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Effects of Antibiotic Growth Promoter and Characterization of Ecological Succession in Swine Gut Microbiota S

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Ever since the ban on antibiotic growth promoters (AGPs), the livestock death rate has increased owing to pathogenic bacterial infections. There is a need of developing AGP alternatives; however, the mechanisms by which AGP enhances livestock growth performance are not clearly understood. In this study, we fed 3-week-old swine for 9 weeks with and without AGPs containing chlortetracycline, sulfathiazole, and penicillin to investigate the effects of AGPs on swine gut microbiota. Microbial community analysis was done based on bacterial 16S rRNA genes using MiSeq. The use of AGP showed no growth promoting effect, but inhibited the growth of potential pathogens during the early growth stage. Our results showed the significant increase in species richness after the stabilization of gut microbiota during the post-weaning period (4-week-old). Moreover, the swine gut microbiota was divided into four clusters based on the distribution of operational taxonomic units, which was significantly correlated to the swine weight regardless of AGP treatments. Taxonomic abundance analysis indicated a negative correlation between host weight and the abundance of the family *Prevotellaceae* species, but showed positive correlation to the abundance of the family *Spirochaetaceae*, *Clostridiaceae*_1, and *Peptostreptococcaceae* species. Although no growth performance enhancement was observed, the use of AGP inhibited the potential pathogens in the early growth stage of swine. In addition, our results indicated the ecological succession of swine gut microbiota according to swine weight. Here, we present a characterization of swine gut microbiota with respect to the effects of AGPs on growth performance.

Keywords: Gut microbiota, MiSeq, mothur, swine, antibiotics, growth promoter

Introduction

Antibiotics have been used as a feed additive to protect livestock and also to enhance growth performance. Long-term use of antibiotics can cause the dissemination of antibiotic resistance genes in the gut of livestock animals [16], consequently producing antibiotic-resistant zoonotic pathogens [18]. Antibiotic resistance can spread to other animals and humans directly by contact and indirectly *via* the food chain, water, air, and manured and sludge-fertilized soils [29]. In addition, antibiotics are poorly adsorbed in the gut of the animals, and the majority is excreted unchanged in feces and urine, leading to environmental pollution [37]. Therefore, the use of antibiotics as a growth promoter has been banned, especially in the European Union and recently in the Republic of Korea owing to the subsequent pollution [14, 17] and the dissemination of antibiotic resistance genes to the environment [16].

Ever since the ban on antibiotic growth promoters (AGPs), death of livestock animals has increased owing to pathogenic bacterial infections, especially in early post-weaning pigs [5]. Therefore, there is a high demand for developing AGP alternatives. For example, natural immune systems (*i.e.*, cytokines) were used to protect livestock animals from pathogens [28] and several known probiotic strains were used as a feed additive to increase the growth performance of livestock [1]. Recently, it was reported that the use of plant extracts, tannins, effectively reduced the damage caused by diseases and consequently improved the growth performance [35]. Although these AGP alternatives

were found to be effective in improving livestock growth performance, the mechanisms by which AGP or these alternatives enhance livestock growth performance have not been understood yet.

The use of AGPs has been thought to influence gut microbiota by enhancing higher metabolic capacities, digestion, and absorption of nutrients [32]. This hypothesis, however, has many contradicting reports. Previous studies showed that commonly used AGPs, such as tylosin and chlortetracycline, altered the swine gut microbial community [23, 36]; however, links to growth effects were yet to be discovered. Castillo *et al*. [6] reported that the types of feed additives have less effect on bacteria counts in gastrointestinal tracts but significantly changed the community structure, and changed metabolic activities such as carbohydrase activity as a result. It was also reported that the use of AGPs increased microbial functional genes related to energy production and conversion [27]. On the contrary, it was reported that the use of AGPs increased intestinal antibiotic-resistant bacteria but did not enhance growth performance [15] or change colonic microbial communities [19]. These discrepancies could be due to the analytical methods. Since the development of sequencing technology, next-generation sequencing is taking the place of culture-based molecular typing methods, especially for 16S rRNA gene-based microbial community analysis. To date, pyrosequencing has been preferably used for sequencing one or two hypervariable regions of 16S rRNA genes, mostly due to its relatively longer sequencing capacity when compared with other next-generation sequencing platforms. Recently, the use of Illumina sequencers, such as HighSeq 2500 and Miseq, has been suggested for microbial ecology studies to utilize far deeper sequencing capacities, allowing the analysis of more than a few hundred samples at one run [3, 25].

