Effects of *Fritillaria Taipaiensis* Long Term Single Planting on the Bacterial Diversity and Main Bio-chemical Factors in the Rhizospheric Soils

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Abstract

Fritillaria taipaiensis is a traditional Chinese herbal plant. Germplasm degradation usually appears during its extended years of cultivation. Aiming to explore the mechanisms of the germplasm degradation, the bacterial diversities, bio-chemical characters in the rhizospheric soils of *F. taipaiensis* with different cultivation years from 1 to 5 were analyzed. The result showed the Chao1 and the ACE indices of the bacterial community had no significant difference among samples while the Shannon and Simpson indices declined with the cultivation year; the dominant bacteria phylum was Proteobacteria, the remaining phyla were Chloroflexi, Nitrospirae, Planctomycetes, Actinobacteria, Gemmatimonadetes, Firmicutes, Verrucomicrobia, Bacteroidetes, and Acidobacteria; the relative abundances of the dominant bacterial genera changed obviously among samples; the bacterial community structures in the rhizospheric soils with cultivation years of 2 and 4 were significantly different from that in other samples; the content of organic matter, available N, available P, available K and the activities of urease, alkaline phosphatase in samples decreased with the cultivation years; the soil pH increased with the cultivation years to unsuitable for *F. taipaiensis* growth; the distance-based redundancy analysis showed the pH, organic matter content, available phosphorus content and urease activity were the primary factors in determining bacterial community composition, the intra group beta diversity of the rhizospheric bacteria increased after a initial decline with the cultivation year. These results indicated long term continuous cropping changed the bacterial community structure, fertility condition and soil enzyme activities in the *F. taipaiensis* rhizosphere soils, which could badly affect its growth.

1. Introduction

Chuan BeiMu is a kind of the precious traditional Chinese herbal medicine, its bulbs contain multiple compounds such as alkaloids, steroids, terpenoids, purine and fatty acid, which can cool down body temperature, relieve coughing and asthma, and resolve phlegm [1-4]. *Fritillaria taipaiensis* P. Y. Li, which has important economic value, was collected in Chinese Pharmacopoeia as one of the medical material provenances of Chuan BeiMu in 2015 [5]. In recent years, the production of *F. taipaiensis* has been popularized in suitable areas [6-8]. However, in the actual production, the seedlings of *F. taipaiensis* may appeared the problems such as growth potential reducing [9], disease accumulation [10], and quality decline [11] after 3 to 4 years' continuous planting, which badly affected its production and quality. In response to this situation, current researches mainly focus on the fertilization control and the planting technology improvement [12-13].

Soil contains different and wide variety of microbial communities, and the interaction between soil microorganism and soil factors plays an important role in the process of soil organic matter degradation and soil nutrient cycling [14-15]. Meanwhile, the quality of soil can also be reflected by the soil enzyme activities and the microbial community composition [15-16]. Researches have demonstrated continuous cropping and long-term single plating pattern could affect the diversity and structure of the microorganism population in the planting soil [17-19], these microorganism and then could directly or indirectly affect the growth of plants by changing the chemical properties of soil [20]. In addition, the

pathogenic microorganisms accumulated during the long-term single planting can also hinder the growth of plants [21-24]. The studies on the effects of rhizospheric soil microorganisms to solve the problems of quality decline during the *F. taipaiensis* cultivation have not been deeply progressed at present.

In this study, the population structure compositions, richness and evenness of the bacteria in the rhizospheric soils, in which *F. taipaiensis* was planted for 1 to 5 years, were compared and analyzed. Moreover, we analyzed the chemical properties and enzyme activities of the rhizospheric soils with different cultivation years to explore the influence of plants and soil microorganisms on soil environment.

2. Materials And Methods

2.1 Sampling Site Location

The sampling site is a private planting base located on Lanying Village, Wuxi County, Chongqing, China (31°23'56.11" N, 109°50'29.93" E-31°35'26.57" N, 109°00'11.96" E; 2 274~2 290 m above sea level), with the annual average temperature of 7.2 °C, and the annual average precipitation of 1 100~1 300 mm, the soil in the study site was classified as mountain yellow-brown soil (fine-loamy, mixed, mesic Aridic Haplustalf) [25]. Experimental plots were located in a field established in 2008 that had been cropped continuously. The field was 20 × 20 m for the *F. taipaiensis* with each cultivation year, and the fields were natural wastelands with the same soil basement condition before planting. The fertilization of the field was N-P-K 15:15:15, 225 kg hm⁻² for each year.

