1   **Composition variation of agarwood-associated microbial communities from**

2   ***Aquilaria sinensis***

3   **Qigui Mo [1], Chenyang Fan [1], Gao Zhou [1], Huiying Fu [1], Youwei Wang [1,2] ***

4   **Running Head: Endophytic bacteria and fungi of agarwood**

5   **ABSTRACT**   Agarwood, derived from *Aquilaria sinensis* and *Aquilaria malaccensis,* is of medicinal

6   and ecological value and religious importance as incense. The existing imbalance between short supply

7   and increasing demand of this product remains to be solved. Thus, the biologically artificial agarwood-

8   inducing methods commonly called whole-tree agarwood-inducing techniques (agar-wit) have been

9   established to dramatically improve agarwood yield within a short period. However, several studies

10   reported a lower content of ethanol-soluble extractive in the agar-wit agarwood than in the natural

11   agarwood. To further understand the role of microorganisms in agarwood formation, we investigated

12   and contrasted the endophytic bacteria and fungi between different types of agarwood from *A. sinensis*

13   through high-throughput sequencing. Results showed that the same dominant phyla of bacteria

14   consisting of *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* were shared by the natural agarwood

15   and agar-wit agarwood. Meanwhile, *Ascomycota* and *Basidiomycota* constituted the similar dominant

16   fungal phyla of these two kinds of agarwood. However, the principal microbial communities at the

17   genus or order level evidently varied from natural agarwood to agar-wit agarwood. Moreover, the

18   bacterial communities are closely connected with terpenoid and carbohydrate metabolism, which

19   indicated that the bacterial communities also play a vital role in agarwood formation. In conclusion, the

20   higher concentrated abundance of the dominant microbial communities in agar-wit agarwood than in

21   natural agarwood may promote agarwood formation, however, the low evenness of microbial

22   communities also lowers the content of ethanol-soluble extractive.

23   **KEYWORDS** natural agarwood, agar-wit agarwood, high-throughput sequencing, bacterial diversity,

24   fungal diversity

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25   **IMPORTANCE** Agarwood has become an indispensable product in modern life because of medicinal

26   value, ecological and religious importance as incense. Nevertheless, the enormous demand for

27   agarwood markedly exceeds the supply because of the dramatically declining population of genus

28   *Aquilaria*. Agarwood formation occurring slowly and infrequently in a natural environment, so various

29   artificial techniques were developed to promote the formation of agarwood, such as the physical

30   methods and chemical methods. However, these techniques still are insufficient to compensate for the

31   agarwood shortage. In this case, a novel biological method called the whole-tree agarwood-inducing

32   technique (Agar-wit) induces agarwood production. However, several studies have shown that

33   agarwood harvested from biological technology contains lower content of ethanol-soluble extractive

34   compared with natural agarwood. So to further expose and understand the endophytic bacteria and

35   fungi in agarwood formation is important for the improvement of biological method.

36   **INTRODUCTION**

37   *Aquilaria sinensis* (Lour.) Gilg (*Thymelaeaceae*) is mainly distributed in the provinces of Hainan,

38   Guangdong, Fujian, and Yunnan in China (1, 2), and *Aquilaria malaccensis* Lam is native to Malaysia

39   (3). These plants are sources of agarwood, which is the dark resinous wood harvested from the tree

40   (China Pharmacopoeia 2015, first section). Agarwood is highly prized for its aphrodisiac, sedative,

41   cardiotonic, and carminative effects and its ability to relieve gastric problems, coughs, rheumatism, and

42   fever (4). Agarwood (Chen Xiang in China), used as a traditional Chinese medicine to dispel damp

43   toxin, was first documented in Mingyi Bielu in the Wei and Jin Dynasties. Traditional use was

44   complementally recorded in Bencao Gangmu in the Ming Dynasties. Recently, many chemical

45   ingredients of agarwood have revealed significant anticancer (5) and anti-inflammatory activities (6).

46   Moreover, agarwood has been used as incense for centuries in Buddhist, Hindi, and Islamic ceremonies

47   (4). DingWei, a famous writer in the Song Dynasty, provided crucial contributions to the development

48   of agarwood as incense. In Tianxiang Zhuan, he was the first to classify agarwood into four grades and

49   12 shape categories depending on the fragrance, appearance, and formation. In a word, agarwood has

50   become an indispensable product in modern life.

