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ENVIRONMENTAL BIOTECHNOLOGY

Fungal communities are more sensitive to mildew than bacterial communities during tobacco storage processes

Jiaxi Zhou[1,2,3] · Jing Liu[4] · Dongfei Wang[3] · Yibin Ruan[3] · Shuang Gong[3] · Jianyu Gou[4] · Xiao Zou[1]

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Abstract

Mildew poses a significant threat to tobacco production; however, there is limited information on the structure of the abundant and rare microbial subcommunities in moldy tobacco leaves. In this study, we employed high-throughput sequencing technology to discern the disparities in the composition, diversity, and co-occurrence patterns of abundant and rare fungal and bacterial subcommunities between moldy and normal tobacco leaves collected from Guizhou, Shanghai, and Jilin provinces, China. Furthermore, we explored the correlation between microorganisms and metabolites by integrating the metabolic profiles of moldy and normal tobacco leaves. The results showed that the fungi are more sensitive to mildew than bacteria, and that the fungal abundant taxa exhibit greater resistance and environmental adaptability than the rare taxa. The loss of rare taxa results in irreversible changes in the diversity, richness, and composition of the fungal community. Moreover, rare fungal taxa and abundant bacterial taxa played crucial roles in maintaining the stability and functionality of the tobacco microecosystem. In moldy tobacco, however, the disappearance of rare taxa as key nodes resulted in reduced connectivity and stability within the fungal network. In addition, metabolomic analysis showed that the contents of indoles, pyridines, polyketones, phenols, and peptides were significantly enriched in the moldy tobacco leaves, while the contents of amino acids, carbohydrates, lipids, and other compounds were significantly reduced in these leaves. Most metabolites showed negative correlations with *Dothideomycetes*, *Alphaproteobacteria*, and *Gammaproteobacteria*, but showed positive correlations with *Eurotiales* and *Bacilli*. This study has demonstrated that abundant fungal taxa are the predominant biological agents responsible for tobacco mildew, while bacteria may indirectly contribute to this process through the production and degradation of metabolites.

Key points

- *Fungi exhibited greater sensitivity to mildew of tobacco leaf compared to bacteria*
- *Rare fungal taxa underwent significant damage during the mildew process*
- *Mildew may damage the defense system of the tobacco leaf microecosystem*

Keywords Abundant taxa · Rare taxa · Co-occurrence network · *Fungi* · *Bacteria* · Moldy tobacco

Introduction

As an essential raw material for cigarettes, high-quality tobacco leaves are a critical strategic resource for cigarette enterprises, and tobacco storage security is crucial to ensure the quantity and quality of the raw materials (Luo et al. 2015). Mildew is a potentially significant threat to tobacco storage management. During the mildewing process, the molds grow rapidly on the tobacco leaves and absorb and decompose sugars, proteins, starch, and other nutrients from the leaves. Additionally, the molds release green, blue, and black pigments and a strong mildew stench (Welty and Vickroy 1975). This not only results in significant economic

✉ Xiao Zou
xzou@gzu.edu.cn

1 Department of Ecology / Institute of Fungus Resources, College of Life Sciences, Guizhou University, Guiyang, China

2 Postdoctoral Research Workstation of China Tobacco Guizhou Industrial Co. Ltd, Guiyang, China

3 China Tobacco Guizhou Industrial Co. Ltd, Guiyang, China

4 Guizhou Tobacco Company Zunyi Branch, Zunyi, China

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losses for tobacco farmers and cigarette manufacturers but also causes dissatisfaction among consumers (Yang et al. 2015). In addition, most mildew-causing molds can secrete mycotoxins (such as aflatoxin) into the leaves and release large amounts of mold spores into the environment, thereby threatening human health and environmental safety (Lander et al. 1988; Pauly and Paszkiewicz 2011).

Despite being the primary biological agents causing mildew of stored tobacco leaves and products (Fleurat-Lessard 2017), fungi rarely exist alone in natural ecosystems and instead form mixed communities with bacteria and yeast (Magan et al. 2004). In addition to fungal spores, a diverse array of bacterial cells such as *Sphingomonas*, *Bacillus*, *Pseudomonas*, *Lactococcus*, *Stenotrophomonas*, *Acinetobacter*, and *Methylobacterium* are found within the epidermis and internal tissues of tobacco leaves (Ye et al. 2017; Zhou et al. 2020, 2021). Although bacteria are rarely responsible for causing mildew under dry conditions (Christensen 1972), recent studies have shown that bacteria and fungi can interact to form complex food chains and webs, playing a pivotal role in driving biochemical cycles and maintaining the balance between animal and plant health and ecosystem biodiversity and stability (de Menezes et al. 2017; Deveau et al. 2018; Getzke et al. 2019; Ratzke et al. 2020). However, most studies focus on the role of fungi in the mildew of storage materials.

In natural environments, microbial communities typically comprise a diverse array of microorganisms, with only a selected few exhibiting high abundance, some displaying moderate abundance, and the majority being rare (Pedros-Alio 2012; Li et al. 2020b). The abundant taxa (AT) have broad ecological niches, strong competitive ability, and rapid growth rates, which considerably promote carbon cycling and energy flow (Pedros-Alio 2012; Tian et al. 2020). In contrast, rare taxa (RT) are more metabolically active and genetically diverse (Debroas et al. 2015), providing a virtually inexhaustible source of genetic and functional diversity (Lynch and Neufeld 2015; Jiao et al. 2017b) and making a disproportionate contribution to community diversity and variation (Shade et al. 2014). RT may dominate and play important roles in nutrient cycling (Jousset et al. 2017) and enhance community resistance to environmental disturbances under adverse conditions (Jiao et al. 2017a), thereby providing mechanisms for community persistence and stability (Shade and Gilbert 2015; Xiong et al. 2020). The composition and function of microbial AT and RT have been extensively studied in a variety of habitats, such as plant barks (Dong et al. 2021), soil (Xue et al. 2020), sphagnum (Tian et al. 2020), and marine systems (Wu et al. 2017). However, the investigation of microbial AT and RT in tobacco leaves and their significance in tobacco management practices, particularly under mildewing conditions, remains inadequate.

Metabolites are the terminal products of biological information transmission and are important mediators of intra- and inter-species interactions in the tobacco leaf microecosystem (Schmidt et al. 2019). In this system, microbial interaction can directly or indirectly lead to the degradation or transfer of macromolecules, such as starch, sugars, proteins, and carotenoids, and the formation of small molecules, such as low-carbonyl compounds, low fatty acids, furan compounds, and pyrazines and pyrrole derivatives (Baraniecki et al. 2002; Maldonado-Robledo et al. 2003). Thus far, more than 4000 chemical components have been identified in tobacco leaves and products, including carbohydrates, nitrogenous compounds, heterocyclic compounds, enzymes, organic acids, phenolic compounds, pigments, ether extracts, and minerals (Rodgman and Perfetti 2013), all of which can be exploited by the tobacco leaf microecosystem. For example, monosaccharides and disaccharides found in tobacco leaves can serve as sources of carbon and energy to support the growth and reproduction of molds (Li et al. 2014). Although molds can significantly affect the nutritional composition of tobacco leaves, our understanding of the interactions between metabolites and microorganisms in the mildewed leaves is still limited.

In this study, we investigated the microbial communities in moldy and normal tobacco leaves obtained from tobacco storage warehouses. The main objectives of this study were as follows: (1) to investigate the effects of mildew on the diversity and composition of fungal and bacterial AT and RT in tobacco leaves; (2) to reveal the responses of fungal and bacterial AT and RT towards mildew using co-occurrence network analysis; and (3) to preliminarily reveal the metabolic characteristics of moldy tobacco leaves and explore their relationship with microbial communities using metabolomics.

Materials and methods

Sample collection

A total of 18 samples of moldy (test samples) and normal (control samples) tobacco leaves were collected from tobacco warehouses in Guizhou, Jilin, and Shanghai, China. The samples were collected using sterile gloves and placed in sterile sealed bags. The samples were then transported to the Fungal Resources Research Laboratory of Guizhou University (Guiyang, China) and stored at − 20 °C until further analysis. Additionally, considering the tobacco samples were from the same warehouse, but might not be from the same growing area, therefore, all tobacco samples for the study were stored for at least 5 years to mitigate the potential impact of leaf sources on the microbiome.

