



Effect of EM microbial agent on aerobic composting for dairy cattle manure

G. Qu¹ · Y. Cai¹ · P. Lv¹ · X. Ma¹ · R. Xie¹ · Y. Xu¹ · P. Ning¹

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Abstract

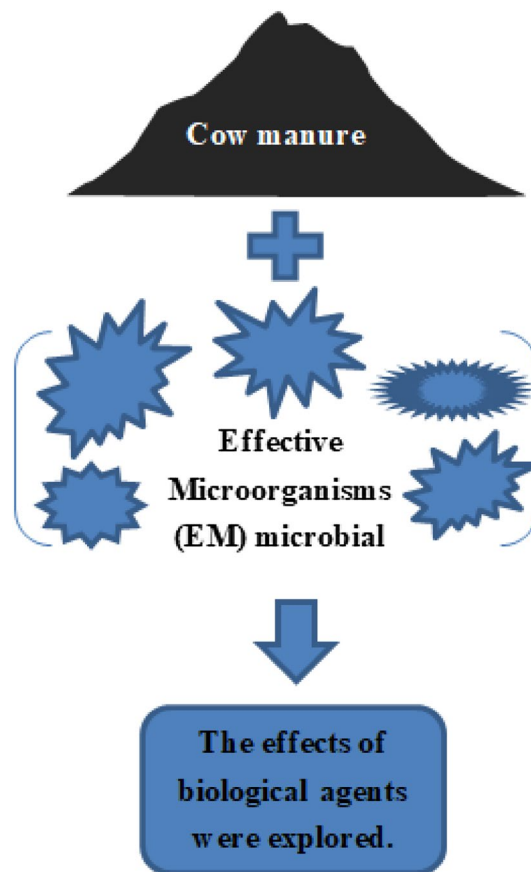
Basing to the controversy on the function of the effective microorganisms (EM) microbial agent in manure aerobic composting, the differences of physical and biomass indexes of the experimental group (EP) (manure with EM inoculant) and the control group (CK) (manure without the EM inoculant) were studied and compared during composting. The results showed that the differences of physical indexes variation between the EP and the CK were similar during the process of composting. The number of operational taxonomic units (OTU) in the EP was lower than that in the CK at the beginning, while it was on the contrary in the end. The current study suggested that the presence of the EM microbial agent did not improve the number of OTU at the initial stage and early warming stage of aerobic composting. And, it intensified the competition with indigenous microorganisms in the piles. Furthermore, no obvious difference effects were observed between the EP and the CK. Considering the composting cost, the addition of the EM bacteria was unnecessary.

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✉ P. Ning
ningpinglab@163.com

¹ Faculty of Environmental Science and Engineering,
Kunming University of Science and Technology,
Kunming 650500, Yunnan, China

Graphical abstract



Keywords Aerobic composting · Dairy manure · Diversity analysis · Effective microorganisms (EM) microbial agent

Introduction

Aerobic composting is one of the most common methods for the treatment of solid organic wastes (Gavilanesterán et al. 2016; Rynk et al. 1992). Through composting, it could realize the harmlessness and resourcefulness for livestock manure, which was one of the technologies to realize the interface between livestock breeding and planting industry (Rasapoor et al. 2016; Yamada and Kawase 2006; Zhu 2006). The traditional composting was usually used to improve compost environmental conditions or increase nutrition method decomposing organic matters by the function of indigenous microorganisms. However, due to less number of effective microorganisms in the early stage, it took much time to propagate to certain quantity of the compost required. Therefore, traditional composting

methods had a series of problems, such as long time, much lower efficiency and so on (Gotaas 1956; Inbar et al. 1993).

For the above questions, some studies found that the addition of certain EM microbial agent could increase the number of effective microorganisms, which could promote the decomposition of organic matter and accelerated the composting process so as to realize the rapid biological composting (Jishao et al. 2015; Li et al. 2013, 2014; Sasaki et al. 2006). For examples, Wang et al. (2014) researched the effect of independently developed microbial inoculum named CC-1 with high lignocellulose degradation ability on cattle manure enhanced composting. The results showed that the composting period was shortened with the addition of CC-1 or the EM inoculum. Jin et al. (Jin et al. 2010) studied the microbial agents and the optimum control parameters for pig manure composting. They chose



BN1 as microorganism agent which was selected from piggery wastes in Beijing areas. The results showed that self-made bacteria BN1 could promote rapid fermentation of pig manure compost. Gao et al. (2014) studied the effects of the cold-adapted complex microbial agents at the low temperature on the cow dung compost fermentation. The experiment was conducted under 0 °C with the cow dung and the straw as experimental materials. The results showed the cold-adapted complex microbial agents could promote the elevation of compost temperature rapidly in the low temperature and ensured the quality of the rotten of compost.

However, there were a lot of controversies on the researches. Some researches viewed that the compost material contained many microbial populations and communities, which could grow rapidly in the appropriate environmental conditions. It was not able to adapt to the composting environment in the start period with the addition of the EM microbial agent. Then it also could increase the competition with indigenous microbial in the adapt period, which caused the lack of indigenous microbial either (Zhang et al. 2016). Li et al. designed three treatments to study the effects of two kinds of microbial agents on the physical–chemical properties of the mixture of corncob and mushroom residue during composting fermentation. The results showed that these two kinds of microbial agents had no effects on the fermentation time of substrate. What is more, the cost of composting was also increased (Qian et al. 2014). Thus, the addition of the EM microbial agent was not worth considering.

The experiments above were performed in the laboratory-scale composting reactors, which could not well simulate the rich composting biomass environment. The physical index of the whole composting was under artificial control, which was difficult to simulate the environment of natural composting.

In this research, the effect of the EM microbial agent on the cow manure composting was studied through optimizing experimental conditions, which was a pilot-scale experimental and the nature composting was adopted. Changes

of physical and biomass in the composting process were analyzed in order to explore whether the EM bacteria had necessity in the treatment of the livestock waste for future.

Materials and methods

Experimental materials

The dairy cattle manure was collected from the collection station in Dengchuan Town, Eryuan County, Dali, China. Expansive agent (Iqbal et al. 2010), CaO, superphosphates (SSP) and the EM microbial agent were purchased from the market. The main conditions and properties of EM microbial agent are shown in Table 1.

