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Diversity and dynamics of the bacterial community involved in pig manure biodegradation in a microbial fermentation bed system

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Abstract Overproduction of livestock manures with unpleasant odors causes significant environmental problems. The microbial fermentation bed (MFB) system is considered an effective approach to recycling utilization of agricultural byproducts and pig manure (PM). To gain a better understanding of bacterial communities present during the degradation of PM in MFB, the PM bacterial community was evaluated at different fermentation stages using 16S rRNA high throughput sequencing technology. The heatmap plot clustered five samples into short-term fermentation stage of 0–10 days and long-term fermentation stage of 15–20 days. The most abundant OTUs at the phylum level were *Firmicutes*, *Actinobacteria* and *Proteobacteria* in the long-term fermentation stage of PM, whereas *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* predominated in the short-term fermentation stage of PM. At the genus level, organic degradation strains, such as *Corynebacterium*, *Bacillus*, *Virgibacillus*, *Pseudomonas*, *Actinobacteria*, *Lactobacillus*, *Pediococcus* were the predominate genera at the long-term fermentation stage, but were found only rarely in the short-term fermentation stage. C/N ratios increased and the concentration of the unpleasant odor substance 3-hydroxy-5-methylisoxazole (3-MI) decreased with prolonged period of fermentation. Redundancy analysis (RDA) demonstrated that the relative abundance of *Firmicutes*, *Actinobacteria*, *Acidobacteria* and *Proteobacteria* had a close relationship with degradation of 3-MI and increasing C/N ratio. These results provide valuable additional information about bacterial community composition during PM biodegradation in animal husbandry.

Introduction

The large quantities of animal manures produced in the livestock industry lead to serious environmental problems (Ni et al. 2012). Pig manure (PM)—a major source of livestock manure—causes extensive concern (Chadwick et al. 2015). The disposal of PM attracts public attention worldwide. Intensive livestock production is the main cause of the release of 3-hydroxy-5-methylisoxazole (3-MI, CAS: 83–34-1) to the environment. 3-MI (also referred to as skatole) is commonly found in feces and sewage, and is formed by the bacterial degradation of tryptophan (Jensen et al. 1995; Nakai et al. 1999). Skatole has an unpleasant odor, and leads to extensive pollution due to its persistence, mobility and potential health impacts (Deslandes et al. 2001), with 3-MI derivatives being highly toxic to microorganisms and animals, as well as harmful to the pork industry (Arora et al. 2015). There is a need for environmentally sustainable and cost-effective approaches to treatment of PM for pig rearing. A new type of PM treatment facility using a microbial fermentation bed system (MFB system) developed by author's research team has obtained satisfactory results (Zheng et al. 2011).

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The MFB system, also known as the deep-litter-system, breeding pig on litter, or in situ decomposition of PM, comprises deep-bedded groove structures on which the animals are raised on a thick layer of litter (Tam 1995; Morrison et al. 2007; Neumann et al. 2015). A large scale MFB equipped with an automatic control system can raise more than 2000 pigs at a time, and is also a productive system for decomposing PM (Liu et al. 2011). The pig feces are mixed with the litter to decompose, without waste emission or spread of odors (Menalled et al. 2005; Rashad et al. 2010; Karadag et al. 2013; Costa et al. 2015). In large scale MFB on pig farms, microorganisms such as bacteria, fungi and actinomycetes play pivotal roles in the degradation of pig feces and composting process (López-González et al. 2014). Ubiquity in the environment, metabolic versatility, and the ability to adapt to extreme conditions were the major factors influencing the effectiveness of bacteria in compost (Liu et al. 2011; Li et al. 2013). Arthurson (2011) reported that bacterial communities in fresh manure were different from those in manure stored in deep pits. However, the structures of bacteria community of PM in the MFB system remain obscure.

The main objective of this investigation was to gain a better understanding of the dynamics of bacterial communities in PM under in situ fermentation in the MFB system, and provide appropriate information for biodegradation of PM. In the light of the above considerations, bacterial community dynamics at different fermentation stages in the MFB system were evaluated using 16S rRNA high throughput sequencing technology, along with physicochemical analysis of organic carbon, nitrogen, phosphorus, and potassium concentrations, and odor substance (3-MI).

