Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine

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Abstract
The use of antibiotics in swine production for the purpose of growth promotion dates back to the 1950s. Despite this long history of use, the exact mechanism(s) responsible for the growth-promoting effects of antibiotics in swine remain largely unknown. It is believed, however, that growth promotion is due to antibiotics having a direct impact on the gut microbiota. In this study, the effect of two antibiotics on the swine gut microbiota over a 19-week monitoring period was investigated using Illumina-based sequencing. A shift in the relative abundance of several taxa and in 26 operational taxonomic units (OTUs) was observed in pigs fed subtherapeutic concentrations of tylosin (44–11 mg kg\(^{-1}\) feed). Only minor alterations were noted with the administration of chlortetracycline at 5.5 mg kg\(^{-1}\) feed. The most notable changes in the relative abundance of taxa and OTUs were noted between suckling piglets and postweaned pigs. Diversity was also reduced in the gut microbiota of suckling piglets as measured using the Shannon, Chao1, and phylogenetic diversity indices. These results show that the effect of antibiotics on the swine gut microbiota is variable based on dosage and duration and that the swine gut microbiota exhibits considerable resilience to long-term changes due to antibiotic perturbations.

Introduction
Antibiotics have been used for decades in agricultural production to increase the growth rate and feed efficiency of food animals as well as to treat disease (Cromwell, 2002). Despite this long period of usage, the specific mechanism(s) responsible for the growth-promoting benefits of antibiotics remains largely elusive. Currently, it is believed that antibiotics promote animal growth through a direct impact on the gut microbiota related to a reduction in subclinical disease and harmful metabolites produced by intestinal bacteria or an increase in nutrient absorption and availability in the gut (Dibner & Richards, 2005). The use of antibiotics in livestock, however, selects for antibiotic-resistant bacteria and resistance determinants, which can be passed to humans through the food chain, the release of animal waste into the environment, or from direct contact with animals (van den Bogard & Stobberingh, 2000; Marshall & Levy, 2011).

Therefore, the continued use of antibiotics in agriculture, particularly for nontherapeutic reasons (i.e. growth promotion), is a source of controversy. There is also a question of whether these benefits attributed to antibiotics remain under modern swine production practices (Holt et al., 2011; Holman & Chénier, 2013). A ban on antibiotic use in livestock production has been in place since 2006 in the European Union, and it seems probable that similar restrictions may be imposed in Canada and the United States in the future (Mathew et al., 2007; Marion et al., 2013). As a result, there is the need for alternatives to antibiotics in agriculture. The development of an effective replacement for antibiotics in swine production, however, requires a better understanding of the response of the gut microbiota to antibiotic exposure (Allen et al., 2013).

The gut microbiota of the pig is comprised of a large and diverse number of microorganisms which contribute to the health of the animal (Lamendella et al., 2011).
structure of the gut microbiota is largely determined by factors such as diet, age, genetics, and in some cases antibiotic exposure (Scott et al., 2013). High-throughput sequencing (HTS) technologies targeting the 16S rRNA gene have revolutionized the way that the gut microbiota can be analyzed and allow for a much greater depth of coverage than had been possible with culture-dependent and clone library methods (Hamady & Knight, 2009). To date, only a few studies have examined the impact of antibiotics on the gut microbiota of swine using HTS, and none have monitored the temporal changes in the gut microbiota in farrow-to-finish swine production (Kim et al., 2012; Looft et al., 2012, 2014a; Poole et al., 2013).

Although agricultural usage of antibiotics is not well monitored in Canada or the United States, tylosin and chlortetracycline are two of the most commonly employed antibiotics in swine production (Deckert et al., 2010; Apley et al., 2012). For this reason, in the present study, the impact of the subtherapeutic administration of these two antibiotics on the swine gut microbiota from suckling to finishing was evaluated. It is hypothesized that the continuous administration of tylosin and chlortetracycline at subtherapeutic doses in feed reduces the gut microbial diversity in swine and alters the microbial community.

Materials and methods

Animals, experimental design, and sample collection

This is a companion study using the same pigs and sows as in Holman & Chenier (2013). The experimental design was also the same with the exception that samples from three males and three females per treatment group were used in the present study (n = 6). In addition, the fecal samples used in the current work were taken at suckling (3 weeks), weanling (6 weeks), starting (9 weeks), growing (12 weeks), and finishing (19 weeks). In total, 94 fecal samples were sent for Illumina sequencing: 6 samples per ing (12 weeks), and finishing (19 weeks). In total, 94 fecal samples were sent for Illumina sequencing: 6 samples per experiment (250 bp) following manufacturer's instructions (Illumina).

