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Dynamic characteristics and co-occurrence patterns of microbial community in tobacco leaves during the 24-month aging process

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Abstract

Purpose: Microorganisms are important in tobacco aging. These are used to improve the quality of tobacco leaves after threshing and redrying. However, the response of microbial community to the storage environment and time during the tobacco aging process has been less explored. This study aimed to characterize the dynamic changes in microbial community composition and diversity in tobacco leaf samples.

Methods: In this study, 16S and ITS rRNA gene amplicon sequencing techniques were used to characterize the composition, diversity, and co-occurrence of the microbial community in tobacco leaves stored in two different cities during the 24-month aging. Furthermore, the activities of several enzymes were measured spectrophotometrically, and the correlation between the microbiota and enzyme activity was analyzed by network analysis.

Results: Shannon diversity and Chao richness of bacterial communities gradually increased during the first 18 months, whereas those of the fungal community decreased. The relative abundance of *Proteobacteria* decreased, whereas that of *Actinobacteria* and *Bacteroidetes* increased. The proportion of *Ascomycota* gradually increased during the first 18 months and then rapidly decreased, whereas the proportion of *Basidiomycota* exhibited a completely opposite pattern. The change in the composition of bacterial community and dominant genera in leaves was not significant between Guiyang city and Maotai city storerooms, but that in the fungal community was significant. The network analysis revealed that fungal networks were more complex and compact than bacterial networks, and a strong negative correlation existed between bacteria and fungi. Moreover, the bacterial microbiome showed a strong positive association with amylase activity, while the fungal microbiome positively correlated with cellulase activity.

Conclusions: This study demonstrated a significant spatiotemporal heterogeneity in the composition of the microbial community during tobacco aging and highlighted the possible influence of the interactions and enzyme activity on microbial diversity and composition. The findings provided a scientific basis for using microorganisms to regulate and control tobacco aging.

Keywords: Co-occurrence patterns, Enzymatic activity, Microbial community diversity, Network analysis, Tobacco aging

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Introduction

Flue-cured tobacco is an important non-edible economic crop, but unaged tobacco is not suitable for making cigarette products directly because it has a sharp, disagreeable odor and an undesirable aroma, and produces irritating smoke (Zhao et al. 2007). The aging process takes approximately 2 years and is typically used to improve the quality of flue-cured tobacco because it can greatly improve the aroma and color, reduce smoke irritation, and improve the overall tobacco quality (Su et al. 2011). Increasing evidence shows that microorganisms are crucial in the aging process. Microbial metabolism can promote the transformation of proteins, starch, cellulose, and other macromolecules in the tobacco leaf substrate, thus increasing the contents of aromatic compounds such as ionone (Maldonado-Robledo et al. 2003) and reducing the contents of harmful components such as nicotine and tobacco-specific nitrosamines (Liu et al. 2015; Vigliotta et al. 2007).

Modern molecular biology techniques can be used to improve the understanding of tobacco-associated microbial community structure and function. Microbial community diversity has been investigated in tobacco and tobacco products using culture-independent molecular biology techniques, such as denaturing gradient gel electrophoresis (Zhao et al. 2007), 16S rRNA clone libraries (Su et al. 2011), restriction fragment length polymorphisms (Huang et al. 2010), and Illumina MiSeq sequencing (Law et al. 2016). *Sphingomonas*, *Bacillus*, *Pseudomonas*, *Lactococcus*, *Stenotrophomonas*, *Acinetobacter*, *Methylobacterium*, *Erwinia*, and *Pantoea* were identified as the predominant bacterial populations; and *Neophaeosphaeria*, *Cladosporium*, *Lodderomyces*, *Tilletiopsis*, *Symmetrospora*, *Xeromyces*, *Wallemia*, *Rhodotorula*, *Cercospora*, *Alternaria*, *Cryptococcus*, and *Aspergillus* were identified as the prevalent fungal groups (Villemur et al. 2009; Ye et al. 2017; Wang et al. 2018; Zhang et al. 2020; Zhou et al. 2020).

Alternatively, the structure and function of microbial communities are usually dynamic and affected by environmental conditions (Kinkel 1997). Storage conditions also significantly affect the composition of tobacco microbial communities (Larsson et al. 2008). During the tobacco aging process, tobacco leaves (e.g., sources and varieties) and storage conditions (e.g., temperature and humidity) provide a variety of niches for microbial growth and reproduction, and these factors determine the tobacco microbe species, abundance, and evolution (Chopyk et al. 2017). Therefore, tobacco companies usually transfer local tobacco leaves to other cities for aging. However, the response of microbial communities to different storage environments remains unclear.

Microorganisms are the main driving force behind the circulation and transformation of the tobacco leaves through the whole process of aging. However, a dry environment is a necessary prerequisite for tobacco fermentation or aging. In industrial production, the moisture content of tobacco leaves should not exceed 13% and the relative humidity of the environment should not exceed 70% during the aging process (Lian et al. 1998), restricting the growth of most microorganisms. Furthermore, the relationships between microbial taxa also shape the microbial community structure (Barberán et al. 2012). The network analysis of significant taxon co-occurrence patterns can help reveal the structure of complex microbial communities across spatial or temporal gradients. Recently, the network analysis has been widely applied to reveal ecological linkages among microorganisms in complex ecosystems, such as in biogas digesters (Rui et al. 2015), sludge (Zhao et al. 2014), plants (Cao et al. 2020), soil (Shetty et al. 2017), and humans (Sun et al. 2011). However, the existence of direct or indirect interactions among microbial taxa in tobacco leaves during aging remains unclear. This study characterized the dynamic changes in microbial community composition and diversity in tobacco leaf samples collected from five different periods in Guiyang city (GY) and Maotai city (MT) for 2 years by employing high-throughput sequencing technology. Additionally, the network analysis was also used to reveal the correlation among microbial taxa and enzyme activities that coexisted in tobacco leaves during the aging process.

Materials and methods

Sample collection

Tobacco leaves were sampled from a warehouse located in GY and MT, Guizhou Province, China, and collected by China Tobacco Guizhou Industry Co., Ltd. (Guiyang, China) between July 2015 and July 2017. Guiyang is about 1100 m above sea level, with a monthly mean relative humidity ranged from 70.0 to 89.2%, and a monthly mean temperature of 24.3 °C in summer and 4.1 °C in winter. Maotai is about 400 m above sea level, with a monthly mean relative humidity ranged from 67.3 to 84.8%, and a monthly mean temperature of 27.2 °C in summer and 5.4 °C in winter.

