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Deodorization of Pig Slurry and Characterization of Bacterial Diversity Using 16S rDNA Sequence Analysis[§]

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The concentration of major odor-causing compounds including phenols, indoles, short-chain fatty acids (SCFAs) and branched chain fatty acids (BCFAs) in response to the addition of powdered horse radish (PHR) and spent mushroom compost (SMC) was compared with control non-treated slurry (CNS) samples. A total of 97,465 rDNAs sequence reads were generated from three different samples (CNS, n = 2; PHR, n = 3; SMC, n = 3) using bar-coded pyrosequencing. The number of operational taxonomic units (OTUs) was lower in the PHR slurry compared with the other samples. A total of 11 phyla were observed in the slurry samples, while the phylogenetic analysis revealed that the slurry microbiome predominantly comprised members of the *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla. The rarefaction analysis showed the bacterial species richness varied among the treated samples. Overall, at the OTU level, 2,558 individual genera were classified, 276 genera were found among the three samples, and 1,832 additional genera were identified in the individual samples. A principal component analysis revealed the differences in microbial communities among the CNS, PHR, and SMC pig slurries. Correlation of the bacterial community structure with the Kyoto Encyclopedia of Genes and Genomes (KEGG) predicted pathways showed that the treatments altered the metabolic capabilities of the slurry microbiota. Overall, these results demonstrated that the PHR and SMC treatments significantly reduced the malodor compounds in pig slurry ($P < 0.05$).

***Keywords*:** metagenomics, microbial diversity, pyrosequencing, 16S rDNA, malodor-reducing additive

Introduction

In recent years, livestock production has become a main contributor to the issue of odor pollution. Epidemiological studies have shown that the residential exposure to livestock malodors can affect the health and well-being of rural citizens (Blanes-Vidal *et al.*, 2012). Increased public concern, legislation and environmental regulations have focused on pollution and present a major dilemma to the livestock industry. Hence, odor management by reducing the emission of offensive odorants is currently considered a potential approach for sustainable livestock production. Generation of malodors is a complex process that involves many bacterial species producing a wide range of volatile compounds under currently used slurry storage conditions.

Pig slurries contain incompletely digested nutrients and generate unpleasant odors. Anaerobic slurry fermentation by complex microbiomes in a storage pit is a major cause for odor generation (Williams and Evans, 1981). Mackie *et al.* (1998) reported the presence of 200 odorous compounds, such as phenols, indoles, volatile fatty acids (VFAs), sulfide and ammonia, in livestock waste. Since the odor threshold concentrations of phenols and indoles are low, they are considered crucial for odor generation (Parker, 2008). Odorous compounds are produced through fermentation of various potential substrates including undigested organic compounds in slurry. When protein components were added to slurry in excess, the concentrations of branched chain fatty acids (BCFAs) and aromatic compounds were increased, whereas those of VFAs were increased after carbohydrates were added (Miller and Varel, 2003). Additionally, the concentration of ammonium nitrogen was increased in the slurry of pigs fed protein-enriched diets (Sutton *et al.*, 1999).

Horseradish contains various antibiotic substances and significant amounts of peroxidase, which can oxidize and convert aromatic compounds such as phenols and indoles to odorless non-toxic polymers. Peroxidase has been used in wastewater treatment and recently for malodor reduction in swine manure (Parker *et al.*, 2012). Reportedly, minced horseradish roots can be used for the removal of phenols from pig slurry but are limited by transport and storage (Govere *et al.*, 2007). Spent mushroom compost (SMC) of Flammuliua velutipes consists of abundant carbohydrates, such as corn cob meal and wheat bran (Williams *et al.*, 2001). Several studies evaluated the reduction of malodorous compounds in pig slurries by the addition of fermentable carbohydrates (Miller and Varel, 2003; Le *et al.*, 2008; Li *et al.*, 2009; Neifar *et al.*, 2012). However, microbial changes in pig slurries have not been evaluated, even though the environments of pig intestine and slurries could be improved.

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Several research studies related to the treatment of pig slurries for malodor control have been published (Govere *et al.*, 2005, 2007; Ye *et al.*, 2009). Diminishing malodorous compounds by reducing the amount of dietary crude protein has been confirmed (Hobbs *et al.*, 1999). Manipulations of dietary additives have resulted in decreased malodorous compounds in waste (Sutton *et al.*, 1999). Most studies have focused on measuring odor and gas intensity with proposed attempts to reduce odors; however, understanding the basic microbiology of pig slurry storage has not been well evaluated despite its importance. As a consequence, the determination of gaseous compounds, manipulation of precursor compounds generating odors, and development of effective biological control systems are needed for efficient odor control. In this study, we determined the modulatory effects of short-term nutritional enrichment practices using powdered horseradish (PHR) and SMC treatments of pig slurry storage for controlling odorous compounds. The effects of PHR and SMC treatments on pig slurry were characterized by measuring odorous compounds, along with microbiome changes, using pyrosequencing of the 16S rDNA gene sequences (Ahn *et al.*, 2012a).

Materials and Methods

Sample preparation

Pig slurry samples were obtained from pigpen pits of finishing pigs fed a basal diet (digestible energy, 3,450 kcal/kg; crude protein, 16.5%; total lysine, 0.87%). The three treatments, repeated four times, consisted of PHR [final 0.03% with 112 ml 3% hydrogen peroxide solution (Sigma- Aldrich)], SMC (Flammuliuavelutipes, final 1%) and CNS. The amount of hydrogen peroxide used was decided according to a previous report (Ye *et al.*, 2009). Additionally, optimal PHR and SMC dosages for deodorization of pig slurries were determined in a previous study (data not shown). Each sample was obtained from the 20-L pilot chamber that resembled a pigpen pit. After treatment with additives, the mixtures were incubated in the 20°C chamber for 14 days with continuous air flow (15 ml/min), because odors in pig farms are the most problematic from April to October in Korea when the pig slurry is moored at the pit for 2 weeks at a mean temperature of 19.92°C (http://www.kma.go.kr/weather/climate/average_world_monthly.jsp). Samples were collected from 10 cm below the slurry surface and stored at -20°C before using.

