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Study on Bacterial Diversity and Community Structure in Grape Rhizosphere Soil Based on High-throughput Sequencing

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Abstract:

In this study, we used the rhizosphere soil of Crimson seedless grape vines with large planting area in Shihezi, Xinjiang as research material, sequenced the bacterial 16S rRNA gene (V4) in different depths of the soil with the grape vines planted for 5, 8, 10, 12 and 15 years by using Illumina MiSeq sequencing platform, and analyzed the diversity and community structure of soil bacteria through bioinformatics related methods. The results showed that 196,690 OTUs were obtained from 45 grape vine rhizosphere soil samples, and the dominant bacterial phyla in the rhizosphere soil were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes* and *Actinobacteria*, and the main dominant bacterial genera were *Bacteroides*, *Sphingomonas*, *Prevotella_9*, *Lactobacillus* and *Enterococcus*. Redundancy analysis (RDA) showed that soil total potassium and total phosphorus had the greatest influence on bacterial community structure. Correlation analysis showed that all dominant bacterial communities, except for *Actinobacteria,* were significantly related to soil physicochemical properties. Alpha diversity analysis showed that in 15-25cm deep soil, Shannon index, Chao1 and ACE indices were the

highest, indicating that layer had the highest bacterial diversity and community richness; Chao1 and ACE indices of 8-year-old vine group were the highest, indicating that group had the highest bacterial community richness; so, grape vine planting years and soil depth had some influence on the diversity and richness of bacterial communities. Beta diversity and PCoA (principal coordinates analysis) analysis showed that bacterial community structure presented significant difference between different samples. Clustering results also showed that soil depth had some influence on bacterial community structure. Linear discriminant analysis suggested that the 15-year-old vine group had the highest level of biomarkers and classification, and the 15-year-old vine group also had more unique bacterial community (biomarkers) than other age groups, especially in the lower soil layer (25-35cm).

Keywords: grape; rhizosphere soil; high-throughput sequencing; bacterial diversity.

Introduction

Xinjiang has a long history of grape cultivation, and it is the earliest region where grapes are planted and wine is brewed in China. The "golden" region recognized worldwide for grape growth lies between 30° and 40° north latitude, while Xinjiang lies between 73°40′ - 96°18′ east longitude and 34°25′ - 48°10′ north latitude, so the vast majority of Xinjiang is located in the golden region for grape growth. Because of its unique ecological and climatic conditions, Xinjiang is rich in high-quality grapes, which are excellent for eating directly, making raisin or making wine. As the key factor determining the value of grape products, grape quality is closely related to many ecological factors and planting techniques, and ecological factors can directly affect the diversity of soil microbial community. Soil is the basic guarantee for human survival, the "converter" of nutrient elements, the "purifier" of toxic substances and the "regulator" of ecosystem, while soil microorganisms play a leading role in these processes. The study of soil microorganisms is helpful to clarify the response of soil microorganisms to environmental changes and deepen the understanding of the relationship between aboveground and underground parts in terrestrial ecosystems[1]. Most microorganisms living in soil are bacteria, which play a key role in the growth of plants. Soil microbial diversity plays an important role in maintaining the dynamic balance of soil ecosystem and promoting the absorption and utilization of root nutrition. In turn, vines planting affect the diversity and structure of soil microorganisms by changing the soil environment[2]. As a perennial liana,

grape vines cannot be cleared of and re-planted every year, and their fertilization and irrigation methods are limited, so the microbial diversity in grape growth soil is significantly affected[3]. Grape growth, quality formation and immunity improvement are closely related to the community structure and quantity of microorganisms in the habitat, especially in the rhizosphere. Up to now, there are relatively few studies on bacterial diversity and colony structure in vineyard soil. Therefore, the study of bacterial diversity and community structure in vineyard soil has practical guiding significance for rationally formulating soil management measures, promoting the optimization of rhizosphere microbial structure, and achieving high yield and high quality of grapes[4]. This study aims to compare the rhizosphere bacteria of Crimson seedless vines of different ages (5 years, 8 years, 10 years, 12 years, and15 years) with large planting areas in Xinjiang, and to explore the state and diversity of microbial community structure in grape rhizosphere, so as to provide reasonable guidance for vineyard management, soil fertility improvement, yield increase, and quality enhancement.

1 Materials and methods

1.1 Location

Soil samples were taken on August 15, 2018 in the experimental base of grape planting in Shihezi Grape Research Institute, Xinjiang. The shortest age of grape vines was 5 years and the longest was 15 years. The geographical coordinates of sample taking places were 44°17′ - 44°35′ north latitude, 86°11′ - 86°20′ east longitude, and the altitude was 480m. The annual average temperature is 6.5 - 7.2℃, the annual precipitation is 125.0 - 207.7mm, and the annual sunshine hours are 2721 - 2818 h. The vineyard soil type is sandy loam, and the main cultivated variety is Crimson seedless grapes for fresh eating.

1.2 Sample collection

From the same variety (seedless Crimson) of grape plants with different ages (5, 8, 10, 12 and 15 years), three plants were randomly selected for each age group. Around 20 cm from each grape plant, three points were selected take soil samples. After removing debris about 5cm from the surface, the upper layer (5-15cm), middle layer (15-15 cm) and lower layer (25-35cm) of soil were taken with earth boring augers at each point. With disposable sterilized rubber gloves, the soil was manually mixed, subpackaged and marked to make it into soil samples (about 500 g each) through quartering method. Each sample was divided into two parts, which were immediately put into two sterilized self-sealing bags with labels denoting

the sampling time, place, soil depth and serial number. The samples were coded with a two-digit system, where the first digit indicated the replicate number and the second indicated the depth (1, 2 and 3 denoted the depth of 15, 25 and 35 cm, respectively). There were 5 age groups of grape vines, each group had 3 plants, and for each plant the soil was taken at three depths; so, in total there were 45 soil samples (5 x 3 x3). The fresh soil was immediately screened by 20 mesh sieve and divided into two parts, in which one was stored with liquid nitrogen and sent to Beijing Compass Biotechnology Co., Ltd for high-throughput sequencing, and the other was air-dried and stored for determining the physicochemical properties of soil. (See table 1 for sample coding information)

