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Microbial community analysis of mildewed cigar tobacco leaves from high-throughput sequencing data

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Abstract

Background Cigar tobacco leaves contain abundant bacteria and fungi that are vital for their quality. In this study, the microbial communities were analyzed in the artificial mildewed cigar tobacco leaves of different mildew stages (healthy control, early stage, middle stage and late stage).

Results For cigar wrapper tobacco leaves, there was an increased bacterial genera abundance of *Terribacillus*, *Bacillus* and *Micrococcus*, while there was an increased fungal genera abundance of *Aspergillus*, *Penicillium* and *Mucor*. For cigar filler tobacco leaves, there was an increased bacterial genera abundance of *Staphylococcus*, while there was an increased fungal genera abundance of *Aspergillus* and *Trichomonascus*. Microbial communities (bacterial and fungal) showed significantly different compositions in both cigar wrapper and filler tobacco leaves from different mildew stages. The top important microbial communities (bacterial and fungal) in cigar wrapper and filler tobacco were *Sphingomonas*, *Aerococcus*, *Wallemia* and *Trichomonascus*, respectively.

Conclusion This study provided evidence for the great changes in microbial communities during the mildew process of cigar wrapper and filler tobacco. The effects of the dominant bacterial genera and fungal genera on tobacco mildew should be explored in depth, whose findings may be applied to develop strategies for controlling tobacco mildew.

Keywords Tobacco mildew, Cigar tobacco leaves, Bacterial community, Fungal community

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Introduction

Tobacco is one of the most widely used cash crops. Cigar is a special tobacco product wrapped with tobacco leaves but not wrapping paper. In recent years, cigar consumption has been increasing for the decreased prices and diversified tastes (Wang, 2020; Wang et al 2022). High-quality tobacco leaf raw materials are important for the development of cigarette enterprises, and tobacco storage safety is the key to ensuring the quantity and quality of raw materials (Zhou et al 2024). Cigar tobacco leaves need air-curing, fermenting and aging for cigar products, while tobacco leaves for cigarettes are flue-cured (Zhang et al 2023). Fresh tobacco leaves must be stored for two to three years for naturally aging or artificial fermentation for four to eight weeks before being used in cigarette production (Keller, 1929). During the storage process for aging or artificial fermentation, factors such as storage environment factors (temperature, humidity, etc.) (Tang et al 2020), the content of chemical components (total sugar, protein, starch, biological enzymes, etc.) in tobacco leaves (Yang et al 2021), moisture content and pH of tobacco leaves, and surface microorganisms of tobacco leaves are interconnected and constrained to form a specific ecosystem (Chulze 2010; Liu et al 2019; Long et al 2020; Stevenson et al 2017). Ecological conditions have important impacts on the development of high-quality tobacco leaves (Ma et al 2018; Tang et al 2020). Amount of studies demonstrated that during cigar tobacco leaves fermentation process, the balance of bacterial and fungal communities significantly changed. Tao et al. found that many microorganisms such as *Bacillus*, *Pseudomonas*, *Enterobacter*, *Sphingomonas* and *Methylobaterium* degrade macromolecular organic matter and produce small fragrant matter (Tao et al 2022). For the bacteria community in the fermentation process, *Staphylococcaceae* and *Lactobacillales* were dominant in the early stage, while *Actinomycetales* was dominant in the late stage (Wan et al 2020).

Mildew is a serious problem that commonly happens during the storage of tobacco leaves. Due to the strong hygroscopicity of tobacco leaves, the probability of mildew formation will largely increase as the storage time goes by. Many types of fungi are able to infect tobacco leaves across the whole stages from leaf-growth, harvesting, curing, aging, to storage (Li et al 2023; Long et al 2020; Pan et al 2021). Once mildew occurs on leaves, fungi will absorb nutrients and grow. As the consequence, the fungi destroy the content ratio of nitrogen, sugar, organic acids and aroma components that are closely related to the sweetness and mellowness, and finally ruin the senses of cigars (Zhou et al 2022). This causes huge economic losses to tobacco industry and consumer. To make matters worse, there was still no effective strategy in controlling tobacco leaf mildew, which calls for further investigation to elucidate the cause underline the mildew, such as the change patterns of microbial communities (bacterial and fungal). Since the imbalance of microbial communities would affect the ecosystem in the tobacco leaves, and thereby induce mildew.

To this end, we here applied high-throughput sequencing technique to examine the composition and diversity of microbial communities in cigar tobacco leaves with varying degrees of mold growth, and identify the evolution of microbial ecology in cigar tobacco leaves during the mildew process, hope for gaining a better understanding of the essential roles of bacteria and fungi in cigar tobacco mildew and providing a reference for the prevention and control of cigar tobacco mildew.

Materials and methods

The mildew process of cigar tobacco leaves was induced in the condition of 32℃ and 90% (KCl) humidity. The cigar wrapper tobacco leaves were from Sumatra of Indonesia. The cigar filler tobacco leaves were from Danjiangkou of China. According to the comprehensive conditions of mildew degree, tobacco leaf color and fungal colony size, the mildew samples of cigar wrapper leaf (JY) and cigar filler leaf (JX) were divided into three groups: early stage, middle stage and late stage. The healthy tobacco wrapper and filler leaves were set as the control group. The mildew process of cigar tobacco leaves was induced in the condition of 32℃ and 90% (KCl) humidity. The character of the four groups of tobacco leaves was described as follows: Control group means that the tobacco leaves have no milk and no music smear (examined by microscopy); Early stage means that the tobacco leaves have no apparent mold growth visible to the naked eye, but have mycelium that can be detected under microscopic examination; Middle stage means that the tobacco leaves have obvious mildew, and can be used after removing the damaged part; Late stage means that the tobacco leaves have noticeably mildewed spots, turn black in color, emit a strong suffocating musty smell, and completely lost their use value. The samples of cigar wrapper leaves were the normal group (JY-control), the early stage group (JY-early), the middle stage group (JY-middle) and the late stage group (JY-late). Similarly, the samples of cigar filler groups were named JX-control, JX-early, JX-middle and JX-late. There were three replicates in each group of cigar wrapper and filler leaves, and a total of 24 samples were finally collected and immediately stored at −20℃.