51   Nevertheless, the enormous demand for agarwood markedly exceeds its supply because of the

52   dramatically declining population of the genus *Aquilaria* caused by illegal overexploitation (7). This

53   genus has been listed in Appendix II of the Convention on International Trade in Endangered Species

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54 of Wild Fauna and Flora since 2004 (1). The factors mentioned above explain the price inflation of

55 agarwood products to US$ 100,000/kg for superior, pure material (4). Natural agarwood forms around

56 trunk wounds, particularly external wounds caused by lightning strikes, animal attack, which easily

57 induce the wounds to infect with microorganisms (8). As agarwood formation occurs slowly and

58 infrequently in the natural environment, various artificial techniques have been developed to promote

59 agarwood formation. Such techniques include physical methods where tree trunks and branches are

60 wounded using axes, fire, knives, or nails (9) and chemical methods where the wounds are intentionally

61 treated with some chemical reagents initially (e.g., inorganic salt, acids, and phytohormone) (10).

62 However, these techniques are insufficient to compensate for the agarwood shortage. In this case, a

63 novel biological method called the whole-tree agarwood-inducing technique (agar-wit) induces

64 agarwood production by inoculating certain elicitors into the xylem part of *Aquilaria* trees through

65 simple transfusion sets (12). The prepared inducer is then transported throughout the whole tree

66 gradually by transpiration force and initiates a series of defensive responses to produce resin (12). A

67 remarkable biological inoculation is the fungal inoculum, which is mainly fermentation liquid of fungi,

68 such as *Menanotus flavolives* (13), *Botryosphaeria dothidea* (14), and *Lasiodiplodia theobromae* (12).

69 As reported, such inoculum is capable of stimulating agarwood formation. Given the fungal mobility

70 and plant transpiration, agarwood may form on the trunk and branches of the entire tree, causing a far

71 higher production of agarwood at a lower cost of labor and time than that induced by previous artificial

72 techniques. However, several studies have shown that agarwood harvested from biological technology

73 contains lower content of ethanol-soluble extractive compared with natural agarwood (15-17). Zou (18)

74 considered the possible role of endophytic bacteria in agarwood formation, but failed to determine the

75 bacterial community structure by PCR–DGGE methods. Thus, the bacterial and fungal communities of

76 agarwood from *A. sinensis* must be investigated to refine the current agar-wit technique.

77 In this study, the major bacterial and fungal communities of natural agarwood and agar-wit agarwood

78 were investigated using high-throughput sequencing without the prepared isolation and cultures. The

79 variation of dominant microbial communities between these two agarwood types was analyzed.

80 Functional profiles were also used to investigate the role of bacterial communities in the metabolic

81 activity of agarwood formation. Finally, the relationship between the variation of dominant microbial

82 communities and agarwood types was characterized.

83 **RESULTS**

84   General analyses. After data filtration, a total of 64474 and 37850 high-quality sequences of all

85   samples were obtained for the fungal primer SSU0817F/1196R and bacterial primers 799F/1392R and

86   799F/1193R, respectively. Then, these effective sequences were clustered to OTUs with 97% similarity,

87   which is a common parameter for describing microbial community structure (19, 20). After the

88   chimeric, mitochondrial, and chloroplast sequences were removed, the OTU table generated 30 OTUs

89   and 185 OTUs for fungal and bacterial communities, respectively. These OTU tables were used for

90   downstream analyses.

91   First, rarefaction curves were applied to evaluate and compare data from the current study (Fig. S1).

92   With increasing read number, the curves tended to stabilize and be saturated for both bacterial and

93   fungal taxonomies; this finding suggested that the sequenced numbers were adequate to reflect

94   the vast majority of microbial types, and the structures of bacterial and fungal communities associated

95   with different agarwood samples could be reasonably characterized by a sufficient saturated OTU

96   number (21).

97   **Bacterial and fungal diversity analyses.** Chao and Shannon indices were related to microbial

98   communities' richness and diversity and are usually used to preliminarily compare the different types

99   of samples (22-24). In our study, the Chao and Shannon indices of bacteria (Fig. 1a and 1b) in natural

100 agarwood samples were higher than those in agar-wit samples, and the same trend was found in fungal

101 communities (Fig. 1c and 1d). These results indicated that the microbial types in natural agarwood,

102 including bacteria and fungi, were more abundant and complicated than those in the agar-wit samples.

103 In both natural agarwood and agar-wit samples, the values of Chao and Shannon indices of bacterial

104 types (Fig. 1a and 1b) were significantly higher than those of fungal types (Fig. 1c and 1d), which

105 suggested that the bacterial communities showed higher abundance than the fungal communities in

106 agarwood samples from two different production methods.

