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Effects of harvest maturity on microbial community composition, enzyme activities, and phenolic acids in upper tobacco leaves after curing

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Abstract. The upper leaves of flue-cured tobacco are an important raw material for cigarette production, and harvest maturity is one of the important factors affecting tobacco leaf quality. This study used Illumina MiSeq high-throughput sequencing, conventional physicochemical assays, and gas chromatography-mass spectrometry analysis to investigate the effects of different harvest maturities on the microbial community composition, enzyme activities, phenolic acid contents, and main aroma and chemical components of tobacco leaves after curing. The results showed that Proteobacteria, Actinobacteria, and Ascomycota were the dominant bacteria and fungi in the cured tobacco leaf samples. At the genus level, *Unclassified_k_Fungi* was the dominant fungal genus in the conventional harvesting treatment; *Altemaria, Leptosphaerulina*, and *Stagonosporopsis* were the dominant fungal genera in the 7-d delayed harvesting treatment, *Aspergillus*, *Symmetrospora*, and *Filobasidium* were the dominant bacterial and fungal genera in the 14-d delayed harvesting treatment. Correlation analysis revealed that the dominant bacterial and fungal communities at the genus level showed a high correlation with the enzyme activities, phenolic acid contents, aroma component contents, and chemical component contents in the tobacco leaves. For example, the content of the neutral aroma substance neophytadiene was significantly positively correlated with *Aspergillus*, *Symmetrospora*, and *Curtobacterium*, while the nicotine content was extremely significantly negatively correlated with *Aspergillus*, *Symmetrospora*, and *Pseudomonas* and significantly positively correlated with *Massilia*. Finally, we found that the enzyme activities, phenolic acid contents, and aroma component contents in the tobacco leaves in the 14-d delayed harvesting treatment were higher, the nicotine content was the lowest, and the chemical composition was more consistent. In the microbial community, *Aspergillus*, *Symmetrospora*, and *Filobasidium* promoted the improvement of tobacco leaf quality to a certain extent. Our study evaluated the effect of harvest maturity on tobacco leaf quality from multiple perspectives and emphasized the important role of the endophytic microbial communities in the development of tobacco leaf quality. The objective is to offer scientific support for the ensuing modification of the harvest period for flue-cured tobacco and the use of the microbial community to enhance the quality of tobacco leaves.

Keywords: Illumina MiSeq high-throughput sequencing, upper tobacco leaves, microorganisms, phenolic acids

1. INTRODUCTION

The upper leaf of tobacco is an important raw material for the tobacco industry. Currently, the output of upper tobacco leaf accounts for 30-40% of the total tobacco leaf production in China. The quality of the upper leaf determines its industrial usability (Han, 2022). However, the upper leaves of flue-cured tobacco exhibit such problems as a compact tissue structure after curing, thicker leaves with poor opening, higher contents of protein, starch, and nicotine, and an inconsistent chemical composition (Chen *et al.*, 2019). In addition to variety, cultivation measures, and curing processes, harvest maturity is an important factor affecting tobacco leaf quality (Gu, 2021). The implementation of the strategy of "reducing ash and harm" in cigarettes has changed the suitability of available tobacco leaves. The

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industry tends to use the upper leaves with more intense flavor to produce Class I and II cigarettes (Li, 2014; Ye, 2018). However, unreasonable harvesting practices and insufficient harvest maturity greatly reduce the availability of the upper leaves of flue-cured tobacco (Shen, 2022). The quality evaluation of tobacco leaves mainly includes four components: appearance quality, chemical composition, sensory quality, and physical characteristics (Shi *et al.*, 2024; Yu, 2003; Zhang and Zhang, 2011). In addition, safety and aroma substances are two important aspects used to evaluate the quality of tobacco leaf raw materials (Liu *et al.*, 2024). Safety, appearance quality, and sensory quality all depend on the interaction between the chemical components and neutral aroma components of tobacco leaves. Each index related to the chemical composition can reflect the quality of tobacco leaves from different aspects (Tobacco Research Institute of Chinese Academy of Agricultural Sciences, 2005). Microorganisms are present throughout the entire process of tobacco growth, curing, and fermentation. Microbial metabolism can promote the production of a variety of enzymes. Microorganisms work synergistically with enzymes to convert polymer compounds (Huang *et al.*, 2015), degrade harmful substances in cured tobacco leaves, increase the aroma and smoking flavor of tobacco leaves, and improve the quality of cured tobacco leaves (Li *et al.*, 2012; Xue *et al.*, 2019). Studies have shown that flue-cured tobacco leaves mainly contain several types of microorganisms, including bacteria, fungi, actinomycetes, and yeasts (Huang *et al.*, 2010), with bacteria accounting for the largest proportion, followed by fungi, actinomycetes, and yeasts with relatively low abundance (Gong *et al.*, 2023; Wu *et al.*, 2022). Ascomycota was found to be the dominant fungal phylum. Proteobacteria and Firmicutes were the dominant bacterial phyla, with a great influence on the style and aroma of tobacco leaves (Zhang *et al.*, 2020), and microorganisms such as *Bacillus*, *Paenibacillus*, *Enterobacter*, and *Pantoea* were the dominant bacterial genera in tobacco leaves. These types of bacteria have strong vitality and can secrete a variety of hydrolytic enzymes to effectively reduce the nicotine content (Su *et al.*, 2011). *Bacillus* can degrade protein and starch in cured tobacco leaves and convert them into small-molecule aroma components, such as ketones and aldehydes, reducing the burnt taste generated in the combustion of tobacco leaves and resulting in more consistent contents of chemical components in tobacco leaves (Wu *et al.*, 2021; Huang *et al.*, 2022). *Actinomyces* and *Pseudomonas* bacteria can reduce the contents of protein, total nitrogen, soluble nitrogen, and nicotine, reduce the accumulation of nitrosamines and nitrite, and increase the contents of free amino acids, ammoniacal nitrogen, and phthalamine nitrogen (Lei, 2007; Gutierrez-Albanchez *et al.*, 2021).

Previous research results show that peroxidase (POD), polyphenol oxidase (PPO), and lipoxygenase (LOX) play important roles in phenylalanine degradation, terpenoid degradation, and lipid metabolism, thus affecting the polyphenol content in cured tobacco (Welty *et al.*, 2021; Bouthour *et al.*, 2012). The content of organic acids is also an important indicator used to determine the quality of tobacco leaves. Under the combined action of enzymes or light, volatile fats can be converted into various aroma substances, such as alcohols, aldehydes, ketones, and acids, which significantly affect tobacco aroma and smoking flavor (Wu, 2010, 2022). There are numerous reports on the microbial structure and enzyme activity in cured tobacco leaves, but the correlations of microorganisms, phenolic acids, and enzyme activities with the contents of aroma substances and chemical components in cured tobacco leaves have been rarely reported. Therefore, this study used Illumina MiSeq high-throughput sequencing to analyze the effect of harvest maturity on the microbial composition in upper tobacco leaves after curing and to evaluate the correlation between microbial communities and phenolic acids, enzyme activities, aroma substances, and chemical components of tobacco leaves. The purpose of this study is to provide a scientific reference for adjusting the harvest time of upper leaves and for the screening and utilization of microorganisms in actual tobacco production.

2. MATERIALS AND METHODS

2.1. Experimental design

The test material was the six upper leaves of Yunyan 99. The test location was Daba Village, Yongde County, Lincang City, Yunnan Province (longitude 99°, latitude 24°), which has an altitude of 1 500 m, annual mean temperature of 17.4°C, annual precipitation of 1 283 mm, and annual average sunshine hours of 2 196.1 h. Three treatments were set up in the experiment, including conventional harvesting (based on the local conventional harvesting time, CSD1), 7-d delayed harvesting (CSD2), and 14-d delayed harvesting (CSD3). All samples were B2F grade first-cured tobacco leaves. Except for harvest time, production management measures were implemented in accordance with the local technical specifications for high-quality flue-cured tobacco production.

2.2. Sample parameters and measurement methods

2.2.3. Determination of phenolic acids in first-cured tobacco leaves

The content of phenolic acids was determined by high-performance liquid chromatography (HPLC) (Mesarović *et al.*, 2017) using an Agilent high-performance liquid chromatograph and a Thermo Scientific data processing system. The mobile phases were acetic acid:water (A) and ultrapure water (B), the detection wavelength was 280 nm, the column temperature was 30°C, and the flow rate was 1.0 mL min^{-1}.

2.2.4. Determination of enzyme activity in first-cured tobacco leaves

PPO activity was determined using an enzyme-linked immunoassay (ELISA) kit (Shanghai Ruifan Biotechnology Co., Ltd.) (Li *et al.*, 2023), POD activity was detected using a plant POD kit (Shanghai Yaji Biotechnology Co., Ltd.) (Wang *et al.*, 2023), LOX, phenylalanine ammonia lyase (PAL) and α-Amylase (AMY) activities were determined using a plant LOX kit, plant PAL kit, and AMY kit (Shanghai Saipeisen Biotechnology Co., Ltd.) (Viswanath *et al.*, 2020; Zhang *et al.*, 2023), and detection of the above enzymes was performed using the trace method.

2.2.5. Determination of aroma components in first-cured tobacco leaves

The aroma components of the samples were detected by referring to the method of Zhang (2012) using gas chromatography-mass spectrometry (GC-MS) (7890B-5977A GC/MSD, Agilent, USA).

2.2.6. Determination of chemical components of first-cured tobacco leaves

YC/T 159-2002, YC/T 160-2002, YC/T 161-2002, YC/T 217-2007, and YC/T 162-2011 were used to analyze total sugars, plant alkaloids, total nitrogen, potassium oxide, and chloride ions, respectively.

