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Characterization of microbial community succession during vermicomposting of medicinal herbal residues

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ABSTRACT

Large amounts of medicinal herbal residues (MHR) are produced in the world annually due to the increasing demand for herbal products. In this study, vermicomposting was used to stabilize MHR. Four inoculating density of earthworms was studied, specifically, 0 (W1), 60 (W2), 120 (W3) and 180 (W4) earthworms per kilogram of substrate. The C:N ratios of vermicomposts in W2, W3 and W4 were less than 20 by the end of the first week, while the value for W1 was 30.92. This indicates that earthworms promote the stabilization of MHR. In the initial stage, richness and diversity of the microbial community decreased due to earthworm inoculation, and then began to increase. The dominant phyla were Proteobacteria, Bacteroidetes, Basidiomycota and Ascomycota in the substrates. The abundance of the dominant phyla varied according to earthworm density, indicating that earthworms change the microbial composition. The results suggest that MHR can be stabilized by vermicomposting.

Keywords: Microbial community; Vermicomposting; Medicinal herbal residues; High-throughput pyrosequencing

1. Introduction

The herbal pharmaceutical and cosmetic industries are an essential part of the healthcare system in Asian countries. However, herbal treatments are regarded as a complementary or alternative medical system in most Western countries (Zhou et al.,

2016). In recent years, the growing concern over the side effects of synthetic pharmaceutical and personal care products has resulted in a greater demand for herbal products in the market. As a result, a large amount of medicinal herbal residues (MHR) are produced annually. For instance, in China, the amount of MHR is approximately 30 million tons per year (Meng et al., 2017). In India, approximately 400,000 to 500,000 MT of raw herb materials are consumed annually for herbal product manufacturing (Singh and Suthar, 2012a). Large amounts of MHR are disposed of through stacking in the open, sanitary burial or burning, which cause serious environmental pollution (Meng et al., 2017). Safe management of this waste has become a societal challenge. Vermicomposting, the process in which worms convert organic wastes into a more stable product - vermicompost, is a process of bio-oxidation and stabilization of organic matter, which involves complex interactions between earthworms and microorganisms (Raphael and Velmourougane, 2011). This method is widely used to stabilize a variety of organic wastes. Vermicompost can be used as a nutrient source and as a soil conditioner in agricultural applications (Lavelle et al., 2006). Many types of lignocellulosic wastes, such as bagasse (Bhat et al., 2015), disposable paper cups (Karthika et al., 2015) and urban forest leaf litter waste (Suthar and Gairola, 2014), have been successfully converted into vermicompost using the vermicomposting process. It has been reported that MHR were also successfully stabilized by vermicomposting (Singh and Suthar, 2012a; Singh and Suthar, 2012b; Kumari et al., 2011). *Eisenia fetida* is regarded as one of the most suitable earthworm species for developing a

vermicomposting system in temperate regions because of its tolerance to a broad range of environmental conditions, i.e., pH, moisture content, and temperature (Fernández-Gómez et al., 2010).

Complex microbial communities play a key role in the vermicomposting system. The bioactive ingredients of herbal medicines are often the secondary metabolites of plants extracted by decoction. Generally, the extraction efficiency of the ingredients is approximately 50%; thus, MHR still contain the active ingredients (Zhou et al., 2016). Some of the active ingredients possess antimicrobial properties, and they may affect the bacteria and fungi present in the vermicomposting mass. Therefore, an understanding of the diversity and structure of the microbial community is necessary for proper functioning of a vermicomposting system, and characterizing the shifts in the structure of the microbial community may be helpful for predicting the microbial populations responsible for the degradation of the recalcitrant components in the MHR. However, only a limited number of studies on vermicomposting of MHR are available to date. Singh and Suthar (2012a) studied the vermicomposting of herbal pharmaceutical industry waste using *E. fetida*. They claimed that the noxious industrial waste can be converted into value-added products for soil fertility. The earthworms showed high growth and cocoon production rates in vermibeds. The end products contained greater numbers of bacteria, fungi and actinomycetes than those contained in the initial substrates. Singh and Suthar (2012b) reported that vermicomposting of herbal pharmaceutical industry waste caused significant changes in vermibeds characteristics.

A decrease in pH, electrical conductivity, organic C, C:P ratio and C:N ratio was observed, and an increase in total N, available P and exchangeable K was found. Kumari et al. (2011) performed bioconversion of herbal industry waste using *Eudrilus eugeniae*, and observed that the C:N ratios of the end products were less than 20. In addition, there was a reduction in TOC of the vermibed materials. The vermicomposts promoted the plant growth of pea and marigold. The diversity and structure of the microbial community during vermicomposting of MHR have remained relatively uncharacterized.

High-throughput pyrosequencing is a second-generation DNA sequencing platform for investigating the microbial diversity of environmental samples (Ronaghi et al., 1998). It can provide enough sequencing depth to cover complex microbial communities (Zhang et al., 2015). High-throughput pyrosequencing was used to analyze microbial communities in this study. The aims of this study are to (1) evaluate the performance of the vermicomposting system in stabilizing MHR and (2) characterize the shifts in the diversity and structure of the microbial community during the vermicomposting process.

2. Materials and Methods

2.1 Substrates and earthworm species

The MHR were collected from a Traditional Chinese Medicine pharmacy in

Changchun, Jilin Province, China. The plant species and their components of the MHR are shown in Table 1. Cow dung was collected from a farm located near Changchun. The plants and wastes were air - dried to a moisture content of 14% and then stored in dry plastic containers for further vermicomposting operations. *E. fetida* was purchased from Shenyang Ruida Company, Liaoning Province, China. Adult earthworms were chosen for the study. The earthworms were adapted to the experimental conditions by keeping them in plastic pots containing cow dung and chopped MHR (the substrate in the present study) at 20 ºC for 15 days. The earthworms were washed with water to remove adhesive residues and mucus before being inoculated to the substrates.

