Soil microbial communities of three major Chinese truffles in Southwest China

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1 **Soil microbial communities of three major Chinese truffles in Southwest China**

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17 One-sentence summary: Soil microbial communities in *T. pseudoexcavatum*, *T. sinoaestivum*, and
18 *T. indicum* produced in Sichuan, China were disclosed by barcoded pyrosequencing to search
19 microbes potentially attributing to the formation of truffle fruit bodies.

20

21 **Abstract:**

22 *Tuber pseudoexcavatum*, *T. sinoaestivum*, and *T. indicum* are the three most important truffles
23 growing in Southeast China while their cultivation is still of low efficiency, with one important
24 reason being the composition and function of bacterial and fungal community from soils around
25 the fruit bodies and ectomycorrhiza of these truffles remained unclear. The aim of this study was
26 to disclose the microbial communities in truffle-producing soils in Huidong County, Sichuan,
27 China, by barcoded pyrosequencing. Approximately 350,000 quality-controlled sequences were
28 obtained and grouped into 14,025 bacterial operational taxonomic units (OTUs) and 4,385 fungal
29 OTUs, which included 29 bacterial and 7 fungal phyla, respectively. Bacterial genus
30 *Acidobacterium* and fungal genus *Modicella*, *Pseudogymnoascus*, and *Mortierella* were
31 significantly more abundant in control than in truffle-producing soils ($P < 0.05$), while the
32 bacterial genus *Sphingomonas* (Alphaproteobacteria) and arbuscular mycorrhizal fungal *Glomus*
33 were significantly enriched in truffle-producing soil than in control ($P < 0.05$), indicating their
34 different roles within truffle grounds. Notably, some non-fungal organisms detected by 18S rDNA
35 pyrosequencing were of high abundance, among which Cercozoa and Ochrophyta were
36 significantly ($P < 0.05$) more abundant in truffle soils than in control, indicating their interactions
37 with truffles.

38 **Key words:** *Tuber. pseudoexcavatum* *Tuber. sinoaestivum* *Tuber. indicum* microbial communities
39 truffle

40

41 Introduction:

42 Truffles are ectomycorrhizal (ECM) fungi belonging to the *Tuber* genus (Ascomycota,
43 Pezizales). Truffles produce hypogeous fruiting bodies that are highly appreciated worldwide as
44 foods or seasoning for their richness of amino acids and the unique aroma and flavor. Due to the
45 low yields, the demand for truffles exceeds their supply, especially for *Tuber melanosporum*
46 (Périgord black truffle), one of the most popular truffles over the world (Mello et al. 2006; Wang
47 et al. 2008; Yun and Hall 2004). The genome sequencing of *T. melanosporum* expanded our
48 understanding of the reproduction of *T. melanosporum* and inspired the biological investigation of
49 other *Tuber* spp. (Martin et al. 2010). *T. indicum*, *T. pseudoexcavatum*, and *T. sinoaestivum*,
50 mainly distributed in Sichuan, Yunnan and Tibet, are the three most important commercial truffle
51 species in southwest China. They serve as a good complement to *T. melanosporum*
52 (Garcia-Montero et al. 2008). Unfortunately, local farmers, motivated by high reward, tend to pick
53 Chinese truffles before they reach maturity, and this causes a serious threat to the sustainable
54 production of truffles in China. At the same time, there is difficulty in the cultivation of *Tuber* spp.
55 and the development of their fruiting body in pure culture has not been achieved successfully so
56 far (Kues and Martin 2011; Rubini et al. 2011; Rubini et al. 2007).

57 Microbial communities are believed to be associated with the maturation of truffle ascocarps
58 and affect their aroma (Barbieri et al. 2005; Barbieri et al. 2007; Splivallo et al. 2015). Some
59 microbes, referred to as rhizosphere “mycorrhization helper bacteria” that promote
60 ectomycorrhizal infection have been reported and received special attention for their potential
61 application in stimulating mycorrhizal root colonization and eventually the truffle production
62 (Garbaye 1994). Ectomycorrhizal fungi biodiversity has been characterized in *T. macrosporum*, *T.*

magnatum, *T. melanosporum* and *T. borchii* production sites (Benucci et al. 2014; Guerin-Laguet et al. 2013; Iotti et al. 2010; Leonardi et al. 2013; Murat et al. 2005; Pruett et al. 2008), but non-ectomycorrhizal fungal communities in truffles soil have not been investigated adequately. To our knowledge, no research on associative microbial communities, especially on both bacterial and fungal communities, that participate in the formation and maturation of Chinese truffles has been reported.