107 **Cross-OTU comparisons in natural agarwood and agar-wit samples.** To determine the relation

108 between these two kinds of samples, we conducted cross-OTU comparison by using Venn diagrams

109 (25). The overlaps were present in two different types of microorganisms between natural and agar-wit

110 agarwood (Fig. 2a and 2b). The bacterial and fungal OTUs enriched in the natural agarwood

111 successfully colonized the agar-wit agarwood, as 77 out of 185 OTUs of bacteria included in natural

112 agarwood were also enriched in the agar-wit agarwood (Fig. 2a), and the fungal OTUs in the agar-wit

113 agarwood were almost completely replicated from natural agarwood (Fig. 2b). These results indicated

114   that the bacterial species in agarwood obtained from whole-tree agarwood-induction technology were

115   stabilized and only slightly varied relative to that in natural agarwood.

116   A total of 77 and 17 OTUs were clustered to the common region, which were all shared by two

117   different agarwood samples for bacterial and fungal types, respectively (Fig. 2c and 2d). A total of 77

118   OTUs for bacteria mainly consisted of *Proteobacteria* and *Actinobacteria* (Fig. 3a), and 17 OTUs for

119   fungi were mainly composed of *Ascomycota* (Fig. 3b). These same OTUs were enriched in two

120   different agarwood samples with different abundances. The majority of *Proteobacteria* bacterial OTUs

121   were allocated to *Burkholderiaceae* (15.09%) and *Sphingomonadaceae* (13.47%), whereas most of the

122   *Actinobacteria* bacterial OTUs corresponded to *Microbacteriaceae* (9.18%) and *Solirubrobacterales*

123   (9.14%). The *Ascomycota* fungal OTUs contained *Coniochaetaceae* (29.92%) and *Sordariomycetes*

124   (29.79%, OTUs 36 and 28). These results indicated that the shared microbial communities by natural

125   agarwood and agar-wit agarwood were concentrated on several types.

126   **Microbial community composition in natural agarwood and agar-wit agarwood.** In this study, we

127   investigated the bacteria at different taxonomic levels, and taxonomies with < 1% abundance were

128   defined as others (Fig. 3a and 3c). The dominant phyla consisted of *Proteobacteria*, *Actinobacteria*,

129   and *Acidobacteria*, which constituted the main sections of the bacterial community structure of natural

130   agarwood and agar-wit agarwood (Fig. 3a). The agar-wit technology distinctly caused an increasing

131   ratio of *Proteobacteria* in the agar-wit agarwood from 47.87% to 64.04% compared with natural

132   agarwood. Inversely, the relative abundance of *Actinobacteria* decreased from 44.08% of natural

133   agarwood to 30.06% of agar-wit agarwood and showed a reverse trend (Fig. 3a). Similar to

134   *Arabidopsis* (26) and rice (25), an increased pool of *Proteobacteria* was found in the bacterial

135   communities. However, the difference was that the bacterial community composition was simpler in

136   agarwood, and the three bacterial phyla above achieved a total relative abundance of more than 97.60%.

137   The simpler bacterial community composition of agarwood might be due to the lack of soil

138   microorganisms that could influence each other directly compared with *Arabidopsis* and rice. More

139   detailed variation in relative phylum abundance between two different samples could be embodied by a

140   more specific order level (Fig. 3c). Compared with natural agarwood, the substantially increasing ratio

141   of *Burkholderiales* and *Sphingomonadales* and the sharply decreasing ratio of *Solirubrobacterales* in

142   agar-wit agarwood samples were the main reasons for the change in the phyla of *Proteobacteria* and

143   *Actinobacteria* (Fig. 3c). Interestingly, *Xanthomonadales*, which belongs to the phylum *Proteobacteria*,

144   sharply decreased in agar-wit agarwood than in natural agarwood, which was not observed with the

145   phylum *Proteobacteria*. Similarly, the variation tendency of *Micrococcales* was vastly increasing and

146   thus opposite to that of phylum *Actinobacteria* (Fig. 3c). The variation in relative abundance of

147   *Xanthomonadales* and *Micrococcales* may be due to the cooperation and competition among the

148   different microbial types, which have been thoroughly investigated and utilized (27, 28). More details

149   were showed in Table S1. 65.95% OTUs were assigned to genus or species.