DNA extraction and amplicon sequencing of microbial communities

10 g of each sample were weighed into a sterile homogenization bag with 90 ml of sterile PBS buffer, and the bead beated for 10 min in a sterile beating homogenizer. Then the buffer was filted with sterile double-layer gauze. The filtrate was centrifuged for 10 min at 12,000 r/min at 4℃. The precipitate was rinsed with sterile water, centrifuged at 12,000 r/min for 5 min, and rinsed twice. Finally, the precipitate was collected for microbial high-throughput sequencing. Genomic DNA was extracted according to the instructions of the FastDNA® SPIN Kit for soil (MP biomedicals, USA). The concentration and integrity of genomic DNA were determined via spectrophotometry (NanoDrop 2000c, Thermo Scientific, USA) and 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of bacterial 16S ribosomal RNA (rRNA) gene were amplified with the universal primers of 16S rRNA were 338F (5'-ACTCCTACGGGAGGC AGCA-3') and 806R (5'-ACTCCTACGGGAGGCAGC A-3') (Salas-González et al 2021). The nuclear ribosomal DNA internal transcribed spacer (ITS) 3–4 regions of fungi were amplified with the universal primers of ITS3F (5'-GCATCGATGAAGAACGCAGC-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') (Gade et al 2013). Then, 2% agarose gel electrophoresis was used to detect the polymerase chain reaction (PCR) products, and the PCR products were purified, quantified, amplified, and sequenced on an Illumina Novaseq 6000 platform (Meiji Biomedical Technology Co., Ltd).

Statistical analysis

Raw reads were quality-filtered under specific filtering conditions to obtain high-quality clean reads using fqtrim (v0.94), then 97% sequence identity was utilized to cluster the clean reads to the operational taxonomic unit (OTU) using Usearch software (Edgar 2013). Basic local alignment search tool (BLAST) searches were used for sequence alignment, and the feature sequences were annotated using the UNITE database and Greengenes database for each representative sequence (McGinnis & Madden 2004).

For measuring the operational taxonomic unit (OTU) level α-diversity of the species, Chao1, and Simpson were employed to analyze the α-diversity of the microbiota

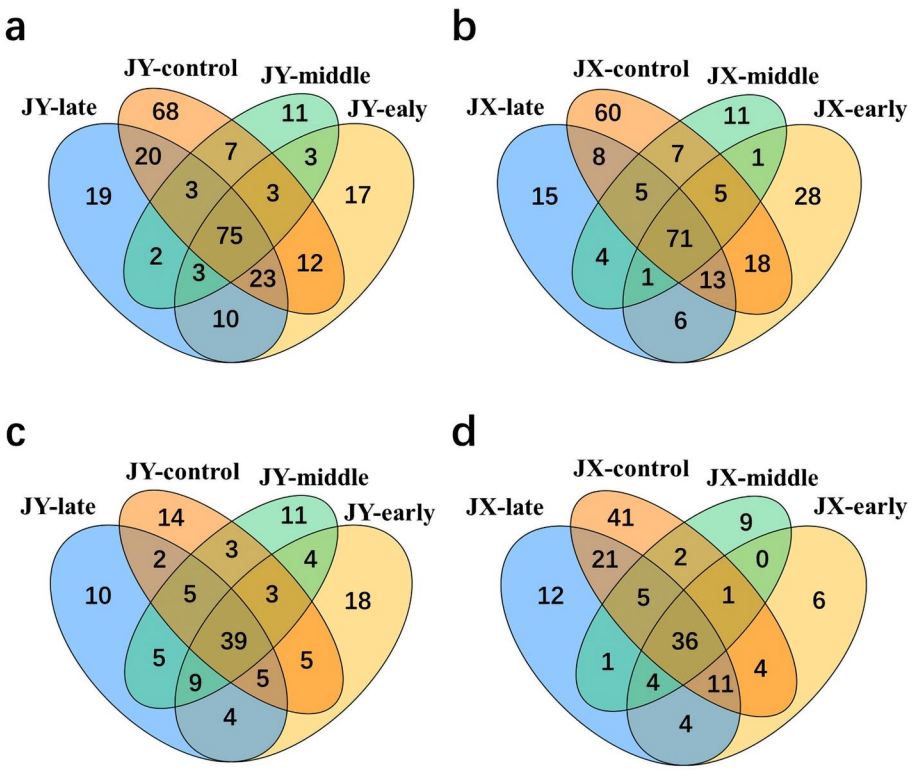

Fig. 1 Venn diagram depiciting number of genera identified distribution of bacterial communities for cigar wrapper tobacco leaves (**a**) and cigar filler tobacco leaves (**b**); Fungal comminites for cigar wrapper tobacco leaves (**c**) and cigar filler tobacco leaves (**d**). JY is the abbreviation of cigar wrapper tobacco leaves, JX is the abbreviation of cigar filler tobacco leaves. The normal group (control), mildew early stage (early), mildew middle stage (middle) and mildew late stage (late)

of tobacco leaves in these samples using QIIME2 (Ver. 2022.08) software. Principal coordinate analysis (PCoA) and Adonis were performed based on the Bray–Curtis distance using R software (Shi et al 2020). In the Vegan package (https://cran.r-project) R3.3.1, a Venn diagram, community heatmap and phylogenetic maps were generated from the relative abundances of OTUs.

Result

Effects of mildew on microbial changes in cigar tobacco leaves

After the quality control processes, a total of 1,441,879 high-quality sequences were obtained from the JY groups and the JX groups (average number of sequences 60,078.3 per sample). Fig. 1a and b showed us the numbers of bacterial species in each group of JY and JX samples, respectively. A total of 619 bacteria genera were identified in JY samples, and 211, 146, 107 and 155 genera of which were identified in samples of JY-control, JY-early, JY-middle and JY-late, respectively. A total of 558 bacteria genera were identified in JX samples, and 187, 143, 105 and 123 genera of which were identified in samples of JX-control, JX-early, JX-middle and JX-late, respectively. Fig. 1a and b also showed us the Venn diagram of bacterial community composition among the groups of JY and JX samples, respectively. It was found that the numbers of non-repetitive bacterial species decreased across the different mildew stages. In both JY and JX samples, the bacterial genera numbers of moldy groups were significantly lower than that of control groups. Compared with control groups, the number of bacterial genera decreased with the mildew degree increasing, but suddenly raised in the later stage.