Materials and methods

Sample collection and treatment

The MFB-equipped piggery was located in a farm in Fuqing County, Fujian Province, China. The breeding area of the fermentation bed was 2000 m^2, with 1800 pigs. The mattress materials comprised agricultural byproducts, including otherwise worthless mushroom-planted, mash and rice-husk materials. The depth of the mattress was 0.8 m. The temperature and humidity were controlled at 28–32°C and 70–90%, respectively. Generally, it is necessary to plow the surface litters once every 2–3 days, and the pigs' daily rooting behaviors also help to loosen the litter.

PM at different phases was collected from the MFB by the five-point sampling method. Fresh PM (PM1), and PM at fermentation times of 5 days (PM2), 10 days (PM3), 15 days (PM4) and 20 days (PM5) were collected at depths from the surface down to 20 cm. A part of the samples was pooled and homogenized for detection of bacterial diversity. The other part was prepared for basic litter properties tests. Total nitrogen (N), total carbon ©, phosphorus (P), and potassium (K) concentrations are shown in Table 1.

HPLC analysis

Samples (2 g) were dissolved in 10 mL methanol in 50 mL sterilization tubes, and the mixture was vortexed for 30 s, then transferred to a 40 °C water bath for 20 min, blended every 5 min. After centrifugation at 12,000 g for 15 min, the supernatant was stored at 4 °C prior to HPLC analysis by Agilent Technologies 1100 HPLC (Palo Alto, CA). Standard 3-MI (Sigma, St. Louis, MO) was dissolved in methanol. After passing through a 0.22 μm-pore filter (Millipore, Bedford, MA), 10 μL filtrate was subjected to the reversed phase HPLC C18 column (4.6 × 50 mm, 5 μm, Welch, Shanghai, China). The column was eluted with solutions of acetonitrile and Milli-Q water: 0–3.5 min, 40%; 4.5–9.0 min, 75%; 11.0–15 min, 40% at a flow rate of 1 mL min^{-1}. The eluate was monitored at 270 nm (Dehnhard et al. 1991).

DNA extraction and sequencing

Community genomic DNA from samples was extracted using the cetyltrimethyl ammonium bromide (CTAB) method. The concentration and purity of genomic DNA was monitored by 1% agarose gels. The V4 hypervariable regions of the 16S rRNA genes were amplified using primers 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′- GGA CTA CHV GGG TWT CTA AT-3′). All PCR were carried out in 30 μL reactions with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Waltham, MA), including 0.2 μM forward and reverse primers, and 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min. All the PCR reactions were done in triplicate, and then an equal volume of 1× loading buffer (contained SYB green) was mixed with the PCR products. Samples with a bright main band between 400 bp and 450 bp were purified with a GeneJET Gel Extraction Kit (Thermo Scientific) for further experiments. The purified PCR amplicons were sequenced using the Illumina Miseq Platform at Novogene Bioinformatics Technology (Beijing, China).

Paired-end reads from the original DNA fragments were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/ FLASH/) to get raw tags. Qualities filtering on the raw tags were performed according to the QIIME (V1.7.0, http://qiime. org/index.html) quality control process. Chimeras were detected and removed using USEARCH software based on the UCHIME algorithm (http://www.drive5.com/usearch/ manual/ uchime_algo.html).

Table 1 The basic properties of pig manure (PM) samples in a microbial fermentation bed (MFB) system. *TC* Total carbon, *TN* total nitrogen

Sample ID	Fermentation time (d)	TN (%)	TC (%)	C/N	Phosphorus (%)	Potassium (%)	Skatole (µg g^{-1})[a]
PM1	0	2.39	24.8	10.4	2.17	1.89	4.2
PM2	5	2.15	24.0	11.2	3.10	1.60	2.7
PM3	10	2.09	24.8	11.9	2.51	2.20	1.6
PM4	15	2.15	24.9	12.8	2.23	1.90	1.0
PM5	20	1.95	31.1	18.4	1.69	1.63	0.7

[a] 3-Hydroxy-5-methylisoxazole (3-MI)

Bioinformatics analysis

Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs) by UPARSE software (UPARSE v7.0.1001, http://drive5.com/uparse/). Taxonomic information for each OTU was annotated by the RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/). The taxon abundance of each sample was generated into phylum, class, order, family, and genera levels. All analyses from clustering to alpha (within sample) and beta diversity (between samples) were performed with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Weighted UniFrac was employed for principal component analysis (PCA). Hierarchical clustering of samples was completed using UPGMA. Venn diagram, heatmap and redundancy analysis (RDA) were constructed by Hierarchical clustering. The sequences were deposited in the sequence read archive (SRA), under accession numbers SRR4473783, SRR4473830, SRR4473831, SRR4473832 and SRR4473833.