150   At present, fungi are considered as the most important microbial factor promoting agarwood

151   formation. Therefore, studies on fungal communities existing in the resinous region of *A. sinensis*

152   emerged in succession over the years (14, 18, 29, 30). In our study, high-throughput sequencing

153   without the prepared isolation and cultures was applied to characterize the fungal community

154   composition in natural agarwood and agar-wit agarwood. The chloroplast, *Amoebozoa* OTUs, and other

155   not belonged to fungi were filtrated, and then 30 OTUs, which included 1 singleton, were obtained.

156   Based on the results, *Ascomycota* and *Basidiomycota* constituted the main fungal community

157   composition with a total relative abundance of more than 99.89% (Fig. 3a and 3b). Consistent with

158   Premalatha's (30) report, the phylum *Ascomycota* occupied the majority of sequence numbers with

159   78.55% relative abundance in natural agarwood and 91.68% relative abundance in agar-wit agarwood

160   (Fig. 3b). On the contrary, the relative abundance of *Basidiomycota* was 21.34% in natural agarwood,

161   which sharply decreased to 8.32% in agar-wit agarwood (Fig. 3b). At the taxonomic order level, the

162   proportion of phylum *Ascomycota* in agar-wit agarwood indicated a rise relative to natural agarwood

163   owing to the increasing percentage of *Sordariales* and another unclassified order. Actually, these two

164   fungal types on the order level belonged to the same class, *Sordariomycetes* (Fig. 3d). Similar to other

165   bacterial communities, the antagonism could manage the relative abundance of some fungal types. The

166   sharply decreasing *Capnodiales* ratio in agar-wit agarwood was mainly attributed to the lost vivosphere,

167   which was compressed by other dominant fungal types. The final taxons of all fungal OTUs were

168   showed in Table 1. Only 8 OTUs were assignable to genus or species. To supplement this, 10 fungal

169   strains were isolated and identified, and all strains were assigned to genus or species. 6 strains belonged

170   to *Sordariomycetes* (Table S2), which also were the most abundant of microbial types in the culture-

171   independent approach (Fig. S2). *L. theobromae*, a strain usually used in whole-tree agarwood-inducing

172   technique, was isolated from both the natural agarwood and agar-wit agarwood.

173   **Comparative analyses between natural agarwood and agar-wit agarwood.** In the heatmap of

174   bacterial and fungal distributions, we used color intensity to compare the top 50 most abundant genera

175   present in the natural agarwood and agar-wit agarwood (Fig. 4). Lower evenness was found in the

176   bacterial communities of the agar-wit agarwood than in the natural agarwood (Fig. 4a). The bacterial

177   types in the agar-wit agarwood focused on several genus levels, such as *Gryllotalpicola*, *Burkholderia-*

178   *Paraburkholderia*, *Sphingomonas*, and *Acidothermus* (Fig. 4a). A same trend was found in the fungal

179   communities of the natural and agar-wit agarwood (Fig. 4b). In particular, the distribution revealed a

180   highly concentrated relative abundance of *Sordarionmycetes* (Fig. S3).

181   The highly concentrated relative abundance of some microbial communities caused variation in the

182   dominant microbial communities between natural agarwood and agar-wit agarwood (Fig. 5). The main

183   dominant bacterial communities at the genus level contained *Jatrophihabitans*, *Acidibacter*, and an

184   unclassified genus belonging to the family *Elev-16S-1332* in natural agarwood (Fig. 5a and Fig. S4).

185   However, the highly abundant population was changed to *Burkholderia-Paraburkholderia*,

186   *Sphingomonas*, *Gryllotalpicola*, and *Acidothermus* in agar-wit agarwood. Actually, the relative

187   abundance of the bacterial communities at the genus level tended to be thoroughly distributed in natural

188   agarwood, which showed a higher evenness in bacterial communities than that in agar-wit agarwood. A

189   variation in the dominant microbial communities was also found within fungal types on the order level

190   between natural agarwood and agar-wit agarwood (Fig. 5b). The dominant fungal communities

191   changed from *Sordariomycetes*, *Agaricomycetes*, and *Dothideomycetes* in natural agarwood to

192   *Sordariomycetes* in agar-wit agarwood.