Similarly, the fungal genera were isolated from the same samples, classified and identified with fungal OTUs, recorded the species number and analyzed by Venn diagram. A total of 317 fungal genera were obtained from JY samples, and 75, 86, 78 and 78 genera of which were obtained from samples of JY-control, JY-early, JY-middle and JY-late, respectively (Fig. 1c). A total of 339 fungal genera were obtained from JX

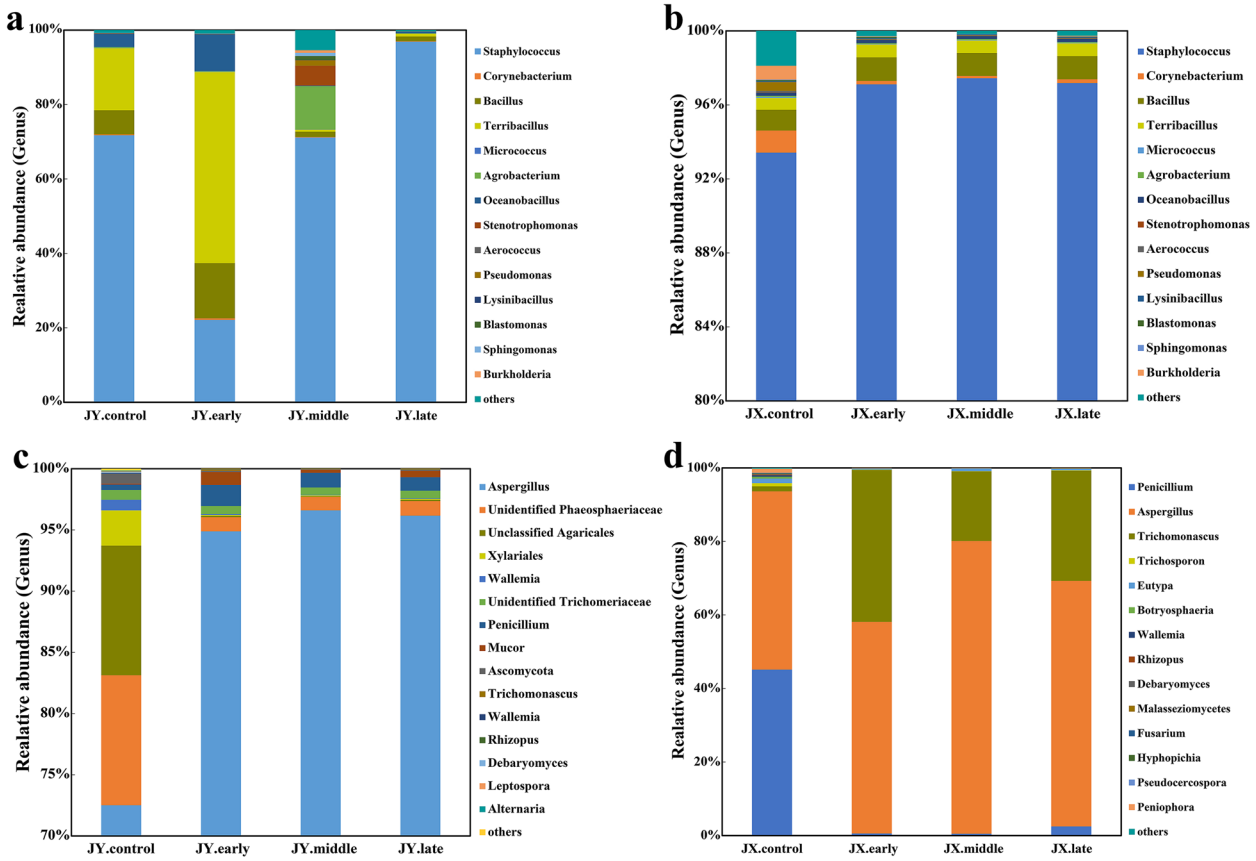

Fig. 2 Relative abundance of bacterial community on genera level in different samples: cigar wrapper tobacco leaves (**a**) and cigar filler tobacco leaves (**b**); relative abundance of fungal community on genera level in different cigar leave samples: cigar wrapper tobacco leaves (**c**) and cigar filler tobacco leaves (**d**). JY is the abbreviation of cigar wrapper tobacco leaves, JX is the abbreviation of cigar filler tobacco leaves. The normal group (control), mildew early stage (early), mildew middle stage (middle) and mildew late stage (late)

samples, and 121, 66, 58 and 94 genera of which were obtained from samples of JX-control, JX-early, JX-middle and JX-late, respectively (Fig. 1d). Interestingly, the change trend of fungal species number was quite opposite to that of bacterial species number in JY samples, while the change trend for fungal species number was the same as that of bacterial species number in JX samples.

Composition analysis of microbial communities in cigar tobacco leaves during mildew process

Figure 2a showed us the composition of bacterial communities in JY leaves. The dominant bacteria genera were *Staphylococcus* (71.73%), *Terribacillus* (16.71%), *Oceanobacillus* (3.52%), *Stenotrophomonas* (0.08%), *Agrobacterium* (0.25%) and *Bacillus* (6.46%) in the leaf samples of JY-control. In the leaf sample of JY-early, the relative abundance of *Staphylococcus* decreased to 22.12%, while *Bacillus* increased to 14.83%. In the leaf samples of JY-middle, the relative abundance of *Staphylococcus* restored to the original level on healthy leaves, and the relative abundances of *Oceanobacillus*, *Stenotrophomonas* and *Agrobacterium* increased to 9.8%, 5.33% and 11.6%, respectively. In the leaf samples of JY-late, the relative abundance of *Staphylococcus* was 96.8%. These results indicated that the functional diversity of bacterial community was gradually dismissed in JY mildewed leaves.