Odorous component analyses

The odorous components were quantified using gas chromatography (6890N, Agilent Technologies) equipped with a flame ionization detector. For determination of phenols and indoles, the samples were prepared according to previously reported methods (Jensen *et al.*, 1995) and measured using a DB-5 column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies). The gas chromatograph parameters were as follows: split mode, 5:1; inlet and detector temperature, 250°C; injection volume, 2 μl. The oven temperature program comprised an initial temperature of 50°C, a 5-min hold; 10°C/min ramp to 220°C, and a 2-min hold. VFAs were extracted using 25% HPO3 solution (Sigma-Aldrich) and analyzed under the following conditions using a HP-INNOWax column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies): split mode, 10:1; inlet and detector temperature, 250°C; and injection volume, 0.2 μl. The oven temperature program comprised an initial temperature of 80°C, a 5-min hold, 20°C/min ramp to 120°C, 0-min hold, 10°C/min ramp to a final temperature of 205°C, and a 2-min hold.

Slurry metagenomic DNA extraction and retrieval of 16S rDNA sequences

Total metagenomic DNA was extracted from the pig slurry samples using the Qiagen Stool Kit (QIAGEN Inc.) following the manufacturer's instructions for bacterial metagenomic DNA extraction. To amplify 16S rDNA, the extracted metagenomic DNA (100 ng) was used for polymerase chain reaction (PCR) with the universal prokaryotic primers, 27F 5-AGAGTTTGATCMTGGCTCAG and 518R 5-ATTACC GCGGCTGCTGG, which amplify an approximately 500-bp fragment of the V1-V3 region of 16S rDNA (Ahn *et al.*, 2012b). The temperature profiles for the 50-μl PCR reaction were as follows: 5 min at 94°C; 35 cycles of 30 sec at 94°C, 45 sec at 55°C, 90 sec at 72°C, and hold at 8°C. PCR products were examined on a 1.5% agarose gel, and the main amplicon band of 500 bp was excised using a razor blade. The PCR amplicon was purified using the gel elution method with the Xprep Gel & PCR Purification Kit (JMC-R&D) and prepared for pyrosequencing using the 454 GS Junior (Roche), after labeling with MID according to the manufacturer's protocol.

Phylogenetic analysis

16S rRNA sequence data generated by the 454 GS Junior (Roche) were demultiplexed, quality filtered and analyzed using QIIME v1.8.0 (Caporaso *et al.*, 2010a). Reads were truncated at any site containing more than three consecutive bases receiving a quality score of < 1e-5, and any read containing one or more ambiguous base calls was discarded. The primer sequences were excluded, and sequences low in quality or shorter than 200 bp in length were removed from the data sets. Initially, all chimeric sequences were identified and excluded from downstream analysis using a QIIME-based wrapper in USEARCH 6.1 (Edgar *et al.*, 2011). Operational taxonomic units (OTUs) were assigned using a threshold of 97% pairwise identity using the uclust-based method with open-reference option in QIIME (Edgar, 2010). Sequence prefiltering (sequences with < 60% pairwise identity to any reference sequence were discarded) and reference-based OTU picking were performed using a representative subset of the Greengenes bacterial 16S rRNA database (13_8 release) to remove incomplete and unannotated taxonomies (McDonald *et al.*, 2012a). OTUs were classified taxonomically using a QIIME-based wrapper of the Ribosomal Database Project (RDP) classifier (Wang *et al.*, 2007a) against a representative subset of the Greengenes 16S rRNA database 13_8 release (McDonald *et al.*, 2012a; Oh *et al.*, 2012) with a 0.80 confidence threshold for taxonomic assignment.

Bacterial 16S rRNA gene sequences were aligned using PyNAST (Caporaso *et al.*, 2010b) against a template align-

ment of the Greengenes core set filtered at 97% similarity. From this alignment, chimeric sequences were identified and removed using ChimeraSlayer (Haas *et al.*, 2011) before downstream analysis. Any OTU representing less than 0.001% of the total filtered sequences was removed to avoid inclusion of erroneous reads, which can lead to inflated estimates of diversity (Bokulich *et al.*, 2013).

Alpha-diversity estimates, including non-parametric richness estimators Chao1 and the Shannon index, were calculated for each sample, and beta-diversity estimates were calculated within QIIME using weighted UniFrac distance (Lozupone and Knight, 2005) between samples for bacterial 16S rRNA reads (evenly sampled at 1,000). Principal coordinates were computed from the resulting distance matrices to compress dimensionality into principal coordinate analysis (PCoA) plots, allowing visualization of sample relationships. To determine potential differences in phylogenetic or species diversity among the sample classifications (CNS, PHR, and SMC), analysis of similarities (ANOSIM; Clarke, 1993) with 999 permutations was used between sample groups based on weighted UniFrac distance matrices (Lozupone and Knight, 2005). One-way ANOVA was used to determine which taxa differed among the sample groups (CNS, PHR, and SMC). Distributions of individual bacterial genera among the three pig slurries were visualized using Venn diagrams generated at http://bioinformatics.psb.ugent.be/webtools/Venn/.

Defining the community correlation of pig slurry microbiota

To define the functional correlation of microbial community in the pig slurries, an in silico metagenome was generated using PICRUSt (Langille *et al.*, 2013). Reference OTUs from QIIME were subjected to PICRUSt analysis to sort individual genes using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways represented in varying proportions among each generated metagenome (www.genome.jp/kegg/pathway.html). Comparisons of predicted pathway abundance among the CNS, SMC, and PHR treatments were visualized using a heat map and gplot packages in R. Pearson's r test was computed for each treatment to test significant correlations of the pig slurry treatments with various taxa.

Statistical analyses

Slurry odor components were analyzed statistically using the GLM procedure of the Statistical Analysis System (SAS, 1996). The effects of additives were compared and examined for significant differences between the treatment and control means using Duncan's multiple range tests. A P-value < 0.05 was determined to indicate statistical significance for all variables measured. The other statistical analyses were performed using R software (version 3.0.1).

Results and Discussion

Odorous compound analyses

Concentrations of phenols, indoles, and VFAs were measured in the slurries incubated for 14 days after addition of 0.03% PHR and 1% SMC. After PHR treatment, phenol concentration was decreased significantly by 9%, and the p-cresol concentration, a major odorous component in phenols, was decreased by 10%. After SMC treatment, indole concentration was decreased by 25%, and skatole concentration, a major odorous component in indoles, was decreased by 32% (Table 1). Reportedly, phenols including phenol, p-cresol and 4-ethylphenol in slurry were reduced 45% after treatment with 10% minced horseradish root and hydrogen peroxide (Govere *et al.*, 2007). The reduction of phenols, but not indoles, was initiated by the degradation of phenolic compounds caused by the peroxidase in horseradish, which was consistent with a previous report (Govere *et al.*, 2007).