2.3. Determination of the diversity of endophytic microbial communities in first-cured tobacco leaves

2.3.1. Genomic DNA extraction from tobacco leaves

Total DNA from each sample was extracted using a Plant Tissue DNA Extraction Kit (Qiagen, 69104). After extraction, the concentration and purity of the extracted DNA were examined using a NanoDrop 2000.

2.3.2. Amplification and sequencing

The ITS1 region of fungal 18S rRNA was amplified using primers ITS1F (5'-CTTGGTCATTTAGGGAAGTAA-3') and ITS2R (5'-GCTGCGTCTTCATCGATGC-3'). The first PCR amplification of the 16S rRNA gene was performed using bacterial primers 799F (5'-AACMGGATTAGATACCCKG-3') and 1392R (5'-ACGGGCGGTGTRC-3') (Hanshew *et al.*, 2013). The primers used for the second PCR amplification were 799F (5'-AACMGGATTAGATACCCKG-3') and 1193R (5'-ACGTCACCCTTCC-3') (Di, 2017). PCR products were recovered using the AxyPrep DNA Gel Recovery Kit (AXYGEN) by cutting the gel, then eluted with Tris-HCl, and detected by 2% agarose electrophoresis. The samples were sent to Shanghai Meiji Biomedical Technology Co., Ltd. for high-throughput sequencing on the Illumina MiSeq platform.

2.3.3. Sequencing data processing and analysis

Trimmomatic software was used for the quality control of the original sequence, and FLASH software was used for sequence splicing; the obtained sequences were filtered by Usearch software (Version 7.0), and chimeric sequences were removed to obtain valid sequences. UPARSE software was used to classify the operational taxonomic units (OTUs) at 97% similarity. The representative sequences were annotated with the RDP classifier software and the SILVA database, and Mothur software was used to draw the dilution curve. The library coverage (Coverage), Shannon, Simpson, ACE, and Chao1 indices were calculated to evaluate the species diversity and abundance index, and the Bray-Curtis distance algorithm established by QIIME software was used for principal coordinate analysis (PCoA). Collinear network analysis of species diversity was performed using Tax4fun software (Tax4fun 0.3.1). Vegan software was used for redundancy analysis (RDA) to analyze the relationships among samples, flora, enzyme activity, phenolic acids, and pH. The relationship between different factors and microbial species composition was calculated by Spearman correlation coefficients, and a correlation heatmap was used to assess the association between microbial taxonomy and enzyme activity, phenolic acid, and pH, as well as the synergic effect on tobacco leaf quality.

2.3.4. Data processing

All data were analyzed by univariate analysis using IBM SPSS Statistics (2016) and Microsoft Office Excel (2019), and graphing was performed using Hiplot software.

3. RESULTS AND ANALYSIS

3.1. Analysis of the operational taxonomic unit (OTU) abundance, alpha diversity, and beta diversity of fungi and bacteria in first-cured tobacco leaves under different harvest maturities

The OTU abundance and alpha diversity of fungi and bacteria in the first-cured tobacco leaves with different harvest maturities are shown in Fig. 1, Table 1, and Fig. 2. With an increasing number of sequences, the dilution curve based on the Shannon index tended to become flat, indicating that the sequencing number was sufficient to support the subsequent data analysis (Fig. 1). The results of one-way analysis of variance (ANOVA) (Table 1) showed that there were no significant differences among the CSD1, CSD2, and CSD3 treatments in the OTU abundance and alpha diversity of fungi and bacteria ($p < 0.05$). A total of 1 736 fungal and 6 343 bacterial OTUs were detected in the three treated tobacco leaves, respectively; The OTUs of fungi and bacteria shared by the three treatments were 226 and 756, accounting for 20.79 and 19.26% of the total, respec-

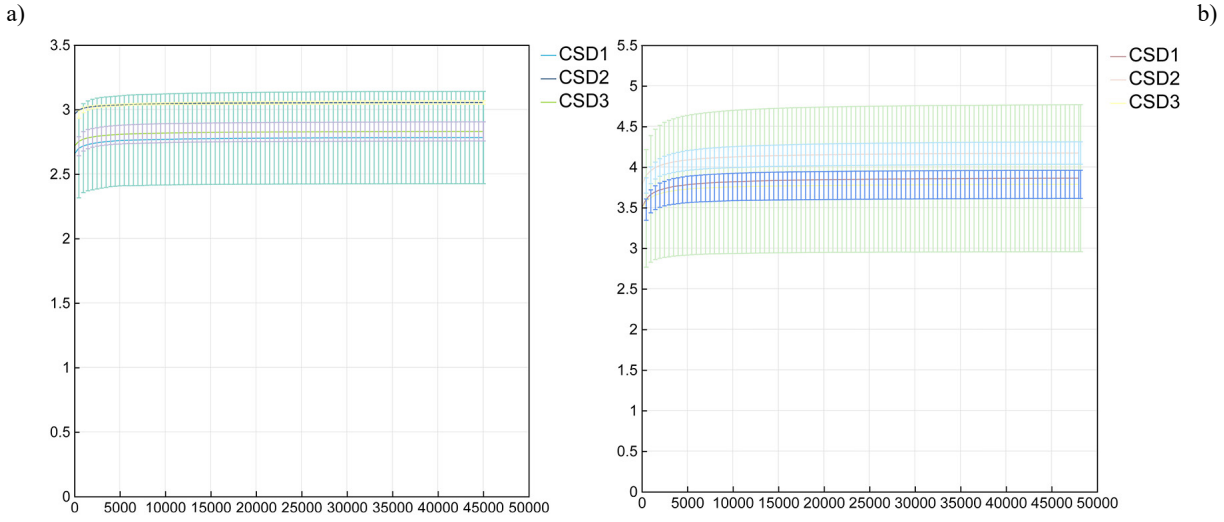

Fig. 1. Shannon index dilution curves for bacteria and fungi under various treatments: a) fungi, b) bacteria.

Table 1. Alpha diversity and OTU abundance of fungus in basic flue-cured tobacco leaves under various treatment conditions

	Microbial diversity index		CSD1	CSD2	CSD3
Fungi	Count of the original sequences		77778±11088ab	61978±14870c	91038±4313a
	OTU abundance		295a	140c	229b
	Alpha diversity index	Shannon	2.78±0.36bc	3.05±0.01a	2.83±0.07b
		Simpson	0.14±0.08a	0.08±0.01c	0.11±0.01b
	Richness index	Ace	410.51±128.17a	312.47±228.26c	384.52±13.47b
		Chao1	397.21±115.88a	298.95±214.95c	376.20±13.47b
	Coverage (%)		1.00±0.00a	1.00±0.00a	1.00±0.00a
Bacteria	Count of the original sequences		74575±1414a	69973±7771b	73124±6316ab
	OTU abundance		2462a	2289ab	1582b
	Alpha diversity index	Shannon	4.17±0.14a	3.86±0.90b	3.78±0.17bc
		Simpson	0.05±0.01bc	0.06±0.04ab	0.08±0.01a
	Richness index	Ace	1378±285.14a	1286±704.49b	914.33±195.18c
		Chao1	1334±256.10a	1242±674.05b	909.16±181.71c
	Coverage (%)		1.00±0.00a	1.00±0.00a	1.00±0.00a

Mean ± standard deviation values, with distinct lowercase letters denoting significant differences in treatment efficacy ($p \leq 0.05$).

tively. There were 295, 140, and 229 unique fungal OTUs in the CSD1, CSD2, and CSD3 treatments. The number of unique fungal OTUs in the CSD2 treatment decreased by 110.72 and 63.57%, compared with the CSD1 and CSD3 treatments (Fig. 2a). The number of unique bacterial OTUs was 926, 770, and 564. Compared with the CSD1 and CSD2 treatment, the number of bacterial OTUs unique to the CSD3 treatment was reduced by 39.09 and 26.75%. (Fig. 2b). The results suggest that, with the increasing harvest maturity, the abundance of fungal OTUs in the cured tobacco leaves gradually decreased. The abundance of bacterial OTUs showed a high-low-high trend.

3.2. Community species composition, relative abundance, and relationship of fungi and bacteria in first-cured tobacco leaves with different harvest maturities

3.2.1. Community species composition and relative abundance of fungi and bacteria in first-cured tobacco leaves with different harvest maturities

Five phyla of fungi were detected under the CSD1, CSD2, and CSD3 treatments: Acomycota, Basidiomycota, Mortierellomycota, Mucoromycota, and Unclassified_k_Fungi; these five phyla accounted for 99.89, 99.91, and 99.94% of the CSD1, CSD2, and CSD3 treatments, respectively. Among them, the phylum Unclassified_k_Fungi had the highest relative abundance in the CSD1

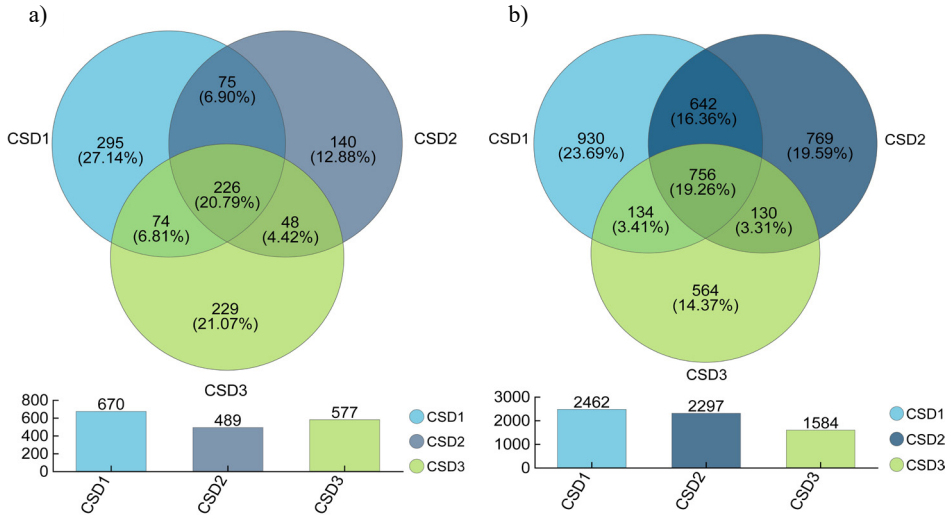

Fig. 2. Venn diagram showing the distribution of OTUs: a) Fungi and b) bacteria in tobacco leaves that have been cured to varying harvest maturities.