Results

Physicochemical properties of the manure related to the fermentation stages in the MFB system

The physicochemical properties of the manure samples are listed in Table 1. Organic matter, total nitrogen and phosphorus were different in PM exposed for different times. The C/N ratios increased from 10.4 (PM1) to 18.4 (PM5) with fermentation of PM. Concentrations of phosphorus and potassium varied among these samples. PM2 and PM3 had the highest content of phosphorus (3.10%) and potassium (2.20%), respectively. PM5 had the lowest content of phosphorus (1.69%) and potassium (1.63%). A gradient levels of skatole appeared in those samples, ranging from 4.2 µg g^{-1} to 0.7 µg g^{-1}. Among them, PM4 and PM5 had low concentrations of skatole.

Bacterial diversity and richness

After quality and chimera filtering, a total of 315,761 valid reads were obtained from five samples, with number of sequencing reads ranging from 57,239 (PM4) to 76,417 (PM1) at 3% distance (Table 2). PM1 has the highest value of OTUs (2011), followed by PM2 (1760), PM3 (1758), PM4 (1599) and PM5 (1599). All rarefaction curves tended to approach a plateau, suggesting that the sequencing depth for all samples was reasonable to cover bacterial community diversity (Fig. 1). The alpha diversity including Shannon, Simpson, Chao1 and ACE index could reflect the variation in microbial diversity among those samples. The Shannon indexes of PM1, PM2, PM3, PM4 and PM5 were 8.177, 7.83, 7.761, 5.5576 and 6.311, respectively (Table 2).

Taxonomic compositions

Bacterial diversity richness and phylogenetic distribution were analyzed at different stages (Fig. 2). Bacterial community abundance was significantly different among the five PM samples (Fig. 3). The most abundant taxa throughout 31 phyla were *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* (Fig. 3a). The most abundant OTUs associated with the PM4 library were sequences related to *Firmicutes* (65.5%), *Actinobacteria* (22.5%) and *Proteobacteria* (7.4%). The most abundance sequences in the PM1, PM2 and PM3 library were those related to *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Fig. 3a).

The most abundant bacteria in PM4 at the genus level were *Lactobacillus* (44.9%), *Corynebacterium* (18.9%) and *Virgibacillus* (1.2%) (Fig. 3b). *Lactobacillus* was the dominant genus in PM4, sharply increasing from 1.3% in PM3 to 44.9% in PM4. The relative contents of genus *Corynebacterium* were 18.9% (PM4) and 16.5% (PM5), but this genus was rarely seen in the other litters. The relative abundance of *Pediococcus* in PM4 was also the highest among those samples, increasing from 0.1% in PM3 to 6.5% in PM4. PM5 had the highest amount of genus *Bacillus* (3.1%) and *Virgibacillus* (16.9%). The relative abundance of *Pseudomonas* in samples was also calculated,

Table 2 Similarity-based operational taxonomic units (OTUs) and species richness estimates of the bacterial phylotypes at a 3% dissimilarity level for the PM samples in the MFB system

Sample ID	Valid read	Cluster distance (0.03)				
		OTU	Shannon	Simpson	Chao1	Coverage
PM1	76,417	2011	8.177	0.987	1992.418	0.994
PM2	72,754	1760	7.83	0.98	1714.149	0.995
PM3	69,945	1758	7.761	0.987	1763.661	0.994
PM4	57,239	1599	5.557	0.903	3506.913	0.989
PM5	75,406	1599	6.311	0.952	1532.672	0.994
Total	351,761	8727	–[a]	–	–	–

[a] Not available

and the amounts of genus *Pseudomonas* increased to 4.5% in PM5 samples.

Relationship between bacterial communities among PM samples

A Venn diagram of the common and unique OTUs among PM libraries revealed that the number of OTUs shared among all libraries was 666 (Fig. 4). The PM1 library shared 96 and 60 OTUs with PM2 and PM3, and 66 and 44 common OTUs with PM4 and PM5. The shared OTUs accounted for >41.75% of OTUs in each group, and 3.7–8.4% of OTUs were unique in each sample.