193   **Bacterial functional profile.** The PICRUSt tool has been used to predict the functional profile of the

194   16S rRNA gene and achieves a high correspondence with the reference genome across several

195   microbial types (31, 32). This method thoroughly compensates for the weakness of 16S rRNA, which

196   cannot directly provide a functional profile (31). By annotating the Cluster of Orthologous Group

197   (COG) function, we obtained 25 functional classifications, in which general function prediction only

198   and function unknown were the two largest clusters. This result was very similar to a previous study

199   (33). After removing the two above-mentioned functional classifications that tentatively held no

200   connection with agarwood formation, we compared the top 18 most abundant functional classifications

201   (Fig. 6a). As a result, two annotated functions related to agarwood formation, "Carbohydrate transport

202   and metabolism" and "Secondary metabolites biosynthesis, transport and catabolism," shared a high

203   relative abundance in the agarwood samples. These two metabolic activities were more active in agar-

204   wit agarwood than in natural agarwood (Fig. 6a). After annotating the Kyoto Encyclopedia of Genes

205   and Genomes (KEGG) function, we obtained above 52.64% relative abundance of metabolic pathways

206   (Fig. 6b). Terpenoid and polyketide metabolism is an important biosynthetic pathway of sesquiterpenes

207   in this study, and besides, carbohydrate metabolism also showed a prominent abundance in two types

208   of agarwood (Fig. 6c). Most of the COG and KEGG functions were higher in agar-wit agarwood than

209   in natural agarwood probably because of the variation in the dominant bacterial communities.

DISSCUSSION

211   Agarwood are more and more popular in our daily life as medicine and incense nowadays. Majority of

212   techniques, mainly including physical, chemical, and biological methods have been established to

213   promote agarwood yield (9-11). Expressly, the biological technique, which mainly inoculated fungal

214   fermentation liquid into *A. sinensis* trees, has been considered as the most promising method to obtain a

215   far higher production of agarwood at a lower cost of labor and time (12-14). So previous studies on

216   microbiomes from *A. sinensis* mainly focused on fungal communities across the white wood and

217   resinous region (14, 29, 30). In the present study, we successfully characterized the bacterial and fungal

218   community structure in the natural and agar-wit agarwood through high-throughput sequencing. We

219   also found that bacterial communities were deeply involved in agarwood formation.

220   Actually, bacterial communities are drew less attention compared to fungal communities in

221   agarwood formation. Premalatha and Kalra (30), who proposed that pathogenic fungi are the primary

222   causative factor in agarwood formation, attempted to investigate the impact of endophytic fungi on the

223   resinous and white wood of *A. malaccensis*. Zou (18) considered that endophytic fungi and bacteria

224   were involved in agarwood formation, but failed to reveal the bacterial community structure by PCR–

225   DGGE methods. In this study, we found the higher bacterial communities' richness and diversity in

226   both types of agarwood than fungal communities (Fig. 1). Remarkably, we detected a high bacterial

227   pool from *A. sinensis* by high-throughput sequencing, which was typically ignored in the past.

228   The diversity of microbial communities between the natural and agar-wit agarwood were different in

229   current study. The microbial communities richness and diversity analyses all showed the higher values

230   in the natural agarwood than agar-wit agarwood (Fig. 1). Venn diagrams indicated that the microbial

231   species existing in agarwood obtained from whole-tree agarwood-induction technology were relatively

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232   fixed and only slightly changed relative to that in the natural agarwood (Fig. 2). Although Zou (18)

233   considered that fungal species were significantly abundant in agarwood samples compared with white

234   wood, other sufficient reports shared different ideas. Mohamed (29) found no difference in fungal

235   species between agarwood and white wood. Similarly, Tian et al (14) reported that most of the fungi on

236   the genus level colonized the white wood from resinous sections. Combined with our results, we boldly

237   speculate that the methods to promote agarwood formation, physical, chemical, and microbial infection

238   methods, enhanced the abundance of key microbial species rather than transforming the microbial

239   community structure.

240   Fungal infection technology promoting agarwood formation is becoming increasingly developed, but

241   this method still has a noticeable deficiency. Several studies proved that the content of ethanol-soluble

242   extractive, a crucial indicator in evaluating agarwood product quality in the Chinese Pharmacopeia

243   (2015), was lower in the agarwood product from agar-wit technology than in the natural agarwood

244   samples (15-17). Therefore, the role of bacterial infection in agarwood formation is increasingly being

245   valued. We successfully characterize the bacterial community composition of agarwood products

246   through high-throughput sequencing, an advanced method that allows the investigation of microbial

247   types without the prepared isolation and cultures (25, 34). Although the richness and diversity of

248   microbial communities and cross-OUT comparison analyses showed the difference of microbial

249   communities in the natural and aga-wit agarwood, these two types of samples shared the same

250   dominant phyla for both bacterial and fungal communities (Fig. 3a and 3b). On order level, the

251   abundances of most microbial categories were different, which might cause the different values of

252   microbial communities' richness and diversity.