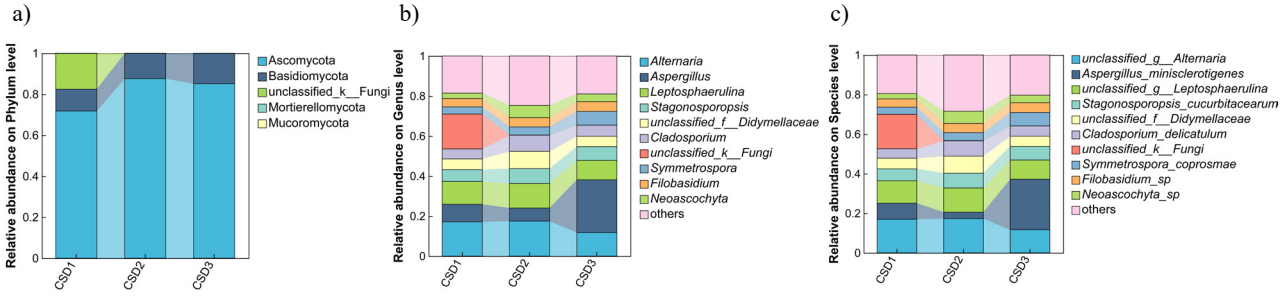

Fig. 3. Histogram of fungal community species richness at different harvest stages of flue-cured tobacco leaves: a) phylum, b) genus, c) species.

treatment, accounting for 17.43% (Fig. 3a). There were 11 fungal genera with relative abundances greater than 1% at the genus level. Among them, the relative abundance of the three genera: *Unclassified_f_Didymellaceae*, *Cladosporium*, and *Neoascochyta* in the tobacco leaves in the CSD2 treatment was significantly higher than that in the other two treatments, with values 1.60, 1.51, and 2.29 times that of the CSD1 treatment and 1.67, 1.45, and 1.60 times that of CSD3 treatment ($p<0.01$), respectively. The relative abundance of three genera (*Aspergillus*, *Symmetrospora*, and *Filobasidium*) in the CSD3 treatment was significantly higher than that in the CSD1 and CSD2 treatments, with values 3, 1.91, and 1.17 times that of the CSD1 treatment, respectively, and 4.02 times, 1.68, and 1.05 times that of the CSD2 treatment ($p <0.01$) (Fig. 3b). There were 11 fungal species with relative abundances greater than 1% at the species level. *Unclassified_k_Fungi* was a species unique to the CSD1 treatment. There were 5 species whose relative abundance in the CSD2 treatment was significantly higher than that in the CSD1 and CSD3 treatments, including *Unclassified_g_Leptosphaerulina*, *Stagonosporopsis cucurbitacearum*, *Unclassified_f_Didymellaceae*, *Cladosporium delicatulum*, and *Neoascochyta* sp., with values 1.08, 1.25, 1.60, 1.61, and 2.29 times that of CSD1, respectively, and 1.26, 1.08, 1.68, 1.51, and 1.60 times that of CSD3 ($p<0.05$). The relative abundance of two species (*Aspergillus minisclerotigene* and *Symmetrospora coprosmae)* in the CSD3 treatment was significantly higher than that in the CSD1 and CSD2 treatments, with values 3.15 and 1.90 times that of the CSD1 treatment, respectively, and 7.91 and 1.68 times that of the CSD3 treatment ($p<0.05$) (Fig. 3c).

A total of 29 phyla of bacteria were detected in the tobacco leaf samples in the three treatments, of which 11 bacterial phyla had a relative abundance of more than 1%. Proteobacteria, Actinobacteria, and Firmicutes were the dominant phyla in the tobacco leaf samples, accounting for 95, 97, and 98% of the CSD1, CSD2, and CSD3 treatments, respectively. The abundances of the three types of dominant bacteria in the CSD3 treatment were increased, compared to those in the CSD1 and CSD2 treatments. The relative abundance of Actinobacteriota increased by 66.67% ($p<0.01$) and 7.14%, compared to the CSD1 and CSD2 treatments, respectively, and the abundance of Firmicutes increased by 700 and 33.33% ($p<0.01$) (Fig. 4a). The relative abundance at the genus level (Fig. 4b) identified 53 genera with relative abundances greater than 1% in the three treatments, and *Pseudomonas*, *Pantoea*,

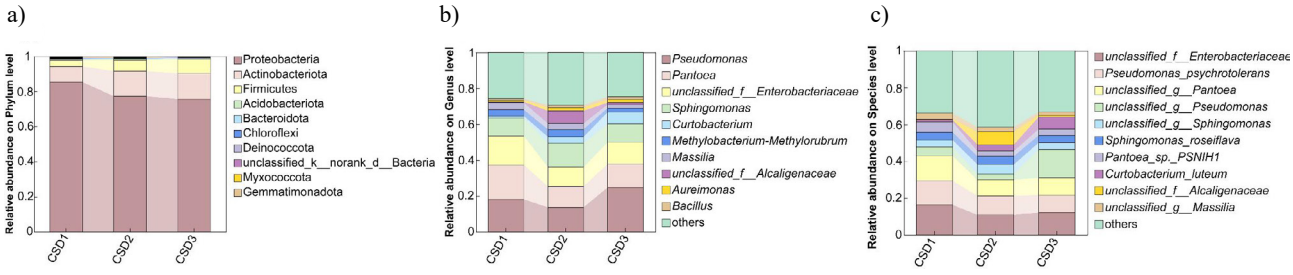

Fig. 4. Histogram of flue-cured tobacco leaves' bacterial community richness at various harvest maturity stages: a) phylum, b) genus, c) species.

Unclassified_f_Enterobacteriaceae, and *Sphingomonas* were the dominant genera in the tobacco leaf samples. *Sphingomonas, Bacillus*, and *Pseudomonas* play important roles in amino acid biosynthetic pathways and the formation of aroma substances in tobacco leaves. Among several genera, *Pseudomonas*, which has a positive effect on the degradation of nicotine content and enhancement of flavor and aroma in tobacco, had the highest relative abundance (25%) in the CSD3 treatment, representing an increase of 38.89 and 78.57% ($p<0.01$), compared to that in the CSD1 and CSD2 treatments, respectively. The relative abundance of bacteria at the species level is shown in Figure 4c. There were 11 bacterial species with a relative abundance above 1%. In particular, *g_Pseudomonas* and *Curtobacterium luteum* increased by 200, 400, 500, and 100% ($p<0.01$) in the CSD3 treatment, compared to their values in the CSD1 and CSD2 treatments, indicating that the relative abundance of functional species in the CSD3 treatment was high.

3.2.2. Community composition and relationship of fungi and bacteria in first-cured tobacco leaves with different harvest maturities

The species composition and sample distribution proportions of fungi and bacteria in the tobacco leaves with different harvest maturities are shown in Figs 5 and 6. Among fungi, Ascomycota had the highest distribution proportion in the CSD2 treatment (36%), Basidiomycota had the highest distribution proportion in the CSD3 treatment (39%), and the distribution proportion of unclassified_k_Fungi in the CSD1 treatment was 99% (Fig. 5a). At the bacterial phylum level, Proteobacteria had the highest proportion (36%) in the CSD1 treatment, and the distribution proportions of Actinobacteria (39%) and Firmicutes (44%) were significantly higher in the CSD3 treatment than in the other treatments.