2.2 Vermicomposting system and sampling method

Cow dung was used as an amendment to lower the high C:N ratio of the MHR so that they were suitable for vermicomposting. Chopped MHR were mixed with cow dung in a ratio of 3:2 (on a dry weight basis) according to our preliminary studies. The chemical characteristics of the initial mixed substrate are as follows: pH 7.0 ± 0.05; total organic carbon (TOC) (401.73 ± 10.58) g kg^{-1} dry matter; Total nitrogen (TN) (11.17 ± 0.64) g kg^{-1} dry matter; C:N ratio 35.97 ±2.85; and cellulose, hemicellulose and lignin contents (32.67 ± 1.15) % (w/w), (30.23 ± 0.25)% (w/w), and (17.33± 1.15)% (w/w), respectively. Vermicomposting was carried out in plastic pots that were 28cm in height and 18cm in diameter. A total of 12 plastic pots were used. One kilogram of mixed substrates was placed into each plastic pot. The experiment was classified into four

groups: (i) the control group, which contained the mixed substrates without earthworms, denoted as the W1 group; (ii) pots with the mixed substrates and 60 individuals of *E. fetida*, denoted as the W2 group; (iii) pots with the mixed substrates and 120 individuals of *E. fetida*, denoted as the W3 group; and (iv) pots with the mixed substrates and 180 individuals of *E. fetida*, denoted as the W4 group. Each group was composed of three pots. The initial mixed substrates were denoted as W0. All of the pots were covered with a pierced lid for aeration and placed in the laboratory. The room temperature was kept at approximately 25 ºC. The moisture content of the substrate was maintained at approximately 55-65% by periodic sprinkling with an adequate quantity of tap water (Singh and Suthar, 2012a). The duration of the experiment was 49 days. Samples were obtained weekly after inoculating earthworms to the substrate. Samples for each group were denoted as Wi1, Wi2, Wi3 and Wi4, where i is sampling week. The samples were frozen at -80 ºC for chemical composition and microbial community structure analysis.

2.3 Chemical composition

TOC was determined by using the potassium dichromate and concentrated sulfuric acid oxidation method (Nelson and Sommers, 1996). TN was measured by the semi-micro Kjeldahl method (Jackson, 1973). The C:N ratio of each sampling date was calculated with the corresponding TOC and TN values. The pH was determined using a digital pH meter. The vermicompost was suspended in double-distilled water at a ratio of 1:10 (w/v). The aqueous solution was agitated mechanically for 20 min and filtered

through a Whatman no. 1 filter paper. The filtrate was used to determine the pH (Kumari et al., 2011). The content of cellulose, hemicellulose and lignin were determined by the Van Soest method (Van Soest et al., 1991).

2.4. Microbial community structure analysis

The genomic DNA was extracted in triplicate from 0.2 g of samples using the E.Z.N.A. Soil DNA Isolation Kit (OMEGA Biotek., USA) according to the manufacturer's instructions, and the purity was confirmed for the subsequent analysis of microbial community by 1% agarose gel electrophoresis. Two primer sets were chosen for bacteria and fungi, respectively, to amplify bacterial and fungal DNA fragments. The bacterial V3-V4 region of 16S rRNA genes was amplified using primers V338F (5'-ACTCCTACGG GAGGCAGCA -3') and V806R (5'- ATGCAGGGACTACHVGGGT WTCTAAT-3') (Zhang et al., 2015). The fungal ITS sequence of 18S rRNA genes was amplified using primers ITS_1737F (5'- GGAAGTAA AAGTCGTAACAA GG-3') and ITS_2043R (5'- ATGCAGGCTGCGTTCTTCA TCGATGC-3') (Bellemain et al., 2010). All PCR reactions were performed according to Chen et al. (2015). Purified PCR products were used for high-throughput pyrosequencing. Pyrosequencing was carried out by Majorbio Bio-Pharm Biotechnology Co., Ltd., Shanghai, China using Illumina Miseq PE250. Sequences were analyzed by the Quantitative Insights into Microbial Ecology (Caporaso et al., 2010). All of the sequences were clustered into Operational Taxonomic Units (OTUs) based on a 97% identity threshold by the SILVA database

(Quast et al., 2013). A representative sequence from each OTU was selected for downstream analysis. Richness and diversity indexes (abundance-based coverage estimator (ACE), Chao 1 and Shannon) were calculated.

2.5. Statistical analysis

Data analysis was performed using SPSS16.0. Two-way ANOVA was used to determine the effect of the sampling date and the earthworm density on the chemical composition of the substrate. All results were expressed as the mean ± standard deviation (SD). A result was considered statistically significant when the P value was less than 0.05.

3. Results and Discussion

3.1. C:N ratio

The C:N ratio showed a decreasing trend during the whole vermicomposting process (Fig. 1). The decrease of W2, W3 and W4 was significantly larger than that of W1 ($p<0.001$). The C:N ratio of the initial substrate (at the beginning of the inoculation of earthworms) was 35.97 ± 2.85. The values of the C:N ratios in W1, W2, W3 and W4 were 30.92 ± 4.34, 18.25 ± 1.06, 17.53 ± 2.03 and 19.19 ± 1.02, respectively, by the end of the first week. The inoculation of earthworms significantly promoted the decrease of the C:N ratio ($F_{(3, 64)} = 272.898$, $p<0.001$). Moreover, the promoted effect of the