Table 1 Sample coding information

Sample code			Soil depth	Age/year	Latitude	longitude	Elevation	Remarks
GM1.1	GM1.2	GM1.3	5-15cm					
GM2.1	GM2.2	GM2.3	15-25cm	15				
GM3.1	GM3.2	GM3.3	25-35cm					
HY1.1	HY1.2	HY1.3	5-15cm					There were 5 age groups of grape vines, each group had 3 plants, and for each plant the soil was taken at three depths; so, in total there were 45 soil samples (5 x 3 x3).
HY2.1	HY2.2	HY2.3	15-25cm	10	North	East		
HY3.1	HY3.2	HY3.3	25-35cm					
XZ1.1	XZ1.2	XZ1.3	5-15cm					
XZ2.1	XZ2.2	XZ2.3	15-25cm	8			480m	
XZ3.1	XZ3.2	XZ3.3	25-35cm					
ZN1.1	ZN1.2	ZN1.3	5-15cm		44°17′ - 44°35′	86°11′ - 86°20′		
ZN2.1	ZN2.2	ZN2.3	15-25cm	5				
ZN3.1	ZN3.2	ZN3.3	25-35cm					
WH1.1	WH1.2	WH1.3	5-15cm					
WH2.1	WH2.2	WH2.3	15-25cm	12				
WH3.1	WH3.2	WH3.3	25-35cm					

1.3 Determination of soil physicochemical properties properties

The air-dried soil samples were tested for the following physicochemical indices: organic matter (SOM), total nitrogen (TN), total phosphorus (TP), total potassium (TK), nitrate nitrogen (XN), ammonium nitrogen (AN), available phosphorus (SP), available potassium (SK), pH value (1:5) and electrical conductivity (EC). The soil properties were determined in

accordance with the methods in the textbook *Soil Agrochemical Analysis* compiled Bao Shidan[5]. The 3 samples taken from the same layer of soil around each grape plant were mixed into one sample to determine the physicochemical properties. There were 5 ages groups, and for each group the soil involved 3 depths, so there were 15 soil samples were determined for physicochemical properties. The soil physicochemical indices were analyzed using SPSS Statistics 22.0.

1.4 Bacterial 16S rRNA gene sequencing

1.4.1 DNA extraction and PCR amplification of 16s rRNA gene

Soil genomic DNA extraction kit (centrifugal column type) was used for DNA extraction of the samples by CTAB method. Then, the purity and concentration of DNA were detected with 1% agarose gel electrophoresis. A proper amount of sample DNA was taken in a centrifuge tube, where the sample was diluted to 1 ng/μl with sterile water. Using diluted genomic DNA as template, specific primers with Barcode were selected according to the sequencing region to be used; Phusion® high-fidelity PCR master mix with GC buffer (New England Biolabs) was used together with NEB high-efficiency and high-fidelity enzymes to carry out PCR, so as to ensure the amplification efficiency and accuracy. The 16S rRNA gene (V4) was amplified with 515F-806R (5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-GGACTACHVGGGTWTCTAAT-3'), and compared with the Barcode. According to the concentration of PCR products, the samples were mixed up for isoconcentration, and then the PCR products were purified by electrophoresis with 1×TAE buffer and 2% agarose gel. The sequences with main bands between 400-450 bp were selected, and the target bands were recovered by tapping. The product purification kit we used was GeneJET gel recovery kit (Thermo Scientific). Ion plus fragment library kit 48rxns (Thermofisher) was used to construct the library, which was then quantified and qualified by Qubit, and finally sequencing was carried out with Ion S5TMXL (Thermofisher).

1.4.2 Biological information analysis

a. sequencing data processing

Cutadapt(V1.9.1)[6] was used to cut off the low-quality part of the reads; then, based on the reads obtained from Barcode, the data for each sample were separated. After cutting off Barcode and the primer sequences, raw reads were obtained. The raw reads were then detected with UCHIME Algorithm[7] and compared with gene annotation database to remove chimeric sequences[8], so as to get the final clean reads.

b. OTU clustering and annotation

Sequence clustering was carried out to all clean reads of the samples by using Uparse v7.0.1001[9] at the default identity of 97% to obtain OTUs (Operational Taxonomic Units). Representative sequences were selected for the OTUs to carry out specie annotation; then, specie annotation analysis was done by using Mothur method and SILVA[10] SSUrRNA database[11] to obtain taxonomic information, and the community composition of each sample was analyzed at each classification level. Using MUSCLE[12] (Version 3.8.31) software, the phylogenetic relationships of all OTUs' representative sequences were obtained. Finally, the data of each sample were homogenized.

Qiime software (Version 1.9.1) was used to calculate OTU abundance, Alpha diversity and UniFrac distance. R software (Version 2.15.3) was used to do rarefaction curve plotting, redundancy analysis (RDA), intergroup variation analysis of Alpha diversity index, and intergroup variation analysis of Beta diversity index. LEfSe software was used for LDA Effect Size analysis at the default LDA score of 4.

2 Results and analysis

2.1 Soil physicochemical properties

The test results of physical and chemical indicators of the soil are shown in Table 2. The content of soil organic matter (SOM) was 42.102 - 57.650 g/kg; the contents of total nitrogen (TN), total phosphorus (TP) and total potassium (TP) in soil were 2.913 - 4.292 g/kg, 1.081 - 1.304 g/kg and 14.318 - 16.944 g/kg, respectively. The contents of nitrate nitrogen (XN) and ammonium nitrogen (AN) in soil were 10.169 - 97.959 mg/kg and 7.505 - 23.665 mg/kg, respectively. The contents of available phosphorus (SP) and available potassium (SP) were 30.393 - 103.853 mg/kg and 107.620 - 175.180mg/kg, respectively. Soil EC values ranged from 185 μs/cm to 454μs/cm. The soil pH value was between 7.65 and 8.02, indicating the soil was alkaline.

The analysis of variance showed that vine age had significant effect on total potassium and available phosphorus ($P<0.05$), and root depth had significant effect on nitrate nitrogen and available potassium ($P<0.05$). Among the physicochemical indices, organic matter had a significant effect on total nitrogen and potassium ($P< 0.01$), while total nitrogen had a significant effect on total potassium and nitrate nitrogen ($P<0.05$). Ammonium nitrogen was

closely related to nitrate nitrogen ($P<0.05$), and nitrate nitrogen had a significant effect on pH value ($P<0.05$).