At the genus level of fungi, the distribution proportion of *unclassified_k_Fungi* under the CSD1 treatment was 99%, which was extremely significantly higher than those under the CSD2 and CSD3 treatments ($p<0.001$). The distribution proportions of six fungal genera (*Neoascochyta*, *Cladosporium*, *unclassified_f_Didymellaceae*, *Stagonosporopsis*, *Leptosphaerulina*, and *Alternaria*) in the CSD2 treatment were significantly higher than those in the CSD1 and CSD3 treatments ($p<0.05$), accounting for 49, 43, 45, 37, 37, and 38%, respectively, The distribution proportions of 3 fungal genera (*Symmetrospora*, *Filobasidium*, and *Aspergillus*) in the CSD3 treatment were significantly higher than those in the CSD1 and CSD2 treatments ($p<0.05$), accounting for 47, 36, and 63%, respectively (Fig. 5b). At the genus level of bacteria, three genera (*Massilia*, *unclassi-*

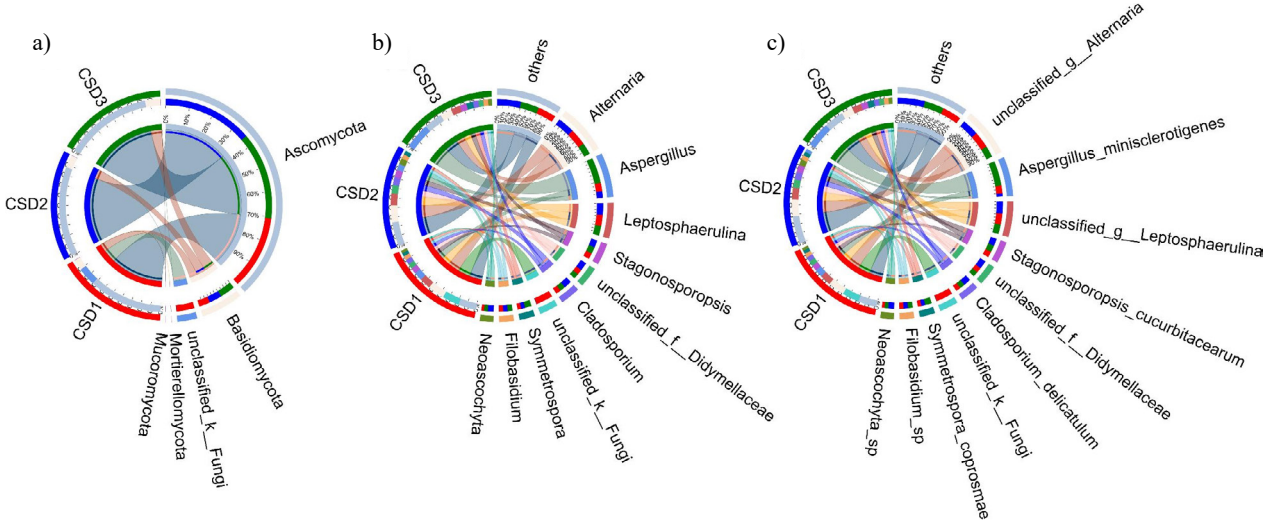

Fig. 5. Circos connection between tobacco leaf samples with varying harvesting maturity stages and the make up of fungal species: a) phylum, b) genus, c) species.

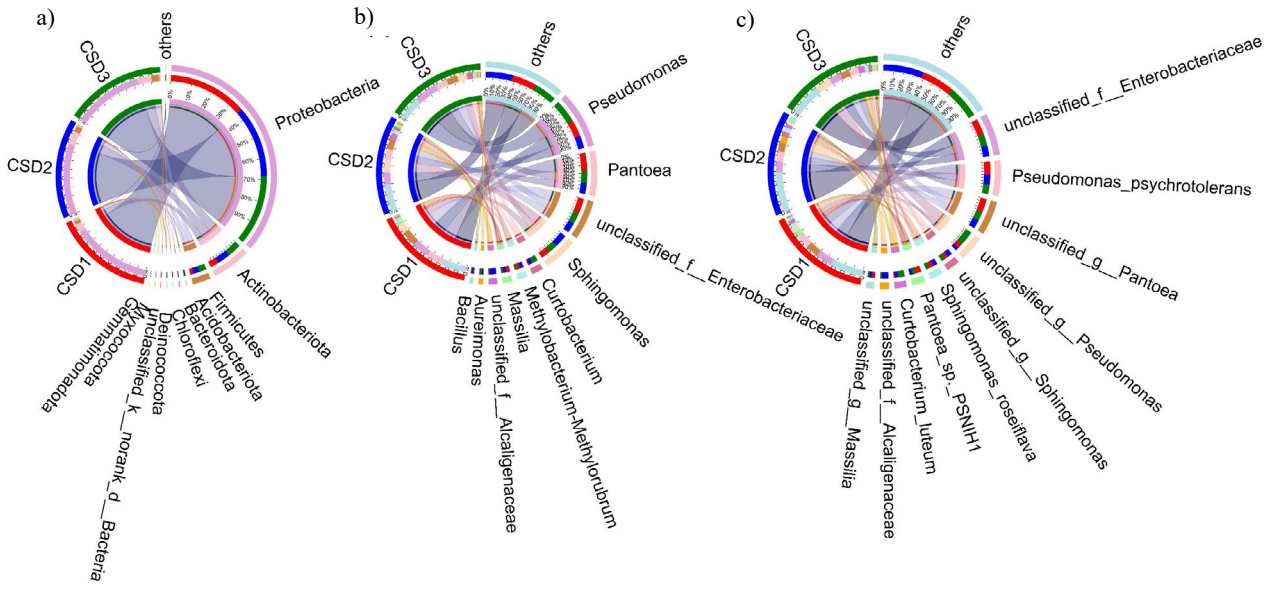

Fig. 6. Circos connection between tobacco leaf samples with varying harvesting maturity stages and the make up of Bacterial species.

fied_f_Enterobacteriaceae, and *Pantoea*) had the highest proportion in the CSD1 treatment, accounting for 42, 41, and 44%, respectively ($p<0.05$). The distribution proportion of four genera (*Aureimonas*, *unclassified_f_Alcaligenaceae*, *Methylobacterium-Methylorubrum*, and *Sphingomonas*) in the CSD2 treatment was significantly higher than that in the other treatments ($p<0.05$), accounting for 42, 83, 42, and 40%, respectively. The distribution proportions of three genera (*Bacillus*, *Curtobacterium,* and *Pseudomonas*) in the CSD3 treatment were significantly higher than those in the other treatments ($p<0.05$), accounting for 43, 59, and 44%, respectively (Fig. 6b).

At the species level of fungi, the distribution proportion of *unclassified_k_Fungi* in the CSD1 treatment was 99%, which was significantly higher than that in the CSD2 and CSD3 treatments ($p<0.001$). The distribution proportions of five species (*Cladosporium delicatulum*, *unclassified_f_Didymellaceae*, *Stagonosporopsis cucurbitacearum*, *unclassified_g_Leptosphaerulina*, and *unclassified_g_Alternaria*) in the CSD2 treatment were significantly higher than those in the CSD1 and CSD3 treatments ($p<0.05$), accounting for 44, 45, and 37, 37, and 38%, respectively. The distribution proportions of three species (*Filobasidium* sp., *Symmetrospora coprosmae*, and *Aspergillus minisclerotigenes*) in the CSD3 treatment were significantly higher than those in the CSD1 and CSD2 treatments ($p<0.05$), accounting for 36, 47, and 69%, respectively (Fig. 5c). At the species level of bacteria, five species (*unclassified_g_Massilia*, *Pantoea*_sp._*PSNIH1*, *unclassified_g_Pantoea*, *g_Pseudomonas_psychrotolerans*, and *unclassified_f_Enterobacteriaceae*) exhibited significantly higher distribution proportions in the CSD1 treatment than in the other treatments ($p<0.05$), accounting for 42, 47, 42, 40, and 41%, respectively. The distribution proportions of three species (*unclassified_f_Alcaligenaceae*, *Sphingomonas_roseiflava*, and *unclassified_g_Sphingomonas*) in the CSD2 treatment were significantly higher than those in the other treatments ($p<0.05$), accounting for 83, 36, and 41%, respectively. The distribution proportions of two species (*Curtobacterium luteum* and *unclassified_g_Pseudomonas*) in the CSD3 treatment were significantly higher than those in the other treatments, accounting for 59 and 65%, respectively (Fig. 6c).

3.2.3. PCoA, cluster analysis, and collinear network analysis of the fungal and bacterial communities of first-cured tobacco leaves with different harvest maturities

The results of the principal coordinate analysis (PCoA) of the fungal and bacterial community compositions of the cured tobacco leaves in the CSD1, CSD2, and CSD3 treatments are shown in Fig. 7. Principal component 1 (PC1) and principal component 2 (PC2) explained 54.61 and 27.37% (fungi) and 42.58 and 29.5% (bacteria) of the differences between the samples; the explanation of the total variance for all the samples were 81.98% (fungi) and 72.08% (bacteria). Bacterial and fungal communities in the CSD3 treatment were mainly distributed in the positive PC2 area, and samples in the CSD1 and CSD2 treatments were mainly distributed in the negative PC2 area, indicating that there were significant differences between CSD3, CSD1, and CSD2 in the fungal and bacterial species composition of the cured tobacco leaves.

3.2.4. Differences in apparent botanical traits and SPAD values of fresh tobacco leaves with different harvest maturities

As shown in Fig. 8, there were significant differences in apparent botanical traits among the treatments with diffe-rent harvest maturities. The CSD3 treatment showed brown leaves, leaf shrinkage, and yellow leaf veins. With the

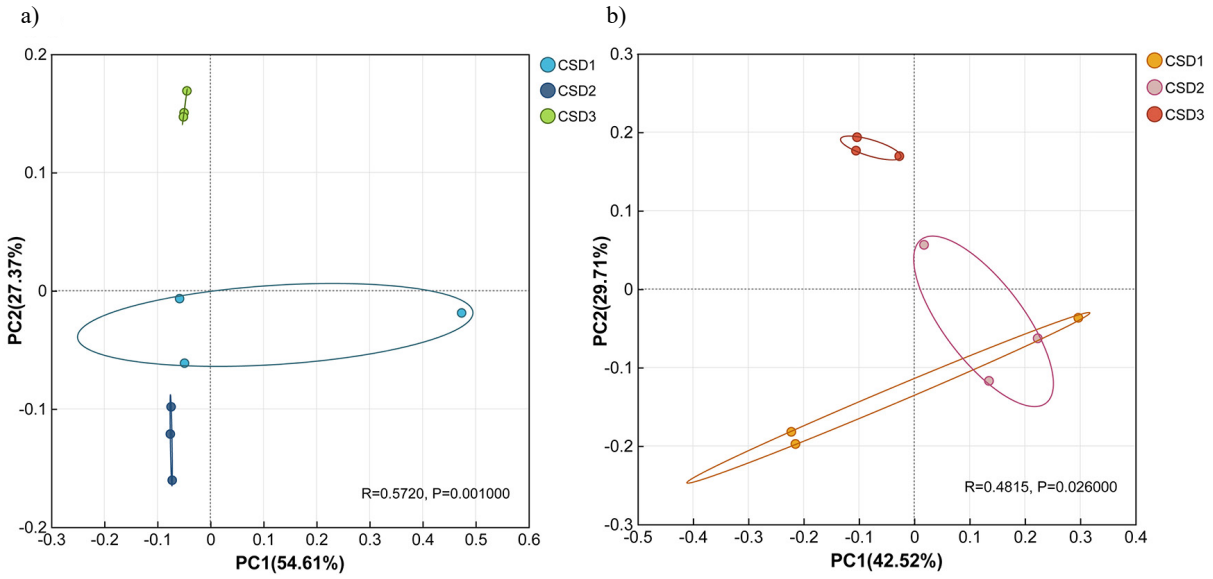

Fig. 7. PCoA study on the composition of fungal and bacterial communities in flue-cured tobacco leaves with different harvest maturity: a) fungi, b) bacteria.