Next generation amplicon sequencing is an effective tool to investigate microbial diversity in mixture samples such as stool and soil (Elahi and Ronaghi 2004; Siqueira et al. 2012). Prokaryotes associated with *T. aestivum* ectomycorrhizae and the surrounding soil were investigated using 454-pyrosequencing, and several uncultivable bacterial genera belonging to *Pseudonocardineae* were identified, indicating their function in modulation of competition with other symbiotic and saprotrophic microbes, facilitation of root penetration and/or accessing mineral nutrients in the soil (Gryndler et al. 2013). However, High-throughput amplicon sequencing has not been used to investigate associative microbial communities of *T. pseudoexcavatum*, *T. indicum* or *T. sinoaestivum*.

In this study, with the aim to disclose microbial communities in soil from truffle grounds and search microbes potentially attributing to the formation of fruit bodies of the three Chinese truffles in Huidong County, Sichuan, China, we analyzed microbial diversity associated with these three truffle species by pyrosequencing of 16S rRNA gene V3-V4 region of bacteria and 18S rRNA gene V4 region of fungi.

Materials and methods

85 Sampling sites

86 Soil samples were collected from *T. pseudoexcavatum*, *T. sinoaestivum* and *T. indicum*
87 grounds at Tangtangzhen, Xueshanxiang, and Xintianxiang in Huidong, respectively
88 (26°22'-26°35' N, 102°34'-103°57' E, Liangshan Yi Autonomous Prefecture, Sichuan, China) in
89 January 2015. Ascocarps were collected in Tangtangzhen and Xintianxiang but not in
90 Xueshanxiang (Supplementary 1). In Huidong, the mean annual temperature is 16.2 °C and the
91 mean annual precipitation is 1,099.7 mm. The sampling sites were mixed forests of *Pinus*
92 *yunnanensis* Franch and *P. armandii* Franch. with sandy loam soil at altitudes from 2,506 m to
93 2,624 m above sea level. The major vegetation type of forests where the samples were collected
94 was *Pinus armandii*, along with a small amount of *Platycarya strobilacea*, *Pyracantha fortuneana*,
95 and sparse patches of herbaceous species.

96 Sampling method and storage

97 Samples were collected in Tangtangzhen (TTZ), Xueshanxiang (XSX) and Xintianxiang (XT)
98 where ascocarps of *T. pseudoexcavatum*, *T. sinoaestivum* and *T. indicum*, respectively, had been
99 found during the last three years. Control samples (TTZ-CK, XSX-CK, and XT-CK) were
100 collected from nearby soils (less than 10 meters apart) where truffles were not present. Three soil
101 samples that were less than 5 m to each other within productive spot were collected. Each sample
102 was composed of 200 g cylindrical soil cores immediately surrounding (< 5 cm) the fruiting point
103 (20 cm in depth and 8 cm in diameter), which was characterized by scanty vegetation. Soil
104 samples and ascocarps were stored in sterile plastic bags less than 3 days at 4 °C during transport
105 to the laboratory. Prior to storage at -80 °C, forest debris was removed from the samples.

106 DNA extraction and amplicon sequencing

107 DNA was extracted from 200 mg soil with E.Z.N.A.TM Soil DNA Kit (OMEGA Bio-Tek,
108 U.S.A) following manufacturer's instructions. Ascocarps collected from Tangtangzhen and
109 Xintianxiang were identified as *T. pseudoexcavatum* and *T. indicum*, respectively, by
110 morphological and molecular identification in Soil and Fertilizer Research Institute, Sichuan
111 Academy of Agricultural Sciences. The concentration and quality of the extracted DNA were
112 determined using a Qubit® 2.0 (Invitrogen, USA). The primers for the bacterial 16S rRNA gene
113 and fungal 18S rRNA gene amplicon sequencing were barcoded as described (Supplementary 2)
114 (Langenheder and Szekely 2011; Sun et al. 2014). PCR reactions were performed in a total
115 volume of 50 µL containing 5 µl of 10× PCR buffer, 0.5 µL dNTPs (10 mM each), 0.5 uM each
116 primer, 2.5 U Platinum Taq (Thermo, USA) and 10 ng of template DNA. The PCR amplification
117 program included an initial denaturation at 94 °C for 3 min, 5 cycles of denaturation at 94 °C for
118 30 s, annealing at 45 °C for 20 s, and extension at 65 °C for 30 s, followed by 20 cycles of
119 denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 30 s, and a
120 final extension at 72 °C for 5 min. For each sample, three technical replicates of PCR reactions
121 were combined and quantified by agarose gel electrophoresis. Before sequencing, the PCR
122 products were normalized in equal concentration to construct the PCR amplicon libraries.
123 Sequencing was carried out on Illumina MiSeq 2000.