Additionally, Subair *et al.* (1999) suggested that a decrease in skatole concentration induced by SMC addition might result from a relative reduction in protein degradation caused by the increased proportion of carbohydrates in slurry. For example, increased carbohydrate levels derived from sugar beet pulp in feed were sufficient to eliminate skatoles and

Table 1. Effects of slurry additives on the odorous compound concentrations of pig slurries (mg/L)

	CNS	PHR	SMC
pH	$7.36^{a} \pm 0.06$	$7.27^{a} \pm 0.08$	$7.01^{b} \pm 0.13$
Phenols[1)]	$49.61^{a} \pm 7.85$	$26.74^{b} \pm 2.56$	$53.14^{a} \pm 6.57$
Phenol	$2.09^{a} \pm 0.20$	$1.59^{a} \pm 0.18$	$1.83^{a} \pm 0.15$
p-Cresol	$47.52^{a} \pm 7.83$	$25.14^{b} \pm 2.48$	$51.31^{a} \pm 6.44$
Indoles[1)]	$3.79^{a} \pm 0.19$	$4.43^{a} \pm 0.45$	$2.48^{b} \pm 0.13$
Indole	$1.14^{a} \pm 0.07$	$1.08^{a} \pm 0.09$	$0.73^{b} \pm 0.06$
Skatole	$2.65^{b} \pm 0.14$	$3.35^{a} \pm 0.38$	$1.75^{c} \pm 0.09$
SCFA[1)]	$7545^{b} \pm 674.16$	$5717^{c} \pm 420.35$	$10737^{a} \pm 330.87$
Acetic acid	$4305^{b} \pm 282.30$	$3059^{c} \pm 262.23$	$5506^{a} \pm 210.44$
Propionic acid	$1867^{b} \pm 246.67$	$1325^{c} \pm 99.18$	$2805^{a} \pm 56.40$
Butyric acid	$1373^{b} \pm 152.33$	$1333^{b} \pm 72.07$	$2426^{a} \pm 91.90$
BCFA[1)]	$1743^{a} \pm 122.29$	$1168^{b} \pm 42.35$	$1648^{a} \pm 72.23$
I-Butyric acid	$659^{a} \pm 48.73$	$439^{b} \pm 18.14$	$641^{a} \pm 29.25$
I-Valeric acid	$1085^{a} \pm 73.61$	$728^{b} \pm 25.60$	$1007^{a} \pm 43.04$

[1)] Phenols, sum of Phenol and p-Cresol; Indoles, sum of Indole and Skatole; SCFA, sum of Acetic acid, Propionic acid and Butyric acid; BCFA, sum of I-Butyric acid and I-Valeric acid

[a,b,c] Figure with different superscripts within the same row are significantly different ($P < 0.05$)

Table 2. Summary of the pyrosequencing data obtained from the pig slurry samples

Samples	Number of reads		Number of OTUs[a]	Good's Coverage[a]	Richness estimator[a]		Diversity index[a]	
	Raw	Chimera removed			Chao1	ACE	Shannon	Simpson
CNS.1	14,105	647	628.0	0.93	1,181	1,247	6.84	0.970
CNS.2	13,315	612	618.0	0.92	1,218	1,301	6.87	0.971
PHR.1	7,932	364	417.0	0.95	919	890	6.08	0.923
PHR.2	7,845	360	366.0	0.95	731	737	5.80	0.911
PHR.3	7,640	351	315.0	0.96	624	674	5.37	0.902
SMC.1	15,592	716	807.0	0.92	1,450	1,274	7.50	0.981
SMC.2	16,416	754	860.0	0.91	1,479	1,576	7.56	0.982
SMC.3	14,620	671	703.0	0.93	1,186	1,193	7.32	0.979

[a] Calculated at a 97% sequence similarity cut-off

indoles (Hawe *et al.*, 1992). Additionally, SCFAs, but not BCFAs, were significantly increased in SMC pig slurries (Table 1). Reportedly, a diet of fermentable carbohydrates increased the formation of SCFAs in the hind gut and, thereby, reduced the growth of skatole-forming bacteria, which resulted in decreased skatole levels in the slurry (Overland *et al.*, 2011). This was consistent with the lower pH (7.01) of SMC pig slurries compared with the pH 7.27 of PHR pig slurries and pH 7.36 of the CNS in this study. The SMC polysaccharides, including hemicelluloses, celluloses and lignins, can be considered easily fermentable (Williams *et al.*, 2001). Thus, addition of fermentable carbohydrates into the pig slurry moderated odor generation in this study (Table 1).

PHR treatment showed significant effects on the concentrations of SCFAs and BCFAs, which were reduced by 24% and 33%, respectively (Table 1). Hypothetically, peroxidase and peroxide reactions generate dissolved oxygen and activate aerobic bacteria in the slurry. Since these activated aerobic bacteria can decompose the odorous compounds, the VFAs in pig slurry were reduced.

16S rDNA sequence analysis

To reveal the genomic information of the dominant bacteria responsible for malodor generation in pig slurry, 16S rDNA was amplified from the extracted DNA samples using universal 16S rDNA primers and sequenced using a 454 GS Junior pyrosequencing platform, which produced 97,465 reads (46.9 Mbp). A total of 4,476 reads (4.5%) were found to be chimeric and were removed from further analyses (Table 2). Reportedly, duplicates from pyrosequencing reads can lead to incorrect interpretation of the species abundance in metagenomic studies (Niu *et al.*, 2010). The average read length was 403 bp, and 92,989 reads were used for microbiome analysis after quality filtering. Good's coverage values, calculated at a 97% similarity cut-off, indicated that the number of pyrosequencing reads obtained was sufficient to capture the bacterial diversity in the slurry samples. Good's coverage index for the observed OTUs identified in the pig slurry ranged from 0.91–0.96 (Table 2).