Table 2 Determined results of soil physicochemical properties

Sample name	Vine age	SOM g/kg	TN g/kg	TP g/kg	TK g/kg	XN mg/kg	AN mg/kg	SP mg/kg	SK mg/kg	pH(1:5)
XZ1-1	8	56.114	3.519	1.190	14.318	68.682	12.475	52.012	169.549	7.650
XZ2-2	8	44.901	3.350	1.218	16.126	25.951	7.741	51.353	134.765	7.960
XZ3-3	8	48.673	3.388	1.081	15.634	15.752	13.948	30.393	109.034	7.970
HY1-1	10	56.325	3.635	1.304	14.596	20.492	8.217	51.151	150.267	7.980
HY2-2	10	48.873	3.099	1.213	16.137	10.169	12.223	60.305	113.655	8.020
HY3-3	10	46.306	3.337	1.202	15.602	17.006	11.755	32.386	107.620	7.950
ZN1-1	5	50.009	3.491	1.210	15.286	97.959	12.929	66.403	137.999	7.780
ZN2-2	5	49.532	3.656	1.191	15.950	20.016	13.698	70.132	136.654	8.010
ZN3-3	5	57.650	4.291	1.202	15.021	55.443	13.968	58.797	144.423	7.950
GM1-1	15	50.192	3.690	1.181	16.110	94.021	23.665	58.070	175.180	7.930
GM2-2	15	42.102	2.913	1.175	16.620	23.825	7.505	52.726	123.404	7.880
GM3-3	15	47.079	3.279	1.172	16.944	31.092	12.941	66.349	127.107	7.980
WH1-1	12	52.106	4.292	1.128	15.145	82.015	16.694	78.060	124.151	7.980
WH2-2	12	51.632	3.450	1.273	15.017	30.671	14.592	87.228	123.411	7.990
WH3-3	12	56.373	3.692	1.176	15.664	45.021	18.244	103.853	124.967	7.910

Note: Vine age: the ages of grape plants; The second number of the sample No. represents different soil depths (1, 2 and 3 represent the depths of 15, 25 and 35 cm, respectively); SOM: soil organic matter content; TN: total nitrogen content in soil; TP: total phosphorus content in soil; TK: total potassium content in soil; XN: nitrate nitrogen content in soil; AN: ammonium nitrogen content in soil; SP: available phosphorus content in soil; SK: available potassium content in soil; EC: conductivity.

2.2 Analysis of sequencing data

After filtering out low-quality and short sequences, 3500718 original sequences and 2784482 valid sequences were obtained from 45 soil samples, each of which has 61877 valid sequences on average, with the minimum number of 41610 and the maximum number of 78996 for each sample. Based on the abundance of OTUs at 97% level, we used QIIME software to process the OTUs of the valid sequences, and obtained a total of 196,690 OTU classifications, among

which the highest number of OTUs classifications in single sample were 5,538. Then, the data of all samples were statistically analyzed in the terms of soil depth, which showed that the highest abundance of bacterial species was 70,921 OTUs, occurring in the middle layer (15-25cm); the lowest abundance of bacterial species was 60,738 OTUs, occurring in the lower layer (25-35cm); and the data were also analyzed in the terms of vine age, which showed that the 15-year-old vine group (GM) had the highest OTUs, which was 41783, while the 5-year-old vine group (ZN) had the lowest OTUs, which was 35729. See Table 3 for sequence number and OTU number of each soil sample, and see Table 4 for sequence number and OTU number of soil samples in terms of soil depth and vine age.

QIIME software (Version 1.9.1) was used to generate rarefaction curves for 45 samples (Fig. 1). The rarefaction curves reveals the representativeness of each sample, which can be used to evaluate whether the current sequencing depth was enough to reflect the microbial diversity contained in the community samples. It can be seen from Fig. 1 that when the sequencing amount exceeded 40,000 reads, there were still new OTUs appearing, but the curve became flat, indicating that the sampling was basically reasonable, which meant the confidence of the bacterial community structure in the real environment was relatively high and the sequencing depth can truly reflect the bacterial community diversity in the soil; in other words, the current sequencing depth was enough to reflect the diversity of bacterial communities contained in the community sample.

Table 3 Sequence number and OTU number of soil samples

Sample_name	Total_tag	Taxon_Tag	OTU_num
ZN1.1	80092	65906	5075
ZN1.2	80114	68832	4842
ZN1.3	80022	73343	1631
ZN2.1	86442	74653	4988
ZN2.2	80205	63175	5538
ZN2.3	73134	61470	3814
ZN3.1	87581	78996	1964
ZN3.2	80307	66873	5457
ZN3.3	77075	65333	2420
WH1.1	80145	56823	5050

WH1.2	80210	73321	2587
WH1.3	93790	67134	5293
WH2.1	80260	55083	5081
WH2.2	80065	67741	5229
WH2.3	80056	54780	4372
WH3.1	80307	72976	2092
WH3.2	77894	61717	5032
WH3.3	80323	63756	4970
GM1.1	70505	49359	4807
GM1.2	71962	56620	4879
GM1.3	77461	57675	4653
GM2.1	80025	62805	5325
GM2.2	57533	48800	2284
GM2.3	67978	46734	4413
GM3.1	80687	59963	5203
GM3.2	80179	57891	5204
GM3.3	79520	61695	5015
HY1.1	86489	63378	5194
HY1.2	80192	66268	5402
HY1.3	87013	64966	5368
HY2.1	54703	47374	3762
HY2.2	80053	65717	5178
HY2.3	80326	73929	2494
HY3.1	80062	71119	2836
HY3.2	80113	59049	5032
HY3.3	80319	72656	2755
XZ1.1	80082	69332	4470
XZ1.2	80248	70308	3725
XZ1.3	74304	54404	4795
XZ2.1	63961	41669	4301

XZ2.2	77477	57168	5330
XZ2.3	60952	41610	4252
XZ3.1	80086	60298	4883
XZ3.2	80181	61700	5202
XZ3.3	70285	50083	4493
Total	3500718	2784482	196690
Average	77794	61877	4371

Table 4 Sequence number and OTU number of soil samples in the terms of soil depth and vine age

Sample_Name	Total_tag	Taxon_Tag	OTU_num
Upper layer (5-15cm)	1171427	929734	65031
Middle layer (15-25cm)	1166733	945180	70921
Lower layer (25-35cm)	1162558	909568	60738
ZN (5-year）	724972	618581	35729
XZ (8-year）	667576	506572	41451
HY (10-year）	709270	584456	38021
WH (12-year）	733050	573331	39706
GM (15-year)	665850	501542	41783