DNA amplification and Illumina sequencing

Total DNA was extracted from each fecal sample using the ZR Fecal DNA Miniprep kit (Zymo Research, Irvine, CA) as previously described (Holman & Chenier, 2013). The primers 515-F (GTGCCAGCMGCCGCGGTAA) and 806-R (GGACTACVSGGGTATCTAAT) were used to target the V4 region of the 16S rRNA gene of both Bacteria and Archaea (Caporaso et al., 2011). The forward primer contained a unique 8 bp barcode to allow for pooling of samples prior to sequencing. Amplification and sequencing steps were performed at Molecular Research LP (Shallowater, TX). Briefly, the 16S amplicons were generated using HotStarTag Plus Master Mix Kit (Qiagen, Valencia, CA). The PCR program consisted of a 3 min initial denaturation at 94 °C followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min, with a final extension of 5 min at 72 °C. The size and specificity of PCR amplicons were verified using 2% agarose gel electrophoresis and samples were then pooled together in equal proportions and prepared for sequencing according to the standard protocol for the Illumina TruSeq DNA library preparation kit (Illumina, San Diego, CA). Sequencing was carried out using an Illumina MiSeq system (2 × 250 bp) following manufacturer’s instructions (Illumina).

Data processing and analysis

Sequenced 16S rRNA gene amplicons were processed using the QIIME software package (version 1.8.0) (Caporaso et al., 2010). The two paired-end reads were joined together, and the sequences were demultiplexed and quality filtered with the removal of sequences containing base calls with a Phred score of < 20, any mismatched primer sequences, or a total length of < 200 bp. Sequences were assigned de novo to operational taxonomic units (OTUs) at ≥ 97% similarity with chimera filtering using the USEARCH algorithm (version 6.1.544) within QIIME (Edgar et al., 2011). Taxonomy was assigned using the naïve Bayesian RDP classifier and the GREENGENES 13.5 database and a confidence cutoff of 0.8 (DeSantis et al., 2006; Wang et al., 2007). Low-abundance OTUs (< 0.005% of total reads) were removed as recommended by Bokulich et al. (2013) to reduce the number of
spurious OTUs. To allow for comparisons between samples and treatment groups, the OTU table was subsampled and rarefied to 12 500 reads per sample for alpha and beta diversity analyses with the loss of one sample from the finishing chlortetracycline group. Sequences were submitted to the NCBI Sequence Read Archive (SRA) under project accession number SRP041290 (http://www.ncbi.nlm.nih.gov/sra).

**Statistical analysis**

\(\alpha\)-Diversity was calculated within QIIME using the Shannon index (Shannon, 1948), Chao1 (Chao, 1984), and phylogenetic diversity (PD whole tree) (Faith, 1992). Good’s coverage was also measured. \(\beta\)-Diversity was calculated using unweighted and weighted UniFrac (Lozupone & Knight, 2005) and displayed using principal coordinate analysis (PCoA). Statistical comparisons of unweighted and weighted UniFrac distances between treatment groups and between different sampling times were made using ANOSIM (analysis of similarities) with 999 permutations.

Due to the fact that the same pigs were sampled repeatedly over the course of the study, a two-way repeated measures ANOVA with post hoc Tukey’s honestly significant difference (HSD) comparisons was performed using PROC GLM in SAS (SAS Inst., Inc., Cary, NC) to compare proportions of taxa as well as diversity indices between groups of pigs. Comparisons of OTU abundance were calculated using the nonparametric Kruskal–Wallis test with the false discovery rate (FDR) correction (Benjamini & Hochberg, 1995). All results were considered significant at \(P < 0.05\).

**Results**

**Sequence analysis**

A total of 5 880 818 raw sequences were obtained from the Illumina MiSeq sequencing. Following quality filtering and demultiplexing, 5 401 765 sequences remained with an average length of 256 bp. Upon chimera removal, the 4 950 142 sequences left were clustered into OTUs (\(\geq 97\%\)) using the de novo method. The removal of low-abundance OTUs (< 0.005%) yielded a total of 1281 OTUs for analysis. While the average number of sequences per sample was 31 152, all samples were rarefied at 12 500 sequences per sample to retain as many samples as possible. Good’s coverage was > 97.5% for all samples.

