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Original Article

Analysis of fungal composition in different layers of Bantou agarwood-forming trunk of *Aquilaria sinensis* revealing presence of *Aspergillus*-inhibiting substances in agarwood sites

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ABSTRACT

Objective: The objective of this study was to analyse fungal composition and exploit application potential in the Bantou (BT) agarwood-forming trunk of *Aquilaria sinensis*.
Methods: BT agarwood is a naturally formed agarwood that was collected after cutting. Total genomic DNA of the fungi in BT agarwood was extracted by the hexadecyltrimethy ammonium bromide (CTAB) method, followed by PCR amplification and library construction. The effective tags were obtained by the HiSeq2500 platform, and the data were subjected to bioinformatics and statistical analyses.
Results: A total of 7 850 040 effective tags were obtained, Ascomycota was the most abundant fungus at the phylum level, with a relative abundance of 56.36%–61.44%, followed by Basidiomycota, with a relative abundance of 10.49%–20.39%. Dothideomycetes, Agaricomycetes and Sordariomycetes were dominant at the class level, accounting for 26.21%–33.88%, 8.40%–17.66%, and 18.41%–24.11%, respectively. *Lignosphaeria*, *Phaeoacremonium* and *Hermatomyces* were dominant at the genus level, with relative abundances of 6.25%–7.64%, 1.95%–9.05% and 1.5%–5.4%, respectively. Diversity and richness analysis showed that the fungal composition in the agarwood formation sites (agarwood layer, upper agarwood layer and lower agarwood layer) were significantly lower than those in the decomposing layer and the healthy layer. That is, the fungal diversity and richness were significantly reduced during agarwood formation by the action of open wounds. The fungal community structure in the decomposing layer and agarwood formation sites obviously differed from that in the healthy layer. The number of *Aspergillus* taxa in agarwood formation sites decreased significantly (healthy layer is 0.5%, decomposing layer is 0.022%, upper agarwood layer is 0.012%, agarwood layer is 0.01%, and lower agarwood layer is 0.013%), indicating that agarwood may contain potential substances to inhibit the growth of *Aspergillus*.
Conclusion: Agarwood from agarwood formation sites contains potential substances that inhibit *Aspergillus*, which provides valuable information for the control of the genus of *Aspergillus*.

1. Introduction

Agarwood is resin-containing wood formed in wounded *Aquilaria* or *Gyrinops* trees of the family Thymelaeaceae. Among the *Aquilaria* spp., *A. sinensis* (Lour.) Gilg is the primary producer and mainly found in southern China (Abdul Kadir, Azizan, & Othman, 2020; Chen et al., 2022). Agarwood, listed in the *Chinese Pharmacopoeia*, is a well-known aromatic traditional Chinese medicine with diverse uses in medicine, incense, perfume and aromatherapy (Shivanand, Arbie, Krishnamoorthy, & Ahmad, 2022; Zhang et al., 2023; Liu, Wei, Gao, Zhang, & Lyu, 2017). The primary active compounds of agarwood have been determined to have neuroprotective, sedative, antianxiety, antirestless, antibacterial and anti-inflammatory actions (Dong et al., 2022; Wang et al., 2021). Agarwood is rarely formed in normal trees unless exposed to specific external factors that cause wounding, such as lightning strikes, animal grazing, insect attack, or fungal infection (Wang et al., 2021). Traditional methods of agarwood production include cutting, nailing, boring holes, or cutting a piece of a trunk (Zhang

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et al., 2021). In this study, Bantou agarwood (BT agarwood) was formed in the trunk of *A. sinensis* with the partial-stem-breaking method. After three years, resinous formation occurs under physical injury in the natural environment. In the wild, because the main stem and side branches of BT agarwood are cut off, holes of different heights will form on the surface of the cut parts under long periods of erosion by rain. The longer the holes extend, the better the quality of agarwood formed (Li et al., 2023). Therefore, it is speculated that the formation quality of agarwood may be related to microbial infection. Earlier studies from our laboratory suggested that fungi isolated from BT agarwood can accelerate agarwood formation (Chen et al., 2017, 2014; Chen, Liu, Liu, Peng, & Wei, 2017). However, the fungal composition and the role of agarwood formation are still unclear.