The grade of all samples was C3F (the third-grade tobacco leaves with yellow-orange color in the middle of the plant), and all tobacco plants were harvested from Baoshan, Yunnan Province, China, in 2014. In total, 200 kg tobacco leaves were aged for 24 months in a tobacco carton (1080-mm long × 650-mm wide × 685-mm high), maintained at room temperature (6–32 °C), and sampled after 0, 6, 12, 18, and 24 months. During each of the five sampling periods, 1.5 kg tobacco leaves were arbitrarily collected from 15 cm below the top of the cartons by the five-point method. A total of 10 samples were obtained as follows: 0 months (GY-0 and MT-0), 6 months (GY-6 and MT-6), 12 months (GY-12 and MT-12), 18 months

(GY-18 and MT-18), and 24 months (GY-24 and MT-24). All samples were packed in sterile plastic bags and transported to the Institute of Fungal Resources at Guizhou University for storage at – 20 °C. In the following experiment, 500 g tobacco leaves were weighed and ground with liquid nitrogen; then, 100 g ground tobacco leaves were weighed for DNA extraction, and the rest were tested for enzyme activity.

Determination of enzymatic activity in tobacco leaves during the aging process

According to the color reaction between the degradation products (glucose, maltose, and galacturonic acid) and 3, 5-dinitrosalicylic acid (DNS), the activities of amylase, cellulose, and pectinase were measured spectrophotometrically (Malhotra et al. 2000; Wang et al. 2008; Su et al. 2016). Each sample was repeated three times. Independent sample *t* test and one-way analysis of variance (ANOVA) were employed to assess the statistical significance of enzymatic activities under different spatiotemporal conditions using SPSS Statistics version 19 (IBM, IL, USA). In all cases, one unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar under the assay conditions (Minjares-Carranco et al. 1997). The enzyme activity was expressed in units of U g^{-1}.

DNA extraction and MiSeq sequencing

The microbial genomic DNA was extracted using the E.Z.N.A. soil kit (Omega Biotek, GA, USA), and the DNA concentration and purity were monitored by electrophoresis on 1% (w/v) agarose gels. The primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V3–4 variable region of the bacterial 16S rRNA gene (Xu et al. 2016). The fungal ITS gene was amplified with the primers ITS1F (5′-CTTGGTCATT TAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCT TCATCGATGC-3′) (Adams et al. 2013). The amplification products were sequenced using the Illumina HiSeq2500 PE250 platform (Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China). Libraries were constructed according to the TruSeq DNA Sample Prep Kit protocol (Illumina, CA, USA). All Illumina HiSeq sequencing data were deposited into the NCBI Sequence Read Archive database under BioProject accession number PRJNA513397, https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA513397.

Data analysis

Raw reads from original DNA fragments were merged and quality-filtered using FLASH version 1.2.7 (Magoč and Salzberg 2011) and Trimmomatic version 0.32. The UPARSE version 7.1 was used for chimera removal, and

operational taxonomic units (OTUs) were clustered with a ≥ 97% similarity (Edgar 2013). Bacterial gene sequences were annotated with taxonomic information using the RDP classifier against the SILVA 132 16S rRNA database (Rognes et al. 2016). The OTU taxonomic information of fungi was obtained by aligning each representative sequence against the Unit 8.0 ITS database (Kõljalg et al. 2013). The standard sequence number was compared with that of the sample with the least number of sequences to normalize the OTU abundance. Alpha diversities of the bacteria and fungi, including Chao and Shannon values, were calculated at the OTU level using QIIME. Beta diversity analysis was performed based on the Bray–Curtis distance of the relative abundance of OTUs in nonmetric multidimensional scaling (NMDS). Analysis of similarity (ANOSIM) was applied to test the differences in the changes in bacterial and fungal community compositions across two sites. These data were analyzed on the free online platform of Majorbio Cloud Platform (www.majorbio.com).

Network analysis

For network analysis, the OTUs were grouped at the genus level, and only those genera with more than 10 sequences in at least three samples were used in the following analysis. The correlation between the microbial communities (bacterial and fungal genera) and enzyme activities was examined by calculating all possible pairwise Spearman's rank correlations using SPSS 19. A valid interaction event was considered to be a robust correlation if the Spearman's correlation coefficient (r) was > 0.6 or < – 0.6 and statistically significant ($P < 0.05$) (Zhao et al. 2014; Rui et al. 2015). The correlation matrix constructed by Spearman's correlations was imported into Cytoscape version 3.5.1 to explore and visualize the network (Shannon et al. 2003). In the correlation network, each node represented one genus or an enzyme, and each edge represented a strong and significant correlation (Zhao et al. 2014). A set of the network topological parameters, such as the degree, closeness centrality (CC), and betweenness centrality (BC), were calculated using the Cytoscape plugin NetworkAnalyzer (Assenov et al. 2008). Furthermore, the keystone taxa for the bacterial and fungal network (Sporns et al. 2007; Gonzalez et al. 2010) were explored based on high centrality (BC and CC) scores; the supergeneralist of the network and a candidate for a hub of the network was the node with higher BC and CC values (Zhao et al. 2019b).

Results
Analysis of the enzyme activity

Starch, cellulose, and pectin worked as the important components of tobacco leaves, so its degradation process

was closely related to the activities of enzymes. The independent sample t test showed no significant difference in enzyme activities between GY and MT storerooms, but one-way ANOVA showed that enzyme activities changed significantly with aging time (Fig. 1). The amylase activity increased significantly at first and then gradually decreased, and the maximum value appeared after 12 and 18 months in MT ($6.74\,U\,g^{-1}$) and GY ($7.95\,U\,g^{-1}$) storerooms, respectively. The pectinase activity in the later 12 months was significantly higher than that in the first 6 months, with the highest activity after 12 months [$5.76\,U\,g^{-1}$ (GY) and $5.56\,U\,g^{-1}$ (MT), respectively]. In contrast, the cellulase activity decreased significantly and was the lowest after 12 months ($5.76\,U\,g^{-1}$) in the GY storeroom.

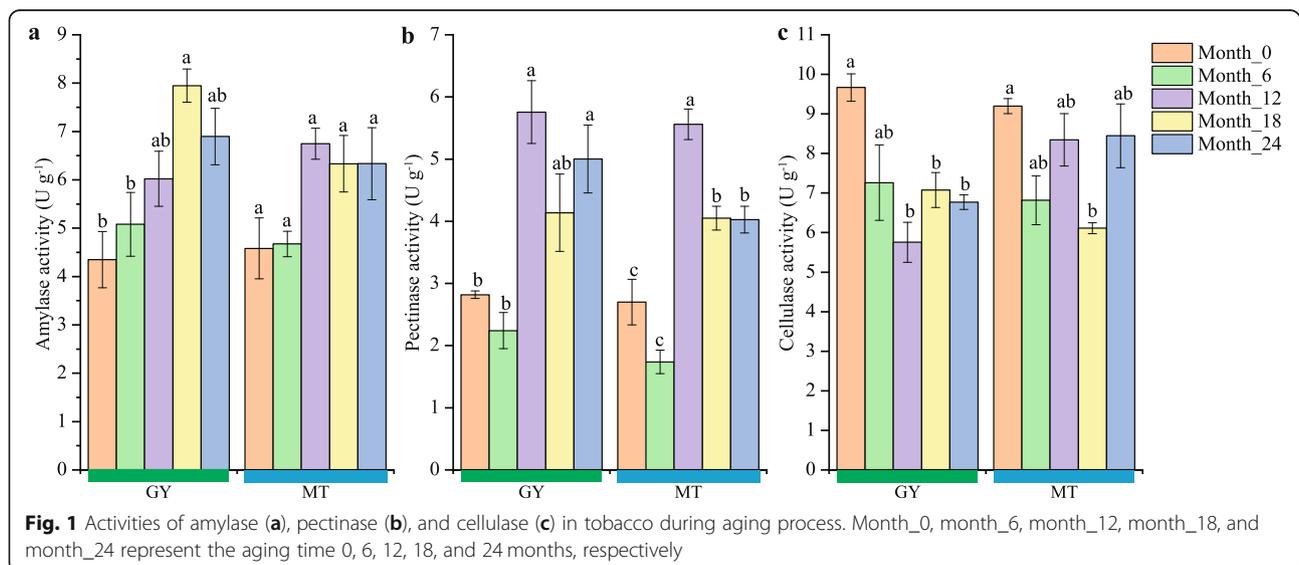## Diversity of bacterial and fungal communities