124 **Sequence data analysis**

125 The sequence reads were sorted into 6 libraries based on their respective barcodes. To
126 minimize the effects of random sequencing errors, the raw sequence reads were checked for their
127 quality. Prinseq was used to remove low-complexity sequences (Schmieder and Edwards 2011).
128 Chimeras were removed by chimeras.uchime (Edgar et al. 2011). The sequences were grouped

129 into operational taxonomic units (OTUs) using the 97% identity threshold (Edgar 2010). OTUs
 130 were classified using Ribosomal Database Project (RDP) classifier in Mothur and the database
 131 provided by the RDP (<http://rdp.cme.msu.edu/>) (Wang et al. 2007) and Silva high quality
 132 ribosomal RNA databases (<http://www.arb-silva.de/>). Rarefaction curves were used to estimate
 133 coverage. Alpha diversity (the phylogenetic distance whole tree, Chao1 and abundance-based
 134 coverage estimator (ACE) of richness, observed species, and Shannon's diversity index) and beta
 135 diversity (PcoA, UniFrac) were determined using the Mothur software (<http://www.mothur.org/>)
 136 (Yatsunenko et al. 2012; Lozupone and Knight 2005). One-way ANOVA was applied to analyze
 137 differences between samples. Relationships between samples were analyzed using principal
 138 coordinates analysis (PcoA). A Venn diagram with shared and unique OTUs was used to depict
 139 similarities between microbial communities.

140

141 **Results**

142 **Numbers of OTUs**

143 To determine the composition of the bacterial and fungal communities colonizing *T.*
 144 *pseudoexcavatum*, *T. sinoaestivum* and *T. indicum* truffle soils, we carried out 16S and 18S rRNA
 145 pyrosequencing. After trimming, the 16S and 18S sequence reads had a mean length of 400–600
 146 bps and an average quality score of 37. Approximately 350,000 quality controlled sequences were
 147 obtained after removal of chimeras and sequences shorter than 200bp. The sequences were
 148 clustered into 4,385 fungal OTUs and 14,025 bacterial OTUs at the 97% similarity threshold. The
 149 number of bacterial OTUs in *T. pseudoexcavatum* soil (TTZ), *T. sinoaestivum* soil (XSX), and *T.*
 150 *indicum* soil (XT) were 7,062, 6,781 and 5,541, respectively. A total of 1,279 OTUs were shared

151 with all three truffle soils (Fig. 1A). A total of 4,385 fungal OTUs were obtained, with 1,510,
152 2,615 and 1,702 OTUs in TTZ, XSX and XT, respectively. A total of 415 fungal OTUs were
153 shared by all three truffle soils (Fig. 1B).

154 **Taxonomic classification of bacteria**

155 A total of 29 bacterial phyla were identified, out of which 20 were detected in all 18 samples.
156 Proteobacteria (40-59%), Acidobacteria (8-23.5%), unclassified (7-10.9%), Bacteroidetes
157 (1.7-7%), Planctomycetes (4-11.7%), and Chloroflexi (1.8-6.4%) were the most abundant phyla in
158 all soil samples. Proteobacteria were more abundant in truffle soils than in control soils, whereas
159 Acidobacteria were less abundant in truffle soils than in control soils (Fig. 2A).

160 Among the 65 bacterial classes, Alpha-proteobacteria (23%) and Acidobacteria (17.6%)
161 were the most abundant. The abundance of Spartobacteria in Verrucomicrobia phylum varied
162 considerably (ranged from 1.3% to 15.6% in the samples). Acidobacteria and Acidimicrobiia were
163 less abundant in truffle soils than in control (Fig. 2C).

164 At genus level, *Azospira* (3.1-5.7%) was abundant in all samples. *Sphingomonas* (1.7-12.5%),
165 *Escherichia-Shigella* (2.4-5.3%) and *Blastocatella* (4.4-5.4%), within the class
166 Alphaproteobacteria, Gammaproteobacteria, Acidobacteria, respectively, were significantly more
167 abundant in truffle soils than in control soils (ANOVA, $P < 0.05$, Table 1). The *Acidobacterium*
168 (8.3-14.8%) were significantly more abundant in control soils than in truffle soils.

169 **Bacterial community diversity and UniFrac analysis**

170 Chao1 index in XT was significantly higher than that of *T. pseudoexcavatum* and *T.*
171 *sinoaestivum* soil samples, as well as than in all control soils (ANOVA, $P < 0.05$, Table 2).
172 According to the ACE and Shannon indices XT had significantly higher diversity than other

173 samples ($P < 0.05$), in spite of lowest sequence numbers. Coverage index showed that sequence
174 efficiency of XT was higher than other samples.

175 UniFrac analysis was performed to estimate the differences in bacterial communities between
176 the samples (Fig. 3). PcoA 3D plot and weighted sample tree showed that the communities in each
177 truffle soil sample were far from the control soil samples in both PcoA 3D plot and weighted
178 sample tree figures (Fig. 3, Supplementary 3). Furthermore, bacterial communities in *T.*
179 *pseudoexcavatum*-truffle soil (TTZ) were far different from *T. pseudoexcavatum* and *T.*
180 *sinoaestivum* soil samples (Supplementary 3).