To date, studies of the fungi in agarwood are mainly based on isolation and culture by tissue separation methods. However, fungal isolation and culture are complicated, time consuming and labour intensive. Notably, limited by the isolation conditions, the isolated species are few, and the uncultured strains are not easy to isolate and thus cannot fully reflect the fungal diversity. Due to their particularity, a comprehensive analysis of the fungi in BT agarwood has not been carried out to date. High-throughput sequencing is the optimal method for application in the studies of fungal ecology and provides new insights into fungal composition and diversity in different environments. Currently, the technology has been widely used to analyse the microbial diversity in soil, deep sea, and food (Liu, Dong, Han, Zheng, & He, 2016; Quijada, Hernández, & Rodríguez-Lázaro, 2020; Zhang et al., 2016).

In this study, the fungal composition and distribution in five layers of BT agarwood were comprehensively revealed. The aim of this work was to evaluate the potential fungi in agarwood after agarwood formation.

2. Materials and methods

2.1. Sample collection and processing

A total of 90 samples from five layers of 18 *A. sinensis* trees from six regions, including Haikou, Wanning, Danzhou, Ledong, Dongguan and Huazhou regions, in Hainan and Guangdong Provinces were collected (Fig. S1). Three trees were collected from each region. A branch sprouting from the side branches of a 10-year-old tree in *A. sinensis* in the different regions was cut off. After three years, agarwood was formed with the partial-stem-breaking method, and woody samples were collected (Fig. 1a and b). Five layers were cut from the BT agarwood (Fig. 1c), i.e., layers A, B, C, D, and E, where A is the decomposing layer, B is the upper agarwood layer, C is the agarwood layer, D is the lower agarwood layer and E is the healthy layer. Layer A had open wound sites; layers B, C, and D had agarwood formation sites; and layer E was a healthy site. The samples taken from *A. sinensis* were approved by the State Forestry Administration. All the procedures related to plants in this study complied with the International Union for Conservation of Nature Policy Statement on Research on Endangered Species and the Convention on Trade in Endangered Species of Wild Fauna and Flora and other national and international norms and legislation.

2.2. DNA extraction, PCR amplification and library construction

Total genomic DNA was directly extracted from each layer of the samples by using the hexadecyltrimethy ammonium bromide (CTAB) method (Yu et al., 2019). DNA concentration and purity were assessed on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL for PCR amplification. The primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) were used for amplification (Jiang et al., 2022). All PCRs were performed in 30 reactions with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs (Beijing) Ltd., Beijing, China), 0.2 μmol/L forward and reverse primers, and approximately 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s and at 72 °C for 5 min. Amplified products were cleaned and purified using the Gene JET Gel Extraction Kit (Beijing Tiangen Biochemical Technology Co., Ltd., Beijing, China). The sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit following the manufacturer's recommendations, and index codes were added. The quality of the library was assessed on a Qubit 2.0 Fluorometer (Thermo Scientific, Beijing, China) and Agilent Bioanalyzer 2100 System. Subsequently, the library was sequenced on an Illumina HiSeq 2500, and 250 bp paired-end reads were generated at Novogene Biotech Co., Ltd. (Beijing, China) (Guo et al., 2016).

2.3. Bioinformatics analysis

Paired-end reads from the original DNA fragments were merged using FLASH (Magoč & Salzberg, 2011), a very fast and accurate analysis tool designed to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end of the same DNA fragment. The splicing sequences were called raw tags. The raw tags were filtered under specific filtering conditions to obtain high-quality clean tags using the QIIME software package (Bokulich et al., 2013; Caporaso et al., 2010). The tags were compared with the reference database (Unite Database; https://unite.ut.ee/) using the UCHIME algorithm (https://www.drive5.com/usearch/manual/uchime_algo.html) to identify chimaera