2.2 Sample Collection

In May, 2017, with the permission of the planting base owner, *F. taipaiensis* rhizospheric soils (6~10 cm below the ground) with different cultivation years from 1 to 5 (Y1, Y2, Y3, Y4, Y5) were sampled from 3 sample plots (3×3 m) randomly selected in the field by multiple mixture sampling method [13]. The soils were collected by shaking root method [13] and then were placed into the aseptic bags in ice box transferred into laboratory after removed the litters and residual cover. 3 parallel soil samples were taken for each experimental sample and then were sifted by 2 mm sieve, divided into two parts, of which one was ground to be used for soil physical-chemical analysis after room temperature air drying, while the other one was stored in -80 °C ultra-low temperature freezer after freeze drying to be used in soil total DNA extraction.

2.3 Soil Enzyme Activity and Chemical Analysis

The soil enzyme activities were determined reference to the previous method [27], the invertase activity was determined by 3,5-dinitrosalicylic acid colorimetric method, expressed in terms of the glucose content in 1 g dry soil 24 h after treatment; the protease activity was determined by ninhydrin colorimetry, expressed in terms of the amino acid content in 1 g dry soil 24 h after treatment; the urease activity was determined by sodium chlorate-sodium phenol colorimetric method, expressed in terms of the amino nitrogen content in 1 g dry soil 24 h after treatment; the catalase activity was determined by pyrogallol

colorimetry, expressed in terms of the theogallin content in 1 g dry soil 20 min after treatment; both activities of acid phosphatase and alkaline phosphatase were determined by sodium phenylene phosphate colorimetric method, expressed in terms of the phenol content in 1 g dry soil 24 h after treatment.

In the determination of soil chemical indicators, pH was determined by pH analyzer, organic matter content was determined by potassium dichromate oxidation-outer heating, available phosphorus content was determined by anti-colorimetric method of molybdenum and antimony, available nitrogen content was determined by diffusion method [26], and available potassium content was determined by ammonium acetate-atomic absorption spectrometry [27].

2.4 Soil DNA Extraction and High Throughput Sequencing

0.25 g *F. taipaiensis* rhizospheric soil sample was used for DNA extraction by CTAB method [28]. The genomic DNA after diluted to 1 ng μl^{-1} was amplified by PCR via high-fidelity DNA polymerase and Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs) by using specific primer 515F (5'-GTGCCACCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') for the bacterial 16sRNA gene V4 sequence, the amplification condition was pre-denaturation at 98°C for 2 min, denaturation at 98°C for 30 s, annealing at 50°C for 30 s, extending at 72°C for 1 min, after 30 cycles, and then extending 72°C again for 5 min to end the PCR reaction. The PCR products were tested by 2% agarose gel electrophoresis and then were extracted by gel extraction kit (Qiagen). The DNA library was constructed by using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina) and then was sequenced by HiSeq2500 PE250.

2.5 Data Analysis

The data of each sample was separated from the off machine data according to the Barcode sequence and PCR amplification primer sequence, and then reads of each sample were assembled by FLASH (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>) after removed the Barcode sequence and PCR amplification primer sequence to obtain the Raw data. The Raw data were filtered and spliced to get Clean data refer to the Tags quality control process in QIIME (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html). And then, the chimeras in the Clean data were eliminated by comparing with UCHIME (Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) and database (Unite database, <https://unite.ut.ee/>) to obtain the Effective tags. The depth of sequencing was 40 000 reads at least in each original library, the operational taxonomic unit (OTU) was analyzed and clustered by using the UPARSE software (version 7.0.1090) based on 97% similarity. According to the result of OTU clustering, the representative sequence of each OTU was annotated by a species by using QIIME (version 1.9.1). The relative abundance was analyzed via Mothur (version.1.44.0) in the SS rRNA database of SILVA (<http://arb-silva.de/>)