181 Fungal taxonomic classification

182 A total of seven fungal phyla were identified, of which five were identified in all 18 samples.
183 Ascomycota (10-33%) and Basidiomycota (3.7-11%) were the dominant phyla in all soil samples.
184 Zygomycota (1.2-37.8%) were significantly more abundant in control than in truffle soils
185 (ANOVA, $P < 0.05$), while Glomeromycota (1.1-10.7%) and Chytridiomycota (0.4-4.8%) were
186 more enriched in truffle soils than in the control (Fig. 2B).

187 A total of 30 fungal classes were identified. Leotiomycetes (2.7-19.2%) and Dothideomycetes
188 (3.0-9.4%) were abundant in all samples. Mucoromycotina (1.2-35.3%) was significantly
189 abundant in control soils than in truffle soils ($P < 0.05$). Glomeromycetes (1.1-10.7%) was more
190 abundant in truffle soils than in control soils (Fig. 2D).

191 At genus level, *Glomus* (0.72-16.1%), belonging to class Glomeromycota, was significantly
192 enriched in three truffle soils as compared with their respective control soil samples (ANOVA, $P <$
193 0.05, Table 3). *Modicella* (0.21-25.3%), *Pseudogymnoascus* (0-17.0%), and *Mortierella*
194 (0.04-37.4%) were significantly enriched in control in comparison with the truffle soil samples (P

195 < 0.05).

196 **Fungal community diversity and UniFrac analysis**

197 The Shannon index of XT was higher than in the other soil samples (ANOVA, $P < 0.05$,
198 Table 2). In XSX ACE, Shannon and Chao1 indices were lower than in other truffle soils.
199 Coverage index showed that sequence efficiency was balanced in our samples for fungal
200 sequences.

201 In both the PcoA 3D plot and weighted UniFrac tree, *T. pseudoexcavatum* control soil
202 (TTZ-CK) was quite close to *T. indicum* control soil (XT-CK), and *T. indicum* truffle soil samples
203 were close to *T. sinoaestivum* truffle soil samples (Fig. 3, Supplementary 3). Fungal communities
204 in each truffle soil sample were different from their corresponding control samples.

205

206 **Discussion**

207 *Tuber* species emit toxic metabolites that can affect the herbaceous cover and roots of host
208 plants in particular Circular zones called 'brûlé' (Streiblova et al. 2012) (Splivallo et al. 2007).
209 Brûlé, where the fruit bodies of *T. melanosporum* usually are collected, is an area devoid of
210 vegetation. The relationship of microbial communities and ectomycorrhizal fungi within brûlé is
211 interactive and complicated. Soil biodiversity can have a positive or negative effect on fungal
212 growth, distribution and fruiting body formation by altering fungal fitness. In addition,
213 communities of ectomycorrhizal fungi, for example *T. aestivum* and *T. melanosporum*, can also
214 alter forest soil biogeochemistry (Mello et al. 2013; Menta et al. 2014). It was also reported that
215 the diversity of soil bacterial communities inside and outside the brûlé was different. Firmicutes,
216 Actinobacteria, and some genera belonging to class Flavobacteriaceae had higher relative

abundances inside the brûlé compared with outside part (Mello et al. 2013). Furthermore, the dynamics of fungal populations seem to be correlated to Brûlé formation (Napoli et al. 2010). The word 'Brûlé' is not used commonly in China, while 'truffle producing ground', the word used in this study instead, represents the small area where Chinese truffles and ectomycorrhizae grow and is characterized by scanty vegetation. To understand the factors associating with truffle growing in this specific area, soil properties were investigated by our group before. It was found that some parameters were different between truffle soils and control. For example, the most suitable pH values for Chinese truffle growing ranged from 5.53 to 6.64, and higher than in control. So did total nitrogen. However, the values of silt in truffle soils were lower than in control. Additionally, most properties in truffle soils were relatively similar to each other, while total nitrogen etc. varied within truffle soils. Based on this, the aim of the current study was to investigate microbial community that may make contribution to truffle growing.