A total of 429,490 bacterial and 565,872 fungal sequences were obtained and clustered into 1586 and 885 OTUs, respectively, from 10 tobacco leaf samples collected in 5 periods during the aging process. As shown in Fig. 2 a and b, the Shannon and Chao indices of bacteria increased from 3.93 and 391.63 to 5.39 and 839.39 during the first 18 months, respectively, in the GY storeroom, but decreased to 4.86 and 724.26 by 24 months. In the MT storeroom, Shannon's index of bacteria fluctuated from 5.11 to 5.26. However, the Chao index was the highest after 24 months (651.21) and the lowest at the beginning (355.00). The fungal diversity and richness in the GY storeroom decreased significantly from 3.15 and 451.29 to 1.91 and 284.69 during the first 6 months, respectively, and reached the lowest after 18 months (1.38 and 232.14, respectively). In the MT storeroom, the fungal diversity increased from 2.38 to 2.63 in the first 6 months, then decreased to 0.33 after 18 months,
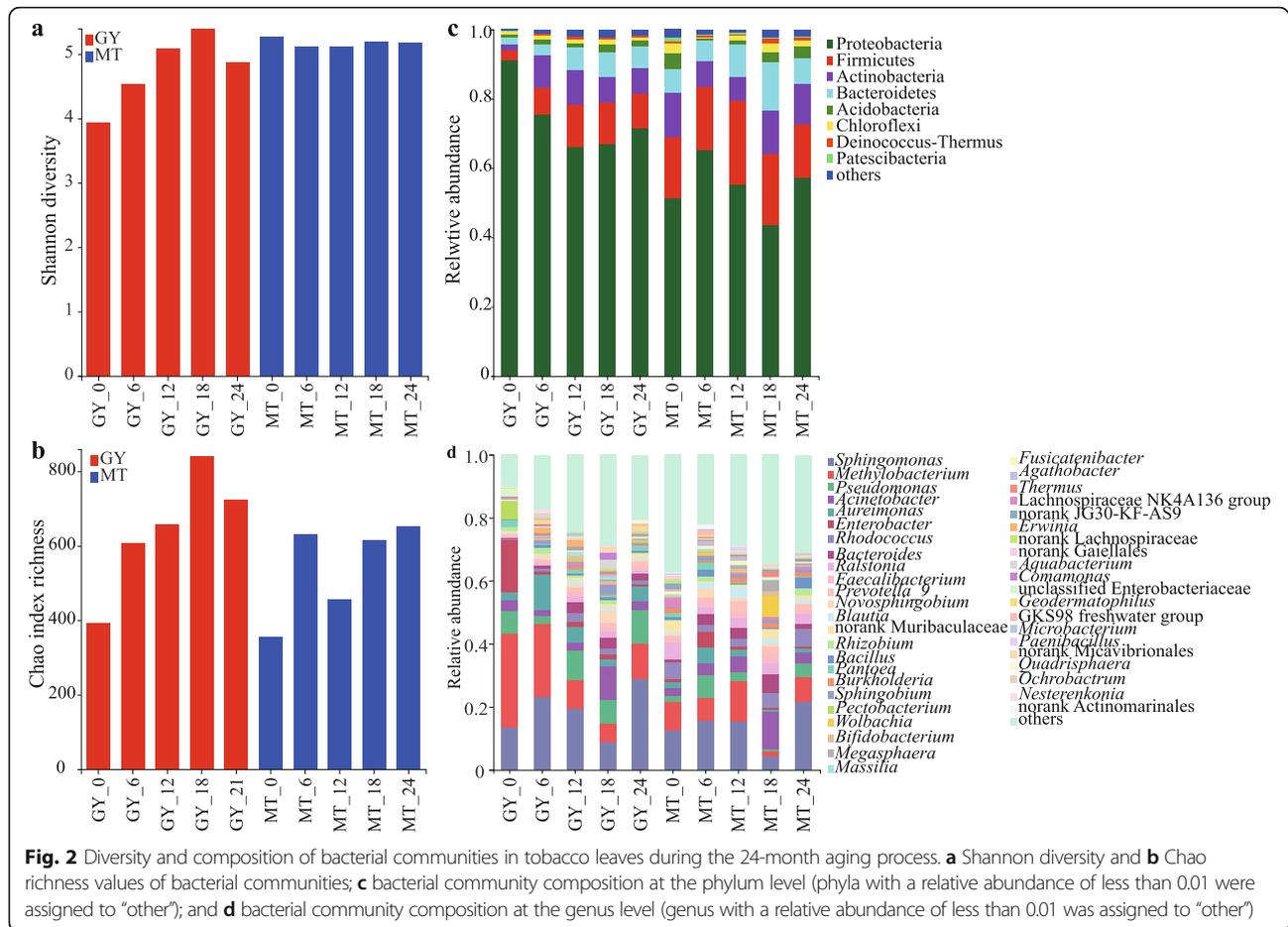
and finally recovered to 1.97 after 24 months (Fig. 3 a and b). However, the richness dropped significantly from 417.84 to 12.5 in the first 18 months before recovering to 144.05. In general, the Shannon and Chao indices of the fungi in the MT storeroom were lower than those in the GY storeroom, but the difference was not significant. The aforementioned results indicated that bacteria and fungi worked together in the early stage of tobacco aging, while the latter was dominated by bacteria.

Bacterial and fungal community composition

Analyses of NMDS based on Bray–Curtis distance demonstrated that the bacterial and fungal communities in GY and MT storerooms could be divided into two groups (Fig. 4). Furthermore, ANOSIM analysis demonstrated significant differences in the changes of fungal community composition between the two storage rooms ($P = 0.017 < 0.05$), but no statistically significant differences were identified for bacterial communities ($P = 0.110 > 0.05$).

Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, Deinococcus–Thermus, and *Patescibacteria* were the main bacterial groups in the tobacco aging system, and the dominant bacterial phyla were *Proteobacteria* (43.65–91.14%) and *Firmicutes* (2.84–23.96%) based on the average relative abundance. Obviously, the proportion of *Proteobacteria* gradually decreased during the aging process, and its average relative abundance was higher in the GY storeroom than in the MT storeroom. The relative abundance of *Proteobacteria* decreased from 91.14% at the beginning to 66.14% by 18 months in the GY storeroom, and then slightly recovered to 71.61% by 24 months. Similarly, the relative abundance of *Proteobacteria* decreased from 65.21% after 6 months to 43.65% after 18

Fig. 1 Activities of amylase (**a**), pectinase (**b**), and cellulase (**c**) in tobacco during aging process. Month_0, month_6, month_12, month_18, and month_24 represent the aging time 0, 6, 12, 18, and 24 months, respectively

Fig. 2 Diversity and composition of bacterial communities in tobacco leaves during the 24-month aging process. **a** Shannon diversity and **b** Chao richness values of bacterial communities; **c** bacterial community composition at the phylum level (phyla with a relative abundance of less than 0.01 were assigned to "other"); and **d** bacterial community composition at the genus level (genus with a relative abundance of less than 0.01 was assigned to "other")
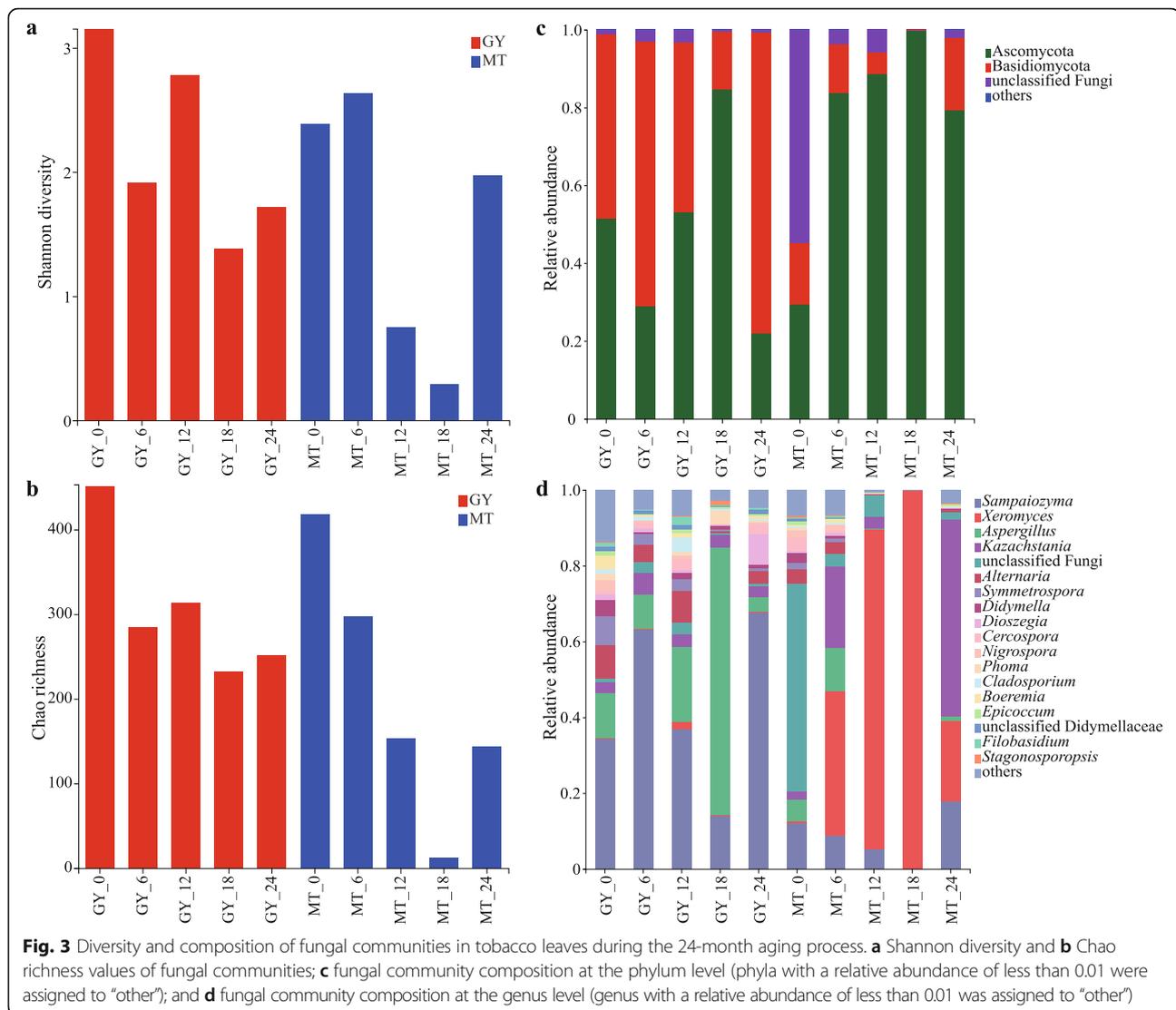
months in the MT storeroom and then recovered to 57.17% by 24 months (Fig. 2c). Contrary to *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* gradually increased in relative abundance, and their average abundance in the MT storeroom (34.03–47.06%) was higher than that in the GY storeroom (6.73–28.71%). A total of 317 bacterial genera were detected, of which 44 taxonomic groups had a relative abundance greater than 1% (Fig. 2d). *Sphingomonas* and *Methylobacterium* were the most dominant genera. The relative abundance of *Sphingomonas* after 0, 6, 12, 18, and 24 months of tobacco leaf aging was 13.46%, 23.14%, 19.58%, 8.86%, and 29.05%, respectively, in the GY storeroom, and 12.69%, 15.65%, 15.43%, 4.54%, and 21.50%, respectively, in the MT storeroom. The relative abundance of *Methylobacterium* decreased gradually from 29.98% after 0 months to 5.91% after 18 months in the GY storeroom, and from 12.64% after 12 months to 1.42% after 18 months in the MT storeroom. In addition, the abundance of *Enterobacter* decreased from 13.68% and 4.765% to 0.71% and 0.38% throughout the entire aging process in GY and MT storerooms, respectively. The relative abundance of *Acinetobacter* showed a consistent trend between GY

and MT storerooms, first increasing and then decreasing, with the highest after 18 months (10.61% and 11.98%, respectively).

