

Research on the Microbial Community Structure and Evolutionary Changes During the Industrial Fermentation Process of Cigar Tobacco Leaves

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Abstract: Cigar tobacco leaves host a diverse bacterial community on their surfaces, playing a vital role in the fermentation process. This study aimed to investigate and analyze the bacterial community dynamics during the artificial fermentation of cigar tobacco leaves, recognizing the advantages of artificial over natural fermentation. The research focused on elucidating the structural composition of the bacterial community on the leaves, exploring their metabolic activities, understanding how they influence the characteristics of the leaves during artificial fermentation, and identifying potential metabolic pathways. Significant shifts in the microbial community were observed post-fermentation, with a marked increase in diversity. Notably, genera like *Oceanobacillus*, *Terribacillus*, and *Corynebacterium* exhibited substantial growth during the mid-phase of fermentation, suggesting their importance in the process. The insights derived from this study shed light on the artificial fermentation process of cigar tobacco leaves, offering opportunities for enhancing safety and quality.

Keywords: Cigar tobacco leaves, functional microorganisms, Microbiomics.

1. Introduction

The fermentation of cigar tobacco leaves is a vital procedure in the cigar manufacturing process, playing a pivotal role in enhancing the quality of the leaves. (Jia *et al.* 2023), Tobacco leaves must undergo a specific fermentation or aging period to substantially enhance their quality and processing characteristics, ensuring compliance with the standards of commercial cigarette production(Zhou *et al.* 2016). The fermentation of cigar tobacco leaves primarily involves natural and artificial processes. Typically, the moisture content of the leaves is adjusted to approximately 30% before fermentation, which is conducted under controlled temperature and humidity conditions, either artificially or naturally(Di Giacomo *et al.* 2007). Natural fermentation depends on enzymatic hydrolysis and microbial fermentation conducted by tobacco and microorganisms existing in the fermentation environment, at self-ignition temperatures and suitable conditions. This fermentation process can span from 1 to 2 years, exhibiting unstable quality that is susceptible to external environmental influences(Li *et al.* 2020; Rivett and Bell 2018; Zhang *et al.* 2013). Artificial fermentation can achieve equivalent fermentation outcomes and concurrently shorten the fermentation duration. Consequently, cigar tobacco leaves are typically subjected to artificial fermentation, wherein enzymes, microorganisms, and other factors collaboratively facilitate the conversion of biomass in the tobacco leaves.

The fermentation of cigar tobacco leaves is a crucial procedure that eliminates noxious odors, decreases harmful substances, and enhances the distinctive flavor of the tobacco leaves, thereby ameliorating their quality(Li 2020). Many studies have shown that microbes play a crucial role in the fermentation process of tobacco(Zhou *et al.* 2020). The metabolic activities of microbial communities, including protein degradation, amino acid biosynthesis and metabolism, and carbohydrate degradation, enhance the production of

volatile aromatic compounds in tobacco leaves while decreasing green odors and irritant compounds(Liu *et al.* 2021). Tobacco leaves host a vast and intricate microbial community, with *Bacillus*, *Pseudomonas*, and *Agrobacterium* being the predominant bacterial genera. These microorganisms exhibit diverse biological functions; for instance, *Pseudomonas* is capable of nicotine degradation, *Bacillus* can break down carotenoids, and *Agrobacterium* plays a role in enhancing the synthesis of aromatic compounds(Hu *et al.* 2022). The application of microbial inoculants during tobacco leaf fermentation can enhance both the quality of the leaves and the quantity and quality of their fragrance. While traditional isolation methods fall short in separating all microbial species within tobacco leaves, this limitation constrains the development of beneficial microbial inoculants for tobacco fermentation. In contrast, metagenomic sequencing technology offers a solution by detecting the genomic DNA of all microorganisms in the sample. This approach provides a comprehensive insight into the composition of the tobacco leaf microbial community, aiding in the selection of functional surface microorganisms and offering theoretical guidance to improve the quality of cigar tobacco leaves.

2. Materials and Methods

2.1. Collection of cigar tobacco leaf samples

Cigar tobacco leaf samples from Hainan, the region of origin, were collected as follows: The fermentation process commenced by rehydrating the leaves at 85% relative humidity and 35°C for 24 hours. Subsequently, a 5-day fermentation period followed in an environment maintained at 70% relative humidity and 40°C. Samples were obtained at distinct stages: before rehydration (A), on the first day (B), the third day (C), and the fifth day (D) of fermentation, with each stage consisting of three independent biological replicates. These samples were promptly stored at -80°C in an ultra-low temperature freezer for subsequent analysis.