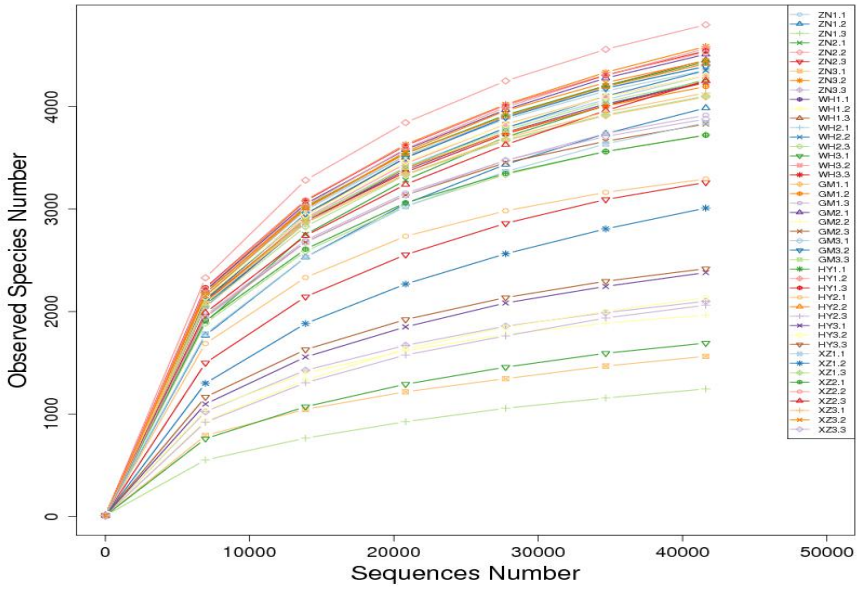

Fig. 1 Rarefaction curves at the OTUs similarity of 97%

The OTUs similarity of the samples shown in Fig. 1 is 97%. The abbreviations ZN, XZ, HY, WH, GM represent the vine ages of 5, 8, 10, 12 and 15 years, respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil depths of 5-15, 15-25 and 25-35 cm, respectively.

2.3 Analysis of bacterial community structure in soil

Fig. 2 shows the level of ten bacterial phyla in rhizosphere soil. In this study, based on the abundance from high to low, the top 10 bacterial phyla were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, *Nitrospirae*, *Tenericutes* and *Fusobacteria*, accounting for 22.3%, 14.9%, 14.5%, 10.1%, 9.6%, 6.7%, 6.4%, 2.4% and 1.1, respectively. *Proteobacteria* had the highest abundance in soil samples, with the abundance higher than 10%.

As for the average abundance of *Firmicutes*, *Bacteroidetes*, and Fusobacteria, the 5-year-old vine group (ZN) showed the highest value, while the 15-year-old vine group (GM) showed the lowest value, and the abundance varied greatly. The abundance of *Proteobacteria* did not change obviously with the vine age. There was no obvious regularity showed between the abundance changes of *Actinobacteria*, *Chloroflexi*, *Nitrospirae* and *Tenericutes* and the vine age. However, the vine age had certain influence on the abundance of *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Gemmatimonadetes* and *Acidobacteria*.

In the perspective of the vertical distribution of soil, *Firmicutes* showed the highest abundance at 5-15 cm depth and the abundance gradually decreased with the increase of depth, while *Chloroflexi* and *Nitrospirae* showed the lowest abundance at 5-15cm depth and the abundance gradually increased with the increase of depth. *Gemmatimonadetes* and *Fusobacteria* had the highest abundance at the depth of 25-35cm, and their abundance had little change at the depths of 5-15cm and 15-25cm. The abundance of *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Acidobacteria* showed no significant relationship with soil depth. So, soil depth had some influence on the abundance of *Firmicutes, Chloroflexi, Nitrospirae, Gemmatimonadetes* and *Fusobacteria*.

Fig. 3 shows the top ten bacterial genera in rhizosphere soil. At the level of genus classification, there were many kinds of bacteria, and the unclassified bacteria with low abundance were the most important microflora, accounting for more than 90%, indicating that there are a large number of unknown microbial resources to be discovered in the soil. The 10 dominant bacterial genera with high relative abundance were *Bacteroides* having the highest abundance (2.37%), *Sphingomonas* (1.06%), *Prevost*_9 (0.98%), *Lactobacillus* (0.97%), *Enterococcus* (0.56%), *Anaerococcus* (0.36%) *Sutterella* (0.31%), *Helicobacter* (0.24%), *Phascolarctobacterium* (0.23%), and *Butyricicoccus* (0.18%). By analyzing the sample data

of the top three dominant bacteria, it was found that *Sphingomonas* had the highest abundance in the upper layer (5-15cm) and the lowest abundance at the lower layer (25-35cm); *Prevotella_9* had the highest abundance at the lower layer (25-35cm); and *Bacteroides* showed little difference of abundance in different layers. The analysis of variance (ANOVA) showed that the vine age had a significant effect on the abundance of *Anaerotruncus* ($P< 0.05$), and that the soil depth had a significant effect on the abundance of *Enterococcus* ($P< 0.05$).

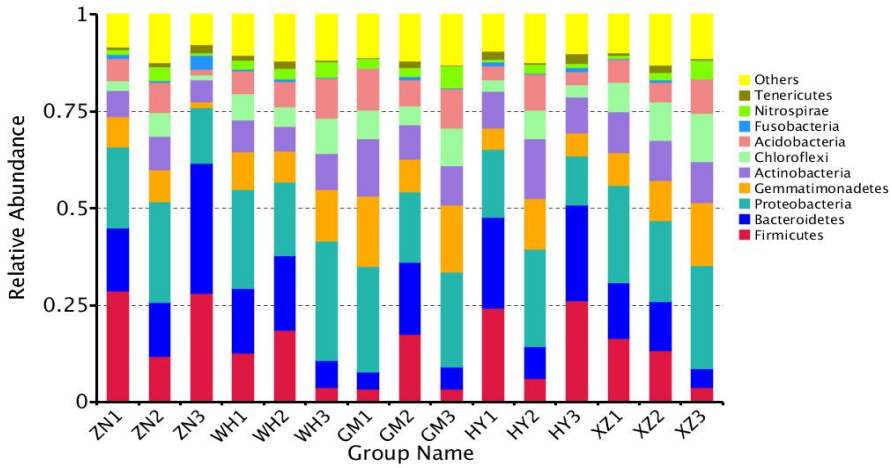

Fig. 2 The relative abundance of top ten bacterial phyla in rhizosphere soil

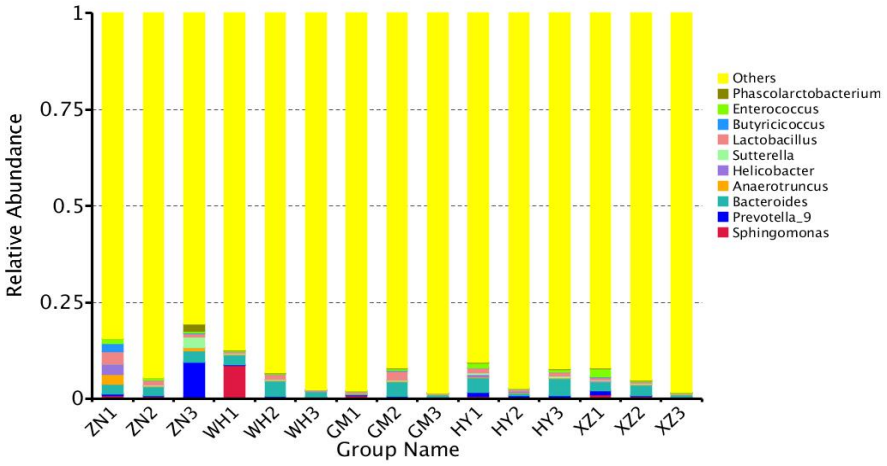

Fig. 3 The relative abundance of top ten bacterial genera in rhizosphere soil

Fig. 2 shows the relative abundance of top ten bacterial phyla in grape rhizosphere soil, and Fig. 3 shows the relative abundance of top ten bacterial genera in grape rhizosphere soil. The abbreviations ZN, XZ, HY, WH, GM represent the vine ages of 5, 8, 10, 12 and 15 years,

respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil depths of 5-15, 15-25 and 25-35 cm, respectively.