To examine the diversity of the microbial community in the treated pig slurry, OTUs were identified in the microbiome of pig slurry. The total number of high quality singleton 16S OTUs recovered from each treatment is listed in Table 2. The average numbers of high quality OTUs for each treatment were as follows: CNS, 623 ± 7.0; PHR, 366 ± 51.0 and SMC, 790 ± 80.0. Rarefaction analysis indicated a high level of bacterial diversity was obtained for subsequent analysis of slurry additive treatments (Fig. 1). The Chao1 estimate for PHR treatment (758 ± 149) was in close agreement with the observed number of OTUs (366 ± 51.0). Specifically, rarefaction curves of CNS and SMC did not become asymptotic at 7,800 reads in the PHR, whereas SMC approached saturation, indicating that the OTU diversity was almost completely covered. Ni *et al.* (2013) showed that the number of sequence reads belonging to unobserved OTUs did not affect the alpha-diversity of a given metagenome or the genomic coverage of rare species. The total abundance of the observed

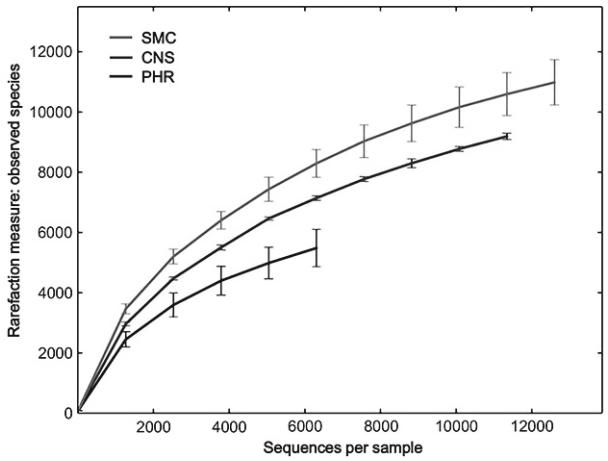

Fig. 1. Rarefaction analysis curves of bacterial 16S rDNA pyrosequences spanning the V1-V3 region from the pig slurry samples. Sequences were associated with OTUs using a pairwise sequence similarity threshold of 97%.

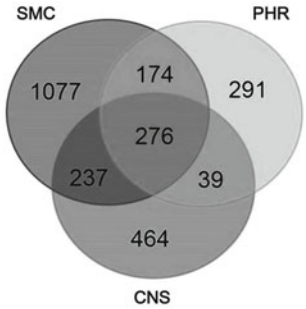

Fig. 2. A Venn diagram showing the distribution of all 805 OTUs identified by 16S rDNA pyrosequencing among the three pig slurry treatments, which revealed a shared community comprising 106 OTUs found in all three samples.

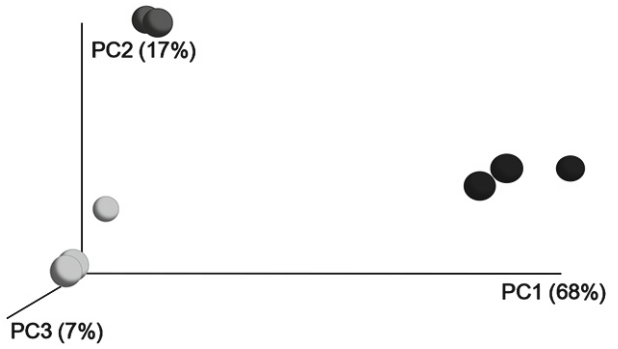

Fig. 3. Principal coordinates analysis (PCoA) based on weighted UniFrac distance matrices revealed the treatment effect on slurry bacterial communities. The clustering pattern was recaptured with only 1,000 sequences/sample, with the CNS (red), SMC (green), and PHR (blue) largely clustering with each other.

OTUs and their associated centroids distributed across treatments were indicated in the Venn diagram (Fig. 2). The overlapping OTU clusters among the treatments (CNS, SMC, and PHR) were calculated and were high in quality after removing singleton OTU sequences. CNS and SMC showed the largest OTU overlap among the treatments when compared with PHR (Fig. 2). At the OTU level, a total of 276 OTUs (10.7%) were in common among the three treatments, and 1,832 OTUs (71.6%) were identified in each treatment, indicating that the microbial diversity was influenced significantly by the treatments.

Bacterial taxonomic distribution in the pig slurry

To compare the overall composition of the microbiota among the pig slurry samples, UniFrac distances (Lozupone and Knight, 2005; Lozupone *et al.*, 2011) were calculated and compared among sample aliquots. Principal coordinate analysis (PCoA) based on weighted UniFrac distances for all samples (n = 8) revealed three distinct clusters, and each cluster contained samples obtained from each treatment (Fig. 3). The PCoA plot of weighted UniFrac distances showed significant sample clustering depending on the treatment. ANOSIM confirmed the samples from the individual treatment clustered based on the treatment condition (r = 0.9592, $P = 0.005$). Interestingly, the average weighted UniFrac distances calculated based on the experiment were found to be significant ($P < 0.05$), indicating that the microbiota in pig slurry samples were more similar within the individual of origin. A recent study on swine gut microbiota demonstrated a relationship between phylogenetic similarity and co-occurrence, with the phylogenetic group containing similar functional properties (Lamendella *et al.*, 2011).

A total of 11 phyla were distributed among all slurry samples (Fig. 4A), and the majority were represented by five phyla (83.4%): Bacteroidetes (32.3%), Proteobacteria (22.2%), Firmicutes (17.2%), Synergistetes (7.4%), and Tenericutes (4.3%). Lentisphaerae, Verrucomicrobia, Actinobacteria, Spirochaetes, Planctomycetes, and TM7 were considered the less abundant phyla. In addition, 12.5% of the sequence reads were unclassified as other bacterial clones. Firmicutes phylotypes belonging to the order Clostridia (84.0%) accounted for the majority of the classified OTUs (Fig. 4B). Four of the Proteobacteria classes (α, β, γ, and δ) were represented in all three slurries (Fig. 4C); OTUs classified as β-Proteobacteria (73.0%) accounted for the majority of the Proteobacteria phyla among the three pig slurries. Microbes can comprise up to 100 trillion cells, 10-fold more than eukaryotic stem and somatic cells (Turnbaugh *et al.*, 2007). The majority of microbes in the animal gastrointestinal tract (GIT) belong to the Firmicutes and Bacteroidetes phyla (Marchesi, 2010). Bacteroidetes, Firmicutes, and Proteobacteria phyla are also encountered in the fecal microbiota of mammals, such as mice (Savage *et al.*, 1968), dogs (Middelbos *et al.*, 2010), pigs (Leser *et al.*, 2002), and ruminants (Tajima *et al.*, 1999; Leng *et al.*, 2011). In the present study, the taxonomic distribution was consistent with the phylum distribution observed previously in the GIT and fecal microbiota (Snell-Castro *et al.*, 2005; Middelbos *et al.*, 2010; Allen *et al.*, 2011; Lamendella *et al.*, 2011; Looft *et al.*, 2012).