Figure 2b showed us the composition of bacterial communities in JX leaves. The dominant bacterial genera were *Staphylococcus* (93.4%), *Corynebacterium* (11.93%), *Bacillus* (1.12%), *Terribacillus* (0.64%), *Pseudomonas* (0.49%) and *Burkholderia* (0.74%) in the leaf samples of JX-control. And the relative abundance of *Staphylococcus* increased to around 97% in the early, middle and late stages of JX mildewed leaves. Compared with the JY leaves, the relative abundances of bacterial species decreased in JX leaves, but they had a similar overall change trend of bacterial genera during mildew process. The compositions of bacterial communities were nearly similar among the three groups of JX-early, JX-middle and JX-late.

Figure 2c showed us the composition of fungal communities in JY leaves. The dominant fungal genera were *Aspergillus* (72.51%), *unidentified members of the family Phaeosphaeriaceae* (10.61%), *order Agaricales* (10.59%), *order Xylariales* (2.87%), *Wallemia* (0.87%), *Unidentified Trichomeriaceae* (0.8%) and *Penicillium* (0.44%) in the leaf samples of JY-control. During the mildew process, the relative abundance of *Aspergillus* increased, while that of *Unidentified Phaeosphaeriaceae* and *Unclassified Agaricales* decreased. There was no significant difference in the composition of fungal communities between early, middle and late stages. With the mildew degree increasing, fungi with pathological and symbiotic nutrition patterns gradually reduced or even disappeared, such as *animal pathogenic bacteria*, *parasitic fungi*, *plant pathogenic bacteria* and *endophytic fungi*. However, the proportion of *Aspergillus* gradually increased in the mildew process, accounting for more than 90%, which showed that saprophytic nutrition is an important feature of fungal communities in mildewed tobacco leaves.

Figure 2d showed us the composition of fungal communities in JX leaves. It was observed that the dominant fungal genera were *Penicillium* (45.1%) and *Aspergillus* (48.5%) in the leaf samples of JX-control. In the leaf samples of the three stages of JX mildewed leaves, the dominant fungal genera were *Aspergillus* and *Trichomonascus*. Unlike the situation in JY mildewed leaves, *Aspergillus* did not achieve complete dominance and *Trichomonascus* also occupied certain positions in JX mildewed leaves.

The analysis of microbial diversity in cigar tobacco leaves during mildew process

A-diversity index was applied to test the differences in microbial communities between the four groups of JY leaves and JX leaves, respectively. Of note, the results of α-diversity analysis were in accord with the results shown in Venn diagram. Consisted with the number of OTUs, Chao1 index of bacteria in JY mildewed leaves was significantly lower than that in JY healthy leaves (Fig. 3a), while the analysis of Simpson index showed the opposite result (Fig. 3b). The results of bacterial diversity analysis (Chao1 and Simpson) in JX leaves (Fig. 3c and d) were similar to those in JY leaves. The diversity of bacterial community decreased with the degree of mildew going, and then increased inversely in the late stage. As shown in Fig. 4a and b, the results of fungal diversity analysis (Chao1 and Simpson) had no significant difference among the groups of JY-control, JY-early, JY-middle and JY-late. However, in JX leaves, the situation of differences for the fungal diversity was similar to that for bacterial diversity (Fig. 4c and d).

β-diversity analysis was also applied to test the differences in microbial communities among the four groups of JY leaves and JX leaves, respectively. The PCoA result showed that the bacterial communities in these four groups of JY leaves were totally different (Fig. 5a), while the bacterial communities in these four groups of JX leaves were mutually overlapped with each other (Fig. 5b). The PCoA results of fungal communities demonstrated that JY leaves (Fig. 5c) and JX leaves (Fig. 5d) have a similar pattern. Specifically, the fungal communities in control group were distinctly different from that in mildew groups (Fig. 5c), and the fungal communities in

