Diversity of Bacterial and Fungal Communities in Wheat Straw Compost for *Agaricus bisporus* Cultivation

Guangtian Cao¹

Department of Horticulture, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, Zhejiang, China; and College of Standardisation, China Jiliang University, Hangzhou 310018, Zhejiang, China

Tingting Song¹, Yingyue Shen, Qunli Jin, Weilin Feng, Lijun Fan, and Weiming Cai²

Department of Horticulture, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, Zhejiang, China

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Abstract. The *Agaricus* genus represents the most popular edible mushroom in the world. Wheat straw often is used as the substrate for mushroom cultivation following pretreatment to degrade the lignocellulosic biomass in agricultural waste. In this study, we investigated the changes in bacterial and fungal microflora of wheat straw substrate during different phases of composting. We collected samples of the raw material (M1), phase I aerobic fermentation (F1, F2, F3), and phase II after-fermentation (AF1) for high-throughput 16S rRNA and internal transcribed spacer (ITS) sequencing to analyze the microbial diversity in the substrate during composting. Our data revealed that among the five stages, 365 operational taxonomic units (OTUs) were shared, with *Firmicutes*, *Proteobacteria*, and *Actinobacteria* being the predominant bacterial phyla. In addition, *Thermobispora*, *Thermopolyspora*, *Ruminiclostridium*, *Thermobacillus*, and *Bacillus* were the predominant genera in F3 and AF1, with the species *Thermobispora bispora* and *Pseudoxanthomonas taiwanensis* being predominant in F2. Both principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS) plots showed that the bacterial communities of five stages could be distinguished from each other based on their composting time. The Shannon and Simpson indexes of F2 were lower than M1 ($P < 0.05$), and the clustering dendrogram showed that the bacterial communities in AF1 were similar to F3, with *Micromonosporaceae*, *Streptosporaceae*, *Thermomonosporaceae*, and *Vulgatibacteraceae* representing the differential bacterial families by linear discriminant analysis with effect size (LEfSe) analysis. The analysis of fungal communities showed that 384 OTUs were common among the five stages, with 1054 and 454 OTUs unique to M1 and AF1, respectively. *Ascomycota* and *Basidiomycota* were the two predominant phyla in all stages, and *Chytridiomycota* was predominant in F2, F3, and AF1 stages. PCA and NMDS plots showed that the clusters of F2 and AF1 were more dispersed than the other stages. No differences were observed in alpha diversity between the stages, and samples of F1, F2, and F3 were closer to AF1 in the clustering dendrogram. By LEfSe analysis, *Mycothermus thermophilus*, *Gonapodya polymorpha*, and *Phaeophleospora eugeniae* were identified as the predominant fungal species in AF1.

China, Malaysia, India, and Ireland are leading in global mushroom production (Hanafi et al., 2018). *Agaricus* genus is the most popular edible mushroom in the world,

which is cultivated on the agricultural waste, including straw, wheat, and hay base (Rinker, 2017; Treuer et al., 2018). A previous study has reported the application of agroindustrial biomass from agricultural waste for the production of energy and promoting the growth of mushrooms (Hanafi et al., 2018). Pala et al. (2012) also suggested that high-quality mushrooms could be produced when agricultural waste was used as the mushroom substrate. Harith et al. (2014) reported that agrowaste is rich in carbon and nitrogen, which contributes to the production of better mushroom fruiting bodies. In addition, the micro-organisms during fermentation dramatically influence the *Agaricus bisporus* (*A. bisporus*) production, which more studies are needed to be conducted.