A total of 6 phyla and 389 genera of fungi were detected from 10 tobacco leaf samples. The dominant fungal phyla were *Basidiomycota* and *Ascomycota* in the GY storeroom. The relative abundance of *Basidiomycota* decreased significantly from 68.08% after 6 months to 15.06% after 18 months and then recovered rapidly to 77.28% after 24 months. In contrast, the abundance of *Ascomycetes* increased significantly from 28.97% after 6 months to 84.55% after 18 months and then decreased to 22.06% after 24 months (Fig. 3c). In the MT storeroom, *Ascomycota* was the most dominant phylum; its relative abundance gradually increased after treatment, reaching the maximum (more than 99%) after 18 months. In addition, after 18 months, the proportion of *Basidiomycota* also increased (18.74% after 24 months) with a decrease in the abundance of *Ascomycota*. At the genus level, 18 taxonomic groups had a relative abundance greater than 1% for all samples (Fig. 3d). The dominant genera in different storerooms were different. In the GY storeroom, *Sampaiozyma* was the most
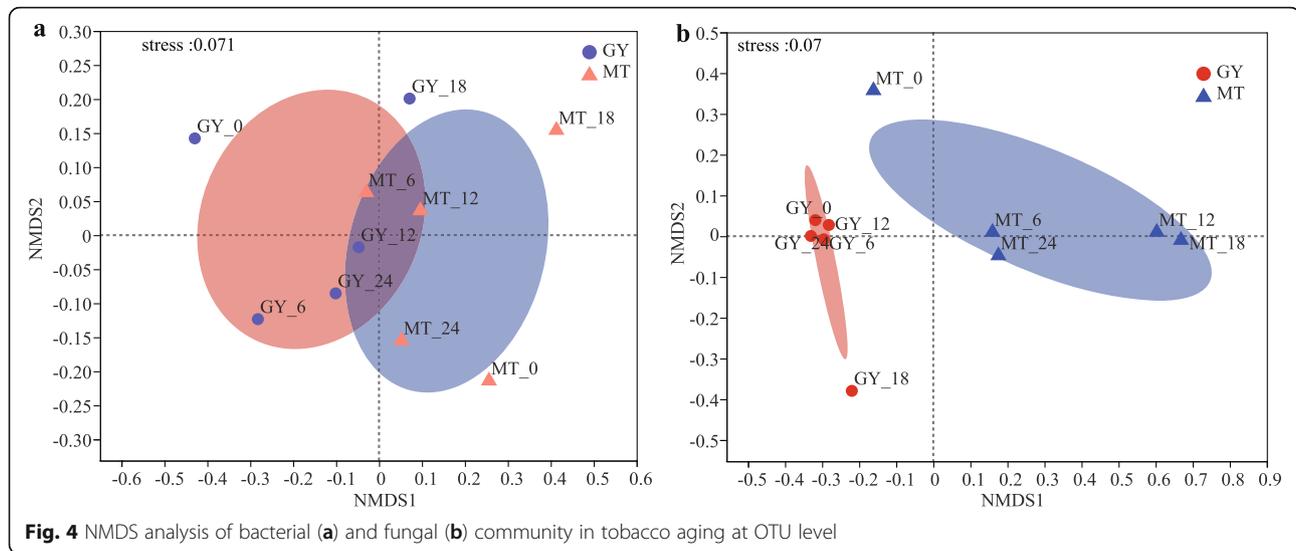
Fig. 3 Diversity and composition of fungal communities in tobacco leaves during the 24-month aging process. **a** Shannon diversity and **b** Chao richness values of fungal communities; **c** fungal community composition at the phylum level (phyla with a relative abundance of less than 0.01 were assigned to "other"); and **d** fungal community composition at the genus level (genus with a relative abundance of less than 0.01 was assigned to "other")

dominant genus in the whole aging process except *Aspergillus* after 18 months. However, *Xeromyces* had a distinct advantage in the MT storeroom ranging from 6 to 18 months. In addition, Fig. 3d shows that the abundance of most genera of *Ascomycota*, such as *Alternaria* and *Cladosporium*, gradually decreased during the aging process in the two storerooms, whereas the abundance of *Dioszegia* belonging to *Basidiomycota* gradually increased in the GY storeroom.

Co-occurrence among bacterial and fungal taxa

The bacterial community co-occurrence patterns during the aging process were inferred based on strong and significant correlations ($P < 0.05$, $r > 0.6$). A total of 97 strong positive correlations and 24 strong negative correlations were identified from 46 bacterial genera (Fig. 5a). The average network path length was 2.914 edges with a diameter of six edges; the network density and clustering coefficient were 0.116 and 0.438, respectively. The co-occurring taxa were mainly distributed in *Proteobacteria* (39.13%, ratio of targeted nodes to total nodes), *Firmicutes* (36.99%), *Bacteroidetes* (10.87%), *Actinobacteria* (8.70%), *Acidobacteria* (2.17%), and *Deinococcus–Thermus* (2.17%). The top five genera (*Burkholderia*, *Blautia*, *Fusicatenibacter*, *Rhodococcus*, and *Methylobacterium*) were identified based on high centrality (BC and CC) scores. Taxa that individually or in a guild exerted a considerable influence on microbiome structure and functioning, irrespective of their abundance across space and time, were highly connected (Berry and Widder 2014). Furthermore, genera from different phyla (interphylum) had high co-occurrence (69.17%, ratio of targeted edges to total edges). The incidence of positive occurrence was the highest between the members of *Firmicutes* and *Proteobacteria* (up to 18.33%), such as between *Megamonas* and *Ralstonia*, between *Blautia* and *Aquabacterium*, *Novosphingobium*,

Fig. 4 NMDS analysis of bacterial (**a**) and fungal (**b**) community in tobacco aging at OTU level

and *Sphingobium*. However, the incidence of negative occurrence was the highest between *Proteobacteria* and *Actinobacteria* (up to 5.00%), such as between *Burkholderia* and *Aureimonas*, *Pectobacterium*, and *Stenotrophomonas*. For intraphylum correlation, *Firmicutes* had a high incidence of positive occurrence (10.83%), whereas *Proteobacteria* (7.50%) had a higher incidence of the negative occurrence. In addition, *Methylobacterium* and members of other genera had a high negative incidence (5.00%). For example, negative correlations were found between *Methylobacterium* and *Lactobacillus*, *Bifidobacterium*, *Rhodococcus*, *Bacteroides*, *Novosphingobium*, *Faecalibacterium*, and *Thermus*.

The correlation network analysis of fungi identified 552 strong positive correlations and 4 strong negative correlations from 488 fungal genera ($P < 0.05$, $r > 0.6$; Fig. 5b). The average network path length was 1.678 edges with a diameter of five edges. The fungal network had a higher network density and clustering coefficient (0.493 and 0.740, respectively) compared with the bacterial network, indicating that the fungal microbiome associations were more tightened than the bacterial microbiome associations. The co-occurring taxa were mainly distributed in *Ascomycota* (82.61%) and *Basidiomycota* (19.42%). According to the combined score of high CC and high BC, the top five genera identified were *Nigrospora*, *Boeremia*, *Alternaria*, *Cladosporium*, and *Corynespora*. They played critical roles as keystone taxa in the tobacco during the aging process. Furthermore, the incidence of co-occurrence within a phylum (intraphylum) was the highest among genera from *Ascomycota* (65.83%). In the fungal network, *Xeromyces* showed significant and strong negative correlations with other genera, such as *Neoascochyta*, *Microdiplodia*, and *Phoma*.