High-throughput sequencing

Microbial DNA extraction was performed following the method described by Zhou et al. (2022). The internal transcribed spacer 1 (ITS1) region of fungal rRNA was amplified using the ITS1F and ITS2R primers by an ABI GeneAmp® 9700 PCR thermocycler (ABI, Los Angeles, CA, USA), while the V3-V4 hypervariable region of bacterial 16S rRNA gene was amplified using the 338F and 806R primers (Adams et al. 2013; Xu et al. 2016). PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The PCR reactions were conducted using the following program for bacterial 16S rRNA gene: 95 °C for 3 min, 27 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR reactions were conducted using the following program for fungal ITS1 region: 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, Fitchburg, WI, USA). The purified PCR products were sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using paired-end sequencing by Shanghai Meiji Biological Technology Co., Ltd. (Shanghai, China).

The raw sequences were quality-controlled (with an average quality score > 20) and spliced using Fastp v.0.19.6 and FLASH v.1.2.11 software, respectively (Magoc and Salzberg 2011; Chen et al. 2018). The optimized sequences were then classified into operational taxonomic units (OTUs) at a 97% similarity threshold using UPARSE v.7.1 software (Edgar 2013). The most abundant sequence from each OTU was selected as the representative sequence, and the Ribosomal Database Project classifier was used to classify fungi and bacteria based on Unite 8.0 (https://unite.ut.ee/) and Silva 138 (https://www.arb-silva.de/) databases, respectively, with a confidence threshold of 70% (Wang et al. 2007). To reduce spurious OTUs, any OTU consisting of < 2 sequences was removed.

Molecular ecological network analysis

To gain further insight into the importance of interspecific interactions in the tobacco leaf microecosystem, we constructed co-occurrence networks for fungal and bacterial communities of the moldy and normal tobacco leaves based on the relative abundance of OTUs. The Molecular Ecological Network Analysis (MENA) pipeline (http://129.15.40.240/mena/) was used to perform network analysis, with threshold identification based on the recommended Pearson correlation coefficient and random matrix theory modeling (Deng et al. 2012; Shi et al. 2016; Qian et al. 2020). Network visualization was generated using the Gephi v.0.9.2 software (https://gephi.org/). The topological role of each node in a network was defined by its "within-module connectivity value" (*Zi*) and "among-module connectivity value" (*Pi*) (Guimera`and Nunes Amaral 2005). All the nodes were classified into four subcategories: modular hubs (highly connected nodes within a module, $Zi > 2.5$), network hubs (highly connected nodes within or among the modules, $Zi > 2.5$ and $Pi > 0.62$), peripherals (nodes with only a few links within or among the modules, $Zi < 2.5$ and $Pi < 0.62$), and connectors (highly connected nodes among the modules, $Pi > 0.62$) (Poudel et al. 2016). Modular hubs, network hubs, and connectors can be used to identify keystone species that play important roles in the stability and resistance of microbial network structure and function (Fan et al. 2018); thus, the OTUs associated with these nodes are defined as keystone species.

Metabolomic analysis

The tobacco leaf samples were weighed (50 mg) and placed in a 2-mL centrifuge tube with a 6-mm grinding bead. Thereafter, 400 μL of extraction solution (4:1 methanol to water, v/v) containing 0.02 mg/mL L-2-chlorophenylalanine (internal standard) was added to the tube. Subsequently, the mixture was cryogenically ground at − 10 °C and 50 Hz for 6 min, followed by low-temperature ultrasonic extraction at 5 °C and 40 kHz for 30 min. Thereafter, the extract was left undisturbed for 30 min at − 20 °C and then centrifuged at 13,000 rpm at 4 °C for 15 min. Finally, the supernatant (200 μL) was transferred to a sample bottle with an inner cannula for liquid chromatography-mass spectrometry (LC–MS) analysis.

An ultra-high-performance liquid chromatography system (Thermo Scientific, Waltham, MA, USA) coupled with a Fourier transform mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used for metabolite detection. The Acquity UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters, Milford, MA, USA) was utilized for chromatographic separation. The injection volume was set at 2 μL and the column temperature was maintained at a constant value of 40 °C. Spectral detection was performed in both positive and negative ion modes, with the spray voltage being adjusted accordingly. The capillary temperature was maintained at 325 °C, and the sheath gas and auxiliary gas flow rates were set to 50 and 13 arb, respectively. The data were analyzed using Progenesis QI software (Waters Corporation, Milford, MA, USA), and metabolite identification and annotation were conducted using the HMDB v.4.0 (http://www.hmdb.ca/) and KEGG public databases (https://www.genome.jp/kegg/) (Li et al. 2020a). Unsupervised

principal component analysis and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed using SIMCA v.14.1 software (Umetrics, Umeå, Sweden) to observe the overall distribution of the metabolites between the moldy and normal tobacco leaf samples. Significant differences in metabolites were identified between the moldy and normal tobacco leaf samples by combining the variable influence on projection (VIP) values of OPLS-DA and Student's *t*-test *p*-values that were adjusted by false discovery rate (FDR) (Xin et al. 2019). Metabolites with VIP > 1 and $p < 0.05$ were considered significantly altered.

Statistical analysis

In this study, we classified all the OTUs into AT, intermediate taxa (IT), and RT based on their relative abundance (> 1%, 0.01–1%, and < 0.01%, respectively) (Jiao and Lu 2020; Dong et al. 2022). To assess microbial community composition in different samples, we used the ggplot2 R package to map species stacks (Wickham 2016) and the UpSetR R package to visualize shared and unique taxa among the different samples (Gehlenborg 2019). We used the Vegan R package to calculate the Shannon and Chao indexes to assess the α-diversity of the samples (Oksanen et al. 2022), while we used the Picante R package to compute the Bray–Curtis distances and visualize them using PCoA to assess the β-diversity of the samples (Kembel et al. 2010). Similarity analysis was used to assess the difference in the microbial community structure between the moldy and normal tobacco leaves.

To explore the species characteristics and ecological adaptability of different microbial subcommunities, we calculated the Levin niche breadth index for AT, IT, and RT using the Spaa R package (Zhang 2016). We further explored the relationship between the niche breadth and relative abundance of species through Spearman correlation analysis. The psych package was used to compute Spearman's correlations between the microbial taxa and metabolites (Revelle 2021), which were then visualized as heatmaps. All statistical analyses were performed using R v.4.0.5 (https://www.r-project.org) and RStudio v.1.1.463 (https://www.rstudio.com/) software. Non-parametric statistical tests (Kruskal–Wallis or Wilcoxon test) were used to analyze microbial composition, α-diversity index, niche breadth index, and network properties between the moldy and normal tobacco leaves.

Results

Effects of tobacco leaf mildew on the fungal and bacterial community diversity

A total of 1,202,609 ITS sequences (51,464–73,943 sequences per sample) with an average length of 232 bp and 83,764 16S rRNA sequences (28,952–54,535 sequences per sample) with an average length of 421 bp were generated from the 18 tobacco samples (Supplemental Table S1). After quality-filtering and rarefying based on the minimum sequencing depth of any individual sample (51,354 reads per fungal sample and 7426 reads per bacterial sample), a total of 924,372 remaining ITS sequences were assigned to 599 fungal OTUs, while 133,668 remaining 16S rRNA sequences were assigned to 756 bacterial OTUs. Among the identified fungal OTUs, fungal AT accounted for only 5.34%; however, these accounted for > 97% of the total sequence count. Conversely, fungal IT and RT constituted 9.68% and 84.97% of the OTUs, respectively, while their contribution to the overall sequence count was 1.84% and 0.8%, respectively. Among the 756 bacterial OTUs, bacterial AT accounted for only 7.14% of the OTUs but contributed to a significant proportion (93.67%) of the total sequences, while bacterial IT and RT accounted for 18.78% and 74.08% of the OTUs, respectively, but contributed to smaller proportions (4.63% and 1.70%, respectively) of the total sequences (Table 1). Interestingly, in comparison to normal tobacco leaves, moldy tobacco leaves exhibited a significant increase in the number and proportion of OTUs associated with

Table 1 The number of OTU and sequences of all, abundant, and rare communities in moldy and normal tobacco leaf samples

Kingdom	Groups	OTU			Sequences		
		All	Normal	Mildew	All	Normal	Mildew
Fungi	Abundant	32 (5.34%)	32 (5.35%)	30 (35.72%)	899,928 (97.36%)	440,718 (95.35%)	459,210 (99.36%)
	Intermediate	58 (9.68%)	58 (9.70%)	28 (33.33%)	17,010 (1.84%)	14,180 (3.07%)	2830 (0.61%)
	Rare	509 (84.98%)	508 (84.95%)	26 (30.95%)	7434 (0.80%)	7288 (1.58%)	146 (0.03%)
Bacteria	Abundant	54 (7.14%)	52 (9.14%)	54 (8.84%)	125,211 (93.67%)	62,265 (93.16%)	62,946 (94.18%)
	Intermediate	142 (18.78%)	137 (24.08%)	135 (22.09%)	6192 (4.63%)	3509 (5.25%)	2683 (4.02%)
	Rare	560 (74.08%)	380 (66.78%)	422 (69.07%)	2265 (1.70%)	1060 (1.59%)	1205 (1.80%)

The numbers outside the parentheses denote the number of OTUs or sequences, respectively, while those inside indicate their respective proportions in the sample

bacterial RT, while demonstrating a significant decrease in those related to fungal RT (Table 1).

