Comparison of Fecal Microbial Communities between White and Black Pigs

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Abstract Meat from black pigs (BP) is in high demand compared with that from modern white pig (WP) breeds such as Landrace pigs owing to its high quality. However, the growth rate of black pigs is slower than that of white pig breeds. We investigated differences in the fecal microbial composition between white and black pigs to explore whether these breeds differed in the composition of their gut microbial communities. The swine gut microbiota was investigated using Illumina's MiSeq-based sequencing technology by targeting the V4 region of the 16S rRNA gene. Our results showed that the composition of the gut microbiota was significantly different between the two pig breeds. While the composition of the WP microbiota shifted according to the growth stage, fewer shifts in composition were observed for the BP gut microbiota. In addition, the WP gut microbiota showed a higher *Firmicutes/Bacteroidetes* ratio compared with that of BP. A high ratio between these phyla was previously reported as an obesity-linked microbiota composition. Moreover, the WP microbiota contained a significantly higher abundance of cellulolytic bacteria, suggesting a possibility of higher fiber digestion efficiency in WP compared to BP. These findings may be important factors affecting growth performance and energy-harvesting capacities in pigs. Our findings of differences in the gut microbiota composition between the two breeds may provide new leads to understand growth rate variation across pig breeds.

Keywords cellulolytic microorganisms · feces · growth rate · gut flora · pig breeds

Introduction

The gut microbiota constitutes one of the most complex mammalian microbial communities and has a major impact on the health status of animals (Fisher, 2012). Sequence analysis of the 16S rRNA gene has been widely used to elucidate the diversity and composition of microbial communities within several animal gut systems, including that of pigs (Ley et al., 2008; Lamendella et al., 2011; Isaacson and Kim, 2012). Recent studies have implicated the gut microbiota as a critical determinant of nutrient uptake, energy regulation, and ultimately, weight and metabolic disorders (Tilg, 2010; DiBaise et al., 2012; Krajmalnik-Brown et al., 2012). Moreover, recent reports of a possible association between the gut microbiota and obesity have placed the focus on the significance of the microbiota for well-being (Ley et al., 2005; Turrone et al., 2008; Tilg, 2010). While large amounts of research have been conducted to date on the human microbiome, only a limited amount of information has been collected about swine gut microbiota (Lamendella et al., 2011).

The breed of the pig is one of the most important sources of variation in the sensory quality of pork (Candek-Potokar et al., 1997), which is also influenced by physiological functions such as growth rate and fat deposition (Wood et al., 2004). For example, white pigs (WP), such as Landrace, are noted for their rapid growth, and their weight at weaning is higher than that of other breeds (Taylor et al., 2005). In contrast, black pigs are known for their slower growth rate and lighter carcass weight when compared with those of the other commercial pig breeds (Hwang et al., 2004a). Moreover, there is a high amount of consumer demand for meat from black pigs (BP) because of their redder meat, higher unsaturated fatty acid content, and higher intramuscular fat content compared with those of commercial white breeds such as Landrace pigs (Jin et al., 2001; Hansen et al., 2006; D.H. et al., 2009). Recently, it has been reported that the pig breed affects the composition of the gut microbiota, and the fecal microbial composition was found to differ between foreign breeds and native pigs (Yang et al., 2014). Although the growth of livestock animals can be affected by a number of factors, including

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genetics, age, antibiotics, and the health status of the animal (Turnbaugh et al., 2006; Looft et al., 2012; Shetty et al., 2013), information about non-genetic factors such as the gut microbiota may also be useful for improving the growth rate of livestock animals.

In the present study, we compared the gut microbiota composition between WP and BP. To date, various studies have reported that feed additives such as organic acids and probiotics could enhance the growth performance of pigs, which is an improvement that has been suggested to be likely mediated through the modulation of the gut microbiota (Zentek et al., 2013; Lahtinen et al., 2014). While BP are favored due to their high-quality meat, their growth rate is lower than other breeds. To our knowledge, the comparison of intestinal microbiota between white and black pigs has not previously been performed, and this is the first study that has investigated the relationship between pig breed and intestinal microbial community by 16S rRNA gene sequencing using Illumina's MiSeq technology. The work presented here provides fundamental information on the differences in the fecal microbial composition between white and black pigs, and these differences are discussed with respect to growth rate.