Based on PCA supported by Weighted UniFrac distance, the five samples were divided into two significant groups in the PCA plot (Fig. 5). PM1, PM2 and PM3 grouped to the left of the plot along the first principal component (PC1), while PM4 and PM5 gathered on the right of PC1, which represented 87.24% of the total variation. PM1 and PM2 gathered together along PC2, whereas the PM3 exhibited a noticeable
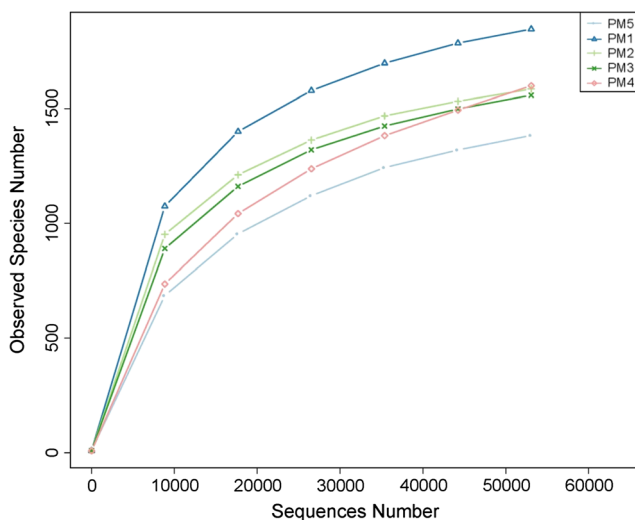
separation on the lower half of the graph, accounting for 6.56% of total variation.

A hierarchically clustered heatmap was prepared based on the top 35 abundant bacterial communities at genus level. The heatmap plot gives an overall view of the identified connections among PM samples. As shown in Fig. 6, the five samples were classified into two groups. The first group was composed of short-term fermentation samples (PM1, PM2 and PM3). PM1 and PM2 libraries grouped firstly together, and then clustered with PM3. The other group was composed of long-term fermentation samples (PM4 and PM5).

Among the five samples, PM1 had highest relative abundance of *Paludibacter* (1.3%), *Ruminococcus* (2.5%) and TYRC22 (1.7%). *Acinetobacter* (8.7%), *Myroides* (6.4%), *Sphingobacterium* (0.9%) and *Bacteroides* (3.1%) exhibited relatively higher abundance in PM2. All those predominant strains in PM1 and PM2 were rare in long-term fermentation
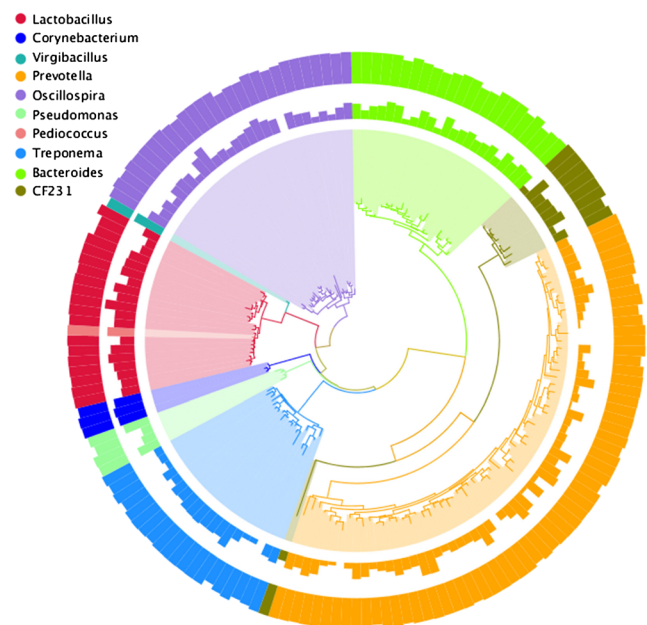

Fig. 1 Rarefaction curves based on 16S rRNA sequences among the different samples

Fig. 2 Bacterial diversity richness and phylogenetic distribution on phylum (*inner circle*), class (*middle circle*), and order level (*outer circle*)