The diversity and richness of fungal communities in moldy tobacco leaves were significantly lower compared to those in normal tobacco leaves (Supplemental Fig. S1A). Surprisingly, the Shannon and Chao indices of fungal RT were significantly higher than those of fungal AT and IT in normal tobacco leaves ($p < 0.05$, Supplemental Fig. S2A-B). However, the Chao index of fungal RT was significantly lower than that of fungal AT in moldy tobacco leaves (Fig. 1A–C, Supplementary Fig. S2C–D). In contrast to the fungal community, there were no significant differences in bacterial community diversity and richness observed between moldy and normal tobacco leaves at any level (AT, IT, RT or All) (Fig. 2A–C, Supplementary Fig. S1D). However, the diversity and richness indices of bacterial RT were significantly higher than those of bacterial AT in both moldy and normal tobacco leaves (Supplementary Fig. S3). The PCoA analysis showed mildew had no significant effect for tobacco fungal and bacterial communities in the overall community level (Supplemental Fig. S1B and E). However, it showed significant effects on fungal RT ($p < 0.05$, Figs. 1D–F and 2D–F), this suggested that variations in microbial communities within moldy tobacco leaves may primarily be attributed to the differences in fungal RT. Moreover, fungal communities were notable disparities among the three sites ($R^2 = 0.379$, $p = 0.001$, Supplemental Fig. S1C and F), this implied that the fungal community structure of tobacco leaf might be more susceptible to alterations induced by storage conditions.

Furthermore, mildew had a significant impact on the niche breadth of fungal and bacterial communities (Figs. 1G–I and 2G–I). Among the fungal and bacterial communities, taxa with high abundance exhibited the highest niche breadth indices, while those with low abundance showed the lowest indices (Figs. 1G and 2G). Moreover, the niche breadth of fungi and bacteria in moldy tobacco leaves was significantly lower than that in normal tobacco leaves (Figs. 1H and 2H). Additionally, the relative abundance of individual OTUs was positively correlated with the Levin niche breadth index ($p < 0.001$) (Figs. 1I and 2I).

Effects of tobacco leaf mildew on the fungal and bacterial community composition

The community composition of fungi and bacteria in moldy and normal tobacco leaf samples exhibited high similarity in composition with fungal AT and bacterial AT, respectively (Fig. 3), indicating that the microbial communities in tobacco leaf samples were primarily regulated by AT. However, a few differences were observed in the distribution of the dominant microflora among the subgroups. For instance, *Eurotiomycetes*, *Wallemiomycetes*, and *Microbotryomycetes* were predominantly distributed within fungal AT, whereas *Agaricomycetes* and *Dothideomycetes* were observed in fungal RT and IT (Fig. 3A). Furthermore, the abundances of *Dothideomycetes*, *Sordariomycetes*, *Microbotryomycetes*, *Tremellomycetes*, and *Agaricomycetes* in moldy tobacco leaves were significantly lower than those in the normal tobacco leaves (Supplemental Fig. S4). Specifically, the majority of bacterial AT were classified as *Gammaproteobacteria*, whereas *Bacilli*, *Actinobacteria*, *Bacteroidia*, and *Clostridia* were predominantly found in bacterial IT and RT subcommunities (Fig. 3B). Compared to the normal tobacco leaves, the relative abundances of *Alphaproteobacteria*, *Actinobacteria*, and *Clostridia* decreased, while those of *Gammaproteobacteria*, *Bacilli*, and *Bacteroidia* increased in the moldy tobacco leaves; however, these changes were not statistically significant (Supplemental Fig. S5). Altogether, these results indicate that the community composition of fungi and bacteria in tobacco leaves was dominated by AT having a wide range of niche widths.

Additionally, the microbial communities in different samples were further subjected to comparative analysis. In Guizhou tobacco leaves, the relative abundance of OTU210 and OTU254 in the fungal community as well as OTU704 in the bacterial community exhibited significantly higher abundance in moldy tobacco leaves than in normal ones. Conversely, in Shanghai tobacco leaves, there was a significant decrease in the relative abundance of OTU264 and OTU252 in the fungal community as well as of OTU197 and OTU31 in the bacterial community in moldy tobacco leaves compared to normal ones. Similarly, in Jilin tobacco leaves, the relative abundance of OTU482 and OTU285 in the bacterial community in moldy tobacco leaves was significantly lower than in normal ones (Supplemental Fig. S6). Moreover, a total of 10 fungal OTUs were shared between the moldy and normal tobacco leaves, including *Aspergillus* (OTU331, OTU324, OTU260, OTU133, OTU327, OTU259, and OTU25), *Xeromyces* (OTU210), and *Wallemia* (OTU426 and OTU191). Normal tobacco leaves from Guizhou, Shanghai, and Jilin had individual 289, 74, and 35 unique OTUs; unfortunately, these OTUs were almost absent in moldy tobacco leaves (Supplemental Fig. S7 and Supplemental Data 1). Furthermore, a total of 90 bacterial OTUs shared between the moldy and normal tobacco leaves, which represented over 90.21% of the total sequences. Moreover, 23, 30, and 40 unique bacterial OTUs were observed in normal tobacco leaves from Guizhou, Shanghai, and Jilin, respectively, but they were entirely absent in moldy tobacco leaves. Surprisingly, 26, 82 and 32 unique bacterial OTUs were detected in the moldy samples from these three regions, this suggested they might be more adapted to the mildew environment than other taxa (Supplemental Fig. S8 and Supplemental Data 2). Remarkably, the number of shared