earthworms is dependent on the sampling time ($F_{(7, 64)}$ = 288.763, p<0.001), and there was a significant interaction between the earthworm treatment and the sampling time ($F_{(21, 64)}$ =6.422, p<0.01). The C:N values in W1, W2, W3 and W4 were 27.52 ± 3.43, 15.74 ± 1.25, 12.60 ± 0.24 and 14.84 ± 1.54, respectively, by the end of the second week. The C:N ratio is one of the most widely used indices for compost maturation. The ratio reflects mineralization and stabilization of the organic residues during the decomposition process. A C:N ratio below 20 is indicative of an acceptable compost maturity (Morais and Queda, 2003). The C:N ratio of the vermicompost obtained at the end of the second week was within the acceptable limit. However, in the control group, the ratio was 27.52 ± 3.43, which was greater than 20, indicating that the substrate was not stable in the group without earthworms. The earthworms promoted stabilization of the lignocellulosic MHR. The loss of carbon through the decomposition of organic carbon and the addition of nitrogen through the production of mucus, enzymes, and nitrogenous excrement by earthworms lowered the C:N ratio of the substrate (Suthar, et al., 2012). The C:N ratios in the groups with earthworms were less than 15 by the end of the third week, while the ratio was 24.48 ± 2.42 in the control. The earthworms promoted the stabilization of the substrates. A C:N ratio of 15 or lower is preferred for agronomic utilization of composts (Suthar et al., 2012). The richness, diversity and structure of the microbial community of samplings taken in the first three weeks were analyzed.

3.2. Richness and diversity of the microbial community

Raw pyrosequencing data were demultiplexed and quality filtered by Trimmomatic according to the method described by Bolger et al. (2014). Overlapping reads were merged by FLASH. After removal of low quality sequence reads, the number of sequence reads for each group was determined and is shown in Table 2. At a cut-off of 97% similarity, the number of OTUs from each group is shown in Table 3. In the initial substrate, the number of OTUs was 1282 and 154 for bacteria and fungi, respectively. By the end of the first week, the number of OTUs for bacteria was 1544, 1505, 1223 and 1584 in W1, W2, W3, and W4, respectively. The corresponding numbers for fungi were 126, 142, 130 and 120. This result indicates that the inoculation of earthworms affects the microbial communities. The number of fungal OTUs decreased in W3 and W4 compared with W1 during the first week, and the deceasing extent was related to the earthworm density (Table 3). Moody et al. (1995) reported that earthworms can selectively feed on particular species of fungi. Aira et al. (2006) assumed that microorganisms, especially fungi, are an important part of the earthworm diet. Our results obtained in the first week are consistent with those of Moody et al. (1995) and Aira et al. (2006). The feeding strategy of earthworms caused the reduction in the fungi population in the initial stage. By the end of the second week, the number of OTUs in the groups inoculated with earthworms began to increase compared to their corresponding groups in the first week, especially for fungi. The trends of the ACE and Chao 1 indices were parallel with the OTU numbers (Table 3). In the first week, the

richness of fungi showed a decreasing trend with an increase in the inoculating density in the first week. By the end of the third week, higher richness appeared in the groups with earthworms. Our results are in agreement with those of Singh and Suthar (2012a), who reported that the populations of bacteria, fungi and actinomycetes in the end product were higher than the populations in the initial materials during vermicomposting of herbal pharmaceutical industry waste spiked with cow dung. The shift in the structure of the microbial community in the presence of earthworms indicates that microbes adapted to the environment and that a new microbial community was established.

The above findings were further supported by the Shannon-Wiener index (H') (Table 3). Higher Shannon-Wiener values reflect a higher microbial diversity. For bacteria, the Shannon-Wiener index showed an increasing trend with time. The values of the index for bacteria in the group with earthworms were higher than 6.0, except for W13, indicating a higher microbial diversity. Some studies reported that the Shannon-Wiener diversity index values for bacteria in solid waste treatment were 2.07-2.53 (Xi et al., 2015), 3.07 and 3.43 (Cerda et al., 2017) and 5.61 (Cardinali-Rezende et al., 2016). For fungi, the Shannon-Wiener index decreased by the end of the first week and then began to increase. By the end of the second week, the index for W3 increased significantly compared to the corresponding values in the first week. The decreasing C:N ratio was especially significant at the end of the first week compared to the initial materials. The

rapid decrease in the C:N ratio in the groups with earthworms can be attributed to the higher microbial diversity and to the higher microbial activity.

The rarefaction curves of both bacteria and fungi for each group suggested that the sequencing capability was not large enough to capture the complete diversity of these communities, as the curves did not reach the plateau by increasing the sample size (Fig. 2). This result is similar to that of Qiu et al. (2012). However, the data could sufficiently show the differences in species diversity among different groups at different sampling times, indicating that a reasonable number of individual samples was taken.

3.3. Similarity of the microbial community

A cluster analysis based on pyrosequencing taxonomy was performed to better understand the phylogenetic profiles corresponding to the microbial communities of each group for the first three weeks of samples. Bacteria in the samples were grouped into three statistically significant clusters of W0, W13 and the remaining samples, reflecting that the bacterial community of the initial substrate was different from that of the other samples (Fig. 3a). By the end of the first week, the bacterial homology coefficients of W11, W12 and W14 were within 78%, except for W13, indicating that the effect of earthworms on the microbial community did not appear within one week. The similarity values for the groups without earthworms (W11, W21 and W31) were within 80%, while the similarity values of the groups with earthworms were lower than 80%, implying that earthworms played an important role in shaping the structure of the

bacterial community during vermicomposting. For W2 and W4, the bacterial homology coefficient of W22 and W32 was 83%, and the coefficient of W24 and W34 was 85%. For W3, the bacterial homology coefficient of W23 and W33 was 82%. For fungi, the microbial cluster analysis also showed that the fungi community of the initial substrate was different from that of the other samples (Fig. 3b). By the end of the first week, the fungi communities of W11, W12, W13 and W14 were different from each other. Until the end of the third week, the fungal homology coefficient of W33 and W34 was 84%. The results indicate that the processing period, earthworm presence and inoculating density influence the microbial community succession.

The non-metric multidimensional scaling (NMDS) allows visualization of how the samples are plotted together (Fig. 4) and provides insight into the level of dispersion among the samples. The NMDS analysis showed that the microbial community structure of the vermicomposting mass differed from that of the initial substrate. For bacteria, the samples were clustered into three groups: W0, W13 and the remaining samples. The samples of W11, W12, W14, W21, W22, W23, W24, W31, W32, W33 and W34 were scattered, which indicated that the samples were different from each other. The fungi were also separated into three groups (W0, W13 and W14, and the remaining samples). The NMDS analysis further corroborated the results shown in the dendrograms. The effect of earthworms and the processing time on the bacterial and fungal assemblages of the samples was evident.