Wheat straw is a lignocellulosic substrate composed of cellulose, hemicellulose, and lignin and is the second largest biomass feedstock in the world (Saha and Cotta, 2006). Hua et al. (2016) found that micro-organisms could enzymatically digest the cell walls of plant biomass. Among the lignocellulolytic and nonlignocellulolytic microbes present on natural substrates, the former shows biodegradability (Tejirian and Xu, 2010). In a recent study, *Proteobacteria* was reported to be the dominant phylum in different fermentative stages of lignocellulosic material (Xiao et al., 2018). Compared with chemical and physical biomass pretreatment methods, biological pretreatment demonstrates an outstanding delignification ability with low energy consumption and the absence of toxic substances. During aerobic fermentation, temperature in the wheat straw substrate varies over time. Microbial activity leads to temperature increments, as their metabolic activity produces energy by the codigestion of lignocellulosic biomass. In addition, ligninolytic fungi produce highly active ligninolytic enzymes, which further delignify the plant biomass (Čilerdžić et al., 2017; Rouches et al., 2017; Saparrat and Guillén, 2005; Šnajdr and Baldrian, 2006). A study confirmed that the micro-organisms degrade about 40% of the dry matter of in the compost, and the dry matter holds potential valuable nutrients of *A. bisporus* (Straatsma et al., 1994). Thus, changes in the *Agaricus* sp. of fungi are crucial to mushroom production. In this study, we investigated the changes in microflora of the wheat straw substrate used for the cultivation of *A. bisporus*.

Materials and Methods

Preparation of wheat straw substrate. Wheat straw was composted in the ventilation fermentation chamber of the Horticulture Institute, Zhejiang Academy of Agricultural Sciences in 2016. Composting windrows (15 × 2 × 1.8 m) consisted of 600 kg of wheat straw mixture, 40 kg of rapeseed cake, 15 kg of CaSO₄·2H₂O, 5 kg of Ca(H₂PO₄)₂·H₂O, 4 kg of (NH₄)₂SO₄, and 6 kg of urea (CH₄N₂O).

Sample collection. The substrate mixture was moistened by manual spraying, and ≈200 g of samples (M1) were collected in triplicate before initiating the self-heating composting phase. To enhance the composting process, the windrows were turned on the fifth (F1), ninth (F2), and 12th (F3) day of Phase I. During turning, water and CaCO₃ were added manually to maintain the moisture content (60% to 70%) and pH (6–8). At the end of each stage, samples (≈200 g each) were collected in triplicate. The three samples collected at five different points (at all depths from the four edges and center) were pooled and mixed thoroughly. Phase II, consisting of 6 d, was characterized by a rapid increase in temperature up to 60 °C for 8–9 h, followed by stabilization of the compost temperature to 45 to 50 °C for 5 d, and

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¹These authors contributed equally to this work.

²Corresponding author. E-mail: caiwm527@126.com.

Fig. 1. Operational taxonomic unit analysis of bacterial communities in wheat straw substrates (A–C). Top 10 bacterial phyla (D) and genera (E) of bacterial communities in wheat straw substrates. Principal component analysis (PCA) (F) and nonmetric multidimensional scaling (NMDS) (G) plots of bacterial communities in wheat straw substrates.

gradual cooling to 25 °C. When the composting process was complete, samples (AF1) were collected as described for further analysis. Before analysis, all samples were dried using a vacuum freeze dryer (VirTis Company, New York, NY) and stored at –80 °C.

High-throughput sequencing of 16S rRNA. Total genomic DNA was extracted from a 0.5-g mixture of wheat straw using the Power Soil DNA Extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to manufacturer's instructions. The 16S rRNA amplicon sequencing was performed as

described previously with modifications (Sun et al., 2018; Xiao et al., 2016). To summarize in brief, the specific primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') targeting the V4 hypervariable regions of 16S rRNA were used on the Illumina MiSeq platform at Novogene Cooperation (Beijing, China), with the HiSeq. 2500 (PE250) sequencing system. The ITS amplicon sequencing used the specific primer pair ITS1-F (5'-CT-TGGTCATTTAGAGGAAGTAA-3') and ITS1-R (5'-GCTGCGTTCTTCATCGATGC-3').

The split_libraries_fastq.py script in Quantitative Insights Into Microbial Ecology (QIIME V1.7.0) was used for the filtering of raw reads. OTUs with 97% similarity were clustered according to the UPARSE method.

Biostatistics analysis. Unweighted and weighted UniFrac (QIIME) analysis were used to compare the similarity or difference among the different wheat straw sequencing libraries. The Wilcoxon test was used to assess alpha and beta diversity parameters. PCA and NMDS analyses were performed using R

software (Version 2.15.3; Foundation for Statistical Computing, Vienna, Austria). LEfSe tool was accessed online at <http://huttenhower.sph.harvard.edu/galaxy/>.