Substantial reports have indicated the vast diverse gut microbiota and their complex interactions with the host's physiological functions. In this study, we applied Illumina sequencer MiSeq to sequence the V4 region of the bacterial/archaeal 16S rRNA genes to investigate the effects of AGPs on swine gut microbiota.

Materials and Methods

Swine Feeding Trials

Approximately 3-week-old swine were selected from a farm in Jeju, Republic of Korea, where no AGPs were used. Three swine were raised in one pen and fed with standard commercial feed mixed with AGPs containing chlortetracycline, sulfathiazole, and penicillin (2:2:1) at 0.2%. Another set of 3-week-old swine were raised in a separate pen as a control and fed with the same commercial feeds without AGPs. Trials were conducted for 9 weeks. Fresh fecal samples were collected from each swine weekly during the 9 weeks of trials. Body weight was measured at the same time when fecal materials were sampled.

DNA Extraction and 16S rRNA Gene Sequencing

Total DNA was extracted from 150 mg of fecal materials using a MO BIO Power Fecal DNA isolation kit (MO BIO Laboratories Inc., CA, USA). PCR was carried out to amplify the V4 region of the 16S rRNA gene of bacteria and archaea as previously described [25] Two microliters of the total DNA from each sample was used as a template, and amplification was done in triplicates using the Maxime PCR PreMix Kit (iNtRON Biotechnology Inc., Republic of Korea) with the following conditions: 95ºC for 2 min; 30 cycles of 95ºC for 20 sec, 55ºC for 15 sec, and 72ºC for 1 min; and 72ºC for 5 min. Obtained PCR products were further gel-purified using an AccuPrep Gel Purification kit (Bioneer Inc., Republic of Korea). All obtained DNA was quantified using Qubit (Invitrogen, CA, USA), and equimolar purified amplicons were pooled and stored at −20°C until sequenced. Amplicons were sequenced using the Illumina MiSeq platform at Macrogen Inc. (Seoul, Republic of Korea) according to the manufacturer's instructions.

Sequence Processing and Analysis

Fastq files obtained from MiSeq paired-end sequencing were assembled using "pear" software [44]. The resulting fastq files were converted to fasta files, aligned to the silva database [33], screened, and filtered by mothur pipeline [38]. Artificial erroneous reads were corrected using the pre.cluster mothur subroutine, and chimeric sequences were removed by using uchime [10]. Taxonomic classification was done using the Ribosomal Database Project [7] training set ver. 9, followed by non-archaeal/bacterial sequence removal based on the taxonomic classification results. Prior to the cluster analysis, all singleton sequences were removed as suggested previously [9] using the mothur split.abund subroutine. To normalize the number of reads per sample, 5,000 sequences were randomly picked from each sample using the subsample mothur subroutine. Samples containing less than 5,000 reads were removed from the further analysis. Operational taxonomic units (OTUs) were calculated at a distance of 0.03 using mothur subroutine cluster.split, and OTU consensus taxa were determined using the classify.otu mothur subroutine. The weight of hosts, number of reads, OTUs, and diversity indices are summarized in Table S1. Microbial community dissimilarity was analyzed based on the Yue and Clayton theta coefficient calculated by the tree.shared mothur subroutine.

Sequence data used in this study were deposited to Sequence Read Archive with the accession number SRP045387.

Statistics

Correlations between the host weight and taxonomic abundance were analyzed based on Pearson's *p*-value, and one-way ANOVA and Student's *t*-test were applied to analyze the effects of AGPs on weight gain, microbiota shift, and taxonomy. Significant differences between microbial communities were examined based on a *p*-value obtained using analysis of molecular variance (AMOVA). Metastats analysis [12] was also used to conduct differential abundance tests between the read abundance and treatments.

Results and Discussion

Sequencing Results

We sequenced 60 samples and obtained a total of 2,778,689 reads using MiSeq. After removal of erroneous reads, a total of 2,302,715 reads remained. Removal of singleton sequences reduced the number of unique sequences from 1,333,535 to 52,228, which not only removed possible Illumina artifacts [4, 9] but also lessened the computational workload prior to clustering. While 15,000–1,000,000 reads were obtained for most of the samples (Table S1), some of the samples collected during the 5–6 weeks trials had fewer reads, ranging approximately from 800 to 2,000 reads, and thus samples collected during the 5–6 weeks trials were removed from the downstream analyses. The number of resulting sequences can vary depending on the condition of DNA extractions, tags used along with 16S rRNA primers, or specimen conditions.

