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Effects of the Antibiotics Growth Promoter Tylosin on Swine Gut Microbiota

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Tylosin has been used as a livestock feed additive and antibiotic growth promoter for many years. However, the mode of action by which tylosin enhances animal growth is unclear. We used high-throughput sequencing of 16S rRNA genes to investigate the effects of tylosin as a feed additive on swine gut microbiota. No significant difference in the rate of weight increase was observed between control and tylosin-treated pigs during a 10-week feeding trial. However, tylosin-treated pigs showed rapid increases in the relative abundance of the phylum Firmicutes. Increases in Firmicutes species are associated with (so-called) obese-type gut microbiota. The abundance of species of four families of the phylum Firmicutes (*Streptococcaceae*, *Peptococcaceae*, *Peptostreptococcaceae*, and *Clostridiaceae*) correlated positively with host weight gain. The abundance of *Streptococcaceae* family bacteria was least affected by tylosin treatment. Distribution analysis of operational taxonomic units (OTUs) showed that both control and tylosin-treated pigs exhibited similar OTU alterations during growth. However, the tylosin-treated group showed distinctive alterations in gut microbiota when the host weighed approximately 60 kg, whereas similar alterations occurred at around 80 kg in the control group. Our results suggest that use of tylosin accelerates maturation of swine gut microbiota rather than altering its composition.

Keywords: 16S rRNA gene, antibiotics, growth promoter, swine gut microbiota, tylosin

Introduction

In the livestock industry, antibiotics are used as feed additives to prevent bacterial infections and enhance the growth of livestock animals; in the latter context, they are referred to as antibiotic growth promoters (AGPs). For decades, the excessive use of AGPs has led to the presence of undigested antibiotic residues in meat products [9] and animal feces [20]. Moreover, an increase in the levels of antibiotic-resistant bacteria in animal feces has also been reported [14, 17]. These findings led to complete bans on the use of AGPs in animal feed in many developed countries, including those of the European Union and the USA. However, the banning of AGPs has caused secondary problems, including increased death rates among livestock animals at early stages of growth, due to pathogenic bacterial infections.

To reduce mortality rates among young livestock animals, alternatives to AGPs have been developed. For pigs, these include immunoglobulins [28], zinc oxide [15], and probiotics [13]. However, studies examining these methods have produced conflicting data [44]. For example, essential oils are known to have strong antimicrobial activity [42], but are unable to control pathogenic bacteria when mixed with livestock feed [6]. Since the mechanisms by which antibiotics enhance the growth of livestock animals are unclear, it has been difficult to develop genuinely useful alternatives.

Reports of experiments using chickens as model organisms indicate that the use of tylosin, a macrolide antibiotic that inhibits growth of gram-positive bacteria and a limited number of gram-negative bacteria, can reduce levels of gram-positive pathogens (*i.e.*, *Clostridium perfringens*) [4].

However, the use of tylosin could unbalance gut microflora, leading to increased host susceptibility to pathogenic infections [35]. Although there have been reports of the growth enhancement effects of tylosin from mouse model experiments [34], no such effect was observed in pigs [48]. Neither were growth promotion effects observed in pigs using other AGPs, such as chlortetracycline [14, 47]. Previously, it has been reported that the use of tylosin significantly changes swine gut microbiota [21]; however, whether these changes impact growth promotion or protection from pathogenic bacteria is not yet understood.

In this study, the effects of tylosin on swine growth and gut microbiota were investigated. Whereas the majority of animal studies have been conducted with a minimum of a few dozens of animals, we chose to use a relatively controlled environment (*i.e.*, one animal per pen), to rule out the effects of cohabitation [33] and coprophagy, which significantly affect gut microbial composition. This study provides information potentially useful in understanding the effects of tylosin in growth promotion.

Materials and Methods

Feeding Trials

Six pigs (Landrace) of approximately 100 days old at the beginning of the study were each raised in separate pens. IRB approval was obtained from the Animal Care and Use Committee of Jeju National University (2015-0002). All animals were fed a standard corn and soybean meal-based commercial diet until the end of the experiment. Pigs were provided with ad libitum access to feed and water. For three pigs, feed was supplemented with tylosin (Sigma Inc., USA) at 45 mg/kg, whereas the other three pigs acted as controls with no tylosin supplementation. The duration of the experiment was 10 weeks. Fresh fecal samples were collected from each pig once per week and body weights were measured at the same time as fecal material collection.