Results

OTUs analysis of bacterial communities. Among the five stages, 365 core OTUs were observed (Fig. 1A). M1, F1, F2, F3, and AF1 had 58, 17, 8, 8, and 29 unique OTUs, respectively. F1, F2, and F3 shared 474 OTUs (Fig. 1B). M1 and AF1 shared 409 OTUs, whereas 255 and 239 OTUs were unique in M1 and AF1, respectively (Fig. 1C).

Top 10 bacterial phyla and genera. Figure 1D lists the top 10 phyla identified by relative abundance in the five stages of the substrate. *Firmicutes*, *Proteobacteria*, and *Actinobacteria* were the top three phyla. Moreover, *Bacteroidetes* was also dominant in M1. Interestingly, the relative abundance of *Firmicutes* increased significantly to the dominant phyla at F1 and then decreased gradually at F2-3 and AF, and *Actinobacteria* increased over time. Orders of *Bacillales*, *Clostridiales*, *Rhizobiales*, and *Streptosporangiales* dominated different fermentation stages, with the *Streptosporangiales* increasing significantly over time (Supplemental Fig. 1A).

Figure 1E lists the top 10 genera based on relative abundance, and only a few of them were shared across the five stages of the substrate. *Actinobacter*, *Bacillus*, *Sphingobacterium*, and *Rhizobium* were the top four genera in M1. *Bacillus*, *Thermobacillus*, *Rhizobium*, and *Ruminiclostridium* were the top four genera in F1. *Firmicutes*, *Thermopolyspora*, *Thermobispora*, *Bacillus*, and *Thermobacillus* were the top five genera in F2, and *Thermobispora*, *Thermopolyspora*, *Ruminiclostridium*, *Thermobacillus*, and *Bacillus* were the top five genera in F3 and AF1.

PCA and NMDS plots of bacterial communities. The PCA plot showed that the microflora community of five stages could be distinguished from each other according to the time point, and the M1 samples were separated from the fermentation and after-fermentation phase samples (Fig. 1F). Similarly, the NMDS plot also confirmed the aforementioned results, which the samples from the five stages could be distinguished from each other.

Dominant bacterial species. The top 10 bacterial species identified in the five stages of the substrate are listed in Fig. 2. Bacterial species identified in straw substrate F2 accounted for 22% of the bacterial composition, which was less than 20% for the samples from the other stages. *Acinetobacter* sp. NIPH 2171, *Rhizobium larrymoorei*, and *Acinetobacter schindleri* were the predominant species in M1, whereas F3 and AF1 were dominated by *Thermobispora bispora*. *Bacillaceae bacterium* NS1-3 and *Bacillus thermocloaceae* represented about 50% of the bacterial species identified in F1. In addition, *Pseudoxanthomonas*

taiwanensis was the predominant species in F2.

Alpha and beta diversity of bacterial communities. The parameters of alpha diversity at the five stages are listed in Fig. 3A–C. The Shannon index of F2 was significantly lower than that of M1 or F1 ($P < 0.05$), and M1 had an increasing trend compared with F3 and AF1 ($P = 0.052$, $P = 0.087$). Likewise, the Simpson index of F2 was significantly lower than that of M1 ($P < 0.05$) and had a decreasing trend than F1 ($P = 0.066$). Furthermore, the PD_Whole tree of F2 was significantly ($P < 0.05$) lower than that of F1.

Based on the unweighted UniFrac distance, F2 was significantly greater ($P < 0.05$) than F1 (Fig. 3D), and the cluster dendrogram of the relative abundance in phylum level showed that AF1 was similar to F1 or F3 (Fig. 3F). Based on the weighted UniFrac distance, F2 were significantly greater ($P <$

0.05) than the other stages (Fig. 3E), and the cluster dendrogram showed that AF1 was similar to F3, whereas M1 was distinguished from the other phases (Fig. 3G). Interestingly, with time the relative abundance of the *Firmicutes* phylum decreased, whereas that of *Actinobacteria* increased.