Fig. 8. Variations in botanical characteristics across various harvesting ripeness treatments

delay of harvesting time, the soil plant analysis development (SPAD) values of the fresh tobacco leaves gradually decreased in the different treatments. The mean values of SPAD under the CSD1, CSD2, and CSD3 treatments were 30.62, 19.68, and 9.88, respectively (Fig. 9), the ranges were 4.5, 3.3, and 7.8, respectively, and the coefficients of variation were 5.65, 6.96, and 32.18%, respectively. The SPAD value of the CD3 treatment was quite different, decreasing by 67.73 and 49.80%, compared to those of CSD1 and CSD2, respectively, indicating that the maturity in the CSD3 treatment was significantly higher than those of CSD1 and CSD2.

3.3. Association analysis of the fungal community composition, enzyme activities, and phenolic acids in first-cured tobacco leaves with different harvest maturities

3.3.1. Differences in enzyme activities and phenolic acid contents in first-cured tobacco leaves under different harvest maturities

The differences in enzymatic activity and phenolic acid contents in the first-cured tobacco leaves with different harvest maturities are shown in Figs 10-11. The activities of POD, LOX, PAL, and AMY in the CSD3 treatment were all higher than those in the CSD1 and CSD2 treatments, with values of 6.07, 3.01, 19.55, and 1.13 U g^{-1}, respectively, and were increased by 57.66, 22.36, 43.75, 52.70, 31.10, 21.86, 67.81, and 66.18% ($p<0.05$), compared to the CSD1 and

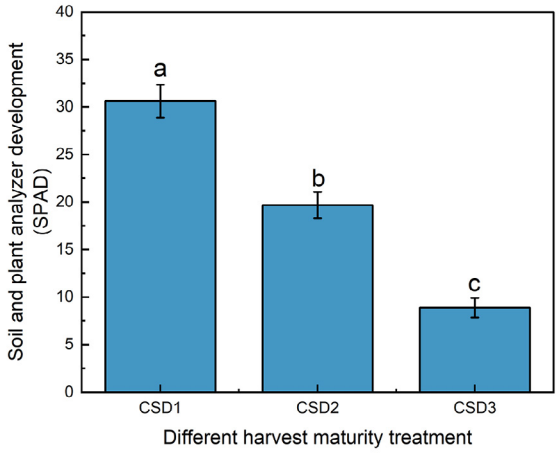

Fig. 9. SPAD values treated with different harvesting maturity.

other two treatments, at 3.59 and 0.37 μg g^{-1}, which were increased by 37.09, 117.58, 66.76, and 50.34% compared to those in CSD1 and CSD2 ($p < 0.05$). There were no significant differences in the contents of coumalic acid and ferulic acid among the three treatments (Fig. 11).

3.3.2. Spearman correlation analysis of the fungal and bacterial community composition, enzyme activities, and phenolic acids in first-cured tobacco leaves with different harvest maturities

The results of the Spearman association analysis of the fungal communities (genus level) and the enzyme activities and phenolic acids in the first-cured tobacco leaves with different harvest maturities are shown in Figs 12-13.

There was a substantial positive correlation between *Pseudopithomyces* and LOX. LOX and PH showed a substantial positive correlation with *Nigrospora*. POD, LOX,

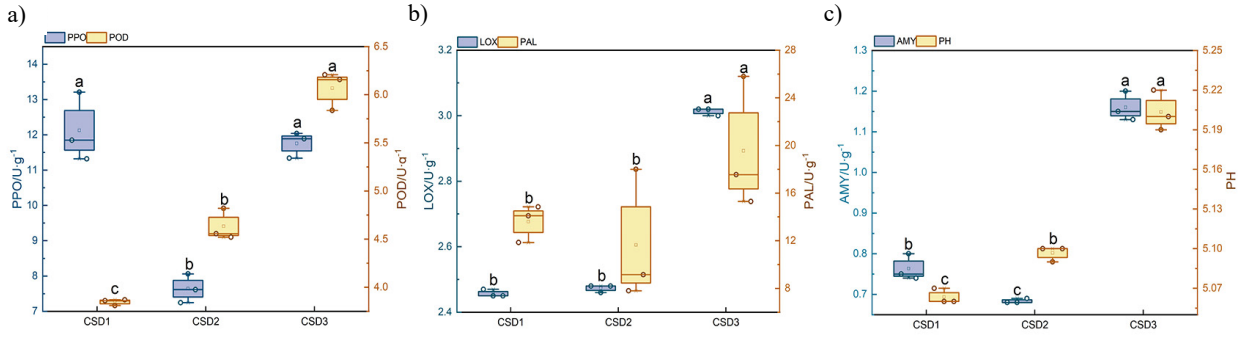

Fig. 10. Box diagram of enzyme activity of flue-cured tobacco leaves treated with different harvest maturity. PPO – Polyphenol oxidase, POD – Peroxidase, LOX – Lipoxygenase, PAL – Phenylalanine Ammonia-Lyase, AMY – α-Amylase.

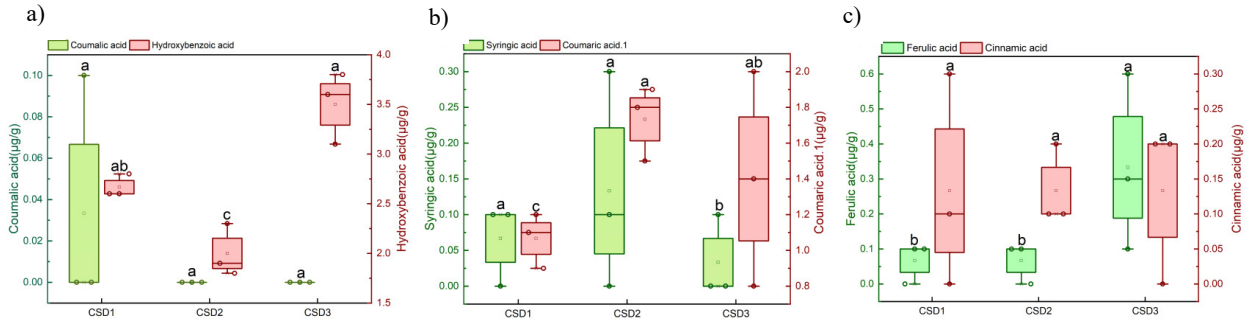

Fig. 11. Box plot of phenolic acid content in flue-cured tobacco leaves treated with different harvest maturity.

CSD2 treatments. The pH value increased with the increasing harvest maturity (Fig. 10). The contents of syringic acid and coumaric acid in the CSD2 treatment were significantly higher than those in CSD1 and CSD3, with values of 0.13 and 1.73 μg g^{-1}, respectively, which were 85.71, 61.68, 333.33, and 28.57% higher than those in CSD1 and CSD3. The contents of hydroxybenzoic acid and ferulic acid in the CSD3 treatment were significantly higher than those in the

and PH all showed a substantial positive correlation with *Symmetrospora*. LOX and PAL showed a substantial positive correlation with *Aspergillus*. AMY and *unclassified_o_Xylariales* showed a strong positive connection. Significantly unfavorable correlations were found between PAL and *Stemphylium*, *Phoma*, and *Cercospora*. There was a substantial negative correlation between AMY and *Unclassified_f_Botryti*s, *Epicoccum*, and *Didymellaceae*

