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# Pyrosequencing-based Analysis of Fecal Microbial Communities in Three Purebred Pig Lines[§]

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**This study examined the fecal bacterial diversity of 15-week-old pigs from three purebred lines: Duroc, Landrace, and Yorkshire. Taxon-dependent and -independent analyses were performed to evaluate differences in the fecal bacterial communities and to identify bacterial genera that can be used to discriminate breeds, following high-throughput pyrosequencing of 16S rRNA genes. Among the breeds evaluated, Landrace had the most diverse bacterial community composition. *Prevotella*, *Blautia*, *Oscillibacter*, and *Clostridium* were detected in all samples regardless of breed. On the other hand, *Catenibacterium*, *Blautia*, *Dialister*, and *Sphaerochaeta* were differentially detected among breeds, as demonstrated by the canonical loading plot. The discriminant analysis of principal components plot also showed clear separation of the three purebred pig lines, with a certain degree of similarity between Landrace and Yorkshire pigs and a distinct separation between Duroc pigs and the other two breeds. Other factors not related to breed, such as season or time of sampling and pen effects, may contribute to shaping the gut microbiota of pigs.**

***Keywords***: pyrosequencing, 16S rRNA genes, microbiome, pig breeds

## Introduction

The mammalian gastrointestinal tract is abundantly populated by a community of organisms constituting the gut microbiome (Gibson and Roberfroid, 1995). The gut microbiome is recognized as an integral part of gut development, influencing many digestive functions and mucosal immunity (Gibson and Roberfroid, 1995), and is therefore described as an important organ of the body (O'Hara and Shanahan, 2006). High-throughput next-generation sequencing of 16S rRNA genes has provided abundant information on the composition of bacterial communities from diverse environments and conditions (Jung *et al*., 2011). The advent of high-throughput next-generation sequencing has facilitated characterization of the metagenomes and microbiomes of both human samples and animal models, such as pigs. Previous pig microbiomic reports focused on the effects of diverse factors, such as diet (Lu *et al*., 2013), administration of antibiotics and feed additives (Kim *et al*., 2012a), and exposure to pathogens (Dowd *et al*., 2008), on the gut microbiome. However, there have been few studies on the variations in gut bacterial communities among purebred pigs.

Gut microbiomic composition may be shaped by two main factors (Ley *et al*., 2006): (1) host genetics and (2) environmental factors. Previous studies have identified the presence or absence of certain genes or loci (Rehman *et al*., 2011), as well as differential expression of functional genes (Brodziak *et al*., 2013) that control the composition of the gut microbiota; however, the results have often been contradictory and inconclusive (Loh *et al*., 2008). Previous studies demonstrated the important interaction between genetic and environmental factors in shaping the gut microbiomic composition (Hildebrand *et al*., 2013).

Swine breeds have phenotypically distinct genetic makeups that influence many physiological traits and digestive functions. These genetic factors are distinguishable among breeds as observable characteristics and traits, including morphology (de Sevilla *et al*., 2008), reproduction (Tantasuparuk *et al*., 2000), digestive capacity (Guixin *et al*., 1995), and physiology (Shan *et al*., 2010), and have been commonly utilized in crossbreeding methods to improve digestive efficiency and overall productivity (Ibáñez-Escriche *et al*., 2011). The three purebred pig lines used in this study, Duroc, Landrace, and Yorkshire, are the most commonly used commercial pigs for crossbreeding and production purposes by farmers and hog raisers due to their favorable growth characteristics and performance (Ibáñez-Escriche *et al*., 2011). This work was limited to investigating the similarities and differences in the fecal microbial composition among three purebred pig lines (Duroc, Landrace and Yorkshire) using pyrosequencing of 16S rRNA genes to explore possible links between the breed of pig and the fecal bacterial community. To our knowledge, this is the first study to characterize and compare the fecal microbiomes of purebred pigs using this method.