3.4. Composition of the microbial community

The relative abundance of bacteria and fungi taxonomic groups is shown in Fig. 5. For bacteria, 33 phyla comprising 65 genera were identified (Fig. 5a). At the phylum level, in all groups, the dominant phyla were Proteobacteria, Bacteroidetes, Firmicutes, Chloroflexi and Actinobacteria, which accounted for 83-93% of the total bacteria. Proteobacteria was the most abundant phylum in all of the groups, followed by Bacteroidetes. Proteobacteria and Bacteroidetes composed 69%, 79%, 84% and 80% of the bacterial community in the groups W11, W12, W13 and W14, respectively, indicating that earthworms stimulated the growth of Proteobacteria and Bacteroidetes in the first week. The stimulation effect was also observed in the second and third weeks. The relative abundance of Chloroflexi and Actinobacteria showed an increasing trend in the treatments with earthworms, while the abundance of Firmicutes showed a decreasing trend. The dominant phyla categorized as Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria have also been reported in other compost studies (Wang et al., 2017; Zhang et al., 2016). These species are generally involved in the degradation of recalcitrant organics such as lignocellulose. Proteobacteria and Actinobacteria are two major taxa involved in the decomposition of lignin (DeAngelis et al., 2011). Bacteriodetes genera are considered to be effective degraders of macromolecules including cellulose and chitin (Manz et al., 1996). Firmicutes have been reported to produce cellulases, lipases, proteases and other extracellular enzymes. The predominance of Firmicutes reflected the ability of the vermicomposting system to

metabolize a variety of substrates including protein, lipids, lignin, cellulose, sugars and amino acids (Lim et al., 2014). Actinomycetes produce extracellular peroxidases, i.e., lignin peroxidase-type enzymes (Varma et al., 2017). For fungi, 5 phyla comprising 21 genera were identified (Fig. 5b). At the phylum level, in the initial substrate, Zygomycota and Ascomycota were the dominant phyla, accounting for 76% of the sequences. Ascomycota was the dominant phylum in the groups without earthworms throughout the first three weeks, accounting for 85-93% of the fungal sequences. In groups with earthworms, Ascomycota was the dominant phylum in W12, W22, W24, W32, W33 and W34, accounting for 85-92% of the sequences. It has been reported that Ascomycota is widely distributed during the aerobic composting process (Langarica-Fuentes et al., 2014). The high relative abundance of Ascomycota in the substrates indicates that this phylum could rapidly grow under high C:N ratio conditions (the initial value was approximately 36). Basidiomycota and Ascomycota were the dominant phyla in W13, W14 and W23, accounting for 89-99%. Basidiomycota secretes enzymes that are necessary for lignin degradation through oxidative biotransformations (Schmidt-Dannert, 2016).

At the genus level, in the initial substrate, the dominant bacteria were Gammaproteobacteria_unclassified, *Bacteroides*, Enterobacteriaceae_unclassified and *Pseudomonas*, with the relative abundances of 9.33%, 4.54%, 3.94% and 3.73%, respectively. The dominant fungi in the initial substrate were *Mortierella*, *Trichosporon*, *Emericella* and *Aspergillus*, with the relative abundances of 44.54%, 12.26%, 10.07%

and 8.05%, respectively. The inoculation of earthworms significantly influenced the microbial composition. By the end of the first week after the inoculation of earthworms, *Bacteroides*, *Pseudomonas*, *Cellvibrio*, *Aspergillus*, *Trichosporon* and Nectriaceae_unclassified were the dominant genera in the groups with earthworms, while in the group without earthworms, the dominant genera were Anaerolineaceae_uncultured, Gammaproteobacteria_unclassified, *Mortierella* and *Trichosporon*. *Bacteroides* is a mesophilic bacterium and is commonly found in composting systems. Some species of *Bacteroides* have been reported to possess cellulolytic ability. For instance, cellulosomes were found in *Bacteroides cellulosolvens* (Van Dyk and Pletschke, 2012). It has been reported that some species of *Pseudomonas* could produce a complete set of cellulose degrading enzymes. For instance, *Pseudomonas* sp. CL3 could produce cellulase consisting of endo-β-1, 4-D-glucanase, exo-β-1, 4-D-glucanase and β-1, 4-D-glucosidase. In addition, *Pseudomonas* sp. CL3 can also produce xylanase (Cheng and Chang, 2011). A significant increase in the genus *Cellvibrio* belonging to Proteobacteria was found in the groups with earthworms. Some species of *Cellvibrio* were active in the degradation of polysaccharides. For instance, *Cellvibrio japonicus* is a powerful saprophyte microbe and has a robust ability to degrade polysaccharides such as cellulose, xylan xyloglucan, mannan, arabinan and pectin (Gardner, 2016). *Aspergillus* has been reported to exhibit typical cellulose hydrolysis, which accelerates the degradation of MHR and promotes the stabilization of the substrate (Zhou et al., 2016). Note that the relative abundance of *Trichosporon*

increased in the groups with earthworms compared to the control. Some species of *Trichosporon* showed cellulolytic activity. For example, *Trichosporon cutaneum* demonstrated excellent performance for cellulose degradation and had a high tolerance to lignocellulose-derived inhibitors; in addition, it can utilize various sugars, such as glucose, xylose, sucrose, cellobiose, and lactose, as well as corn stover hydrolysate and corncob residues hydrolysate (Wang et al., 2016). By the end of the third week, the dominant genera in the groups with earthworms were Cytophagaceae_uncultured, *Cellvibrio*, Xanthomonadales_uncultured, *Flavobacterium*, *Aspergillus*, Ascomycota_unclassified and Sordariomycetes_ unclassified, while the dominant genera in the groups without earthworms were Cytophagaceae_uncultured, Anaerolineaceae_uncultured, BIrii41_norank, *Aspergillus*, *Trichosporon*, and Nectriaceae_unclassified. This result indicates that the microbial community composition was affected by the presence of earthworms and by the processing time. The temperature was reported to be one of the key environmental factors for microbial community succession (Wang et al., 2017). In this study, the substrates were pre-composted for two weeks prior to the inoculation of earthworms into the substrates. There is no thermophilic stage during vermicomposting of MHR spiked with cow dung. The room temperature was kept at approximately 25 ºC during the experimental process; therefore, the temperature cannot be a factor for microbial community succession in this study. The difference in the richness, diversity and composition of the microbial community in the groups with earthworms and the control can be attributed to the

inoculation of earthworms.