Homogeneity and heterogeneity of microbial ecological habitat in Chinese truffle and other Tuber species grounds

In our study, bacterial phylum Proteobacteria had greater representation in the truffle fruiting point soils compared with control soils, whereas the bacterial phylum Acidobacteria had higher relative abundances within control soils compared with truffle soils. Bacterial communities from soils adhering ECMs and ascocarps of *T. melanosporum* were dominated by proteobacteria, bacteroidetes and Actinobacteria (Antony-Babu et al. 2014), while Actinobacteria, Proteobacteria and Acidobacteria were dominant in both two types of soils (Gryndler et al. 2013). It was reported that Acidobacteria, Actinobacteria and Planctomycetes were of low abundance or even absent in the ascocarp but highly abundant in the soil (Antony-Babu et al. 2014), while in our study,

239 Acidobacteria were less abundant in all truffle soils than in control soil samples.

240 For fungal phylum, Glomeromycota and Chytridiomycota were significantly more abundant
241 in truffle soils compared with control soils, while Zygomycota had higher abundance in control
242 compared with truffle soils. The number of fungal phylum Zygomycota increased inside the *T.*
243 *melanosporum* brûlé (from 5 to 32%) while Basidiomycota decreased (from 61 to 23%) in the
244 investigation by Napoli et al (Napoli et al. 2010). Ascomycota were the dominant fungal phylum
245 and their number decreased moving from inside the *T. melanosporum* brûlé to outside, while the
246 number of Basidiomycota increased (Mello et al. 2011). In our study, both Ascomycota and
247 Basidiomycota were abundant, and similarly, Basidiomycota were more abundant in non-truffle
248 soils than in truffle soils, and this might suggest that Basidiomycota played important roles in the
249 ground of *T. melanosporum* and these three Chinese truffles. *Mortierella*, belonging to phylum
250 Zygomycota, were significantly enriched in control soils compared with the truffle soils ($P < 0.05$),
251 while previous study showed they were an equal abundance inside and outside the *T.*
252 *melanosporum* brûlé (Mello et al. 2011). It suggested that although truffle brûlé might affect soil
253 microbial structure, the exact effect might depend on different Tuber species or geographical
254 location. Our findings may disclose specific microbial ecological habitat of Chinese truffle
255 grounds.

256 **Both bacterial and fungal communities showed differences between truffle-producing and**
257 **non-producing soils**

258 UniFrac analysis showed that both bacterial and fungal communities of fruit body -producing
259 soils were different from their respective control soils in this study. Different signal intensities of
260 bacteria were presented inside versus outside the *T. melanosporum* brûlé by using DGGE and

261 DNA microarrays (Mello et al. 2013). Mello et al also found that the ratios of ECM: non-ECM
262 fungi were different inside and outside the *T. melanosporum* brûlé by the use of DGGE and
263 molecular cloning of ITS. The present study supports the previous findings that both bacterial and
264 fungal communities displayed different tendency inside the brûlé from outside part.

265 The ecological impact of brûlé, which is the specific small area characterized by scanty
266 vegetation where truffle grows, is still under exploring. The present study disclosed the differences
267 of microbial community between *tuber* spp. growing and non-growing soils. Many studies have
268 demonstrated that some plants perceived specific truffle volatiles and their growth could
269 potentially be inhibited (Splivallo et al. 2011). Previous work on the composition of both fungal
270 and bacterial communities inside and outside *T. melanosporum* brûlé revealed the differences and
271 suggested that truffles have an efficient strategy of ensuring their survival by spreading their
272 metabolites, mainly volatile organic compounds (VOCs) characterized by allelopathic effects on
273 the herbaceous plants and the microorganisms of the rhizosphere (Mello et al. 2013; Napoli et al.
274 2010). Some microbes like bacterial genus *Sphingomonas* (Alphaproteobacteria) and arbuscular
275 mycorrhizal fungal genus *Glomus* were significantly more abundant among these three Tuber
276 grounds compared with non-truffle soils, while some were not. This might be due to the inhibition
277 by truffle metabolites, which may not be effective on all microbes. Additionally, microbial
278 communities and ectomycorrhizal *Tuber* spp. within burned area were linked since the former
279 might have an impact on truffle development, while few researches have focused on the dominant
280 microbes in fruiting grounds of different *Tuber* spp so far. In this study, some bacteria like
281 *Sphingomonas* (class Alphaproteobacteria), *Escherichia-Shigella* (class Gammaproteobacteria),
282 and *Blastocatella* (class Acidobacteria) were in greater number in truffle fruiting point, and their

283 potential relation to the development of truffle fruiting bodies remains to be disclosed.

284 Unlike *Tuber* spp., which produces ECM with symbiosis hosts, arbuscular mycorrhizal fungi
285 (AMF) penetrates the cortical cells of the roots of vascular plants and cannot easily be recognized
286 by eyes. It is widely known that AMF are critical links in plant-soil continuum and play a critical
287 role in alleviating plant stress (Zhang et al. 2016). AM fungi also help plants to capture nutrients
288 such as phosphorus, sulfur, nitrogen and micronutrients from the soil. It is believed that the
289 development of the AM symbiosis played a crucial role in the initial colonization of land by plants.
290 Furthermore, AM fungi are affected by various saprotrophic soil microorganisms, changing the
291 root physiology and releasing organics into the soil (Gryndler et al. 2000). New study has recently
292 explored the AMF communities, which thrive in a *Tuber melanosporum* brûlé, and disclosed that
293 Glomerales were the dominant fungi inside and outside the brûlé (Mello et al. 2015). In our study,
294 fungal genus *Glomus* was dominant and significantly more abundant in truffle-producing soils
295 than control soils, showing possible correlation of this fungus with truffle presence.

