

ORIGINAL ARTICLE

Microbial community and metabolic function analysis of cigar tobacco leaves during fermentation

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Abstract

Cigar tobacco leaves (CTLs) contain abundant bacteria and fungi that are vital to leaf quality during fermentation. In this study, artificial fermentation was used for the fermentation of CTLs since it was more controllable and efficient than natural aging. The bacterial and fungal community structure and composition in unfermented and fermented CTLs were determined to understand the effects of microbes on the characteristics of CTLs during artificial fermentation. The relationship between the chemical contents and alterations in the microbial composition was evaluated, and the functions of bacteria and fungi in fermented CTLs were predicted to determine the possible metabolic pathways. After artificial fermentation, the bacterial and fungal community structure significantly changed in CTLs. The total nitrate and nicotine contents were most readily affected by the bacterial and fungal communities, respectively. FAPROTAX software predictions of the bacterial community revealed increases in functions related to compound transformation after fermentation. FUNGuild predictions of the fungal community revealed an increase in the content of saprotrophic fungi after fermentation. These data provide information regarding the artificial fermentation mechanism of CTLs and will inform safety and quality improvements.

KEYWORDS

artificial fermentation, chemical compositions, cigar tobacco leaves, function prediction, microbial community

1 | INTRODUCTION

Cigar tobacco has a long history of cultivation (Bauk, 2016). Unlike tobacco leaves for cigarettes, which are flue-cured, tobacco leaves for cigars are air-cured, and this difference is the main reason for the unique flavor and aroma characteristic of cigars (Burns, 1998; Sun et al., 2019). In general, after air-curing, cigar tobacco leaves (CTLs) must additionally be fermented, to retard pungent and irritating odors, before being used for cigar products (Frankenburg, 1950; Huang et al., 2010). Tobacco leaves are fermented by natural aging

or artificial fermentation (Lawrence & Bahnson, 1914). The quality of naturally aged tobacco is better than that of artificially fermented tobacco, but natural aging takes a long time (typically, 1–2 years) (Keller, 1929; Liu et al., 2016). The temperature and humidity of the aging room are easily influenced by external weather conditions, which may induce tobacco mildew and insect damage (Li et al., 2007). Compared with natural aging, artificial fermentation can shorten the fermentation cycle to 4–8 weeks, by controlling moisture and temperature (Keller, 1929). Thus, artificial fermentation can be beneficial in reducing storage costs compared with natural aging.

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Tobacco aging is complex and involves the enzymatic action of microorganisms and chemical interactions in tobacco leaves (Zhao et al., 2007). The microbial community and its activity are important in improving tobacco quality. An integrated study of microbial community changes in CTLs may suggest the mechanism responsible for the sensory quality of CTLs during the fermentation process. Tobacco leaves contain many microorganisms (Zhou et al., 2020). *Bacillus*, *Pseudomonas*, *Enterobacter*, *Sphingomonas*, *Pantoea*, and *Methylobacterium* are the dominant genera in tobacco leaves (Huang et al., 2010; Zhou et al., 2020). These bacteria degrade macromolecular organic matter in tobacco leaves during fermentation. For example, *Pseudomonas* spp. can effectively degrade nicotine (Zhong et al., 2010) and *Bacillus* spp. can produce small fragrant substances by decomposing macromolecular substances, such as carotene (Maldonado-Robledo et al., 2003). The fungal species identified in tobacco leaves are less diverse and less numerous than the bacterial species. *Aspergillus*, *Phoma*, *Alternaria*, *Monographella*, and *Cladosporium* are the major fungal genera in tobacco (Liu et al., 2020; Shanyi et al., 2018). Much of the recent literature concerning microorganisms associated with tobacco leaves has focused on the natural aging of flue-cured tobacco leaves (Huang et al., 2010; Maldonado-Robledo et al., 2003; Zhao et al., 2007; Zhou et al., 2020); CTLs have not been as extensively studied. Data from systematic research on the microbial community in CTLs during artificial fermentation would be valuable in cigar manufacturing.

In this study, the microbial community diversity of CTLs planted in Hainan, China, was investigated, and the major chemical contents of unfermented CTLs (UFD-CTLs) and fermented CTLs (FD-CTLs) were identified using the MiSeq platform (Illumina, San Diego, CA, USA) and continuous flow analyses, respectively. The functions of CTLs were assessed using the Functional Annotation of Prokaryotic Taxa (FAPROTAX) software. The data may inform quality improvements to CTL fermentation.

2 | MATERIALS AND METHODS

2.1 | Tobacco growth, curing conditions, and sample collection

Cigar tobacco (H382) was planted at Fan-yang, Hainan, China, at the end of December 2018 and harvested in March. The cultivated area was 6.8 hm². The freshly harvested CTLs were placed in a barn for air-curing. After 10 weeks, 15 kg of CTLs of the same grade was randomly selected from the barn and sent to the laboratory immediately. Based on previous studies (Du et al., 2016; Yu et al., 2008), 5 kg samples of CTLs were evenly stacked and placed in separate cabinets (HWS-P400C) at 40°C and 70% humidity. The experiments were repeated three times for three cabinets. Before artificial fermentation, CTLs were first wetted with sterile water within 2 days, until the moisture content was 35%–40% (Tian et al., 2018). The CTLs were sampled after 0 days (UFD-CTLs) and 1 month (FD-CTLs) of artificial fermentation (Yu et al., 2008). Each sample contained three independent biological replicates from the three cabinets, and

for each biological replicate, 600 g CTLs were taken from the cabinet using a five-point sampling method (Zhou et al., 2020). The samples were immediately stored at -80°C until further analysis.

2.2 | Determination of chemical contents of CTLs

The chemical contents of CTLs were analyzed using a continuous flow analytical system (Lu et al., 2020). The contents of total nitrate (TN) and protein were determined according to the Tobacco Industry Standard YC/T161-2002. The contents of water-soluble sugar (WSS), nicotine, and starch were determined according to Tobacco Industry Standards YC/T159-2002, YC/T468-2013, and YC/T216-2013, respectively. For each sample, three independent biological replicates from three cabinets were collected separately for chemical analysis, and 500 g of CTLs was used for each biological replicate (Furci et al., 2019). All the tests were conducted three times.