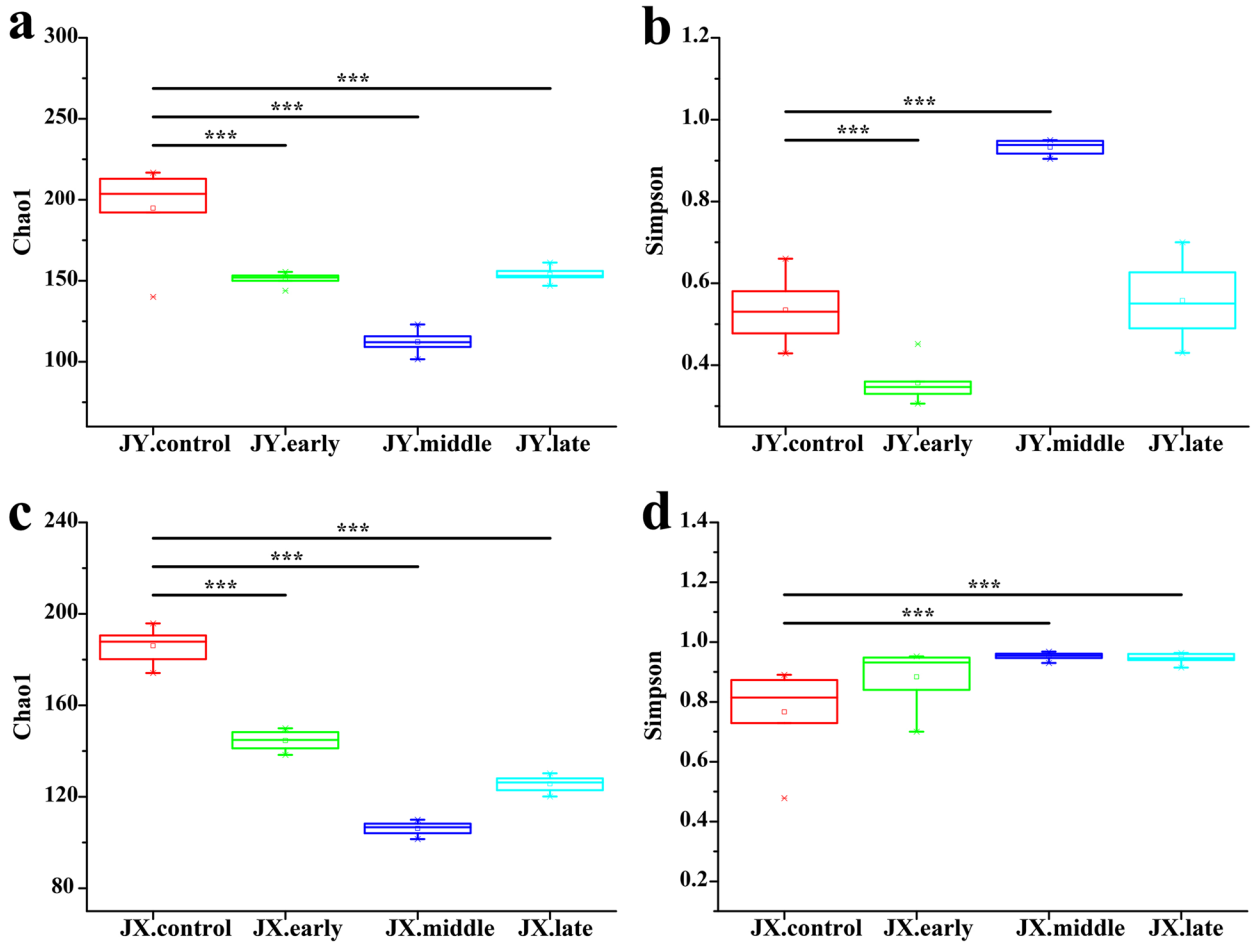

Fig. 3 Alpha diversity index of bacterial community in cigar leave samples with different mildew levels: Chao1 index of cigar wrapper tobacco leaves (**a**) and cigar filler tobacco leaves (**c**); Simpson index of cigar wrapper tobacco leaves (**b**) and cigar filler tobacco leaves (**d**). JY is the abbreviation of cigar wrapper tobacco leaves, JX is the abbreviation of cigar filler tobacco leaves. The normal group (control), mildew early stage (early), mildew middle stage (middle) and mildew late stage (late). The statistical significance between each two groups was tested using T-test. *** $p < 0.001$

mildew groups has high similarity (early, middle and late) (Fig. 5d).

The similarities and differences of microbial communities in cigar tobacco leaves during mildew process

The identified microorganisms were assessed by random forest model to show the importance of bacterial and fungal genera in JY and JX leaves during mildew process. The relative abundances of the top 15 bacteria and fungus were included and analyzed to create the heat map, respectively (Fig. 6). The top important bacterial genera in the mildew process of JY leaves and JX leaves were *Sphingomonas* and *Aerococcus* respectively, while the top important fungal genera in the mildew process of JY leaves and JX leaves were *Wallemia* and *Trichomonascus* respectively.

Figure 6a showed us the top 15 bacterial genera in JY leaves. Compared with JY-control leaves, JY-early leaves had more amount of *Corynebacterium*, *Bacillus*, *Terribacillus*, *Micrococcus*, *Oceanobacillus* and *Aerococcus*, and JY-middle leaves had more amount of *Agrobacterium*, *Stenotrophomonas*, *Pseudomonas*, *Blastomonas*, *Sphingomonas* and *Burkholderia*. Fig. 6b showed us the top 15 bacterial genera in JX leaves. The amount of these bacterial genera was totally different between JX-control leaves and JX mildewed leaves. Moreover, the amount of these bacterial genera in JX-early leaves was more than that in leaves of JX-middle and JX-late. Interestingly, the amount of different dominant bacteria genera was totally opposite between JY-control leaves and JX-control leaves. Fig. 6c showed us the top 15 fungal genera in JY leaves. The amount of major dominant fungal genera in JY-control leaves was higher than that in JY mildewed

