The diversity, structure and function of microbial communities changes across aging process of tobacco leaves

Fan Wang\textsuperscript{1,2,6}, Yongming Jin\textsuperscript{2,5,6}, Xiaona Chen\textsuperscript{2,5}, Yao Zhang\textsuperscript{4,6}, Xinglin Jiang\textsuperscript{1}, Ge Zhang\textsuperscript{2}, Guoqiang Chen\textsuperscript{2}, Mingjun Yang\textsuperscript{5}, Feifan Leng\textsuperscript{1}, Hongtao Li\textsuperscript{4,*}, Lijun Wu\textsuperscript{1,*} and Haibo Zhang\textsuperscript{2,*}

\textsuperscript{1} Technology Center of China Tobacco Yunnan Industrial Co., Ltd, Kunming, 650106, People’s Republic of China
\textsuperscript{2} Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, 266101, People’s Republic of China
\textsuperscript{3} The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kongens Lyngby, Denmark
\textsuperscript{4} Technology Center of China Tobacco Shandong Industrial Co., Ltd, Qingdao, 266101 People’s Republic of China
\textsuperscript{5} School of Life Science and Engineering, Lanzhou University of Technology, Lanzhou, 730050, People’s Republic of China
\textsuperscript{6} These authors contributed equally to this work.
\textsuperscript{*} Authors to whom any correspondence should be addressed.
E-mail: lihongtao022@126.com, wallis8@126.com and zhanghb@qibebt.ac.cn

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Abstract

Microbial communities that inhabit aging tobacco leaves play a key role in improving products quality. A better understanding of microbial communities on the aging of tobacco leaves could provide an important microbial repository for the industrial applications. Here, we examined the structural and compositional changes of microbial communities throughout the aging process of by tobacco leaves 16 S and ITS rRNA amplicon sequencing techniques and identified the potential metabolic pathways of bacteria and fungi using Functional Annotation of Prokaryotic Taxa (FAPROTAX) and Fungi Functional Guild (FUNGuild), respectively. The results showed that the diversity and structure of the microbial communities keep changing along with the aging process went on. The richness and diversity of bacterial community decreased, while the richness of fungal community was in an inverse trend. At the phylum level, the bacterial community was dominated by \textit{Proteobacteria}, \textit{Actinobacteria}, and \textit{Firmicutes}, while \textit{Ascomycota} and \textit{Basidiomycota} were the dominant species in the fungal community. In the bacterial community, metabolic functions related to the carbon and nitrogen cycles which response to the degradation of harmful components, and the metabolism of aromatic hydrocarbons showed extremely dynamic at different aging periods. The change of the main nutritional mode of the fungal community also led to an increase in the abundance of saprophytic fungi. These results provide information on the succession of microbial community structure and function in the whole process of tobacco aging and suggest that the aging process of tobacco leaves can be a natural microbial collection for target microorganism and their metabolites. It also enables the further investigation of coordination mechanisms between beneficial microbial regulation and pathogenicity during aging process.

1. Introduction

Tobacco (\textit{Nicotiana tabacum} L.) belongs to the Solanaceae family, which is widely planted in the world and has important economic value. China is the world’s largest producer and consumer of tobacco (Wang \textit{et al} 2015b). As the main type and raw material of cigarette production, the safety and quality of tobacco leaves have strict requirements. Unaged fresh flue-cured tobacco is not suitable for cigarette production due to their strong unpleasant odors and irritating smoke (Huang \textit{et al} 2010, Wang \textit{et al} 2015a). Hence, fermentation or aging is usually employed in cigarette production to reduce irritating odours and promote tobacco quality.
aging is a long and complicated dynamic process, which is closely related to enzymes, microorganisms, and other effects in tobacco leaves (Jensen and Parmele 1950).