2.3 | DNA extraction and sequencing

Genomic DNA was extracted based on previous studies (Su et al., 2011; Ye et al., 2017; Zhang, Acuña, et al., 2019). For each biological replicate, 60 g of CTLs was ground with liquid nitrogen and then divided into three equal parts, namely the technical replicates; then, the DNA extracted from the three technical replicates of each sample was pooled into one DNA sample to minimize any potential DNA extraction bias. The DNA extraction steps were performed following the manufacturer's instructions for the E. Z. N. A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The integrity of the extracted DNA was checked on 1% agarose gel, and DNA concentration and purity were measured using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The V5–V7 regions of the bacterial 16S rRNA gene were amplified using the universal primers 799F (5'-AACMGGATTAGATACCKG-3') and 1193R (5'-ACGTCATCCCCACCTTCC-3') (Wang et al., 2018). The fungal internal transcribed spacer gene was amplified using the universal primers ITS1F (5'-CTTGGTCATTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3'). The PCR system and program were chosen based on a previous study (Ye et al., 2017). The PCR products were extracted and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) following the manufacturer's instructions. Finally, the purified amplicons were sequenced (2 × 300) using the Illumina MiSeq platform following the standard protocols of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.4 | Processing of sequencing data

All raw gene sequencing reads were checked and merged using Trimmomatic and FLASH software, respectively (Bolger et al., 2014; Magoč & Salzberg, 2011). The sequences were identified as

the same operational taxonomic units (OTUs) with 97% similarity, and the chimeric sequences were eliminated using UPARSE (version 7.1, <http://drive5.com/uparse/>) (Edgar, 2013). Based on the SILVA database, each sequence was classified and noted using the RDP Classifier (<http://rdp.cme.msu.edu/>) with a confidence threshold of 70% (Maidak et al., 2000; Pruesse et al., 2007).

2.5 | Statistical analyses

All experimental results were expressed as mean values \pm standard errors of the means (SEM). Data with a p -value < 0.05 were considered statistically significant. Statistical calculations and analyses were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA, USA) and SPSS 19 software for Windows (IBM Corporation, Armonk, NY, USA). The bacterial functions of CTLs were predicted using FAPROTAX (<http://www.zoology.ubc.ca/louca/FAPROTAX/lib/php/index.php?section=Instructions>) (Liang et al., 2019). The trophic mode of the fungi was annotated using FUNGuild software (<http://www.stbates.org/guilds/app.php>) (Nguyen et al., 2016). Multivariate analysis was conducted, using CANOCO software (version 4.52), via detrended correspondence analysis (DCA) and redundancy analysis (RDA). DCA revealed first axis lengths < 3.5 (1.9104 for bacterial analysis and 1.0841 for fungal analysis). Consequently, RDA was the appropriate linear model for analysis.

3 | RESULTS

3.1 | Major chemical component changes in CTLs after fermentation

The chemical contents, including those of TN, protein, nicotine, starch, and WSS, were analyzed before and after fermentation. The chemical composition of CTLs during fermentation changed under the action of microorganisms (Figure 1). All chemical contents decreased at the end of the CTL fermentation process. Significant decreases in the TN content occurred in FD-CTLs, unlike in UFD-CTLs ($p < 0.01$). The contents of protein, nicotine, starch, and WSS also significantly decreased after fermentation, compared with those in UFD-CTLs ($p < 0.05$).

3.2 | Effects of fermentation on structural changes in CTL microbiota

A total of 273,699 bacterial sequencing reads and 430,876 fungal sequencing reads were obtained from all CTLs using Illumina sequence analysis. To account for potential biases between samples with uneven sequencing depth, all read counts from the remaining samples were rarefied to the read depth of the sample with the lowest read number. Specifically, bacterial and fungal communities in each sample were rarefied to 25,762 (the minimum number of bacterial sequences) and 68,080 sequences (the minimum number of fungal

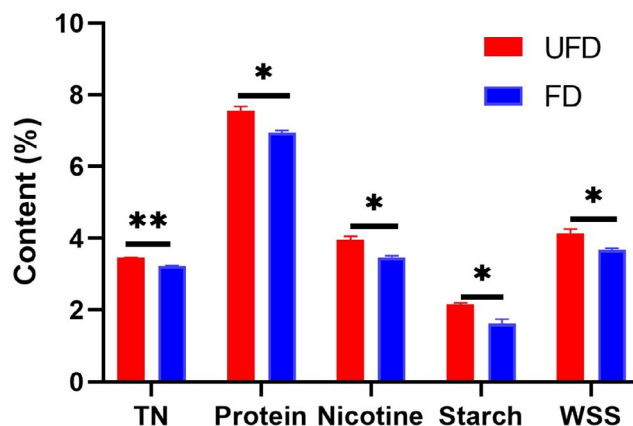


FIGURE 1 Chemical components in UFD-CTLs and FD-CTLs. Data are expressed as means \pm SEM ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus UFD-CTLs

sequences), respectively (Glowska et al., 2020; Huang et al., 2020; Schloss & Handelsman, 2006); 154,572 valid bacterial sequences and 408,480 valid fungal sequences remained for subsequent analysis (Table A1 in Appendix 2). Illumina sequence analysis indicated 1,041 bacterial OTUs and 270 fungal OTUs with 97% similarity. The rarefaction curves (Figure A1 in Appendix 1) of all groups tended to be flat with an increase in the number of OTUs, and the coverage of all samples was more than 99% for the bacterial and fungal sequences (Table A1 in Appendix 2). The collective data revealed that the detection rate of the microbial community in CTLs was close to saturation and that most of the microorganisms in the CTLs were covered by the sequencing data. Community richness (ACE and Chao estimators) and diversity (Simpson and Shannon indices) were determined to reflect the alpha diversity of CTL microbiota. The decrease in ACE and Chao indices of bacteria and fungi indicated a decrease in the microbial richness of CTLs after fermentation (Figure 2). Additionally, the diversity of the microbial community in CTLs showed a downward trend, but this was not significant after fermentation.

3.3 | Differences in microbial communities before and after fermentation of CTLs

For bacterial diversity and succession, 1041 OTUs were classified into 21 phyla, 41 classes, 125 orders, 249 families, and 533 genera with 97% similarity. Proteobacteria and Actinobacteria were the predominant phyla in UFD-CTL and FD-CTL samples, with the latter accounting for a larger proportion before fermentation and the former accounting for a larger proportion after fermentation (Figure 3a). Before fermentation, the distribution of bacteria was relatively scattered at the order level. After fermentation, the distribution was dominated by the orders of Betaproteobacteriales and Corynebacteriales, accounting for 71.29% of the sequences, which increased by 41.66% compared with that of UFD-CTLs (Figure 3c). For fungal diversity and succession (Figure 3b,d), 7 phyla, 19 classes, 45 orders, 88 families, and 139 genera were classified with 97%