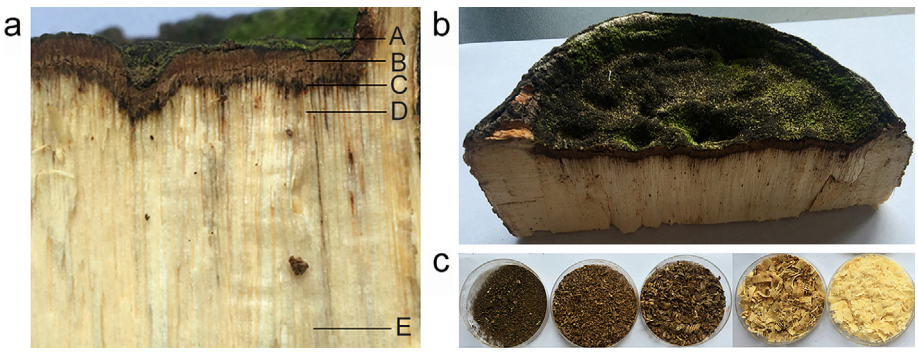

Fig. 1. Representative sample collection. BT agarwood was cut from *A. sinensis* trees (a and b) (A, decomposing layer; B, upper agarwood layer; C, agarwood layer; D, lower agarwood layer; E, healthy layer); BT agarwood was cut into five layers (c).

sequences (Edgar, Haas, Clemente, Quince, & Knight, 2011), and the chimaera sequences were removed (Haas et al., 2011). Then, the effective tags were finally obtained. Sequence analysis was performed by UPARSE software (UPARSE v7.0.1001, http://drive5.com/uparse/) (Edgar, 2013). Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTU). The representative sequence for each OTU was screened for further annotation. For each representative sequence, the Unite Database (Kõljalg et al., 2013) was used on the basis of the Blast algorithm, which was implemented by QIIME software (Version 1.9.1) (https://qiime.org/scripts/assign_taxonomy.html) to annotate taxonomic information. To study the phylogenetic relationship of the different OTUs and the differences in the dominant species in the various samples (groups), we conducted multiple sequence alignments using MUSCLE software (Version 3.8.31; https://www.drive5.com/muscle/) (Edgar, 2004). The OTU abundance was normalized using a standard of sequence numbers corresponding to the sample with the fewest sequences. Subsequent analysis of the alpha and beta diversities was performed on the basis of the output normalized data. Alpha diversity was applied to the analysis of the complexity of species diversity for a sample by using four indices, namely, observed species, Chao 1, Shannon, and Simpson indices. These indices were calculated with QIIME (Version 1.9.1) and displayed with R software (version 2.15.3). R software (version 2.15.3) was used to analyse beta diversity index differences between groups, and a *t*-test was used. LEfSe software was used for LEfSe analysis, and the screening value of the LDA score was set as 4 by default. For species with significant differences between groups, a *t*-test was performed by R software, and a graph was generated.

2.4. Statistical analysis

Anosim (analysis of similarities) is mainly used to analyse the similarity between data groups, which is usually expressed by the *R*-value and permutation test. *R*-values are between −1 and 1, and if *R*-value > 0, it indicates a significant difference between groups. If $R = 0$, it indicates that there is no difference between groups, and if *R*-value < 0, it indicates that there is a significant difference within groups. In addition, the permutation test is a commonly used test method. A *P* test indicated the reliability of the statistical analysis, and $P < 0.05$ indicated statistical significance.

A *t*-test was used to find the species difference between layers at different taxonomic levels (phylum, class, family, genus, species); if $P \leq 0.05$, the species were considered significantly different.

3. Results

3.1. Diversity and richness analysis of fungal composition in five layers of BT agarwood from A. sinensis

A total of 7 850 040 effective tags were obtained. Alpha-diversity analysis indices were used to analyse the diversity, richness and coverage of fungal composition in five layers (Table 1). Goods coverage was over 99.6%, which showed that the sampling depth satisfied the analysis requirements. A high Shannon value indicated high fungal microbiome diversity in each sample. A high Chao 1 value indicated high fungal microbiome richness in each sample. Shannon's diversity index showed that the fungal diversity from high to low was in the healthy layer (E) (Shannon = 4.352), followed by the decomposing layer (A) (Shannon = 3.876), upper agarwood layer (B) (Shannon = 3.187), agarwood layer (C) (Shannon = 3.114), and lower agarwood layer (D) (Shannon = 3.101). The results showed that fungal diversity in the agarwood layers was significantly lower than that in the decomposing and normal layers (Fig. 2A). The results indicated that fungal diversity in healthy wood changed during the formation of agarwood under the action of open wounds. In other words, fungal diversity was significantly reduced during agarwood formation (agarwood sites). The Chao 1 richness index showed that the fungal richness from high to low was in the decomposing layer (A) (Chao 1 = 622.030), followed by the healthy layer (E) (Chao 1 = 576), agarwood layer (C) (Chao 1 = 465.755), lower agarwood layer (D) (Chao 1 = 453.020) and upper agarwood layer (B) (Chao 1 = 423.045). The greatest fungal richness was found in the decomposing layer (open wound sites). This was followed by the healthy layer (E), but the fungal richness was significantly reduced in the agarwood formation sites (Fig. 2B). The Chao 1 index indicated that the richness of fungi in the upper agarwood layer, agarwood layer and lower agarwood layer (agarwood formation sites) was significantly lower than that in the decomposing and healthy layers. In other words, the fungal population changed after agarwood formation, and the richness of the fungal composition obviously decreased. Combined analyses of fungal diversity and richness showed that fungal composition in the agarwood formation sites were significantly lower than those in the decomposing and healthy layers. That is, fungal diversity and richness were significantly reduced from open wound sites to agarwood formation sites.