Influence of treatments on the pig slurry microbiota

The bacterial species richness (Chao1) values of PHR-treated (758 ± 149.3) and SMC-treated (1371 ± 161.4) pig slurries differed from the CNS value (1200 ± 26.1). Bacterial diversity in the SMC-treated pig slurry, represented by species richness (Chao1), was increased by the addition of SMC containing abundant carbohydrates. Species richness in DGGE analysis of feces was increased in pigs fed whole crop rice (Wang *et al.*, 2007b). SCFA concentration, which was associated with bacterial species richness, was increased in SMC-treated pig slurries (Table 1). Increasing the fiber content in the diets fed to pigs resulted in higher levels of VFAs and phenols in feces (Ziemer *et al.*, 2009). Moreover, addition of PHR could decompose malodor compounds, which results in reduced SCFAs and BCFAs in treated pig slurries. Horseradish roots contain large quantities of peroxidase enzymes and possess a unique ability to oxidize aromatic compounds to free radicals or form non-toxic, odorless polymers (Klibanov *et al.*, 1983).

When compared with CNS (Fig. 4A), PHR and SMC treatments decreased the proportions of Firmicutes ($P < 0.001$), Synergistetes ($P = 0.073$), and Verrucomicrobia ($P < 0.001$) and increased the proportion of Proteobacteria ($P < 0.001$). Additionally, significant differences were observed among treatments in the abundances of Bacteroidetes ($P = 0.027$) and Tenericutes ($P = 0.070$) phyla. Moreover, no significant differences were observed in the phyla Lentisphaerae ($P = 0.269$), Spirochaetes ($P = 0.114$), Actinobacteria ($P = 0.255$), Planctomycetes ($P = 0.146$) or TM7 ($P = 0.323$). These phyla were shown previously to constitute the majority of the common bacterial groups found in different ecological niches, including soil, ocean, freshwater and the GIT of animals (Bowers *et al.*, 2009; Marchesi, 2010). This indicates that the metabolic potential of some phyla allows others to dominate collectively, while the other less abundant phyla encounter extreme environments.

The responses of the most abundant bacteria at the phylogenetic levels of class, order and family were observed in a series of heat maps (Supplementary data Figs. S1, S2, and S3). At the class level, Bacteroidia, Clostridia, Synergistia, Betaproteobacteria, Sphingobacteria, Mollicutes, Deltapro-

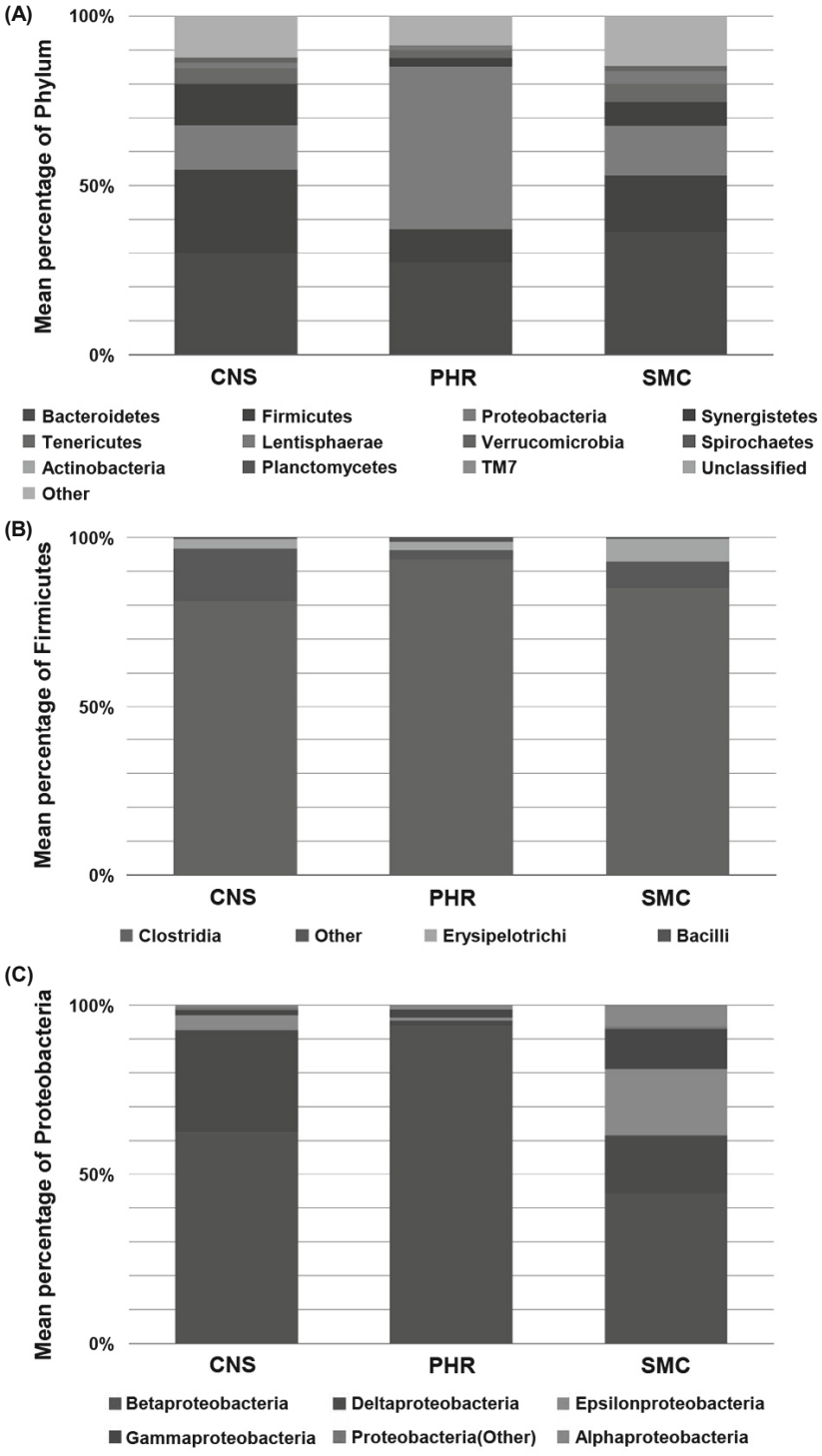

Fig. 4. Relative abundance of bacterial 16S rDNA genes from pig slurry samples at the phylum level (A). Breakdown of *Firmicutes* (B) and *Proteobacteria* (C) OTUs as determined by the RDP classifier.