Fig. 3 Relative abundance of operational taxonomic units (OTUs) of predominant bacterial compositions at **a** phylum and **b** genus levels in the microbial fermentation bed (MFB) system
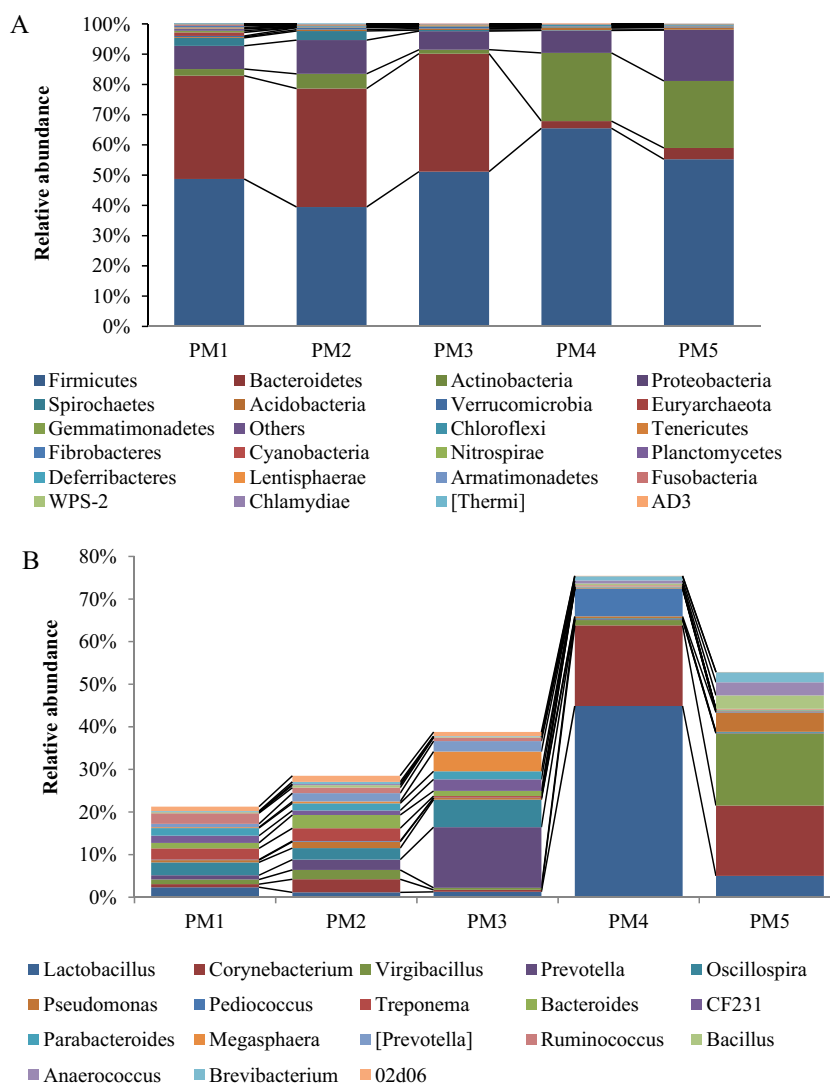
PM. Although PM3 shared several of the same predominant bacteria with PM1 and PM2, the dominant strains were *Prevotella* (2.4%), *Anaerovibrio* (1.1%), *Dialister* (0.8%), *Mitsuokella* (0.6%) and *Megasphaera* (4.6%). Sequences belonging to SMB53, *Clostridium*, *Lactobacillus*, *Pediococcus*, and *Corynebacterium* were the most dominant bacterial genus in the PM4 sample but were rare in the other samples.

The bacterial genera *Pseudomonas* (4.5%), *Bacillus* (3.1%), *Virgibacillus* (16.9%), *Anaerococcus* (3.1%), *Caldicoprobacter* (0.9%) and *Ignatzschineria* (0.9%) were rare in PM1, PM2 and PM3, but had relatively higher abundance in PM5. The abundant genus *Corynebacterium* was detected in both PM4 and PM5 samples. The results of the PCA and heatmap analyses indicated that the bacteria varied at different periods of PM fermentation.

RDA indicated that skatole content was the main determinant factor for the bacterial diversity of the five PM samples (Fig. 7). PM1 had high potassium and skatole content, and low C/N ratio. At the phylum level, *Euryarchaeota* (1.2%) and *Fibrobacteres*

(0.6%) were the most abundant bacteria in PM1 among five samples, while the *Firmicutes*, Actinobacteria and Acidobacteria were predominant bacteria in PM4 and PM5. The relative abundance of *Firmicutes*, *Actinobacteria*, *Acidobacteria* and *Proteobacteria* had a close relationship with degradation of 3-MI and increasing C/N ratio.