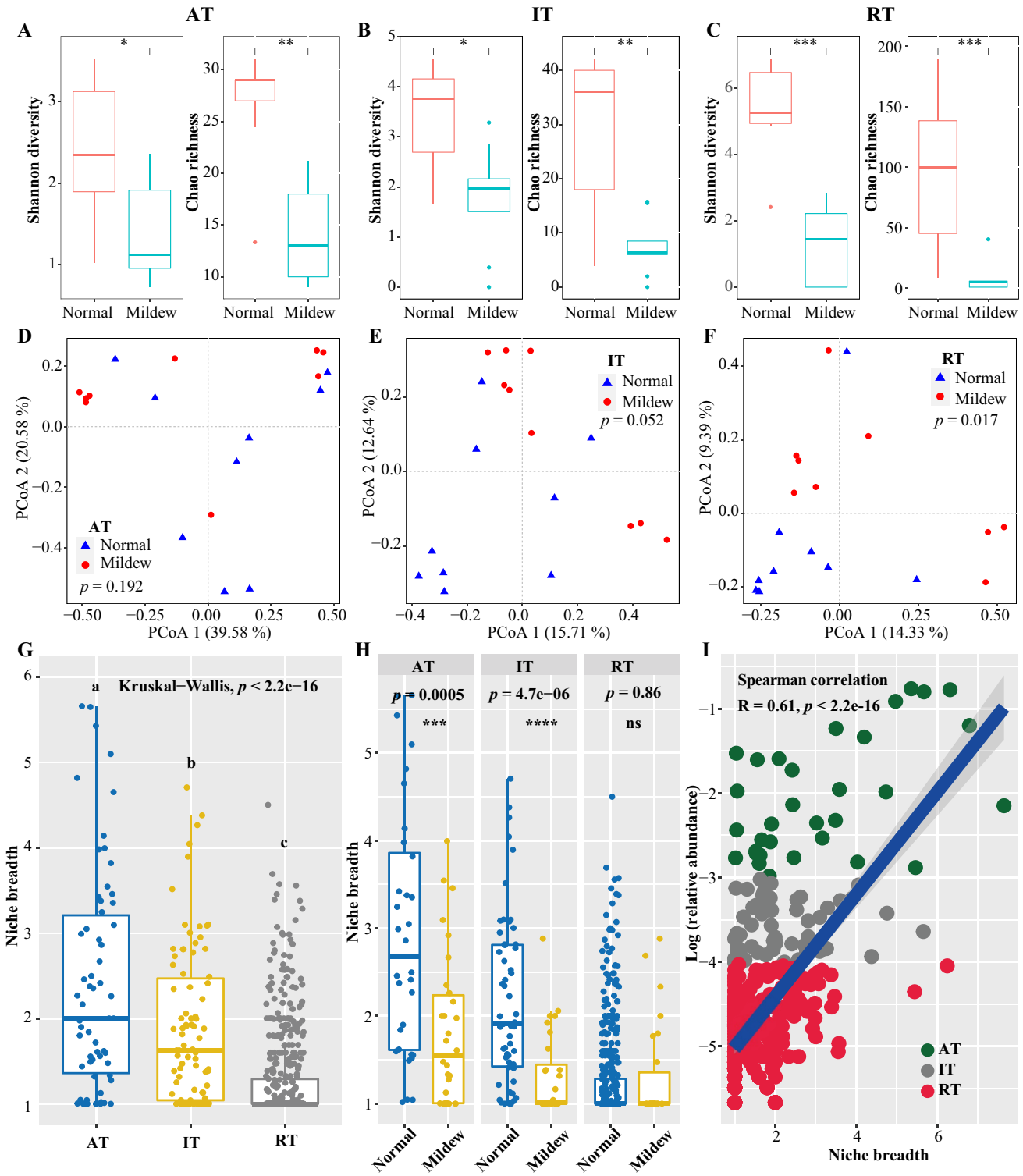

Fig. 1 The α-diversity (**A**–**C**), β-diversity (**D**–**F**), and Levin niche breadth (**G**–**I**) of fungal communities in the moldy and normal tobacco leaf samples. Asterisks "*," "**," and "***" indicate significance differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. "ns" indicates not significant ($p > 0.05$)

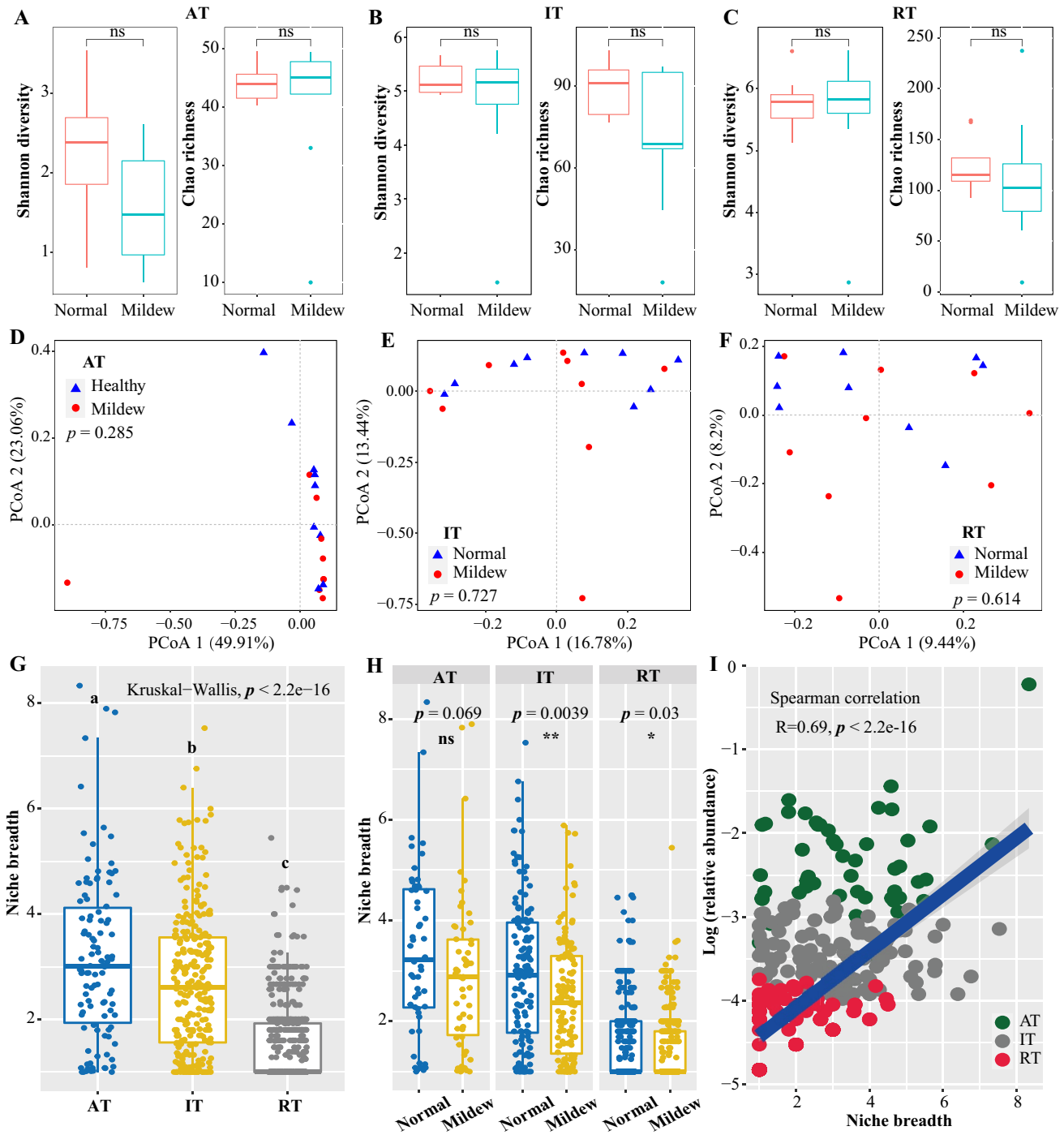

Fig. 2 The α-diversity (**A**–**C**), β-diversity (**D**–**F**), and Levin niche breadth (**G**–**I**) of bacterial communities in the moldy and normal tobacco leaf samples. Asterisks "*" and "**" indicate significance differences at $p < 0.05$ and $p < 0.01$, respectively. "ns" indicates not significant ($p > 0.05$)

community members were significantly higher than the number of unique members in each sample across all the samples. These findings suggested that moldy and normal tobacco leaves may share a similar set of core bacterial functional communities.

Effect of mildew on the molecular ecological network of fungal and bacterial communities

To explore the impact of mildew on ecological interactions among the microbial populations in tobacco leaves, we

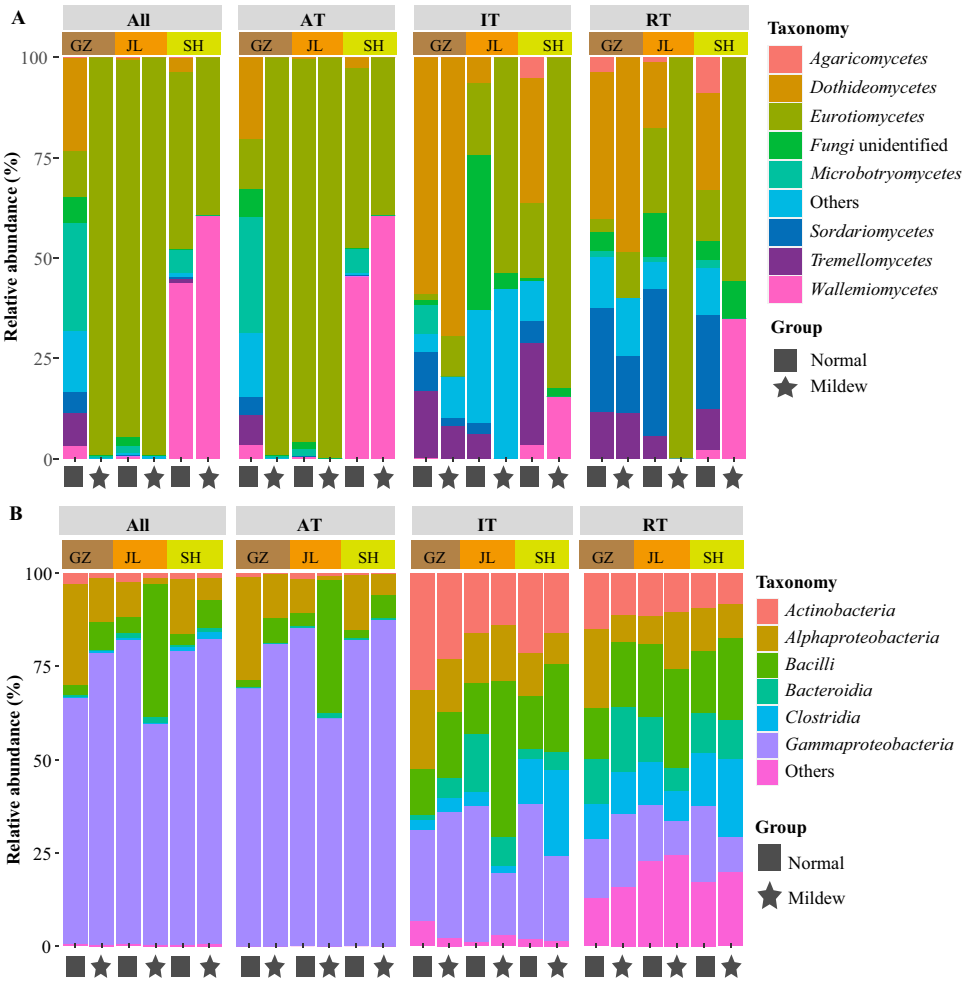

Fig. 3 Taxonomic composition of the fungal (**A**) and bacterial (**B**) communities in the moldy and normal tobacco leaf samples. Taxa accounting for < 1% of the total reads were grouped as "Others." GZ, Guizhou province; JL, Jilin Province; SH, Shanghai; All, all taxa; AT, abundant taxa; IT, intermediate taxa; and RT, rare taxa