LEfSe analysis and ternary plot of bacterial communities. The cladogram depicts the fermentative microbial strains distinguishing the five stages (Fig. 4A). As expected, M1 was represented by strains of *Bacteroidetes* and *Proteobacteria* phyla, consisting the *Sphingobacteriaceae* family in the former, and *Enterobacteriaceae*, *Moraxellaceae* and *Pseudomonadaceae* families in the latter. The AF1 stage included strains from the *Actinobacteria* and *Proteobacteria* phyla, in which the former was represented by the families *Micromonosporaceae*, *Streptosporaceae*, and *Thermomonosporaceae*,

Fig. 2. The top 10 bacterial species identified in the five stages of wheat straw substrate. “Total (%)” represents the known species in all the sequences.

Fig. 3. Alpha and beta diversity of bacterial communities in the wheat straw substrates. The parameters of alpha diversity in the five stages are listed in A–C. The parameters of beta diversity in the five stages are listed in D and E. Based on unweighted and weighted unifrac distance, the cluster dendrogram of relative abundance in phylum level are listed in F and G.

and the latter by the family *Vulgatibacteraceae*. The distinct families in F1, F2, and F3 were *Bacillaceae*, *Family XVIII* and *Xanthomonadaceae*, and *Ruminococcaceae*, respectively.

The ternary plot analysis (Fig. 4B–E), showed that the *Thermobispora* and *Thermopolyspora* genera were represented in AF1

and F3 compared with F1, F2, or M1 stages. *Acinetobacter* and *Sphingobacterium* were the top two genera in M1, and *Bacillus* was the topmost genus in F1.

Top fungal phyla and genera. The dominant phyla and genera of fungi in the five fermentation stages are shown in Fig. 5A and B. *Ascomycota* and *Basidiomycota* were

the top two phyla in all stages except for AF1, and *Chytridiomycota* was represented in the F2, F3, and AF1 stages. *Pyrenophora*, *Alternaria*, and *Sporobolomyces* were the predominant genera in stage M1; *Gibellulopsis* and *Alternaria* dominated F1; *Microidium*, *Chaetomium*, *Gonapodya*, *Gibellulopsis*, and *Trichoderma*

A

Cladogram

B

Ternaryplot

D

C

E

Fig. 4. The cladogram linear discriminant analysis with effect size analysis (A) and ternary plot of bacterial communities (B–E).

dominated F2; *Chaetomium*, *Gonapodya*, and *Gibellulopsis* were predominant in F3; and *Microidium*, *Chaetomium*, *Mycothermus*, and *Gonapodya* were represented in the AF1 stage. On the basis of these data, greater fungal diversity was observed in the F2 and F3 stages. It is also worth mentioning that 75% relative abundance of fungi were unknown in F1.

OTUs analysis of fungal communities. There were 384 OTUs in common among the five stages (Fig. 5C). The number of unique OTUs in stages M1, F1, F2, F3, and AF1 were 90, 391, 265, 195, and 328, respectively. Only 594 OTUs were shared between M1 and AF1 (Fig. 5D), and 1054 and 454 OTUs were unique to M1 and AF1, respectively. Meanwhile, 603 unique OTUs

were common among the stages F1, F2, and F3 (Fig. 5E). In addition, 641, 530, and 370 OTUs were unique to F1, F2, and F3, respectively.

PCA and NMDS plots of fungal communities. The PCA plot distinguished the F2 and AF1 stages from the other three stages, which were clustered together (Fig. 5F). Similarly, the NMDS plot showed

Fig. 5. Top 10 phyla (A) and genera (B) of fungal communities in wheat straw substrates. Operational taxonomic unit analysis of fungal communities in wheat straw substrates (C–E). Principal component analysis (PCA) (F) and nonmetric multidimensional scaling (NMDS) (G) plots of fungal communities in wheat straw substrates.

that samples of F2 and AF1 were more dispersed than those of the other stages (Fig. 5G).