Materials and Methods

Swine feeding and fecal sampling. A total of six pigs (3 white and 3 black pigs) were used in this study, which were each aged approximately 3 weeks at the beginning of the study. The animals were ear-tagged and were raised in a local pig farm in Jeju Island, Korea. All animals were fed with standard commercial diet based on corn and soybean meal until the end of the experiment. Access to feed and water was given *ad libitum*. Feeding trials were conducted for 9 weeks. The animals were weighed weekly, and fresh fecal samples were collected from each pig during the early growing stage (3–5 weeks of age) and the late growing stage (10–12 weeks of age) after weighing the animals. Fecal samples were collected by rectal swabbing. The samples were immediately transported to the laboratory on ice and stored at 20°C until further analysis.

DNA extraction and 16S rRNA gene sequencing. Total DNA was extracted from fecal swabs by using a PowerFecal® DNA Isolation Kit (MO BIO Laboratories, USA). Polymerase chain reaction (PCR) was carried out to amplify the V4 region of the 16S rRNA gene of bacteria and archaea as previously described (Kozich et al., 2013). Two microliters of the total DNA from each sample was used as a template, and amplification was performed in triplicate using a Maxime™ PCR PreMix Kit (iNtRON Biotechnology, Korea) under the following conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 20 s, 55°C for 15 s, and 72°C for 1 min, and 72°C for 5 min. Obtained PCR products were further gel-purified using an AccuPrep® Gel Purification Kit (Bioneer, Korea). All obtained DNA was quantified using a Qubit® fluorometer (Thermo Fisher Scientific, USA), and equimolar purified amplicons were pooled prior to sequencing. Amplicons

were sequenced using MiSeq platforms (Illumina, USA) at MacroGen Inc. (Korea), according to the manufacturer's instructions.

Sequence processing and analysis. FASTQ files obtained from MiSeq paired end sequencing were deposited at short read archives with the registration number SRP043400. Paired end reads were assembled using PEAR software (Zhang et al., 2014). The resulting FASTQ files were converted to FASTA files, aligned to rRNA sequences in the SILVA database (Quast et al., 2013), screened, and filtered by a Mothur pipeline (Schloss et al., 2009). Artificial erroneous reads were corrected using the pre.cluster Mothur subroutine, and chimeric sequences were removed by using UCHIME (Edgar et al., 2011). Taxonomic classification was performed using Ribosomal Database Project (Cole et al., 2009) training set version 9, followed by the removal of non-archaeal/bacterial sequences based on the taxonomic classification results. Prior to the cluster analysis, all singleton sequences were removed as suggested previously (Degnan and Ochman, 2012) by using the Mothur split.abund subroutine. To normalize the number of reads per sample, 5,000 sequences were randomly picked from each sample by using the Mothur sub.sample subroutine. Operational taxonomic units (OTUs) were calculated at distance of 0.03 by using the Mothur cluster.split subroutine, and OTU-consensus taxa were determined using the Mothur classify.otu subroutine. Similarity analysis between microbial communities was performed using the Mothur tree.shared subroutine based on the Yue and Clayton theta coefficient (JC and MK, 2005).

Distribution analysis of operational taxonomic units. All OTUs were grouped into two growth stages (weeks 3–6 and weeks 10–12) and average abundance was calculated within the growth stages; OTUs where the average abundance was lower than 10 were removed. Cytoscape (Shannon et al., 2003) was used to conduct a network analysis between the two breeds at different growth stages. A circular yFiles layout (yWorks, Germany) was applied to analyze factors influencing the distribution of OTUs. **Statistical analysis.** Significant differences between microbial communities were examined based on P-values obtained by analysis of molecular variance (AMOVA) and Student's *t*-test. Metastats analysis (Foster, 2003) was also used to conduct differential abundance tests between read-abundance and treatments.