The quality and value of tobacco leaves can be improved by adjusting certain microorganisms and fermentation conditions to appropriate levels during aging process (Ye et al 2017). This aging process could reduce the content of macromolecular substances such as protein, starch, and pectin and degrade harmful substances of tobacco leaves, such as organic amines and alkaloids. Besides, it can effectively enrich the aroma substances in tobacco leaves, and further improve the taste and smoking quality of cigarettes (Li et al 2003, Wang et al 2018a). Microorganisms promote the aging of tobacco and reduce the harmful substances in tobacco through the action of the enzymes and metabolites produced by themselves (Zhou et al 2020). Therefore, the change of microbial community in the process of fermentation is closely linked to the content of relevant chemical indexes. It has been found that Bacillus megaterium could convert tobacco cambranoids into hydroxyl analogs, which can improve the natural odor and taste of tobacco leaves and minimize irritation to the throat and lungs (El Sayed et al 2008). The added thermophilic strains of Bacillus can accelerate the formation of ideal aroma (English et al 1967), while the microbial system composed of Bacillus sp. and Geotrichum sp. can convert lutein into tobacco-scented compounds (Maldonado-Robledo et al 2003). In addition, the system of Trichosporon asahii and Pseudococcus amylolyticus can significantly increase the yield of tobacco aroma compounds by fermentation (Rodriguez-Bustamante et al 2005). After applying Bacillus Amylolyticusfciens in the aging process of tobacco, it was found that the content of specific nitrosamines in tobacco decreased significantly (Wei et al 2014). A study also found that Pseudomonas and Arthrobacter bacteria could efficiently degrade nicotine, a toxic compound that seriously affects human health and represents more than 90% of the total alkaloid content of tobacco (Gurusamy and Natarajan 2013). And literature sources reported that Pseudomonas possessed ability to produce a variety of valuable metabolites from nicotine (Liu et al 2015, Wang et al 2015b).

Microbial communities play a crucial role in a wide range of natural processes that affect ecosystem function. However, the microbial communities are so complex that only a small proportion can be cultured on laboratory culture medium due to the high species diversity and the low culturability of the microbial communities. It is impracticable to fully simulate the natural environment in which they live and the positive or negative interactions among the members of the community in laboratory (Butler and O’Dwyer 2018, Goldford et al 2018, Wolfe et al 2014). The wide studies of microbial communities on different tobacco leaves benefited from the development of next-generation sequencing (NGS) technique. Early uncovering uncultured microorganisms on specific tobacco leaves mainly depended on the low degree of development techniques. For example, Zhao et al (2007) applied 16 S rRNA amplification and denaturing gradient gel electrophoresis (DGGE) techniques to analyze the changes of leaf microorganisms in different aging stages of tobacco leaves and identified three kinds of uncultured bacteria. Huang et al (2010) analyzed bacterial community changes in unaged and aged tobacco by 16 s rRNA amplification and applied restriction fragment length polymorphism (RFLP) and resulted that Bacillus spp. and Pseudomonas spp. were two dominant genera in unaged tobacco leaf and more uncultured bacteria species were found in aging than in unaged one. Then, studies were also carried out in a small number of batches or at a specific fermentation time, but employed the high-throughput technology resulted in more bacteria found in tobacco leaves (Wang et al 2018a).

Recently, the changes in microbial community on tobacco leaves during aging process become attractive, since microorganisms has been considered to play an important role in regulating of tobacco aging and improving the quality of tobacco leaves. Zhou et al (2020 and 2021a) investigated the microbe community on Yun 87 and an unknown tobacco leaves every 6 months during 24 month aging process. They concluded that composition of the microbial community highly affected by the aging location and highlighted the vital role of bacterial and fungi in the aging of tobacco leaves. In fact, most of previous studies were carried out using one or two tobacco samples and displayed the limited information of microbial communities on aging tobacco leaves for analyzing the microbial action. Thus, there is still a strong preference for revealing the changes of microbial community succession throughout the fermentation process. Eventually, a greater understanding of microbial diversity and succession of an ecosystem is essential to culture of unculturable microbiota and provide opportunities for dissecting the mechanisms of microbial community formation (Wolfe and Dutton 2015). Therefore, in this study, bacterial 16 S rRNA gene and fungal ITS amplifiers sequencing technology were applied to investigate the diversity and structural changes of microbial community in the whole aging process of two types of tobacco leaves collected in the same aging storeroom. The microbial function of bacterial and fungal community was then predicted. These would reflect the characteristic information of microorganisms in tobacco aging, and provided a guidance for screening and isolating strains with specific functions.

2. Materials and methods

2.1. Sample collection and processing

The two tobacco samples used in this study were Honghuadajinyuan (H) planted at Nanjian, Yunnan (latitude and longitude coordinates, 24°7'5" N 100°24' E; altitude, 2,027 m) and K326(K) planted at Qujing, Yunnan.
(latitude and longitude coordinates, 25°61′N 103°46′E; altitude, 2,356 m). Two tobacco samples were aged at Kunming, Yunnan (latitude and longitude coordinates, 25°19′N 102°42′E; altitude, 1,891 m). The aging temperature kept at 20 °C–32 °C and the humidity was at 50%–65%. Aging flue-cured tobacco samples were collected every three months for total 24 months, then the samples (about 1.0 kg) were sealed in sealing bags for full mixing, named H1–H7, K1–K7 and immediately stored at −20 °C for further analysis.