253   In particular, unlike in most previous studies, the comparative analysis of *A. sinensis* between the

254   white wood and the resinous region was performed to determine the most important microbial

255   communities in agarwood formation (14, 18, 29, 30). In our study, we compared the bacterial and

256   fungal types between the natural agarwood and agar-wit agarwood and found a variation in the

257   dominant microbial communities between these two kinds of agarwood samples. In the cross-OTU

258   comparative analysis, we summarized that the bacterial and fungal species enriched in the natural

259   agarwood successfully colonized the agar-wit agarwood. This result indicated no difference in

260   microbial types between these two kinds of agarwood. In the current study, the primer

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261   SSU0817F/1196R, which showed a fungal-specific characteristic, was employed to accurately

262   characterize the fungal communities without any other false-positive results (35). However, the

263   taxonomic identification from this primer was restricted (35). In our study, most of the fungal

264   communities were characterized on the order level or above (Fig. S3). Actually, next-generation

265   sequencing technology (NGS) have many constrains (36). The fungal DNA may not be recovered from

266   all genotypes in the culture-independent approaches (36). Besides, PCR amplification steps may prefer

267   to the bulk DNA extracts, and hinder the identification of some other genotypes (37). Finally, fungal

268   sequences obtained from the culture-independent approaches are short and variable, which restrict the

269   assignment of fungal sequences to genus or species (38). So the culture-independent methods are

270   usually used to reflect the microbial communities, and the culture-dependent methods are used to

271   identify the microbial taxons. These might be the reasons why no genus or species overlapped between

272   the culture-dependent and culture-independent approaches in this study. Despite all these shortcomings,

273   a lower evenness of fungal communities was found in the agar-wit agarwood than in natural agarwood

274   at the genus level (Fig. 4b). Bacterial communities also showed the lower evenness in agar-wit

275   agarwood and focused on several genus levels (Fig. 4a). The lower evenness and higher concentrated

276   relative abundance of microbial communities resulted in variation of the dominant microbial

277   communities between natural agarwood and agar-wit agarwood (Fig. 5). Given the above results, the

278   highly concentrated relative abundance of the dominant microbial communities in agar-wit agarwood

279   might be a key factor in promoting agarwood formation relative to that in natural agarwood. However,

280   the decreased evenness of microbial communities also caused the lower content of ethanol-soluble

281   extractives.

282   The role of bacterial communities in agarwood formation is unknown, we proved that bacterial

283   communities were deeply involved in agarwood formation in current study. The annotated COG and

284   KEGG functions showed that bacterial communities shared the abundant functions of terpenoid and

285   carbohydrate metabolism in natural and aga-wit agarwood. Sesquiterpenes comprise the main

286   component of agarwood (39). Sesquiterpene synthesis begins with some complex pathways from

287   glycolysis (40, 41). Thus, carbohydrate metabolism with high relative abundance can provide a

288   material for sesquiterpene synthesis in agarwood formation (Fig. 6c). The increased level of terpenoid

289   and carbohydrate metabolism benefits agarwood formation in agar-wit agarwood samples. Several

290   bacteria have been reported to participate in the synthesis of terpenes (42-45). In this study, an

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291   *Mycobacterium* OTU and three *Streptomyces* OTUs were identified (Table S1), which might be the

292   probable contributors for the COG and KEGG functions of terpenoid and carbohydrate metabolism. In

293   this section, the results of the functional profile of the 16S rDNA gene demonstrated that bacterial

294   communities also played a critical role in agarwood formation.

295   In summary, we successfully characterized the bacterial and fungal community composition in natural

296   agarwood and agar-wit agarwood. In these two kinds of agarwood products, the dominant phyla of

297   bacteria consisted of *Proteobacteria*, *Actinobacteria*, and *Acidobacteria*. Meanwhile, the same

298   predominant fungal phyla included *Ascomycota* and *Basidiomycota*. The variation of the dominant

299   microbial communities between natural agarwood and agar-wit agarwood has been described at the

300   taxonomic level of genera or order. The functional profile predicted using the PICRUSt tool indicated

301   that bacterial communities were deeply involved in agarwood formation. We inferred that the highly

302   concentrated relative abundance of the dominant microbial communities in agar-wit agarwood can

303   more effectively promote agarwood formation than that in natural agarwood. However, the low

304   evenness of microbial communities also results in the low content of ethanol-soluble extractive. We

305   believe that this study will make it easier to understand the role of bacterial communities in agarwood

306   formation.