DNA Extraction and 16S rRNA Gene Sequencing

Total DNA was extracted from approximately 150 mg of fecal material using a MOBIO Power Fecal DNA isolation kit (MOBIO Laboratories Inc., USA). PCR was carried out to amplify the V4 region of the 16S rRNA gene of Bacteria and Archaea as previously described [23]. Two microliters of the total DNA from each sample was used as a template, and amplification was performed in triplicates using the Maxime PCR PreMix Kit (iNtRON Biotechnology Inc., Republic of Korea) with the following conditions: 95°C for 2 min; 30 cycles of 95°C for 20 sec, 55°C for 15 sec, and 72°C for 1 min; and 72°C for 5 min. All obtained DNA was quantified using a Qubit fluorometer (Invitrogen Inc., USA), and equimolar amounts of amplicons were pooled and sent to Microgen Inc. (Seoul, Republic of Korea) for sequencing using the MiSeq platform (Illumina), according to the manufacturer's instructions.

Sequence Processing and Analysis

Sequence data were processed as described previously [46]. Briefly, fastq files were assembled using "PEAR" software [50], the mothur pipeline [39] was used to align sequences to the Silva database [36], and UCHIME [11] was used to remove chimeric sequences. Taxonomic classification was performed using the Ribosomal Database Project [3] with training set ver. 10. Operational taxonomic units (OTUs) were calculated with a sequence similarity distance of 0.03 using the mothur subroutine "cluster.split." Samples were grouped according to weight using the mothur subroutine "merge.groups," and then subjected to microbial community analysis based on the Yue and Clayton theta coefficient calculated by the mothur subroutine "tree.shared." Nonmetric multidimensional scaling was performed using the mothur subroutine "nmds," and correlations between bacterial composition and bacterial community shifts were assessed using the mothur subroutine "corr.axes." Network analysis comparisons of samples from control and tylosin-treated pigs were performed using OTUs commonly identified in each sample replicate. Cytoscape software [41] was used to draw a network map of consensus OTUs.

Statistical Analyses

Correlation analysis was conducted based on Pearson's *p*-value. Comparisons of ecological indices were based on analysis of variance.

Results and Discussion

Sequence Data Processing

Sequence data obtained in this study were deposited to the short read archives (PRJNA304066). A total of 4,413,600 sequences from 66 samples (range 32,023–113,576 reads per sample) were obtained in this study. After removing erroneous reads, a total of 2,422,598 reads remained, 34,834 of which were unique. The number of reads in each sample was normalized based on a minimum number of reads (23,842), which resulted in a total of 30,261 unique sequences. After normalization, a total of 3,926 OTUs were obtained (range, 315–732 per sample). Results of rarefaction analysis indicated sufficient sequencing depth, even after the normalization process (Fig. S1). Table S1 summarizes the number of reads, OTUs, and ecological indices obtained in this study. Comparison of ecological indices indicated no significant difference between tylosin-treated and control pigs (Fig. S2).

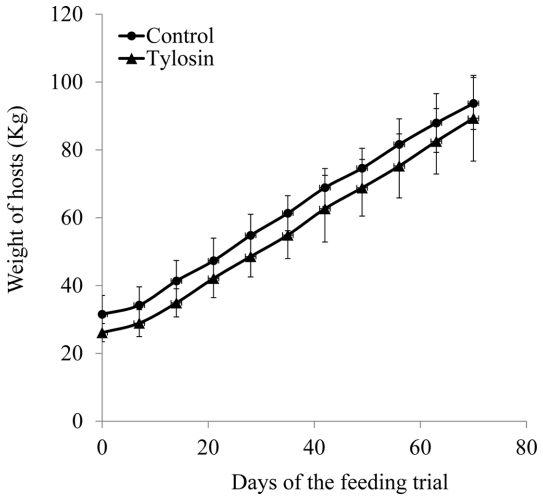

Fig. 1. Average weight of pigs used in this study during the feeding trial.

Effects of Tylosin on Pig Growth Performance

Each animal was weighed weekly for 10 weeks. The rate of weight increase was almost identical in control and tylosin-treated pigs (Fig. 1). Although tylosin has been used as a method to increase the growth performance of livestock animals, there have been reports that it does not induce growth rate increases in swine [14, 48]. In contrast, antibiotics have proven useful in controlling pathogenic bacterial infections in chickens in polluted environments, leading to improvements in their health status and increased growth rates [25]. Several mechanisms by which antibiotics may enhance the growth of livestock animals have been suggested, including prevention of subclinical infections and reduction in microbial nutrient use [8, 37]. However, the idea that gut microbiota compete with their hosts for nutrients has been questioned, since recent studies applying high-throughput next-generation sequencing approaches reported an association between gut microbiota and obesity [7, 29, 32, 38, 45], indicating that gut bacteria help hosts to absorb nutrients more effectively, rather than compete for them [24, 31]. Therefore, the current understanding of how antibiotics increase host weight gain is that they are likely to modify gut microbial composition, thereby influencing metabolic activity.