### 2.2. DNA extraction and amplification sequencing

The samples were processed under aseptic conditions and the total genome DNA of the tobacco microbial community was extracted by CTAB method (Umessa et al. 2016). The primers in table 1 were used to amplify bacteria and fungi with the barcode respectively after the testing for DNA purity. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs) as described in the instruction manual. The PCR products were sheared and recovered for library construction. After the library passed the test, Ion S5™ XL sequencing platform was used for computer sequencing (Zhai et al. 2019). The sequence data have been submitted to the NCBI (National Center for Biotechnology Information) database with accession number PRJNA734186.

| Table 1. Primer pairs used for amplification. |
|----------------------------------------------|
| **Primer pairs** | **Primer sequence (5′ to 3′)** | **References** |
|------------------|-------------------------------|---------------|
| Bacteria         |                               |               |
| 799 F            | AACMGGATTAGATACCCCKG          | Wang et al.2018b |
| 1193 R           | AGTCTACCCCCACCTTCC            |               |
| Fungi            |                               |               |
| ITS1-F           | CTTGGTCATTAGAGGAGTAA          | Adams et al.2013 |
| ITS1-R           | GCTGGTTCTTCATCGGTGC           |               |

### 2.3. Sequence data processing and analysis

The low-quality portion of reads was cut by Cutadapt (version stable) (Martin 2011), and then the resulting reads were separated according to barcode sequence to obtain the raw data for each sample. According to Shi et al. (2020), the UCHIME software (version 4.1) (Edgar et al. 2011) combined with the species annotation database were employed to get valid data. Sequences analysis were performed by Uparse software (version 7.0.1090) and classified into OTUs (Operational Taxonomic Units) with 97% identity (Edgar 2013). Species annotation of bacteria and fungi were performed by the Silva database based on RDP classifier (Version 2.2) (Quast et al. 2013) and Unite database (Abarenkov et al. 2010) respectively. The data were homogenized and the alpha diversity were calculated by QIME (version 1.9.1) (Caporaso et al. 2010). All data were visualized using an online tool platform (https://magic.novogene.com/).

### 2.4. Network analysis

Network analysis is performed to assess species interactions and identify potential core microorganisms at different periods. The correlations between microbial OTUs from different periods of aging process were assessed using the spearman correlation coefficient. Correlation networks were calculated in R software (version 4.1.0) using the Hmisc package (Harrell Jr and Harrell Jr 2019). The multiple test corrections were carried out by the Benjamini and Hochberg False Discovery Rate method (Benjamini and Hochberg 1995). Correlation coefficients >0.6 and corresponding p < 0.01 were considered to generate networks with robust correlation. The network graph was obtained after filtering the nodes for self-connection, and the network structure was explored and visualized by using the undirected network and Fruchterman-Reingold layout in Gephi software (version 0.9.2), which was used to calculate the relevant topological parameters of the network (Bastian et al. 2009).

### 2.5. Functional potential of microbial communities

In this study, FAPROTAX (Sansupa et al. 2021) was employed to predict ecological relevant functions of bacterial and archaeal taxa derived from 16 S rRNA amplicon sequencing of our tobacco samples. Meanwhile, the classification of fungal function can be realized by linking fungal community with functional guild by bioinformatics method, and FUGuild is a tool for predicting fungal function here (Nguyen et al. 2016). The analysis was all performed using the free online platform of the Novomagic according their instructions (https://magic.novogene.com). The top 10 and top 35 function notes were used for making relative abundance histogram and heat map of microbial communities during the aging of tobacco leaves, respectively.
3. Results

3.1. Species clustering results
In this study, the bacteria and fungi of H and K samples were sequenced to determine the composition of microbial community. After quality and length filtering, 1139 and 1356 OTUs were obtained at a 97% similarity level respectively. The commonality and specificity of OTUs among different samples were analyzed based on the clustering results. The results showed that there was little difference in the number of core microorganisms in the bacterial community between the H and K samples. However, significant differences in the core microorganisms’ taxa were observed as aging proceeds in the same sample. The samples with the highest number of special bacteria OTUs were H2 and K4, respectively (figures 1(a), (b)). In the fungal community, the number of common microorganisms in H sample was higher than in K sample. Meanwhile, the special microbial communities were changed with as the aging progress went on (figures 1(c), (d)). In addition, a higher number of common microorganisms were identified in the fungal community (figures S1(a), S1(b) (available online at stacks.iop.org/ERC/4/095012/mmedia)).