MATERIALS AND METHODS

308   **Agarwood samples.** Two kinds of agarwood samples were collected from mature *A. sinensis* (Lour.)

309   Gilg trees in Haikou, China (E109°27′26.08″, N19°42′20.02″), in June 24, 2017. Natural agarwood

310   samples were cut off at 2–3 cm thickness from the exposed section where agarwood formed naturally.

311   Agar-wit samples were then collected from the bottom of the trunk into which fungal inducers were

312   injected 24 months earlier. All samples were cut with a sterilized knife or saw, placed in clean sealable

313   bags immediately, and transported back to the laboratory under 4 °C. Excess white wood was removed,

314   and the remaining agarwood was surface sterilized with 75% ethanol and finally stored in an ultra-low-

315   temperature freezer (Haier biomedical, Qingdao, China) for endophytic microbial diversity analysis.

316   **DNA extraction.** A series of experiments, including DNA extraction, PCR amplification, and

317   pyrosequencing, were carried out on the agarwood samples by Majorbio Bio-Pharm Technology Co.,

318   Ltd. (Shanghai, China). Briefly, agarwood samples were separately milled into powder under liquid

319   nitrogen. Total DNA was extracted from 0.5 g powdered agarwood in accordance with a previously

320   described CTAB (Cetyltrimethylammonium Ammonium Bromide) method (30). Total DNA was

321   quantified by agarose gel electrophoresis and then used for PCR amplification.

322   **PCR amplification of the V5–V7 region of the fungal 18S and bacterial 16S rRNA genes.** The

323   V5–V7 region of the 18S rRNA fungal-specific primers (SSU0817F: 5′-

324   TTAGCATGGAATAATRRAATAGGA-3′ and 1196R: 5′-TCTGGACCTGGTGAGTTTCC-3′) were

325   designed for endophytic fungal diversity analysis (35). Meanwhile, to accurately assess the endophytic

326   bacterial community, two sets of amplification primers were designed. The first one was 799F/1392R

327   (799F: 5′-AACMGGATTAGATACCCKG-3′ and 1392R: 5′-ACGGGCGGTGTGTRC-3′), and the

328   second was 799F/1193R (799F: 5′-AACMGGATTAGATACCCKG-3′ and 1193R: 5′-

329   ACGTCATCCCCACCTTCC-3′) (26, 45, 46). PCR was performed on ABI GeneAmp® 9700 (Applied

330   Biosystems, Foster City, USA) with the following conditions: 2.5 mM dNTPs, 5 μM forward and

331   reverse primers, and 0.4 μL FastPfu polymerase. The cycling process proceeded as follows: 3 min for

332   95 ℃, 27 cycles at the same temperature for 30 s, annealing at 55 °C for 30 s, 72 °C for 15 s, and then

333   extension at 72 °C for 10 min.

334   **Illumina MiSeq sequencing and data analysis.** PCR amplification products of the V5–V7 region

335   of fungal 18S and bacterial 16S rRNA genes were sequenced by Illumina MiSeq sequencer. The

336   Illumina raw sequences were filtered by QIIME (47). Specific barcodes were set to assign the different

337   agarwood samples (48). Chimeric sequences were checked and removed. The taxonomic levels of the

338   bacteria and fungi in this study depended on the OTUs (Operational taxonomic units) at 97% similarity,

339   which was defined on the basis of trimmed sequences. These OTUs were assigned based on the Silva

340   database (http://www.arb-silva.de). The abundance of each OTU in tables described the level of fungal

341   and bacterial phylotypes in different samples. To determine the most representative microbial

342   community, mitochondrial and chloroplast OTUs, as well as those with < 0.1% abundance in different

343   samples, were filtered manually (49). Meanwhile, OTUs were rarefied to the lowest sequence number.

344   The simplified OTU tables were used for the following analysis. Rarefaction curves, Chao and

345   Shannon indices, Venn diagrams, community barplots at different taxonomic levels, and community

346   heatmap analysis were employed to analyze the microbial community structure of the agarwood

347   samples. Bacterial functional profile was used to compare the gene function at different levels derived

348   from the variational microbial community abundance. The raw sequences of all samples reported in

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349 this study have been submitted to the National Center for Biotechnology Information Short Read

350 Archive (accession no. SRP127004) under BioProject PRJNA422987.

351 **Statistical analysis.** The results were reported as means ± SD. Data were analyzed using IBM SPSS

352 Statistics 20. Statistically significant data were further analyzed using one-way analysis. Significant

353 difference was considered at $p < 0.05$.