Co-occurrence between bacterial and fungal taxa

The co-occurrence network of bacterial and fungal microbiomes was constructed to understand the microbial cross-kingdom associations. A total of 376 pairs of significant and strong correlations were identified from 90 genera taxa, including 45 bacterial genera and 45 fungal genera (Fig. 6a). The incidence of negative occurrence was higher than the incidence of positive occurrence in the bacteria–fungus network; they accounted for 95.48% (ratio of targeted edges to total edges) and 4.52%, respectively. For example, *Megamons*, *Burkholderia*, *Prevotella_9*, *Collinsella*, and *Cutibacterium* showed a negative correlation with *Mycosphaerella*, *Golubevia*, *Immersiella*, and *Plectosphaerella*. *Pectobacterium* positively correlated with *Boeremia* and *Malassezia*, *Bacillus* with *Kazachstania* and *Sordaria*, and *Methylobacterium* with *Fusarium*. Overall, these results indicated that the most bacteria–fungi interactions might be mutually exclusive during tobacco aging.

Relationship between the microbial microbiome and enzyme activities

The co-occurrence network of amylase, cellulase, and pectinase with bacterial and fungal microbiomes was constructed to further characterize the effects of enzyme activity on the dynamics of microbiomes in tobacco leaves during the aging process. In the microbiome–enzyme network, 54 strong positive correlations and 21 strong negative correlations were identified between 75 taxa and 3 enzyme activities (Fig. 6b). Not surprisingly, 80% of the taxa were closely related to the activity of amylase, with positive correlations for bacteria and negative correlations for fungi. Starch, accounting for 2–6% of the total biomass of tobacco leaves, was an important carbon source for microbial growth and reproduction

Fig. 5 (See legend on next page.)
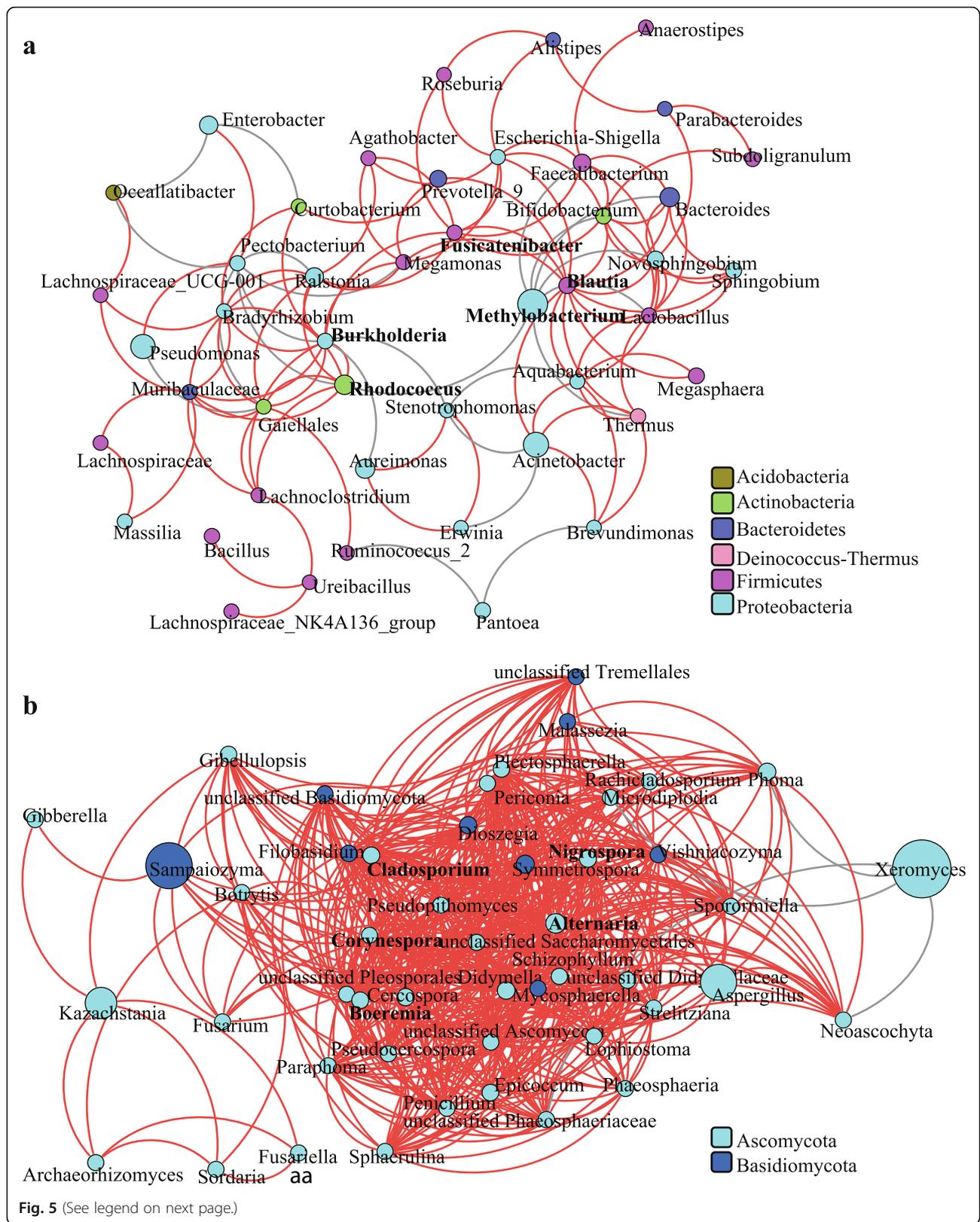
(See figure on previous page.)