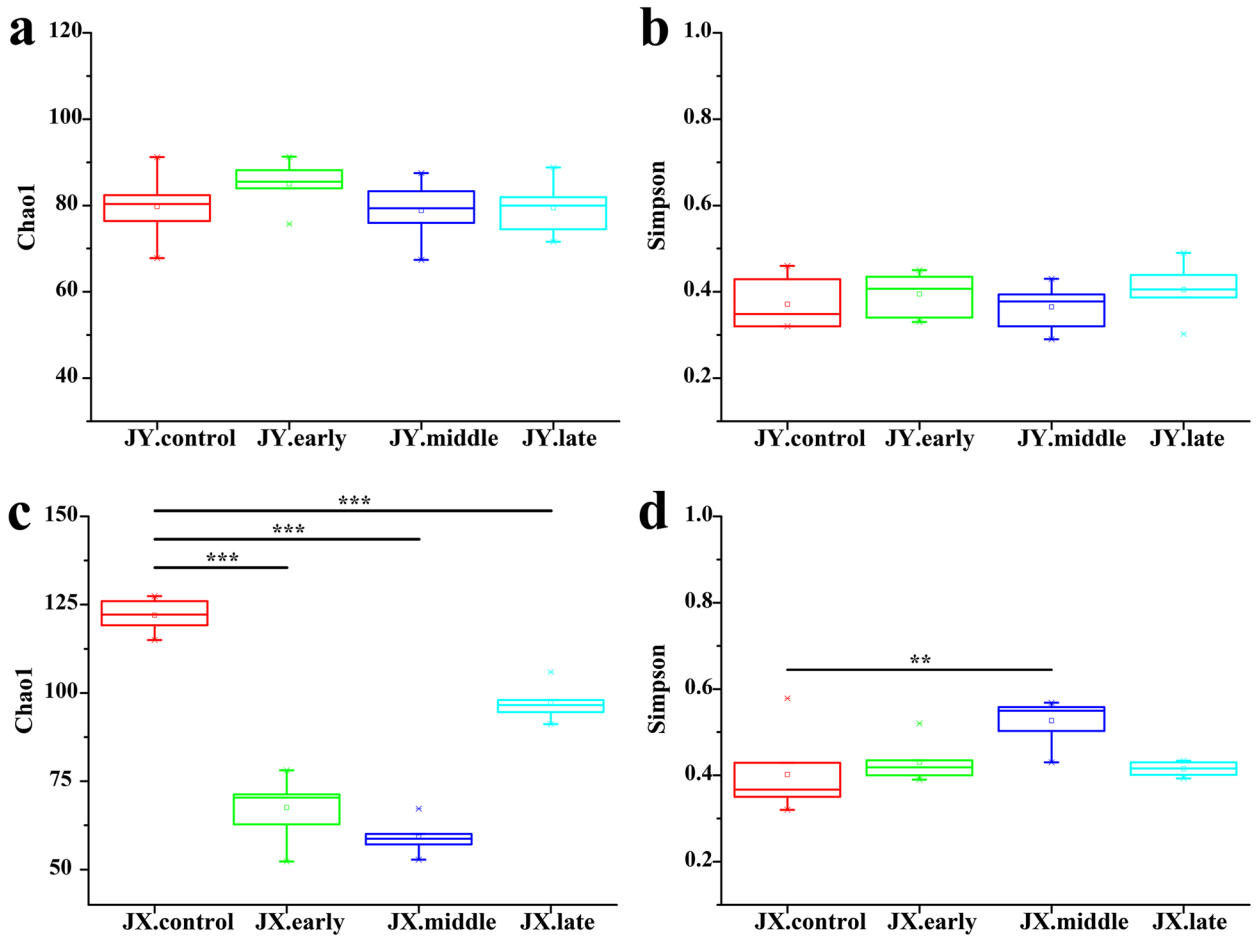

Fig. 4 Alpha diversity index of fungal community in cigar leave samples with different mildew levels: Chao1 index of cigar wrapper tobacco leaves (**a**) and cigar filler tobacco leaves (**c**); Simpson index of cigar wrapper tobacco leaves (**b**) and cigar filler tobacco leaves (**d**). JY is the abbreviation of cigar wrapper tobacco leaves, JX is the abbreviation of cigar filler tobacco leaves. The normal group (control), mildew early stage (early), mildew middle stage (middle) and mildew late stage (late). The statistical significance between each two groups was tested using T-test. $^{**}p < 0.01$ and $^{***}p < 0.001$, respectively

leaves. Among the three groups of JY mildewed leaves, the amount of *Penicillium*, *Mucor*, *Trichomonascus* and *Rhizopus* was highest in JY-early mildewed leaves, and the distributions of dominant fungal genera were similar in mildewed leaves of JY-middle and JY-late. Fig. 6d showed us the top 15 fungal genera in JX leaves. It was observed that almost all the fungal genera exhibited the highest amount in JX-control leaves, and the major fungal genera showed similar distributions in the three groups of JX mildewed leaves. However, compared with JX-late mildewed leaves, the amount of *Trichomonascus* was higher in JX-early mildewed leaves, and the amount of *Aspergillus* was higher in JX-middle mildewed leaves.

The phylogenetic maps of OTUs in each group were constructed by graphical phylogenetic analysis. As shown in Fig. S1, the dominant bacterial phylums were concentrated in *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Planctomycetes* and *Proteobacteria*. Interestingly, *Proteobacteria* was dominant in all four groups of JY leaves. Moreover, the diversity of bacterial communities in the three groups of JY mildewed leaves was lower than that in the JY-control leaves, and the branches of phylogenetic tree in the three groups of JY mildewed leaves were more concentrated than that in the JY-control leaves. In Fig. S2, it was observed that *Proteobacteria* occupied the dominant position of bacteria communities in all four groups JX leaves. And the community diversity, the number of branches and the number of bacterial strains were all decreased in the four groups of JX leaves than that in the four groups of JY leaves. For the fungal evolution map in JY leaves, the fungal classes in the JY mildewed leaves were totally different form that in the JY-control leaves (Fig. S3). Compared with the JY-control leaves, the diversities of fungal communities decreased in the JY