Alpha and beta diversity of the fungal communities. Figure 6A–D lists the alpha diversity of fungi in the different stages, and Fig. 6E and G list the beta diversity. No significant differences were observed in the alpha diversity (Shannon index, Simpson index, PD_Whole tree and observed species) of fungi ($P > 0.05$). On the basis of the unweighted UniFrac, M1 had a significantly lower beta diversity of fungi than F2 ($P < 0.05$) and had a decreasing trend than AF1 ($P = 0.086$). In contrast, in the weighted UniFrac, F2 had a significantly greater beta diversity of fungi than M1 or F3 ($P < 0.05$). Furthermore, the cluster dendrogram based on

weighted UniFrac indicated that F1 samples were closer to AF1 (Fig. 6F), and samples F1, F2, and F3 were closer to AF1 than M1 (Fig. 6H).

Ternary plot and LEfSe analysis of fungi. Figure 7A–D shows that the *Chaetomium*, *Mycothermus*, and *Gonapodya* genera were represented in AF1 compared with M1, with the latter two genera also being closer to AF1 compared with F1, F2, or F3. Furthermore, *Pyrenophora* and *Alternaria* were representative of M1 compared with AF1, F1, F2, or F3. Compared with F1, *Chaetomium* and *Gonapodya* were closer to F2 and F3 (Fig. 7D). Furthermore, *Gibellulopsis* was closer to F1 and F3, compared with F2 samples.

LEfSe analysis showed that the distinct species in AF1 were *Mycothermus thermo-*

philus, *Gonapodya polymorpha*, and *Phaeo-phleospora eugeniae* (Fig. 7E). In M1 the fungal species were *Chaetomium thermophilum*, *Pyrenophora teres*, *Alternaria tenuissima*, and *Pyrenophora graminea*, and F3 was represented by *Sporobolomyces ruberrimus*. And in F1, Capnodiaceae were the prominent order (Supplemental Fig. 1B).

Discussion

Substrate preparation is the most critical step in *A. bisporus* cultivation, which has led to decades of research on the degradation of wheat straw. Composting for mushroom substrate involves the synergistic action of bacteria and fungi. The decomposition of cellulosic plant material and microbial

Fig. 6. Alpha and beta diversity of fungal communities in the wheat straw substrates. The parameters of alpha diversity in the five stages are listed in A–D. The parameters of beta diversity in the five stages are listed in E and G. Based on unweighted and weighted unifrac distance, the cluster dendrogram of relative abundance in phylum level are listed in F and H.

activity are required for mushroom substrate production (Zhang et al., 2014). Few studies have focused on bacteria from different composting stages, although the structure and diversity of microflora have not been well characterized (Singh et al., 2012; Vajna et al., 2012). Valdez–Vazquez et al. (2017)

reported that wheat straw compost was composed of hydrogen producers of the phyla *Firmicutes* and *Proteobacteria*. Phyla of *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* also have been reported to be found in compost (Jurado et al., 2014; Partanen et al., 2010). Our study suggests that

Firmicutes, *Proteobacteria*, and *Actinobacteria* are the predominant phyla in different fermentation stages. In addition, the orders of *Bacillales*, *Clostridiales*, *Actinomycetales*, and *Thermoanaerobacterales* have been reported to be present throughout the composting of plant biomass (Antunes et al., 2016).

Fig. 7. The ternary plot (A–D) and linear discriminant analysis with effect size analysis (E) of fungal communities in the wheat straw substrates. LDA = linear discriminant analysis.

Similarly, the predominant orders we observed were *Bacillales*, *Clostridiales*, *Rhizobiales*, and *Streptosporangiales*, with the latter increasing over composting time. Furthermore, we observed that the relative abundance of *Firmicutes* decreased and *Actinobacteria* increased over time. Studies reported that the *Actinobacteria*, which are cellulose decomposers, always exist and peak in the later stages of composting, suggesting a critical role of *Actinomycetes* in the compost ecosystem (Wang et al., 2014; Zhang et al., 2014). In addition, thermotolerant *Actinobacteria* are observed in thermophilic conditions (Zhang et al., 2014). Tortora et al. (2007) also reported that thermophilic *Actinobacteria* can survive at relatively high

temperatures ranging from 40 to 80 °C. Although the diversity of *Actinomycetes* has been found to increase with the composting process (Peters et al., 2000), the *Actinobacteria* in mushroom substrates have not been identified (Vajna et al., 2012). However, the composition of *Actinobacterial* communities have been reported to vary during various stages of composting (Xiao et al., 2011).