The Mothur software (version.1.44.0) was used to calculate the Goods-coverage, Chao1, ACE, Shannon and Simpson indices. The QIIME software (version 1.9.1) was used to calculate the Unifrac distance and plot the unweighted pair-group method using arithmetic average (UPGMA) cluster tree. The R-software (version 2.15.3) was used to plot the Rank abundance curve. Meanwhile, the relative abundance, the Alpha diversity of OTUs was analyzed to get the information such as the specific species abundance in one sample, the common and the specific OTUs among the different samples and groups by Tukey-test. Non-Metric Multi-Dimensional Scaling (NMDS) analysis, distance-based redundancy analysis (db-RDA) and permutational MANOVA analysis (Adonis) were performed by vegan bag on R-software.

The data were analyzed by single factor (one-way AMOVA) and Duncan for variance analysis and multiple comparison via SPSS 22.0 and Excel 2003 ($\alpha=0.05$).

3. Results

3.1 Alpha Diversity Analysis

The Rank abundance curves (Fig 1) which were drawn according to the relative abundance of the OTUs showed: the curve of sample Y2 had the maximum abscissa span, which indicated that the *F. taipaiensis* rhizospheric soil with 2 years of cultivation had the maximum bacterial species richness; and the curve of sample Y1 had the softest changing trend in ordinate which indicated the *F. taipaiensis* rhizospheric soil with 1 year of cultivation had the maximum bacterial species evenness; the curve of sample Y4 had the minimum abscissa span and the maxim ordinate changing trend which indicated the *F. taipaiensis* rhizospheric soil with 4 years of cultivation had the minimum bacterial species richness and evenness.

As shown in Table 1: Goods_coverage showed the species coverage detected in each sample was above 97%; there was no significant difference in the Chao1 and ACE indices between the samples, which reflected the species richness of the samples with different cultivation years had no significant change; meanwhile, the Shannon and Simpson indices of the samples showed a trend of declining with the cultivation year, which indicated the reduction of bacterial community evenness, and the dominant species gradually concentrated in some specific species.

In the horizontal direction, the wider the abscissa span, the higher the species richness; in the vertical direction, the smoother the curve, the higher the species evenness.

Table 1. Alpha diversity indices

Sample	Goods_coverage	Chao1	ACE	Shannon	Simpson
Y1	0.978 a	4807.988 a	4849.949 a	10.199 a	0.998 a
Y2	0.976 a	4870.574 a	5021.414 a	9.934 ab	0.996 ab
Y3	0.978 a	4354.877 a	4565.658 a	9.505 b	0.995 b
Y4	0.975 a	4530.162 a	4793.504 a	8.944 c	0.985 c
Y5	0.975 a	4987.040 a	5117.301 a	9.678 b	0.993 b

Different lower-case letters indicate significant differences ($P < 0.05$) among the six treatments, the same as follow. Goods_coverage: indicator reflects the depth of sequencing, the closer the value is to 1, the more reasonable the sequencing depth, indicating that the sequencing depth has covered all species in the sample; Chao1: provides an estimation of the actual number of OTUs in the sample as an indicator of species richness, the higher value means higher species richness; ACE: an indicator of species richness, the higher value means higher species richness, with a different algorithm to Chao1; Shannon: an index quantifies the entropy of the distribution, which depends on the evenness of the proportional abundances of OTUs in the population, the higher value means higher species evenness; Simpson: an indicator of species evenness, the higher value means higher species evenness with a different algorithm to Shannon.