2.4 The effect of soil physicochemical properties on bacterial community

Redundancy analysis (RDA) at phylum level was carried out to reveal the relationship between soil physicochemical factors and bacterial community (Fig. 4). The first two axes of RDA analysis explained the 94.41% variations to the bacterial community, in which the first axis explained 91.83% and the second axis explained 2.58%. Soil total potassium had the most significant effect on bacterial community, followed by soil total phosphorus, and the effects of soil physicochemical properties on bacterial community were in the order of soil total potassium > total phosphorus > ammonium nitrogen > total nitrogen > available phosphorus > organic matter > pH > nitrate nitrogen > available potassium.

Correlation analysis was made between the top 10 dominant bacterial communities (phylum level) and soil physicochemical properties (Table 5), and the results showed that all the dominant bacterial communities, except for *Actinobacteria*, were significantly positively related to soil physicochemical properties. *Firmicutes* community was positively significantly correlated with the contents of total potassium and total phosphorus ($P < 0.05$); *Bacteroidetes*, *Gemmatimonadetes* and *Acidobacteria* communities were positively significantly correlated with total phosphorus content ($P < 0.05$) and extremely significantly correlated with total potassium content ($P < 0.01$); *Proteobacteria* community was significantly positively correlated with nitrate nitrogen content ($P < 0.05$); *Chloroflexi* community was extremely significantly correlated with total phosphorus content ($P < 0.01$) and significantly correlated with total potassium content ($P < 0.05$); *Fusobacteria* community was significantly positively correlated with total phosphorus content ($P < 0.05$); *Nitrospirae* community was extremely correlated with total potassium and total phosphorus ($P < 0.01$); and *Tenericutes* was significantly positively correlated with total phosphorus content ($P < 0.05$). The correlations between those dominant bacterial communities and other physicochemical properties (organic matter, total nitrogen, ammonium nitrogen, available phosphorus, available potassium and pH value) were not significant.

Table 5 Correlation between dominant bacterial communities (phylum level) and soil physicochemical roperties

	Proteobacteria	Bactero idetes	Firmic utes	Gemmat imonade tes	Actinob acteria	Acidob acteria	Chlorof lexi	Nitrospi rae	Teneri cutes	Fusoba cteria
SOM	0.360	0.219	0.649	0.087	0.354	0.530	0.197	0.212	0.796	0.611
TN	0.161	0.587	0.865	0.356	0.347	0.631	0.361	0.452	0.956	0.891
TP	0.068	0.011*	0.027*	0.022*	0.744	0.038*	0.007**	0.003**	0.047*	0.016*
TK	0.552	0.008**	0.032*	0.005**	0.058	0.008**	0.039*	0.004**	0.087	0.149
XN	0.498	0.867	0.716	0.896	0.376	0.916	0.554	0.332	0.560	0.610
AN	0.028*	0.101	0.081	0.186	0.897	0.065	0.385	0.057	0.218	0.285
SP	0.193	0.979	0.610	0.871	0.325	0.135	0.782	0.106	0.534	0.861
SK	0.535	0.979	0.735	0.797	0.922	0.781	0.374	0.114	0.562	0.865
PH	0.816	0.966	0.784	0.777	0.568	0.689	0.556	0.152	0.592	0.623

Note: * indicates significant correlation at $P < 0.05$; * * indicates significant correlation at $P < 0.01$.

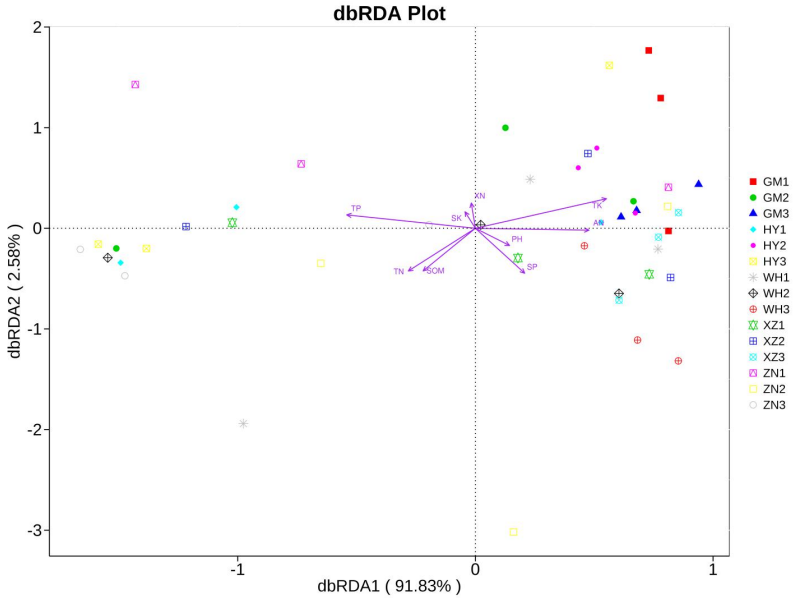

Fig. 4 Redundancy analysis of soil physicochemical properties and bacterial communities (phylum level)

The abbreviations ZN, XZ, HY, WH, GM represent the vine ages of 5, 8, 10, 12 and 15 years, respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil depths of 5-15, 15-25 and 25-35 cm, respectively.