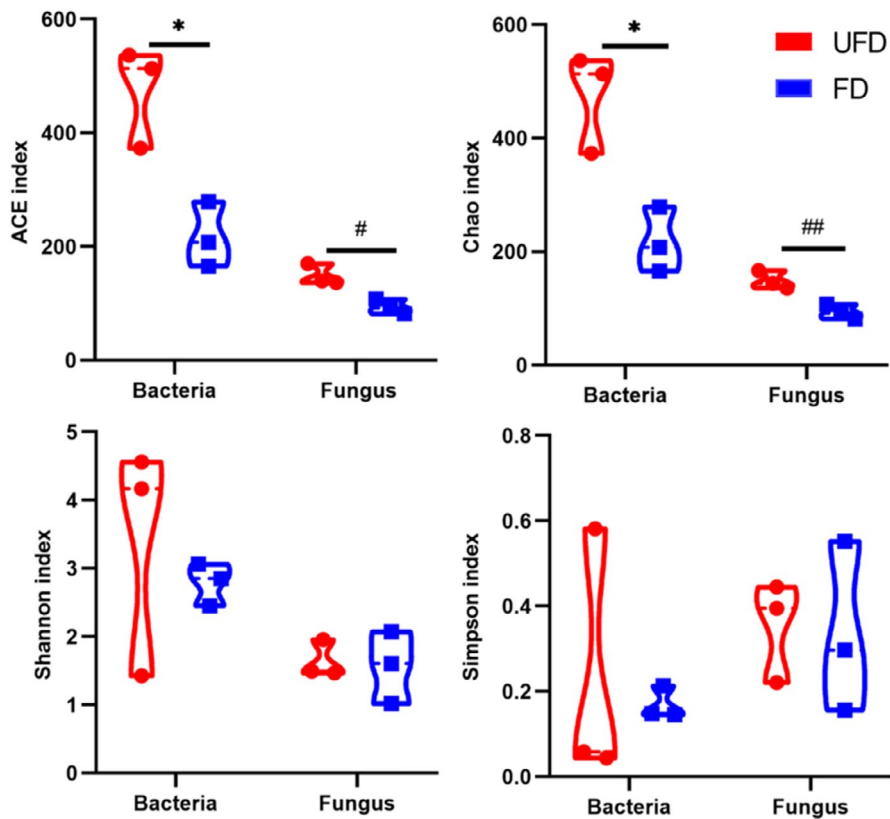


FIGURE 2 Alpha diversity index of microbial community in CTLs. Data are shown as means \pm SEM ($n = 3$). * denotes significance level in bacterial community, * $p < 0.05$; # denotes significance level in fungal community, # $p < 0.05$, ## $p < 0.01$; & denotes significance level between the bacterial and fungal community, && $p < 0.01$, &&& $p < 0.001$

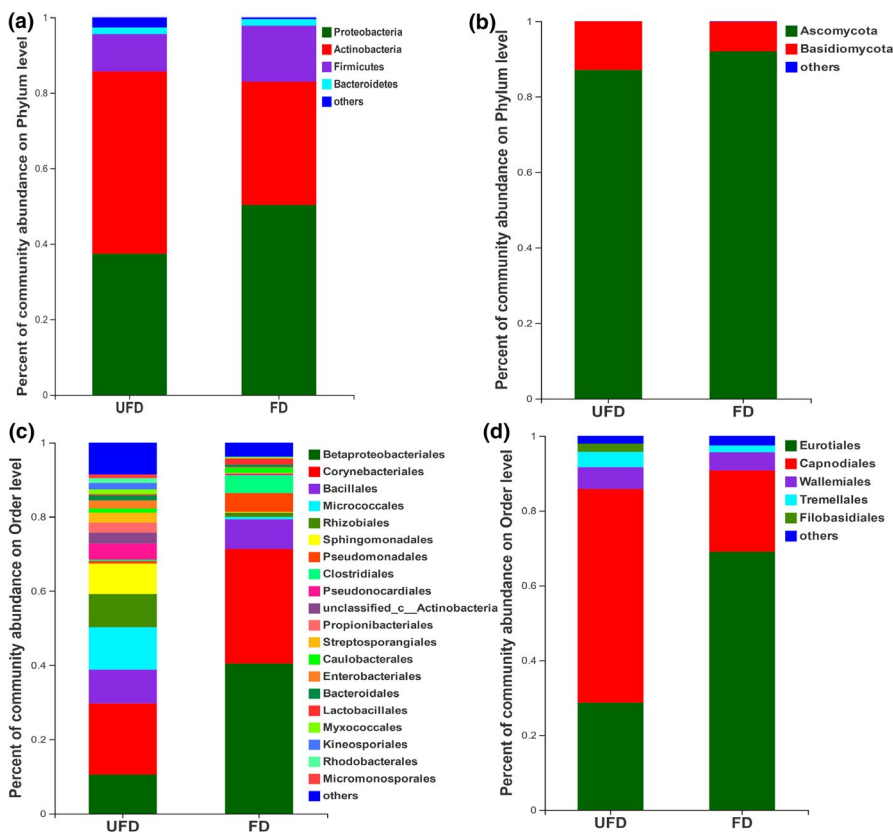


FIGURE 3 Bacterial (a) and fungal (b) compositions at the phylum level in CTLs. Bacterial (c) and fungal (d) compositions at the order level in CTLs

similarity. At the phylum level of fungal composition, Ascomycota was the dominant phylum throughout the fermentation period, whereas Capnoidiales and Eurotiales were the most abundant orders

in the fungal community of CTLs. Capnoidiales showed a high relative abundance in UFD-CTLs but were replaced by Eurotiales after fermentation.

To assess the overall distribution of the microbial community at the genus level, the 50 most abundant genera of the bacterial and fungal communities were determined; they are depicted in Figures A2 and A3 in Appendix 1. These results further illustrated that fermentation changed the relative abundances of the microbial communities in CTLs. To better understand the effects of fermentation on the relative abundance at the genus level, a statistical comparison of the relative abundances of the predominant genera of UFD-CTLs and FD-CTLs (top 20) was conducted (Figure 4). The results revealed that the abundances of seven bacterial genera changed after fermentation. After fermentation, the relative abundances of *Ralstonia* and *Pseudomonas* increased, whereas those of *Methylobacterium*, *Pseudonocardia*, *Aureimonas*, *Planococcus*, and *Nocardiopsis* remarkably decreased. In the fungal community, the abundances of only four genera changed significantly. The relative abundance of *Aspergillus* significantly increased in FD-CTLs by 40.48% compared with that in UFD-CTLs. However, the relative abundances of *Cladosporium*, unclassified *Basidiomycota*, and *Penicillium* significantly decreased and that of *Cladosporium* decreased by 35.47% after fermentation. After the removal of OTUs with <0.01% relative abundance, the relative abundances of *Ralstonia*, *Pseudomonas*, *Romboutsia*, *Chryseobacterium*, and *Comamonas* in the bacterial community of CTLs significantly increased after fermentation (Table A2 in Appendix 2), whereas the relative abundances of *Aspergillus* and *Phaeosphaeriaceae* in the fungal community significantly increased and that of *Cladosporium* significantly decreased (Table A3 in Appendix 2).