3.2 Beta Diversity Analysis of the Bacterial Communities in the Rhizospheric Soils

As shown in Fig 2, the bacterial phyla with the top 10 relative abundances in the *F. taipaiensis* rhizospheric soils with different cultivation years were Proteobacteria (34.16%~54.82%), Acidobacteria (10.19%~18.16%), Bacteroidetes (9.49%~13.75%), Verrucomicrobia (4.91%~7.21%), Firmicutes (0.18%~6.05%), Gemmatimonadetes (2.87%~5.93%), Actinobacteria (1.51%~4.55%), Planctomycetes (2.05%~3.06%), Nitrospirae (1.01%~2.48%) and Chloroflexi (0.96%~1.83%). The dominant phylum was Proteobacteria which had a rising relative abundance from Y1 to Y4 with the cultivation year. And the relative abundance of Firmicutes, Actinobacteria and Chloroflexi had a downward changing trend from Y1 to Y4, in which the relative abundance of Firmicutes had great variation among samples, declined from 6.05% in Y1 to 0.65% in Y3. Meanwhile, the samples were clustered by unweighted pair group method with arithmetic means (UPGMA). Samples Y4 and Y5 could be grouped together but did not belong to the same cluster with samples Y1, Y2 and Y3, which revealed that the samples with 4 and 5 years of cultivation had the similar bacterial community structures but were different from the samples with 1, 2, and 3 years of cultivation. The samples Y1, Y2, and Y3 could be grouped in a large cluster, in which the samples with 1 and 3 years of cultivation could be further grouped in a smaller cluster.

Different lower-case letters indicate significant differences ($P < 0.05$) among the six treatments, the same as follow.

The bacterial genera with the top 15 relative abundances in the *F. taipaiensis* rhizospheric soil were *Methylothera*, *Pseudomonas*, *Lactobacillus*, *Gemmatimonas*, *Ralstonia*, *Bryobacter*, *Sphingobium*,

Flavobacterium, *Collimonas*, *Sphingomonas*, *Candidatus*, *Rhizomicrobium*, *Pedobacter*, *Bacteroides* and unculturable bacterium RB41 (Table 2). The relative abundances of *Methylothera*, *Pseudomonas* and *Sphingobium* showed a tendency of ascending with the cultivation year, the relative abundance of *Methylothera* in Y4 was 16.07 times of that in Y1, and the relative abundance of *Pseudomonas* and *Sphingobium* in Y5 was 5.21 and 4.15 times of that in Y1, respectively. The relative abundances of *Lactobacillus*, *Gemmatimonas*, *Bryobacter* and *Bacteroides* declined with the cultivation year, the relative abundance of *Gemmatimonas*, *Bryobacter* and *Bacteroides* in Y5 declined by 4.67, 2.29, and 99 times compared with that in Y1, respectively, and the relative abundances of *Lactobacillus* in Y4 and Y5 were absent. The relative abundances of *Flavobacterium*, *Collimonas* and *Pedobacter* showed a downward trend after a initial rising, the relative abundance of *Flavobacterium* in Y4 and the relative abundance of *Collimonas* in Y3 was 5.89 and 12 times of that in Y1, respectively. While in Y5, the relative abundances of these 2 genera declined significantly when compared with those in Y3 and Y4, respectively. The relative abundances of *Pedobacter* had no obvious change in Y1, Y2 and Y3 but increased suddenly to a high level in Y4 and then declined obviously in Y5. Meanwhile, the relative abundances of *Candidatus* also had no significant change in Y1, Y2 and Y3 but declined obviously in Y4 and Y5. All the result above showed that *F. taipaiensis* rhizospheric soils with different cultivation years had significant difference in the dominate bacteria community on the genus level.

Table 2. Relative abundance (%) of bacterial genera in the samples with different cultivation years

Genus	Relative Abundance (%)				
	Y1	Y2	Y3	Y4	Y5
<i>Methylothera</i>	0.61 c	2.78 b	2.89 b	8.90 a	3.98 b
<i>Pseudomonas</i>	0.92 c	2.13 bc	2.61b	2.18 bc	4.79 a
<i>Lactobacillus</i>	2.68 a	1.40 ab	0.78 b	0.00 b	0.00 b
<i>Gemmatimonas</i>	2.52 a	0.92 b	0.90 b	0.37 b	0.54 b
<i>Ralstonia</i>	1.26 b	1.26 b	1.01 b	2.39 a	1.98 a
<i>Bryobacter</i>	2.24 a	2.29 a	2.11 a	0.63 c	0.98 b
<i>Sphingobium</i>	0.53 b	1.75 a	1.73 a	2.04 a	2.20 a
<i>Flavobacterium</i>	0.35 c	0.32 c	1.13 b	2.06 a	0.62 bc
<i>Collimonas</i>	0.17 b	0.39 b	2.04 a	0.26 b	0.22 b
<i>Sphingomonas</i>	1.06 bc	1.70 a	1.41 ab	1.00 c	1.23 bc
<i>RB41</i>	1.09 b	0.67 c	0.64 c	0.72 bc	1.66 a
<i>Candidatus</i>	1.42 a	1.50 a	1.48 a	0.43 b	0.75 b
<i>Rhizomicrobium</i>	1.31 a	1.10 a	1.40 a	0.24 c	0.67 b
<i>Pedobacter</i>	0.33 a	0.39 a	0.37 a	1.24 b	0.39 a
<i>Bacteroides</i>	0.99 a	0.61 a	0.23 b	0.01 c	0.01 c