3.2. The succession of microbial community during aging
The taxonomic information of bacterial community was obtained by species annotation analysis of the representative sequence of OTUs obtained by clustering. The identified sequence bacteria of 14 samples belonged to 20 phyla, 39 classes, 87 orders, 164 families, and 376 genera, and fungi included 9 phyla, 25 classes, 74 orders, 177 families, and 287 genera. The top species in terms of abundance at the different taxonomic levels for each sample were chosen to produce species relative abundance cumulative maps (figure 2) and heat map of species abundance clustering (figure S2).

Proteobacteria were dominant bacteria during fermentation of both samples at the phylum level, accounting for 88.7% of the total bacterial community. The changing trend of its relative abundance was relatively stable in the early aging period of H samples and decreased from 91.9% to 85.7% after 15 months. However, the relative abundance of Proteobacteria in K samples first increased to 93.3% at six months, then decreased to 73.2% at 12 months, and finally increased again to 93.9% after aging. In addition, Actinobacteria, Firmicutes, and Bacteroidetes accounted for 5.4%, 3.7%, and 1.9% of the bacterial community, respectively. Firmicutes in the two samples show an increasing trend at first and then decreasing, but there is no significant difference in relative abundance before and after the change. The changing trend of Actinobacteria in the two samples was just the
opposite, gradually increasing in H samples and decreasing in K samples (figure 2(a)). At the genus level, the bacterial communities during aging were composed of Pseudomonas (22.9%), Sphingomonas (19.9%), Methylobacterium (7.2%), Massilia (4.7%), Pantoea (3.5%), Acinetobacter (2.5%), and Stenotrophomonas (2.1%). Sphingomonas and Methylobacterium, which belong to Alphaproteobacteria, have significantly lower relative abundance in both samples. The relative abundance of other communities, which belong to Gammaproteobacteria was significantly higher at the class level. In H samples, the relative abundance of bacteria of these genera decreased with the aging process. In contrast, in K samples, Sphingomonas (50.3%–15.8%) and Methylobacterium (19.7%–7.0%) are the dominant bacteria at the initial aging stage, then Pseudomonas (1.7%–50.8%) gradually become the dominant bacteria with the progress of fermentation (figure 2(c)).

As shown in figure 2(b), the dominant species of the fungal community obtained by clustering are similar, but their relative abundance is significantly different. Ascomycota (62.8%) and Basidiomycota (6.9%) were the dominant fungal groups in the aging process at the phylum level. In addition, many sequences (28.7%) were not attributed to any phylum, therefore, at the phylum level, the species diversity of fungal communities were significantly lower than that of bacterial community. The relative abundance of Ascomycota decreased after aging compared to before in both H (67.6%–59.1%) and K samples (79.1%–54.83%), but remained the dominant phyla in the fungal community. Basidiomycota in H samples (27.1%–29.8%) and K samples (17.6%–36.7%) increased as this process progressed. In addition, the trends in the relative abundance of Ascomycota and Basidiomycota during the aging process were precisely the opposite. At the genus level, the fungal communities were dominated by Alternaria (20.1%), Aspergillus (19.9%), Symmetrospora (5.2%), Cladosporium (4.8%), Phoma (4.2%), Boeremia (3.2%), and Epicoccum (3.0%). The relative abundance of all groups of bacteria decreased slightly during the aging of H samples except for Aspergillus, which increased in relative abundance (17.4%–22.5%). The relative abundance of Alternaria decreased significantly (74.8%–9.6%) in the K samples aging, while the relative abundance of other genera increased to become the dominant genera in the later stage of aging (figure 2(d)).

The sequences of the species with the highest relative abundance obtained from all samples were used to construct evolutionary trees to reveal the phylogenetic relationship of bacteria and fungi. The results showed that at the phylum level, the bacterial communities in the samples were mainly Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes, while the fungal communities were mainly Ascomycota and Basidiomycota at the phylum level (figure S3). This is consistent with the analysis above.