Experimental operation and optimization

The research was a pilot-scale experiment. Two cow manure piles were set as the control (CK) and the experimental (EP) groups, respectively. Each pile was composed of 3 t fresh cattle manure, 1.2 t tobacco wastes, 150 kg CaO and 100 kg SSP. Twenty-five kilograms of the EM microbial agent was added into the EP. The composting piles were blended and turned, and then static composting was started. It would be turned every four days until composting ending. The specific experimental group operation is shown in Fig. 1.

Analysis methods

In the CK and EP, two cow manure piles' groups were set, respectively. The mean data values of these two were taken as the final result, respectively.

The temperature in the depth of 15 cm and 30 cm was measured by thermometer. Five points that circled around piles were selected in the half height of composting, and the thermometers were inserted in perpendicular to the piles' surface. There were five temperature data every time for every pile. The water content was determined according

Table 1 Main conditions and properties of additive microbe

EM agents	State	Color	Main agents	Effective agents
Functional agents	Solid powder	Yellowish-brown	<i>Bacillus lichenius</i> , <i>Bacillus subtilis</i> , <i>Bacillus amyloliquefaciens</i> , <i>Lateral Bacillus</i>	2×10^{10} CFU/g

Fig. 1 The specific experimental operation on cow manure composting

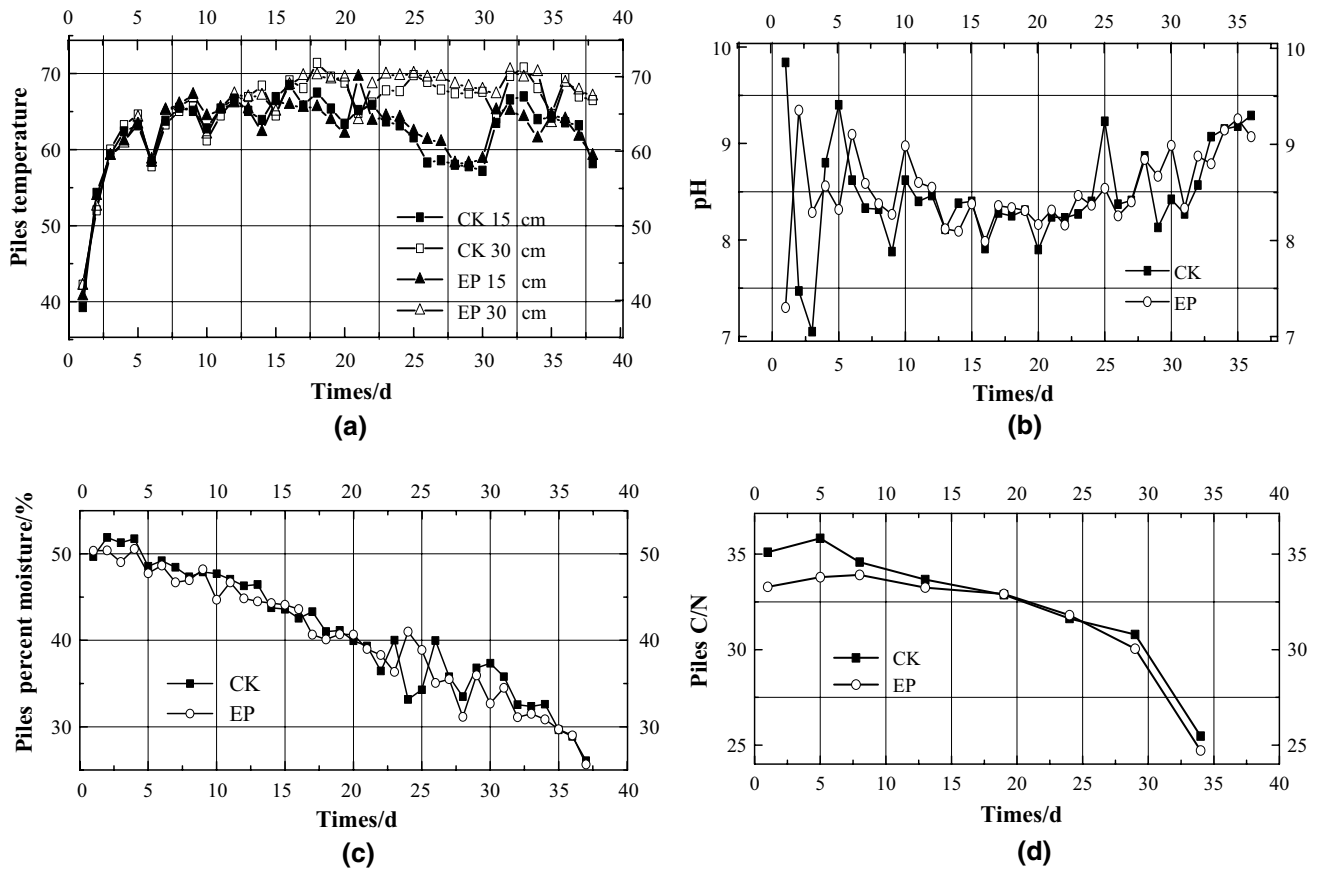
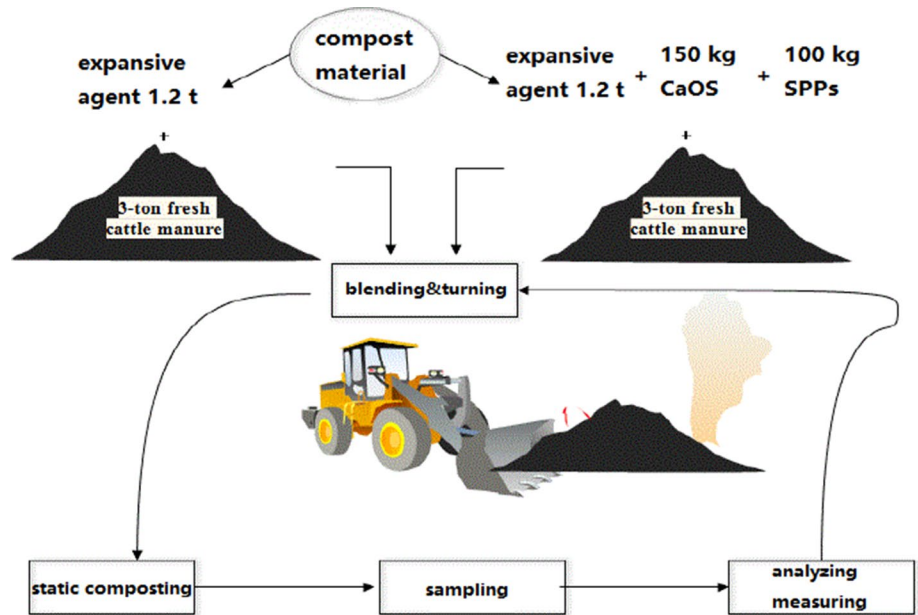