3.4 | Relationships between microbial community and chemical components

RDA was performed to determine the relationship between the chemical components and the microbial communities (Figure 5). The chemical components explained most of the variations in the

bacterial and fungal community structure in CTLs. The first ordination RDA axis that was strongly related to TN explained 67.23% of the variability in the bacterial community (Figure 5a). The first ordination RDA axis that was strongly related to nicotine explained 94.39% of the variability in the fungal community (Figure 5b). These findings indicated that a close relationship existed between the microbiome and chemical composition in the CTLs. TN and nicotine were the most probable factors to be influenced by microbial communities during the fermentation of CTLs.

3.5 | Functional prediction of bacterial and fungal communities

A total of 52 functional assignments for 1041 OTUs were obtained using the FAPROTAX tool. Functional group chemoheterotrophy was most abundant in the bacterial community of CTLs, but its abundance decreased after fermentation (Figure 6a). In contrast, the abundant functions in unfermented samples, such as aromatic compound degradation, ligninolysis, hydrocarbon degradation, aliphatic non-methane hydrocarbon degradation, and aromatic hydrocarbon degradation, which are related to compound transformation, increased in the fermentation process. Similarly, functions associated with the carbon cycle, such as phototrophy, photoheterotrophy, aerobic anoxygenic phototrophy, methylotrophy, and methanol oxidation, decreased after fermentation. Also, the number of plant pathogens increased during the fermentation process, and this might have negatively affected the quality of CTLs. However, animal parasites or symbionts, another functional group that may negatively affect CTLs, were maintained at a low level throughout the fermentation. At the end of fermentation, the functional groups related to chemoheterotrophy and carbon metabolism decreased, but those related to compound transformation and pathogens increased.

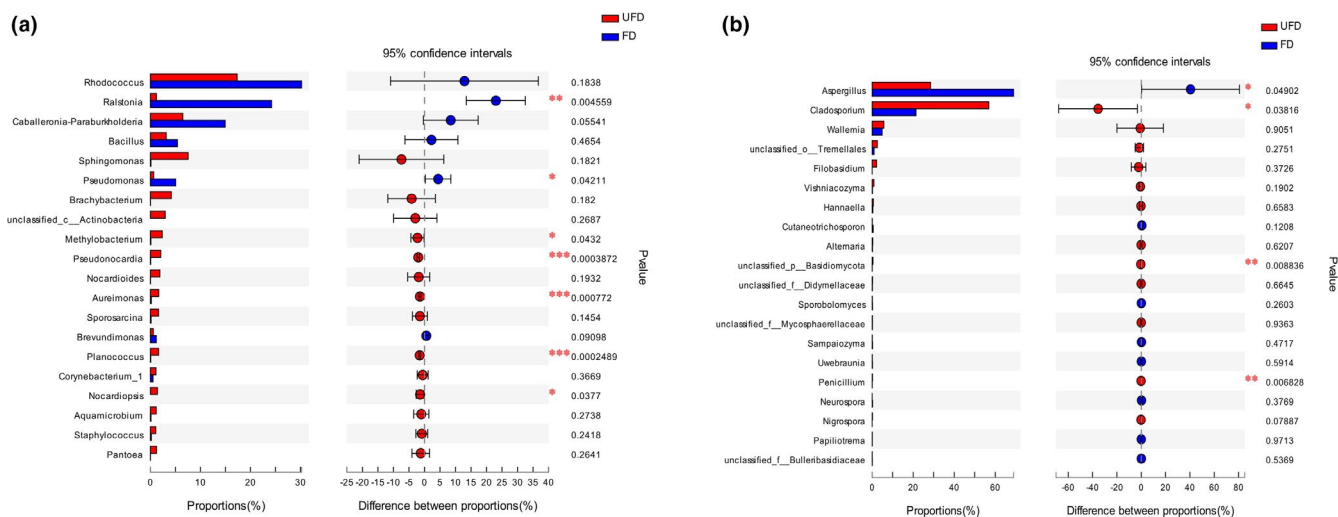


FIGURE 4 Mean proportions of 20 dominant genera in different groups of CTL samples: (a) bacteria and (b) fungi. Welch's *t* test was performed to assess the difference between two groups of CTL samples. **p* < 0.05, ***p* < 0.01, ****p* < 0.001

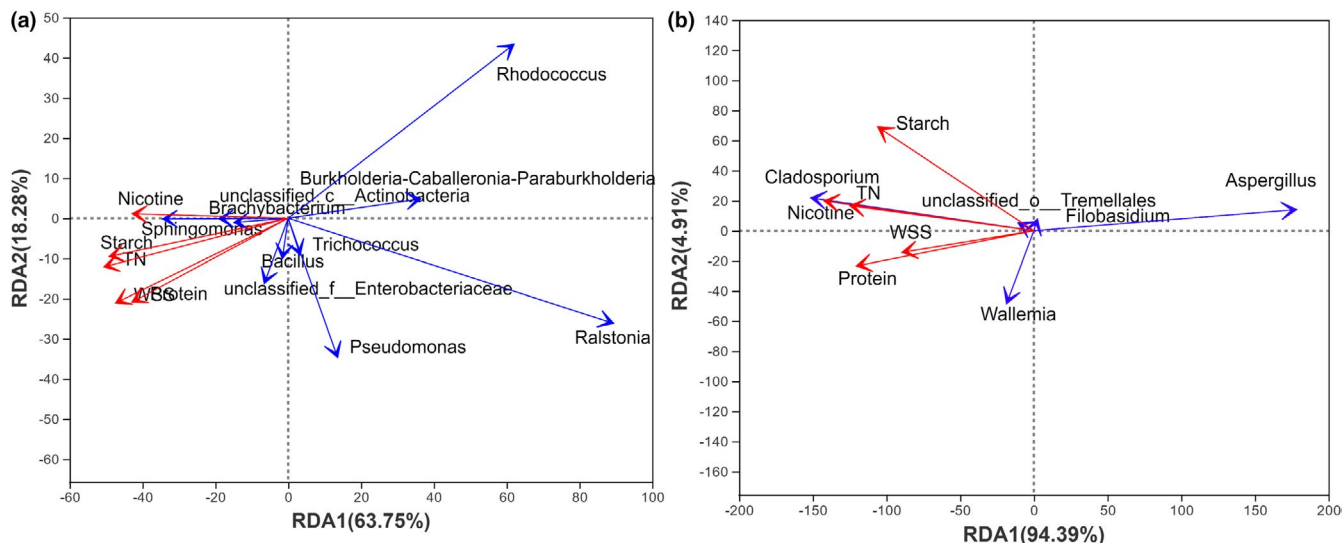


FIGURE 5 Ordination diagram of RDA for qualitative variables between microbial structure and major chemical compounds in CTLs for (a) bacteria and (b) fungi