The heat map of the bacterial genera with the top 35 relative abundances in each sample showed a significant difference between the dominate bacterial communities in the *F. taipaiensis* rhizospheric soils with different cultivation years (Fig 3A). As shown in the Venn diagram, the samples with different cultivation years shared 2 928 common OTUs, the number of the unique OTUs in each sample from Y1 to Y5 was 339, 490, 213, 281 and 332, respectively (Fig 3B). It indicated that the sample Y2 was greatly different with the other samples in bacterial community structures.

On the left side of (A) is species clustering tree. The values corresponding to the heat map are Z-values obtained by standardizing the relative abundances of the species in each row. The Z-value of a sample in a certain classification means the difference between the relative abundance of the sample and the average relative abundance of all samples divided by the standard deviation of all samples in the classification.

3.3 NMDS and Adonis Analysis

As shown in Fig 4, there was a certain coincidence in the distribution ranges of the points representing sample Y1 and Y3. However, the points representing these two samples were far from the other samples,

indicating the rhizospheric bacterial communities of Y1 and Y3 were similar but were significantly different from the others. The above result was similar to that of UPGMA clustering. The further analysis by Adonis showed there were significant differences in rhizospheric bacterial communities between Y1 and Y2, Y2 and Y3, Y1 and Y5, Y3 and Y5 ($p=0.001$) (Table 3).

The differences between the samples or the groups were expressed by the distance in figure, and the nearer distance showed the more similar bacterial community structure between the two samples. NMDS can accurately reflect the difference between samples when Stress < 0.2.

Table 3. Adonis analysis between groups

Vs Groups	Df	Sums of Sqs	Mean Sqs	F. Model	R ²	<i>p</i>
Y1/Y2	1	0.128	0.128	3.454	0.463	0.001
Y1/Y3	1	0.157	0.157	4.589	0.534	0.100
Y1/Y4	1	0.479	0.479	15.058	0.790	0.100
Y1/Y5	1	0.279	0.279	7.781	0.660	0.001
Y2/Y3	1	0.079	0.079	2.557	0.390	0.001
Y2/Y4	1	0.324	0.324	11.478	0.742	0.100
Y2/Y5	1	0.192	0.192	5.941	0.598	0.100
Y3/Y4	1	0.358	0.358	14.044	0.778	0.100
Y3/Y5	1	0.212	0.212	7.208	0.643	0.001
Y4/Y5	1	0.142	0.142	5.255	0.568	0.100

Df: Degree of freedom; Sums of Sqs: Total variance; Mean Sqs: Mean square, Sums of Sqs/Df; F. Model: F inspection value ; R²: The explanation degree of the differences among different groups of samples, i.e. the ratio of grouping variance to total variance, the larger R² means the higher the explanation of the difference; *p*: $p < 0.05$ indicates that the reliability of the test is high.

3.4 Soil Bio-chemical Analysis

As shown in Table 4, the pH of the rhizospheric soils decreased after a initial increasing with the cultivation year. The pH turned from weak acidity in sample Y1 to weak alkalinity in sample Y4 gradually, and then declined slightly in sample Y5. The organic matter content in the samples declined with the cultivation year, indicating the microorganism in the *F. taipaiensis* rhizospheric soil dissolved the organic matters, and the organic matter had not been effectively supplemented. The content of available phosphorus showed a reduction with the cultivation year, which corresponded to the gradual decrease of phosphatase activity. The available potassium content in samples varied greatly with the cultivation year. The content of available nitrogen in the sample Y2 was significantly higher than that in sample Y1, and

then declined with the cultivation year, which had a similar changing trend with the urease activity. The protease activities in the Y1, Y2 and Y3 samples had no significant change, and then rose in the Y4 and Y5 samples. The invertase activities in the samples Y2 and Y3 were lower than that in the sample Y1, but then apparently increased in the samples Y4 and Y5. The catalase activity in the samples also increased after an initially decrease with the cultivation year.