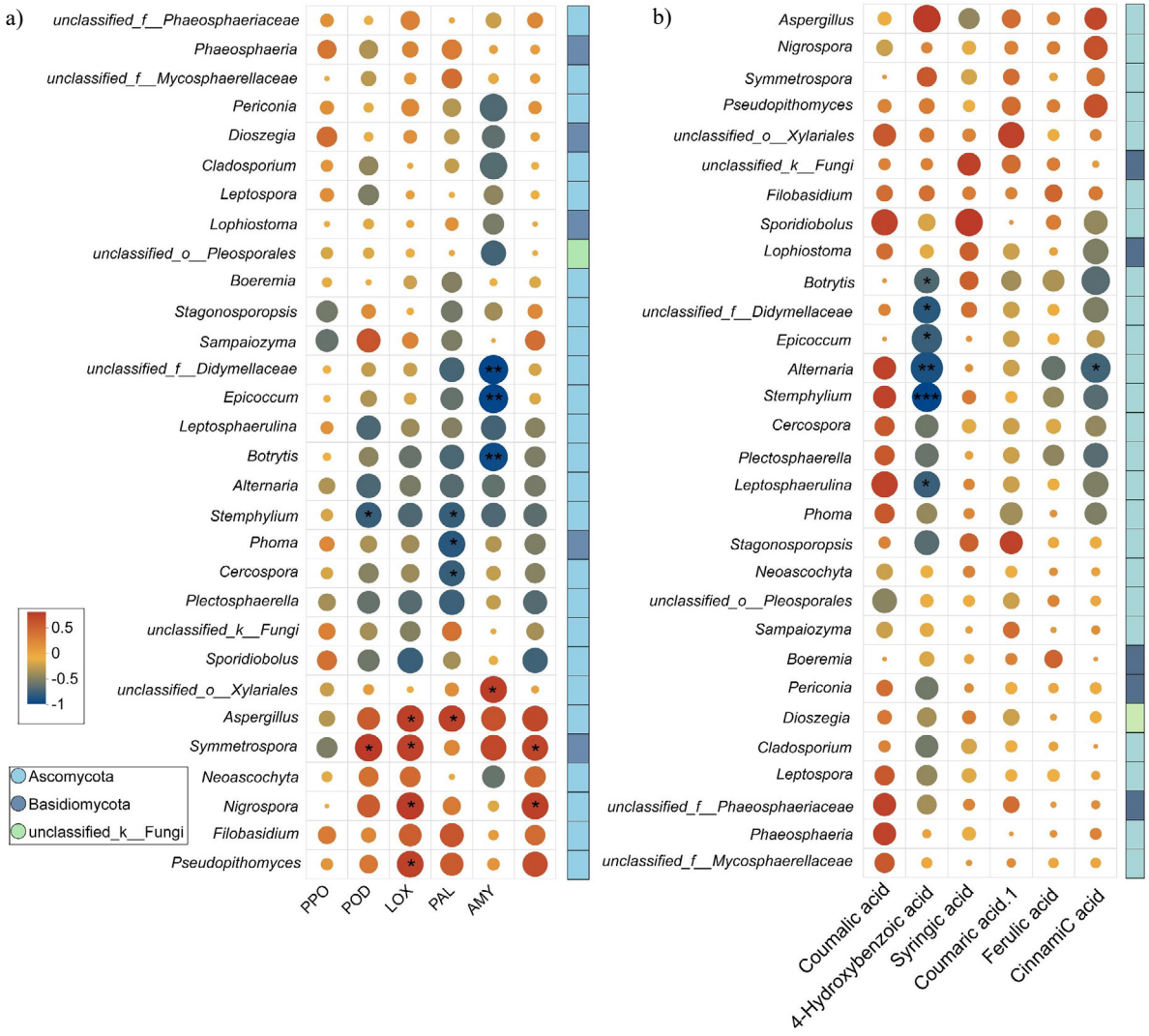

Fig. 12. Spearman correlation analysis of enzyme activity: a) phenolic acid, b) concentration and fungal community (genus level) of flue-cured tobacco leaves at varying harvest maturity levels.

(Fig. 12a). There was a substantial positive correlation between *Comamonas* and PPO. *Massilia* had a negative correlation with POD, LOX, and PH and a positive correlation with PPO. The relationships between *Allorhizobium*, *Neorhizobium*, *Pararizobium*, and *Rhizobium* and POD, LOX, AMY, and PH were substantially inverse. There was a substantial negative correlation between *Kocuria* and PPO. There was a substantial negative correlation between *Pseudomonas* and PPO and a favorable correlation with AMY. *Curtobacterium* showed a strong positive correlation with both PH and LOX. *Terribacillus* and *Exiguobacterium* had a negative correlation with PPO and a positive correlation with POD, LOX, and PH. *Unclassified_f_Erwiniaceae* and AMY showed a strong positive connection (Fig. 13a).

In varied degrees, there was additionally a negative correlation between coumalic acid and *Botrytis*, *unclassified_f_Didymellaceae*, *Epicoccum*, *Alternaria*, *Stemphylium*, and *Leptosphaerulina*. Additionally, there was a negative correlation between *Alternaria* and cinnamic acid (Fig. 12b). The analysis showed a substantial positive correlation between *Pseudomonas* and syringic acid and 4-hydroxybenzoic acid. Cinnamic acid showed a substantial positive correlation with both *Curtobacterium* and *Terribacillus*. A substantial negative correlation was found between *Corynebacterium* and coumalic acid (Fig. 13b).

3.4. Differences and correlation among the contents of aroma components and chemical components in first-cured tobacco leaves with different harvest maturities

3.4.1. Differences in the contents of aroma components and chemical components in first-cured tobacco leaves with different harvest maturities

The differences in the contents of aroma components in the first-cured tobacco leaves with different harvest maturities are shown in Table 2. The CSD3 treatment had higher levels of cembranoids, carotenoid degradation products,

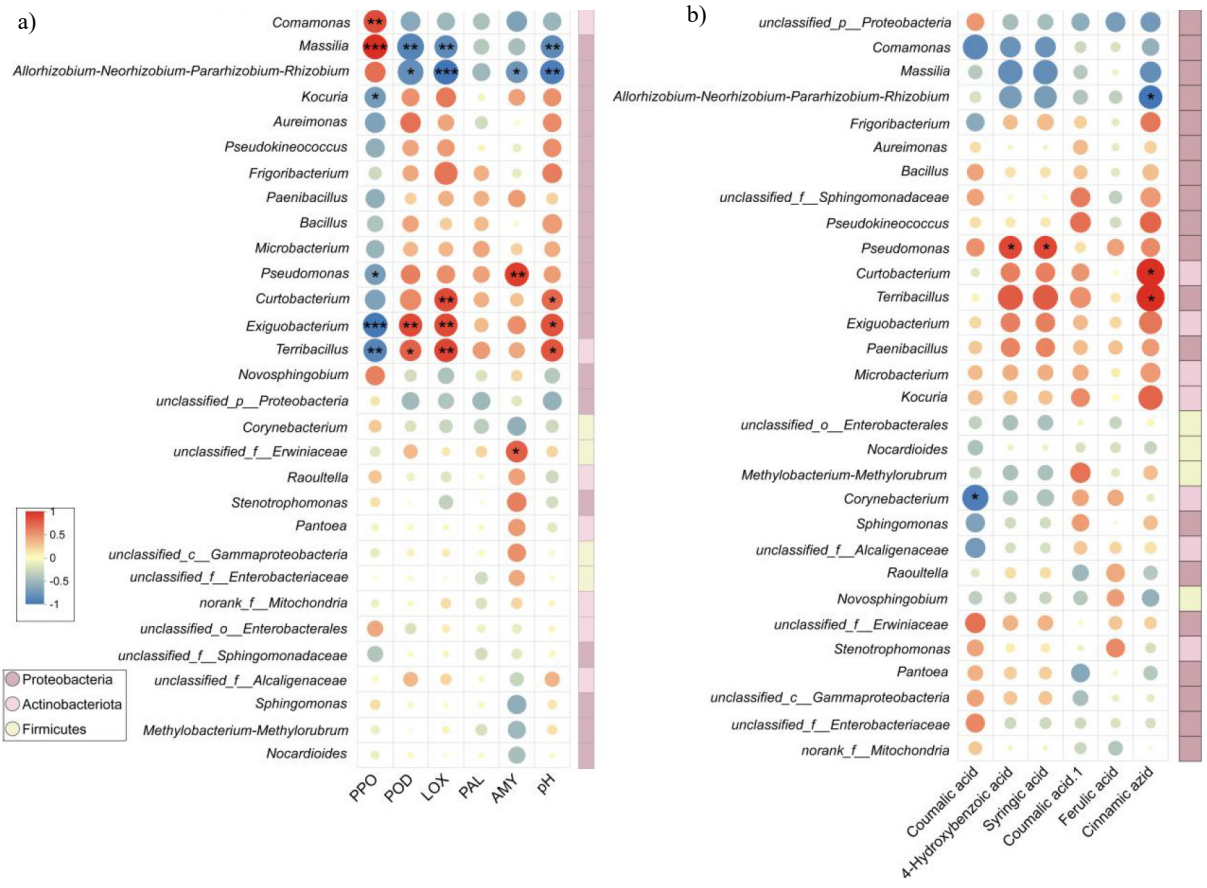

Fig. 13. Spearman correlation analysis of enzyme activity (a), phenolic acid (b) concentration and Bacteria community (genus level) of flue-cured tobacco leaves at varying harvest maturity levels.

phenylalanine, and neophytadiene than CSD1 and CSD2. They were 22.58, 27.47, 10.23, and 201.69 μg g^{-1} ($p<0.05$), respectively, which were increased by 5.12, 17.44, 12.29, and 21.35% ($p<0.05$) and by 20.30, 17.95, 34.25, and 8.42% ($p<0.05$) compared to the CSD1 and CSD2 treatments, respectively. The nicotine content was reduced by 36.33 and 39.87% ($p<0.05$), respectively, compared to the CSD1 and CSD2 treatments.

The differences in the chemical component contents in the first-cured tobacco leaves with different harvest maturities are shown in Table 3. The contents of reducing sugars, total nitrogen, total alkaloids, and potassium oxide in the CSD3 treatment were lower than those in the CSD1 and CSD2 treatments by 6.45, 35.82, 76, and 19.71% ($p<0.05$) and by 14.76, 50, 97.6, and 19.71% ($p<0.05$), respectively. There was no significant difference in the total sugar and potassium oxide contents.

