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Molecular Analysis of Bacterial Diversity in Pig Deep Litter System

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Deep litter system is widely applied to pig production. The beddings have complex microbial communities which play an important role in fermentation efficiency. To obtain the bacterial community in pig deep litter system, we utilized the traditional culture-dependent method and molecular biology technique. Using plate count method *Microbacterium* (35.33%) and *Arthrobacter* (20.30%) were remarkable in total culturable bacteria. By 16S rRNA gene library method, the sequences are mostly distributed in 3 phyla: Proteobacteria (38.40%), Firmicutes (28.18%), Bacteroidetes (16.57%) and affiliated to 10 genera: *Clostridium* (19.89%), *Castellaniella* (4.70%), *Comamonas* (2.76%), *Rhodanobacter* (2.21%), *Acinetobacter* (1.38%), *Planctomyces* (1.38%), *Nitrosomonas* (1.10%), *Devosia* (1.10%), *Gemmatimonas* (1.10%), and *Steroidobacter* (1.10%). In addition, an Illumina next generation sequencing named MiSeq was used to observe the V4/V5 hypervariable region of 16S rDNA sequence. Proteobacteria, Firmicutes, and Bacteroidetes accounted for 83.7% of all sequences. The 10 genera described above were also presented and new 4 genera were added. *Clostridium* was increased to 25.5% as the predominant bacteria. The results were consistent with that of 16S rRNA gene library analysis in general. It is the first report which provides information on bedding microbiota in pig deep litter system taking advantage of culture and culture-independent approaches.

Key words: Bacteria community; Deep litter system; MiSeq; 16S rRNA gene.

With the development of intensive livestock production, since the 1950s Western Europe countries have paid attention to the slatted floor system for growing pigs, instead of the straw based litter system. However, since the 1980s, people have been interested in the litter system because this method can improve animal welfare[1] and reduce odour nuisance[2, 3]. The litter system is an ancient technology and nowadays deep litter system has been developed as a popular method for pig production. It has a floor based on the mixed bedding which is constituted by sawdust, rice hull, corn stalk, etc[4, 5]. Hence, using this system the pig wastes are able to ferment in situ. Compared to the slatted or solid floor system, deep litter system can reduce the cost of wastes treatment and the environmental risk[6, 7]. The former studies of deep litter system focus on the behaviour, welfare, growth performance of pigs[8-10] and gaseous emissions from this process[11, 12]. The bedding has a complex microbial community which plays an important role in deep litter system. For example, microbes are closely related to waste fermentation efficiency. But only a few studies about it were reported in scientific literatures. Therefore, more knowledge on the microbial population will contribute to improve the effect of deep litter system.

It is well-known that plate count analysis is a traditional, cultivation-dependent method of microbial population and the classic cultivation-

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based method was strongly biased since only a small proportion (0.01-10%) of microorganisms in natural environment could be cultivated and identified[13]. Molecular biology brings new culture-independent methods to study the microbial community[14-16]. The 16S rRNA gene library has been widely used for the research of bacterial community[17, 18]. However, this method needs to analyse lots of clones to obtain the accurate bacterial community in complex environment. The work is labor-intensive and time-consuming. The Next Generation Sequencing technologies which extremely increase sequencing throughput, such as Roche 454 pyrosequencing and Illumina MiSeq, have a light insight in microbial diversity. The MiSeq is a widely popular method of microbial community because of low-cost and fast-sequencing. With this method just short but highly variable regions of the 16S rDNA sequence (e.g. the V3, V4 or V5 hypervariable regions) were applied to sequencing for bacterial communities[19]. The previous study demonstrated that V4/V5 region could supply higher classification accuracy than other regions[20]. Although MiSeq technology is increasingly used in microbial community research, there is no microbiota report related to deep litter system by this method. The aim of this research was to study the microbial community in a pig deep litter system which was used in 12 months and collected from the hoggery in Changsha, China. Three different methods were used: (1) dilution plate method; (2) 16S rRNA gene library method; (3) high throughput sequencing method (MiSeq) of partial 16S rRNA gene. Combining the results of the three methods, we have obtained the microbiota in pig deep litter system for the first time.