2.2. PCR amplification and Illumina Miseq sequencing

PCR amplification, library construction, and sequencing were commissioned to Wuhan Huada Genomics Co., Ltd. The bacterial primers used were “forward primer ACTCCTACGGGAGGCAGCAG, reverse primer GGACTACHVGGGTWTCTAAT” targeting the V3-V4 region. The fungal primers used were “forward primer CTTGGTCATTTAGAGGAAGTAA, reverse primer GCTGCGTTCTTCATCGATGC” targeting the ITS1 region.

2.3. Sequence data processing and data analysis

Following PCR product purification and fluorescence quantification, the forward reads from paired-end sequencing undergo initial pairwise assembly. Concurrently, a sequence quality check is conducted, filtering out reads containing ‘N’ and low-complexity sequences (consecutive 10 ATCG). Subsequent to the removal of primers and adapters, a window-based quality trimming approach is employed: utilizing a window size of 30 bp, if the average quality value

within a window falls below 20, the read sequence is truncated from that window, and reads below 75% of the original read length are discarded. The optimized sequences are then analyzed online using the microbial amplicon analysis platform by Wuhan Huada Genomics Co., Ltd. The Usearch software is employed for operational taxonomic unit (OTU) clustering analysis, where sequences sharing $\geq 97\%$ similarity are grouped into a single OTU for biological information analysis, with each OTU representing a distinct species. Taxonomic classification of OTU representative sequences is achieved using the RDP classifier Bayesian algorithm, with examination of community composition at various taxonomic levels for each sample. The distribution of sequence lengths is evaluated through R scripts to determine the length distribution of high-quality sequences across all samples.

3. Results and Discussion

3.1. Reliability analysis of Illumina MiSeq sequencing results

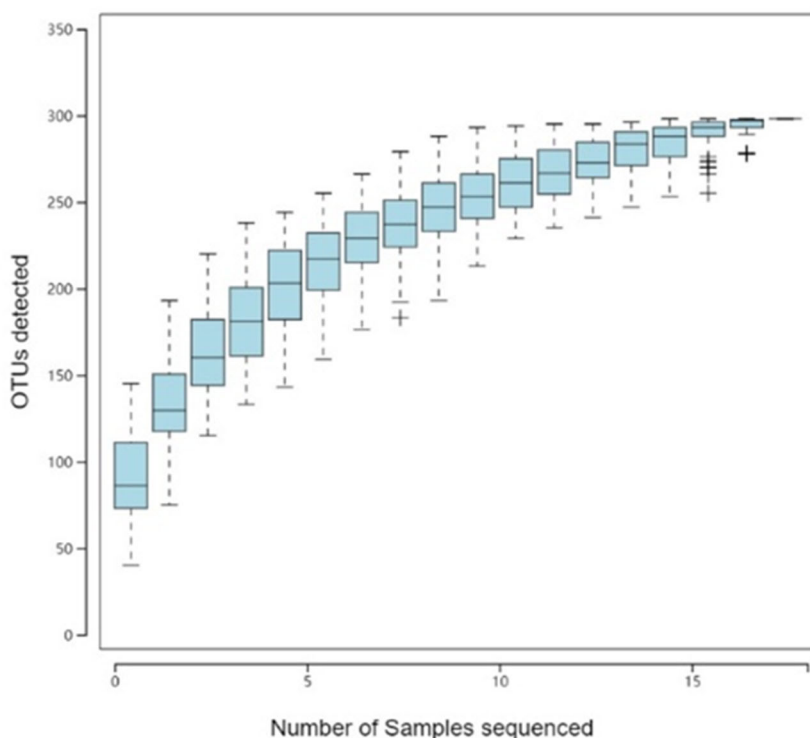


Figure 1. Species accumulation curve

The species accumulation curve (Fig.1) reflects the effect of sample size on species diversity; with a small sample size, the discovered species are not comprehensive and cannot represent the entire community structure. As the sample size increases, more species are discovered, providing a better representation of the community structure. In practical analysis, if the curve shows a sharp increase towards the end, it indicates insufficient sampling. Increasing the sample size can further reveal new species. When the rising trend at the end of the curve levels off, it signifies sufficient sampling. In our experimental results, the trend at the end of the curve leveled off, indicating that the sampling size was sufficient.

3.2. OUT analysis

Figure 2 shows a Venn diagram at the bacterial OTU level

in the fermentation process of cigar tobacco leaves. A total of 276 bacterial OTUs were identified across the four samples, with 52 OTUs shared among the samples. In the bacterial OTU analysis, the RT sample had 159 bacterial OTUs, of which 37 were unique. The FI sample had 203 bacterial OTUs, with 39 unique OTUs. The FM sample exhibited only 4 unique bacterial OTUs, the least among the samples, alongside 166 shared OTUs. The FT sample contained 155 shared bacterial OTUs, with 20 unique OTUs. Overall, there are significant differences in bacterial composition among cigar tobacco leaf samples at different fermentation times.



Figure 2. Venn diagram of bacterial OTUs in cigar tobacco leaves.