4. Conclusions

Vermicomposting could be utilized to stabilize MHR mixed with cow dung, and earthworms promoted the decomposition process compared to traditional composting. The richness and diversity of the microbial community decreased in the initial stage and then began to increase due to the presence of earthworms. Earthworms exerted a remarkable effect on the microbial composition during vermicomposting of MHR. The abundances of the dominant phyla Proteobacteria, Bacteroidetes, Basidiomycota and Ascomycota were affected by the earthworm inoculating density. Earthworms improved the microbial community by enhancing the growth of lignocelluloytic bacteria and fungi. The processing period also affected the microbial community composition.

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Figure Captions:

Fig. 1. C:N ratios of the substrates during vermicomposting. W1, W2, W3 and W4 denote inoculating density of 0, 60, 120 and 180 earthworms per kg substrate.

Fig. 2. Rarefaction curves of sequences of bacterial 16S rRNA (a) and fungal 18S rRNA (b) depicting the effect of 3% dissimilarity on the number of OTUs identified in the samplings. W0: initial mixed substrate; Wij: i denotes sampling week, and j denotes earthworm inoculating density. j ranging from 1 to 4 means inoculating density of 0, 60, 120 and 180 earthworms per kg substrate.

Fig. 3. Cluster analysis based on pyrosequencing taxonomy of bacteria (a) and fungi (b). W0: initial mixed substrate; Wij: i denotes sampling week, and j denotes earthworm inoculating density. j ranging from 1 to 4 means inoculating density of 0, 60, 120 and 180 earthworms per kg substrate.

Fig. 4. NMDS dimension analysis based on pyrosequencing taxonomy of bacteria (a) and fungi (b). W0: initial mixed substrate; Wij: i denotes sampling week, and j denotes earthworm inoculating density. j ranging from 1 to 4 means inoculating density of 0, 60, 120 and 180 earthworms per kg substrate.

Fig. 5. Composition of bacterial (a) and fungal (b) communities at genus level. W0: initial mixed substrate; Wij: i denotes sampling week, and j denotes earthworm inoculating density. j ranging from 1 to 4 means inoculating density of 0, 60, 120 and 180 earthworms per kg substrate.

Table 1 Composition of MHR

Plant species	Common name	Parts
Paeonia lactiflora Pall.	Chinese peony	roots
Glycyrrhiza uralensis Fisch.	Liquorice	roots
Panax notoginseng	Notoginseng	roots
Angelica sinensis (Oliv.) Diels	Chinese angelica	roots
Bupleurum chinens DC.	Radix stellaviae	roots
Leonurus heterophyllus Sweet	Herba Leonuri	leaves and stems
Mentha haplocalyx Brig.	Herba Menthae	leaves and stems
Taraxacum mongolicum Hand.-Mazz.	Dandelion	stems
Cassia tora L.	Semen Cassiae	seeds

Table 2

Statistics of sequence reads. W0: initial mixed substrate; Wij: i denotes sampling week, and j denotes earthworm inoculating density. j ranging from 1 to 4 means inoculating density of 0, 60, 120 and 180 earthworms per kg substrate

Treatment	Bacteria		Fungi	
	Number of Reads	Average length (bp)	Number of Reads	Average length (bp)
W0	22208	441.12	24086	267.24
W11	22208	439.8	24086	249.61
W12	22208	440.52	24086	259.73
W13	22208	442.44	24086	232.26
W14	22208	441.45	24086	225.05
W21	22208	439.32	24086	250.16
W22	22208	439.45	24086	251.88
W23	22208	439.62	24086	255.99
W24	22208	440.02	24086	263.51
W31	22208	439.3	24086	259.56
W32	22208	440.03	24086	254.97
W33	22208	439.51	24086	251.83
W34	22208	440.01	24086	254.36

Table 3

Comparison of the operational taxonomic units (OTUs) richness and diversity indices of 16S rRNA and 18S rRNA gene libraries for clustering at 97% identity. W0: initial mixed substrate; Wij: i denotes sampling week, and j denotes earthworm inoculating density. j ranging from 1 to 4 means inoculating density of 0, 60, 120 and 180 earthworms per kg substrate. Data in parenthesis are confidence intervals for indices estimators.