3.2. Composition of fungal in five layers of BT agarwood in A. sinensis

At the phylum level, the top 10 fungal taxa in all five layers were Ascomycota, Basidiomycota, Glomeromycota, Mortierellomycota, Chytridiomycota, Rozellomycota, Mucoromycota, Kickxellomycota, Zoopagomycota, and Entorrhizomycota (Fig. 3A). The dominant phylum was Ascomycota, with a relative abundance of 50.12%–61.44%, followed by Basidiomycota, with a relative abundance of 10.49%–20.39% (Table S1). At the class level, the top 10 fungal taxa in all layers were Dothideomycetes, Agaricomycetes, Sordariomycetes, Eurotiomycetes, Lecanoromycetes, Leotiomycetes, Saccharomycetes, Agaricostilbomycetes, Tremellomycetes and Orbiliomycetes (Fig. 3B). Dothideomycetes, Agaricomycetes and Sordariomycetes were dominant at the class level, accounting for 26.21%–33.88%, 8.40%–17.66%, and 18.41%–24.11%, respectively, of the total abundance (Table S2). At the order level, the top 10 fungal taxa in all layers were Trechisporales, Pleosporales, Togniniales, Chaetosphaeriales, Hypocreales, Polyporales, Sordariales, Agaricales, Eurotiales and Botryosphaeriales (Fig. 3C). The dominant order was Pleosporales, with a relative abundance of 19.06%–26.46%, followed by Trechisporales, with a relative abundance of 5.13%–12.45% (Table S3). At the genus level, the top 10 fungal genera in all layers were *Lignosphaeria*, *Phaeoacremonium*, *Pyrigemmula*, *Medicopsis*, *Hermatomyces*, *Perenniporia*, *Lophiostoma*, *Talaromyces*, *Alternaria*, and *Mycothermus* (Fig. 3D). *Lignosphaeria, Phaeoacremonium* and *Hermatomyces* were dominant at the genus level, with relative abundances of 6.25%–7.64%, 1.95%–9.05% and 1.5–5.4%, respectively (Table S4).

3.3. Difference in community structure between five layers from BT agarwood in A. sinensis

The differences in the community structures of layers A and B, A and C, A and D, B and C, B and D, and C and D were not obvious. In contrast, the differences in the community structures between layers A and E (*R*-value = 0.249, $P = 0.001$), B and E (*R*-value = 0.272, $P = 0.001$), C and E (*R*-value = 0.251, $P = 0.001$), and D and E (*R*-value = 0.168, $P = 0.001$) were obviously significant (Fig. 4). The results showed that the community structure of the open wound sites and agarwood formation sites obviously differed from that of the healthy sites. In other words, the fungal community struc-

Table 1
Overview of diversity and richness indices of fungi in BT agarwood from *A. sinensis*.