Changes in Taxonomic Composition of Swine Gut Microbiota in Response to Tylosin

The taxonomic composition of each sample is summarized at the phylum and family levels in Figs. S3 and S4, respectively. For the majority of animals, the overall fecal microbiota primarily comprised Firmicutes and Bacteroidetes.

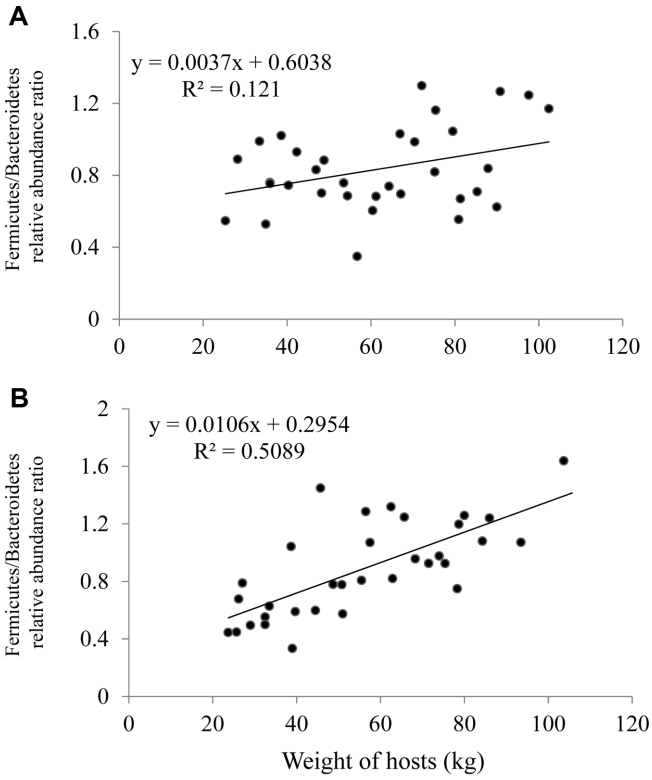

Fig. 2. Correlation between F/B ratio and weight for control (**A**) and tylosin-treated pigs (**B**).

One animal in the control group (C1) showed high abundance of bacteria of the phylum Proteobacteria, the majority of which are unclassified at the family level. Higher abundance of bacteria of the phylum Spirochaetes (family *Spirochaetaceae*) was observed in both one control and one tylosin-treated animal; thus, growth of Spirochaetes bacteria may not be inhibited by tylosin, consistent with a previous report [21].

The ratio of the relative abundance of Firmicutes and Bacteroidetes bacteria (F/B ratio) has been suggested to differentiate between lean and obese types in humans [26, 27]. The postulated explanation for these findings is that Firmicutes species metabolize available energy sources more effectively than Bacteroidetes species, and consequently promote weight gain [18]. Our results demonstrate a moderate increase in F/B ratio over time in the control group (Fig. 2). In contrast, a sharp increase in F/B ratio was observed in the tylosin group. Although the F/B ratio has been used as an obesity indicator in a number of studies, the results are conflicting [10, 40], and there have been disagreements over their statistical interpretation [30, 49]. Nevertheless, our results suggest that the use of tylosin

