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| 보 문 |

Comparison Analysis of Swine Gut Microbiota between Landrace and Yorkshire at Various Growth Stages

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두 돼지 종의 다양한 성장단계에 따른 장내미생물 비교분석

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In this study, we conducted a next generation sequencing based microbial community analysis to investigate gut microbiota of the two commercially most available swine breeds, Yorkshire and Landrace. Bacterial 16S rRNA gene was amplified from fecal DNA using universal primer sets designed for V4 regions. Our comparison analysis of the gut microbiota of the two breeds suggested that their gut microbiota changed depending on the growth stages, while the difference between the two breeds was insignificant. However, there was a limited number of genera, the abundance of which was found to be different between the breeds. Those included the genus *Xylanibacter* in the Yorkshire samples, which was previously reported as a fiber digesting bacteria, likely increasing energy harvesting capacity of swine. In addition, others included opportunistic pathogens mostly found in the Yorkshire samples while the Landrace samples had significantly more prevalent *Clostridium_IV* species that were known to play a key role in systemic immunity of hosts. While microbial community shifts was found to be associated with growth stages, the difference between the two breeds seemed to be insignificant. However, there were several bacterial genera showing differential abundance, which may affect growth of hosts.

***Keywords*:** growth promoter, gut microbiota, swine

Pork is one of the popular agricultural products and breed is an important factor for livestock market in Korea. Purpose of developing new breeds is mostly related to meat production. Recently, the use of antibiotic growth promoters (AGPs) as a feed additive was banned in Korea. In general, the banning of the AGPs has caused increased death rates of livestock animals, especially at their early ages (Casewell *et al.*, 2003). Therefore, there is a rising demand of developing AGP-alternatives. It has been reported that oral feeding of probiotics could improve the growth of livestock animals (Konstantinov *et al.*, 2008) or chemical treatments such as lysozymes also enhanced the growth of livestock (Oliver and Wells, 2013). Moreover, natural products such as plant extracts (Castillo *et al.*, 2006) and cytokine (Lowenthal *et al.*, 2000) were also reported to be candidates of the AGP-alternatives. However, these seem to have presented conflicting data, thus there is a need of developing novel approaches (Thacker, 2013).

Cross-breeding of the livestock animals has been conventionally practiced in order to create agriculturally productive breeds, aiming higher quality of meat, faster growth, and more immunized animals. Recent studies have indicated that mammalian's health was significantly associated with their gut microbiota. For example, high abundance of the phylum *Firmicutes* could induce obesity (Ley *et al.*, 2006), and some gut microbial species can inhibit colonization of pathogenic bacteria (Kamada *et al.*, 2012).

While studies have indicated the importance of gut microbiota, little has been achieved to enhance agricultural productions related to livestock animals. In this study, we have conducted next generation sequencing based gut microbial community analysis to obtain fundamental knowledge of swine gut microbiota. Our results should lead to further understanding of swine physiological functions provided by those gut microbiota. Here we revealed and compared gut microbiota of the two commercially popular breeds, Landrace and Yorkshire,

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towards novel approaches of livestock growth enhancement.

Materials and Methods

Fecal material sampling

Swine fecal materials were provided by Seungjin GGP (Haenam, Korea). Approximately 20 swine were raised in each pen according to growth stages; weanling, grower, finisher and sow. Two breeds, Yorkshire and Landrace, were selected for this study. Five fecal materials were collected from each pen and kept in an ice-box during transportation. Fecal materials were stored under -20℃ until processed. Total 40 fecal samples were obtained in this study.

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from the fecal materials using the MOBIO Power Fecal DNA isolation kit (MO BIO Laboratories Inc., USA). The v4 region of 16S rRNA gene was amplified by polymerase chain reaction (PCR), as previously described (Kozich *et al.*, 2013). Briefly, 2 μl of the total DNA from each sample was used as a template and PCR was done in triplicate using the Maxime PCR PreMix Kit (iNtRON Biotechnology Inc., Korea) with the following reaction conditions: 95℃ for 2 min, 30 cycles of 95℃ for 20 sec, 55℃ for 15 sec, and 72℃ for 1 min, and 72℃ for 5 min. All purified PCR products were quantified using Qubit (Invitrogen, USA). The equimolar amplicons were pooled and stored at -20℃ until sequenced. Illumina MiSeq platform (Illumina, USA) was used to sequence the v4 region of the 16S rRNA gene. The construction of the gene library and sequencing were conducted at Macrogen Inc. (Korea) according to the manufacturer's instructions.