In our study, PCA and NMDS plots revealed that the bacterial communities of the five stages of composting were clearly clustered according to the different stage when the samples were collected. The relative abundance of top 10 genera in the five fermentation stages were different from each other, except at F3 and after-fermentation.

The temperature of substrate for *A. bisporus* cultivation changes from 80 °C in phase I to between 50 and 60 °C in phase II, and finally drops to 45 °C in phase III, which impacts the composition of microflora (Gerrits, 1988; Grimm and Wösten, 2018). Meanwhile, the alpha diversity of fermentative stage II was lower than that of the other stages, suggesting poorer microflora in stage II. We speculate that the diversity of micro-organisms in substrate was decreased by the heat stress of phase I. In this study, LEfSe and ternary plots revealed that the *Thermobispora* and *Thermopolyspora* genera were predominant at the fermentation stage III and after-fermentation stage of wheat straw substrate. Vajna et al. (2012) reported that *Bacillus*, *Geobacillus*,

Ureibacillus, *Pseudoxanthomonas*, and *Thermobispora* appeared at the end of composting for oyster mushroom substrate, and *Thermus*, *Bacillus*, *Geobacillus*, *Thermobacillus*, and *Ureibacillus* were predominant in the mature substrate. The type of cultivation, components of substrate, fermentation time, and type of wheat straw material (ligninolytic composition) were identified as important factors that influence the diversity of microorganisms in the substrate.

In thermophilic fermentation processes, bacteria are responsible for initiating degradation of materials, whereas fungi begin to play a role during the cooling and curing stages (Dhaeseleer et al., 2013; López-González et al., 2015; Neher et al., 2015). Fungi occupy an important position in biological biomass pretreatment due to their strong ligninolytic activity (Stajić et al., 2016). Studies found that white-rot fungi could effectively degrade lignin by producing extracellular enzymes, such as lignin peroxidase, manganese peroxidase, and laccase, and the fungal strain, substrate, and culture conditions influence the pretreatment efficiency (Wan and Li, 2012). In our study, the F1 stage had the fewest types of the top 10 identified fungal genera, and no differences in fungi were observed in the alpha diversity of five stages. Antunes et al. (2016) reported that fungi are not detected above 65 °C, which supports our observations. However, over time the relative abundance of *Chytridiomycota* phylum increased in the fermentation of wheat straw substrates, and *Mycothermus thermophilus*, *Gonapodya polymorpha*, and *Pyrenophleospora eugeniae* were the predominant species in the after-fermentation stage. Kertesz and Thai (2018) reported that the thermophilic fungus *Mycothermus thermophilus*, *Thermophilic proteobacteria*, and *Actinobacteria* play a key role in the processes of composting. Because of the limited research about fungal communities in composting, more studies focusing on this aspect are needed to better understand the wheat straw substrate of *A. bisporus*.

To conclude, on the one hand, the microflora diversity of substrates at different fermentation stages changed dramatically, which included the alpha and beta diversity (considering the bacterial types and relative abundance) of F2 and that differed dramatically from the other stages. It is also worth noting that the relative abundance of *Firmicutes* phylum decreased, whereas that of the *Actinobacteria* increased over time. Members of *Micromonosporaceae*, *Streptosporaceae*, *Vulgaribacteraceae*, and *Thermomonosporaceae* comprised distinguished microbiota of the substrate at after-fermentation stage. However, *Microidium*, *Chaetomium*, *Mycothermus*, and *Gonapodya* comprised the predominant fungi at after-fermentation phase. Thus, considering the relative abundance, the *Chytridiomycota* ratio increased, whereas that of *Basidiomycota* decreased over time, with the exception of the material phase and *Mycothermus thermophilus*,

Gonapodya polymorpha, and *Pyrenophleospora eugeniae* were the distinguished fungi in AF1.

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Supplemental Fig. 1. Top ten orders (A) and classes (B) of fungal communities in wheat straw substrates.