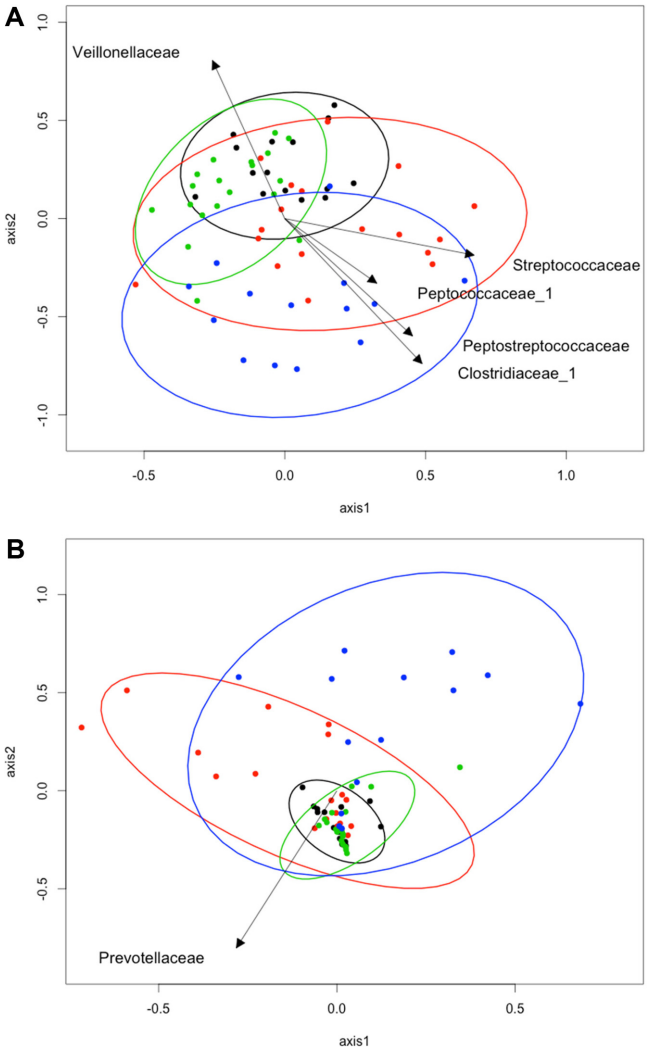

Fig. 3. Nonmetric multidimensional scaling (NMDS) analysis according to weight of hosts and tylosin treatment for Firmicutes species (**A**) and Bacteroidetes species (**B**).
Black and red indicate control pigs of 20–60 kg and 60–100 kg, respectively. Green and blue indicate tylosin-treated pigs of 20–60 kg and 60–100 kg, respectively. Ellipses were drawn to include 90 percentiles of samples. Arrows indicate a positive correlation of specific taxa to NMDS coordinates.

slightly shifted the swine gut microbiota to an obese type, despite the lack of a significant difference in rate of weight gain.

We investigated which families of Firmicutes and Bacteroidetes bacteria were responsible for the observed microbial community shifts. Five families of Firmicutes and one of Bacteroidetes were significantly correlated with microbial community shifts ($p < 0.05$) (Fig. 3). Among the Firmicutes families, *Veillonellaceae* species were found to be more abundant in the early stage of growth, whereas species of the families *Streptococcaceae*, *Peptococcaceae*, *Peptostreptococcaceae*, and *Clostridiaceae* were more abundant in the late stage growth. Whereas the abundance of species of the family *Streptococcaceae* was less significantly correlated with tylosin treatment, the use of tylosin increased the abundance of *Peptococcaceae*, *Peptostreptococcaceae*, and *Clostridiaceae* family bacteria, which is unexpected, since tylosin is a macrolide antibiotic that inhibits growth of gram-positive bacteria. Tylosin is proven to be effective in stopping diarrhea [2]; however, it has been reported that its use also increases the abundance of several groups of bacteria expected to be sensitive targets [43], likely due to natural selection of bacteria that obtained resistance genes [16, 17]. Therefore, long-term treatment with tylosin could change the gut microbiota irrespective of target species.

The distribution of Bacteroidetes species also showed clear differences during the late stage of growth. Only one family, *Prevotellaceae*, was found to have a significant negative correlation with growth, which is consistent with our previous study [22]. Although a significant difference was observed between the fecal microbiota of control and tylosin-treated pigs at the late stage of growth ($p < 0.001$), no other specific taxa showed a significant correlation with the microbial shifts, suggesting that the abundance of diverse Bacteroidetes bacteria was only moderately affected by tylosin treatment. In addition, our results show that ellipses drawn for the early growth stage samples are smaller than those for the late growth stage in both Firmicutes and Bacteroidetes, suggesting that gut microbiota diversity was increased in the late stage of growth (Fig. 3). Among Firmicutes species, there was no significant difference between control and tylosin-treated pig samples in the size of the ellipse (Fig. 3A). In contrast, among Bacteroidetes species, the ellipse for tylosin-treated samples was larger than that for control samples at the late stage of growth (Fig. 3B). It has been reported that swine gut microbiota contain abundant *Veillonellaceae* and *Prevotellaceae* species (which are likely to assist in the digestion of lactose [1]) during the nursing period [12]. Further studies are needed to investigate role of *Peptococcaceae*, *Peptostreptococcaceae*, and *Clostridiaceae* family bacteria in swine growth. Our results suggest that the use of tylosin has potential to decrease the time required for gut microbiota to adjust to weaning.