MATERIALS AND METHODS

Deep litter samples collection

The bedding of deep litter system was collected from the hoggery in Changsha City, Hunan Province, China in April, 2013. In deep litter system, there was a floor space allowance of approximately 1 m^2 per pig. The bedding was constituted by sawdust and rice hull with 1:1 ratio, the thickness ranging from 60 to 70 cm. The bedding has been used for 12 months and approximately 1 kg of fresh middle-level bedding (20 cm from top) was collected from 5 different locations in a pig deep litter systems. The basic information of each sample was showed in Table 1.

Calculation and isolation of microorganisms

To count the total culturable bacteria, each 10 g of the frozen sample was added to 90 ml of sterilized water. The mixture was homogenized by a shaker to disperse bacteria and serial ten-fold dilutions. Volume 0.1 ml of undiluted samples and different diluents (ranging from 10^{-2} to 10^{-7}) were distributed onto the plates of beef peptone agar medium (beef extract, 3 g; peptone, 5 g; agar, 15 g; distilled water, 1,000 ml; pH 7.0). Then the plates were incubated at 37°C for 48-72 h. Cell densities were estimated by colony-forming units (CFU) method. Multiple nucleotide sequence alignments were performed using latest version of Clustal (Clustal Omega)[21], and phylogenetic trees were constructed by MEGA 6 software[22] with a neighbor joining method.

Total DNA extraction and purification

A modified DNA extraction method was developed based on the procedure as previous described[23]. Firstly the bedding was grinded with liquid nitrogen. A 5 g sample was added to 12 ml of 4% SDS and 100 µl of Proteinase K (20 mg/ml) and then was incubated in a 70°C water bath for 1 h with gentle inversion every 15–20 min. The mixture was centrifuged for 10 min at 8,000×g. The supernatant was transferred to 50 ml centrifugal tube containing ~20 ml of 10% PEG 8000. After centrifugation at 10,000×g for 15 min at 4°C the precipitate was added to 10 ml of 2% CTAB. Then it was vortexed for 10 s and incubated at 65°C for 15 min. The sample was mixed enough with an equal volume of chloroform and isopentyl alcohol (24:1, vol/vol). The mixture was centrifuged at 10,000×g for 10 min. The aqueous phase was transferred to another sterile centrifuge tube containing ~20 ml of 10% PEG 8000. The pellet of DNA was obtained by centrifugation at 12,000×g for 15 min at 4°C, washed with 1 ml of 70% ethanol and dried at room temperature. The total DNA was resuspended in 50 µl sterile deionized water with RNase A (10 mg/L). Then DNA was purified by MagExtractor-Nucleic Acid Purification Kit (TOYOBO). The size and quality of the extracted DNA was determined by agarose gel electrophoresis (1.5% agarose) and the samples were stored at ≤20°C for PCR analysis.

Cloning and analysis of 16S rRNA gene library

To amplify 16S rDNA sequence of bacterial community, the following primers were used: forward primer 27F (52 -AGA GTT TGATCC TGG CTC AG-32) and reverse primer 1492R (52 -TAC CTT GTT ACG ACT-32)(24). A 50 µl of PCR mixture contained 1 µl (100 ng) DNA as template, 1 µl of each primer (5 µM), 5 µl of dNTP (2 mM), 5 µl of 10×PCR buffer (Mg^{2+} plus), 1 µl *Ex* Taq (5 U/µl, TaKaRa), and 37 µl sterile water. The PCR cycle program was: 94°C for 4 min, 94°C for 30 s, 52°C for 30 s, 72°C for 2 min, 30 cycles, then 72°C for 10 min. The PCR products were purified and ligated into the pMD18-T vector (TaKaRa). The ligation products were transformed into Escherichia coli DH10B competent cells. White colonies were selected randomly and the insert size of chosen clones was detected using PCR. Sequencing was done by BGI Biotechnology Co., Ltd. (Beijing, China). To determine the phylogenetic affiliation the nucleotide sequences were used to search against Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/)[25]. Operational Taxonomic Units (OTUs) was divided by Mothur software at 3% difference level and the community richness was analyzed by Chao1 estimator[2]). The representative sequences were submitted to the GenBank database under accession numbers KM456053-KM456177.