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## Materials and Methods

### Sample collection and DNA extraction

Fresh individual fecal samples were collected aseptically from 15 vaginally delivered male pigs (five pigs per breed) at 15 weeks of age. To reduce maternal effects and individual variation (Campbell *et al*., 2012), we repeated the same experiment 2 months later using a new set of individual pigs, also 15 weeks of age, resulting in a total of 30 pigs (10 pigs per breed). Piglets were weaned 3–4 weeks after birth, and all pigs were given the same feed without administration of antibiotics or feed additives. Pigs of the same breed were kept within the same pen in an environmentally controlled private breeding facility (NongHyup, Youngkwang, Korea) during the entire study, without the introduction of new pigs. DNA was extracted from 0.5-g aliquots of each fecal sample using an UltraClean Fecal DNA Isolation Kit (Mo Bio Laboratories Inc., USA).

### Pyrosequencing

Using the DNA extracted from fecal samples, PCR was performed prior to pyrosequencing according to conditions described previously (Yu and Morrison, 2004). The cycling parameters were as follows: initial incubation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec and 55°C for 45 sec, with a final extension at 72°C for 1 min 30 sec. Amplicons were separated by 1.5% (w/v) agarose gel electrophoresis and purified using Gel Extraction kits (Macherey-Nagel, Germany) in accordance with the manufacturer's instructions. Bar-coded amplicon pyrosequencing of 16S rRNA genes was performed as described previously by Jeon *et al*. (2013) using bar-coded fusion primers targeting the V1–V3 variable regions (http://www.ezbiocloud.net/oklbb/1001). Pyrosequencing was performed by ChunLab Inc. (Korea) using Roche 454 GS-FLX Titanium chemistry (454 Life Sciences, USA). Raw sequence reads from each fecal sample were processed and analyzed as described by Jeon *et al*. (2013) using CLCommunity (http://www.chunlab. com).

### Data analysis

Sequence reads were analyzed as described previously (Jeon *et al*., 2013). Briefly, both the proximal and distal primers were trimmed from demultiplexed sequence reads. To minimize the effects of random sequencing errors, sequences were subjected to a quality control process that eliminated reads containing ambiguous base calls and those with less than 300 bases. Chimeras were identified and removed from the dataset using Mothur and the Bellerophon method, a partial-treeing approach (Lamendella *et al*., 2013). Non-specific PCR amplicons that showed no match against the EzTaxon-e database (http://www.eztaxon-e.org) in a BLASTN search were also excluded from subsequent analyses (Kim *et al*., 2012b).

Each pyrosequencing read was processed and assigned taxonomically using the EzTaxon-e database (Chun *et al*., 2007). The bacterial diversity of microbial communities was calculated using pooled sequences from 10 pigs per breed with an Operational Taxonomic Unit (OTU) defined at an identity cut-off of 97% (Na *et al*., 2011). Bacterial community composition and abundance were generated using the CLCommunity software (ChunLab Inc.).

The following statistical analyses were conducted using R software v3.0.2. To compensate for sequencing depth bias per sample, the percent abundances of each taxon within the sample were subjected to square root transformation. Prior to normalization, all bacteria that could not be classified into genera were grouped together in one group and removed from the identified genera. The heatmap (heatmap {vegan}) was created based on the normalized data for 26 differentially abundant genera (>0.1%). For multivariate analysis of bacterial genera, we used the adegenet package in R (Jombart and Ahmed, 2011). A canonical loading plot (loadingplot {adegenet}) was used to identify bacterial genera that could distinguish the pig breeds according to a user-defined threshold, set at the first quartile of the normalized data of bacterial genera. The separation of breeds was evaluated using bacterial genera as variables in discriminant analysis of principal components (DAPC) (dapc {adegenet}) using percent abundance data and square-root transformed data (sqrt {base}). Unlike principal component analysis (PCA) and multidimensional scaling (MDS), DAPC can optimize group variation to reveal differences among breeds. Permutational analysis of variance (PERMANOVA) was performed to identify the most significant factors influencing the microbial communities of pigs in this study. Using the normalized data of the 26 differentially abundant bacterial genera, we calculated PERMANOVA (adonis {vegan}) using 9,999 replicate permutations for all pigs grouped according to seasonal (sampling time), pen, and breed effects.