Operational Taxonomic Unit Distribution Analysis

The distribution and abundance of operational taxonomic units were compared among samples based on the Yue and

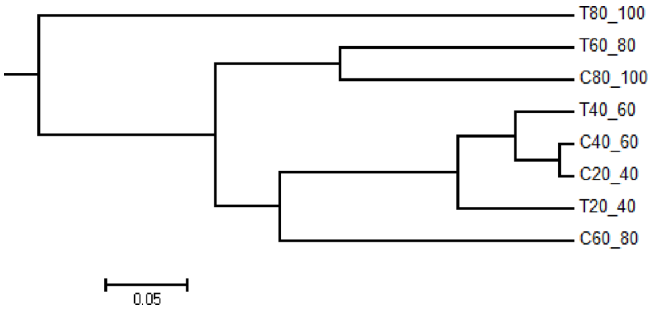

Fig. 4. Tree analysis based on OTU distribution and abundance according to host weight and tylosin treatment.
C and T indicate control and tylosin-treated pigs, respectively, followed by range of host weight (kg).

Clayton theta coefficient of the individual pigs (Fig. S5) and for samples grouped according to weight (Fig. 4). Tight clusters were observed among samples obtained at early growth stages (20–60 kg) regardless of tylosin treatment. Gut microbiota shifts are expected to occur at each growth stage [22]; however, control grower pigs (60–80 kg) did not show a clear shift in microbiota after the early growth stage. By contrast, tylosin-treated grower pigs showed a distinctive difference compared with the early growth stage. Our results suggest that tylosin-treated pigs undergo faster gut microbiota shifts than control pigs. Previously, Kim *et al*. [21] also suggested that tylosin accelerates the development or maturation of the unique "adult-like" fecal microbiome, which is consistent with the results of our analysis (Fig. 4).

The distribution of shared OTUs was visualized by network analysis (Fig. 5). The results demonstrate that the most significant alterations of OTUs occurred in tylosin-treated pigs at 60 kg, whereas in control pigs these occurred at 80 kg (Fig. 5). We have previously reported that dynamic species-level alterations in swine gut microbiota occur between the grower and finisher phases [22]. Kalmokoff *et al*. [19] reported that antibiotics fed at later growth stages (*i.e.*, the grower and finisher phases) did not affect the fecal microbial community composition or the prevalence of resistance genes, suggesting that antibiotics only affect the gut microbial composition if fed in early life. In addition, Cox and Blaser [5] reported that antibiotics had more significant impacts on gut microbiota shifts and host weight gain if they were administered in early life. Although no significant difference in gut microbiota during the early growth stage was observed in this study, antibiotic treatments given from the early growth stage may have caused the gut microbiota shifts observed in the late growth

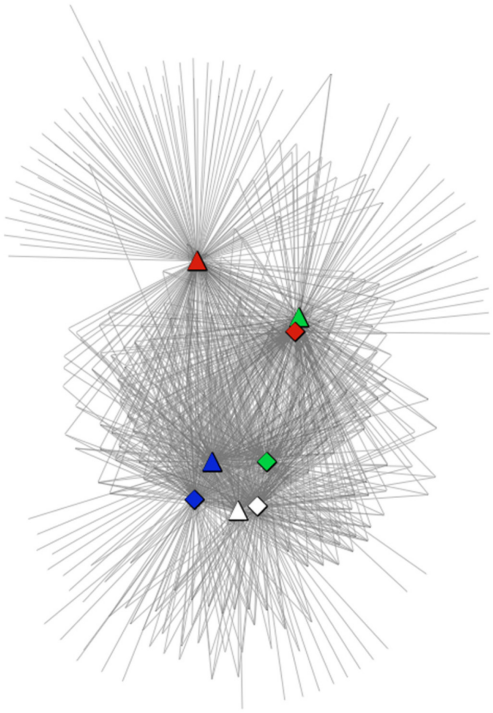

Fig. 5. Network analysis based on operational taxonomic units.
Diamonds and triangles indicate control and tylosin-treated pigs, respectively. Lines indicate shared operational taxonomic units among samples. White, blue, green, and red indicate weight of hosts: 20–40 kg, 40–60 kg, 60–80 kg, and 80–100 kg, respectively.

stage. Further studies with shorter durations of antibiotic supplementation of feed are needed to investigate whether standard gut microbiota shifts over time can be accelerated by disruption of the gut microbiota in early life.

In summary, the use of tylosin as a feed additive did not enhance the growth of swine; however, it did accelerate maturation of the gut microbiota. This indicates that tylosin did not alter the gut microbiota by targeting susceptive strains, but rather accelerated the shift in microbiota that normally occurs over time as the host grows. It is usual for the F/B ratio in swine gut microbiota to gradually increase over time [21, 22]; thus, tylosin-treated pigs showed an accelerated increase in the F/B ratio. Nonetheless, these changes did not affect the rate of weight gain of the swine.

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