Illumina high-throughput sequencing

The V4 plus V5 region of the 16S rDNA sequence was amplified by PCR using the 515F primer (5'-GTGCCAGCMGCCGCGG-3') and the 907R primer (5'-CCGTCAATTCMTTTRAGTTT-3'). The PCR of each sample was repeated 3 times. The PCR productions were mixed and extracted from 2% agarose gels. Then they were purified using the Gel extraction kit (Axygen) and detected by Fluorescence quantitative system (Promega). Next it was pooled in the same concentration and paired-end sequenced on Illumina MiSeq platform. According to the tag sequences of barcode, the reads of different samples could be distinguished. Sequences that had a quality score <20 in a window of 50 bp were removed. Sequences which differed by >2 bp from primer sequences were filtered. Chimeras were removed using UCHIME software[27]. The sequence reads were processed using Mothur[26] software.

RESULTS

The distribution of the total culturable bacteria in the bedding

Using CFU method the number of total culturable bacteria in pig deep litter was 88.67±9.71 $\times 10^6$ cfu/g (Mean±SD). The detected level of various bacteria was in the same order of magnitude of 10^6 cfu/g. The relative abundance of

Table 1. The basic information of each sample

	Temperature(°C)	Organic matter(%)	Moisture content(%)	C/N ratio	pH
Sample 1	43	71.3	58	30:1	6.2
Sample 2	41	74.6	49	26:1	5.8
Sample 3	40	62.5	55	24:1	6.5
Sample 4	48	59.3	52	32:1	6.8
Sample 5	42	66.1	54	34:1	6.1

Table 2. The distribution of culturable bacteria in the bedding of deep litter system

Closest group	Closest genus/species	GenBank accession	Similarity (%)	The various bacteria(10^6 cfu/g)	Ratio (%)
Actinomycetales	Microbacterium sp.	JX514855	98	31.33±4.16	35.33
Actinomycetales	Arthrobacter sp.	HM191728	94	18.00±3.00	20.30
Gammaproteobacteria	Acinetobacter lwoffii	JQ815203	95	16.67±3.06	18.80
Gammaproteobacteria	Citrobacter freundii	KC634232	100	13.33±4.16	15.03
Bacteroidetes	Flavobacterium balustinum	D14016	97	4.67±1.53	5.27
Firmicutes	Bacillus sp.	AB362831	91	3.33±1.16	3.76
Gammaproteobacteria	Stenotrophomonas sp.	HM047512	94	1.33±1.16	1.50

7 types of bacteria was showed in Table 2, ranging from 35.33% to 1.50%. The genus or species were estimated by 16S rDNA sequence analysis. Using the Sequence Match program in RDP database, the results showed all of them shared the similarity from 91% to 100%. *Microbacterium* was the most abundant genus with a frequency of 35.33%. *Arthrobacter*, *Acinetobacter* and *Citrobacter* were the second largest groups (representing 20.30%, 18.80% and 15.03%, respectively) which were followed by *Flavobacterium* (5.27%), *Bacillus* (3.76%), and *Stenotrophomonas* (1.50%). Both of *Microbacterium* and *Arthrobacter* belonged to Actinomycetales. *Acinetobacter lwoffii*, *Citrobacter freundii* and *Stenotrophomonas* falled into Gamma proteobacteria.

The phylogenetic relationship of the 16S rDNA sequences and the references was shown in Fig. 1. The phylogenetic tree included three major clusters: one of them was gram-positive bacteria (the two types of Actinomycetales and *Bacillus*); two of them were gram-negative bacteria.

16S rRNA gene library construction and analysis

As the template total microflora DNA was subjected to PCR amplification the full-length sequence of 16S rRNA gene. The PCR product which is approximately 1,500 bp size was applied to construct the 16S rRNA gene library. The false positive clones were excluded by PCR test. In total 384 sequences were determined by Sanger sequencing. After chimeras check it was still 362 valid 16S rDNA sequences. The rarefaction curve is shown below in Fig. 2. Whereas the 362 cloned sequences were placed into 226 OTUs at

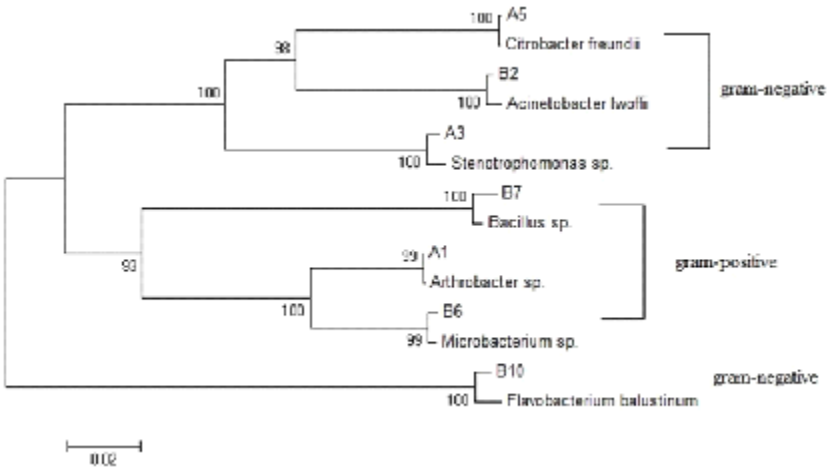

Fig. 1. The phylogenetic relationship of the 16S rRNA gene sequences and references