2.5 Analysis of soil bacterial diversity

2.5.1 Alpha diversity analysis

Community richness indices Chao1 and ACE and diversity indices Shannon and Simpson of each soil sample are shown in Table 6. With the increase of soil depth, Shannon index, Chao1 index and ACE index first increased and then decreased, and all the three indices showed the highest values in soil depth of 15-25cm, indicating that bacterial diversity and community richness were the highest in the middle layer of soil. The 15-year-old vine group had the highest Shannon index and the highest diversity of bacteria communities, while the 8-year-old vine group had the highest Chao1 and ACE indices. The richness of soil bacterial species fluctuated with the planting years of grape vines, and was not distributed linearly. The result of two-way ANOVA showed that Shannon index, Simpson index, Chao1 index and ACE index showed no significant difference ($P > 0.05$), and the Good's coverage was between 97% and 99%, indicating that the microbial species information of all the sample plots was fully reflected and the sequencing results can represent the real situation of soil bacterial communities in the vineyard sample plots.

Table 6 Alpha diversity indices of bacterial communities

Sample name	Observed species	Shannon	Simpson	Chao1	ACE	Good's coverage
GM1	4332	10.49	0.998	5088.94	5213.74	0.97
GM2	3517	9.63	0.995	3992.71	4174.54	0.98
GM3	3998	10.20	0.997	4573.93	4764.99	0.98
HY1	3369	9.36	0.994	4098.01	4098.49	0.98
HY2	4428	10.54	0.998	5429.12	5507.35	0.97
HY3	3007	8.85	0.991	3784.93	3804.54	0.98
WH1	3429	9.21	0.988	4071.70	4220.13	0.98
WH2	3596	9.42	0.993	4473.85	4558.51	0.98
WH3	4137	10.35	0.998	5099.45	5131.99	0.97
XZ1	3898	9.92	0.996	4554.28	4639.57	0.98
XZ2	3994	9.87	0.995	4791.71	4981.00	0.97
XZ3	4077	10.24	0.998	6729.79	5247.01	0.97
ZN1	3376	9.33	0.993	4408.51	4437.90	0.98
ZN2	4456	10.20	0.996	5674.08	5657.94	0.97

2.5.2 Beta diversity analysis

Principal coordinates analysis (PCoA) was carried out to the data of bacterial communities in the soil samples to present their dispersing or clustering. As shown in Fig. 5, the contribution rate of the principal coordinate 1 (PC1) was 74.97%, the contribution rate of the principal coordinate 2 (PC2) was 5.27%, and their total contribution rate was 80.24%. It can be seen from Fig. 6 that, except for XZ3, WH3, GM3, GM1 and HY2, which were relatively close to each other, most of the other samples were far apart, indicating that there was significant difference of bacterial community composition among the samples.

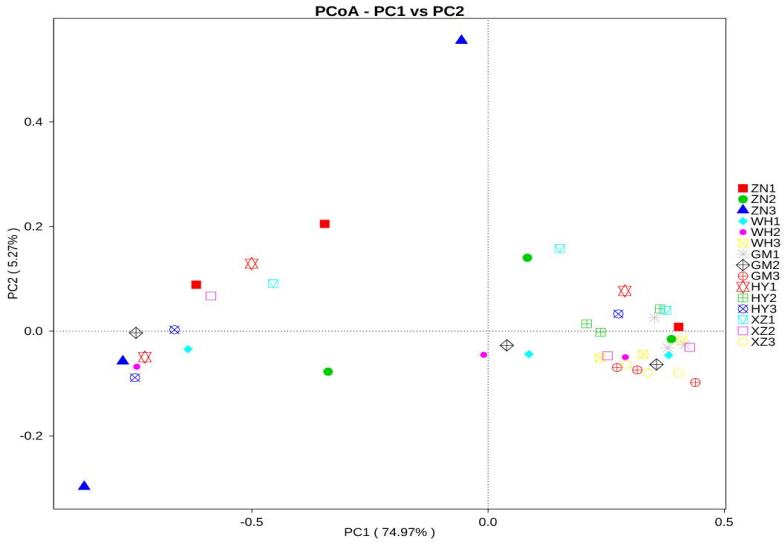

Fig. 5 PCoA analysis of soil bacterial communities

The abbreviations ZN, XZ, HY, WH, GM represent the vine ages of 5, 8, 10, 12 and 15 years, respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil depths of 5-15, 15-25 and 25-35 cm, respectively.

A petal diagram can reflect the number of common and unique OTUs between groups or samples visually, for example, the overlap can presents the common OTUs between two samples. Each petal in the diagram represents a sample, and different colors represent different samples. The core number in the center represents the number of common OTUs of all samples, and the number on each petal represents the specific OTUs number of each sample. As shown in Fig. 6, the number of OTUs shared by 15 groups of samples was 1690, and the number of OTUs unique to each group of samples was quite different. The number of OTUs unique to the 5-year-old vine group (ZN1, ZN2 and ZN3) was 220, 586 and 244, respectively, accounting for 27.7% of the total OTUs number (3794), and the number of OTUs unique to the 15-year-old vine group (GM1, GM2 and GM3) was 62, 62 and 63, which were the lowest, accounting for 4.7% of the total OTUs number.

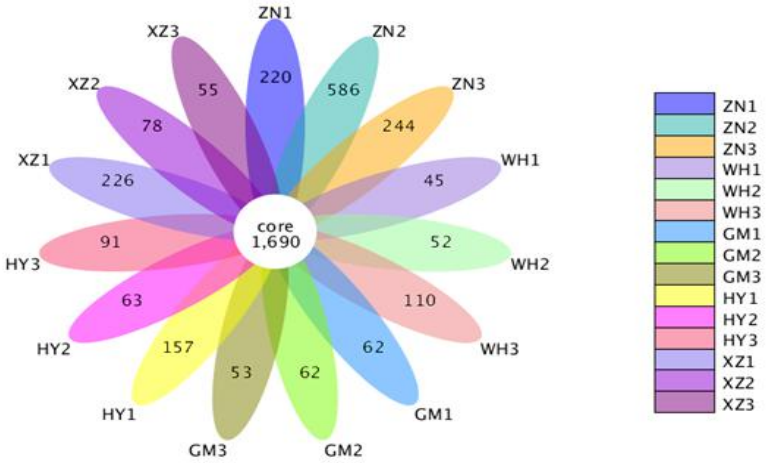

Fig. 6 OUTs-based petal diagram

The abbreviations ZN, XZ, HY, WH, GM represent the vine ages of 5, 8, 10, 12 and 15 years, respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil depths of 5-15, 15-25 and 25-35 cm, respectively.

2.5.3 Clustering analysis of sample similarity

In order to study the similarity between different samples, we clustered the samples and constructed a sample clustering tree. As shown in Fig. 7, the bacterial communities can be divided into three taxa, where ZN3 and ZN2 were respectively clustered into one branch, and ZN1 and other samples were clustered into one big branch. ZN1, ZN2 and ZN3 represent different soil depths of 5-15, 15-25 and 25-35 cm, respectively. Each taxon came from a different soil depth, indicating that soil depth had certain effect on the bacterial community composition.