Discussion

Intensified and large-scale pig production leads to large amounts of manure production, and appropriate manure management is urgently needed to reduce manure emissions and environmental pollution. The MFB system has been introduced to recycle these wastes as a resource. However, research on the bacterial communities and diversity in PM in MFB systems have been lacking to date. Analysis of PM in an MFB system in situ revealed that bacterial community dynamics were related to fermentation stage. OTUs, species richness estimates and heatmap results
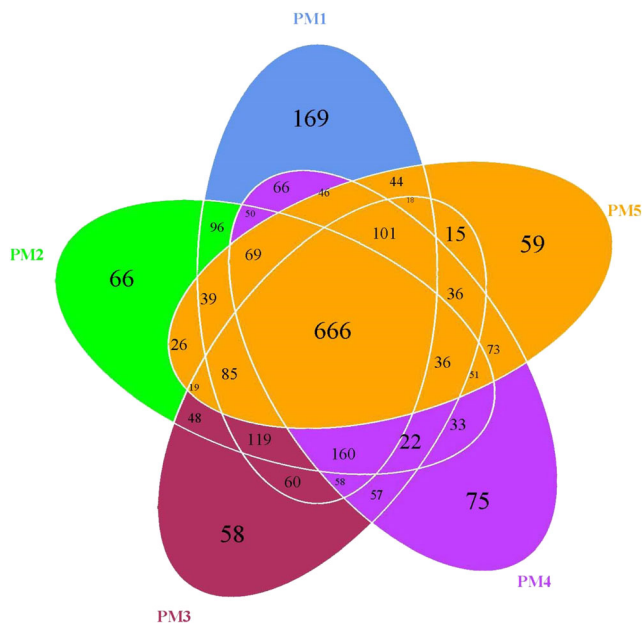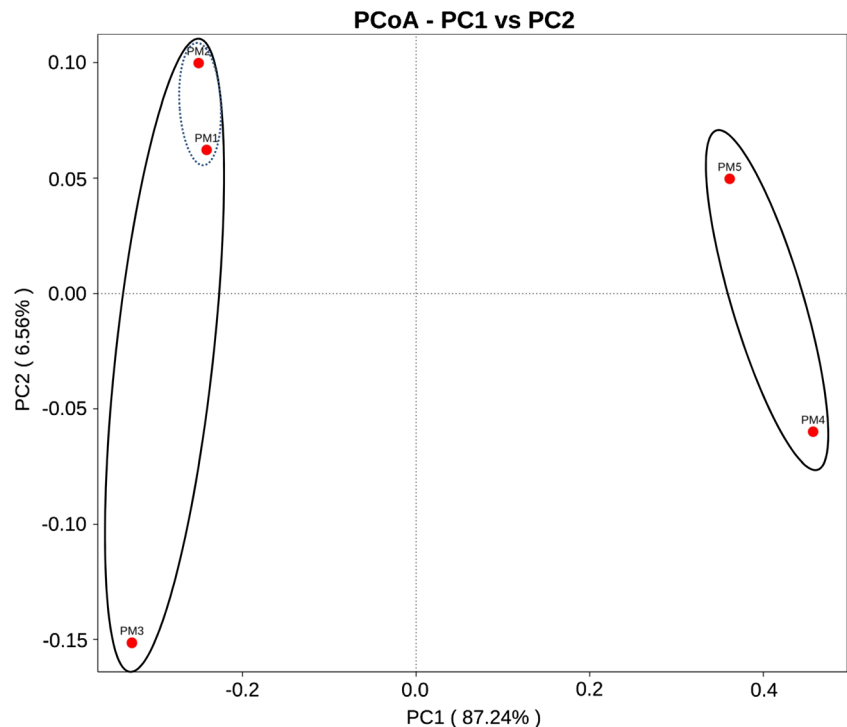
Fig. 4 Venn diagram based on shared OTUs in the different samples

illustrated that PM at different fermentation stages had significantly different bacterial communities. Organic degradation strains such as *Bacillus*, *Virgibacillus*, *Pseudomonas*, *Actinobacteria* and *Lactobacillus* might play important roles in the decomposition of PM. The amount of the unpleasant odor substance 3-MI decreased and C/N increased in the fermented manure, referred to as manure with deep fermentation in the MFB system.