296 Notably, non-fungal organisms ranging from 19% to 38% were detected by pyrosequencing
297 of 18S rRNA gene V4 region, among which Cercozoa and Ochrophyta were dominant and
298 significantly more abundant in all truffle-producing samples than in control (data not shown) ($P <$
299 0.05), suggesting that they may also play important roles in truffle grounds.

300

301 **Method Limitations**

302 Barcoded pyrosequencing were chosen to compare the bacteria and fungi communities from
303 truffle producing ground and non-truffle ground in this study, because traditional culture method
304 might miss some important tax. However, DNA of dead cell could also be detected by

pyrosequencing, which means some microbes exist in truffle ground might be killed and had no significance in this area. Nevertheless, differences of microbial communities between truffle and non-truffle ground are found in previous and our studies (Gryndler et al. 2013) (Mello et al. 2013) (Mello et al. 2011), which suggested some microbes might be active in this specific area and potentially associated with truffle fructification.

We believe that microbes and truffle in natural truffle-growing ground are interacted and this relationship is quite complicated. Although it was reported that truffle might impact soil bacteria communities (Mello et al. 2013) and fungal dynamics (Napoli et al. 2010), or vice versa, i.e., the specific microbial communities would put influence on the growing of the specific truffle species, more direct evidence is in demand.

Conclusion

The present study gives a first glimpse into the microbial communities in *T. indicum*, *T. pseudoexcavatum*, and *T. sinoaestivum* from China, which represents specific microbial ecological habitat of Chinese truffle burned area. Microbes of bacterial genus *Sphingomonas* and fungal *Glomus*, as well as some non-fungal organism (Cercozoa and Ochrophyta), were more abundant within truffle-producing grounds, and their roles playing in the truffle growth remained to be disclosed. Targeted experiments are the next steps and the investigation on bacteria and fungi community living in ascocarps were in our consideration in order to understand the communities directly associated with the growth of these truffle species.

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331

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336

337 ***Conflict of interest statement.*** None declared.

338

339 **Reference**

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460 **Table and figure legends**

461 Table 1. The ten most abundant bacterial genera in the truffle-producing and control soil samples.

462 Average percentages are given into brackets. Each value is the mean of three biological
463 replicates. Genera which were significantly more abundant in one sample than another
464 (one-way ANOVA, $P < 0.05$) are highlighted with bold letters. TTZ = Tangtangzhen
465 sampling site. XSX = Xueshanxiang. XT = Xintianxing. CK = control soil.

466 Table 2. Richness estimates and diversity indices of bacterial and fungal communities in

467 truffle-producing and control soil samples. Each value is the mean of three biological
468 replicates ($\pm$ SE). Letters indicate significant differences ($P < 0.05$) among the bacterial and
469 fungal communities of the analyzed samples. TTZ = Tangtangzhen sampling site. XSX =
470 Xueshanxiang. XT = Xintianxing. CK = control soil.

471 Table 3. The ten most abundant fungal genera in the truffle-producing and control soil samples.

472 Average percentages are given into brackets. Each value is the mean of three biological
473 replicates. Genera which were significantly more abundant in one sample than another
474 (one-way ANOVA, $P < 0.05$) are highlighted with bold letters. TTZ = Tangtangzhen sampling
475 site. XSX = Xueshanxiang. XT = Xintianxing. CK = control soil.

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477 Figure 1. Numbers of shared and site specific bacterial (A) and fungal (B) OTUs. TTZ =

478 Tangtangzhen sampling site. XSX = Xueshanxiang. XT = Xintianxing. CK = control soil.

479 Figure 2. Composition of soil bacterial and fungal communities at phylum and class level in

480 truffle-producing and control soil samples. TTZ = Tangtangzhen sampling site. XSX =

481 Xueshanxiang. XT = Xintianxing. CK = control soil.

482 Figure 3. Principal coordinate analysis based on weighted UniFrac analysis for three truffle soil
483 bacterial and fungal communities. TTZ = Tangtangzhen sampling site. XSX = Xueshanxiang.
484 XT = Xintianxiang. CK = control soil.
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Numbers of shared and site specific bacterial (A) and fungal (B) OTUs. TTZ = Tangtangzhen sampling site.
XSX = Xueshanxiang. XT = Xintianxiang. CK = control soil.

692x338mm (300 x 300 DPI)

Composition of soil bacterial and fungal communities at phylum and class level in truffle-producing and control soil samples. TTZ = Tangtangzhen sampling site. XSX = Xueshanxiang. XT = Xintianxiang. CK = control soil.