3.3. Alpha diversity analysis

During the fermentation of Hainan Wuzhi Shan cigar tobacco leaves, the α diversity analysis of the microbial community reflects the diversity of microorganisms in the samples, mainly including various indices such as the Shannon index, Simpson index, Ace index, Chao index, and Coverage index. The Ace and Chao indices indicate community richness, while the Simpson and Shannon indices reflect community diversity, and the Coverage index reflects community coverage. In this study, high-throughput sequencing was conducted on Hainan Wuzhi Shan cigar tobacco leaf samples from different fermentation periods. Subsampling was performed based on minimum sample number, and clustering was conducted at a 97% similarity level. The results are shown in the table 1.

Table 1. Bacterial α Diversity Analysis

Sample	Chao Index	Ace Index	Shannon Index	Simpson Index	Coverage Index
A	93.11±18.37	115.90±34.16	1.35±0.54	0.39±0.14	99.98%
B	150.43±2.98	149.15±9.06	1.36±0.12	0.36±0.04	99.96%
C	99.57±8.26	126.17±9.92	1.73±0.17	0.30±0.04	99.95%
D	125.34±13.31	137.51±17.18	1.57±0.37	0.39±0.11	99.94%

The study findings reveal dynamic changes in the Ace index and Chao index throughout the fermentation process, with a notable decrease in the early to mid-phase and a significant increase from the mid to late phase. This pattern suggests that the richness of the bacterial community in Hainan Wuzhi Shan cigar tobacco leaves undergoes continuous evolution during prolonged fermentation. This fluctuation may be attributed to the initial microbial community's lack of adaptation to environmental shifts, leading to diminished richness in the mid-fermentation phase characterized by dominant bacterial genera. The Shannon index experiences a sharp rise from the early to mid-fermentation stage, followed by a rapid decline and a subsequent slight ascent towards the late fermentation phase. Conversely, the Simpson index indicates a gradual decline succeeded by an increase, reflecting lower microbial diversity in the early and late fermentation stages and higher diversity in the mid-phase. As fermentation advances, microbial diversity progressively rises before declining, mirroring a consistent trend in community species evenness.

3.4. Bacterial community analysis at the genus level of cigar tobacco leaves

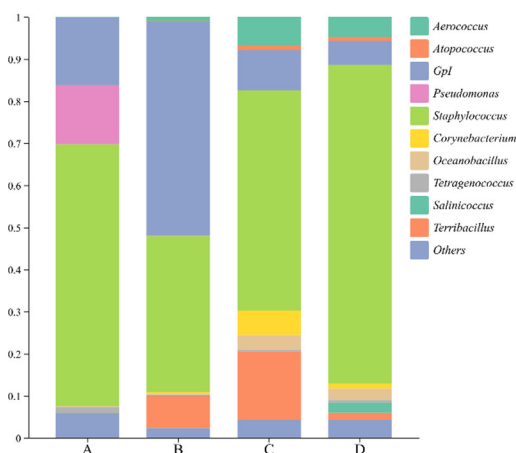


Figure 3. Bar graph of the bacterial community composition in cigar tobacco leaves at the genus level.

The examination of the bacterial community composition in cigar tobacco leaves at the genus level highlights the top 10 genera with average relative abundances exceeding 1% across 12 samples. Notably, these genera encompass *Terribacillus* (0.01% ~ 16.26%), *Salinicoccus* (0.02% ~ 2.41%), *Tetragenococcus* (0.05% ~ 1.43%), *Oceanobacillus* (0.18% ~ 3.48%), *Corynebacterium* (0.01% ~ 5.83%), *Staphylococcus* (37.12% ~ 75.72%), *Pseudomonas* (0.01% ~ 14.07%), *Gpl* (5.64% ~ 50.92%), *Atopococcus* (0% ~ 1.16%), and *Aerococcus* (0.02% ~ 6.75%). *Staphylococcus* emerges as the dominant genus during cigar tobacco leaf fermentation, displaying an initial decrease in abundance at the onset of fermentation compared to the unfermented state; however, it gradually rises to represent 75.72% of the total bacterial species abundance. Conversely, *Gpl* shows the highest abundance in sample A but experiences a notable decline in relative abundance as fermentation progresses, reaching a minimum of 5.64% on the fifth day of fermentation. *Terribacillus*, *Oceanobacillus*, and *Corynebacterium* exhibit peak relative abundance on the third day of fermentation, indicating heightened bacterial community diversity as evidenced by α diversity analysis. Relative to unfermented cigar tobacco leaves, those in the early fermentation stage exhibit notable internal microbial variations and distinct microbial structures compared to other samples, potentially influenced by later-stage environmental changes during fermentation.