Asano et al. (2010) reported that the stable dominant phylum in cattle manure was *Firmicutes*. In the co-composting of cow manure and rice straw, *Bacteroidetes* and *Proteobacteria* were the most abundant phyla (Ren et al. 2016). In the PM samples (PM1), a similar result was observed. The main phylum was *Firmicutes*, which increased from 48.7% in fresh PM to 65.5% in PM4. Since it makes up a large portion of the gut microbiome (Ley et al. 2008), *Firmicutes* from gut flora could be the main source of *Firmicutes* in PM. However, the proportion of *Proteobacteria* showed high variability between different samples, in the range of 7.4% to 16.8%. Lin et al. (2016) reported that most functional proteins in a thermophilic swine manure digester were from *Proteobacteria*. *Actinobacteria* was the second most abundant phylum in samples PM4 and PM5, contributing of 22.2% and 22.6% of all sequences. As *Actinobacteria* had extraordinary ability to decompose organic matter (Ventura et al. 2007), the increase in this phylum may have some correlation with the degradation of organic matter in PM.

As shown in the heatmap of bacterial community composition of the five samples (Fig. 4), the predominant strains changed at each stage of the fermentation process. In long-term fermentation PM, the dominant genera were related to organic degradation strains such as *Corynebacterium*, *Bacillus*, *Virgibacillus*, *Pseudomonas*, *Actinobacteria*, *Lactobacillus* and *Pediococcus*. Genus *Corynebacterium* is widely distributed in soil, water, plants, and food products, and members of genus *Corynebacterium* are capable of degrading aromatic compounds (Shen et al. 2012). PM5 had

Fig. 5 Principal component analysis (PCA) based on OTU level among five samples

Fig. 6 Hierarchically clustered heatmap of bacterial distribution of different communities from the five samples at the genus level

the highest amount of genus *Bacillus* (3.1%) and *Virgibacillus* (16.9%), which are prevalente in varied environments such as soil, bile, water and food (Heyndrickx 1998; Nicholson 2002).

Bacillus spp. and *Virgibacillus* spp. play a key role in bio-composting processes, by producing a variety of enzymes (Hayat et al. 2013; Alkindi and Abed 2016). The relative

Fig. 7 Redundancy analysis (RDA) of the relationship between the PM physicochemical parameters and the relative abundance of bacterial phyla in the five PM samples
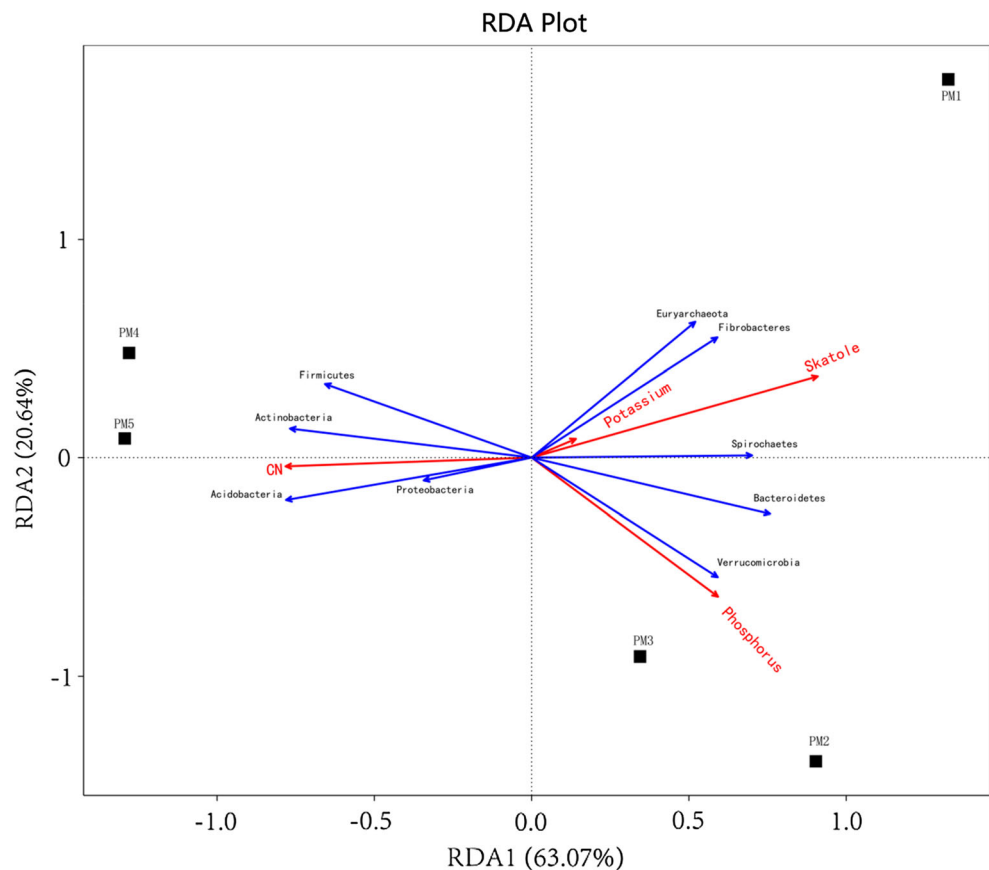
abundances of *Actinomyces* and *Pseudomonas* in PM5 were also calculated; both demonstrated great metabolic diversity and were the most versatile and efficient hydrocarbon degraders (Folsom et al. 1990; Folsom 1997; Song et al. 2009). Liu et al. (2011) reported that *Actinobacteria* was the major decomposer for breaking down organic matter in composting. Prolonging compost time from 0 days to 20 days produces a sudden increase and decrease of *Lactobacillus*, and the appearance of *Pediococcus*. *Lactobacillus* and *Pediococcus* were the main lactic acid bacteria (LAB) in the five samples. LAB have been enumerated in manure and fertilized silage crops, and play important roles in composting. A LAB community comprising mainly *L. fermentum*, *L. plantarum* and *L. paracacei* efficiently accelerated the fermentation of straw, which could efficiently utilize rice straw and lessen its environmental disposal problems (Gao et al. 2008). The genus *Pediococcus* is a close relative of the genus *Lactobacillus*, and is used in treatment of manure. A biotechnological process in which poultry waste manure is inoculated with a starter culture of *Pediococcus* is a key component of composite organic waste and cattle manure fermentation systems (Asano et al. 2010; Probst et al. 2013). *L. plantarum* and *P. acidolactici* have been applied for treatment and recycling of manure as a feed ingredient (Jalil et al. 2001). In conclusion, organic degradation strains as *Corynebacterium*,