Taxonomic Abundance Analysis with Respect to the Swine Weights

All swine were weighed once a week (Table S2), and the weekly weight gain for each individual is summarized in Fig. 1. The deviation between each individual was relatively small during the 9 weeks of trials. While AGP-treated and control swine showed nearly identical growth rate for the first 6 weeks, two control swine showed no weight gain at the 7th week. The total weight difference, however, was not observed between AGP-treated and control swine.

To investigate the cause of decrease in growth rate at the 7th week, a correlation analysis between taxonomic abundance and swine weight was conducted. Regardless of the AGP treatment, the read abundance of the family *Prevotellaceae* showed negative correlation, and the families *Spirochaetaceae*, *Clostridiaceae*_1, and *Peptostreptococcaeae* showed significantly positive correlation to host weight ($p < 0.01$) (Fig. 2). Two control swine that showed no weight gain at the 7th week had significantly more *Spirochaetaceae* species than the other four swine when the hosts weighed

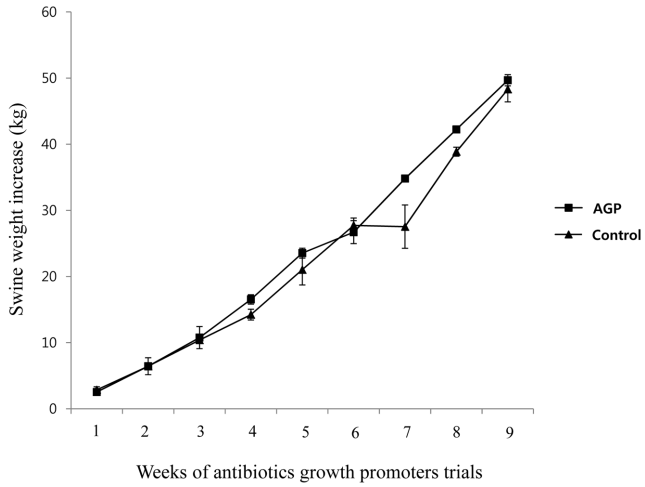

Fig. 1. Weekly changes in weight of swine fed with and without antibiotic growth promoters.

between 30 and 40 kg ($p < 0.05$). All *Spirochaetaceae* species observed in this study were classified into the genus *Treponema*, which is known to cause porcine skin necrosis and ulcers [21]. Moreover, Looft *et al*. [26] reported that the use of carbodox as a feed additive decreased the number of *Spirochaetacea* species, suggesting that one of the effects of AGP in general includes the inhibition of potential intestinal pathogens.

Microbial Community Shifts during Weight Increase

Based on the distribution of OTUs, microbial communities were divided into five clusters, in which AMOVA test results indicated significant difference between each cluster ($p < 0.001$) (Fig. 3). Results in Fig. 3 show that cluster I mostly consisted of samples collected at the early growth stage (days 0 and 7), and the high variation within cluster I indicates the low stability of the gut microbiota during the early growth stage. Previously, gut microbiota stabilization in the early growth stage was reported to take at least two years for human infants [13]. Our results suggest that swine gut microbiota was unstable until the age of 4 weeks, which is supported by the previous study conducted by Thompson *et al*. [39]. On the other hand, cluster II consisted of the second earlier growth stage mostly collected during days 14–21 of trials, whereas clusters III and IV shared samples collected during days 28–56, and cluster V consisted of later growth stage samples collected during days 49–63. Cluster analysis did not show significant differences between samples obtained from AGP-treated and control swine.