teobacteria and other unclassified bacterial classes were among the most abundant in all pig slurries (Supplementary data Fig. S1). The relative abundances of Clostridia ($P < 0.001$), Synergistia ($P < 0.001$), Sphingobacteria ($P = 0.001$), Mollicutes ($P = 0.001$), Deltaproteobacteria ($P = 0.003$), and unclassified Firmicutes ($P = 0.005$) were significantly lower in the PHR treatment, while the class *β-proteobacteria* ($P < 0.001$) was increased in the PHR slurry compared with CNS and SMC pig slurries (Supplementary data Fig. S1). The predominant orders were Bacteroidales, Clostridiales, Synergistales, Burkholderiales, Sphingobacteriales, and unclassified bacteria (Supplementary data Fig. S2). The percentages of Clostridiales ($P < 0.001$), Synergistales ($P < 0.001$), Sphingobacteriales ($P < 0.001$), Desulfovibrionales ($P < 0.001$), and other Firmicutes ($P = 0.001$) were decreased, while Burkholderiales ($P < 0.001$) was increased in the PHR (Supplemen-

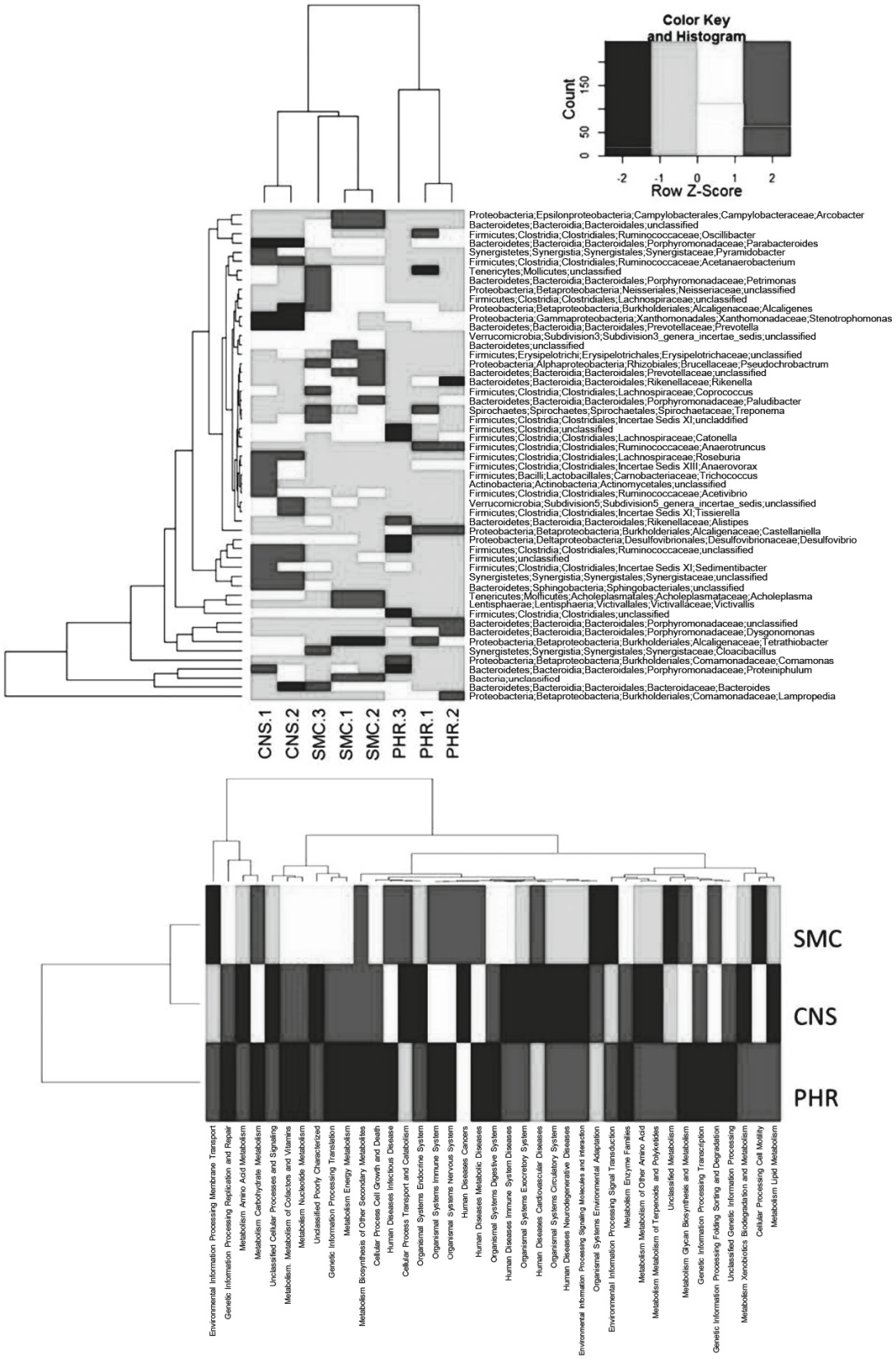

Fig. 5. (A) Heat map displaying the effects of pig slurry additives on the relative abundances of the top-level bacterial genera. (B) Heat map visualization of PICRUSt-predicted community metagenomes of pig slurry compared with respective controls. PHR-treated slurry exhibited enrichment of an increased number of KEGG pathways compared with CNS and SMC pig slurries.

tary data Fig. S2) compared with the CNS and SMC pig slurries. At the family level, the predominant families included Porphyromonadaceae, Synergistaceae, Ruminococcaceae, Alcaligenaceae, IncertaeSedis XI, Desulfovibrionaceae, Acholeplasmataceae, Bacteroidaceae, Victivallaceae, Comamonadaceae and unclassified bacteria (Supplementary data Fig. S3). When compared with the PHR treatment, Synergistaceae ($P < 0.001$), Ruminococcaceae ($P < 0.001$), Sphingobacteriales ($P < 0.001$), IncertaeSedis XI ($P < 0.001$), Desulfovibrionaceae ($P < 0.001$), Acholeplasmataceae ($P = 0.001$), and Lachnospiraceae ($P = 0.003$) proportions were lower, while Comamonadaceae ($P = 0.003$) was more abundant (Supplementary data Fig. S3).