Fig. 2 Changes of temperature, pH, water content and C/N in each reactor. CK represented control group; EP represented experimental group. 15 cm and 30 cm represented the sampling depth of composting piles



to the standard of NY525-2012. The organic matters were measured by potassium dichromate capacity method. OTU change, relative species abundance and diversity of micro-organism (alpha and beta diversity) in the cow manure composting piles were analyzed by 16S rRNA gene sequencing technology (Yamada et al. 2008; Yang et al. 2017).

Results and discussion

Analysis of physical and chemical indexes

Temperature

React temperature was an indicator of microbial activity in the composting system. According to the national composting standard, the maximum temperature should be higher than 50 °C and lasted at least for 10 days or higher than 60 °C and lasted at least for 5 days that could kill all kinds of pathogens and meet the waste harmless health treatment requirement.

The heating rate (Fig. 2a) in the EP group was almost consistent with the CK group. The temperature of each pile could reach higher than 50 °C after 2 days' composting. The temperature of the pile in the depth of 15 cm was above 60 °C and lasted more than 19 days, while the temperature of the pile body in the depth of 30 cm was above 60 °C, which lasted more than 32 days. The internal average temperature in the depth of 30 cm was 5 °C higher than that of 15 cm. According to the statistical analysis, $p(15\text{ cm}) > 0.05$ and $0.01 < p(30\text{ cm}) < 0.05$, it inferred that the temperature in the depth of 15 cm had no significant difference between the EP and the CK while that in the depth of 30 cm had significant difference between the EP and the CK, which might result from turning cow manure piles constantly. The result of statistical analysis is calculated in Table 2. In the

Table 2 Significance analysis of temperature, pH, moisture and C/N in each reactor

Index	<i>F</i>	<i>p</i> value	<i>F</i> crit
<i>T</i>			
15 cm	0.097	0.757	4.105
30 cm	5.201	0.028	4.105
pH	1.045	0.314	4.130
Water content	3.014	0.091	4.113
C/N	2.015	0.084	4.121

T temperature

end of composting, the temperature in the depth of 30 cm in the case of the EP and the CK was nearly the same, which indicated that the addition of the EM agent had rare effects on the piles' temperature. From the view of the maturity state of composting combined with decomposition time, the EM bacteria effects were unapparent.

pH value

The pH values of the CK and the EP (Fig. 2b) fluctuated alternately. In the end of composting, the pH value was stable at about 9.0. In the initial stage of composting, pH value fluctuated significantly, which possibly due to insufficient mix of the CaO. Later, the pH value declined with the composting process, which might result from the volatilization of alkaline gas due to the temperature raising. Then, the pH value still increased owing to the degradation of organic matter and the production of large amounts of water-soluble ammonia at the end of composting. The pH value reflected the microbial activity in the react to a certain extent (Tran et al. 2012; Tubail et al. 2008). When the pH value was between 7.5 and 8.5, the microbial growth rate was the fastest and the maximum composting efficiency would be got (Sánchez-Monedero et al. 2001). Combined with the difference significance analysis, $p(\text{pH}) > 0.05$ indicated that there was no significant difference in the pH value between the EP and the CK at the end of composting. Therefore, the addition of EM bacteria was unnecessary.