Table 4. Bio-chemical analysis along five years of *F. taipaiensis* cultivation

Enzyme activity/Content/pH	Samples				
	Y1	Y2	Y3	Y4	Y5
pH	6.43 c	6.50 c	6.52 c	7.47 a	6.85 b
Enzyme activity/Content	g kg ⁻¹				
Acid phosphatase	0.739 a	0.588 ab	0.484 b	0.452 b	0.694 a
Alkaline phosphatase	0.139 a	0.112 b	0.103 bc	0.089 c	0.066 d
Catalase	0.685 a	0.551 b	0.539 b	0.688 a	0.680 a
Invertase	12.826 c	9.569 d	9.250 d	21.783 b	28.959 a
Organic matter	36.049 a	28.836 b	26.135 b	21.434 c	21.334 c
Protease	0.291 c	0.281 c	0.288 c	0.361 b	0.480 a
Urease	5.418 ab	5.673 a	5.246 ab	4.399 c	4.743 bc
	mg kg ⁻¹				
Available nitrogen	113.012 b	135.315 a	114.056 b	81.324 c	79.224 c
Available phosphorus	150.478 a	96.490 b	97.089 b	44.728 c	39.425 d
Available potassium	405.865 a	174.810 d	210.419 c	169.305 d	302.991 b

Relationship Between the Rhizospheric Bacterial Community and the Soil Bio-chemical Factors

The results of the db-RDA showed the effect of the soil bio-chemical factors on the bacterial community in the *F. taipaiensis* rhizospheric soils with different cultivation years explained 68.36% of the total characteristic value (Fig 5 A). Further analysis showed that the pH ($R^2=0.9005$, $p=0.001$), the urease activity ($R^2=0.7792$, $p=0.001$), the available phosphorus content ($R^2=0.7890$, $p=0.001$) and the organic matter content ($R^2=0.8564$, $p=0.001$) were the dominate factors to affect the rhizospheric soil bacterial community structure, among which the pH was the most influential factor (Supplementary Table S1). The average distance from the point to the center in each group of the PCoA ranking result based on the Bray-curtis distance matrix could represent the beta diversity within group. And the beta diversity of the bacterial community within group showed an upward trend after an initial decrease with the cultivation year (Fig 5 B).

4. Discussion

Previous study has shown that agricultural planting could influence the relative abundances of some specific bacterial communities in the soil which obviously alter the evenness of the bacterial communities in the soil, while the bacterial community richness would be less affected [20]. In this study, the Chao1 and ACE indices which reflect the species richness had no significant change with the cultivation years while the Shannon and Simpson indices which reflect the species evenness declined with the cultivation year could support this phenomenon. The Rank abundances curves also showed the cultivation year could change the species richness and evenness of the bacterial community in the *F. taipaiensis* rhizospheric soil. The artificial disturbance during the planting changed the physical and chemical properties of the *F. taipaiensis* rhizospheric soil. Some studies have shown that the long term fertilization could limit the randomness of the microbial community succession in the cultivated soil and make the soil to be only suitable for the survival of certain kinds of bacteria. As a result, the microbial community structures in the planting soils gradually converged [29]. Meanwhile, the plants can shape their rhizospheric microbiome structure by the root exudates during the growth, and then directionally change the rhizospheric microbial community structure [30]. The downward trend of the intra group beta diversity of the bacterial community in from sample Y1 to sample Y4 could confirm this phenomenon. But the lower intra group beta diversity of the bacterial community in sample Y4 compared with that in sample Y3 may caused by the change of pH. In this study, the pH of sample Y4 was significantly higher than that of sample Y3, and the pH was also the most dominate factor to effect the bacterial community structure in *F. taipaiensis* rhizospheric soil. Studies have shown that the bacterial community diversity in the soil was positively correlated to the pH [31], and the changing trend of the pH from sample Y3 to sample Y5 was consistent with changing trend of intra beta diversity, which was consistent with this result. Both the result of UPGMA and NMDS showed that the bacterial community structure in *F. taipaiensis* rhizospheric soil changed gradually with the cultivation year, the bacterial community structures in the soils with the adjacent cultivation years were closer to each other in clustering. While with the increasing of cultivation year, the distance between samples were enlarged gradually. Meanwhile, the result of Adonis showed there were significant differences between samples Y1 and Y2, Y2 and Y3 and Y3 and Y5, which was consistent with the results of UPGMA and NMDS. However, although the explanation degrees of the differences between Y1 and Y3, Y1 and Y4, Y2 and Y4, Y2 and Y5, Y3 and Y4, and Y4 and Y5 were relatively high, the differences were not significant yet, the reason may due to the sampling method or the internal bacterial community diversity difference in a same sampling field, which needs to be further improved in the future research.