Five layers	Effective tags	Shannon	Chao1	Goods_coverage
A	1 578 228	3.876	622.030	0.996
B	1 551 587	3.187	423.045	0.997
C	1 626 624	3.114	465.755	0.997
D	1 542 805	3.101	453.020	0.997
E	1 550 796	4.352	576.000	0.997
Total	7 850 040			

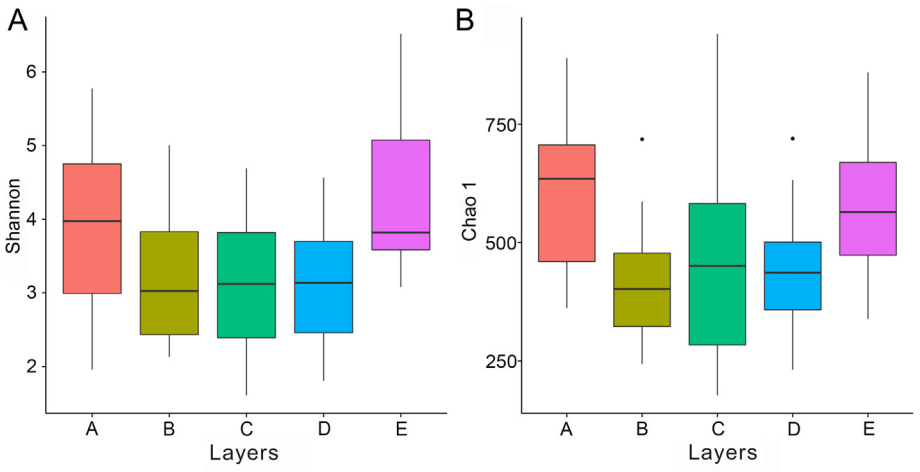

Fig. 2. Diversity and richness indices of fungi in five layers of BT agarwood from *A. sinensis*. Each colour represents a layer. (A) Shannon index for fungal diversity; (B) Chao 1 index for fungal richness; A, B, C, D, and E represent the decomposing layer, upper agarwood layer, agarwood layer, lower agarwood layer, and healthy layer, respectively. The same representations are used in the other Figures.