Treatment	Bacteria				Fungi			
	OTUs	ACE	Chao 1	H'	OTUs	ACE	Chao 1	H'
W0	1282	1506.17 (1459.39, 1565.29)	1507.50 (1449.49, 1585.61)	5.56 (5.53, 5.58)	154	187.33 (171.46, 217.63)	187.48 (168.98, 228.82)	2.61 (2.59, 2.63)
W11	1544	1818.15 (1765.75, 1882.93)	1834.50 (1766.41, 1926.05)	6.13 (6.11, 6.15)	126	182.40 (160.43, 218.38)	145.38 (134.01, 172.84)	2.53 (2.51, 2.55)
W12	1505	1789.17 (1734.77, 1856.44)	1823.30 (1748.61, 1920.88)	6.02 (6.00, 6.04)	142	177.86 (160.67, 210.90)	179.00 (158.50, 224.97)	2.26 (2.24, 2.28)
W13	1223	1570.70 (1503.60, 1653.85)	1616.43 (1522.53, 1739.76)	5.07 (5.04, 5.10)	130	168.85 (150.03, 205.33)	169.38 (147.11, 220.63)	1.89 (1.87, 1.91)
W14	1584	1836.58 (1787.75, 1897.11)	1847.25 (1784.41, 1929.80)	6.10 (6.08, 6.12)	120	272.68 (227.34, 337.18)	190.71 (152.43, 274.19)	1.47 (1.45, 1.5)
W21	1606	1847.04 (1800.07, 1905.39)	1816.98 (1765.49, 1885.11)	6.27 (6.25, 6.29)	151	190.09 (171.67, 224.95)	181.00 (164.17, 219.34)	3.13 (3.11, 3.14)
W22	1514	1776.65 (1725.67, 1839.91)	1766.43 (1705.58, 1846.61)	6.00 (5.98, 6.02)	140	171.71 (155.89, 203.26)	166.11 (150.95, 202.25)	2.19 (2.17, 2.22)
W23	1548	1870.30 (1810.48, 1943.75)	1931.14 (1843.18, 2045.32)	6.20 (6.18, 6.22)	167	193.81 (180.78, 219.16)	189.96 (176.75, 221.04)	3.00 (2.98, 3.02)
W24	1687	1926.97 (1880.46, 1984.66)	1946.51 (1883.50, 2028.00)	6.41 (6.39, 6.43)	193	242.48 (220.79, 281.11)	234.58 (213.07, 279.12)	2.49 (2.47, 2.52)
W31	1642	1908.08 (1857.53, 1970.48)	1915.39 (1851.07, 1999.50)	6.34 (6.33, 6.36)	185	240.73 (216.56, 283.42)	238.26 (211.08, 293.79)	2.73 (2.71, 2.76)
W32	1590	1845.13 (1795.59, 1906.62)	1838.69 (1779.05, 1917.14)	6.34 (6.32, 6.35)	165	206.11 (186.87, 242.29)	210.88 (185.68, 266.79)	2.74 (2.71, 2.76)
W33	1574	1869.71 (1814.07, 1938.25)	1912.77 (1833.89, 2015.58)	6.22 (6.20, 6.24)	226	278.43 (256.47, 316.21)	283.12 (254.89, 338.93)	3.47 (3.45, 3.49)
W34	1623	1863.40 (1816.02, 1922.40)	1902.91 (1835.59, 1991.54)	6.31 (6.29, 6.33)	230	275.32 (255.88, 309.37)	283.26 (256.08, 338.79)	3.32 (3.30, 3.34)

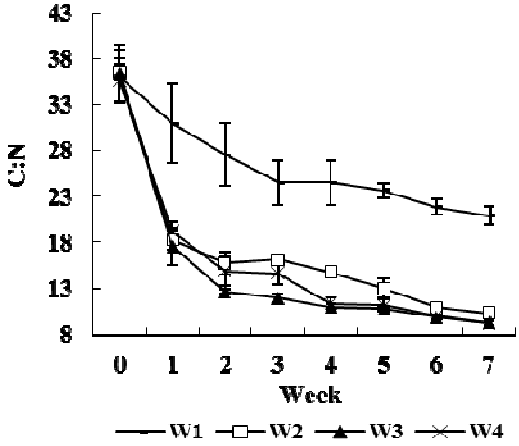
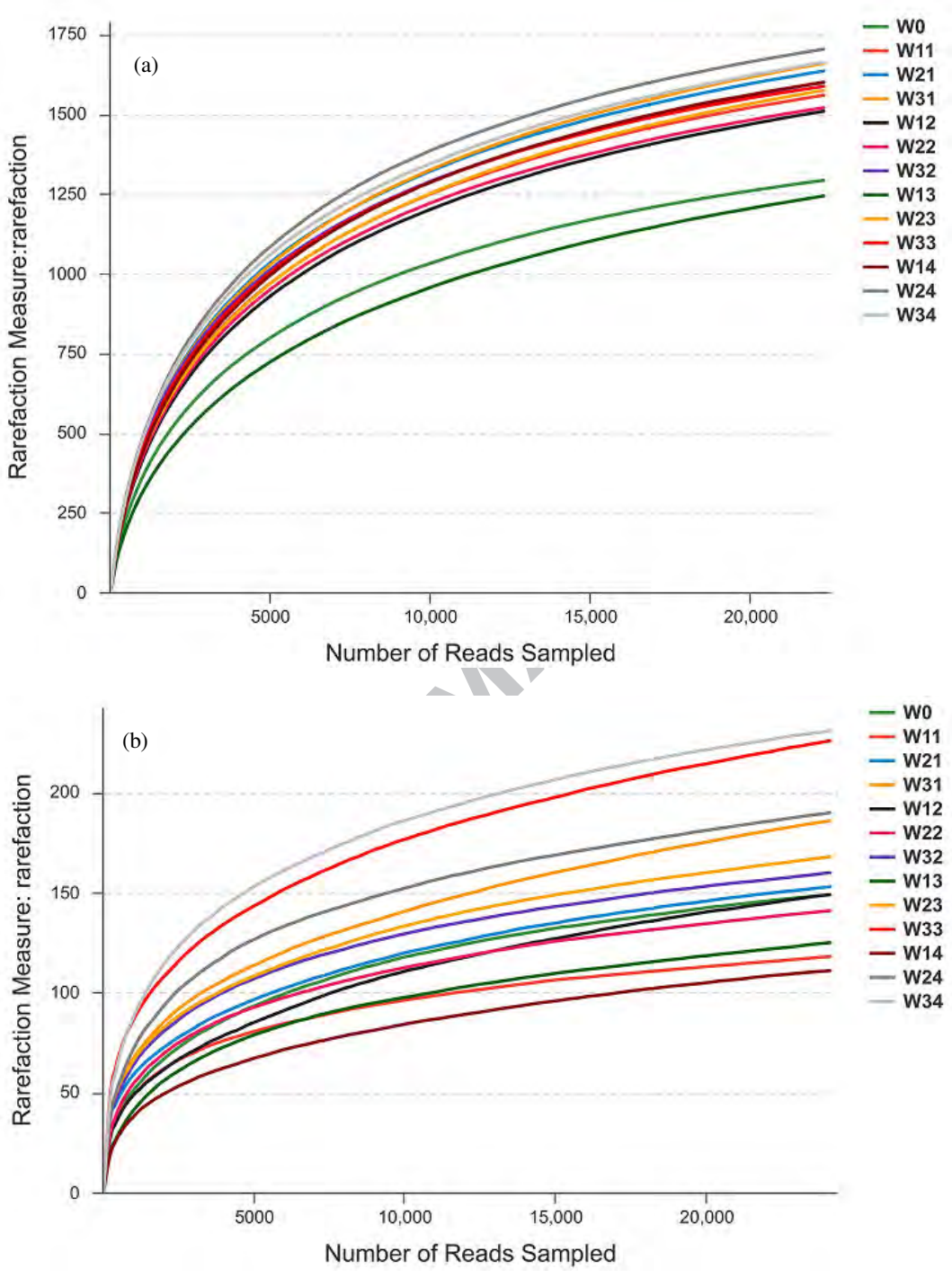