Influence of treatments on the genera of the pig slurry microbiota

The influence of the treatments on the genera of the pig slurry microbiome was evaluated using double hierarchical cluster analysis of the top 53 most abundant genera (≥ 97% of the total number of bacterial genera observed) clustered by treatment (Fig. 5A). Among these genera, the 18 most abundant genera comprised a single distant cluster, and the other 35 genera comprised another main cluster. The average abundance of the top 20 genera and the responses of the taxa to PHR and SMC were significantly associated with treatment type ($P < 0.05$). The top 20 genera accounted for approximately 75% of all phylotypes in the three samples (Table 3). In general, when compared with CNS pig slurry, PHR and SMC treatments decreased the proportions of Proteiniphilum ($P = 0.040$), unclassified bacteria ($P = 0.313$), Synergistaceae ($P < 0.001$), Sphingobacteriales ($P < 0.001$), Tetrathiobacter ($P = 0.021$), Clostridiales ($P = 0.003$), Ruminococcaceae ($P < 0.001$), Sedimentibacter ($P = 0.047$), Desulfovibrio ($P = 0.001$), unclassified Firmicutes ($P < 0.001$), Acholeplasma ($P = 0.154$), and Acetanaerobacterium ($P < 0.001$), while those of Bacteroides ($P < 0.001$), Pyramidobacter ($P < 0.001$) and Comamonas ($P = 0.487$) were increased in the PHR and SMC pig slurries.

Correlation of the bacterial community with the concentration of odorous compounds

The present study showed the presence of high concentrations of SCFAs and BCFAs in the SMC pig slurry. Bacterial fermentation in the pig slurry contributes substantially to the digestion of slurry additives and produces odorous compounds such as phenols, indoles and VFAs in slurry (Mackie *et al.*, 1998). VFAs represent a wide range of compounds produced through in vitro anaerobic fermentation of pig slurry during storage (Mackie *et al.*, 1998). The increased concentrations of SCFAs and BCFAs in the CNS and SMC pig slurries were associated with bacterial species abundance, whereas their concentrations in the PHR treatment were decreased due to disturbance in species abundance (Fig. 2). The bacteria-mediated fermentation of biopolymers in the pig slurry leads to the release of volatile SCFAs. Reportedly, microbial digestive additives contain bacteria or enzymes that eliminate odors and suppress gaseous pollutants by their biochemical digestive processes (Liao and Bundy, 1994).

The 11 bacterial phyla detected in pig slurry have been reported in fecal microbiota and play a fundamental role in protein and carbohydrate metabolism (Leser *et al.*, 2002; Middelbos *et al.*, 2010; Lamendella *et al.*, 2011; Mao *et al.*, 2012). Several researchers showed their potential roles in

Table 3. Relative abundance and number of operational taxonomic units (OTUs) of top 20 genera

Genus	CNS		PHR		SMC	
	Ra	#OTUs	Ra	#OTUs	Ra	#OTUs
G:Proteiniphilum	14.22%	1793	6.50%	471	9.40%	1320.6
K:Bacteria;Unclassified	12.12%	1529	8.40%	609	14.80%	2080
F:Synergistaceae	6.30%	803.5	0.40%	31.3	2.00%	280.6
O:Sphingobacteriales	6.20%	790.5	0.08%	6.3	2.18%	307.3
G:Tetrathiobacter	5.90%	749.5	7.00%	505.3	1.02%	144.3
O:Clostridiales	5.10%	649	2.27%	164.3	5.83%	819.3
F:Ruminococcaceae	4.20%	541.5	1.19%	86	2.18%	306.3
G:Cloacibacillus	4.10%	523.5	2.10%	151	3.86%	543.3
G:Sedimentibacter	4.10%	521.5	1.23%	88.6	0.64%	90.6
G:Desulfovibrio	3.80%	491.5	0.61%	44	2.44%	343
P:Firmicutes	3.80%	483	0.26%	19.3	1.27%	179.3
G:Acholeplasma	3.50%	452.5	1.66%	120	4.17%	587
F:Porphyromonadaceae	2.90%	369	4.57%	328.6	2.64%	372
G:Bacteroides	2.60%	338	6.67%	480	11.00%	1544.3
G:Acetanaerobacterium	1.80%	229	0.06%	4.3	0.17%	24
G:Pyramidobacter	1.70%	221.5	12.00%	8.6	1.03%	145.6
G:Victivallis	1.70%	217	1.35%	97.3	3.91%	550
G:Comamonas	1.70%	215	8.34%	600	1.80%	252
G:Oscillibacter	1.20%	152	1.90%	136.3	1.60%	224.6
G:Petrimonas	1.10%	139.5	0.43%	31	1.40%	195.6
Total	88.04%	11209	67.02%	3982.6	72.34%	10310.3

Ra, Relative abundance; K, kingdom; P, phylum; O, order; F, family; G, genus

microbial degradation of carbohydrates (Flint *et al.*, 2012; McDonald *et al.*, 2012b). Bacteroidetes have become increasingly regarded as efficient consumers of biopolymers such as proteins and carbohydrates (Cottrell and Kirchman, 2000). Bacteroidetes are also proteolytic bacteria capable of fermenting amino acids into acetate (Kindaichi *et al.*, 2004). Firmicutes are syntrophic bacteria, which can degrade VFAs such as butyrate and its analogs (Hatamoto *et al.*, 2007; Sousa *et al.*, 2007). Proteobacteria, formerly known as purple bacteria, are the most physiologically diverse group of bacteria and well-known for utilization of a wide spectrum of carbon sources (Snaidr *et al.*, 1997; Samanta *et al.*, 2012). Additionally, *β-Proteobacteria* are the main consumers of propionate, butyrate and acetate (Ariesyady *et al.*, 2007). Moreover, a recent fecal study using dairy cows reported the fecal VFAs concentrations were significantly related to the Bacteroidetes and Firmicutes species (Mao *et al.*, 2012).