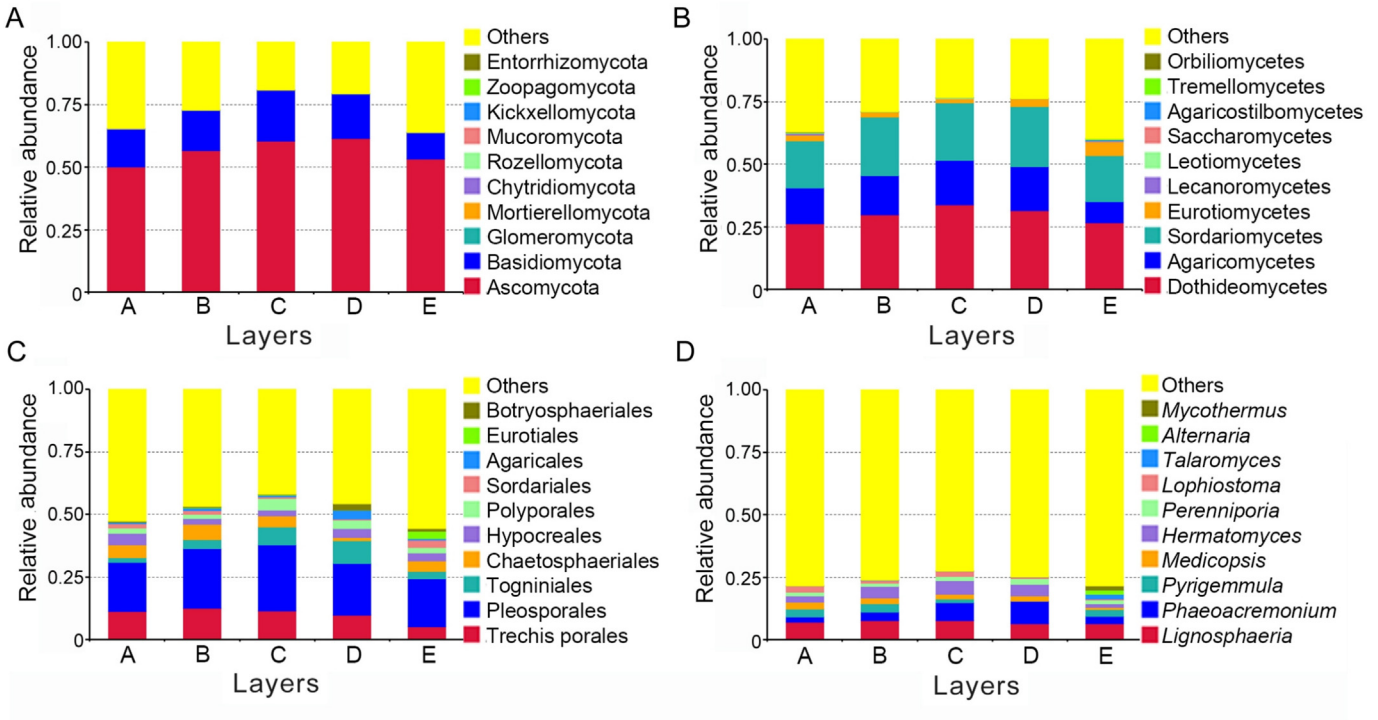

Fig. 3. Relative abundance of fungi at phylum (A), class (B), order (C) and genus (D) levels in five layers of BT agarwood from *A. sinensis*. Each colour represents the percentage of the fungi in the total effective tags of each layer.

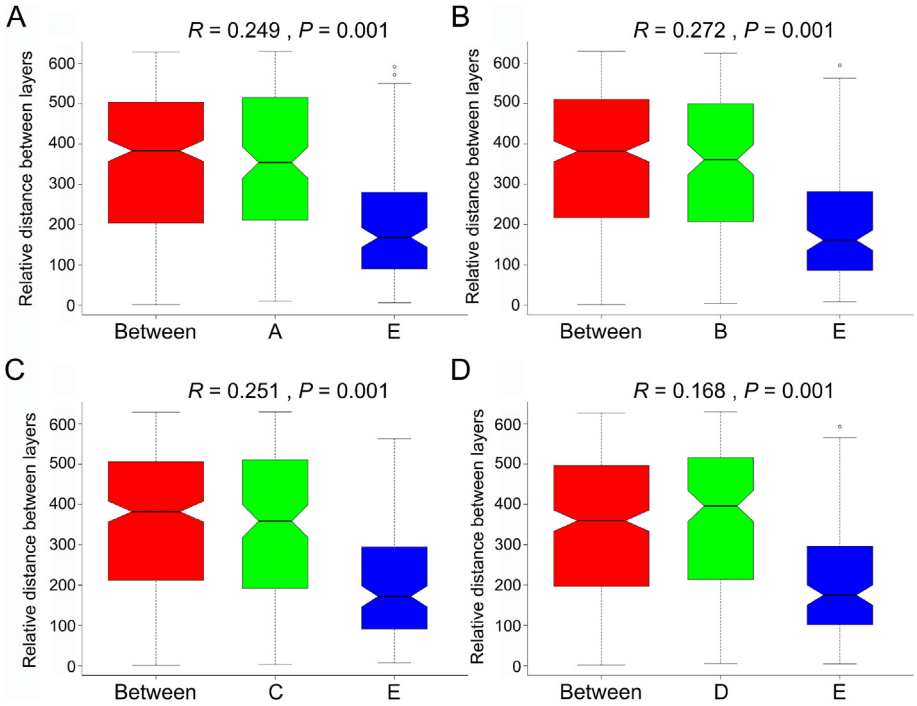

Fig. 4. ANOSIM of fungal differences between two layers of BT agarwood from *A. sinensis*. (A) A and E; (B) B and E; (C) C and E; (D) D and E.

ture showed significant changes after agarwood formation under the action of open wounds.