Among the genera with the top 15 relative abundances, the relative abundances of *Methylobacter*, *Pseudomonas*, and *Sphingobium* had an upward trend with the cultivation year. *Methylobacter* and *Pseudomonas* can absorb and fix CO₂ from the air [32-33], so they can continue to grow under the condition of decreasing organic matter content. *Sphingobium* is resistant to a variety of pesticides and herbicides [34-35] and can degrade various macromolecular organics as its nutrition [36], its increasing relative abundance may intensify the consumption of soil organic matter. *Flavobacterium* is a kind of

important soil saprophytic bacteria, the ammonia produced by the process of its organic matter degradation may have toxic effects on the *F. taipaiensis* [37]. In addition, the ammonia produced by *Flavobacterium* can alkalize the soil, the increasing trend of pH from Y1 to Y4 may also associated to the increasing of *Flavobacterium* relative abundance. The suitable soil environment for the *Fritillaria's* growth is weakly acidic (pH = 6.66) [38], therefore, in the actual planting, the rising soil pH may cause the growth and quality decline of *F. taipaiensis*. The metabolic process of *Ralstonia* can produce alkaline substances that could rise the soil pH [39]. The relative abundance of *Ralstonia* in sample Y4 was obviously higher than those in Y1, Y2 and Y3, and The pH of Y4 was 7.47, the weak alkaline environment can also associated to the increasing of *Ralstonia*, which can badly affect the growth of *F. taipaiensis*. In addition, the relative abundance of *Lactobacillus* which can produce lactic acid, gradually declined to 0% in Y4 and Y5, that process hastened the rising of soil pH. *Bryobacter*, RB41 and *Candidatus* were affiliated to Acidobacteria, the bacteria of this phylum are generally negatively correlated with soil pH [40-41], the decline on the relative abundance of these 3 genera may also associate to the soil pH rising. The significant higher protease activities in Y4 and Y5 compared with those in Y1, Y2, and Y3 may corresponded to the increase of *Collimonas* in Y3, for the *Collimonas's* metabolites had high protease activities [42]. Previous studies showed that when the relative abundance of plant pathogenic fungi *Fusarium* rose, the the growth of *Gemmatimonas* could be seriously inhibited, and the relative abundance of *Pseudomonas* would rise [43]. Therefore, in this study, the decline of the *Gemmatimonas's* relative abundance and the increase of the *Pseudomonas's* relative abundance may corresponded to the accumulation of pathogenic fungi with the cultivation years.