Distribution analysis of host weights in each cluster

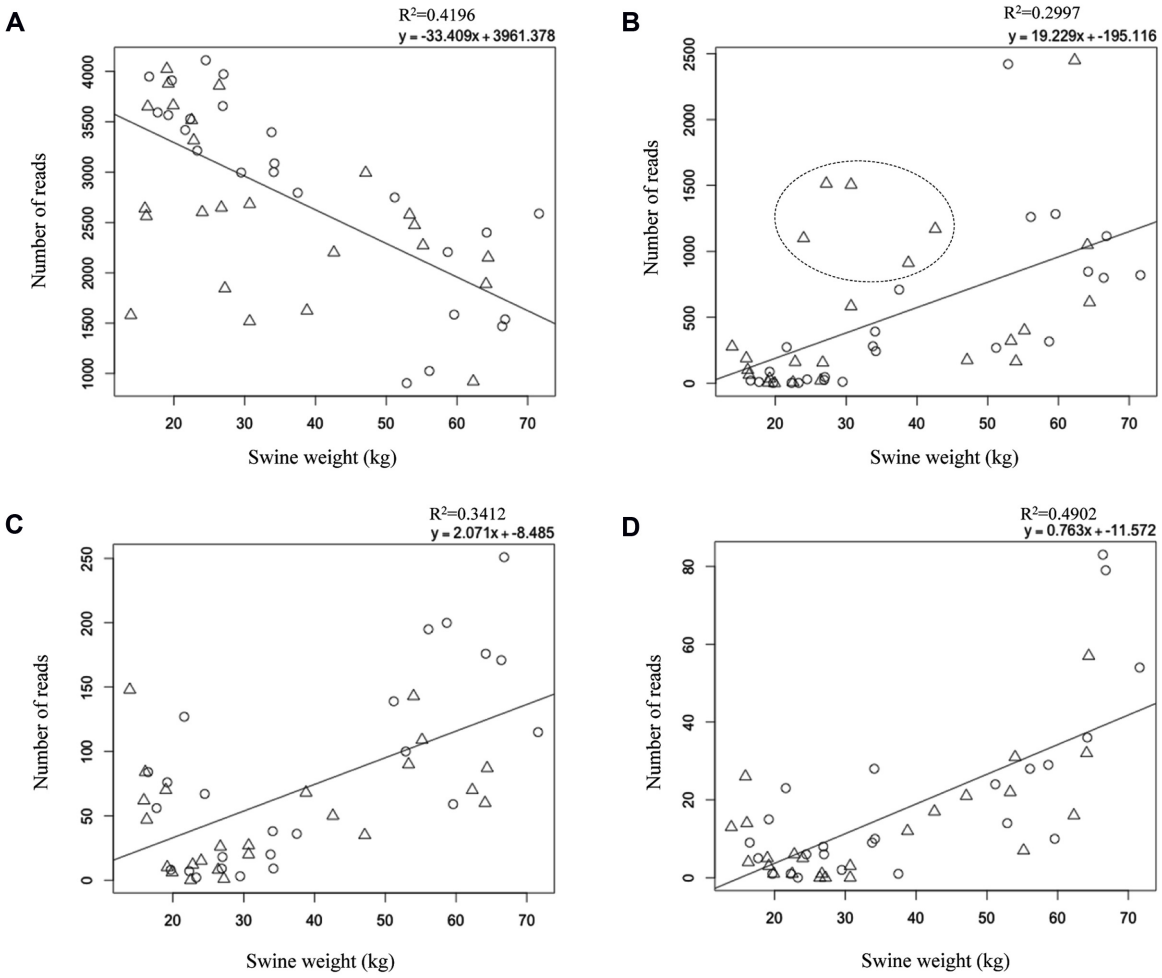

Fig. 2. Significant correlation ($p < 0.01$) between read abundance and host weight among the family *Prevotellaceae* (**A**), *Spirochaetaceae* (**B**), *Clostridiaceae*_1 (**C**), and *Peptococcaceae* (**D**).
Circles denote swine treated with antibiotic growth promoters and triangles are control. Dashed circles indicate read abundance of the *Spirochaetaceae* species obtained for the control swine that did not show weight gain at the 7th week.

indicated that the type of microbiota can be divided into three growth stages (clusters I–II, III–IV, and V) regardless of AGP trials ($p < 0.05$) (Fig. 4). Whereas the microbiota was significantly different between clusters I and II, weight difference was not significant. It can be suggested that significant weight increase occurred once the gut microbiota was stabilized. In addition, species richness also increased significantly after the stabilization of gut microbiota ($p < 0.05$). Interestingly, the inverted Simpson index was significantly different among the clusters ($p < 0.05$). In summary, after the stabilization, the swine gut microbiota started to be colonized with exogenous bacterial species and went through significant ecological changes in structure and diversity. Metastats analysis [12] was conducted to capture significantly abundant species in each cluster. As a result, species in the families *Carnobacteriaceae*, *Clostridiales_Incertae_Sedis_XI*, *Corynebacteriaceae*, *Aerococcaceae*, *Oceanospirillaceae*, and *Oxalobacteraceae* increased as swine gained weight ($p < 0.05$ and $q < 0.05$). It is not clear if these taxa are related to the host's physiological functions, and further studies are needed to understand the roles of each taxon in keeping homeostasis in the gut microbiota.