3.3. Analysis of microbial community diversity

The rarefaction curve showed in figure S4, indicating that most species in the samples are covered. And the rank abundance curve indicated a high species abundance of the sample and the uniform distribution of species (figures S4(b), S4(d)). The tables S1 and S2 provided the information that essentially most species in the samples...
were detected with the coverage of over 99%, and the obtained data could perfectly reflect the composition of bacteria and fungi in the tobacco aging process. Furthermore, the amount of species in the microbial community of the samples were reflected by observed species, Chao1, and ACE index, and the multiplicity and evenness were shown by Shannon and Simpson indices (figures 3(a), (b)). We observed that there was no significant difference in species richness and diversity of bacterial and fungal communities between the two samples ($P > 0.05$). In addition, the richness and diversity of bacterial communities decreased during the aging process of two samples. In contrast, compared with the early aging stage, the richness of fungal community increased and the diversity decreased in the late aging stage of the two samples. Furthermore, the difference analysis of diversity index between groups showed no significant difference in microbial community variety during aging process of the two samples (figure S5).

PCoA analysis using weighted Unifrac was performed to evaluate difference in microbial community composition in the aging process. Samples with a high degree of similarity in species composition in the community are close together. Conversely, they are further apart (Zhou et al 2021b). Clearly, the H samples clustered closer than K samples of the tobacco leaves aging processes (figures 3(c), (d)). In addition, the difference in community structure between groups (H, K) was tested using anosim test. The results revealed no significant difference ($R = 0.054, P = 0.159$) in bacterial community composition between the two samples, while the differences in fungal community structure of the samples were significant with a distinct time succession model ($R = 0.2935, P = 0.007$) in the aging process (figures 3(e), (f)).

UPGMA (Unweighted Pair-group Method with Arithmetic Mean) clustering tree were constructed by relating the relative abundance of species at the phylum level to each sample based on unweighted unifrac matrixs. The results showed that the bacterial communities were clearly clustered into four large branches, and the two samples were scattered in different aging periods, which indicated that the bacterial community structure of the samples changed considerably during the aging process. The species composition results showed that the bacterial communities of both samples were essentially the same during the aging process, consisting mainly of Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes at the phylum level (figure 4(a)). While the fungal community clusters were more dispersed, indicating that the structure of the fungal community changed more significantly. The species composition of the fungal community mainly includes Ascomycota, Basidiomycota, and Mucoromycota (figure 4(b)).

3.4. Differential species analysis
LEfSe (Linear discriminant analysis Effect Size) analysis can identify species with significant variation in abundance among groups and obtain the enrichment of different species among different groups (Segata et al 2011). The aging
process were divided into two stage artificially, early aging stage (Ha: H1–H4, Ka: K1–K4) and later aging stage (Hb: H5–H7, Kb: K5–K7). A threshold of three was set for the LDA (Linear discriminant analysis) score for LEfSe analysis as shown in figure 5. LDA (LAD score > 3.0) result showed a significant alteration of the bacterial community characterized by higher Firmicutes, Microbacteriaceae and Bacteroidales levels in early aging stage, and higher Curtobacterium_flaccumfaciens and Phyllobacterium in later aging stage of H samples (figures 5(a), (c)). For K tobacco samples, the Propionibacteriales, and Microbacteriaceae were more abundant in early aging stage group, while Phyllobacterium and Proteobacteria were more abundant in in later aging stage, which were included in the H samples (figures 5(b), (d)). Fungal communities at the genus level, Leptosphaerulina, Phlebia, Issatchenkia, and Sclerostagonospora, Phlebia, Alloleptosphaeria are the differential species for the pre-aging stages of the H and K samples, respectively (figure S6). In contrast, the late differential species are Toxicocladospormium and Humicola, Boeremia. Finally, when comparison of H and K samples, and the main taxa of bacteria that was different was only Actinobacteria, which just appeared in H sample. Whereas for the fungal community, Pleosporaceae, presented in H sample was the main different taxa.
Table 2. Relevant topological parameters of microbial networks.

|            | H       | Hb      | Ka      | Kb      |
|------------|---------|---------|---------|---------|
| Nodes      | 231     | 255     | 175     | 175     |
| Links      | 385     | 549     | 250     | 303     |
| Average degree | 3.333 | 4.306  | 2.857   | 3.463   |
| Density    | 0.014   | 0.017   | 0.016   | 0.02    |
| Modularity | 0.95    | 0.938   | 0.929   | 0.92    |

3.5. Microbial interaction networks and key microbes

The network was constructed using only positive and significant correlations \(r > 0.6, P < 0.05\) (Dong et al 2021). For both samples, the complexity of interaction networks of later aging stage was higher than that of early aging stage, which was concluded based on more edges, higher average degrees, and more complex network relationships in later aging stage (table 2). The keystone taxa of aging period mainly belong to Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes for bacteria (figure 6) and Ascomycota, Basidiomycota for fungi (figure S7). In summary, the aging process enhances the complexity and interactions of the microbial community network. The interrelated keystone taxa in the early stages of aging mainly belong to bacteria, and in the later stages change to a predominance of fungi (figure 6, tables S3, S4).