Bacillus, *Virgibacillus*, *Pseudomonas*, *Actinobacteria*, *Lactobacillus* and *Pediococcus* from those samples could be involved in the degradation of PM.

LAB also represent potential 3-MI-degrading bacteria. *Lactobacillus* and *Pediococcus* of *Firmicutes* were the main LAB in the five samples. Meng et al. (2013) demonstrated that *Lactobacillus brevis* 1.12 is capable of 3-MI degradation. The large amount of LAB in PM samples might participate in the removal of skatole in PM, and be responsible for the low concentrations of 3-MI. Besides LAB, many species of *Pseudomonas* (phylum *Proteobacteria*) could also degrade 3-MI. *Pseudomonas* was detected in all PM samples, with relative abundance of 0.5%, 1.5%, 0.4%, 0.4% and 4.5% in PM1, PM2, PM3, PM4 and PM5, respectively. *P. aeruginosa* Gs isolated from Mangrove Sediment can use 3-MI as the sole source of carbon and energy (Yin et al. 2006). *Cupriavidus* (phylum *Proteobacteria*) was detected only in PM4 and PM5 samples, with quite low relative abundant of 0.004% and 0.01%, respectively. *Cupriavidus* sp. strain KK10 isolated from soil could transform 3-MI to ring cleavage products under aerobic conditions (Fukuoka et al. 2015). Borowski et al. (2016) investigated microbial-mineral preparation that contained *Pseudomonas* sp., *Bacillus* sp. and *Lactobacillus* sp. for the removal of offensive odors from poultry manure. The changes of genus *Lactobacillus, Pseudomonas* and

Cupriavidus matched with the changes of 3-MI, and might result in the decrease of 3-MI.

This study investigated the characteristics of PM in MFB at different stages, and examined the structures of bacterial communities involved in the composting process. The diversity of bacterial communities and content of 3-MI decreased in this process. Compared with fresh PM, organic degradation strains were the dominate genera in long-term fermentation PM. *Lactobacillus*, *Pseudomonas* and *Cupriavidus*, as potential 3-MI-degrading bacteria, might participate in the removal of skatole in PM. This study provides data to help our understanding of bacterial metabolic activity of PM under in situ fermentation in the MFB system.

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