297x180mm (300 x 300 DPI)

Principal coordinate analysis based on weighted UniFrac analysis for three truffle soil bacterial and fungal communities. TTZ = Tangtangzhen sampling site. XSX = Xueshanxiang. XT = Xintianxiang. CK = control soil.

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Table 1. The ten most abundant bacterial genera in the truffle-producing and control soil samples.

Samples	TTZ-CK	TTZ	XSX-CK	XSX	XT-CK	XT
genera	<i>Candidatus</i> (20.5)	<i>Sphingomonas</i> (9.2)	<i>Acidobacterium</i> (15.6)	<i>Candidatus</i> (11.2)	<i>Acidobacterium</i> (14.8)	<i>Sphingomonas</i> (12.5)
	<i>Acidobacterium</i> (12.2)	<i>Azospira</i> (5.7)	<i>Candidatus</i> (6.3)	<i>Acidobacterium</i> (8.3)	<i>Candidatus</i> (14.0)	<i>Candidatus</i> (10.3)
	<i>Sphingomonas</i> (4.5)	<i>Escherichia-Shigella</i> (5.3)	<i>Pseudomonas</i> (4.5)	<i>Sphingomonas</i> (7.8)	<i>Arthrobacter</i> (4.7)	<i>Blastocatella</i> (5.4)
	<i>Bradyrhizobium</i> (3.2)	<i>Blastocatella</i> (5.2)	<i>Arthrobacter</i> (4.2)	<i>Blastocatella</i> (4.4)	<i>Sphingomonas</i> (4.3)	unclassified <i>Planctomycetaceae</i> (3.5)
	<i>Azospira</i> (3.1)	<i>Rubrobacter</i> (3.1)	<i>Burkholderia</i> (4.1)	<i>Azospira</i> (3.8)	<i>Bradyrhizobium</i> (3.2)	<i>Acidobacterium</i> (3.2)
	<i>DeFluviicoccus</i> (2.4)	unclassified <i>Nitrosomonadaceae</i> (2.9)	unclassified <i>Planctomycetaceae</i> (3.6)	<i>Pseudomona</i> (3.8)	<i>Azospira</i> (3.2)	<i>Novosphingobium</i> (3.0)
	unclassified <i>Planctomycetaceae</i> (2.3)	<i>Ornatilinea</i> (2.6)	<i>Azospira</i> (2.6)	<i>Ornatilinea</i> (3.3)	<i>Novosphingobium</i> (2.7)	<i>Phycisphaera</i> (2.7)
	<i>Bryobacter</i> (2.3)	<i>Candidatus</i> (2.6)	<i>Skermanella</i> (2.3)	<i>Novosphingobium</i> (3.1)	<i>Blastocatella</i> (2.5)	<i>Azospira</i> (2.5)
	<i>Novosphingobium</i> (2.2)	unclassified <i>Chitinophagaceae</i> (2.6)	<i>Bryobacter</i> (2.2)	unclassified	Unclassified <i>Comamonadaceae</i> (2.4)	<i>Escherichia-Shigella</i> (2.4)
	<i>Blastocatella</i> (2.0)	unclassified <i>Rhodospirillaceae</i> (2.4)	unclassified(2.0)	8) <i>Escherichia-Shigella</i> (2.4)	<i>Bryobacter</i> (2.1)	unclassified <i>Gemmatimonadaceae</i> (2.4)

Average percentages are given into brackets. Each value is the mean of three biological replicates. Genera which were significantly more abundant in one sample than another (one-way ANOVA, $P < 0.05$) are highlighted with bold letters. TTZ = Tangtangzgen sampling site. XSX = Xueshanxiang. XT = Xintianxing. CK = control soil.

Table 2. Richness estimates and diversity indices of bacterial and fungal communities in truffle-producing and control soil samples.