3.6. Prediction of microbial metabolic function

FAPROTAX is a database of environmental functions of prokaryotes. The current version classifies the ecological functions of bacteria and archaea in the environment according to the published literature evidences and collates a variety of element cycles and functional classifications into a FAPROTAX database, which has a good prediction effect on the biochemical cycle process of environmental samples (Sansupa et al 2021). Functional annotation of the bacterial OTUs shows a high proportion of functions related to chemoheterotrophy and aerobic chemoheterotrophy (figure 7). Chemoheterotrophy and aerobic chemoheterotrophy increased at first and then decreased in H samples, while this trend was reversed in the K samples (figure 7(a)). As shown in figure 7(b), the functions closely related to nitrogen cycle such as nitrite respiration, nitrite denitrification, and nitrous oxide denitrification. Nitrate denitrification was the most abundant in the late stage of H samples aging, while nitrate reduction, nitrogen respiration, and nitrate respiration were more abundant in the mid-aging stage in K samples. It is worth mentioning that the abundance of hydrocarbon degradation, aromatic hydrocarbon degradation, and aliphatic non-methane hydrocarbon degradation function increased in the H sample at the 21st month of aging, which is associated with the increase of small molecules in the aroma of tobacco leaves during fermentation. Besides, the t-test of function of H and K samples results showed that methanol_oxidation \((P < 0.05)\) and dark_hydrogen_oxidation \((P < 0.05)\) which were in low abundance, performed statistical significance (figure 7(c)).

The results of fungal function showed that the primary nutritional modes of fungi during tobacco aging are obligate and facultative, including pathotroph-saprotroph-symbiotroph, saprotroph, pathotroph, pathotroph-symbiotroph, and pathotroph-saprotroph. In addition, there are still many fungi that are not classified into any nutritional mode group. Pathotroph-saprotroph-symbiotroph decreased gradually from the 3rd to the 13th month of H samples aging and increased to a maximum at 15th months and then decreased. However, saprotroph showed an increasing trend during aging, reflecting a change in the main nutritional mode of fungi from pathotroph-saprotroph-symbiotroph to saprotroph during the H samples aging process. The change of this nutrition mode was more pronounced in K sample, and the main nutrition mode changed from pathotroph-saprotroph-symbiotroph in the early stage to saprotroph and pathotroph in the later stage of aging (figure 8(a)). The functional composition of fungi was further analyzed at the level of economic guild, and the main functional groups identified are animal pathogen-endozyte-plant pathogen-wood saprotroph, undefined saprotroph, endophyte-plant pathogen, plant pathogen-wood saprotroph, fungal parasite-plant pathogen, plant pathogen, wood saprotroph, and lichenized (figure 8(d)). Although a large number of fungal functional types can be annotated, the annotation level is still relatively cursory, such as undefined Saprotroph, which accounts for a large number of fungal functional types. The heat map showed that the fungal function changed significantly in the nutritional model and ecological guild in different aging periods of tobacco leaves (figures 8(b), (d)).

4. Discussion

Compared with the study at a specific time point, it is more meaningful to study the succession of microbial communities in the whole aging process, because the diversity, composition and function of bacterial and fungal
communities change dynamically (Zhou et al 2021a). In this study, high-throughput sequencing was applied to investigate the bacterial and fungal communities on tobacco leaves during aging. The comparison of species among samples indicated that tobacco microbial community diversity and structure were variable during aging times, while many microbial community structures were similar in both samples, which probably result from the same aging environment. With regard to diversity, our results shown that the aging process resulted in the decrease of the richness and diversity of bacterial community, while changes in fungal communities showed an increase in richness and a decrease in diversity. So far, only few examples have investigated the microbial community on tobacco leaves throughout the aging process, but just one described the diversity. They concluded that the diversity and richness of bacteria increased, while the fungal diversity and richness decreased (Zhou et al 2021a). This show a little difference from our results in the diversity of fungal community which may result from the different type of tobacco, the aging storeroom and the climate of the aging location. For the two years aging tobacco leaves, we obtained 1139 and 1356 OTUs for bacteria and fungi, respectively, from 14 tobacco leaf samples collected in 7 periods during the aging process. While Zhou et al found 1586 and 885 OTUs, respectively, from 10 tobacco leaf samples (Zhou et al 2021a), and a total of 2,652 and 656 OTUs from the 15 tobacco samples collected in 5 periods during the aging process (Zhou et al 2020). The much more fungal OTUs