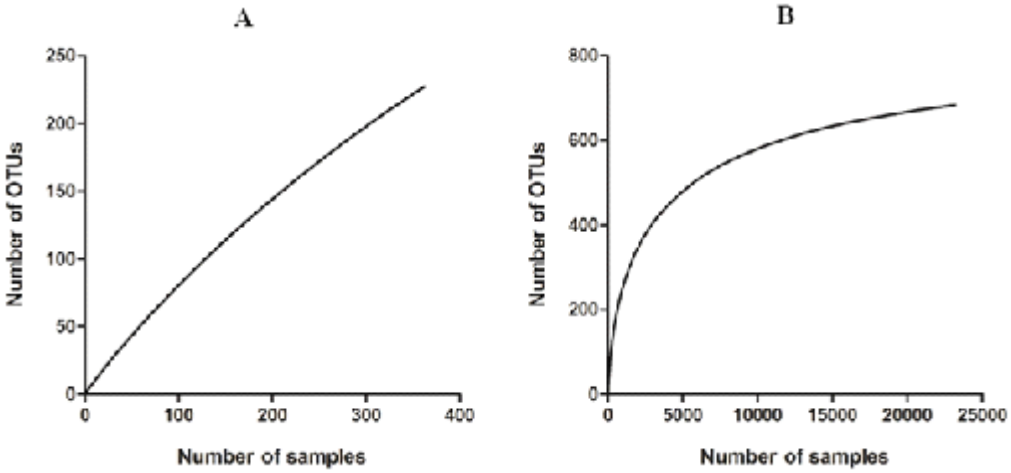

Fig. 2. The rarefaction curve of 16S rDNA library (A) and Miseq (B)

the 3% difference level by Mothur software. The Chao1 richness estimate produced 292 OTUs.

All the sequences were used as a query to search against RDP and classified from phylum to genus. The results showed that most bacteria affiliated with 11 different phyla, mainly distributed in *Proteobacteria*, *Firmicutes*, *Bacteroidetes* with the frequencies of 38.40%, 28.18%, 16.57%. Only 12 clones (3.31%) were not classified by Classifier program (Fig. 3). The library had one dominant genus *Clostridium* with a frequency of 19.89%. The second largest group included genus *Castellaniella*, *Comamonas*, *Rhodanobacter*, *Acinetobacter*, *Planctomyces*, *Nitrosomonas*, *Steroidobacter*, *Devosia*, and *Gemmatimonas* with the frequencies of 4.70%, 2.76%, 2.21%, 1.38%, 1.38%, 1.10%, 1.10%, 1.10% and 1.10%. All of them belonged to Proteobacteria except for *Planctomyces* and *Gemmatimonas*.

Illumina next-generation sequencing analysis

A total of 25,435 valid sequences were obtained by Illumina next-generation sequencing. Among them 2,257 sequences were chimeras and removed in the subsequent analysis. The average length of sequences was 395 bp. The sample community included 683 OTUs with 97% similarity level, with Chao1 richness estimate of 734 OTUs. The rarefaction curve tended to near the saturation plateau.

All sequences were classified from phylum to genus blasted by RDB with the default setting. A total of 22 different phyla or groups were identified and 14 of them had the proportion exceeding 0.1%. Only 0.65% of the sequences were not classified. In this way 10 phyla described above were presented and Firmicutes (35.62%), Proteobacteria (28.54%), and Bacteroidetes (19.55%) were also the most abundant groups. These results were consistent with that of 16S rRNA gene library analysis, although the proportion of phyla were not in agreement by the two methods. Comparing the outcome of library analysis, 5 new groups were added: Cyanobacteria (0.42%), Deinococcus-Thermus (0.38%), Armatimonadetes (0.18%), TM6 (0.14%), and BRC1 (0.13%). At the genus level, *Clostridium* which was increased to 25.53% was still the predominant genus in bedding microbiota. Thirteen genera which showed in Fig. 4 constituted the second largest groups with the percentage from 0.44% to 3.55%.