Sequence processing and analysis

Paired end reads obtained from a MiSeq run were assembled using the 'PEAR' software (Zhang *et al.*, 2014). Index sequences were trimmed, subsequently aligned to similar sequences in the Silva rRNA database (Quast *et al.*, 2013), screened, and finally filtered using the Mothur pipeline (Schloss *et al.*, 2009). Artificial erroneous reads were corrected using the pre.cluster Mothur subroutine, and chimeric sequences were removed using UCHIME (Edgar *et al.*, 2011). Taxonomic classification was done using the Ribosomal Database Project (Cole *et al.*, 2009) training set version 9, followed by non-archaeal/bacterial sequence removal based on the taxonomic classification results. Prior to cluster analysis, all singleton sequences were removed, as suggested previously (Degnan and Ochman, 2012). Operational taxonomic units (OTUs) were calculated at a distance of 0.03, using Mothur subroutine cluster.split, and the OTU-consensus taxa were determined using classify.otu Mothur subroutine. Principle coordinate analysis (PcoA) was done using pcoa Mothur subroutine. Differential abundance tests were carried out using Metastats (Foster, 2003). Analysis of molecular variances (AMOVA) was done for statistical confirmation of group separation between breeds and among growth stages.

Results and Discussion

MiSeq amplicon sequencing

Total 1,693,483 reads were remained after Mothur removed erroneous reads. Number of reads obtained per sample ranged approximately from 23,000 to 75,000, which is far greater than many of the studies published to date. The use of Illumina sequencer for PCR amplicons was suggested only recently (Zhou *et al.*, 2011; Caporaso *et al.*, 2012; Degnan and Ochman, 2012), however, we have obtained high quality and substantial data. While pyrosequencing generally produce 1.5 million reads, Illumina's Miseq produces more than 20 million reads with an equivalent cost, allowing maximizing number of sample pools to a greater extension.

Species diversity comparison

Using the Mothur pipeline, we obtained ecological indices for each sample. Figure 1 shows a comparison of Chao and Shannon diversity indices between two breeds at each growth stage. Chao species richness ranged approximately from 600 to 1,200 at maximum and Shannon species evenness ranged from 3.4 to 5.1 (Fig. 1). The lowest species evenness and richness were observed for weanling pigs and the analysis of the

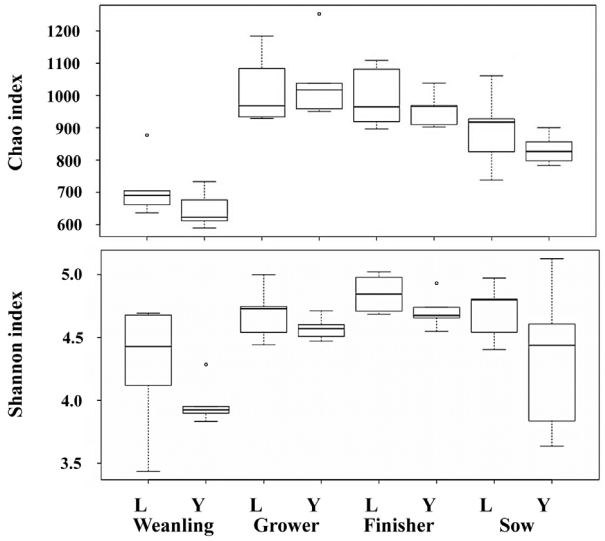

Fig. 1. Ecological diversity indices comparison between the Landrace and the Yorkshire gut microbial communities. L and Y denote Landrace and Yorkshire, respectively.

variance (ANOVA) showed a significant increase from weanling to grower stages ($P < 0.05$) (Fig. 1). It has been reported that swine gut microbiota was stable during the weanling period (Pang *et al.*, 2007), and our results suggested that the unstableness of the early age swine gut microbiota may be due to the low species richness and evenness. On the other hand, no significant difference was observed between the two breeds.

Microbial community comparison

Bacterial composition was investigated at the phylum and family levels (Fig. 2). Relative abundance was obtained by averaging 5 replicates for each breed at each growth stage. Relatively constant bacterial composition was observed at the phylum level (Fig. 2A), while increasing 'unclassified' family and decreasing *Prevotellacea* species were observed at the family level (Fig. 2B). ANOVA did not show significant difference between the breeds nor across each growth stage. It should be noted that the abundance of the family *Prevotellacea* was inversely proportional to the growth of swine, suggesting that the abundance of those may have been related to growth of swine. However, further studies are needed to confirm this.