Fig. 1. C:N ratios of the substrates during vermicomposting

W1, W2, W3 and W4 denote inoculating density of 0, 60, 120 and 180 earthworms per kg substrate.



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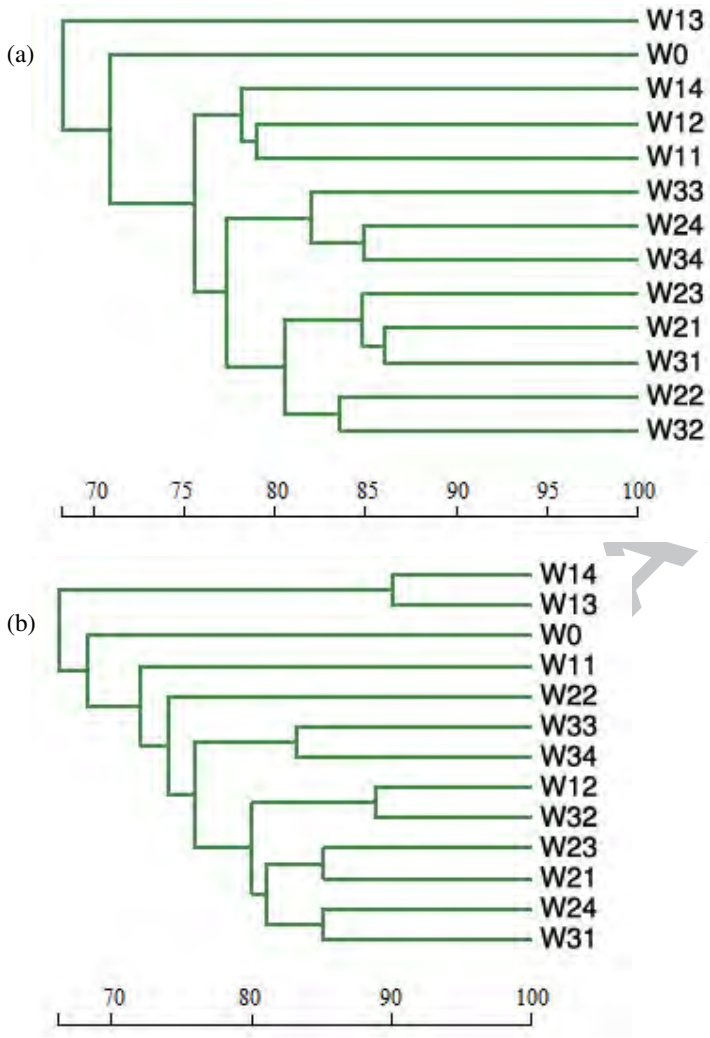

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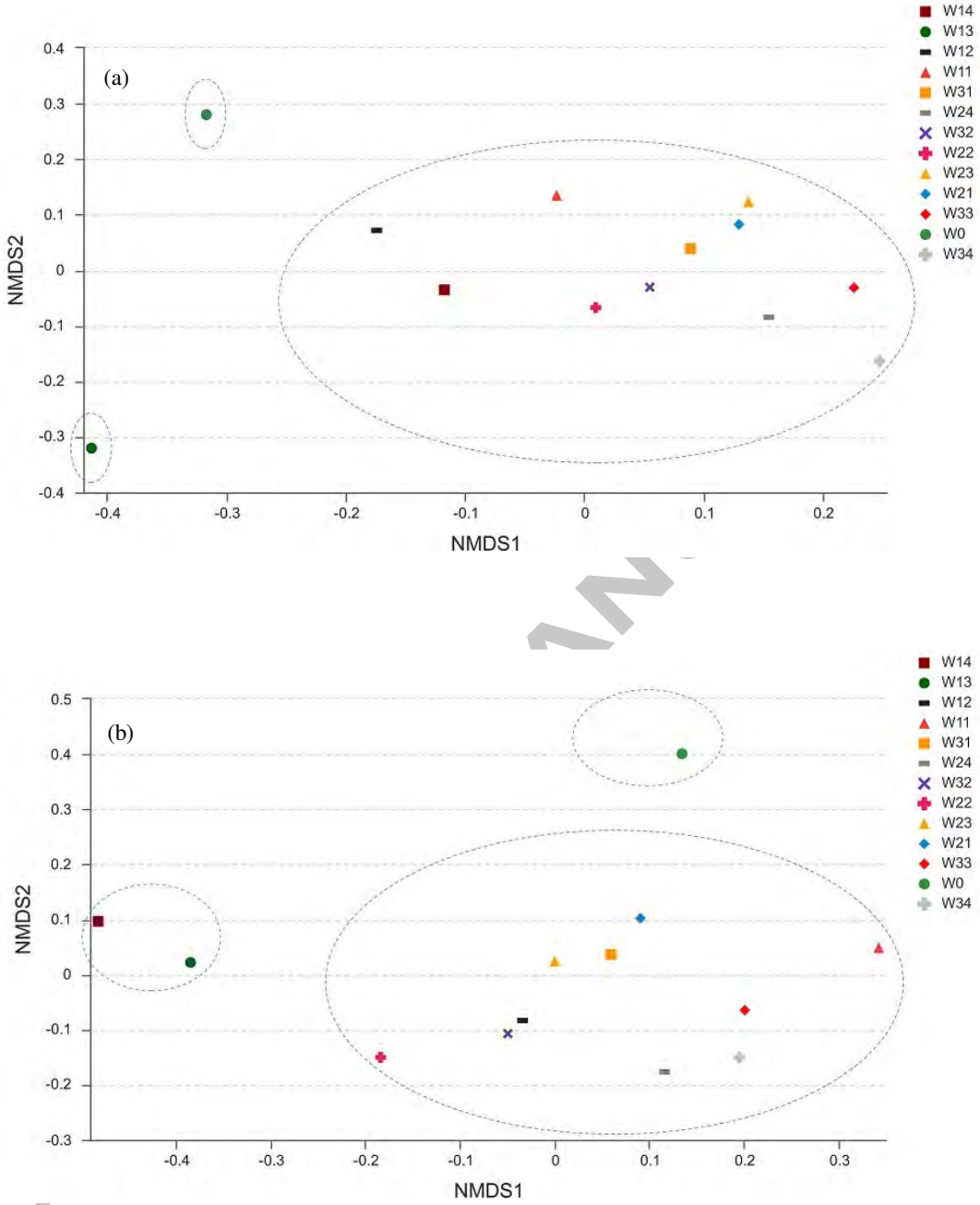