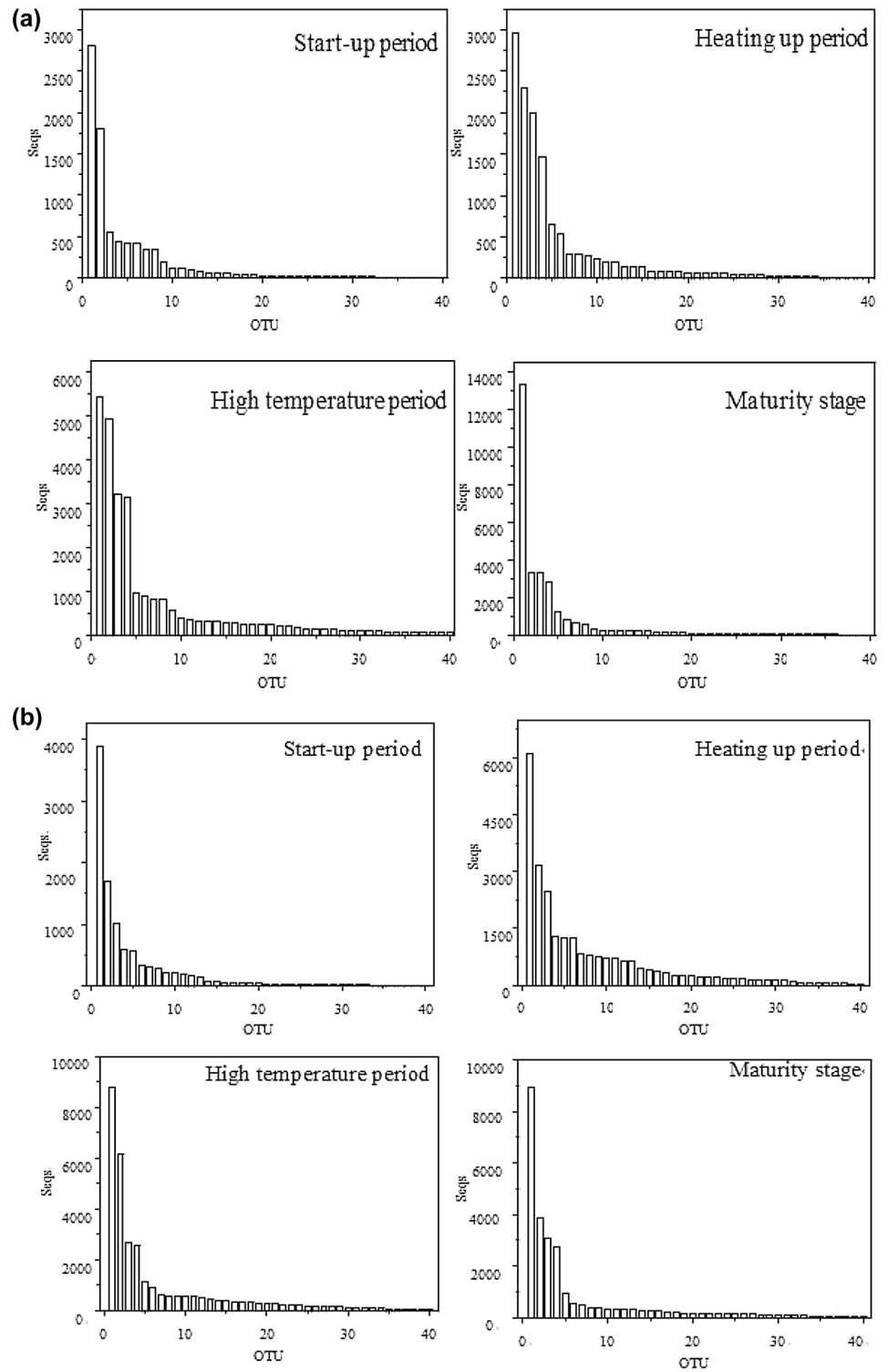
Water content

Water content was another important parameter that affected the composting. The change of the water content during composting was related to the increasing rate of heating period and duration of high-temperature period. The moisture contents (Fig. 2c) of the piles had a little fluctuation. Within 35 days, the moisture of each decreased to 30%. According to the analysis of difference significance, $p(\text{water content}) > 0.05$ indicated that there was no significant difference in the water content between the EP and the CK at the end of composting. Thus adding EM bacteria agent was unnecessary.

C/N ratio

C/N ratio was a more relatively intuitive for evaluating maturity of compost. In the composting process, the C/N ratio was falling continuously and it was generally believed that when the C/N ratio of composting products was close to 20, compost could be considered as basically mature (Gu

Fig. 3 The changes of OTU in the four periods. **a** Changes of OTU in the four periods during CK piles. **b** Changes of OTU in the four periods during EP piles



et al. 2015; Zhang 2013; Zhang et al. 2015). The C/N ratio (Fig. 2d) of the CK was slightly higher than that of the EP

in the start-up and early stage of temperature heating phase. However, the C/N ratio of the CK and the EP was close to 25



during 35 days' composting. The results indicated that there was no significant difference between the EP and the CK. Therefore, adding EM bacteria was superfluous.

Analysis of biomass change

OTU

Operational Taxonomic Units (OTU) might represent a specie (Fang et al. 2013). The changes of 40 kinds of OTU in the CK and the EP were carried out by 16S rRNA gene sequencing technology analysis.

In the EP, there were 28 kinds of OTU increased while only 12 were reduced among the 40 kinds of OTU during the total compost cycle compared with the CK. The changes of OTU between the EP and the CK are shown in Fig. 3(1) and (2). Among the 28 species of OTU increased, 13 kinds of them continuously increased and 15 kinds of them increased firstly then decreased. Of the 12 species of OTU decreased, 6 kinds of them continuously decreased and 6 kinds of them decreased firstly then increased. It could be predicted that 13 species of microbial species increased continuously with composting, which were mostly resistant high-temperature bacteria, like *Thermophilic* and *Actinomycetes*. Fifteen kinds of the OTU increased firstly then decreased subsequently, which were mostly the medium-temperature bacteria. There were a large number of medium-temperature bacteria breeding in the process of piles' temperature heating up. When accessing the high-temperature period, it could not stand the high temperature then gradually decreased, but it was just slightly increased compared to the start-up period. The six species of OTU continued to decrease which were presumed to be the original cryogenic bacterial species in the fecal mass. As composting continued, the temperature of the heap continued rising resulting low-temperature organisms in a gradual decrease.

The six species of OTU decreased firstly then increased which were most likely belong to the EM bacteria added in the start period. In the rapid initiation period, the slight decreasing of the EM bacteria was due to unadaptation of the fecal heap environment. It began to reproduce and quickly consumed the organic matter in the heap until it was domesticated. When the organic matter was completely depleted in the maturity period, the microbial species in the EP were slightly higher than those in the CK. The changes of

the OTU number had no significant difference in composting of the EP and the CK at the end of the composting.