	Sample	Seq num	OTUs num	Shannon_index	ACE_index	Chao 1_index	Coverage_index	Simpson_index
Bacterial alpha diversities	XSX-CK	33572	7695	7.4326±0.3 ^c	25165±5729 ^b	16460±3047 ^b	0.8632±0.03 ^a	0.004202±0.0014 ^a
	XSX	31422	6686	7.4436±0.06 ^c	20093±1656 ^b	13820±633 ^b	0.8791±0.01 ^a	0.003118±0.00049 ^a
	TTZ-CK	24110	6774	7.6992±0.06 ^b	34588±2931 ^a	19198±2556 ^b	0.7344±0.19 ^{ab}	0.001817±0.0006 ^b
	TTZ	34903	7412	7.6359±0.06 ^{bc}	23215±4756 ^b	16018±2253 ^b	0.8787±0.02 ^a	0.002050±0.0001 ^b
	XT-CK	18534	6309	7.6499±0.15 ^{bc}	33018±8347 ^a	18039±2907 ^b	0.7439±0.09 ^{ab}	0.002889±0.0001 ^b
	XT	16496	7405	8.1511±0.10 ^a	42802±8286 ^a	23641±2280 ^a	0.6519±0.11 ^b	0.001741±0.0006 ^b
Fungal alpha diversities	XSX-CK	12692	1709	5.15±0.60 ^{ab}	4643± 262 ^{ab}	3478± 146 ^{bc}	0.91± 0.02	0.04± 0.02
	XSX	9131	1042	4.67±0.08 ^a	3070± 1080 ^a	2161± 447 ^a	0.94± 0.02	0.10± 0.14
	TTZ-CK	12666	1867	5.34±0.21 ^{ab}	6159± 315 ^b	4062± 229 ^b	0.93±0.01	0.03±0.01
	TTZ	17621	2083	5.86±0.20 ^b	4978± 2090 ^b	3947± 1408 ^b	0.93± 0.02	0.02± 0.01
	XT-CK	11547	1579	5.20±0.18 ^{ab}	4413±440 ^{ab}	3117±208 ^{ab}	0.93±0.02	0.04±0.01
	XT	9801	1505	6.00±0.10 ^b	3655± 167 ^{ab}	2812± 244 ^{ac}	0.92± 0.01	0.01±0.00

Each value is the mean of three biological replicates (± SE). Letters indicate significant differences ($P < 0.05$) among the bacterial and fungal communities of the analyzed samples. TTZ = Tangtangzgen sampling site. XSX = Xueshanxiang. XT = Xintianxing. CK = control soil. Letters indicate significant differences ($P < 0.05$) among treatments.

Table 3. The ten most abundant fungal genera in the truffle-producing and control soil samples.

Samples	<u>TTZ-CK</u>	<u>TTZ</u>	<u>XSX-CK</u>	<u>XSX</u>	<u>XT-CK</u>	<u>XT</u>
Genera	<i>Mortierella</i> (20.5)	<i>Glomus</i> (8.3)	<i>Modicella</i> (24.2)	<i>Xylogone</i> (14.3)	<i>Mortierella</i> (30.2)	<i>Glomus</i> (8.6)
	<i>Pseudogymnoascus</i> (13.2)	<i>Lophium</i> (4.5)	<i>Mortierella</i> (10.9)	<i>Glomus</i> (5.3)	<i>Modicella</i> (7.3)	<i>Malassezia</i> (4.9)
	<i>Modicella</i> (6.4)	<i>Septoglomus</i> (1.4)	<i>Xylogone</i> (5.1)	<i>Mortierella</i> (4.4)	<i>Pseudogymnoascus</i> (5.5)	<i>Pseudogymnoascus</i> (3.4)
	<i>Coprinus</i> (5.6)	<i>Fimicolochytrium</i> (1.3)	<i>Pseudogymnoascus</i> (3.8)	<i>Malassez</i> (4.2)	<i>Polyozellus</i> (4.2)	<i>Mortierella</i> (2.5)
	<i>Herpotrichia</i> (0.9)	<i>Malassezia</i> (1.2)	<i>Russula</i> (3.5)	<i>Boeremia</i> (3.0)	<i>Herpotrichia</i> (3.1)	<i>Grosmannia</i> (2.1)
	<i>Glomus</i> (2.2)	<i>Tetracladium</i> (0.9)	<i>Athelia</i> (2.6)	<i>Modicella</i> (2.0)	<i>Glomus</i> (1.8)	<i>Fimicolochytrium</i> (1.9)
	<i>Tetracladium</i> (2.1)	<i>Isaria</i> (0.9)	<i>Trichoderma</i> (2.4)	<i>Thyrea</i> (1.9)	<i>Malassezia</i> (1.5)	<i>Septoglomus</i> (1.7)
	<i>Inocybe</i> (1.6)	<i>Inocybe</i> (0.9)	<i>Exophiala</i> (1.6)	<i>Lophium</i> (1.7)	<i>Lophium</i> (1.3)	<i>Lentinula</i> (1.4)
	<i>Cadophora</i> (1.2)	<i>Powellomyces</i> (0.7)	<i>Cadophora</i> (1.2)	<i>Tuber</i> (1.6)	<i>Trechispora</i> (1.0)	<i>Herpotrichia</i> (1.1)
	<i>Malassezia</i> (1.2)	<i>Xylogone</i> (0.7)	<i>Herpotrichia</i> (0.9)	<i>Pseudogymnoascus</i> (1.3)	<i>Mytilinidion</i> (0.9)	<i>Modicella</i> (1.0)

Average percentages are given in the brackets. Each value is the mean of three biological replicates. Genera that are significantly more abundant in one sample than another (one-way ANOVA, $P < 0.05$) are highlighted with bold letters. TTZ = Tangtangzgen sampling site. XSX = Xueshanxiang. XT = Xintianxing. CK = control soil.