Since nearly half the data are unclassified at the lower taxonomic level, we have conducted operational taxonomic units (OTUs) analysis at the distance 0.03 equivalent to species level. Figure 3 shows the principle coordinate analysis (PcoA) for the both breeds at each growth stage. The first three major determinants (axis 1, 2, and 3) were taken for comparison of each gut microbiota (Fig. 3). While difference between the breeds may seem insignificant, axis1-axis2 comparison showed separations based on growth stages (shown in colors), in addition, sow swine showed higher diversity than other samples collected at different growth stages. In human model study, it was reported that pregnant women possessed diverse gut microbiota depending on their weight (Santacruz *et al.*, 2010), suggesting that sow swine may have various gut microbial communities depending on months of pregnancy. On the other hand, growers and finishers were relatively closed to each other, suggesting the gradual gut microbiota shift according to the growth stage. Analysis of the molecular variance (AMOVA) indicated insignificant variation between the breeds, however, the difference across the growth stages were confirmed ($P < 0.05$) (Supplementary data Table S1). F values obtained from AMOVA also indicated the closeness of microbial communities between growers and finishers (Supplementary data Table S1).

Genus level differential abundance test

Using Metastats (Foster, 2003), we have conducted differential abundance test at the genus level. While it is commonly performed at the species level or the OTU level, results show rather complicated between genera. For example, one OTU may be significantly more abundant than the others, while another OTU in the same genus could be significantly less

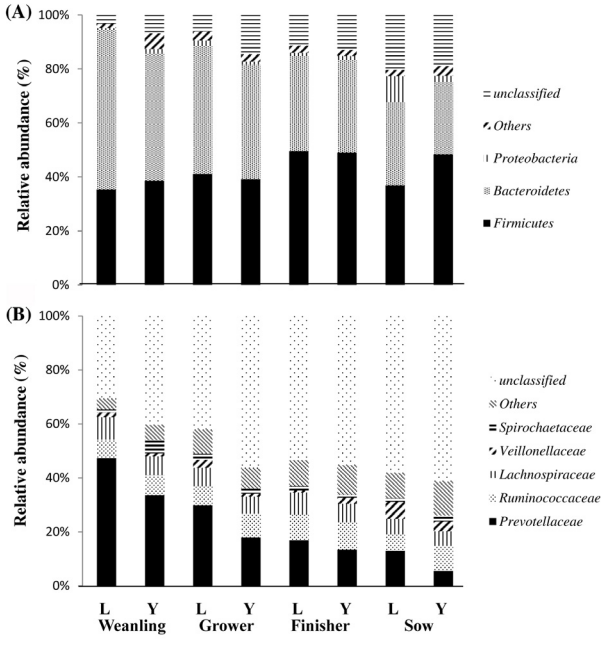

Fig. 2. Comparison of bacterial composition of the Landrace and the Yorkshire at the phylum level (A) and the family level (B).

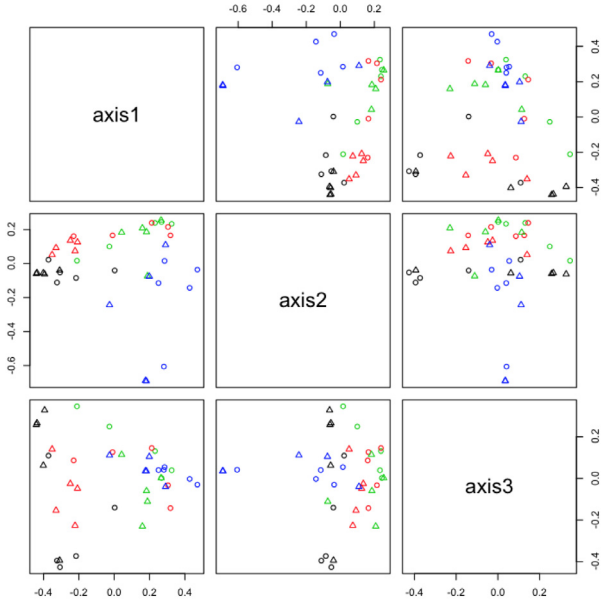

Fig. 3. Principle coordinate analysis for gut microbiota comparison. Circles and triangles denote Landrace and Yorkshire, respectively. Black, red, green, and blue denote each growth stage of weanling, grower, finisher and sow, respectively.