3.4. Species differences between five layers from BT agarwood in A. sinensis

The results of *t*-tests showed that, the significantly different genera between the A and E layers were *Lasiodiplodia*, *Strigula* and *Aspergillus*, and the abundance of *Lasiodiplodia*, *Strigula* and *Aspergillus* in the E layer was greater than that in the A layer. The significantly different genera between layers B and E were *Cyphellophora*, *Strigula* and *Aspergillus*, and the abundance of *Cyphellophora*, *Strigula* and *Aspergillus* in layer E was greater than that in layer B. The significantly different genera between layers C and E were *Lasiodiplodia*, *Strigula*, *Aspergillus*, *Cyphellophora*, and *Acremonium*, and the abundances of *Lasiodiplodia*, *Strigula*, *Aspergillus*, *Cyphellophora*, and *Acremonium* were higher in the E layer than in the C layer. The significantly different genera between layers D and E were *Strigula*, *Aspergillus*, *Cyphellophora*, *Acremonium*, and *Cladophlaphora*. The abundance of *Strigula*, *Aspergillus*, *Cyphellophora*, *Acremonium*, and *Cladophlaphora* in the E layer was greater than that in the D layer (Fig. 5). The results showed that the abundance of different species in the E layer (healthy sites) more than that in the A, B, C, and D layers (open wound sites and agarwood formation sites). The genus *Strigula* was significantly reduced in the A layer (0.15%), B layer (0.015%), C layer (0.027%), and D layer (0.023%) compared to the E layer (1.22%), which showed that agarwood formation led to the abundance of *Strigula* being significantly reduced in the agarwood formation sites. Notably, the genus *Aspergillus* was more in the E layer (healthy sites) (0.5%) than in the A layer (0.022%), B layer (0.012%), C layer (0.01%), and D layers (0.013%), indicating that agarwood formation led to the abundance of *Aspergillus* being significantly reduced in the open wound sites and agarwood formation sites under the action of open wounds. It is possible that the formation of agarwood inhibits the growth of the genus *Aspergillus*.

4. Discussion

The formation of agarwood is very special. Agarwood is formed as a defence reaction of trees either physically or chemically when they are exposed to biotic and abiotic stresses (Al-Hindi et al., 2018). In this study, the results clearly showed that the diversity and abundance of fungi in the agarwood sites were significantly lower than those in the decomposing and healthy layers. The results indicated that the richness and diversity of fungi in agarwood were significantly reduced. The results revealed that agarwood formation significantly affected the fungal community in BT agarwood in *A. sinensis*. Agarwood formation may defend against fungal stresses. In addition, agarwood may contain inhibitory activities that inhibit the growth of fungi. Lei (2015) reported that four compounds isolated from agarwood had inhibitory activities against *Staphylococcus aureus*; five compounds had better inhibitory activities against anti-methicillin-resistant *S. aureus* than others. It was reported that the essential oils of agarwood had better inhibitory activities against *Bacillus subtilis* and *S. aureus* (Chen et al., 2011). In addition, the highest fungal richness was found in the decomposing layer (open wound sites) after cutting but decreased significantly after agarwood formation, perhaps showing that the formation of agarwood may block fungal growth in the external wound.

The fungal community structure in the decomposing sites and agarwood formation sites obviously differed from that in the healthy layer. This result indicated that the fungal community structure underwent significant changes after agarwood formation under the action of open wounds. The abundance of the genus *Aspergillus* in agarwood formation sites decreased significantly, indicating that agarwood may contain potential substances to inhibit the growth of *Aspergillus*. The genus *Aspergillus* is a widely distributed organism on earth that displays a variety of lifestyles, ranging from saprobic to pathogenic on plant diseases (Pennerman, Yin, Glenn, & Bennett, 2020). *Aspergillus* always produces mycotoxins, which are secondary metabolites that pose seri-