constructed co-occurrence networks for fungal and bacterial communities in moldy and normal tobacco leaves. The fungal network in the normal tobacco leaves consisted of 169 nodes and 1746 edges, with AT, IT, and RT accounting for 2.37%, 17.16%, and 80.47% of the OTUs, respectively (Fig. 4A). There were a total of 434 edges between RT and IT nodes, with only 3 and 17 edges connecting AT to IT and RT, respectively (Fig. 4A). Moreover, the node attribute values of RT, including degree, eigenvector centrality, and closeness centrality, were significantly higher than those of AT and IT (Supplemental Fig. S9A), thus suggesting that fungal RT play a crucial role in the normal tobacco network by coexisting with fungal IT and themselves, but rarely with fungal AT. However, compared with the normal tobacco leaves, the fungal network in the moldy tobacco leaves consisted of only 42 nodes and 106 edges. Among these, 40.48%, 33.33%, and 26.19% of the nodes (OTUs) belonged to AT, IT, and RT, respectively (Fig. 4B). The sub-networks linking AT to IT, AT to RT, and IT to RT contained 26, 28, and 25 edges, respectively (Fig. 4B), with no significant differences in the node attributes (Supplemental Fig. S9B). These findings suggest that the contribution of fungal AT is significantly amplified in the moldy tobacco leaves compared to the normal tobacco leaves and that they coexist with fungal IT and RT. However, the fungal networks in the moldy tobacco leaves exhibited significantly lower values for average clustering coefficient (avgCC), average path length (APL), network diameter (ND), and average degree (AD) compared to those in the normal tobacco leaves (Supplemental Table S2), indicating a weaker interaction among the fungal species under mildew conditions. Additionally, the majority of co-occurrence network structures in the normal tobacco leaves exhibited negative correlations (92.55%), indicating competition among the fungal communities. In contrast, the network in moldy tobacco leaves displayed a relatively high proportion of positive correlations between the edges (23.58%), highlighting the role of ecological mutualism or cooperation in the aggregation of fungal communities in the moldy tobacco leaves (Supplemental Table S2). The majority of nodes in both the moldy and normal fungal co-occurrence networks were associated with 5 dominant classes, including *Dothideomycetes*, *Sordariomycetes*, *Eurotiomycetes*, *Microbotryomycetes*, and *Wallemiomycetes*; however, not all the dominant groups played

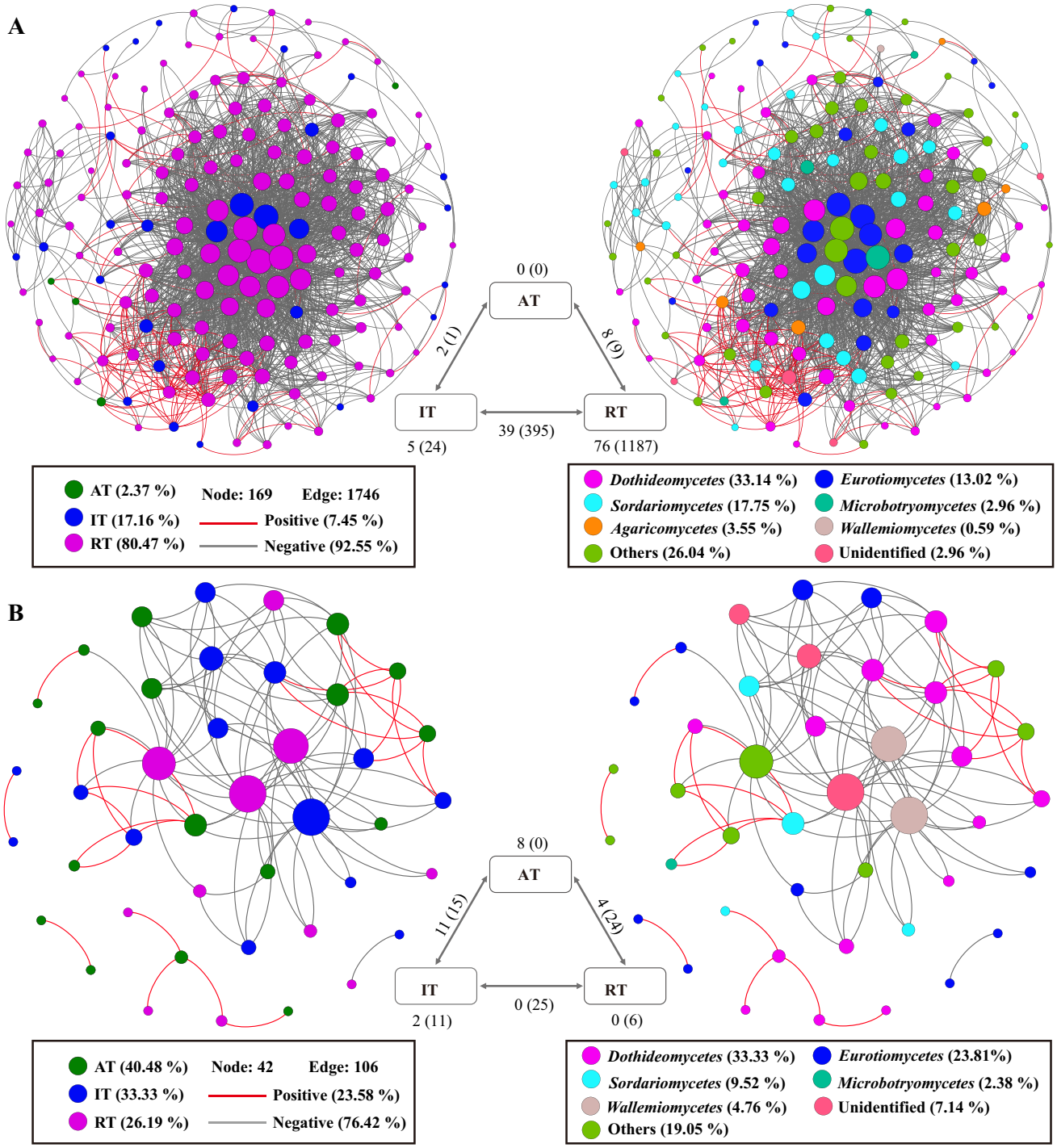

Fig. 4 Co-occurrence network of fungal communities in the normal (**A**) and moldy (**B**) tobacco leaves at the OTU level. The nodes of the network are colored according to different subcommunities (left) and fungal classes (right). The size of a node represents the degree of connection, and the colors of the edges represent the type of interaction (red: positive and gray: negative). The correlation graph in the middle shows the inter-associations between different subcommunities. The numbers outside and inside the parentheses represent positive and negative edge numbers, respectively

significant roles in either of the networks. For example, *Agaricomycetes* did not play any role in the moldy co-occurrence network (Fig. 4).