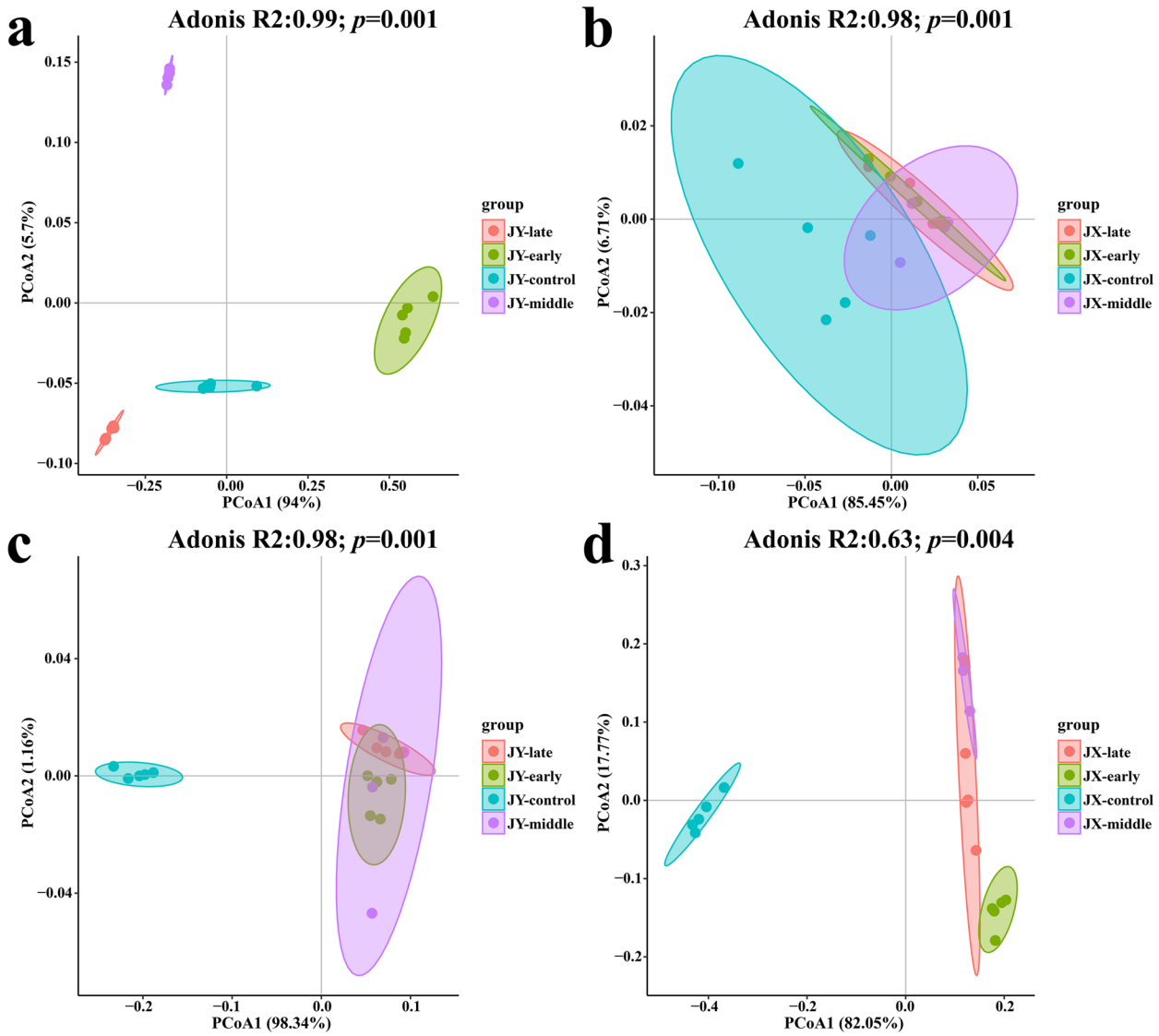

Fig. 5 β-diversity of fungal community in different mildew level cigar tobacco leaves: bacterial β-diversity of cigar wrapper tobacco leaves (**a**) and cigar filler tobacco leaves (**b**). fungal β-diversity of cigar wrapper tobacco leaves (**c**) and cigar filler tobacco leaves (**d**). JY is the abbreviation of cigar wrapper tobacco leaves, JX is the abbreviation of cigar filler tobacco leaves. The normal group (control), mildew early stage (early), mildew middle stage (middle) and mildew late stage (late)

mildewed leaves. Interestingly, *Aspergillus* maintained a dominant position in the mildewed leaves of JY-middle and JY-late, which provided evidence that tobacco mildew is mainly caused by the increase in the number of saprophytic fungi. The findings in fungal evolution maps of JX leaves were in accord with the findings of fungal abundance analysis. In Fig. S4, there were a large number of branches of *Aspergillus* and *Fusarium* in the mildewed leaves of JX-early and JX-middle, but not in the JX-control leaves. By comprehensive comparison, the diversity of fungal strains showed a downward trend with the degree of mildew going.

Discussion

The susceptibility of tobacco leaves to mold contamination is influenced by various factors such as environmental temperature, humidity, and air during grading, purchasing, transportation, storage, and fermentation processes (Menneer et al 2022). For instance, the growth and reproduction speed of mold are affected by temperature, with different types of mold having different optimal temperature conditions for growth. In addition to temperature, relative humidity and moisture content also play crucial roles in the growth and reproduction of mold on tobacco leaves. Oxygen levels also impact

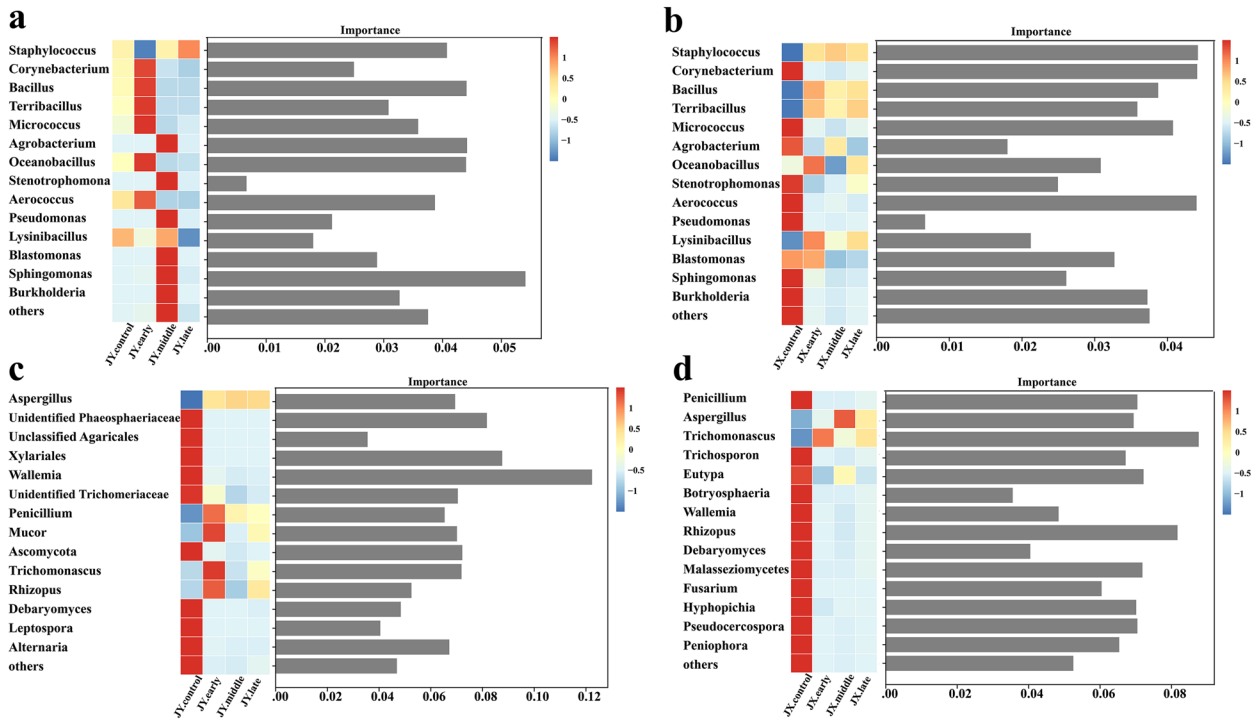

Fig. 6 The heatmap of the relative abundance and the importance of each microbiological: bacterial cluster of cigar wrapper tobacco leaves by Random forest (**a**) and cigar filler tobacco leaves (**b**). Fungal cluster of cigar wrapper tobacco leaves (**c**) and cigar filler tobacco leaves (**d**). JY is the abbreviation of cigar wrapper tobacco leaves, JX is the abbreviation of cigar filler tobacco leaves. The normal group (control), mildew early stage (early), mildew middle stage (middle) and mildew late stage (late)

the reproduction of mold. Some molds are affected by soil microorganisms before tobacco harvesting (e.g. *Fusarium*, *Cladosporium* and *Alternaria*) while others invade stored tobacco after harvesting (e.g. *Aspergillus* and *Penicillium*) (Wang et al 2022; Welty & Lucas 1969). Improper management and inadequate measures for preventing mold can lead to formation of molds on tobacco leaves during various stages including harvesting, initial curing process re-curing leafs transportation storage production.