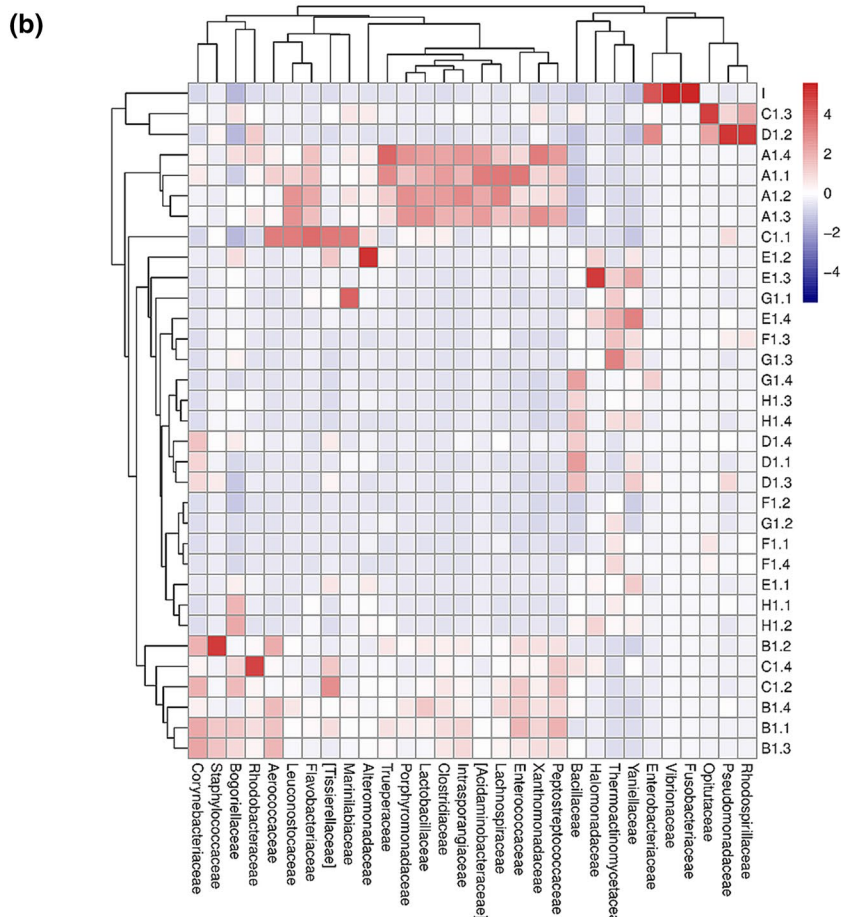
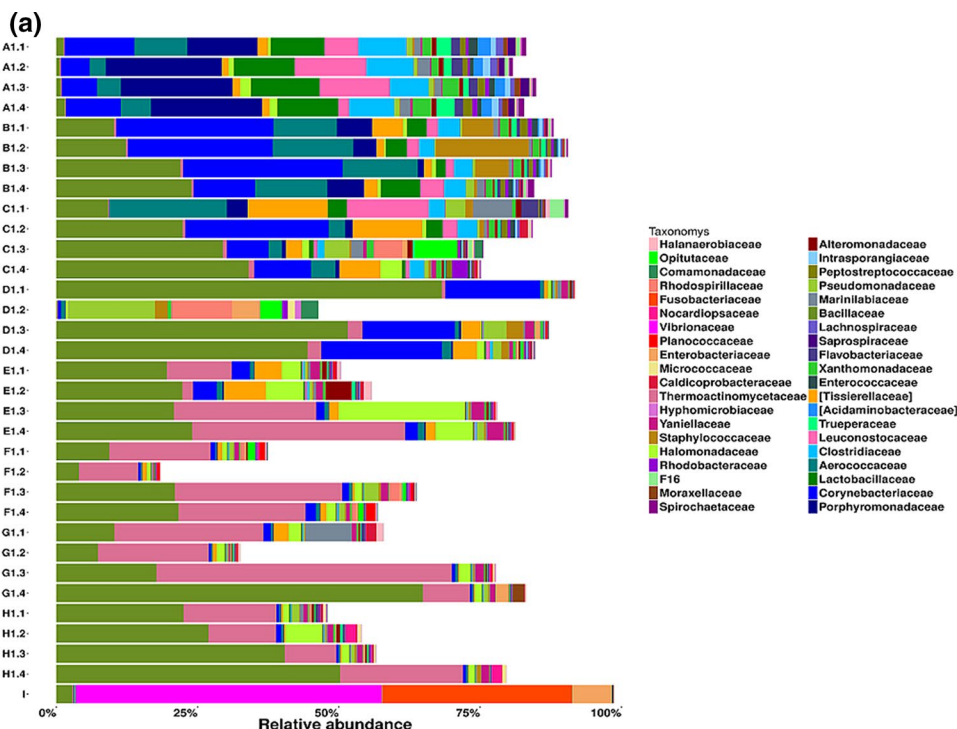
Microbial population relative abundance

In general, the microbial aerobic respiration and metabolism gradually increased at the start-up period and the energy was released continuously to improve the temperature of the compost (Boldrin and Christensen 2010; Budzikiewicz 2004).

In the start-up period of composting, *Corynebacteriaceae*, *Lactobacillaceae*, *Leuconostocaceae* and *Clostridiaceae* were dominant micro floras (Fig. 4a). The kinds of bacteria in the EP and the CK were basically same. The EM microbial agent strains were not detected in the start-up period of the EP, which might result from their unadaptation of the fecal heap environment and the microbial activity had not been activated. At the initial stage of warming, the number of *Pseudomonadaceae*, *Lactobacillaceae*, *Aerococcaceae* and *Staphylococcaceae* increased. The number of *Pseudomonadaceae* in the EP was higher than that in the CK. *Pseudomonadaceae* was one of the main species among the EM microbial agent, which suggested that the EM microbial agent competed with indigenous microorganisms in composting piles. *Pseudomonadaceae* also adapted to the composting environment and propagated as advantage bacteria group rapidly.

During the high temperature and maturity period, *Pseudomonadaceae* and *Thermoactinomycetaceae* were dominant bacteria. *Pseudomonadaceae* belonged to heterotrophic bacteria, which was strictly aerobic respiration and never fermentation. Its content reflected the extent of aerobic compost as well (Budzikiewicz 2004; Palleroni 1992). *Pseudomonadaceae* and *Thermoactinomycetaceae* in the EP were slightly more abundant than those in the CK, which reflected that the degree of aerobic composting in the EP was slightly better than that of the CK. This might be the harsh temperature conditions, which made *Pseudomonadaceae* and *Thermoactinomycetaceae* rapidly propagation and to be the dominant bacteria. The EM microbial agents were mainly *Vibrionaceae* and *Fusobacteriaceae*, and also contained a small amount of *Pseudomonadaceae* and *Enterobacteriaceae* according to the results of analysis on the relative abundance of species. This was generally consistent with the species given in Table 1. However, the above bacteria did not participate in composting process except *Pseudomonadaceae*. *Pseudomonadaceae* as indigenous strains

Fig. 4 Analysis of relative abundance of species. **a** Relative abundance of species at family level (1.1/1.2 as the control group, 1.3/1.4 as the experimental group; a represents the start-up phase samples, **b–d** on behalf of the heating period samples, **e–g** on behalf of the high-temperature samples, **h** on behalf of the maturity period of samples, **i** represents microbial agent). **b** Relative abundance heat map of the top 30 species at family level (the longitudinal cluster represents the sample clustering tree; the horizontal is a species clustering tree, and the Z-score method is used to standardize the relative abundance values)



also existed in the CK. As composting was carried out, it rapidly multiplied into dominant strains as well. Therefore, the EM bacteria mainly supplied *Pseudomonadaceae* in the composting process.