Results and Discussion

MiSeq-based microbial community analysis. Pyrosequencing has been preferably used for microbial community analysis due to its high accuracy and relatively long read length compared with other next generation sequencing platforms (Gilles et al., 2011; Kim et al., 2013). Recently, a MiSeq-based method for the analysis of microbial communities has been established, which was proven to be capable of sequencing more than 300 samples with a few dozens of dual-indexed primer sets (Teeling and Glockner, 2012; Kim et al., 2013). In this study, a total of 1,151,027 and 1,113,192 DNA sequences were obtained for WP and BP fecal samples, respectively. After the removal of

Fig. 1 Growth rate difference between Landrace white pigs (WP) and black pigs (BP).

erroneous reads, 512,957 and 447,814 reads remained for the WP and BP fecal samples, respectively. The number of reads per sample ranged from 7,796 to 51,134, which was normalized by random-sampling of 5,000 reads by using the Mothur sub.sample subroutine. Results from the rarefaction curve analysis suggest that our sequencing efforts should be enough even after the sub-sampling (Supplementary Fig. 1).

Growth rate difference between WP and BP. In general, all pigs appeared to be in a good health condition throughout the experiment. WP and BP were fed *ad libitum* from 18 ± 2.7 to 68 ± 2.9 kg and from 9.4 ± 3.5 to 49.1 ± 11.4 kg live weight, respectively. Weekly weight gain was higher in WP and the difference in growth rate between breeds was significant at 12 weeks old (Fig. 1). In Korea, animal breeders have tried to improve BP productivity by crossing them with commercial breeds such as Berkshire and Landrace since 1910 (Kim et al., 2005), which made BP have a close genetic relationship to WP (Kim et al.,

2002; Kim et al., 2005); yet, the growth rate of BP was lower than that of WP. Therefore, non-genetic factors such as the gut microbiota may play a key role in determining growth rate. It has been reported that certain types of gut microbiota could optimize their host's ability to harvest energy from food (Ley et al., 2005; Turnbaugh et al., 2006). In addition, manipulation of gut microbiota was found to control adiposity (Turnbaugh et al., 2006).

Taxonomy-based gut microbiota comparison. A taxonomy-based analysis was conducted to describe the composition of the gut microbiome between BP and WP and how it changed over time. The results of phylum distributions are shown in Fig. 2. In WP, the three most abundant phyla, *Firmicutes*, *Bacteroidetes*, and *Spirochaetes*, accounted for approximately 95% of all obtained phyla, on average. On the other hand, *Firmicutes* and *Bacteroidetes* were the two most abundant phyla in BP. A significantly higher relative abundance of *Spirochaetes* was observed in WP than in BP from 3 to 6 weeks of age ($p < 0.05$). Recently, it has been reported that obese pigs had a higher abundance of the phyla *Spirochaetes* than did lean pigs (Pedersen et al., 2013). *Spirochaetes* were originally thought to be nonpathogenic; however, some strains may be enteropathogenic and produce clinical syndromes such as swine dysentery and local inflammation in the colon (; Hampson and Ahmed, 2009). Although it is not a desirable way to increase weight of animals, those gut inflammation in the colon could also contribute to weight gain (de La Serre et al., 2010).

In animal models of obesity, the synergy between the dominant gut phyla is shifted, with a significant reduction of *Bacteroidetes* and a corresponding increase of *Firmicutes* (Turnbaugh et al., 2006). In this study, a lower abundance of *Bacteroidetes* species and a correspondingly higher abundance of *Firmicutes* species

Fig. 2 Phylum-level bacterial composition analysis for white pigs (A) and black pigs (B). W and B denote white and black pigs, respectively. The first number with W and B (W1–3 or B1–3) indicates the individual pigs. Numbers (3–6, 10–12) indicates the age of the pigs in weeks.