Figure 6. Microbial community networks during aging. H sample pre-aging (a) and late aging (b). Each node represents an OTU, and the size of each node is proportional to the number of connected edges (i.e., degrees). The connections between nodes show a significant correlation (p < 0.01). The different colors represent the different phyla to which the OTUs belong.
we obtained may indicated that bacteria and fungi did work together in tobacco aging without the OTU number superiority.

The composition of bacterial community on tobacco leaves has been well studied in last twenty years. They all showed that Proteobacteria and Firmicutes were the dominant bacterial phyla (Larsson et al 2008, Huang et al 2010, Su et al 2011, Ye et al 2017, Wang et al 2018a, Wang et al 2018b, Zhou et al 2020, Zhou et al 2021a). And the Sphingomonas, Methylobacterium, Pseudomonas, Acinetobacter, and Bacillus were the most dominant genera found in aging tobacco leaves. Furthermore, we showed that the composition of bacterial community of tobacco leaves across the aging process was relatively similar in both samples. This agrees with a previous report (Zhou et al 2021a) which demonstrated that no statistically significant differences were identified for bacterial communities in tobacco leaves during the 24-month aging process between the two storage rooms \( (P = 0.110 > 0.05) \). These results might provide the clues that the type of tobacco and the storage conditions co-determine the bacterial communities on the tobacco leaves during the aging process.

Despite the investigation in the composition of fungal community on tobacco leaves remains less. They all concluded that Ascomycota is the main dominant flora of the fungal community at the phylum level. However, the dominant flora at the genus level is significantly different. We showed that the dominant flora at the genus level were Alternaria and Aspergillus. While Zhou et al 2021a illustrated that those were Sampaiozyma and Aspergillus in Guiyang city storeroom, the Xeromyces, Sampaiozyma and Kazachstania in Maotai city storeroom. And they also showed that Xeromyces and Wallemia were the dominant fungi in other report (Zhou et al 2020).

**Figure 7.** Prediction of bacterial function. Functional notes of bacteria during the aging of tobacco leaves relative abundance histogram (a) and heat map (b). T-test results of bacterial function between H and K groups (c).

**Figure 8.** Fungal function prediction. Histogram (a) and heat map (b) of fungal nutrition mode during tobacco aging. Statistical significance of fungal function prediction (c). Histogram (d) and heat map (e) of fungal functional composition in ecological guilds.
In the case of the microbial community succession during tobacco aging, our results showed that the microbial community composition changes dynamically, and the relative abundance and flora structure of different microbial communities were also variable. For example, Proteobacteria remains the dominant bacterial phylum before, while after aging, Pseudomonas and Sphingomonas become the main dominant bacteria genera in tobacco aging. The microbial community succession has a profound effect on ecosystem function, and it mostly depends on the environmental changes according to long-term ecological research (Qi et al. 2021). Besides, the aging process of tobacco leaves enhances the interaction and complexity of microbial community networks, resulting in more complex microbial network relationships in the later stages of aging. In this study, with the aging of tobacco, the keystone taxa with strong interactions change from being dominated by bacteria to fungi. However, Zhou et al. (2020 and 2021a) gave a contrary conclusion that the latter period of the tobacco leaves aging process was dominated by bacteria. Hence, it is still necessary to conduct more further studies for confirming this. Microorganisms play a substantial role in the assembly of tobacco aging ecosystem, which can degrade macromolecular compounds like protein, starch, and cellulose, reduce the harmful components like nicotine by degrading nicotine as a life-cycle nutrient (Gurusamy and Natarajan 2013, Liu et al. 2015). They have been reported to degrade nicotine include Pseudomonas (Chen et al. 2008, Ruan et al. 2005), Arthrobacter (Ruan et al. 2006), Pseudomonas putida (Wang et al. 2007), Rhodococcus (Gong et al. 2009), Acinetobacter (Li et al. 2011), Sphingomonas (Wang et al. 2010) and Pseudomonas stutzeri ZCJ (Zhao et al. 2012). In addition, in terms of fungal degradation of nicotine, previous studies have shown that Pellicularia filamentosa and Cunninghamamella echinulate could degrade nicotine (Uchida et al. 2014), (Meng et al. 2010) isolated and identified a highly efficient nicotine-degrading bacteria, Aspergillus oryzae 112822, from tobacco leaves and clarified the pathway of nicotine degradation for the first time. Furthermore, it has been reported that some Pseudomonas have the ability to metabolize chemical pollutants in the environment, such as degrading monocyclic aromatic compounds (Lyra et al. 2021), polycyclic aromatic hydrocarbons (Rabodonirina et al. 2019), petroleum hydrocarbons (Gao et al. 2017), plastics and derivatives (Wierckx et al. 2015).

