compared with the previous results following monkshood treatment with a *Streptomyces* agent [26]: (i) the results of both studies support that all quantitative changes in specific bacteria and fungi in the rhizosphere soil explain the positive effects of *Streptomyces* on cucumber plants; (ii) however, in contrast to the previous study, increases in the relative abundances of Proteobacteria, Bacteroidetes, and Ascomycota were not observed in the present study. These results imply that *Streptomyces* application has a positive effect on different plants, while specific variations on the soil rhizosphere microbiome may be closely associated with the soil type or the plant type.

Microbiomes in the rhizosphere are mainly analyzed with the relative abundance, which may introduce a negative correlation bias and display spurious correlations [43]. In recent years, the more accurate methods of ANCOM and Aldex2 were increasingly used to avoid this bias from compositional data, especially in the studies for human microbiome data [43,44]. To improve the accuracy of our data analysis, the community variation induced by *Streptomyces* application was also analyzed with ANCOM in ASV level (Supplementary Figure S2). The ANCOM results showed trends similar to our analysis based on traditional relative abundance data, in both bacteria and fungi community. This phenomenon can be explained by the similar volume observed in both treatment and control samples.

4.2. Bacterial Co-Occurrence Enhanced by *Streptomyces* in the Rhizosphere

In the present study, we observed increases in the relative abundances of the phylum Actinobacteria and the genus *Streptomyces* in the *Streptomyces*-treated rhizosphere soil of cucumber when compared with those in the control. A similar result was observed in the rhizosphere of monkshood, where the colonization of strain D74 on the root surface of monkshood 7 days after inoculation was verified using the green fluorescent protein labeling technique [26]. Another study found that *Streptomyces* were predominant in wheat roots during the first weeks of inoculation [45]. Although these studies did not last as long as 3 months, their results are consistent with and support our study, where the inoculation

of *Streptomyces* increased the relative abundances of the phylum Actinobacteria and the genus *Streptomyces*.

Here, network analysis results revealed that *Streptomyces* application altered the co-occurrence patterns of bacteria in the rhizosphere soil of cucumber. Following *Streptomyces* application, as the number of links connected with *Streptomyces* increased, the total links in the bacterial co-occurrence network increased. However, changes in bacterial co-occurrence networks could have benefits related to plant growth promotion and pathogen resistance. Indeed, Li and coworkers observed that pathogen invasion was influenced by interactions (competition or facilitation) among microbial communities [46]. Therefore, the establishment of the complex bacterial network also implies that the possibility of pathogen invasion was changed. Additionally, it has been shown that *Streptomyces* can promote plant growth indirectly by stimulating the growth of other plant-beneficial microbes. For example, the application of strain Act12 stimulated an increase in the population of *P. koreensis* in the rhizosphere of ginseng [27], which was consistent with our results that the genus *Pseudomonas* was promoted by 33.82% following *Streptomyces* application. Therefore, the changes in bacterial co-occurrence networks observed here also imply altered interactions between the plant and the rhizosphere microbiome.

Moreover, to avoid the bias from compositional data, more rigorous methods were invented for the network construction, such as SParcc and SPIEC-EASI [47]. However, due to the large calculation volume, these methods are still mainly used in human data analysis, and rarely used to analyze samples from environments and soils.

4.3. Reassembled Rhizosphere Microbiome Function May Be More Conducive to Plant Survival

In the present study, *Streptomyces* application increased the relative abundances of bacteria associated with the carbon cycling function in the rhizosphere soil of cucumber, which implies that more carbon sources—in both type and amount—were provided in the rhizosphere as substrates, via which the host could acquire the capacity to reassemble a preferred rhizosphere microbiome.

With regard to nitrogen cycling, there was a remarkable increase in the relative abundance of bacteria associated with nitrogen fixation after *Streptomyces* application, which suggests that the reassembled bacterial community could import more nitrogen into the rhizosphere zone. In addition, the relative abundances of bacteria associated with nitrification and ammonification increased and reduced, respectively, following *Streptomyces* application. Such conflicting changes suggest that more nitrogen is transformed into nitrate after *Streptomyces* application, which can be more easily assimilated by plant roots. Therefore, the rhizosphere microbiome reassembled by *Streptomyces* application can facilitate the acquisition of more nitrogen (in both quantity and quality) in plant roots.

In summary, the function of the rhizosphere microbiome of cucumber was optimized by *Streptomyces* application, which offers the plants an opportunity to recruit potentially beneficial microbes and reassemble a rhizosphere microbiome that is more conducive to plant survival.

5. Conclusions

The results of the pot experiments in the present study showed that the rhizosphere microbiome diversity and structure shift remarkably after *Streptomyces* application. In particular, the abundances of rhizosphere bacteria increase, while the abundances of fungi decrease. In addition, *Streptomyces* application enriches the bacterial co-occurrence network substantially; however, the effect on the fungal co-occurrence network remains unclear. The functions of the reassembled rhizosphere microbiome are also altered by *Streptomyces* application, leading to an enhancement in the nitrogen use efficiency. Therefore, the application of *Streptomyces* could facilitate the recruitment of beneficial bacteria by cucumber plants. Furthermore, the reassembled and optimized rhizosphere microbiome is more conducive to plant survival.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/d13090413/s1>: Table S1: Major topological properties of the co-occurrence network of the microbiome in the rhizosphere of cucumber; Table S2: Biomass of cucumber after the inoculation with *Streptomyces* Act12; Table S3: Root weight and length of cucumber after the inoculation with *Streptomyces* Act12; Table S4: Root activity after the inoculation with *Streptomyces* Act12; Table S5: Leaf area and SPAD after the inoculation with *Streptomyces* Act12¹ on the 45th day; Figure S1: The rarefaction curve by each sample of the rhizosphere microbiome associated with cucumber plants in *Streptomyces*-inoculated soil (T) and uninoculated control soil (CK) *Streptomyces*. (a) The rarefaction curves of bacteria; (b) the rarefaction curves of fungi.

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