## Results and Discussion

### Bacterial diversity and composition of pig breeds

After quality control and demultiplexing, 146,467 sequences were generated using pyrosequencing with Yorkshire (50161), yielding the highest number of valid sequence reads, compared with Duroc (48457) and Landrace (47849). In addition, the highest number of OTUs with a cut-off of 97% identity was also found in Yorkshire (820), followed by Landrace (779) and Duroc (723). Bacterial diversities were compared among the three pig breeds using diversity and rich-

**Table 1. Diversity indices and summary of the 16S rRNA gene pyrosequencing data**

| Measurement | Duroc ($n$=10) | Landrace ($n$=10) | Yorkshire ($n$=10) |
|---|---|---|---|
| Total no. of valid reads[a] | 48457 [1851, 6745] | 47849 [2992, 7607] | 50161 [3046, 7268] |
| Total no. of OTUs | 723 | 779 | 820 |
| Shannon diversity index (H) | 6.09 | 6.40 | 6.28 |
| Chao1 estimator of species richness[b] | 2975 (2703, 3304) | 3636 (3288, 4053) | 4208 (3853, 4630) |
| ACE estimator of species richness[b] | 4793 (4512, 5102) | 6050 (5686, 6448) | 7208 (6823, 7625) |

Calculations were made based on the OTU definition at >97% sequence identity.
[a] The values in parentheses represent the ranges (of valid reads) in each pig breed.
[b] The values in parentheses represent the 95% confidence intervals, which indicate the precision of the richness estimate.

ness estimators, such as Shannon diversity index, Chao1, and abundance-based coverage (ACE) estimators (Table 1). Landrace had a more diverse bacterial community composition compared with the other breeds. On the other hand, Chao1 and ACE values showed that Yorkshire contained a larger number of less-abundant OTUs compared with Landrace and Duroc pigs. A Venn diagram showed that a greater number of OTUs (at 97% identity cut-off values) was seen in Yorkshire followed by Landrace and Duroc pigs (Supplementary data Fig. S1). Furthermore, these diversity estimates were found to be within the range reported previously for swine bacterial communities (Lu *et al*., 2013). While high bacterial diversity is favorable for overall health and productivity, further analyses of the bacterial community are needed to identify bacterial subpopulations with potential distinct compositions among breeds (Hildebrand *et al*., 2013).

Figure 1A presents the classification of the sequences at the phylum level in each pig breed. The major phyla that showed high abundances were Bacteroidetes, Firmicutes, Proteobacteria, Spirochetes, Tenericutes, Lentisphaerae, and Actinobacteria. Regardless of the breed, the dominance of Bacteroidetes and Firmicutes was consistent with previous reports on swine (Lu *et al*., 2013). These phyla were also found from ornithogenic soils contaminated with penguin feces (Kim *et al*., 2012c). While the fecal bacterial communities in all breeds were dominated by the phylum Bacteroidetes, the bacterial community in the feces of Landrace had a greater abundance of Firmicutes compared with Duroc and Yorkshire. In addition, the phylum Proteobacteria proportion in the feces of Yorkshire pigs (3.40%) was greater than those of the other two pig breeds, Duroc (1.96%) and Landrace (1.34%). At the class level (Fig. 1B), Bacteroidia dominated followed by Clostridia for all breeds, which was similar to the results of other swine microbiomic studies (Lu *et al*., 2013). However, differentially abundant bacteria were found among the breeds. Bacteroidia was more abundant in Duroc (56.99%) than Landrace (47.57%) and Yorkshire (51.32%). In addition, Gammaproteobacteria was more abundant in Yorkshire (2.66%) than Duroc (1.40%) and Landrace (0.59%).