Bacterial networks showed similar structural features in both moldy and normal tobacco leaf samples. In normal tobacco leaves, the bacterial network was composed of

214 edges and 103 nodes, with AT, IT, and RT accounting for 51.46%, 43.69%, and 4.85% of the OTUs (Fig. 5A). Among these, 94, 2, and 8 edges connected AT to IT, AT to RT, and IT to RT, respectively. Furthermore, different subcommunities exhibited similar node topology characteristics (Supplemental Fig. S10A). In moldy tobacco leaves, the bacterial network comprised 176 edges and 73 nodes, with AT, IT, and RT accounting for 53.42%, 45.21%, and

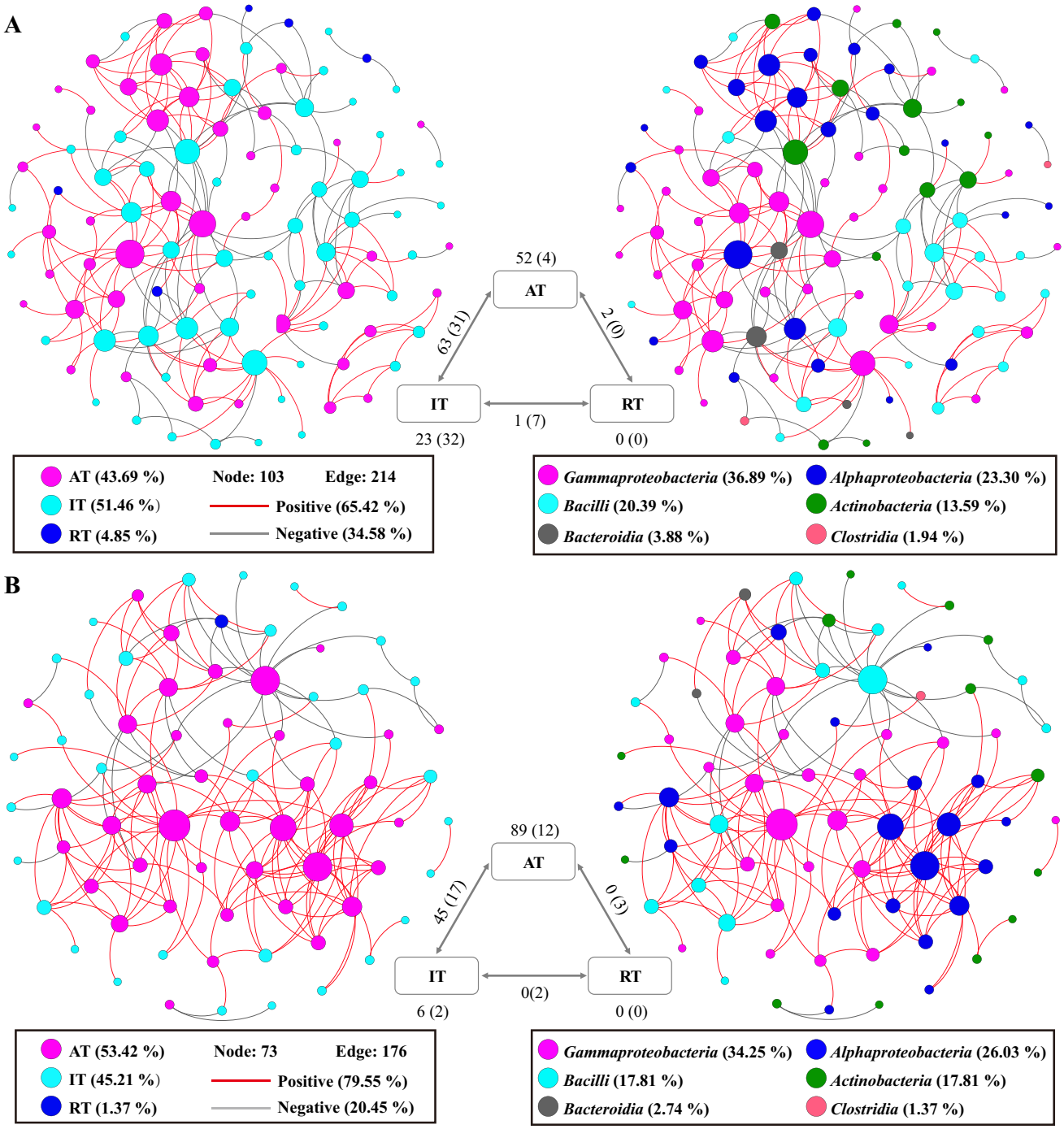

Fig. 5 Co-occurrence network of bacterial communities in the normal (**A**) and moldy (**B**) tobacco leaves at the OTU level. The nodes of the network are colored according to different subcommunities (left) and bacterial classes (right). The size of a node represents the degree of connection, and the colors of the edges represent the type of interaction (red: positive and gray: negative). The correlation graph in the middle shows the inter-associations between different subcommunities. The numbers outside and inside parentheses represent positive and negative edge numbers, respectively

1.37%, respectively (Fig. 5B). Among these, 62, 2, and 3 edges connected AT to IT, AT to RT, and IT to RT, respectively. However, AT exhibited significantly higher degrees, eigenvector centrality, and closeness centrality compared to IT and RT (Supplemental Fig. S10B). These findings indicate that the bacterial network in both moldy and normal tobacco leaf samples was primarily regulated by bacterial AT, which frequently co-occurs with bacterial IT, but rarely with bacterial RT. The proportion of positive links (65.42% and 79.55%) was significantly higher than that of negative links (34.58% and 20.45%) in bacterial networks of both the moldy and normal tobacco leaf samples, indicating a predominantly cooperative rather than a competitive relationship between the bacterial communities (Supplemental Table S2). Moreover, the nodal composition of the two bacterial networks exhibited similarities, with *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacilli*, and *Actinobacteria* being the dominant classes. Notably, the significance of *Actinobacteria* was found to be considerably higher in the moldy tobacco leaf network compared with the normal tobacco leaf network (Fig. 5).

Four *Zi-Pi* plots were constructed to assess the potential topological roles of different subcommunities within the fungal and bacterial networks of the moldy and normal tobacco leaf samples (Fig. 6). Specifically, all fungal OTUs present in the moldy tobacco leaves were categorized as peripherals, whereas 11 nodes (OTUs) were classified as connectors within the normal co-occurrence network (Fig. 6A), all of which belonged to fungal IT and RT. However, two bacterial AT and two bacterial IT OTUs were identified as the module hubs and connectors, respectively, in the normal tobacco leaf network, whereas five bacterial AT OTUs were categorized as the module hubs and connectors in the moldy tobacco leaf network (Fig. 6B). Altogether, these results revealed that the fungal network may exhibit lower stability than the bacterial network under moldy conditions, and both fungal RT and bacterial AT likely play a crucial role in maintaining the functioning of the tobacco leaf microecosystem.

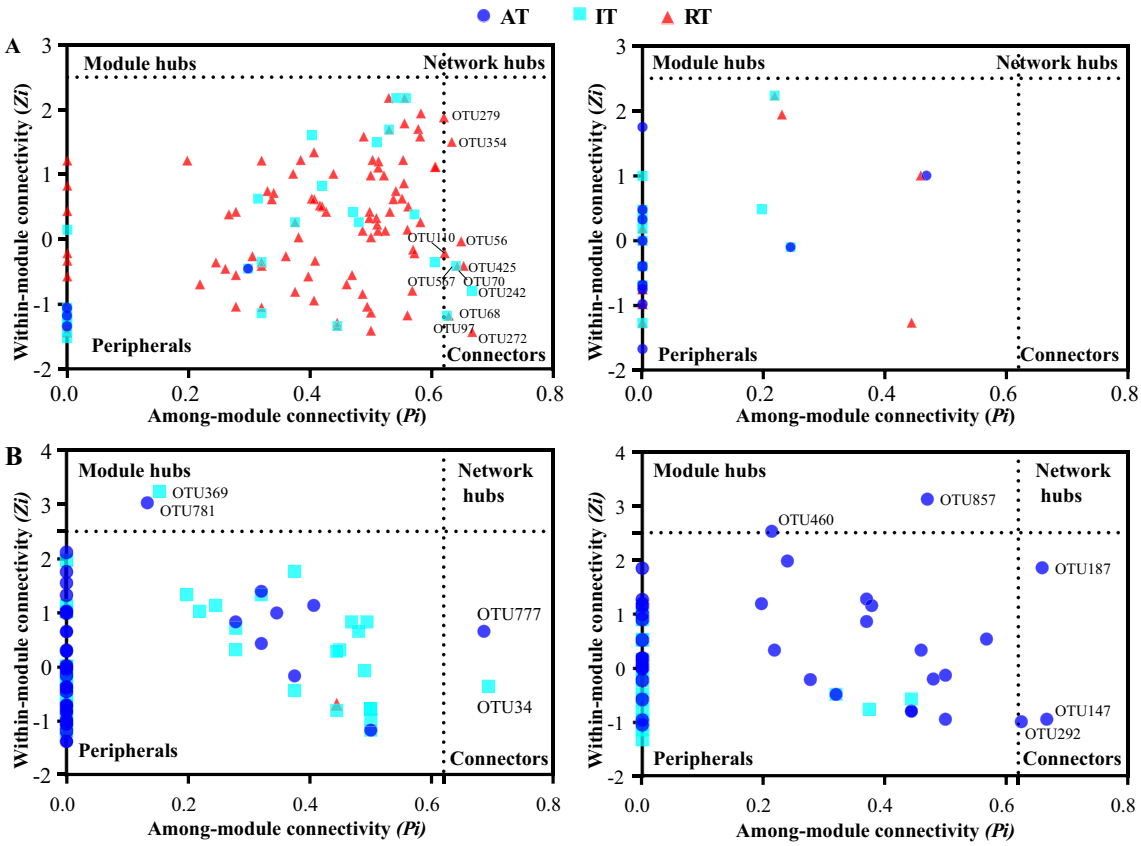

Fig. 6 *Zi–Pi* plot showing the distribution of OTUs based on their topological roles in fungal (**A**) and bacterial (**B**) networks in moldy (right) and normal (left) tobacco leaf samples. Each symbol represented an OTU in the network, with circles, squares, and triangles representing abundant, intermediate, and rare taxa, respectively. The threshold values of *Zi* and *Pi* were 2.5 and 0.62, respectively