While research reports are currently lacking on the role of the *Staphylococcus* genus in the fermentation of cigar tobacco leaves both domestically and internationally, studies have explored its metabolic functions in food processing. For example, the *Staphylococcus* genus has been found to metabolize branched-chain amino acids and sulfur-containing amino acids to introduce a bacon-like flavor during the fermentation of bacon and sausages (Feng *et al.* 2021). *Pseudomonas* bacteria are known for their capability to break down various environmental pollutants, playing a significant role as organic matter decomposers in the environment. Among these bacteria, *Pseudomonas alcaligenes*, a species known for its alkaline production, exhibits the capacity to

degrade polycyclic aromatic hydrocarbons. Additionally, certain species within the *Pseudomonas* genus can degrade harmful compounds present in tobacco, including tobacco-specific nitrosamines (Li *et al.* 2021). Within the predominant fungal genera associated with cigars, numerous strains of *Aspergillus* exhibit the capability to enhance the quality of tobacco leaves. For instance, treatment of the tobacco leaves with *Aspergillus* resulted in a significant increase in the levels of organic acids and petroleum ether extracts, leading to a marked improvement in the sensory smoking score. Nonetheless, *Aspergillus* is a significant genus known to

cause mold formation during the slicing, storage, and curing processes. It is crucial to pay close attention and exercise vigilance concerning this fungus. Identification and targeted control measures for specific species and quantities of *Aspergillus* that may contribute to mold growth in cigars can effectively elevate the quality of cigar products (Jiaxuan *et al.* 2016).

3.5. Bacterial community KEGG metabolic pathway analysis

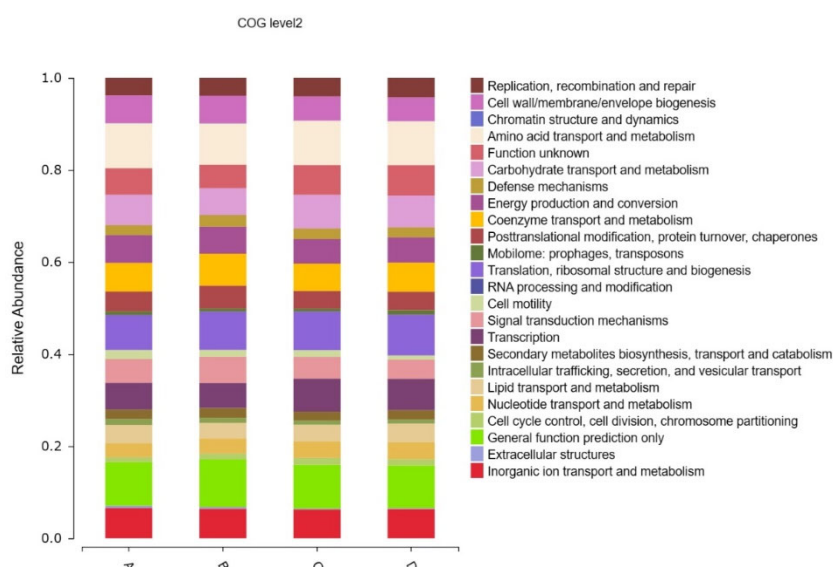


Figure 4. KEGG Metabolic Pathway Functional Annotation Diagram

Figure 4 illustrates the multifaceted roles played by microorganisms within a vast and diverse population, showcasing a range of functions. The utilization of PICRUSt for functional prediction of surface bacteria on tobacco leaves across distinct production regions and applications, utilizing KEGG level 2 functional gene analysis, highlights that bacterial functions during varying fermentation stages predominantly involve amino acid metabolism, auxiliary factors and vitamins, additional amino acid metabolism, carbohydrate metabolism, chitin and keratin metabolism, and lipid metabolism. The notable prevalence of these functions signifies the active engagement of the bacterial community on tobacco leaf surfaces in crucial metabolic pathways. Throughout fermentation, bacterial activities in cigar tobacco leaves primarily revolve around amino acid and carbohydrate metabolism, where carbohydrate metabolism intermediates serve as essential precursors for various syntheses, including the production of aromatic amino acids. Furthermore, amino acids can partake in the Maillard reaction with reducing sugars, playing a pivotal role in generating flavor and bitterness in tobacco products, intimately linked to the sensory attributes of tobacco leaves.

4. Conclusion

Staphylococcus, *Pseudomonas*, and *Aspergillus*, among the dominant bacterial genera within cigar tobacco microbiota, showcase diverse metabolic capabilities. These bacteria not only hold the potential to enhance the sensory characteristics of cigars but also possess the ability to decompose and metabolize certain harmful components present in tobacco. The growth dynamics and metabolic activities of

microorganisms are pivotal in the fermentation process. Understanding the composition of microbial communities and their interrelationships is instrumental in refining cigar manufacturing procedures, enhancing microbial management, and optimizing conditions during the artificial fermentation of cigar tobacco leaves. This comprehension is crucial for elevating the quality and yield of cigar products.

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