From the 417 genera classified by the EzTaxon-e database (Supplementary data Table S1), 26 differentially abundant bacteria (relative abundance >0.1%) were found in the fecal samples (Fig. 2A). Regardless of the breed, the genera *Prevotella*, *Blautia*, *Oscillibacter*, and *Clostridium*, which are generally prevalent in the swine gastrointestinal tract (Kim *et al*., 2011), were also detected in all samples. *Lactobacillus* showed the highest abundance in Landrace, followed by Yorkshire and then Duroc pigs. On the other hand, *Catenibacterium*, *Phascolarctobacterium*, and *Subdoligranulum* were more abundant in Duroc, whereas *Dialister* was more abundant in Yorkshire. These observations may have been due to specific breed differences that influence gut functionality (McKnite *et al*., 2012). However, the functional roles of these bacteria in each of the pig breeds are still unknown. This limitation is due to the inability to culture some bacteria *in vitro*, as well as the scarcity of genomic information regarding these bacteria and their swine hosts.

Canonical loading plot analysis also showed that several bacterial genera were breed-specific. At a more limiting user-

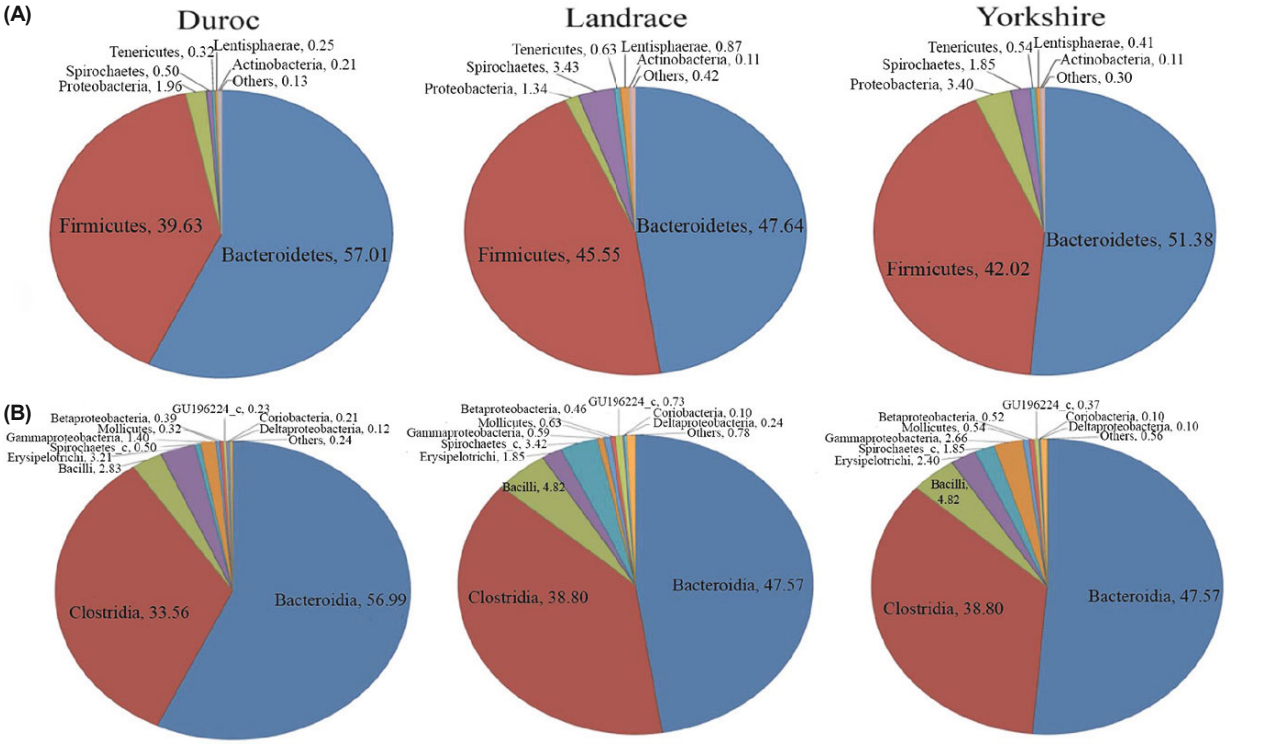


**Fig. 1. Bacterial composition and abundance of the fecal microbiota of Duroc (*n*=10), Landrace (*n*=10), and Yorkshire (*n*=10) pigs at the phylum (A) and class levels (B).**

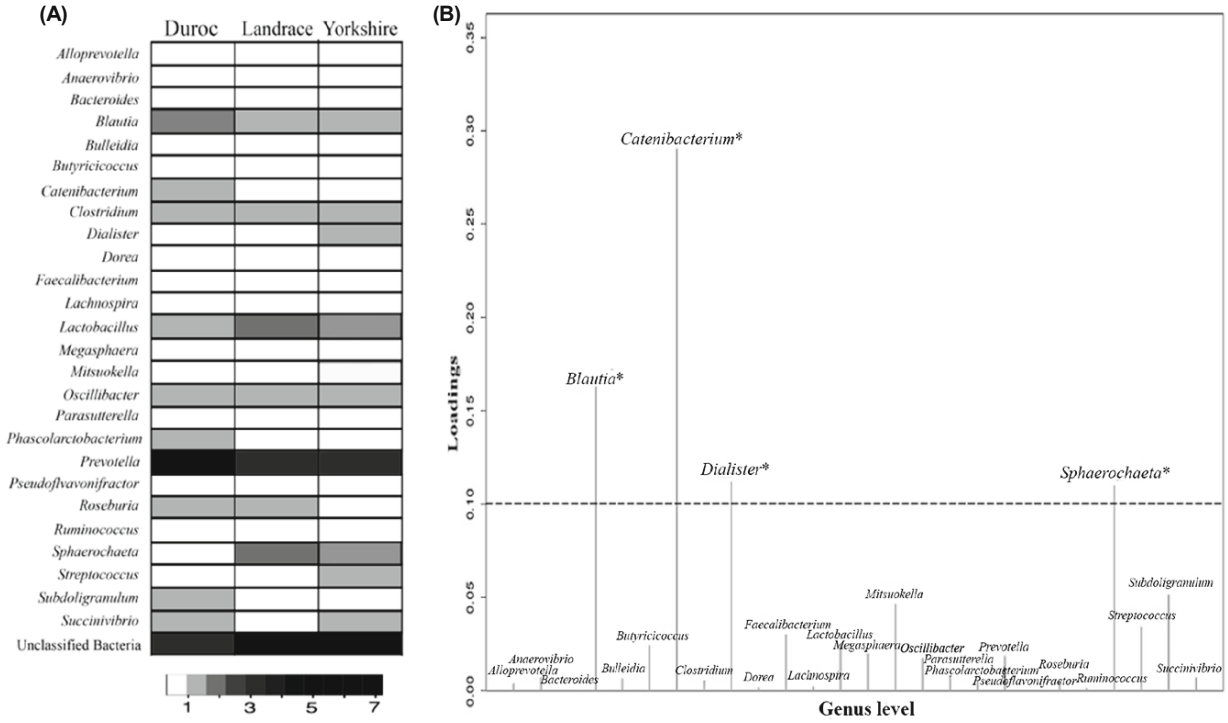


**Fig. 2. Heat map showing normalized values of 26 differentially abundant genera among Duroc, Landrace, and Yorkshire pigs.** (A) Genera with an abundance of >0.1% observed in at least one fecal sample were selected. The plot shows normalized mean abundance values. The relative levels of abundance are color-coded, where white represents the lowest (min=0) and black (max=7) the highest level of abundance. (B) The canonical loading plot of the discriminating bacterial genera used the 26 differentially abundant bacterial genera in the discriminant analysis of principal components and the *adegenet* package in R v3.0.2. At a 0.1 threshold level (broken line), the majority of the separation among Duroc, Landrace, and Yorkshire pigs is attributed to *Catenibacterium*, *Blautia*, *Dialister*, and *Sphaerochaeta*, labeled ( * ).