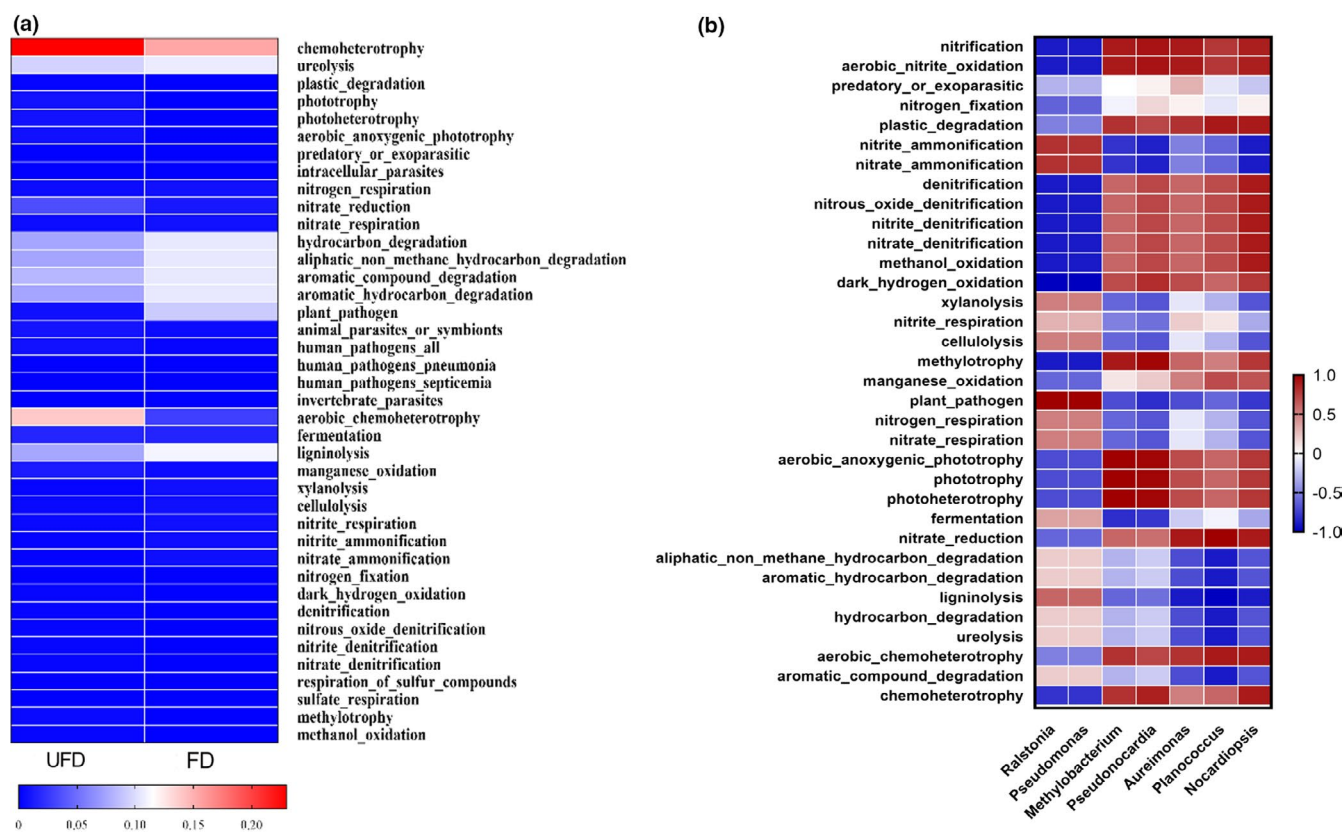


FIGURE 6 Bacterial functional groups in CTLs using FAPROTAX. (a) Top 40 predicted gene families in UFD-CTL and FD-CTLs. (b) Relationship between functional communities and bacteria with significant changes in abundance after fermentation

Figure 6b shows the association between the functional groups and genera with significant changes in abundance after fermentation. *Ralstonia* and *Pseudomonas* were important for compound degradation, which accelerated the degradation of aromatics, hydrocarbons, aliphatic compounds, and lignin. *Methylobacterium*, *Pseudonocardia*, *Aureimonas*, *Planococcus*, and *Nocardiopsis* were important in the carbon and nitrogen cycles. Additionally, *Ralstonia* and

Pseudomonas were closely associated with plant-pathogen function. *Pseudomonas* spp. were associated with pathogens in this study; previous studies have also demonstrated that in the process of plant growth, *Pseudomonas* could not only build a symbiotic relationship with plants as harmless bacteria but also infect them as pathogens (Hirano & Upper, 2000; Visnovsky et al., 2020). There are also many studies showing that *Pseudomonas* spp. are not mainly pathogens

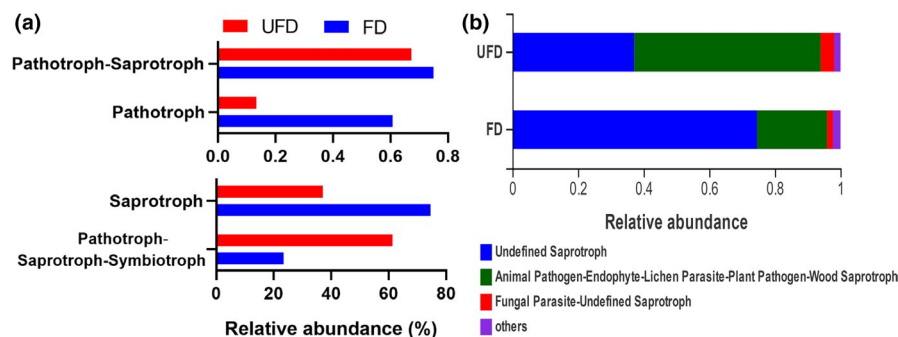


FIGURE 7 Relative abundance of trophic modes (a) and guild modes (b) assigned by FUNGuild for fungal communities

(Kim & Anderson, 2018; O'sullivan, & O'Gara, 1992). Further validation is needed to confirm whether *Pseudomonas* spp. are linked to pathogens or not.

FUNGuild was used to predict the trophic mode of the fungal community in CTLs. The fungal community was classified into four trophic mode groups, with saprophytic and pathotroph-saprotroph-symbiotroph being the dominant trophic modes in the fungal community during fermentation (Figure 7a). The abundance of saprophytic fungi increased from 37.06% to 74.52% after fermentation. The abundance of pathotroph-saprotroph-symbiotroph fungi was 61.35% and 23.44% in UFD-CTLs and FD-CTLs, respectively. Different guild modes in the fungal OTUs of CTL samples were classified (Figure 7b). As the most abundant primary guild mode, undefined saprotroph mode accounted for 37.10% and 74.54% abundance in UFD-CTLs and FD-CTLs, respectively. The second most abundant fungal guild of wood saprotroph accounted for 56.86% abundance of the fungal OTUs in UFD-CTLs but decreased to 21.38% in FD-CTLs.