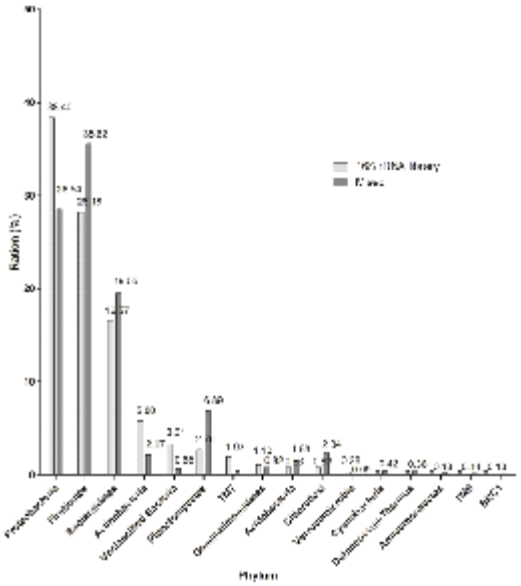

Fig. 3. Relative ration of the common phylum used by 16S rDNA library and Miseq method

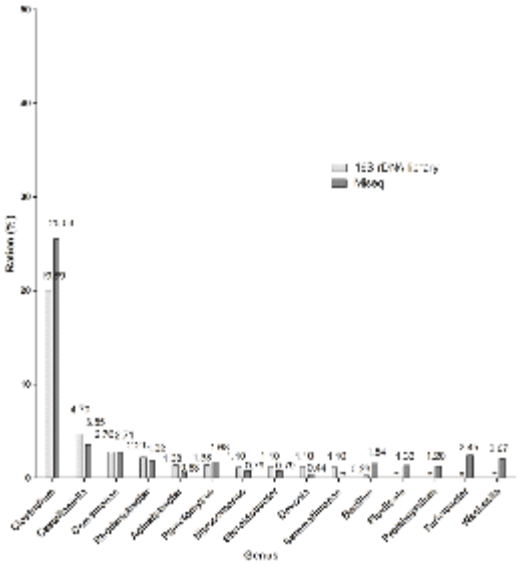

Fig. 4. Relative ration of the common genus used by 16S rDNA library and Miseq method

DISCUSSION

The bedding was collected from five different regions of a pig deep litter systems. The samples were subsequently mixed for DNA preparation. Because the bacterial community represented the bedding microbiota in the different regions of deep litter system as a whole. When PCR-based analysis of microbial diversity was applied in complex environmental samples, it was crucial to obtain highly purified and unbiased DNA. The deep litter bedding included a large amount of humic acids and other contaminants which complicated the follow-up PCR amplification and finally resulted in a bias estimate of diversity[28]. The humic acid contamination was decreased through increasing salt concentration (1.5 M NaCl) in the lysis buffer of CTAB and SDS.

Using dilution plate count, 7 genera/ species were isolated in the bedding of deep litter system. It was known that *Microbacterium* sp. CL-10.9 (GenBank: HQ113212.1) was isolated from swine waste biotreatment and *Microbacterium* sp. could contribute to raise fermentation temperature of swine manure composting. Bacillus was isolated from swine manure composting and it played an vital role in decomposing the organic filth and degrading nitrite, hydrogen sulfide[29]. In our study, *Microbacterium* (representing 35.33%) was most abundant and Bacillus (representing 3.76%) was also found in the beddings of pig deep litter where swine manure was the major waste. *Acinetobacter* is mainly distributed in the water and soil, especially the damp environment. All of *Acinetobacter, Citrobacter*, *Flavobacterium*, and *Stenotrophomonas* belong to the gram-negative bacteria. Most of them are recognized as potential pathogens.