3.4.2. Correlation analysis of the bacterial communities (genus level) and the aroma and chemical components of first-cured tobacco leaves with different harvest maturities

The correlation matrix between the contents of aroma components in the tobacco leaves and the fungal and bacterial species at the genus level is shown in Table 4. The content of cembranoids was significantly negatively correlated with *unclassified_f_Alcaligenaceae*, *Alternaria* and *unclassified_f_Didymellaceae* but significantly positively correlated with *Aspergillus* and *Pseudomonas*. The contents of carotenoid degradation products were significantly positively correlated with *Symmetrospora* and *Pseudomonas* and extremely significantly positively correlated with *Aspergillus* and *Curtobacterium*. The content of Maillard reaction species was significantly negatively correlated with *Stagonosporopsis*. The content of phenylalanine was significantly positively correlated with *Aspergillus*, *Symmetrospora*, and *Pseudomonas*, but significantly negatively correlated with *unclassified_f_Didymellaceae*. The content of other aroma compounds was extremely significantly positively correlated with *Aspergillus*, *Symmetrospora*, and *Pseudomonas*. The content of neophytadiene was significantly positively correlated with *Aspergillus*, *Symmetrospora*, *Curtobacterium*, and *Pseudomonas*. The nicotine content was extremely significantly negatively correlated with *Aspergillus*, *Symmetrospora*, and *Pseudomonas* but significantly positively correlated with *Massilia*.

The correlation matrix between the chemical component contents in the tobacco leaves and the fungal and bacterial species at the genus level is shown in Table 5. There was

Table 2. Variations in components responsible for scent in flue-cured tobacco leaves at several stages of harvest ripeness

Ingredients in fragrances (μg g^{-1})		CSD1	CSD2	CSD3
Cembranoid degradation products	6,8-Nonadien-2-one,8-methyl-5-(1-methylethyl)-,(E)-	21.48±2.86a	18.77±4.09b	22.58±3.45a
Carotenoid degradation products	Linalool	0.24±0.06b	0.21±0.04c	0.28±0.13a
	Damastone	4.38±0.33b	4.33±1.91b	5.54±0.48a
	α_Damarone	1.05±0.02b	1.13±0.01b	1.22±0.01a
	Geranyl acetone	2.19±0.15ab	2.01±0.28b	2.56±0.03a
	MegastigmineA	5.53±0.05b	5.95±0.04b	6.80±0.03a
	MegastigmineB	4.40±0.09b	4.55±0.44b	5.03±0.03a
	MegastigmineC	1.76±0.38b	1.75±0.05b	1.85±0.16a
	3-Hydroxy Damarone	0.56±0.30b	0.54±0.20c	0.61±0.05a
	Solavetivone	1.74±0.02b	1.51±0.01c	1.94±0.04a
	Dihydroactinidiolide	1.30±0.01ab	1.09±0.01b	1.37±0.01a
	3-Oxygen- α- Ionol	0.24±0.11ab	0.23±0.10b	0.28±0.03a
	Funerary aldehyde	0.19±0.02b	0.20±0.02	0.27±0.18a
Maillard reaction products	3-Furaldehyde	1.08±0.02ab	1.05±0.03b	1.10±0.02a
	2-Furanmethanol	0.16±0.01ab	0.13±0.01b	0.17±0.03a
	Furan, 2,3-dihydro-	0.12±0.03b	0.12±0.01b	0.18±0.01a
	2(5H)-Furanone,3-methyl-	0.83±0.03a	0.22±0.02a	0.32±0.02a
	Benzofuran,2,3-dihydro-	0.23±0.01b	0.19±0.02b	0.43±0.02a
	2-Methoxy-4-vinylphenol	1.81±0.13a	2.00±0.12a	1.94±0.12a
	2,3'-Dipyridyl	1.52±0.02a	1.57±0.04a	1.83±0.13a
	2-Furancarboxaldehyde,5-methyl-	0.40±0.44a	0.33±0.03a	0.33±1.31a
	Ethanone,1-(1H-pyrrol-2-yl)-	0.31±0.42a	0.36±0.72a	0.33±0.75a
	Ethanone,1-(3-pyridinyl)-	0.12±0.19a	0.22±0.04a	0.17±0.05a
	Benzaldehyde	0.10±0.03b	0.11±0.16ab	0.13±0.02a
	Benzylalcohol	3.81±0.02b	2.44±0.09c	4.87±0.12a
	Benzeneacetaldehyde	1.00±0.01b	1.15±0.02b	1.41±0.01a
	PhenylethylAlcohol	2.40±0.45a	2.00±0.28ab	1.40±0.71c
	Indole	0.31±0.01a	0.13±0.02b	0.32±0.16a
	2,4-Di-tert-butylphenol	0.36±0.19a	0.51±0.20a	0.53±0.50a
	Dibutyl phthalate	1.13±0.03b	1.27±0.02b	1.56±0.04a
	Phytol	9.52±0.07b	9.43±0.08b	10.86±0.10a
	Nonanal	0.31±0.43c	0.37±0.09b	0.50±0.50a
Phenylalanine degradation products	9,12,15-Octadecatrienoicacid,(Z,Z,Z)-	16.62±0.70b	15.47±0.72c	19.22±0.40a
	n-Hexadecanoicacid	54.75±3.92b	53.42±0.73b	61.25±2.10a
	Thunbergol	16.99±1.89a	14.55±5.17a	20.25±0.69a
	Methyltetradecanoate	143.48±11.57a	141.36±2.01a	150.47±0.70a
Pyridine, 2-(1-methyl-2-pyrrolidin	Pyridine,2-(1-methyl-2-pyrrolidinyl)-	41.24±1.00a	42.31±0.57a	30.25±0.55b
Neophytene	Neophytadiene	166.20±25.86b	186.02±4.35ab	201.69±1.67a

Mean ± standard deviation values, with distinct lowercase letters denoting significant differences in treatment efficacy ($p \leq 0.05$).

Table 3. Variations in the chemical composition of flue-cured tobacco leaves at several stages of harvest maturity

Dispose	Total sugar	Reducing	Total nitrogen	Nicotine	Potassium oxide	Chloride ion
CSD1	30.06±0.69a	31.02±0.29b	1.82±0.01b	2.20±0.06b	1.64±0.01a	0.64±0.03a
CSD2	30.49±0.62a	33.44±1.90a	2.01±0.02a	2.47±0.02a	1.64±0.04a	0.68±0.08a
CSD3	30.11±0.56a	29.14±0.60b	1.34±0.02c	1.25±0.09c	1.37±0.04b	0.57±0.05a

Mean ± standard deviation values, with distinct lowercase letters denoting significant differences in treatment efficacy ($p \leq 0.05$).

Table 4. Correlation matrix between first-cured tobacco leaves' aromatic components and the fungi and bacteria (genus level)

Composition of the microbial community (Genus level)		Product				Other	Neophytene	Pyridine, 2-(1-methyl-2-pyrrolidin
		Cembranoid degradation	Carotenoid degradation	Maillard reaction	Phenylalanine degradation			
Fungi	*Alternaria*	-0.60	-0.25	-0.50	-0.20	-0.22	-0.50	0.30
	Aspergillus	0.57	0.87	-0.13	0.72	0.82	0.77	-0.82
	Leptosphaerulina	-0.57	-0.15	-0.55	-0.33	-0.05	-0.35	0.28
	Stagonosporopsis	-0.22	-0.02	-0.67	-0.12	0.18	0.10	0.12
	Unclassified_f__ Didymellaceae	-0.82	-0.30	-0.50	-0.68	-0.25	-0.25	0.53
	Cladosporium	-0.43	0.07	-0.22	-0.38	-0.13	-0.17	0.45
	Unclassified_k__ Fungi	0.08	-0.52	-0.06	-0.11	-0.39	-0.45	0.29
	Symmetrospora	0.57	0.72	0.02	0.65	0.85	0.70	-0.85
	Filobasidium	0.20	0.28	-0.12	0.00	0.43	0.17	-0.20
	Neoascochyta	-0.50	0.00	-0.23	-0.55	-0.07	0.13	0.32
Bacteria	*Pseudomonas*	0.85	0.57	0.38	0.70	0.72	0.57	-0.83
	Pantoea	0.30	-0.18	0.63	0.08	0.00	-0.32	-0.17
	Unclassified_f__ Enterobacteriaceae	0.35	-0.17	0.57	0.08	-0.03	-0.43	0.05
	Sphingomonas	-0.42	0.05	-0.47	-0.28	-0.22	0.22	0.37
	Curtobacterium	0.48	0.77	0.48	0.35	0.43	0.62	-0.33
	Methylobacterium-Methylorubrum	-0.30	0.18	-0.55	-0.08	0.02	0.25	0.20
	Massilia	-0.45	-0.78	-0.07	-0.53	-0.63	-0.88	0.65
	Unclassified_f__ Alcaligenaceae	-0.50	-0.10	-0.42	-0.55	-0.20	0.22	0.43

a significant positive correlation between the total sugar content and *Sphingomonas*. The reducing sugar content was significantly negatively correlated with *Aspergillus*, *Symmetrospora*, and *Pseudomonas* and significantly positively correlated with *Massilia*. The total nitrogen content was significantly negatively correlated with *Aspergillus*, *Symmetrospora*, and *Pseudomonas* and significantly positively correlated with *unclassified_f_Didymellaceae* and *Massilia*. The total plant alkaloid content was significantly negatively correlated with *Aspergillus*, *Symmetrospora*, and *Pseudomonas* and positively correlated with *unclassified_f_Didymellaceae*, *Symmetrospora*, and *Massilia*. The chloride ion content was significantly positively correlated with *Cladosporium*, *Leptosphaerulina*, *Massilia*, and *unclassified_f_Didymellaceae* and extremely significantly negatively correlated with *Symmetrospora* and *Pseudomonas*. The potassium oxide content was significantly negatively correlated with *Symmetrospora* and *Pseudomonas*.