Effects of AGPs on Gut Microbiota to Enhance Weight Gain

It has been reported that the gut microbiota is associated with obesity in a way of increasing the digestion capacity and energy harvest [40, 41]. In addition, it has been suggested that the gut microbiota was associated with appetite-regulating hormones such as leptin and ghrelin

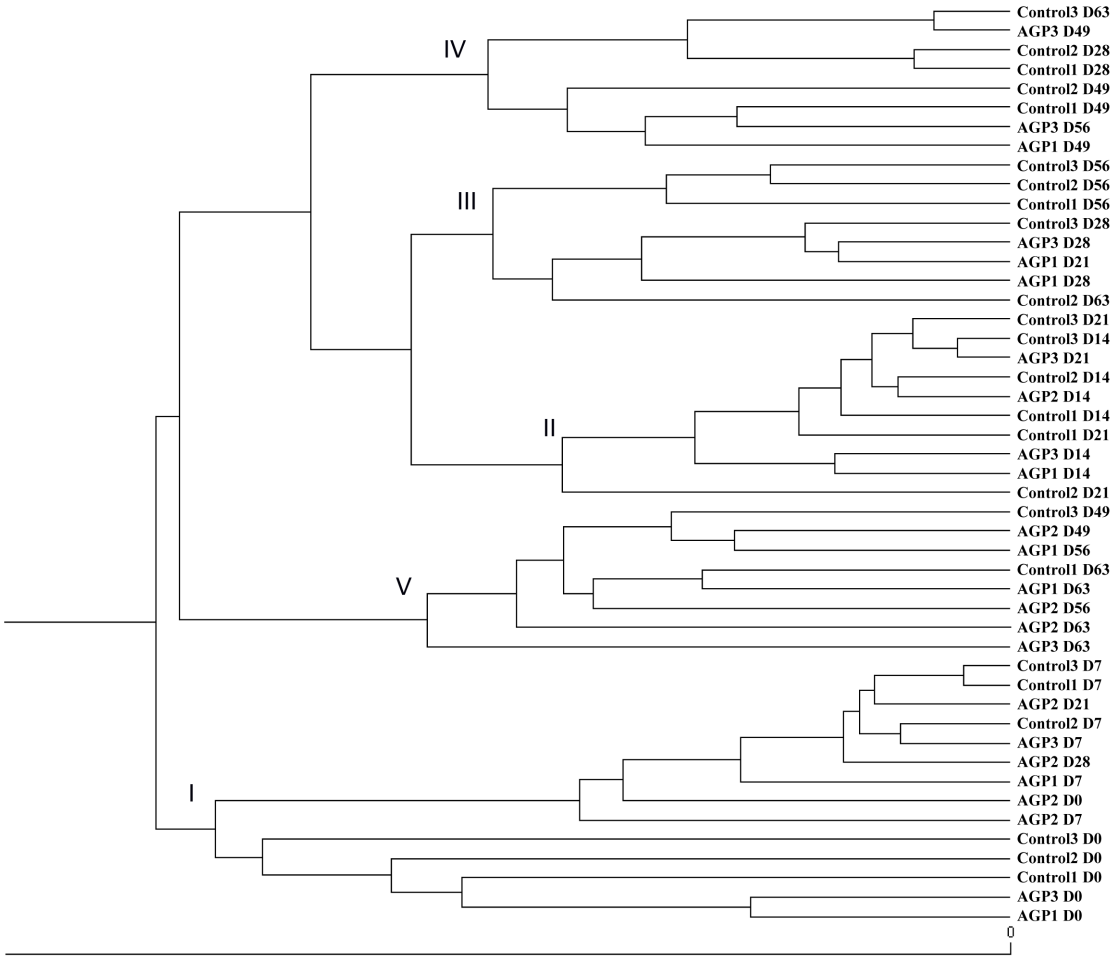

Fig. 3. Cluster analysis of gut microbiota of swine treated with and without antibiotic growth promoters.
AGP indicates AGP-treated swine and control indicates non-treated swine. Sample names are shown with days of AGP treatment. The dendrogram was drawn based on the Yue and Clayton theta coefficient calculated by the mothur tree.shared subroutine.