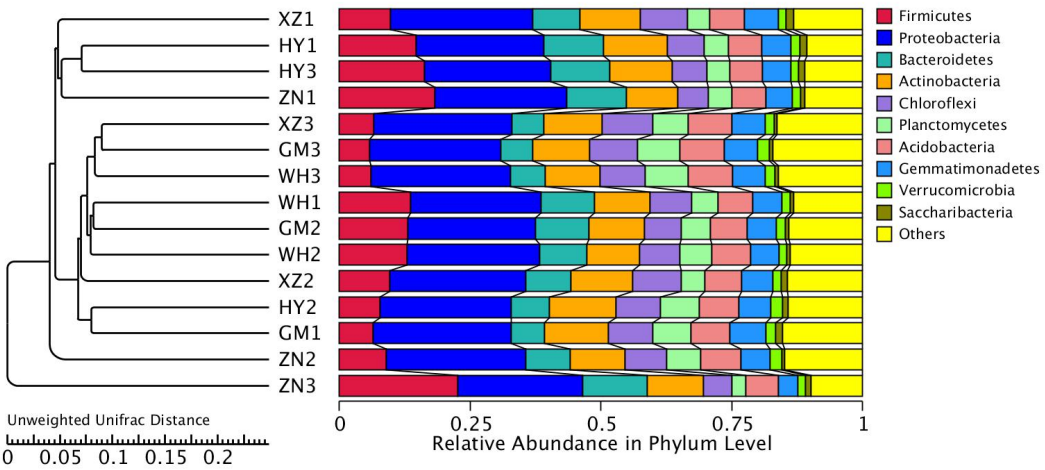

Fig. 7 UPGMA tree based on unweighted UniFrac distance at phylum level

Note: The left part is UPGMA tree structure, and the right part is the relative abundance distribution of species at phylum level. The abbreviations ZN, XZ, HY, WH, GM represent the vine ages of 5, 8, 10, 12 and 15 years, respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil depths of 5-15, 15-25 and 25-35 cm, respectively.

2.5.4 LEfSe (LDA Effect Size) analysis

LEfSe was used to identify bacterial species with significant differences in the terms of vine ages and soil depths. Firstly, the LEfSe analysis was carried out for samples from the same soil depth, and the LDA score of 4 was used to identify statistically significant difference between bacterial taxa. In the lower layer (25-35 cm), there were 21 taxa whose LDA scores were all greater than 4, including 7 taxa of GM (15-year-old vine group), 4 taxa of WH(12-year-old vine group), 6 taxa of XZ (8-year-old vine group) and 4 taxa of ZN (5-year-old vine group) (**Fig. 8**). In the upper layer (5-15cm), there were 4 taxa whose LDA scores higher than 4, including 3 taxa of WH (12-year-old vine group) and 1 taxa of XZ (8-year-old vine group) (**Fig. 9**).No distinct groups were observed at the middle root depth (25 cm).

Then, the LEfSe analysis was carried out for samples from different soil depths, the results found there were 9 bacterial taxa whose LDA scores were higher than 4, indicating they had statistically significant difference. The 9 bacterial taxa included 6 taxa in the lower layer (25-35 cm) of GM (15-year-old vine group), 1 taxon in the upper layer (5-15 cm) of GM (15-year-old vine group), 1 taxon in the lower layer (25-35 cm) of HY (10-year-old vine group) and 1 taxon in the middle layer (15-25 cm) of HY (10-year-old vine group), as shown in **Fig. 10**. Generally, GM (15-year-old vine group) had more biomarkers than the groups of other vine ages, especially in the lower layer (25-35 cm).

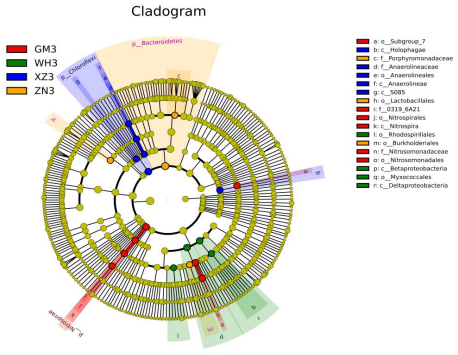

Fig. 8 Cladogram

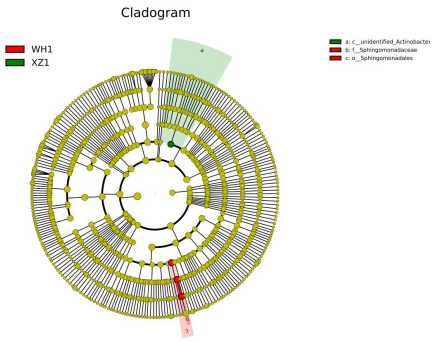

Fig. 9 Cladogram

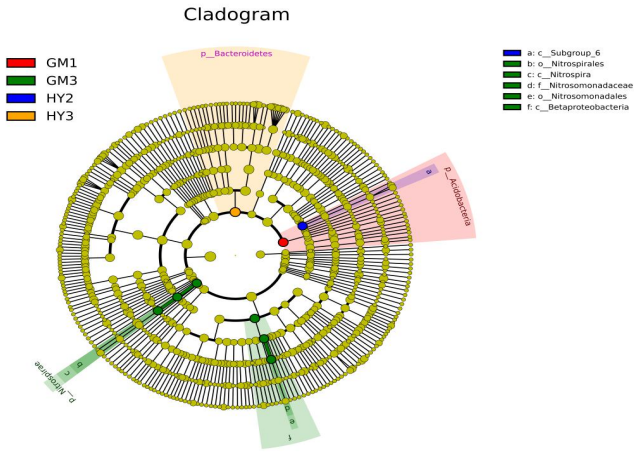

Fig. 10 Cladogram

The polygene distribution of bacterial taxa in grape rhizosphere soil was determined by using Linear Discriminant Analysis (LDA) Effect Size (LEfSe). **Fig. 8** shows the bacterial taxa of the lower layer (25-35 cm); **Fig. 9** shows that of the upper layer (5-15 cm); and **Fig. 10** shows that of all the three layers (5-15 cm, 15-25 cm and 25-35 cm). In the cladograms, circles radiated from inside to outside represent the taxonomic ranks from phylum to genus (or species). Each circle at different taxonomic ranks represents a classification at that level, and the diameter of the circle is proportional to the relative abundance. The coloring principle is that the species with no significant difference are uniformly colored in yellow, and the Biomarkers of different species follow the group for coloring. The red nodes indicate the bacterial taxa that play an important role in the red group, while the green nodes indicate the bacterial taxa that play an important role in the green group. If a certain group in the picture is missing, it means that there is no species with significant difference in that group. The names of species represented by English letters in the figure are displayed in the legend on the right. The abbreviations ZN, XZ, HY, WH, GM represent the vine ages of 5, 8, 10, 12 and 15 years, respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil depths of 5-15, 15-25 and 25-35 cm, respectively. Statistically significant difference is defined by LDA > 4.