defined threshold level (0.1), *Catenibacterium*, *Blautia*, *Dialister*, and *Sphaerochaeta* genera significantly influenced the separation of the three breeds (Fig. 2B). Among them, genus *Catenibacterium* was exclusively found in the Duroc breed (Fig. 2A). *Catenibacterium* is Gram-positive and an obligatory anaerobe that utilizes glucose to produce acetic, lactic, butyric and iso-butyric acids and was exclusively present in pigs fed the dietary fiber inulin (Yan *et al*., 2013) and in healthy companion dogs (Kerr *et al*., 2013). The association among *Catenibacterium* abundance, inulin-fermenting ability, and growth performance in Duroc compared with other breeds should be elucidated further. On the other hand, the *Dialister* genus was specific to Yorkshire, while the *Sphaerochaeta* genus was not found in Duroc. Limited papers link these taxa with breed but they have been previously discovered from a variety of hosts citing their important function in the gut as well as their significance to the environment. *Blautia* was previously found in wild duck eubacterial microbiome (Strong *et al*., 2013). Though *Dialister* was recently linked with pyogenic liver abcess (Song *et al*., 2014), it also has a potential as host-specific fecal indicators of river samples (Jeong *et al*., 2011) and *Spaherochaeta* a commensal in the gastrointestinal tract of pre-weaned calves (Malmuthuge *et al*., 2014) and forage-fed horses (Shepherd *et al*., 2012). It is likely that the specificity of these bacteria to a particular animal breed is due to the various characteristics unique to each purebred pig line, which are controlled by host genetics (Ibáñez-Escriche *et al*., 2011). The importance of these breed-specific genera, however, should be investigated further to elucidate their potential applications.

### Separation of breeds and other effects

While some degree of similarity within the swine microbiota is expected, DAPC plots showed a clear separation among the breeds, indicating that bacterial communities are dissimilar among purebred pigs (Fig. 3). The fecal bacterial communities clustered according to breed, and the overall clustering of the pig gut microbiome suggests the existence of a core microbiota shared by all pigs within the same cluster (Kim *et al*., 2011). The microbiome of the Yorkshire group was similar to that of the Landrace group but clearly distinct from that of the Duroc group. In a previous study (Kim *et al*., 2005), the genetic structures of pig breeds were compared based on microsatellite loci analysis, and the closer relationship between the Landrace and Yorkshire pig breeds was explained by their genetic similarities. This study also suggested that the possible combination or mixture of gene pools between Landrace and Yorkshire was attributed to their closeness, while the Duroc breed was genetically distant from the other pig breeds. A recent study in mice showed that the host strain/breed exerts a marked effect on gut microbiota (Hildebrand *et al*., 2013). Another study using denaturing gradient gel electrophoresis analysis also showed that the microbial composition is different among breeds (Yang *et al*., 2014). Investigation of these similarities and