Correlation between the microbiota and metabolome of the moldy tobacco leaves

To explore the impact of mildew on tobacco leaf metabolism, we conducted non-targeted metabolomics using LC–MS to identify global differences in the metabolic profiles of the moldy and normal tobacco leaf samples. Compared to the normal tobacco leaf samples, 208 compounds exhibited significant upregulation, while 50 compounds showed significant downregulation in the moldy tobacco leaf samples (VIP > 1 and $p < 0.05$) (Supplemental Table S3). Among these, lipids and organic oxygenated compounds were the most abundant metabolites, accounting for 25.97% and 20.16% of the differential metabolites, respectively. Organic oxygen compounds were predominantly composed of carbohydrates (12.79%, with 24 upregulated and 9 downregulated metabolites) and carbonyl compounds (5.43%, with 11 upregulated and 3 downregulated metabolites), while lipids primarily consisted of fatty acids (9.13%, with 18 upregulated and 6 downregulated metabolites), prenol lipids (8.14%, with 18 upregulated and 3 downregulated metabolites), and glycerophospholipids (4.65%, 3 upregulated and 9 downregulated metabolites). Moreover, the contents of amino acids and their analogs (8.14%, with 19 upregulated and 2 downregulated metabolites) and benzene and its derivatives (4.65%, $p < 0.05$, with 11 upregulated and 1 downregulated metabolites) changed significantly in the moldy tobacco leaves, compared with the normal tobacco leaves. Notably, moldy tobacco leaves displayed a significant increase in the concentration of organic heterocycles, organic acids, polyketones, and benzene ring metabolites, including indoles, pyridines, flavonoids, cinnamides, phenols, and peptides.

To further investigate the relationship between the differential metabolites and microbial communities in the moldy and normal tobacco leaf samples, we performed a correlation analysis between the abundant microbial taxa and significant differential metabolites (Supplemental Fig. S11). Remarkably, the OTUs of *Dothideomycetes* (OTU389 and OTU415) and *Alphaproteobacteria* (OTU1207, OTU1354, OTU193, and OTU460) predominantly exhibited significant negative correlations with most metabolites, while they displayed a significant or insignificant positive correlation with pyrrolines and pyrrolidines. The majority of *Gammaproteobacteria* (OTU273, OTU288, OTU292, OTU327, OTU337, OTU777, OTU816, OTU826, and OTU831) and a subset of *Alphaproteobacteria* (OTU375, OTU781, and OTU785) displayed significant negative correlations with benzoxazines, homoisoflavonoids, lactones, tetrapyrroles, tetracyclines, diazanaphthalenes, as well as benzimidazole ribonucleosides and ribonucleotides. In contrast, the majority of *Eurotiales* (OTU133, OTU264, and OTU265) and *Bacilli* (OTU1175, OTU1328, and OTU641) as well as a few *Alphaproteobacteria* (OTU454) were predominantly positively correlation with most differential metabolites, while they displayed a significant or insignificant positive correlation with saccharolipids. The aforementioned findings suggest a strong correlation between the activities of fungi and bacteria and the biosynthesis and catabolism of metabolites in tobacco leaves during mildew.

Discussion

Fungal communities were more sensitive to mildew than bacterial communities

In a tobacco storage ecosystem, the tobacco leaves serve as both an energy source and a habitat for numerous heterotrophic species, including fungi, bacteria, insects, and mites, and microbial activity is the primary contributor to the degradation of stored materials in this ecosystem (Dunkel 1992). In this study, we found that the diversity, composition, and network properties of the fungal communities were significantly altered in moldy tobacco leaves compared to normal tobacco leaves. This is consistent with the results of previous studies that established fungi as the primary causative agents of mildew spoilage in stored and processed foods (Christensen 1972; Fleurat-Lessard 2017). However, no significant alterations were observed in the diversity, composition, and network properties of the bacterial communities between the moldy and normal tobacco leaves, indicating that bacterial communities may exhibit a relatively lower susceptibility to the mold hazard compared to the fungal communities. One possible explanation for these results is that fungi and bacteria employ different mechanisms for community assembly. Previous studies have demonstrated that bacterial communities are primarily influenced by diffusion limitations, whereas fungal communities are dominated by environmental selection (Xiao et al. 2018). In general, tobacco leaves are stored in a dry state (< 13% moisture), which significantly restricts the activities of most microorganisms. However, fungi possess the ability to sense and respond to their environment at the micron scale (Peay et al. 2016). Transient fluctuations in water availability, temperature, pH levels, and foreign compounds exert an influence on fungal interactions and ultimately determine the dominance of fungal communities (Magan and Aldred 2008). Another possible explanation is that physiological variations lead to the allocation of diverse microorganisms to optimal ecological niches based on their nutritional preferences and functional specificities (Qian et al. 2020). It is widely acknowledged that fungi possess a broader range of enzymatic capabilities, exhibit slower biomass turnover rates, and have a higher carbon use efficiency than bacteria (Xiao et al. 2018). In natural ecosystems, fungi play a prominent role in carbon

cycling, owing to their ability to degrade complex substrates (Treseder and Lennon 2015), while bacteria contribute more significantly to nitrogen cycling due to their robust ammonification and nitrification capacities (Huang et al. 2020; Wang et al. 2020). In this study, the metabolomic analysis revealed that the majority of significantly altered metabolites in the moldy tobacco leaves comprised carbohydrates, lipids, carbonyl compounds, and other carbon-containing compounds and derivatives. These results are consistent with the results of a previous study that demonstrated a significant decrease in the total carbon content in moldy tobacco leaves compared to normal tobacco leaves (Zhou et al. 2022). These results suggest that carbon turnover might be a crucial factor driving microbial succession in moldy tobacco leaves.

In addition, compared to bacteria, fungi are more capable of adapting to various stresses and colonizing food resources (Snyder et al. 2019). Additionally, the majority of opportunistic fungi, such as *Aspergillus* and *Penicillium*, and xerophilic fungi adopt a stress-ruderal selected survival strategy to successfully colonize various niches on tobacco leaves (Magan and Aldred 2008; Snyder et al. 2019). Although they generally have a slow growth rate, these fungi are capable of surviving and reproducing under harsh conditions, including high temperatures, low pH, or limited water availability, and they allocate resources to facilitate accelerated reproduction when the conditions improve. Therefore, the differences in physiology and community assembly strategies enable fungi to play a more important role than bacteria in the mildew process of tobacco leaves.

The disappearance of fungal RT may serve as a crucial indicator of mildew occurrence

The ecological functions of the tobacco storage system were modulated by the microbiota inhabiting the tobacco leaves. In normal tobacco leaves, fungal RT exhibited the highest richness and diversity, suggesting that low-abundant microbes are important contributors to species diversity (Lynch and Neufeld 2015). However, significant alterations were observed in both α- and β-diversities of the fungal communities in the moldy tobacco leaves compared to the normal tobacco leaves. Furthermore, the majority (94.88%) of fungal RT were eliminated from the moldy tobacco leaves, suggesting that fungal RT may exhibit increased susceptibility to mildew rather than fungal AT, which exhibited stronger resistance and adaptability towards mildew-induced environmental changes. This can be attributed to their distinct ecological strategies (Lynch and Neufeld 2015). For instance, AT possessed a broad niche breadth, enabling them to competitively exploit a range of resources, effectively adapt to environmental changes, and maintain their persistence and plasticity (Zhao et al. 2022). Conversely, RT, with narrow niche breadths, may exhibit lower competitiveness, growth rate, and resistance against mold-induced environmental changes (Jousset et al. 2017). In contrast, the abundance of AT contributes to the rapid increase in their reproduction rate, which enables them to rapidly occupy more ecological niches (Magan and Aldred. 2008). Therefore, fungal AT demonstrate high resistance and adaptability under unfavorable and complex heterogeneous environments caused by mildew.