It is known that the microbial (bacterial and fungi) community plays an important effect on the quality of cigar tobacco leaves for cigar cigarette. During aging, fermenting or storing, the enzymes of microbe will metabolize the cigar leaf components and release products (Li et al 2020, 2017; Welty RE, 1975). The overgrowth of bacteria and fungi will cause mildew, which badly affects the leaves quality (Zhou et al 2024). In this study, the bacterial and fungal communities in cigar wrapper leaf (JY) and cigar filler leaf (JX) during their mildew process were comprehensively investigated by high-throughput sequencing, which was aimed to reveal the differences in the microbial communities between mildewed JY leaves and mildewed JX leaves, and to identify the dominant bacterial genera and fungal genera in the mildew process of JY leaves and JX leaves.

Consistent with previous finding (Tao et al 2022), the relative abundance of bacterial genera in JY leaves and JX leaves have changed greatly during the mildew process (control, early, middle and late). The α-diversity and richness of bacterial communities in JY leaves and JX leaves significantly decreased after they got mold. Specifically, the relative abundance of *Terribacillus*, *Bacillus* and *Micrococcus* increased in the leaves of JY-early. It was partially explained by that *Bacillus* is the dominant bacteria for degrading starch and cellulose (Wu et al 2021), and *Terribacillus* was negatively correlated with protein. *Staphylococcus* is a significant organism present in aged tobacco. On the one hand, the enrichment of *Staphylococcus* in health tobacoo leaves is important to improve the contents of aroma components, while on the other hand, the proportion of *Staphylococcus* gradually increases once the tobacoo leaves step into mildew process (Perry 1969). In this study, high level of *Staphylococcus* was observed in JY-control leaves, comfirming its health property. When the mildew is being induced, the aroma components begin to be destroyed, which could explain the decrease of *Staphylococcus* in JY-early leaves. With the progressing of mildew, the amount of

Staphylococcus in JY-middle leaves restored to the level of that in JY-control leaves, and reached the peak in JY-late leaves. On the other side, when JX leaves stepped from healthy stage to mildew stage, the abundance of *Staphylococcus* increased, while the abundances of *Corynebacterium*, *Pseudomonas* and *Burkholderia* decreased. The differences in bacterial genera changing between the mildew process of JY leaves and JX leaves might propose that they have distinct bacterial metabolic processes.

In addition, the fungal communities in JY leaves and JX leaves also changed drastically during the mildew process (control, early, middle and late). Among the four groups in JY leaves, the JY-control had the lowest levels of *Aspergillus*, *Penicillium* and *Mucor*, and the highest levels of *Unidentified Phaeosphaeriaceae*, *Unclassified Agaricales* and *Xylariales*. During the mildew process of JY leaves, the amount of *Penicillium* and *Mucor* increased greatly, and *Aspergillus* became the top abundance of fungi. Of note, the fungal diversity of genera was richness in JY-control leaves, and drastically decreased in mildewed JY leaves (early, middle and late). On the other side, the amount of *Penicillium* and *Trichomonascus* in JX leaves significantly decreased and increased from JX-control to JX mildew groups (early, middle and late), respectively. Moreover, the JX-control leaves had higher fungal diversity than JX mildew groups (early, middle and late). Our findings were supported by the works of Chen. et al. and Welty. et al., which reported that the healthy tobacco leaves had higher richness and diversity of fungal community (Chen et al 2020; Welty & Lucas 1969). Similarly, different fungal genera changes were also observed between JY leaves and JX leaves during their mildew process.

As this study showed, the communities of bacteria and fungi changed dynamically during mildew process. Indeed, the diversity, composition and function of bacterial and fungal communities determined the physicochemical environment (El Hadri et al 2021). When the conditions enable fungi to observe a competitive advantage, such as their own material metabolic regulation or advantageous products by other microorganisms, tobacco mildew would grow (Zhou et al 2022). Moreover, the fungal-bacterial interactions may affect the mildew growth of tobacco leaves by regulating diversity and stability of community ecosystem. In addition, the JY leaves and JX leaves had different findings for the alternation of microbe communities (bacterial and fungi), the possible reason may be that these two tobacco leaves have different preparation processes in different environments, as well as their distinct physical and chemical properties.

Conclusion

In conclusion, the present findings revealed that the two parts of cigar tobacco leaves (wrapper and filler) had different communities evolution of bacterial and fungi during the mildew process, and had lower diversity of bacterial and fungi in mildew stage than that in healthy stage. The balance of the dominant bacterial genera and fungal genera in mildewed ciagr tobacoo wrapper and filler leaves should be explored in future studies, whose findings would be of great help in developing effective strategies to control the cigar tobacco mildew.

Abbreviations

JY	Cigar wrapper leaf
JX	Cigar filler leaf
rRNA	Ribosomal RNA
ITS	Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
BLAST	Basic Local Alignment Search Tool
OUT	Operational Taxonomic Unit
PCoA	Principal Coordinate Analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-024-01783-6.

Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions

BW and BC designed the research. MW, YS, XS, ZL and LR performed experiments and analyzed data. JL and BC wrote the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analyzed during the present study are available in the article. Supporting information, the physical properties (Fig.S5), the chemical properties (Table S3) and the nucleotide sequence database and the accession number are available online.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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