Table 1. Differentially abundant genera identified using Metastats

Genera	Abundant in	General characteristics	References
Xylanibacter	Yorkshire	Fiber fermenting and feed efficiency increase	Tremaroli and Backhed (2012)
Arcobacter	Yorkshire	Commensal strains but opportunistic pathogenic to humans	De Smet *et al.* (2011)
Wautersiella	Yorkshire	Opportunistic human pathogens	van der Velden *et al.* (2012)
Stenotrophomonas	Yorkshire	Opportunistic human pathogens	Hauben *et al.* (1999)
Trueperella	Landrace	Swine opportunistic pathogens	Jarosz *et al.* (2014)
Coprococcus	Landrace	No recent reports	NA
Pseudosphingobacterium	Landrace	Isolated from compost	Vaz-Moreira *et al.* (2007)
Treponema	Landrace	Pathogens responsible for swine skin disease	Alvarez-Ordonez *et al.* (2013)
Clostridium_IV	Landrace	Bacteria providing immune	Sarrabayrouse *et al.* (2014)

abundant. Therefore, we conducted the Metastats not using the Mothur pipeline, but using the web interface (hhttp://metastats.cbcb.umd.edu/detection.html) based on the bacterial composition obtained at the genus level taxonomic classification. Table 1 summarizes the genera significantly more prevalent either in the Yorkshire or the Landrace samples. The genus *Xylanibacter* was found to be more prevalent in the Yorkshire and is known to be a fiber digesting bacteria which could increase energy harvesting capacity (Tremaroli and Backhed, 2012). Other than this genus, we did not observe any potential growth promoting strains reported to date. On the contrary, opportunistic pathogens were more profoundly identified in the Yorkshire. Interestingly the Metastats defined the genus *Clostridium_IV* more abundant in the Landrace. The genus *Clostridium_IV* was previously reported to play a key role in systemic immunity (Sarrabayrouse *et al.*, 2014). The less opportunistic pathogens identified in the Landrace could be due to the existence of the prevalent *Clostridium* species, however further studies are needed to confirm this.

Conclusion

We investigated gut microbiota of Yorkshire and Landrace swine. Our results indicated dynamics of the gut microbial community shifts, especially the significant changes between weanling to grower. On the other hand, there was no significant difference between the two breeds when total microbial communities were compared. We, however, observed that several genera were defined to be differentially abundant between the breeds, which suggested that Landrace might be more immune to various opportunistic pathogens when compared to Yorkshire swine, and also suggested that Yorkshire may have higher feed conversion efficiency. Although these results need to be further confirmed, the study presented here provides fundamental information of swine gut microbiota towards the development of livestock related agricultural products.

적 요

본 연구에서는 차세대염기서열분석법(Next Generation Sequencing)을 이용하여 상업적으로 농가에 가장 많이 보급되어 있는 요크셔와 랜드레이스를 포함한 두 종의 장내미생물생태 분석을 실시하였다. 박테리아의 16S rRNA 유전자는 분변샘플로부터 추출한 DNA에서 V4 지역을 증폭할 수 있도록 디자인된 유니버설 프라이머 세트를 이용하여 증폭되었다. 두 종에 대한 장내미생물생태 비교분석은 성장단계에 따라 차이를 보이는 반면, 종에 따른 차이는 거의 없다는 것을 확인하였다. 하지만, 두 종간의 장내미생물생태 내에서 특정 미생물의 수가 차이가 있다는 것을 확인하였다. 요크셔는 특히 섬유질 소화를 통해 에너지 생산률을 높여준다고 보고된 바가 있는 *Xylanibacter* 속(Genus)의 미생물을 많이 포함하는 것으로 나타났다. 또한, 랜드레이스는 숙주 내에서 면역에 중요한 역할을 하는 것으로 알려진 *Clostridium_IV* 종을 상당히 많이 포함하는 것으로 나타났으며, 반면 요크셔는 기회감염미생물들을 많이 포함하는 것으로 나타났다.

본 연구는 요크셔와 랜드레이스를 포함한 두 종간의 장내미생물생태 비교분석을 통해 그 차이점이 종에 의한 차이보다는 성장단계에 따라 큰 차이가 있다는 것을 확인하였다. 하지만 두 종 사이에서 성장에 영향을 미칠 가능성이 있는 몇 장내미생물의 수가 차이가 있다는 것을 확인하였다.

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