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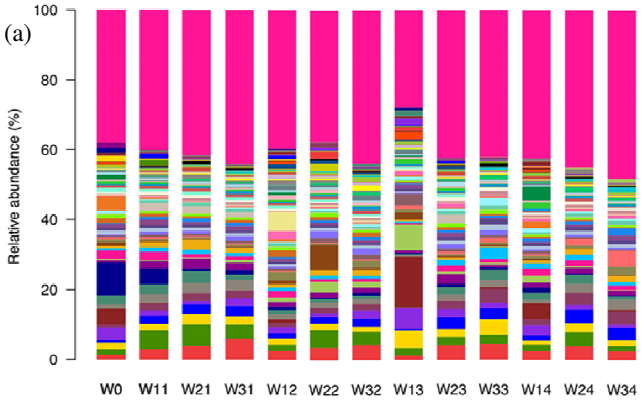
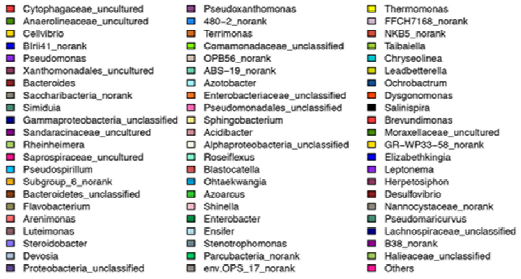
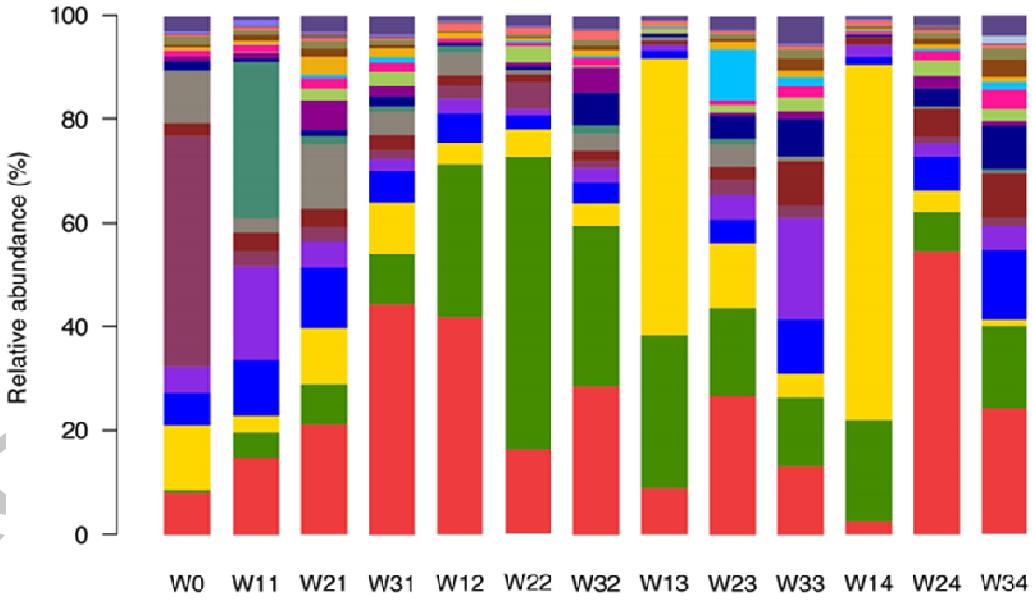
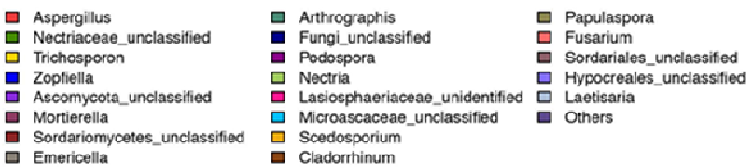
(a)



(b)







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Highlights

- Earthworms promoted the stabilization of medicinal herbal residues.
- The richness, diversity and composition of microbial community were characterized.
- Earthworms enhanced the growth of lignocelluloytic microbes.