Heat map was a kind of graph to represent the data matrix in the numerical color gradient size according to the species abundance or abundance information or sample similarity of a graph clustering (Tiessen et al. 2017). The relative abundance of species at different levels was sorted and the top 30 species were clustered at the family level (Fig. 4b). In the start-up period, *Peptostreptococcaceae*, *Xanthomonadaceae*, *Enterococcaceae*, *Lachnospiraceae*, *Acidaminobacteraceae*, *Intrasporangiaceae*, *Clostridiaceae*, *Lactobacillaceae*, *Porphyromonadaceae* and *Trueperaceae* were the dominant bacteria clusters. The clustering of *Enterococcaceae*, *Lachnospiraceae* and *Acidaminobacteraceae* in the CK was more dominant than those in the EP, indicating that these might be the indigenous flora in the heap. In the early heating period, the clustering of *Marinilabiaceae*, *Tissierellaceae*, *Flavobacteriaceae*, *Leuconostocaceae*, *Aerococcaceae* and *Staphylococcaceae* in the CK were superior to those in the EP, which might be related to the competition between the EM bacteria agent and indigenous microbial in the EP. During the heating period, *Opitutaceae*, *Rhodospirillaceae* and *Pseudomonadaceae* expressed advantage clustering firstly in the EP. Among them, *Pseudomonadaceae* was the main strain in the EM microbial agents, indicating that the EM microbial agents began to multiply and participate in the competition. In the high temperature and maturity period,

the advantage clustering of *Yaniellaceae*, *Thermoactinomycetaceae* and *Halomonadaceae* were main bacteria groups, which mainly existed in the EP. Due to the high-temperature screening among strains after competition, these three kinds of bacteria survived and multiplied. However, in the compost period, the bacteria above did not participate in the composting process, thus the EM bacteria were superfluous.

Diversity analysis

Alpha diversity Alpha diversity was the biological diversity within the sample, which was not related to other samples. The alpha diversity of microorganism, the parameters of calculation were included PD_whole_tree, Chao1, observed_species, Shannon, Simpson and so on (Adams and Frostick 2009). The Chao1 and the observed_specie indexes reflected the richness of community in the sample, which simply referred to the number of species (number of OTU), nor to the abundance of each species in the community. The greater the numerical, the more abundant species were in samples. In the initial and rising stage, the index of the Chao1 and the observed_specie in the EP were larger than those in the CK, indicating the more abundant community abundance in the EP due to the introduction of the EM bacteria. With the composting going, the number of Chao1 and observed_specie index in both EP and CK decreased, which might be due to the increasing temperature for strains to being screened. At the end of composting, both the Chao1

Table 3 Statistical results of Alpha diversity parameters

Sample ID	Group	PD whole tree	Chao1	Observed_species	Shannon	Simpson
A	CK	121	1852	1276	7.07	0.97
	EP	122	2050	1294	6.95	0.96
B	CK	104	1802	1062	6.34	0.95
	EP	107	1886	1087	6.65	0.94
C	CK	76	1323	787	5.94	0.95
	EP	71	1147	684	6.29	0.96
D	CK	46	713	410	5.57	0.91
	EP	53	674	456	5.28	0.92
E	CK	71	1271	745	5.62	0.92
	EP	60	1023	613	5.08	0.89
F	CK	54	717	461	3.98	0.79
	EP	58	694	464	4.81	0.75
G	CK	55	951	546	4.41	0.83
	EP	51	842	483	3.98	0.75
H	CK	62	884	549	4.97	0.91

“A” represents the start-up phase samples; “B, C, D” on behalf of the heating period samples; “E, F, G” on behalf of the high-temperature samples; “H” on behalf of the maturity of sample



Fig. 5 Chart of PCoA and NMDS (Unweight and Weighted Unifrac). **a** Chart of PCoA under Unweighted Unifrac. **b** Chart of PCoA under Weighted Unifrac. **c** Chart of NMDS under Unweighted Unifrac. **d** Chart of NMDS under Weighted Unifrac (1.1/1.2 as the control group, 1.3/1.4 as the experimental group; A represents the start-up phase samples, B–D on behalf of the heating period samples, E–G on behalf of the high-temperature samples, H on behalf of the maturity of samples)