4. DISCUSSION

Maturity has a great impact on tobacco leaf quality. It is used as an important tobacco leaf quality factor in the actual production of flue-cured tobacco (Gong, 2003). In this study, there were significant differences in apparent botanical traits among the treatments with different harvest maturities. Under the 14-d delayed harvesting treatment,

Table 5. Correlation matrix between chemical composition and fungi and bacteria (genus level) of fresh-cured tobacco leaves

Composition of the microbial community (Genus level)		Total sugar	Reducing	Total nitrogen	Nicotine	Potassium oxide	Chloride ion
Fungi	*Alternaria*	-0.20	0.34	0.35	0.58	0.56	0.38
	Aspergillus	-0.13	-0.71	-0.79	-0.68	-0.59	-0.38
	Leptosphaerulina	0.03	0.32	0.37	0.58	0.63	0.25
	Stagonosporopsis	-0.12	-0.03	0.16	0.39	0.08	-0.32
	Unclassified_f__Didymellaceae	0.23	0.59	0.69	0.88	0.58	0.25
	Cladosporium	0.62	0.28	0.49	0.54	0.78	0.53
	Unclassified_k__Fungi	-0.08	0.19	0.06	-0.19	0.15	0.12
	Symmetrospora	-0.52	-0.70	-0.74	-0.59	-0.85	-0.75
	Filobasidium	0.02	-0.14	-0.20	-0.29	-0.07	-0.27
Bacteria	*Pseudomonas*	-0.47	-0.77	-0.83	-0.85	-0.89	-0.77
	Pantoea	-0.55	-0.04	-0.17	-0.36	-0.34	-0.28
	Unclassified_f__Enterobacteriaceae	-0.42	-0.13	-0.03	-0.23	-0.08	-0.20
	Sphingomonas	0.73	0.24	0.37	0.47	0.46	0.47
	Curtobacterium	0.48	-0.49	-0.29	-0.36	-0.20	0.00
	Methylobacterium-Methylorubrum	0.50	-0.01	0.23	0.48	0.40	0.23
	Massilia	-0.03	0.59	0.56	0.44	0.68	0.38
	Unclassified_f__Alcaligenaceae	0.60	0.41	0.50	0.54	0.28	0.18
	Aureimonas	0.15	-0.15	0.03	0.26	-0.22	-0.27
	Bacillus	-0.07	-0.36	-0.41	-0.15	-0.33	-0.17

the leaves were brownish-yellow with a shrunken morphology and yellow veins, which was significantly different from the conventional harvesting and 7-d delayed harvesting treatments. With the delay of harvesting time, the SPAD value of the fresh tobacco leaves in the different treatments gradually decreased (Wang, 2020).

Microorganisms play an important role in the fermentation process of cured tobacco leaves. To study the diversity and function of microorganisms on the surface of first-cured tobacco leaves with different harvest maturities, a total of nine samples subjected to three treatments were selected as the research objects. The samples were analyzed by Illumina MiSeq high-throughput sequencing analysis, which revealed that Ascomycota, Basidiomycota, Mortierellomycota, Proteobacteria, and Actinobacteria were the dominant bacterial and fungal phyla in the tobacco leaf samples. *Aspergillus*, *Leptosphaerulina*, and *Pseudomonas* were the dominant bacterial and fungal genera in the tobacco leaf samples. The relative abundance of *Aspergillus*, *Pseudomonas*, and *Bacillus* in the 14-d delayed harvesting treatment was significantly higher than that in the other two treatments. *Aspergillus* is prone to causing mildew during the fermentation and storage of tobacco leaves, but some species also have a promoting effect on tobacco leaf fermentation (Zhang L. *et al.*, 2018). He *et al.* (2013) showed that *Penicillium verruculosum* can effectively reduce the pectin content and tar content in tobacco leaves. *Bacillus*, *Sphingomonas*, *Pantoea*, and *Pseudomonas* are all bacterial genera that account for a large proportion in cured tobacco leaves (Ding, 2024). Among them, *Bacillus* can secrete a variety of hydrolytic enzymes to convert tobacco cembranes into hydroxy analogs and improve the odor and taste of tobacco leaves (Wei *et al.*, 2024). *Bacillus* can also effectively degrade nicotine, lignin, cellulose, and starch in tobacco stems, have a positive effect on the aging fermentation of cured tobacco leaves, and improve the quality of tobacco leaves (Su, 2015). *Pseudomonas* can reduce the nicotine content in flue-cured tobacco, reduce the irritant effect and unpleasant smoke aroma of tobacco leaves, and improve the smoking quality of tobacco leaves (Li *et al.*, 2017). The relative abundance of *Bacillus* and *Pseudomonas* in the 14-d-delayed harvesting treatment was the highest, accounting for 25%, which was consistent with the results reported by Huang *et al.* (2012) and Wu *et al.* (2021). Moreover, with the delay of harvesting time, the abundance of bacterial OTUs gradually decreased in the samples, and the abundance of fungal OTUs showed a high-low-high trend. This may be related to the water content in tobacco leaves. The higher the maturity of tobacco leaves is, the weaker the water retention ability during the curing process (Song *et al.*, 2014). The water content in tobacco leaves changes during the natural moisture retention process. This may be the cause of the differences in the fungal and bacterial OTU abundance.

When macromolecular compounds on the surface of tobacco leaves work together with enzymes and microorganisms, they can efficiently break down macromolecular substances in cigarettes and change harmful substances, which drastically lowers the amount of harmful ingredients in cigarettes and enhances the quality of tobacco leaves (Zhou, 2018). *Filobasidium* can produce a variety of cell secretion enzymes and degrade macromolecular organic compounds in tobacco leaves to produce aroma substances (Han, 2018). The contents of hydroxybenzoic acid and coumaric acid in the 14-day delayed treatment were significantly higher than those in the other two treatments. These acids cause tobacco leaves to emit a pleasant aroma (Ma and Daugulis, 2014). Related byproducts play a key role in the synthesis of polyphenolic aroma substances in tobacco leaves, such as rutin, caffeic acid, chlorogenic acid, and scopolamine (Zhang Z.N. *et al.*, 2018). In this study, *Aspergillus*, *Symmetrospora*, *Filobasidium*, and *Pseudomonas* showed significant correlations with the enzyme activities and phenolic acid contents in the tobacco leaves ($p < 0.05$). It was speculated that these bacteria may produce some hydrolases. Under the catalysis of these enzymes, polyphenols were produced, thus affecting the content of phenolic acids (Chen *et al.*, 2016), which was generally consistent with the results obtained by Zhou *et al.* (2013) and Breidenbach (2015).

Wang *et al.* (2015) showed that *Bacillus* can degrade macromolecular proteins into amino acids, thereby improving the taste of tobacco leaves. Jiang *et al.* (2021) found that the chemical component contents in tobacco leaves treated with fungal agents changed and the starch content in tobacco leaves decreased to varying degrees. Microbial metabolites contribute to the degradation of starch, protein, and other macromolecules in tobacco leaves into precursor substances to produce their aromas and improve their quality. The results of this study showed that the contents of aroma and chemical components in the flue-cured tobacco leaves were significantly different between the different harvest maturity treatments. The 14-d delayed harvesting treatment showed an increase in the content of cembranoids, carotenoid degradation products, phenylalanines, and neophytadiene and a decrease in the content of nicotine, compared to the conventional and 7-d delayed harvesting treatments. The contents of chemical components in the 14-d delayed harvesting treatment were the closest to the percentage chemical composition of high-quality tobacco leaves. The correlation analysis results for aroma component contents, chemical component contents, and microbial community composition (genus level) in the tobacco leaves showed that *Aspergillus*, *Symmetrospora and Pseudomonas* were highly positively correlated with the content of aromatizing substances in the tobacco leaves ($p<0.05$) and negatively correlated with nicotine. The contents of cembranoids, carotenoid degradation products, phenylalanine, neophytadiene, and other aromatizing substances in the 14-d delayed harvesting treatment were significantly higher than those in the other two treatments ($p<0.05$), while the opposite trend was found for the nicotine content. These results suggest that *Aspergillus*, *Symmetrospora and Pseudomonas*can significantly increase the content of aroma substances in tobacco leaves. Additionally, the nicotine content was reduced. These results emphasized that the endophytic microbial community plays an important role in the development of tobacco leaf quality. In the future, we will further explore the functions of relevant microbial communities using traditional isolation and culture methods and metagenomics.

5. CONCLUSIONS

In this study, the high-throughput sequencing technology was used to analyze fungi and bacteria in tobacco leaves with different maturity. The results showed that Proteobacteria, Actinobacteria, and Ascomycota were the dominant bacteria in the tobacco leaves. *Aspergillus*, *Symmetrospora*, *Filobasidium*, *Pseudomonas*, and *Pseudonocardia* were highly correlated with enzyme activity, phenolic acid content, and aroma component content in the tobacco leaves. The chemical composition content in the tobacco leaves was coordinated and the quality was higher after 14 days of delayed harvest. The results of this study can lay a foundation for formulating strategies to improve tobacco quality, such as adjusting the harvest time and regulating the microbial community.

Conflict of interests: The authors declare no conflict of interest.

6. REFERENCES

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