Compared to simple diversity indices and community structure descriptions, network analysis may provide more profound insights into the interactions among microbial subpopulations (Ziegler et al. 2018). To our knowledge, this is the first study to use correlation-based network analysis to investigate co-occurrence patterns among microbial RT and AT subcommunities in stored tobacco leaves. Network topology can reflect the interactions among microorganisms, and node centrality indicates the potential impact of a species on the co-occurrence of other nodes (Hu et al. 2017). Nodes with high centrality may occupy a core and central position in the network, and the corresponding species may have a strong influence on other interactions (Zhang et al. 2020). Our results show that the majority of nodes in the fungal network of normal tobacco leaves belong to fungal RT (80.47%), and their degree, eigenvector, and closeness centrality values were significantly higher than those of fungal IT and AT, indicating that fungal RT more frequently occupied central positions within the network. In contrast, the fungal network in moldy tobacco leaves exhibited a significant reduction in the proportion of RT (26.19%), along with a decrease in their degree, eigenvector, and closeness centrality values compared to the IT and AT. This indicates that fungal RT are significantly marginalized within moldy tobacco leaves, while fungal AT exert significant influence on the fungal network. Surprisingly, the majority of nodes in all bacterial networks were attributed to the AT and IT, implying that these subcommunities are responsible for maintaining the bacterial network structure.

Microbial keystones are defined as highly connected nodes that strongly influence the community structure and network characteristics, either alone or within a guild, irrespective of their abundance (Banerjee et al. 2018). In this study, the key fungal species in the normal tobacco leaves belonged to the RT and IT, which had low abundance; however, key fungal species were not identified in the fungal network of moldy tobacco leaves. Conversely, most keystone bacterial species in both moldy and normal tobacco leaf samples belonged to the AT. A study demonstrated that RT were just as crucial as AT in upholding ecosystem function (Shi et al. 2016) and that the absence of keystone species could result in the fragmentation of microbial networks (Xue et al. 2020). Our previous studies found that network structures of fungal community in moldy tobacco leaves tend to be fragmented (Zhou et al. 2022). Therefore, it can be

inferred that the maintenance of tobacco storage ecosystems was dependent on the indispensable roles played by fungal RT and bacterial AT and that the disappearance of fungal RT may serve as a crucial indicator for mildew occurrence.

Mildew may have compromised the defense mechanisms within the tobacco microecosystem

Metabolites play a crucial role as signaling molecules for both intra- and inter-species communication (Schmidt et al. 2019). Intracellular metabolic pathways can be altered by diffusible molecules or by direct cell–cell contact (Liu and Kakeya 2020). Microorganisms produce various secondary metabolites that modulate individual and collective responses, ultimately shaping the entire microbial community (Schmidt et al. 2019; Liu and Kakeya 2020). Carbohydrates constitute a significant proportion of tobacco (40–50% by weight), most of which can be hydrolyzed to produce glucose and fructose, which serve as vital energy sources for mold-inducing microorganisms (Tsaballa et al. 2020). Amino acids and carbonyl compounds (alcohols, aldehydes, ketones, essential oils, and other aromatic substances) are important components or precursors of the aromatic compounds in tobacco (Sun et al. 2011). In this study, the proline content in moldy tobacco leaves was significantly lower than that in normal tobacco leaves, which is consistent with the results observed in a previous study (Li et al. 2014). Furthermore, a study demonstrated that proline can enhance plant tolerance to abiotic stresses, such as drought and salt stress (Yamchi et al. 2007). Lipids, including fatty acids, phospholipids, steroids, and glycolipids, were the primary constituents of cell membranes, organelle membranes, and the cuticle and wax of plant epidermis. These compounds play a crucial role in protecting plant tissues against external mechanical damage and pathogen attack (Fischer et al. 2004; Yang et al. 2017); however, their content was significantly lower in moldy tobacco leaves compared to normal tobacco leaves. In addition, polyketones (flavonoids, cinnamic acids, isoflavonoids, and coumarins), phenols (arbutin, caftaric acid, vanillic acid, and vanillin acetate), and polypeptides (threoninyl-isoleucine, asparaginyl-isoleucine, and arginyl-proline), which are known antioxidant and antibacterial compounds (Martens and Mithofer 2005; Sato et al. 2011; Li et al. 2020c; Marchiosi et al. 2020), are not only crucial for human beings but are indispensable for plant growth, development, and survival. In plants, these compounds act as protective agents against abiotic stresses or as phytoantitoxins against pathogens and herbivores (Tsaballa et al. 2020). Interestingly, these compounds were significantly higher in moldy tobacco leaves. Therefore, the significant alteration in the metabolites associated with stress resistance and defense mechanisms in moldy tobacco leaves suggests that microbial activities during mildew growth may seriously compromise the defense system of tobacco leaves. However, the functions of most of these compounds are unknown, necessitating further studies to elucidate their role in mildewing.

Microbial interactions have the potential to affect the production of host metabolites, particularly those related to quorum sensing (Tourneroche et al. 2019). In turn, these metabolites may modulate the composition and activity of microbial communities. For instance, within the gastrointestinal tract, some microorganisms, such as *Clostridium*, *Peptostreptococcus*, and *Lactobacillus*, can metabolize tryptophan into indole and its derivatives. These secondary metabolites subsequently modulate the ecological balance of bacteria, fungi, and viruses or confer a competitive advantage to one community (Roager and Licht 2018). In this study, the majority of fungal taxa exhibited a significant negative correlation with compounds, while only a few taxa of *Eurotiales*, such as *Xeromyces bisporus* (OTU254), *Aspergillus penicillioides* (OTU324, OTU260, and OTU256), and *Aspergillus amstelodami* (OTU133), displayed positive correlations with changes in some metabolites. One possible explanation for this phenomenon is that acclimated species may have secreted antibiotic or antifungal compounds to inhibit the growth of other species, or their secondary metabolites could substitute for the corresponding functional microorganisms.

Surprisingly, we observed positive correlations between differential metabolites and bacterial taxa such as *Bacillales*, *Gammaproteobacteria*, and *Alphaproteobacteria*. A previous study demonstrated the significant influence of bacteria on the production of volatile flavor compounds and flavors in cigar leaves (Zheng et al. 2022a, c). Additionally, *Acinetobacter*, *Sphingomonas*, *Solibacillus*, and *Lysinibacillus* were main carbonyl compound-producing microbes in fermented cigar tobacco leaves (Zheng et al. 2022b). These findings provide evidence for the involvement of bacteria in the occurrence of tobacco leaf mildew, indicating their significant role in both metabolite production and degradation. It is worth noting that while metabolomics can analyze the metabolites of organisms under specific conditions at a particular time, further investigation is necessary to explore the dynamic changes in the tobacco leaf metabolite profiles during mildewing and their correlation with the tobacco leaf microbial community structure.

In summary, we conducted an analysis of the fungal and bacterial AT, IT, and RT in moldy and normal tobacco leaf samples. Our findings indicate that compared to the bacterial communities, the diversity and composition of the fungal communities were more vulnerable to mildew. Additionally, we found that fungal RT and bacterial AT were crucial for maintaining the stability and function of the tobacco leaf ecosystem, while fungal AT exhibited strong resistance and adaptability to mold damage. Furthermore, the metabolomic analysis revealed that mildew may have inflicted irreversible

damage on the defense mechanisms of tobacco leaf microecosystem. The majority of differential metabolites showed a negative correlation with most fungi and bacteria, while only a few *Eurotiales* and *Bacilli* showed a positive correlation. This study demonstrated that fungi are the primary biological agents responsible for tobacco leaf mildew and provided evidence supporting the irreplaceable role of bacteria in the tobacco leaf mildew process. Thus, this study provides novel insights for a comprehensive understanding of the mildew process in a tobacco storage ecosystem and establishes a theoretical foundation for the management of tobacco storage.

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Author contribution JXZ performed the experiments, collected and analyzed the data, and drafted and reviewed the manuscript; JL, DFW, and SG conducted the experiment; YBY and JYG analyzed data; and XZ contributed to the study design and protocol development and reviewed the manuscript. All the authors read and approved the final manuscript.

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Data availability All Illumina HiSeq data in this study have been submitted to the NCBI Sequence Read Archive database under the BioProject accession numbers PRJNA723334 and PRJNA954269. Other available data can be found in the supplementary materials.

Declarations

Ethics approval This article does not contain any studies with human participants or animals.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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