4 | DISCUSSION

Cigar tobacco is air-cured; however, cured but unfermented tobacco is not suitable for cigar products because of its sharp and pungent flavor (Zhao et al., 2007). Artificial fermentation is a more controllable and efficient way of fermenting CTLs compared with natural aging. The microbial quality and functional profile of CTLs during artificial fermentation remain unclear. The data from this study enable a comprehensive understanding of the microbial diversity of UFD-CTLs and FD-CTLs with respect to the bacterial and fungal communities present. Also, the chemical components, bacterial functional groups, and fungal functional classifications in UFD-CTLs and FD-CTLs were analyzed.

The contents of chemical compounds change during fermentation. Primary chemical compounds decrease in content after fermentation. The present findings concerning the chemical components of CTLs were consistent with those of previous studies. After 24 months of natural aging, the content of WSS decreased by 2.63%, whereas the contents of TN, nicotine, starch, and protein decreased slightly (Zhou et al., 2020). Conversely, the contents of chemical components decreased significantly after short-term artificial fermentation (Qiu et al., 2003), consistent with the findings of

this study. We observed that the contents of TN, protein, nicotine, starch, and total sugar in CTLs significantly decreased after 30 days of artificial fermentation. These findings indicate that the chemical contents decrease faster in CTLs during artificial fermentation than during aging. This may be associated with the different activities of microorganisms during different types of fermentation; further research is needed to confirm this viewpoint. The transformation of chemical compounds is closely related to the microbial community in tobacco leaves during the fermentation process (Huang et al., 2010; Liu et al., 2016). The decrease in the contents of chemical components is associated with enzyme activity and microbial metabolism in tobacco leaves. The enzyme activity, such as amylase activity, is high during fermentation (Yamaguchi et al., 2013); this may be the reason for the decrease in starch content in tobacco leaves. The hydrolysis of starch produces WSSs, but the total sugar content does not increase, possibly because microorganisms use these sugars as nutrients to maintain their metabolism. *Bacillus* spp. degrade proteins in tobacco (Chen et al., 2015; Feng et al., 2012). A decrease in protein content can reduce the bitterness and astringent flavor after smoking (Ding et al., 2014; Pu et al., 2013). Nicotine in tobacco is degraded to niacin, methanesulfonic acid, oxidized nicotine, nicotinamide, *n*-methylnicotinamide, and adamantane by *Pseudomonas* spp., *Aspergillus fumigatus*, and *Rhodococcus* spp. (Mu et al., 2020).

The richness and alpha diversity of the microbial community decreased after artificial fermentation in this study. This may be because some microorganisms are not suitable for artificial fermentation conditions and their growth is inhibited (Huang et al., 2010). Actinobacteria and Proteobacteria were the most abundant phyla in the bacterial communities of CTLs, suggesting that the bacteria were stable at the phylum level. Moreover, the dominant phyla were different from those in flue-cured tobacco (Su et al., 2011). The abundance of Actinobacteria is less than that of other phyla in flue-cured tobacco (Su et al., 2011). However, a large number of Actinobacteria were found in CTLs from Mexico (Zhang et al., 2018). The varieties, fertilization methods, growth environments, and curing methods of flue-cured tobacco and cigar tobacco are different; these factors may account for the differences in the bacterial communities in their leaves (Yan et al., 2018). The relative abundances of *Rhodococcus*, *Ralstonia*, *Bacillus*, and *Pseudomonas* increased after fermentation, with those of *Ralstonia* and *Pseudomonas* increasing significantly. These findings indicate that these strains can adapt to fermentation and the complex chemical environment in tobacco

leaves. *Rhodococcus* spp. are gram-positive bacteria that readily degrade various organic matter. Some species of *Rhodococcus* and many species of *Bacillus* and *Pseudomonas* can degrade nicotine effectively (Liu et al., 2015; Mu et al., 2020; Ruan et al., 2005; Zhong et al., 2010). *Bacillus* spp. are widely distributed in nature and are commonly present in tobacco; they are important in tobacco fermentation (Carter, 1990; Huang et al., 2010). *Bacillus* spp. increase the formation of the desired aroma and enhance the quality of tobacco (English et al., 1967) and can degrade starch (Pen et al., 1992), cellulose (Li et al., 2006), and other compounds, making tobacco smoke less irritating. *Bacillus thuringiensis* can also control pests in stored products (Kaelin et al., 1994). Most species of *Ralstonia* are related to plant pathogens. For instance, *Ralstonia solanacearum* can cause bacterial wilt disease (Balamurugan et al., 2020). An increase in the abundance of *Ralstonia* may have been the reason for the increase in the abundance of plant-pathogen functional groups (Figure 6b). We also observed that the relative abundances of *Methylobacterium*, *Pseudonocardia*, *Aureimonas*, *Planococcus*, and *Nocardiopsis* notably decreased at the end of fermentation. These findings indicated that these bacteria could not survive well in the fermentation environment.

Understanding the functional group response to fermentation is essential for the accurate prediction of the potential effects of the fermentation process. Therefore, the functional capabilities of the bacterial communities in CTLs were assessed using FAPROTAX. The results indicated an extensive functional profile of the bacterial community in CTLs. The most abundant functional group was chemoheterotrophs. Chemoheterotrophic microorganisms mainly rely on energy and carbon sources in organic matter to maintain their growth (Glazer & Nikaido, 2007). Most bacteria depend on organic matter as their carbon and energy source in CTLs. Furthermore, many functional groups related to compound degradation participate in the fermentation of CTLs, and the abundance of these functional groups increased after fermentation; this result suggests a close connection between the chemical components and microbial community in CTLs during artificial fermentation, which is in agreement with the results of RDA. The changes in the chemical contents in CTLs can influence not only the quality of CTLs but also the safety (Wang & Ma, 2013) (TsoT, 1972). For instance, in addition to nicotine, a recognized harmful substance (Liu et al., 2015), the protein in tobacco could produce the smell of charred feather and harmful materials, such as quinoline, cyanic acid, and benzopyrene, during smoking when heated (Chen et al., 2015). The activities of microorganisms decreased the nicotine and protein contents in this study, and this was conducive to improving the safety of CTLs.

Although CTLs are mainly fermented by bacteria, fungi also play an important role. At the phylum level, Ascomycota were the most abundant fungi throughout the fermentation. Although Capnodiales and Eurotiales were the predominant orders, *Aspergillus* and *Cladosporium* were the predominant genera throughout the fermentation process. The relative abundances of these genera changed. Capnodiales and *Cladosporium* were the most abundant order and genus before fermentation, respectively. However, Eurotiales and

Aspergillus became the dominant order and genus, respectively, after 30 days of fermentation. The changes in their relative abundances indicated that fermentation changed the fungal trophic mode; this was also reflected in the results of FUNGuild analysis. Previous studies have indicated that saprophytic fungi can promote the decomposition of organic matter (Chen et al., 2020; Zhang et al., 2019). Therefore, an increase in the content of saprophytic fungi would aid the degradation of the chemical components of tobacco leaves. RDA also revealed a strong link between the fungal community and the chemical components.