3 .Conclusion and discussion

(1) In this study, 45 soil samples from vineyards were sequenced by 16S rRNA gene high-throughput sequencing, and 196,690 OTUs were classified at 97% similarity, indicating that there were abundant bacteria in rhizosphere microenvironment, which were not only abundant in number but also rich in species. Statistical analysis of OTUs data of different vine ages and

different soil depths showed that the number of OTUs was the highest in the middle layer (15-25 cm) and the lowest in the lower layer (25-35 cm). The number of OTUS of the vine group with longest age (15 years) was the highest, and that with the shortest age (5 years) was the lowest.

(2) The dominant bacteria in the soil samples were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes* and *Actinobacteria*, among which the most dominant bacterial phylum was *Proteobacteria* which had the highest relative abundance of 22.3%. The soil in the study area is alkaline, so it is speculated that *Proteobacteria* is the main dominant community in alkaline soil, which is consistent with many studies at home and abroad[16]. So far, the enrichment distribution of *Proteobacteria* has been found in the rhizosphere of corn[17], *Arabidopsis thaliana*[18], *Lycium chinensis*[19], strawberry[20], safflower carthamus[21] and alfalfa[22], suggesting *Proteobacteria* can adapt the rhizosphere environment of many different plants. *Firmicutes* and *Actinobacteria* were also dominant phyla. Many *Firmicutes* can produce spores, which can resist dehydration and extreme environment, while the drought and high temperature environment of the study area was beneficial to the growth and reproduction of *Firmicutes*. Through the study, it was found that the abundance of *Firmicutes* was highest in the upper layer soil of the shortest age group, and lowest in the lower layer soil of the longest age group. *Actinomycetes* are an important kind of microorganisms in the rhizosphere environment of plants, and they prefer alkaline environment, while High C/N ratio and low molecular organic matter content in soil can better promote their growth[23]. *Actinomycetes* can produce a large number of different antibiotics, which can regulate the biological balance among plants, pathogenic bacteria and microenvironment, which means *Actinomycetes* can play an important role in promoting plant growth, preventing disease[24-25]. Zhang PP et al. found that 30.9% of the *Actinomycetes* in the rhizosphere soil of Taxus chinensis could inhibit the activity of plant pathogenic fungi, and some of them even showed strong antibacterial activity against many plant pathogenic fungi[26].

(3) In taxonomy of the genus, there are many kinds of bacteria, and the unclassified bacterial taxa with low abundance account for more than 90%, indicating there are a large number of unknown microbial resources to be discovered. In this study, we found *Bacteroides* in the obvious dominant position had the highest abundance, followed by *Sphingomonas*. The average abundance of *Bacteroides* was lowest in the soil of 15-year-old vine group, and the soil depth had little effect on their abundance. With the second highest abundance only to *Bacteroides*, *Sphingomonas* had its highest abundance in the upper layer (5-15cm) and the lowest abundance in the lower layer (25-35cm), and the vine age had little effect on its

abundance. Some studies pointed out that *Sphingomonas* are one of the most effective bacteria for degrading toxic substances in soil, they can promote nutrient absorption in grape rhizosphere and resist various pathogenic bacteria, and some of their strains had the characteristics of nitrogen fixation and dehydrogenation, thus playing an important role in maintaining soil nitrogen balance[27].

(4) Redundancy analysis (RDA) was conducted to understand the effect of soil physicochemical properties on soil microbial communities. The results showed that soil total potassium had the most significant effect on microbial communities, followed by soil total phosphorus. Some studies have revealed that there is a certain correlation between the relative abundance of dominant bacterial communities and soil physicochemical properties[28-30], and those environmental factors greatly affected the bacterial community composition in grape rhizosphere soil.

(5) Correlation analysis was made between the top 10 dominant bacterial communities (phylum level) and soil physicochemical properties. The results showed that there was significant correlation between soil physicochemical properties and all the dominant bacterial communities except for *Actinobacteria*, indicating the microbial community composition was closely related to soil environment. Some researchers pointed out that the distribution and composition of bacterial communities can be explained almost only by habitat characteristics[31].

(6)Alpha diversity analysis showed that Shannon index, Chao1 index and ACE index were the highest in the middle layer (15-25 cm), indicating bacterial diversity and community richness were the highest in that soil depth. The Shannon index of 15-year-old vine group was the largest, indicating the group has the highest bacterial diversity. Chao1 and ACE indices of 8-year-old vine group were the largest, indicating that group had the highest richness of bacterial communities. Generally, the depth of grape rhizosphere soil and different planting years have certain effect on the diversity and richness of bacterial communities.

(7) Beta diversity analysis and PCoA analysis showed that there were significant differences in microbial community composition among samples.

(8) LEfSe (LDA effect size) analysis showed that the vine group with longest age (15 years) in this study had more biomarkers than other groups with shorter ages, especially in the lower layer of soil. With the increase of planting years, the number of biomarkers increased, indicating that continuous cropping of grapes changed the composition of soil bacterial communities. Some studies pointed out that microbial communities in plant rhizosphere is closely related to plant growth time and root exudates[32-34]. Root exudates are the medium of

interaction between plants and rhizosphere microorganisms, and the exudates and s debris from plant root activities are the main nutrient and energy sources of rhizosphere microorganisms, so root exudates are closely related to bacterial communities.

To sum up, according to the research and analysis of high-throughput sequencing, we found in grape rhizosphere soil there were a large number of bacteria, which were not only numerous, but also rich in species. Soil physicochemical properties, grape vine age and soil depth had certain effects on bacterial abundance and community composition. The vine group with longest age had more specific rhizosphere bacteria in the lower layer of soil, and the formation of those specific bacteria may be closely related to the root exudates of vine. Although the root exudates were not measured in this study, according to previous studies, the root exudates were strongly affected by the growth time and growth stage of crops, which in turn affected the succession of bacterial communities.

Although the community composition and diversity of bacteria in vineyard rhizosphere soil were analyzed by high-throughput sequencing technique, it is necessary to make a more in-depth study on the changes of ecosystem functions caused by these bacteria and their abundance, as well as the interaction between rhizosphere exudates and bacteria.

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Author Contributions

ProfessorTongLiu and Dr Feng Xue designed the experiment. Feng Xue analyzed the data and wrote the article. HongMei Chen collected the samples.

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