Fig. 3 *Bacteroidetes* to *Firmicutes* ratio (B/F ratio) of white (WP) and black pigs (BP).

were observed among WP samples (Fig. 3). A high ratio of *Firmicutes* to *Bacteroidetes* abundance has been closely linked with obesity by various studies (Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2006; Guo et al., 2008). However, the relationship between the gut microbial composition and the energy-harvesting capacity of the host is complicated and may not be directly related to the *Bacteroidetes* to *Firmicutes* ratio (Duncan et al., 2008; Murphy et al., 2010). Further studies are needed to elucidate the mechanisms by which the composition of the gut microbiota may influence fat deposition. Nevertheless, with an increasing amount of evidence supporting the association between alteration of gut microbiota composition and obesity, our results suggest that the ratio of the two phyla might contribute to the lower weight gain of BP compared with WP.

Whole microbiota comparison based on operational taxonomic unit distribution. To verify that BP and WP showed differences in their gut microbial communities, a tree diagram was constructed (Fig. 4). All gut microbiota were divided into two clusters (A and B) and two subclusters (I and II). Cluster A was mostly composed of WP at early stage (3 to 6 weeks of age). Cluster B, on the other hand, was divided into two subclusters: subcluster-I contained mostly BP with varying ages and subcluster-II was generally composed of WP at late stage (10 and 11 weeks of age). Analysis by AMOVA suggested that BP and WP have significantly different intestinal microbial communities ($p < 0.001$). Analysis of diversity indices (Supplementary Table 1) showed that WP samples have higher number of OTUs with higher diversity ($p < 0.05$) compared with those of BP. In addition, WP species richness in the early growth stage was significantly higher than that of the late growth stage ($p < 0.01$). Previously, it has been reported that gut microbiota in young swine is still in development, thus difference between individuals could be more noticeable compared to that of adults (Kim et al., 2015). In this study, gut microbiota at late growth stage has been stabilized, which may have minimized bacterial compositional difference between individuals. Our results indicated that WP gut microbiota had higher diversity and species richness

Fig. 4 Cluster analysis of gut microbiota in white and black pigs. Sample names were shown in the following order: pig breed (W/B), replicate number(1-3), and age of the pigs.

than BP, especially in the early growth stage.

A network analysis was conducted to analyze the distribution of OTUs across breeds, individuals, and growth stages. In Fig. 5, continuous white and grey nodes are observed on the network perimeter. The BP nodes are positioned close to each other and linked with highly abundant OTUs. In contrast, the WP nodes are more frequently distributed on the network perimeter and are separately positioned according to the growth stages. Results from the Fig. 5 suggest that the distribution of OTUs was mostly influenced by breed, and only WP nodes were influenced by growth stage. It has been reported that the swine gut microbiota showed significant shifts after stabilization through the weanling period (Thompson et al., 2008), which is in agreement with our results for WP. On the other hand, the BP nodes were positioned close to the early stage WP nodes, suggesting that the growth-stage-dependent shift observed in WP was not observed in BP gut microbiota. Norris et al. (2013) hypothesized that the gut microbiota plays a key role in controlling hosts' appetites, based on previous findings of links between the gut microbiota composition and hosts' behavioral or physiological changes.

Genus level taxonomic classification identified 110 genera in this study. Predominant genera in black pigs were *Prevotella*, whereas white pigs had *Prevotella* and *Treponema* (Supplementary Fig. 2). Since there are quite a few unclassified genera, we conducted OTU-based differential abundance comparison using Metastats analysis (Paulson et al., 2011). Results from the

Fig. 5 Network analysis of operational taxonomic units shared between white pigs (WP) and black pigs (BP). Grey and white nodes indicate BP and WP, respectively. Numbers in the nodes indicate the individual pigs. Squares and rectangles indicate early and late growth stages. Line thickness increases according to their read abundance