In the present study, PHR treatment was shown to be effective in decreasing SCFA and BCFA concentrations ($P < 0.05$; Table 1). Peroxidase has been investigated for the removal of phenolic contaminants from wastewater treatments (Morawski *et al.*, 2001). Peroxidases polymerize phenolic and indolic compounds in the presence of peroxides, rendering them insoluble, non-volatile compounds, thereby reducing the odor (Tonegawa *et al.*, 2003). Moreover, the role of horseradish peroxidase compounds in the initiation and catalysis of indole acetic acid degradation in the presence of oxygen has been reported (Fox *et al.*, 1965). The concentrations of VFAs in this study were similar to the results obtained in previous studies (Subair *et al.*, 1999; Govere *et al.*, 2007).

Interestingly, studies showed that the most abundant bacterial genera, including Proteiniphilum, unclassified bacteria, Synergistaceae, Sphingobacteriales, Clostridiales, Ruminococcaceae, Sedimentibacter, Desulfovibrio, unclassified Firmicutes, Acholeplasma, and Acetanaerobacterium, were decreased in the PHR treatment. Proteiniphilum were reported in anaerobic sludge wastewater as efficient producers of acetic and propionic acids (Chen and Dong, 2005), which correlated positively with the SCFA concentration in the CNS slurry. The increased abundances of Bacteroides, Pyramidobacter, and Comamonas genus in the PHR slurry were correlated positively with PHR treatment. The genus Pyramidobacter comprises strains that produce acetic and isovaleric acids as metabolic end products (Downes *et al.*, 2009). Comamonas was reported to be an efficient consumer of organic acids, amino acids and peptone, but rarely attacks carbohydrates (Ma *et al.*, 2009). To elucidate which of these treatment conditions may drive specific microbial populations across the samples, Pearson's correlation was used to model covariance between the treatments at the bacterial taxonomic levels. Scatter plots and correlation coefficients were generated between the variables and factors to test the strength of the linear relationship, confirming significant correlations between the treatments ($P < 0.05$; Supplementary data Fig. S4). Moreover, the strength of the correlation was influenced markedly and affected significantly by the treatment conditions. Pearson's correlation showed that the PHR slurry had a weak correlation with CNS and SMC pig slurries at the microbiota class level.

Functional correlation of community microbiota

This study used PICRUSt to predict the pathway-related functional gene abundance present in the treatment groups. Compared with CNS and SMC pig slurries, PHR-treated slurry was enriched in a substantially wider range of predicted KEGG pathways, including membrane transport, amino acid metabolism and carbohydrate metabolism, among others (Fig. 5B). In the present study, Burkholderiales ($P < 0.001$) was increased in the PHR (Supplementary data Fig. S2) compared with the CNS and SMC pig slurries. Members of Burkholderiales reportedly degrade aliphatic and aromatic hydrocarbons (Bacosa *et al.*, 2010, 2011). Moreover, bioactive compounds present in the *F. velutipes* have antioxidant and antibacterial activities (Leung *et al.*, 1997), which affect Burkholderiales abundance in the CNS pig slurry.

The predictive capabilities of observed community relationships were investigated by linking phylogenetic structure with function using a systems biology approach mechanism that has shown success in predicting functional differences among complex microbial communities (Roling *et al.*, 2010). The addition of activated sludge and nutrients (bioaugmentation) to slurries resulted in enhanced hydrocarbon removal (Juteau *et al.*, 2003), suggesting that the microbial community contributes to the enhanced removal of hydrocarbons. The results obtained in the present study showed that the enhanced predicted KEGG pathways, including membrane transport, amino acid metabolism and carbohydrate metabolism, in the pig slurry samples were responsible for biodegradation of hydrocarbons (Fig. 5B). Recently, 16S rDNA-based studies have inferred the functional contribution of particular community members by mapping marker genes (Morgan *et al.*, 2012). Similarly, the present study provided information for understanding the mechanisms by which microbial communities generate odorous compounds in pig slurries.

Conclusion

Concentrations of odorous compounds such as phenols, indoles, SCFAs and BCFAs in pig slurries were significantly affected by PHR and SMC treatments compared with CNS-treated pig slurries ($P < 0.05$). The 16S rDNA pyrosequencing results showed sufficient bacterial diversity in the pig slurries. The rarefaction analysis clearly showed that the bacterial species richness varied among the treated samples. The numbers of OTUs (defined at 97% similarity) were lower in the PHR than in the CNS and SMC pig slurries. Taxonomic analysis based on 16S rDNA gene sequences from the pig slurries indicated that the slurry microbiome was highly dominated by members of Bacteroidetes, Firmicutes, and Proteobacteria. A series of genera, including Proteiniphilum, unclassified bacteria, Synergistaceae, Sphingobacteriales, Clostridiales, Ruminococcaceae, Sedimentibacter, Desulfovibrio, unclassified Firmicutes, Acholeplasma, and Acetanaerobacterium, were negatively affected by the PHR and SMC treatments, while increased abundances of Bacteroides, Pyramidobacter, and Comamonas genera were observed in the PHR and SMC treatments. The bacterial community structures in the PHR- and SMC-treated pig slur-

ries were also associated with odorous compound concentrations. The above results substantiated the previous observation that pig slurry is dominated by several specific bacterial groups and showed that the relative abundances of OTUs can change markedly depending on the treatment condition. The differences in microbial communities among the CNS, PHR, and SMC pig slurries were revealed more clearly after PCoA clustering of the 16S rDNA gene sequences. This study was not designed to identify the role of microbes in odor generation. To the authors' knowledge, this is the first study using this method to evaluate the slurry microbiome of pigs treated with PHR or SMC for odor control. The study demonstrated variations in bacterial phylogenetic structure by correlating bacterial abundance with predicted KEGG pathway abundance data. The results constituted a significant and informative characterization of odorous compounds in pig slurry along with bacterial community structure. PHR and SMC treatments in pig slurry caused a sustained reduction in malodor. PHR and SMC could be used as alternative odor controllers in slurry storage by mitigating slurry bacterial communities. Currently, a more efficient odor mitigation strategy is required for manure management in pig farming, due to the importance of environmental welfare undergoing regulation in the livestock industry. Therefore, the results from the present study can be used for understanding the bacterial fermentation of pig slurry and the development of odor mitigation technologies based on control of the bacterial community.

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