Fig. 5 Network of co-occurring bacterial and fungal genera in tobacco leaves during the aging process based on correlation analysis. Networks **a** and **b** were constructed for bacterial and fungal genera, respectively, from five periods during the aging process. Only those genera with more than 10 sequences in at least 3 samples were included in the network analysis. A connection represented a statistically significant ($P < 0.05$) strong positive (Spearman's $r > 0.6$) or negative (Spearman's $r < -0.6$) correlation. The node colors indicate the phyla to which each genus belongs, and the node size is proportional to the abundance of taxa. The edges are colored based on interaction types; positive correlations are indicated in red and negative correlations in gray

and could be directly decomposed and utilized. The cellulase activity showed positive correlations with members of eight fungal genera, such as *Strelitziana*, *Fusarium*, *Sphaerulina*, *Phaeosphaeria*, *Paraphoma*, and *Montagnula*, and *Enterobacter* genus of bacterial phylum, but a negative correlation with *Lachnospiraceae_ND3007_group*. The pectinase activity showed strong negative

associations with most fungal genera such as *Vishniacozyma*, *Golovinomyces*, unclassified *Tremellales*, and unclassified *Pleosporales*, but a strong positive association with *Subgroup_6* of the bacterial kingdom. These results indicated that microorganisms were closely related to biological enzymes during tobacco aging, and most of them had a promoting effect on enzyme activities.
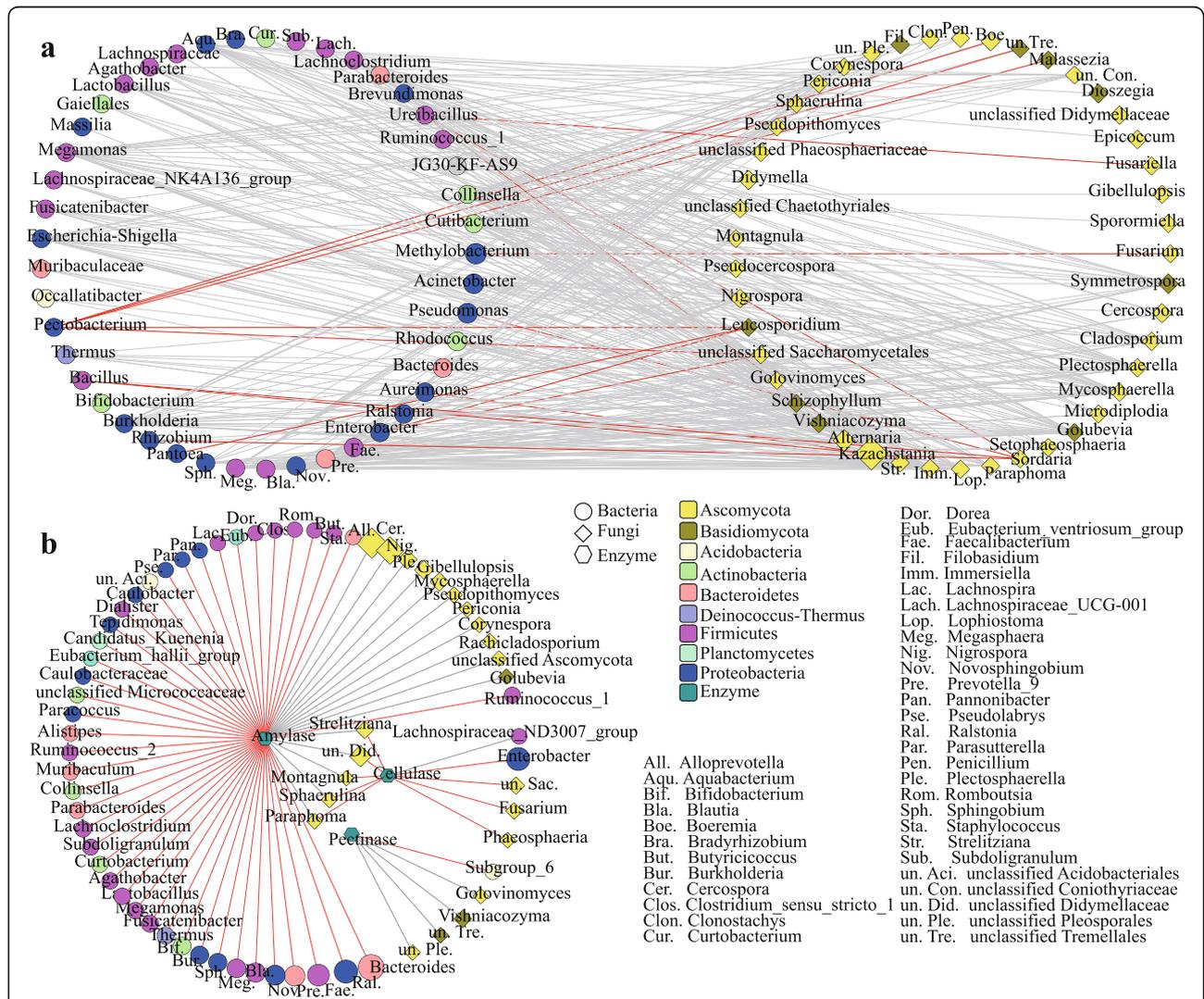
Fig. 6 Network of co-occurrence patterns between bacterial genus, fungal genus, and enzyme activities in tobacco leaves. Networks **a** and **b** were constructed based on the correlation analysis between bacteria and fungi and between microorganism (both bacteria and fungi) and enzyme activities, respectively. A connection represented a statistically significant ($P < 0.05$) strong positive (red, Spearman's $r > 0.6$) or negative (gray, Spearman's $r < -0.6$) correlation. The node colors indicate the phyla to which each genus belongs, and the node size is proportional to the abundance of taxa

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Discussion

This study investigated the microbial community composition, diversity, and co-occurrence patterns in tobacco leaves during the aging process by Illumina HiSeq sequencing based on 16S rRNA and ITS genes. *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, and *Chloroflexi* were abundant in the tobacco leaves during the aging process. Additionally, the most dominant phylum was *Proteobacteria*, which corroborated the results of Huang et al. (2010) and Su et al. (2011); however, this result was different from those of Wang et al. (2018) and Ye et al. (2017), who found that *Firmicutes* was the dominant phylum in flue-cured tobacco.

This study showed that 38.74–62.46% of predominant bacteria belonged to *Sphingomonas*, *Methylobacterium*, *Pseudomonase*, *Acinetobacter*, *Enterobacter*, *Aureimonas*, *Bacteroides*, and *Rhodococcus*. These genera were reported to be important contributors to nicotine degradation or the formation of representative flavor compounds (Zhang et al. 2020). For example, Ma et al. (2016) reported that a strain of *Sphingomonas* sp. isolated from tobacco leaves degraded chlorogenic acid into caffeic acid, shikimic acid, and 3,4-dihydroxybenzoic acid. Moreover, *Enterobacter* and *Methylobacterium* degraded sugars, organic acids, polyols, methanol, formate, and formaldehyde, which were present in the tobacco products (Rachman et al. 1997; Jourand et al. 2004). *Pseudomonas* is also interesting because of its ability to degrade nicotine, as demonstrated for *Pseudomonas* sp. Nic22 (Chen et al. 2008), *Pseudomonas stutzeri* ZCJ (Zhao et al. 2012), and *Pseudomonas* sp. HF-1 (Wang et al. 2013).

Ascomycota and *Basidiomycota* were two main fungal phyla in aged tobacco leaves, and *Sampaiozyma*, *Aspergillus*, and *Xeromyces* were dominant genera. However, this result did not agree with that of Zhang et al. (2020), who showed that *Neophaeosphaeria* and *Cladosporium* were the dominant genera in flue-cured tobacco leaves. Meng et al. (2010) reported that *Aspergillus oryzae* 112822 degraded nicotine to 2,3-dihydroxypyridine through the intermediates nornicotine, myosmine, N-methylnicotinamide, and 2-hydroxy-N-methylnicotinamide. This finding indicated that the fungi also improved the quality of tobacco leaves. However, even more interestingly, most of these detected fungi (*Aspergillus*, *Penicillium*, *Alternaria*, and *Cladosporium*) caused mildew, which reduced the quality and use value of tobacco (Welty and Vickroy 1975).