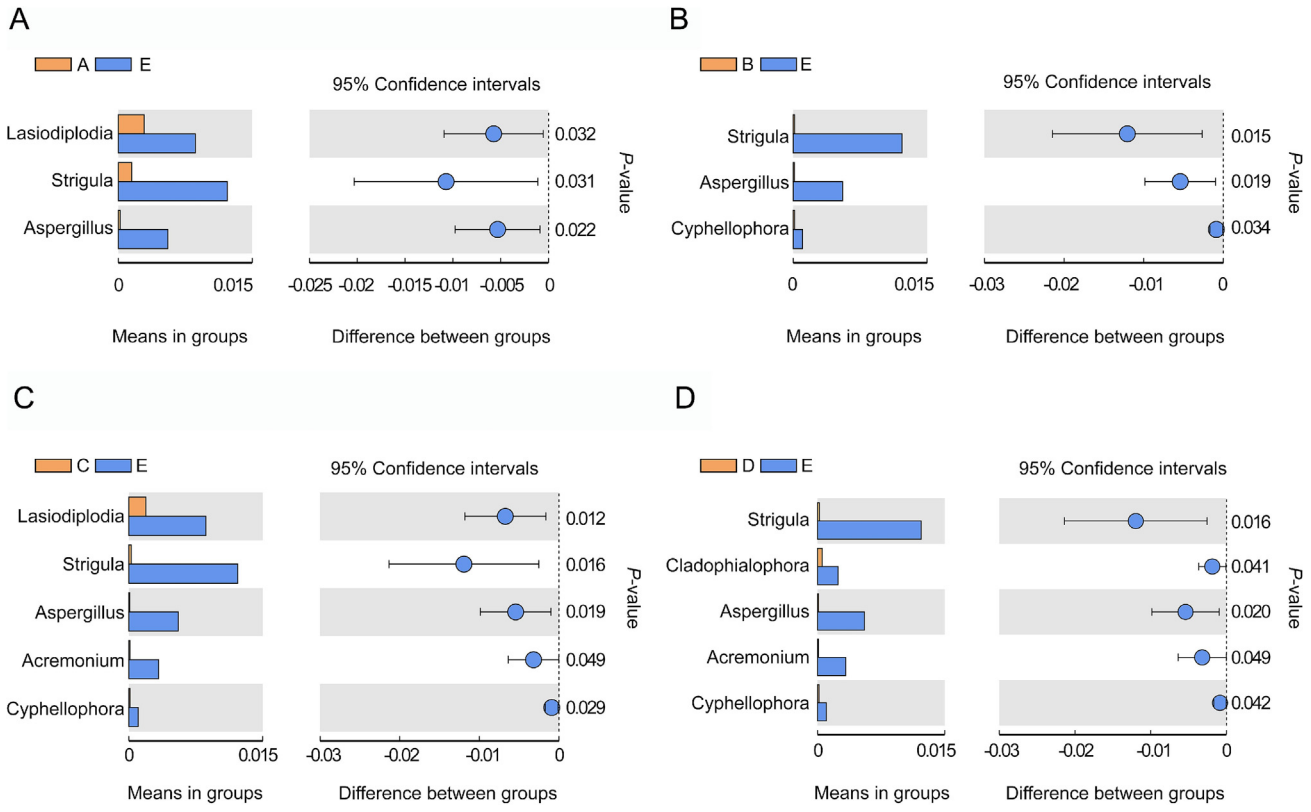

Fig. 5. Fungal differences between two layers by *t*-test analysis. A and E (A), B and E (B), C and E (C), D and E (D).

ous threats to human and animal health (Gizachew, Chang, Szonyi, Torre, & Ting, 2019; Xing et al., 2017). Agarwood has the potential to inhibit *Aspergillus* if it contains certain substances.

A total of 7 850 040 effective tags were obtained in the BT agarwood by high-throughput sequencing. Ascomycota was the most abundant fungus at the phylum level in the agarwood, followed by Basidiomycota. Dothideomycetes, Agaricomycetes and Sordariomycetes were dominant at the class level. *Lignosphaeria*, *Phaeoacremonium* and *Hermatomyces* were dominant at the genus level. In our previous study, 110 strains were isolated from *A. sinensis* by the tissue separation method. Ascomycota is the most prevailing fungus at the phylum level, Dothideomycetes was identified as the major class of agarwood, and *L. theobromae* was the most frequently isolated species from the different layers of *A. sinensis* (Chen et al., 2017). The main fungal taxa that were analysed are basically consistent with those in this paper. The fungi isolated from *A. sinensis* by tissue separation were defective because 99.9% of the fungi could not be isolated and cultured manually. However, high-throughput sequencing revealed a large quantity of fungi, including uncultured fungi. This study is the most comprehensive analysis of fungi in BT agarwood by high-throughput sequencing.

5. Conclusion

The fungal composition and distribution in five layers of BT agarwood were comprehensively revealed. The fungal community structure in the open wound sites and agarwood formation sites obviously differed from that in the healthy sites. The fungal diversity and richness were significantly reduced in the agarwood formation sites. In addition, agarwood from agarwood formation sites has potential substances to inhibit *Aspergillus*, which provides valuable information for the control of the genus *Aspergillus*.

CRediT authorship contribution statement

Xuyu Chen: Writing – original draft. **Yun Yang:** Writing – review & editing. **Yangyang Liu:** Writing – review & editing. **Chun Sui**: Writing – review & editing, Supervision. **Jianhe Wei:** Writing – review & editing, Conceptualization, Project administration.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.chmed.2025.02.001.

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