On the basis of 16S rRNA gene library and MiSeq analysis, Proteobacteria, Firmicutes, and Bacteroidetes were the abundant phylum, representing from 16.57% to 38.40%. *Clostridium* was the predominant genus, representing 19.89% and 25.53%, respectively. It was demonstrated that *Clostridium* was the major bacteria in swine manure composting[30]. Our results were consistent with it because swine manure was the primary waste materials in pig deep litter system. Using dilution plate method *Clostridium* were not found since it usually grows in anaerobic condition. *Castellaniella* is a genus of gram-negative, facultatively anaerobic, motile bacteria from the family Alcaligenaceae of the class Betaproteobacteria. *Comamonas* are able to degrade environmental pollutants, for example, Comamonas testosteroni KF-1 can mineralize sulfophenylcarboxylates (31). In contrast with MiSeq method, the number of sequencing was very few and the rarefaction curve was difficult to approach the saturation plateau. Our results suggest that MiSeq is indeed more reliable and informative and MiSeq could replace 16S rRNA clone libraries for studying the microbial diversity of pig deep litter system. Compared to culture-dependent method and capillary sequencing, high throughput sequencing can outline microbial community at high resolution in complex environment.. Plate count method is obviously biased as only few of microbes in natural environment can be cultured nowadays[32]. PCR-based molecular surveys also have biases associated with the differences in amplification efficiencies and replicate amplifications[33]. In this study each of the methods had its inherent disadvantages, a combined analysis could provide more reliable information of bacterial populations in pig deep litter system.

This research is the first report to study the bacteria community of deep litter using various methods, which helps the regulation of micro-ecosystem and improvement in fermentation efficiency. Next step we plan to investigate the temporal and spatial variability of bacteria in different process of pig deep litter system, which provide the practical guidance for developing highly active germ agent.

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REFERENCES

1. Tuyttens F.A.M. The importance of straw for pig and cattle welfare: A review. *Applied Animal Behaviour Science*, 2005;**92**(3): 261-282.
2. Wang K., Wei B., Zhu S., Ye Z. Ammonia and odour emitted from deep litter and fully slatted floor systems for growing-finishing pigs.

Biosystems Engineering, 2011;**109**(3): 203-210.

3. Jeppsson K.H. SE—Structure and Environment: Carbon Dioxide Emission and Water Evaporation from Deep Litter Systems. *Journal of Agricultural Engineering Research*, 2000;**77**(4): 429-440.
4. Cabaraux J.-F., Philippe F.-X., Laitat M., Canart B., Vandenheede M., Nicks B. Gaseous emissions from weaned pigs raised on different floor systems. *Agriculture, Ecosystems & Environment*, 2009;**130**(3): 86-92.
5. Andersson M. Performance of Bedding Materials in Affecting Ammonia Emissions from Pig Manure. *Journal of Agricultural Engineering Research*, 1996;**65**(3): 213-222.
6. Philippe F.X., Laitat M., Nicks B., Cabaraux J.F. Ammonia and greenhouse gas emissions during the fattening of pigs kept on two types of straw floor. *Agriculture, Ecosystems & Environment*, 2012;**150**(1): 45-53.
7. Philippe F.X., Laitat M., Canart B., Vandenheede M., Nicks B. Comparison of ammonia and greenhouse gas emissions during the fattening of pigs, kept either on fully slatted floor or on deep litter. *Livestock Science*, 2007;**111**(1): 144-152.
8. Morrison R.S., Johnston L.J., Hilbrands A.M. The behaviour, welfare, growth performance and meat quality of pigs housed in a deep-litter, large group housing system compared to a conventional confinement system. *Applied Animal Behaviour Science*, 2007;**103**(1): 12-24.
9. Morrison R.S., Hemsworth P.H., Cronin G.M., Campbell R.G. The social and feeding behaviour of growing pigs in deep-litter, large group housing systems. *Applied Animal Behaviour Science*, 2003;**82**(3): 173-188.
10. Morrison R.S., Hemsworth P.H., Cronin G.M., Campbell R.G. The effect of restricting pen space and feeder availability on the behaviour and growth performance of entire male growing pigs in a deep-litter, large group housing system. *Applied Animal Behaviour Science*, 2003;**83**(3): 163-176.
11. Philippe F.X., Canart B., Laitat M., Wavreille J., Bartiaux-Thill N., Nicks B., Cabaraux J.F. Effects of available surface on gaseous emissions from group-housed gestating sows kept on deep litter. *animal*, 2010; **4**(10): 1716-1724.
12. Philippe F.X., Canart B., Laitat M., Wavreille J., Vandenheede M., Bartiaux-Thill N., *et al.* Gaseous emissions from group-housed gestating sows kept on deep litter and offered an ad libitum high-fibre diet. *Agriculture, Ecosystems & Environment*, 2009;**132**(1): 66-73.
13. Head I.M., Saunders J.R., Pickup R.W. Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms. *Microb Ecol*, 1998; **35**(1): 1-21.
14. Schloss P.D., Hay A.G., Wilson D.B., Walker L.P. Tracking temporal changes of bacterial community fingerprints during the initial stages of composting. *FEMS Microbiology Ecology*, 2003; **46**(1): 1-9.
15. Franke-Whittle I.H., Klammer S.H., Insam H. Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. *Journal of Microbiological Methods*, 2005; **62**(1): 37-56.
16. Ludwig W., Schleifer K.H. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiology Reviews*, 1994;**15**(2): 155-173.
17. Danon M., Franke-Whittle I.H., Insam H., Chen Y., Hadar Y. Molecular analysis of bacterial community succession during prolonged compost curing. *FEMS Microbiology Ecology*, 2008; **65**(1): 133-144.
18. Tian W., Sun Q., Xu D., Zhang Z., Chen D., Li C., *et al.* Succession of bacterial communities during composting process as detected by 16S rRNA clone libraries analysis. *International Biodeterioration & Biodegradation*, 2013;**78**(1): 58-66.
19. Staley C., Unno T., Gould T.J., Jarvis B., Phillips J., Cotner J.B., Sadowsky M.J. Application of Illumina next-generation sequencing to characterize the bacterial community of the Upper Mississippi River. *J Appl Microbiol*, 2013; **115**(5): 1147-1158.
20. Claesson M.J., Wang Q., O'Sullivan O., Greene-Diniz R., Cole J.R., Ross R.P., O'Toole P.W. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Res*, 2010; **38**(22): e200.
21. Sievers F., Wilm A., Dineen D., Gibson T.J., Karplus K., Li W., *et al.* 2011. Fast, scalable generation of high quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*. 2011; **7**: 539.
22. Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*, 2013; **30**(12): 2725-2729.
23. Zhou J., Bruns M.A., Tiedje J.M. DNA recovery from soils of diverse composition. *Appl Environ Microbiol*, 1996;**62**(2): 316-322.
24. Dojka M.A., Hugenholtz P., Haack S.K., Pace N.R. Microbial diversity in a hydrocarbon- and

chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol*, 1998;**64**(10): 3869-3877.

25. Cole J.R., Wang Q., Fish J.A., Chai B., McGarrell D.M., Sun Y., *et al.* Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res*, 2014;**42**(1): D633-642.
26. Schloss P.D., Westcott S.L., Ryabin T., Hall J.R., Hartmann M., Hollister E.B., *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol*, 2009;**75**(23): 7537-7541.
27. Edgar R.C., Haas B.J., Clemente J.C., Quince C., Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, 2011;**27**(16): 2194-2200.
28. LaMontagne M.G., Michel Jr F.C., Holden P.A., Reddy C.A. Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *Journal of Microbiological Methods*, 2002;**49**(3): 255-264.
29. Yi J., Wu H.Y., Wu J., Deng C.Y., Zheng R., Chao Z. Molecular phylogenetic diversity of Bacillus community and its temporal-spatial distribution during the swine manure of composting. *Appl Microbiol Biotechnol*, 2012;**93**(1): 411-421.
30. Yi J., Zheng R., Li F., Chao Z., Deng C.Y., Wu J. Temporal and spatial distribution of Bacillus and Clostridium histolyticum in swine manure composting by fluorescent in situ hybridization (FISH). *Appl Microbiol Biotechnol*, 2012;**93**(6): 2625-2632.
31. Schleheck D., von Netzer F., Fleischmann T., Rentsch D., Huhn T., Cook A.M., Kohler H.P. The missing link in linear alkylbenzenesulfonate surfactant degradation: 4-sulfoacetophenone as a transient intermediate in the degradation of 3-(4-sulfophenyl)butyrate by Comamonas testosteroni KF-1. *Appl Environ Microbiol*, 2010;**76**(1): 196-202.
32. Wagner M., Amann R., Lemmer H., Schleifer K.H. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl Environ Microbiol*, 1993;**59**(5): 1520-1525.
33. Kennedy K., Hall M.W., Lynch M.D., Moreno-Hagelsieb G., Neufeld J.D. Evaluating bias of Illumina-based bacterial 16S rRNA gene profiles. *Appl Environ Microbiol*, 2014;**80**(18): 5717-5722.