However, environmental characteristics frequently affected the microbial community structure and function (Orland et al. 2019). In the tobacco aging process, the microenvironment can be altered by secondary metabolites produced during fermentation. For example, the levels of total organic acid and volatile compounds gradually increase, whereas the levels of nicotine, volatile alkali, total sugar, reducing sugar, and pH values decrease in tobacco leaves (Sun et al. 2011). Di Giacomo et al. (2007) reported that the structure and composition of the microbial community in Italian Toscano cigar changed significantly with the increase in temperature and pH during the fermentation process. This study found that the relative abundance of *Proteobacteria* decreased, whereas that of *Actinobacteria* and *Bacteroidetes* increased. The proportion of *Ascomycota* gradually increased during the first 18 months and then rapidly decreased, whereas *Basidiomycota* exhibited a completely opposite pattern. These results indicated that tobacco aging (or fermentation) is a microdynamic process dominated by microorganisms, whose composition and diversity may be affected by environmental conditions. In addition, this study found that the change in bacterial community structure in tobacco leaves was not significant in two different cities, but the change in fungal community structure was significant. This suggested that the fungi community might be more sensitive to environmental changes compared with bacteria.

Co-occurrence network analysis is useful for revealing common system-level properties of microbial communities in tobacco leaves during the aging process. Co-occurrence analysis of bacterial and fungal taxa from 10 tobacco samples in this study suggested strong within- and between-domain correlations among different groups of microorganisms in the tobacco leaves. In the bacterial community, the less-related interphylum genera had much higher co-occurrence ratios than the intraphylum genera in tobacco leaves. This finding was consistent with those reported by Zhang et al. (2020). However, genera from *Ascomycota* (intraphylum) showed a higher incidence of co-occurrence than other interphylum genera for the fungal community. These results indicated that microbial species might share niches and have synergistic or antagonistic relationships during tobacco aging (Barberán et al. 2012). However, the fungal network had a higher network density and clustering coefficient compared with the bacterial network, implying that the fungal network might have higher connectivity and stability than the bacterial network in the tobacco aging process. This result was consistent with those observed in the soil microbial community (Xiao et al. 2018; de Vries et al. 2018). The bacterium–fungus co-occurrence network showed that the incidence of negative correlation was significantly higher than that of positive correlation, indicating that most bacteria and fungi might be mutually exclusive in the tobacco aging ecosystem, which was similar to the bacterium–fungus interaction in the fermentation of Pu-erh tea (Zhao et al. 2019a). In short, the interactions between microorganisms might be necessary to maintain the stability of the aging ecosystem during the aging process of tobacco leaves.

Microorganisms are known to be the main manufacturers of enzymes in nature. For example, *Aspergillus*, *Trichoderma*, *Alternaria*, *Fusarium*, *Penicillium*, *Rhizopus*, and *Cladosporium* are common cellulolytic fungi that can convert native or derived cellulose to glucose (Jahangeer et al. 2005; Jin et al. 2012). *Phanerochaete chrysosporium* can degrade tobacco stalks by secreting cellulase (Su et al. 2016). The members of Enterobacteriaceae, *Erwinia carotovora* and *Erwinia chrysanthemi*, have been confirmed to degrade and utilize pectin (Abbott and Boraston 2008). During the aging process of tobacco leaves, microorganisms could synthesize and secrete enzymes to promote the degradation or transfer of complex macromolecular compounds. At present, many microorganisms inhabiting the surface of tobacco leaves have been confirmed to have strong enzyme-producing activities. For example, Dai et al. isolated two pectinase-producing strains, *Bacillus subtilis* and *Alcaligenes faecalis*, from the Zimbabwe tobacco strips (Dai et al. 2011). Guan et al. found a bacterial community, which consisted of *Arthrobacter*, *Actinomycetes*, *Klebsiella*, and two unknown bacteria, with the ability to decompose cellulose in aging flue-cured tobacco leaves (Guan et al. 2013). This study showed that the bacterial and fungal microbiomes were significantly associated with the activities of enzymes such as amylase, cellulase, and pectinase. This finding indicated that the microbial community was closely related to enzyme activity during tobacco aging, but the influence of microorganisms on enzyme activity depended on the type of microorganism and enzymes (Han et al. 1999). Residual enzymes in tobacco leaves are crucial in the early stage of aging, while microbial enzymes were important in the late stage of aging (Zhu et al. 2001). Furthermore, environment, time of aging, and enzyme structure affect the types and quantity of microorganisms (Larsen et al. 2019). However, this study only involved cellulase, pectinase, and amylase, with limited research indicators. Future studies should involve more enzymes and tobacco samples to verify the reliability and reproducibility of tobacco in different environments.

Conclusions

This study analyzed the microbial community dynamics and composition in tobacco leaves during the aging process by employing high-throughput sequencing techniques. It demonstrated that tobacco aging was a microdynamic process dominated by microorganisms, Shannon diversity, and Chao richness of bacterial communities gradually increased during the first 18 months, whereas those of the fungal community decreased. The relative abundance of *Proteobacteria* decreased, whereas that of *Actinobacteria* and *Bacteroidetes* increased. The proportion of *Ascomycota* gradually increased during the first 18

months and then rapidly decreased, whereas the proportion of *Basidiomycota* exhibited a completely opposite pattern. The change in bacterial community composition and dominant genera in tobacco leaves was not significant between GY and MT storerooms, but that in fungal community was significant. The network analysis revealed strong within- and between-domain correlations among different groups of microorganisms within the tobacco leaves. Fungal networks are more complex and compact than bacterial networks, and a strong negative correlation exists between bacteria and fungi. Moreover, the bacterial microbiome showed a strong positive association with amylase activity, while the fungal microbiome positively correlated with cellulase activity. These findings revealed the diversity, community composition, and co-occurrence pattern of tobacco bacterial and fungal communities during the aging process, thus providing a reference for tobacco microbial fermentation technology.

Authors' contributions
Jiaxi Zhou and Xiao Zou conceived and designed the experiments. Jiaxi Zhou performed the experiments and analyzed the data. Xiao Zou acquired the funding and administered the project. Xiaomin Zhang and Jing Liu provided resources. Jiaxi Zhou, Lifei Yu, and Xiao Zou wrote, reviewed, and edited the manuscript. The author(s) read and approved the final manuscript.

Availability of data and materials
The authors declare that all materials and data are available.

Competing interest
The authors declare that they have no conflict of interest.

Ethics approval and consent to participate
Not applicable.

Consent for publication
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