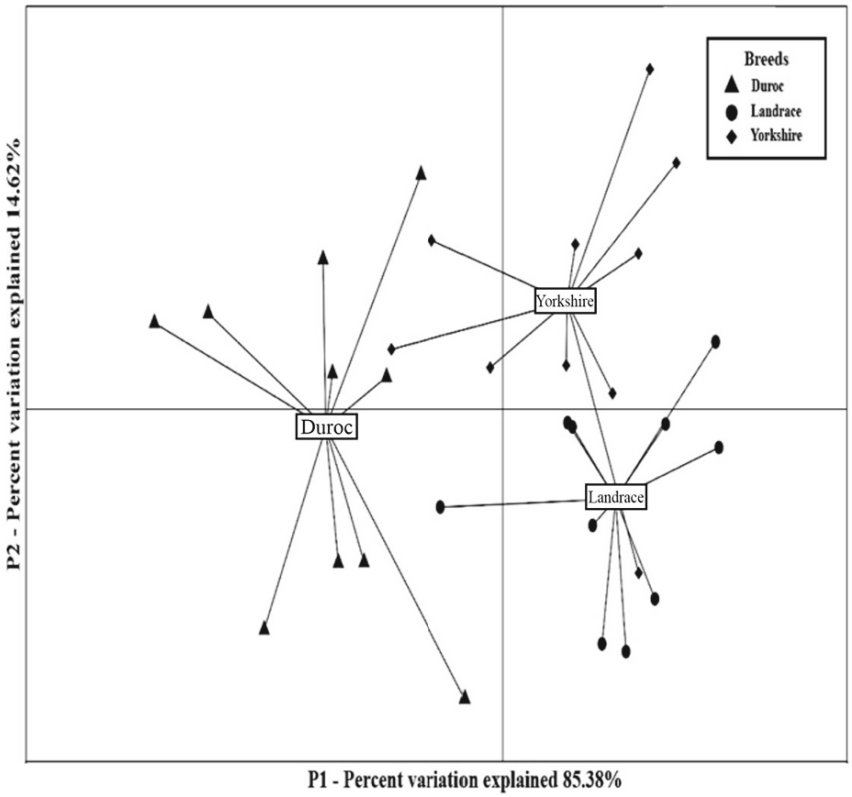


**Fig. 3. Discriminant analysis of principal components among pig breeds.** The 26 differentially abundant bacterial genera represent the number of variables in the model. Individual pig samples for breeding groups are designated with the following symbols: (▲) Duroc, (●) Landrace, and (◆) Yorkshire.

differences in the genetic structure of pigs using microsatellite loci from alleles in association with pyrosequencing may help to elucidate the breed effects that shape the gut microbiome of pigs.

In addition to host genetics, the effects of environmental factors on the gut microbiomic composition were investigated by PERMANOVA analysis. The results indicated that seasonal ($P$=0.0001) and pen effects ($P$=0.0001), in addition to breed, also influenced the gut microbiome of pigs ($P$= 0.2146). The seasonal effects on the microbiota of mammalian species have not been investigated extensively. Previous studies using mice in a highly controlled experimental environment indicated an increasingly similar microbiomic composition after several weeks of co-habitation (Hildebrand *et al.*, 2013). Variations in temperature and humidity also have effects on gut physiology and functionality in pigs (Chmielowiec-Korzeniowska *et al.*, 2012), suggesting that the season affects growth performance and physiology of animals. Nevertheless, it is suggested that genetic effects still play a role in shaping the gut microbiomic composition because pigs were kept in an environmentally controlled facility during our study.

In summary, we used 16S rRNA gene pyrosequencing to evaluate the differences in fecal microbiota of three commercial pig breeds. Analysis using DAPC plots showed a clear separation among the breeds. The microbiomes of Yorkshire and Landrace were more closely related to each other than to that of Duroc pigs, which may be due to gene pool similarities between the two breeds. Variations in the microbiota using an OTU definition cut-off of 95% identity showed that *Catenibacterium*, *Blautia*, *Dialister*, and *Sphaerochaeta* had the greatest influence on the separation of breeds; these bacteria may be linked to functional genes or characteristics unique to the breeds with which they are associated. However, certain environmental factors must also be taken into consideration and controlled in swine farming, including pen and seasonal effects, which can greatly influence the composition of gut microbiota along with the effects of host genetics.

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