5 | CONCLUSION

Chemical composition analysis showed that most of the metabolite levels in CTLs decreased after artificial fermentation. Fermentation changed the bacterial and fungal community structure. RDA revealed that the TN content was most readily affected by the bacterial community, whereas the nicotine content was most susceptible to the effects of the fungal community. FAPROTAX and FUNGuild analyses were used to predict the function of the microbial communities in CTLs. The dominant bacteria in cigars have important application prospects in the degradation of aromatic compounds, hydrocarbons, aliphatic compounds, and lignin. Fungi mainly have a saprotrophic function after fermentation. These results can be used as a basis to develop new strategies to improve the quality of CTLs through artificial fermentation.

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CONFLICTS OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Fang Liu: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (lead); Visualization (equal); Writing-original draft (lead); Writing-review & editing (equal). **Zhiyong Wu:** Formal analysis (supporting); Writing-review & editing (equal). **Xiaoping Zhang:** Software (supporting); Writing-review & editing (supporting). **Gaolei Xi:** Writing-review & editing (supporting). **Zhe zhao:** Resources (supporting). **Miao Lai:** Resources (supporting). **Mingqin Zhao:** Conceptualization (equal); Funding acquisition (lead); Project administration (lead); Supervision (lead).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

All the Illumina 16S rRNA and ITS gene sequence data obtained in this study are available in the NCBI database under the BioProject accession number PRJNA644460: <https://www.ncbi.nlm.nih.gov/>

bioproject/PRJNA644460. All other data are provided in the results section and the appendix of this article.

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APPENDIX 1

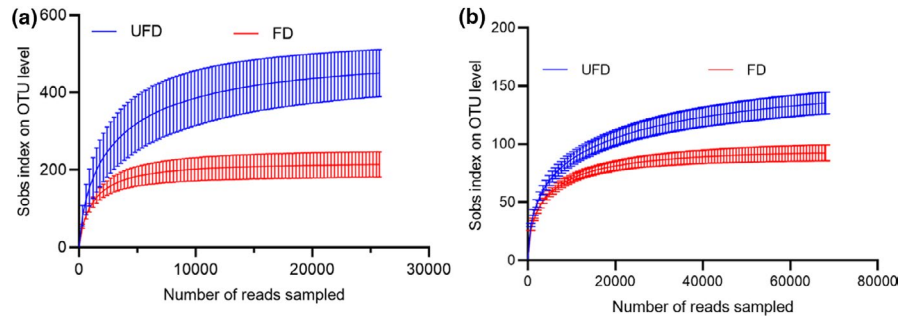


Figure A1 Rarefaction curve of bacterial (a) and fungal (b) OTUs derived from CTLs.

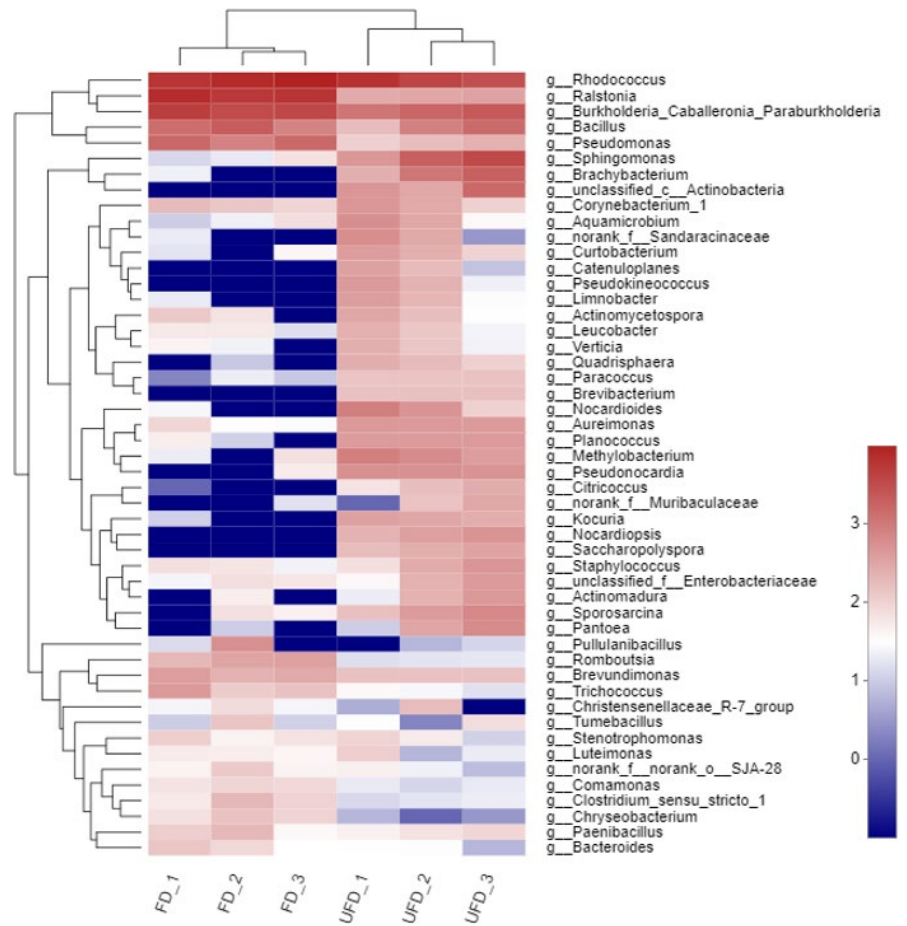


Figure A2 Community heat maps of CTLs at the genus level of the bacterial community (top 50).