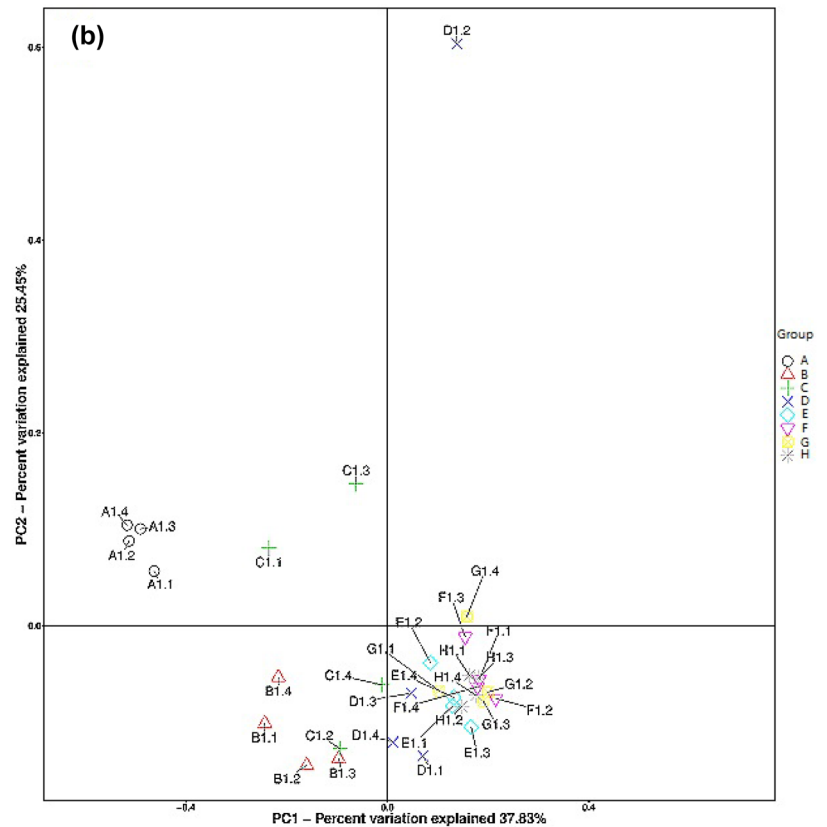
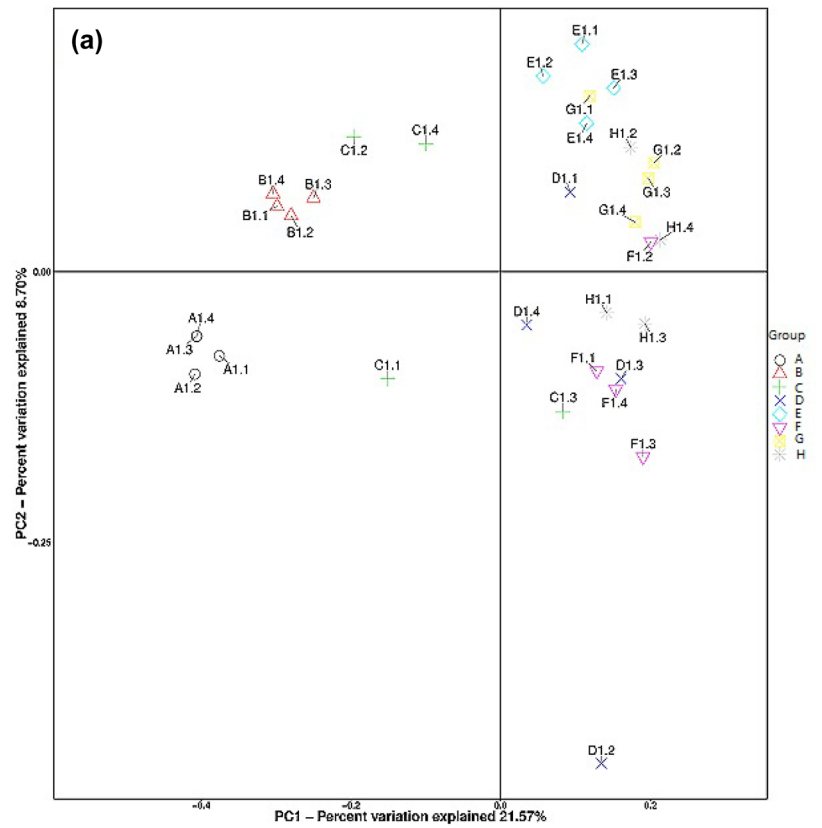
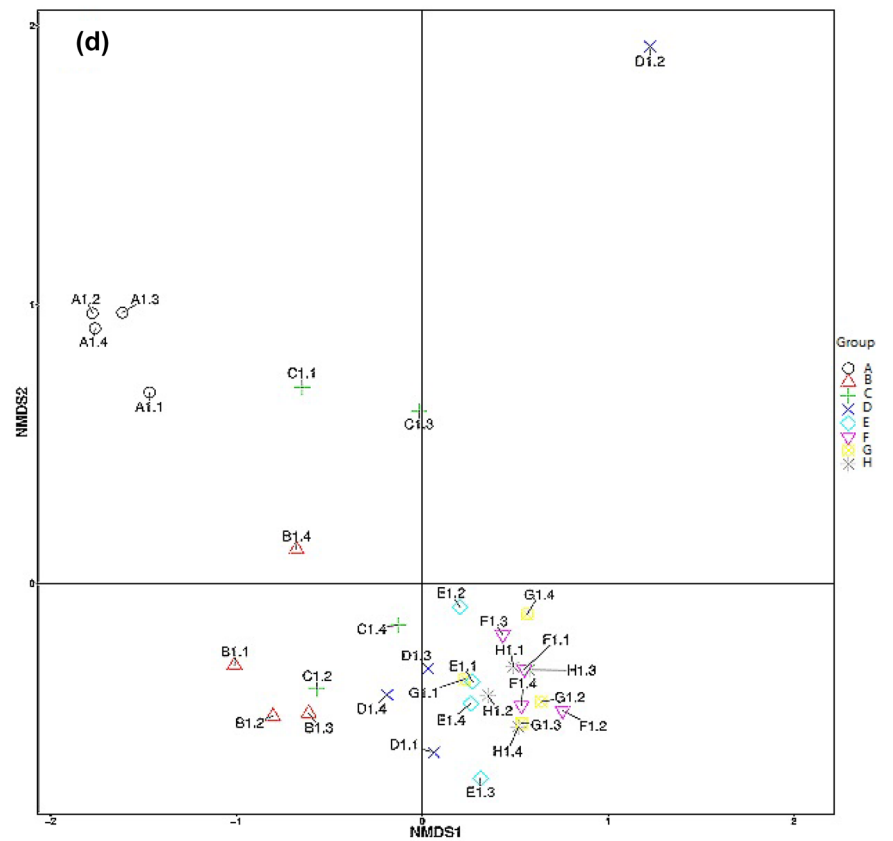
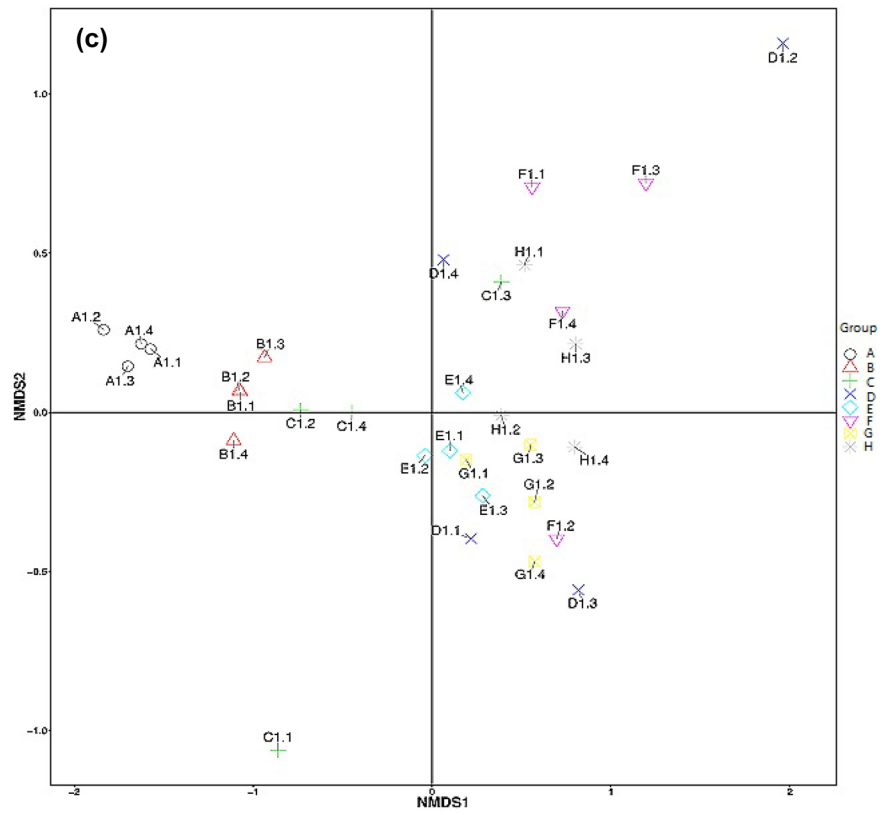