Metastats analysis (Supplementary Table 2) indicated that WP had significantly higher abundances of the genera *Treponema*, *Cellulosilyticum*, *Methanobrevibacter*, *Sporobacter*, *Anaeroplasm*, *Peptococcus*, *Comamonas*, and *Marinospirillum* ($p < 0.05$ and $Q < 0.05$), while BP had significantly higher abundances of the genera *Escherichia*, *Faecalibacterium*, *Sutterella*, *Veillonella*, *Pasteurella*, *Mobiluncus*, *Chlamydia*, and *Odoribacter* ($p < 0.05$ and $Q < 0.05$). Feeds containing high fiber contents have been increasingly used due to greater availability and lower cost (Ziemer et al., 2012). Since pigs do not have any enzymes to hydrolyze non-starch polysaccharides, their intestinal bacteria play the main role in the digestion and fermentation of those dietary carbohydrates (Choct et al., 2010). In this study, we found two abundant potential fiber digesting helpers in WP, *Cellulosilyticum* (Cai et al., 2010) and *Treponema* (Liu et al., 2014). As a result of the bacterial fiber fermentation in colon, short chain fatty acids (SCFA) are produced, which play an important role in maintaining colonic health by supplying energy to colonocytes and protecting them from gastrointestinal disorders (Hijova and Chmelarova, 2007; Cai and Dong, 2010). In addition, energy produced by microflora in the hindgut can satisfy up to 30% of the maintenance energy requirements of the pig (Adeshinwa, 2008). Moreover, it has been reported that the growth performance of pigs can vary depending on the types of fiber content in feeds (Song et al., 2003). Therefore, fiber digestion also could be one of the key factors determining the growth rate of pigs. In addition, a high abundance of *Cellulosilyticum* has been reported to positively correlate with the amount of methane production, suggesting that the genus *Cellulosilyticum* is a key substrate provider for

methanogens (Pramanik and Kim, 2014). Our results showed that the genus *Methanobrevibacter*, a fecal methanogen that utilize acetate, was more prevalent in WP, thus suggesting potentially higher presence of SCFA. Previously, it was suggested that analysis of the fecal methanogen abundance could provide a method to estimate fat storage in pigs, since the abundance of the genus *Methanobrevibacter* was found to be associated with higher weight gain in pigs (Luo et al., 2012). Existence of methanogens may enhance the activity of polysaccharide-utilizing bacterial species through co-colonization in the gut (Samuel and Gordon, 2006), therefore methanogens could also play an important role in probiotics-mediated energy metabolism and adiposity.

While a number of studies have reported how gut microbiota is associated with obesity (Turnbaugh et al., 2006; Sanz et al., 2008; Tilg, 2010; DiBaise et al., 2012), limited numbers of studies have been reported about gut microbiota shift with respect to livestock animal meat quality. Addition of essential oils increased longissimus muscle while reducing noxious gas in fecal output (Yan et al., 2010), suggesting meat quality could be directly or indirectly associated with gut microbiota. In addition, phenyllactic acid reduced number of *E. coli* while partly ameliorating meat quality (Wang et al., 2010). Moreover, feeding bacteria strain, *Clostridium butyricum*, improved quality of meat by increasing polyunsaturated fatty acids (Liao et al., 2015). In contrast, it has been reported that the addition of extruded flaxseed improved meat quality without affecting swine gut microbiota (Holman et al., 2014). These studies suggest further study should be conducted to elucidate functional roles of gut microbiota regarding to meat quality.

In summary, we investigated the gut microbiota composition of BP and WP as a potential contributory factor to the differences in the growth rate between the breeds. In this study, feed and water consumption or fecal output of each animal were not recorded. Recently, it has been reported that gut microbiota is also responsible for gut satiety hormone production (Cluny et al., 2015), thus variation in feeding system may provide additional aspects in revealing functional roles of gut microbiota regarding to growth enhancement. To study gut microbiota with respect to feed efficiency, further study should include more controlled feeding system, such as restricted feeding. Although various factors are involved in swine growth performance, our results suggest that gut microbiota difference is one of the factors that affect growth rate in swine. Further study should prove what causes the gut microbiota difference across breeds. In addition, functional genome study by metagenome shotgun sequencing may reveal functional effects resulting from microbiota differences. These information should provide fundamental knowledge toward developing novel approach in enhancing growth performance of livestock animals.

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