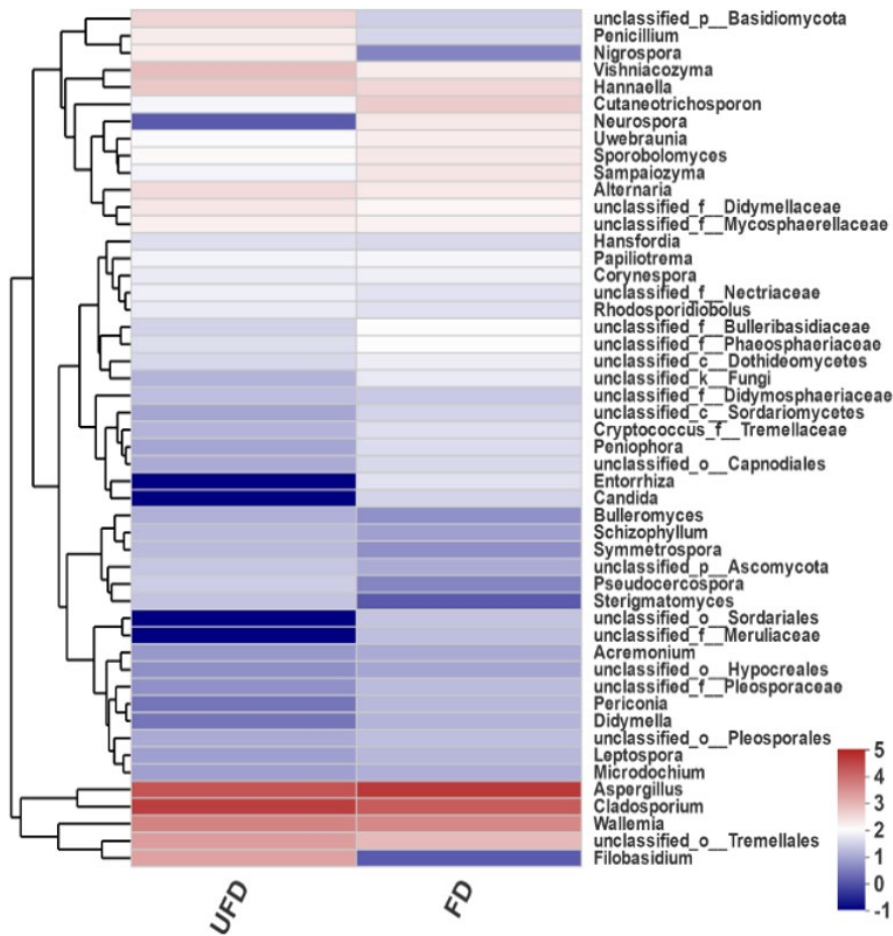


Figure A3 Community heat maps of CTLs at the genus level of the fungal community (top 50).

Sample name	Bacteria		Fungi	
	Sequences	Coverage	Sequences	Coverage
UFD-CTLs-1	37735	0.999231	69640	0.999713
UFD-CTLs-2	57134	0.999387	73062	0.999822
UFD-CTLs-3	25762	0.998370	70339	0.999701
FD-CTLs-1	49260	0.999695	68080	0.999971
FD-CTLs-2	28435	0.999648	69618	0.999971
FD-CTLs-3	28516	0.999790	71840	0.999916

Table A1 Numbers of valid sequences and coverage of 16S rRNA and 18S rRNA gene libraries at 97% similarity from the sequencing analysis

Table A2 Mean proportions of the bacteria genera after removing OTUs with <0.01% in different groups of CTL samples

Name	UFD-mean	UFD-sd	FD-mean	FD-sd	p-value
<i>Rhodococcus</i>	17.34	8.012	30.22	8.29	0.1838
<i>Ralstonia</i>	1.215	0.2086	24.22	4.008	0.004559**
<i>Burkholderia-Caballeronia-Paraburkholderia</i>	6.488	3.11	14.95	3.005	0.05541
<i>Bacillus</i>	3.171	3.546	5.411	2.586	0.4654
<i>Sphingomonas</i>	7.554	8.141	0.1294	0.1171	0.1821
<i>Pseudomonas</i>	0.6618	0.3486	5.062	1.713	0.04211*
<i>Brachybacterium</i>	4.186	4.559	0.02847	0.0493	0.182
<i>Romboutsia</i>	0.06405	0.008234	1.143	0.3311	0.02217*
<i>Corynebacterium_1</i>	1.114	1.016	0.5305	0.1728	0.3669
<i>Trichococcus</i>	0.1009	0.0549	0.9057	0.6644	0.2032
<i>Staphylococcus</i>	1.073	1.128	0.2018	0.0998	0.2418
<i>Aquamicrobium</i>	1.166	1.452	0.1359	0.1298	0.2738
<i>unclassified_f_Enterobacteriaceae</i>	0.9103	1.079	0.2018	0.09421	0.3042
<i>Stenotrophomonas</i>	0.2057	0.2306	0.2834	0.1321	0.6538
<i>Chryseobacterium</i>	0.01747	0.008234	0.4153	0.1614	0.04556*
<i>norank_f_norank_o_SJA-28</i>	0.1048	0.1098	0.2704	0.1793	0.338
<i>Comamonas</i>	0.07375	0.001261	0.3131	0.05439	0.0097**
<i>Enterococcus</i>	0.04852	0.04666	0.2627	0.186	0.2257
<i>Leucobacter</i>	0.5124	0.5929	0.1617	0.08964	0.3527
<i>norank_f_Bacillaceae</i>	0.06987	0.06038	0.1708	0.1688	0.4935
<i>Dyella</i>	0.1863	0.1372	0.06728	0.03522	0.2196
<i>Bradyrhizobium</i>	0.13	0.04666	0.09445	0.06614	0.5644
<i>Caulobacter</i>	0.06211	0.04392	0.1255	0.05051	0.2469
<i>Luteimonas</i>	0.2523	0.247	0.185	0.02642	0.6446
<i>Micrococcus</i>	0.1378	0.06313	0.05176	0.02913	0.1189
<i>Cutibacterium</i>	0.1087	0.1043	0.06211	0.03703	0.5039
<i>Sulfuritalea</i>	0.03494	0.00549	0.1061	0.03607	0.07819
<i>Clostridium_sensu_stricto_1</i>	0.06599	0.02196	0.4542	0.2912	0.1721

*p < 0.05,

**p < 0.01.

Table A3 Mean proportions of the fungi genera after removing OTUs with <0.01% in different groups of CTL samples

Name	UFD-mean	UFD-sd	FD-mean	FD-sd	p-value
<i>Aspergillus</i>	28.47	21.4	68.95	13.08	0.04902*
<i>Cladosporium</i>	56.85	19.48	21.38	5.234	0.03816*
<i>Wallemia</i>	5.753	9.144	4.88	7.625	0.9051
<i>unclassified_o_Tremellales</i>	2.525	1.985	0.966	0.7917	0.2751
<i>Hannaella</i>	0.6101	0.7858	0.3746	0.3374	0.6583
<i>Cutaneotrichosporon</i>	0.0803	0.05276	0.5352	0.3973	0.1208
<i>unclassified_f_Mycosphaerellaceae</i>	0.1689	0.1827	0.1552	0.2112	0.9363
<i>Sporobolomyces</i>	0.1214	0.1009	0.212	0.06445	0.2603
<i>Uwebraunia</i>	0.08911	0.0656	0.1772	0.2537	0.5914
<i>unclassified_f_Phaeosphaeriaceae</i>	0.04309	0.0221	0.09548	0.007344	0.01759*
<i>unclassified_f_Nectriaceae</i>	0.06659	0.07476	0.04798	0.06152	0.756

*p < 0.05.