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360 **AUTHOR CONTRUBUTIONS**

361 Q.G. Mo completed the experiments via culture-independent methods, interpreted data and developed a

362 manuscript draft. C.Y. Fan and H.Y. Fu completed the experiments via culture-dependent methods. G.

363 Zhou supervised the collection of agarwood samples. Y.W. Wang supervised the whole research and

364 rewrote this manuscript.

365 **CONFLICT OF INTEREST**

366 The authors declare that they have no competing interests.

367 **ETHICAL APPROVAL**

368 This article does not contain any studies with human participants or animals performed by any of the

369 authors.

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552 **Figure captions**

553 **FIG 1** Community richness and community diversity indices at a 97% identity threshold of the natural

554 agarwood (NA) and agar-wit agarwood (Agar-Wit). a and c are the Chao indices of bacterial and fungal

555 communities, respectively. b and d are the Shannon indices of bacterial and fungal communities,

556 respectively. The values are presented as means ± SD.

557 **FIG 2** Venn diagrams depicting the shared or unique OTUs for natural agarwood (NA) and agar-wit

558 agarwood (Agar-Wit). The OTU abundance with < 1% are combined and defined as others. a and b are

559 bacterial and fungal OTUs, respectively. c and d are the main shared OTUs in bacterial and fungal

560 types, respectively.

561 **FIG 3** Bacterial and fungal compositions of natural agarwood (NA) and agar-wit agarwood (Agar-Wit)

562 on the phyla (a, b) and order (c, d) levels. a and c are the bacterial communities, and b and d are the

563 fungal communities. Others include the few sequences with < 1% relative abundance.

564 **FIG 4** Heatmap of the bacterial (a) and fungal (b) distributions of the top 50 most abundant genera

565 presented in the natural agarwood (NA) and agar-wit agarwood (Agar-Wit). The relative abundances of

566 bacterial and fungal genera are indicated by color intensity. In the current study, the total fungal

567 community types of these two samples were 25.

568 **FIG 5** Taxon-level analysis of individual genera or orders altered between the natural agarwood (NA)

569 and agar-wit agarwood (Agar-Wit) by Fisher's exact test. a: Bacterial communities at the genus level; b:

570 fungal communities at the order level. The right graph indicates the altered percentage of species

571 abundance in the confidence interval. [**]$0.001 < p < 0.01$, [***]$p < 0.001$.

572 **FIG 6** Abundance and functional predictions of bacterial communities of the natural agarwood (NA)

573 and agar-wit agarwood (Agar-Wit). a: COG functional predictions of OTUs. b and c: KEGG functional

574 predictions of OTUs by using the PICRUSt tool in category levels 1 and level 2, respectively.

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580 **TABLE 1** The final taxon of all fungal OTUs detected from high-throughput sequencing.

Final Taxon	OTU	Abundance (%)	
		NA	Agar-Wit
Agaricomycetes	OTU31	0.095	8.06
Arthrobotrys oligospora	OTU33	0.855	0.839
Ascomycota	OTU2	10.669	0.008
Ascomycota	OTU19	0.576	0.004
Ascomycota	OTU14	0.052	0
Aspergillus	OTU26	1.217	3.655
Basidiomycota	OTU37	0	0.114
Botryosphaeriaceae	OTU30	5.82	2.18
Candida parapsilosis	OTU38	0	0.004
Capnodiales	OTU4	12.303	0.179
Coniochaetaceae	OTU29	7.235	46.194
Corticiaceae	OTU20	13.399	0
Dothideomycetes sp. CRI7	OTU13	0.133	0
Flammispora bioteca	OTU24	1.977	0
Fungi	OTU11	0.112	0
Graphis scripta	OTU5	0.052	0
Herpotrichiellaceae	OTU34	6.203	2.995
Hydnodontaceae	OTU18	2.889	0
Hypocreales	OTU8	0.176	0
Kurtzmanomyces insolitus	OTU12	2.489	0.11
Lecanoromycetes	OTU3	3.791	0.008
Rhizoctonia solani	OTU22	2.093	0
Sordariales	OTU16	7.677	0.509
Sordariales	OTU27	0.047	0.098
Sordariomycetes	OTU36	3.417	25.446
Sordariomycetes	OTU28	15.063	9.518
Sordariomycetes	OTU32	1.221	0.016
Sordariomycetes	OTU9	0.06	0

Tremellaceae	OTU15	0.064	0
Tremellales	OTU10	0.314	0.061

581   NA: natural agarwood; Agar-Wit: agar-wit agarwood.

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