Besides, the microbial community is also a vital role in increasing the content of aroma substances during the aging. Many microorganisms such as Xanthomonadaceae, Enterobacteriales, Pseudomonadaceae, and Sphingomonadaceae can degrade furan compounds to produce aroma substances (Wierckx et al. 2011). Among them, Sphingomonas is a new type of microbial resource which can be used for the biodegradation of aromatic compounds. According to the literature sources, it helped transforming the solanone into important flavor compounds such as norsolane, solanoic acid during aging (Huang et al. 2018). The flavor enhancer produced by the biotransformation of tobacco cembranoids compounds can be added to the tobacco in fermentation to improve the aroma quality of tobacco products (El Sayed et al. 2008, El Sayed and Sylvester 2007). In addition, a system consisting of Bacillus and Geotrichum was found to decompose carotenoids into -ionone (an important compound of tobacco aroma) with tobacco aroma (Maldonado-Robledo et al. 2003). In this study, the mentioned bacteria have been detected in the aging process and the relative abundance of Sphingomonas changed as the aging process progressed.

Here, the function of bacterial communities was analyzed by FAPROTAX and most of the OTUs are related to chemoheterotrophy and aerobic chemoheterotrophy, indicating that bacteria mainly decompose the organic matters in tobacco leaves to obtain nutrients for cell growth and development. Functional changes related to nitrogen cycle revealed that the ability of the microbial community can convert nitrogen compounds like nitrite and nicotine to the non-toxics. The abundance of functions related to human and plant pathogens gradually decreases with time, suggesting that the fermentation process can kill most bacterial pathogens. In contrast, the abundance of some functions concerning the metabolism of aromatic compounds increased in the later phases of aging, which verified the fact that production of aroma substances in the fermentation process of tobacco leaves. In addition, fermentation, ureolysis and ligninolysis, and other functions increased, representing that there were a higher abundance and activity of bacteria related to the decomposition and transformation of organic compounds (Ling et al. 2022).

However, most of the fungal communities in the aging process of tobacco leaves are molds, including Aspergillus, Penicillium, Alternaria, and Cladosporium (Zhou et al. 2020). At the genus level in this study, the fungal community was dominated by Alternaria (20.1%), Aspergillus (19.9%), Cladosporium (4.8%), and Phoma (4.2%), which was consistent with the previously reported tobacco leaf fungal community (Wu et al. 2014). Alternaria always has been the dominant fungal community, because it is distributed in various ecosystems around the world. And most of Alternaria and Aspergillus are promising strains for production antibiotics (Al-Fakh and Almaqtri 2019, Guo et al. 2021). The main functional composition of fungal community also varies at the ecological guilds. As a result, with the aging process, the main nutritional mode of fungal community changed to saprophytic type, and aging caused significant differences in fungal community function. On the other hand, there are still a large number of unknown nutritional ways of fungal OTUs, so it is necessary to strengthen the functional research of fungi.
5. Conclusion

In this study, we displayed the structural, compositional and functional changes of microbial communities throughout the aging process. And the results showed that tobacco leaves are a natural reservoir of microorganisms. And these microorganisms could play an extremely significant role during the tobacco aging and have different metabolic functions in different aging periods, which can degrade harmful substances in tobacco such as nicotine and nitrite, and produce small molecule aroma substances to improve the quality of the tobacco. Therefore, the further study of microorganisms on the tobacco leaves from other storeroom would provide a certain basis for screening and isolation of microbial strains with specific functions. In addition, it also enables the further investigation of aging tobacco leaves to be an experimentally tractable microbial ecosystem, for the study of the mechanisms of microbial community formation and successions.

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Data availability statement

The data generated and/or analysed during the current study are not publicly available for legal/ethical reasons but are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

ORCID iDs

Fan Wang @ https://orcid.org/0000-0003-3301-8189
Mingjun Yang @ https://orcid.org/0000-0003-3954-0592

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