Fig. 5 (continued)



and the observed_specie index of the CK were greater than those in the EP. The results of relative abundance of species indicated that the EM bacteria failed to increase the strains but competed with indigenous microorganisms increasingly in the reaction. The colony abundance in the EP was less than that in the CK, so the EM bacteria were superfluous.

At the initial stage and the early heating period of composting, as shown in Table 3, the PD_whole_tree and Shannon index in the EP were slightly larger than those in the CK while the Simpson index was smaller in the CK. This was related to the EM bacteria added at the initial stage, which made the diversity of the EP be better than the CK. With the composting going, the number of PD_whole_tree and Shannon index in both group decreased, which might be due to the increasing temperature. At the end of composting, both PD_whole_tree and Shannon index of the CK were greater than those in the EP, which might be because the EM bacteria failed to increase the strains but increased competition with indigenous microorganisms in the reaction. The colony abundance in the EP was lower than that of the CK, so the EM bacteria were superfluous.

The PD_whole_tree, chao1, observed_species, Shannon and Simpson index in the EM bacteria were smaller than those of the indigenous microorganism in any pile of any stage, which indicated that the richness and diversity of the community in the EM bacteria were not as good as those of the indigenous microorganism in piles. However, PD_whole_tree and Simpson index of the EM bacteria indicated that the community diversity was also good. With the addition of the EM bacteria, the community diversity of the EP was not greatly improved in the initial stage of composting. Instead, as the compost was done, the diversity of species in the EP alternated smaller than that of the CK. As a result, the addition of the EM bacteria was unnecessary.

Beta diversity Beta diversity was the comparison of biological diversity among samples, which had no related to other samples. Commonly the Unifrac methods and Nonmetric Multidimensional Scaling (NMDS) were used to calculate Unweighted or Weighted matrices (Alotaibi et al. 2014; Zhu and Yu 2009). Then, according to this matrix, the analysis of PCoA and NMDS were carried out. The difference between individuals or groups can be observed through PCoA. The closer the distance between two samples on the axis, the more similar the two samples were.

As shown in Fig. 5a, the PCoA diagram of the sample was analyzed under Unweighted. In the initial stage and the early stage of the heating period, the composition of four samples groups had few differences. However, there were some differences on piles between the initial and the early heating stage period. As the composting continued, not only

the composition of the EP and the CK samples had differences, but each pile' sample also showed a great difference in the late heating period of composting, which might be associated with uneven sampling.

As shown in Fig. 5b, the PCoA diagram of the sample was analyzed under Weighted. Similar to those under Unweighted. In the initial and the early heating stage period of composting, the composition of four samples groups had few differences. However, there were some differences between the initial period and the early stage heating period. With the composting, the difference between the late heating period and the whole high-temperature period of compost was not obvious. However, the differences of the sample composition between the EP and the CK were significant. In the maturity period, the sample composition of the EP and the CK was gradually smaller because of the shorter distance between piles' samples. Considering the maturity time, cost and fertilizer efficiency, the addition of the EM bacteria was superfluous.

NMDS was characterized by the information contained in the sample, which reflected in the multidimensional space in the form of points. The degree of difference between different samples was shown by the distance among points and finally the spatial location map of the sample was obtained.

As shown in Fig. 5c, the NMDS diagram of the sample was analyzed under Unweighted. Similar to that of the PCoA diagram under Unweighted, the samples distance between the EP and the CK was adjacent in the initial and the early heating stage period of composting. However, there was a certain distance between samples in the initial and the early heating stage period of composting indicated that there was a slight difference between the EP and the CK. With the composting, in the late heating period of compost, not only the differences of sample composition in the EP and the CK differences but each pile's sample was also a significant difference, which was consistent with the analysis results of the PCoA under Unweighted.

As shown in Fig. 5d, the NMDS diagram of the sample was analyzed under Weighted. In the initial and the early heating stage period of composting, the samples distance between the EP and the CK was closer. Then, there was a certain distance between the initial stage and the early heating period stage of composting, indicating that there was a slight difference between the EP and the CK. As the composting was continued, the distance between samples in the heating and whole high-temperature composting period was not obvious. What is more, the distance between the EP and the CK was not significant, which indicated that the sample composition on each period and groups was similar in the end of composting. Considering the maturity time, cost and fertilizer efficiency, the addition of EM bacteria was superfluous. The results were consistent with the analysis of the PCoA under Weighted.



Conclusion

The effect of adding EM bacteria was unobvious in the composting process. According to the statistical analysis, the temperature, pH value, moisture and C/N ratio almost satisfied $p > 0.05$, which indicated that there was little significant difference between the EP and the CK. And the results of experiment also showed that the physical and biomass index of the EP and the CK were almost similar at the end of composting. The changes of 40 kinds of OTU in the CK and the EP were analyzed by 16S rRNA gene sequencing technology analysis, which showed that the number of OTU in the EP was slightly less than that in the CK in the end. There was almost few EM bacteria participation except *Pseudomonadaceae* and the alpha diversity of the EP was not significantly improved in initial stage of composting. *Pseudomonadaceae* as indigenous strains also existed in composting piles. The PCoA and NMDS matrices under Unweighted and Weighted were analyzed, and the results of the CK and the EP were similar at the end of composting. Considered of the entire composting time, cost and efficiency, the addition of the EM bacteria was slight significance.

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