[34]. Norris *et al*. [31] hypothesized that gut microbiota could control the host's appetite and consequently cause obesity. In this study, we observed greater abundance of species of the family *Spirocheates* in two control swine during the intermediate growth stage, and these two control swine showed no weight gain in the following week. Although the impact caused by the *Spirocheates* species remains unclear, it might have reduced feed intake of the two control swine as we did not observe any diarrhea symptoms during the study. The use of AGPs was expected to optimize the weight gain by altering the gut microbiota [11]. However, we did not observe significant microbiota alteration or weight difference between AGP-treated and control swine. Our results suggest that stabilization of the swine gut microbiota occurs at the early growth stage (3–4 weeks old), and swine gut microbiota consistently shifts in the growing stage. These results are supported by a previous study [22] in which microbial community shifts in swine were observed at 3-week intervals. In this study, swine gained weight of approximately 800 g per day, and thus these gut microbiota shifts may be required to adjust to hosts' metabolic needs [6]. On the other hand, the application of AGPs during the early growth stage (10-day-old pigs) was found to be effective in enhancing growth performance [30], suggesting that the effect of AGPs might have been clearer if they had been used before the gut microbiota was stabilized. In addition, Upadrasta *et al*. [42] reported that effects of diet supplementation on swine gut microbiota could vary depending on its endogenous microbiota. Therefore, it is important to understand how the endogenous microbiota react with AGPs. As there are various types of AGPs as

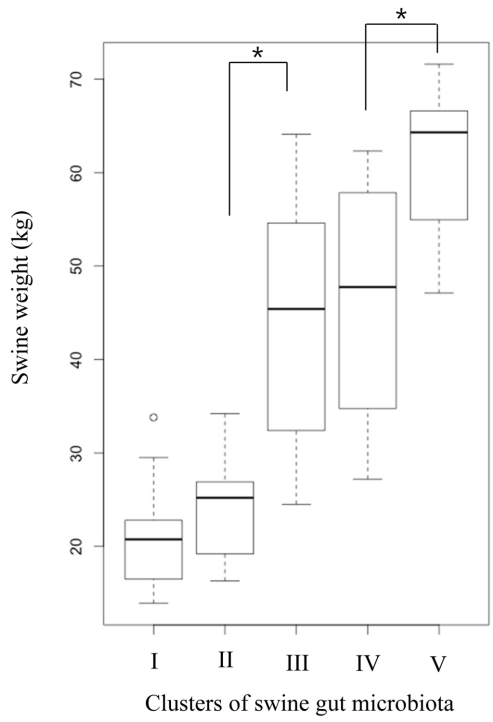

Fig. 4. Weight distribution of swine according to types of microbiota.

Microbiota types (I, II, III, IV, and V) are defined based on distribution of OTUs (Fig. 3). *Significant difference tested with ANOVA ($p < 0.05$).

well as gut microbiota, further studies are needed to understand this point and to make a decision on the type of AGPs to use and which growth stage to apply.

Effects of AGPs on Enteric Pathogens

Although no significant weight difference was observed between AGP-treated and control swine, our results suggest that the inhibition of gut pathogenic bacteria by AGP increased the growth rate of hosts during the intermediate growth stage (30–40 kg). The mechanisms of how AGPs inhibit those pathogenic bacteria are rather complicated. For example, macrolide AGPs designed for gram-positive bacteria reduced diarrhea caused by *E. coli* O157:H7 in swine [8]. Previously, Kamada *et al.* [20] suggested that competition with other metabolically related intestinal bacteria regulated colonization of enteric pathogens. In addition, it was reported that oral feeding of probiotics could stop diarrhea by competing diarrheagenic *E. coli* [2, 24]. One of the effects of antibiotics on gut microbiota could be a perturbation in the gut microbiota, which consequently changes the abundance of opportunistic pathogens [43]. Although AGPs are found effective in controlling pathogens, to develop prolonged protection of livestock animals from pathogenic bacteria, engineering whole gut microbiota, especially the commensals that compete with pathogens, may offer a new approach to this area.

In this study, our results showed the ecological succession of the swine gut microbial community according to host weight regardless of AGPs. Our results also showed that although the use of AGPs did not affect the overall weight gain, it inhibited potential pathogenic bacteria during the early growth stage. Further understanding of the swine gut microbiota should lead to the development of novel approaches with respect to growth performance enhancement.

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