Previous studies have confirmed that pH is one of the most important factors affecting the soil bacterial community structure [44-47]. In this study, the explanation degree of pH exceeded 90%, which was the most significant among the physical and chemical factors affecting the soil bacterial community structure. However, previous studies have shown that, with the increase of continuous cropping years, the pH in the planting soil would gradually decrease, resulting in soil acidification [44-49]. Therefore, the rhizospheric soil pH of *F. taipaiensis* increased with the increase of planting years, which provided a special case. This result improved the soil quality from the side and provided favorable growth conditions for potential rotation plants after harvest. Some studies found that the relative abundances of bacteria in the cultivated soil would decline while the relative abundance of fungi would rise with the cultivation years' increase and the soil microbial population type would turn to fungal type from bacterial type [50-52]. And the total amount of culturable bacteria, the number of phosphorus solubilizing bacteria and the ratio of bacteria/fungi in the rhizospheric soil of *F. taipaiensis* would show a downward trend with the increase of cultivation year [10]. Moreover, he bacteria that declined in the relative abundance include various kind of nitrogen fixing bacteria, potash dissolving bacteria and phosphate dissolving bacteria [51]. Thus, the above conclusion explained the reasons for the decline of the contents of available nitrogen, available phosphorus and available potassium and the activity of alkaline phosphatase in the rhizospheric soil pH of *F. taipaiensis* in this study. Zhang et al [53] found that the soil organic matter content was positively correlated with the activities of urease, acid phosphatase and invertase. Therefore, in our study, the urease activity in the samples showed a downward trend with the increase of cultivation

year, which was consistent with that of the organic matter content. In addition, both of the organic matter content and the urease activity were the important factors affecting the soil bacterial community structure, in which the urease can convert the urea in soil into the available nitrogen [53] and can catalytic the mineralization of the organic phosphorus [54], provide nutrients for plants. The decline of urease activity not only affected the structure of soil microbial community, but also affected the growth of *F. taipaiensis*. The acid phosphatase activity in samples showed a trend of increasing after a initial decline which was contrary to the changing trend of pH, that was corresponding to the previous research [55]. Meanwhile, The reason for the invertase activities' increase in the samples Y4 and Y5 after a initial downward trend may due to the accumulation of some saprophytic fungi which were also important plant pathogen [51]. It is difficult to explain the reason for the catalase activity showed a downward trend from Y1 to Y3 and then an upward trend from Y3 to Y5, however, in our previous study [25], it may due to the accumulation of heavy metals in soil during the process of cultivation.

Moreover, in the actual production, the cultivation of *F. taipaiensis* was mainly began as seed sowing, which usually for 5 years from germination to harvest [56]. And because of this long-term single planting pattern, the plant would release large amount of secondary metabolites and litter falls into the soil which would have toxic effects on the plants and affect the soil microbial structure [57-60].

Conclusion

In summary, long-term single planting pattern cause the obvious change of the bacterial community structure in the *F. taipaiensis* rhizospheric soil, the richness of the soil bacterial community rose while the evenness declined, the relative abundance of the beneficial bacteria decreased and the pathogenic microorganisms accumulated. The decrease of the available nitrogen, available phosphorus and organic matters impoverished the soil. This study can provide a scientific basis for solving the germplasm decline problem appeared in the production of *F. taipaiensis* by combining with reasonable rotation, application of organic fertilizer, development of biological fertilizer and control of harmful pathogens.

Declarations

Data availability

All obtained sequences data and the raw data of the analysis can be downloaded at <https://pan.baidu.com/s/1U21EuYv34yY51LWRAEv6KQ> by the download password of cbrm.

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Competing interests

The authors declare no competing interests.

Author contributions

Nong Zhou conceived the experiment. You Zhou, Maojun Mu and Dongqin Guo conducted the experiment. You Zhou, Hua Zhang and Nong Zhou analyzed the results. You Zhou wrote the manuscript. Nong Zhou, Xiaohong Yang and Min Yang reviewed the manuscript.

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Figures

Figure 1

Rank abundance curve.

Figure 2

UPGMA dendrogram and relative abundance of main bacterial phyla across sampling over years

Figure 3

(A) Heat map showing the relative abundance of the top 35 most abundant bacterial genera in the samples over years and (B) Venn diagram of the OTUs in the samples over years at 97% nucleotide sequence identity similarity, showing the common and specific OTU numbers in the samples.

Figure 4

Non-Metric Multi-Dimensional Scaling (NMDS) analysis.

Figure 5

(A) db-RDA on bacterial communities and soil bio-chemical factors and (B) Intra group beta diversity bacterial communities in the rhizospheric soils of *F. taipaienses* with different cultivation years

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