**Microbial diversity of the swine fecal microbiota**

The rdp classifier and greengenes database was used to assign taxonomy to OTUs from domain to genus level. At the phylum level, a total of 15 different bacterial phyla and one archaeal phylum were identified in each sample (Fig. 1a). On average, Archaea accounted for < 1% of the total sequences per sample (data not shown). The majority of sequences belonged to either Firmicutes or Bacteroidetes, with these two phyla encompassing > 70% of all sequences. Proteobacteria and Spirochaetes dominated the remaining sequences and only 1.2% of sequences could not be classified at the phylum level.

**Fig. 1.** Classification of 16S rRNA gene sequences at the (a) phylum level for the control, tylosin, and chlortetracycline-supplemented pigs (\(n = 6\)) and (b) genus level at each sampling time. In (b), only those genera containing \(\geq 1\%\) of sequences are displayed.
The sequences could be definitively assigned into 65 different genera, with an average of 32.4% of sequences being unclassified at the genus level (Fig. 1b). In terms of relative abundance, the 14 bacterial genera that accounted for more than 1% of sequences were (in decreasing order of abundance): *Prevotella*, *Treponema*, *Lactobacillus*, *Succinivibrio*, *Bacteroidetes*, *Phascolarctobacterium*, SM53 (Clostridiaceae), *Ruminococcus*, Blautia, *Roseburia*, *Streptococcus*, *Faecalibacterium*, *Oscillospira*, and *Campylobacter*.

The only two archaeal genera identified were *Methanobrevibacter* and vadinCA11, which is an uncultured archaeal genus from the phylum *Euryarchaeota*, order *Thermoplasmata*, class *Thermoplasmatales* (Godon et al., 1997).

Several α-diversity indices were calculated using the samples that had been rarefied at 12,500 sequences to account for unequal numbers of sequences between samples. The overall average of all samples for Chao1, a non-parametric estimator of species (OTU) richness, was 1238.6 ± 14.6 (SD of the mean). The average number of observed unique species (OTUs) was 1064.6 ± 21.0. The average value for the Shannon index, which measures both species (OTU) richness and abundance, was 5.543 ± 0.119. The average PD (whole tree) was 189.40 ± 2.75.

The core microbiota, defined as those OTUs found in all samples at all sampling times (including the sows), was comprised of 284 OTUs. While these OTUs represented only 22.2% of the total OTUs, they contained 80.4% of the total sequences (data not shown). The core microbiota of all treatment groups encompassed 309 OTUs that were found in all treatment groups at all times, excluding suckling piglets and the sows. For each diet group, the core microbiota was comprised of 391, 407, and 394 OTUs for the control, tylosin, and chlortetracycline groups, respectively. Finally, the core microbiota of suckling, weanling, starting, growing, and finishing samples was made up of 392, 429, 441, 431, and 449 OTUs respectively.

**Microbial community changes due to the administration of in-feed antibiotics**

Unweighted and weighted UniFrac distances were used to estimate β-diversity and to compare the three diet groups. Analysis of similarities (ANOSIM) of unweighted UniFrac distances indicated that while the control, tylosin, and chlortetracycline diet groups (*P* = 0.022) were significantly different, the relatively small corresponding *R*-value (0.0317) suggests that the diet groups are not well separated from each other. The PCoA plot of the unweighted UniFrac distances visually confirmed that the three diet groups do not form distinct clusters when only microbial community membership is considered (Fig. 2a). In contrast, the ANOSIM of weighted UniFrac distances showed a significant difference between treatment groups (*P* = 0.001) with a higher *R*-value (0.212). Weighted UniFrac takes into account the relative abundance of OTUs, whereas unweighted UniFrac considers only community membership (i.e. presence/absence of OTUs) (Navas-Molina et al., 2013). A PCoA plot of the weighted UniFrac distances shows greater separation between the tylosin-fed pigs and the control pigs as well as the chlortetracycline group (Fig. 2b). The higher *R*-value indicates that tylosin modulates the relative abundance of OTUs rather than determining their presence or absence. Comparisons of the α-diversity metrics Chao1, PD (whole tree), and Shannon indices for each treatment group revealed no significant differences (*P* > 0.05; Supporting Information, Table S1), thus demonstrating that microbial diversity was not affected by antibiotic treatment.

Phyla and genera with a relative abundance of > 0.1% were compared between the antibiotic-supplemented diets and the control diet (Table S2). Changes in the relative abundance of specific OTUs can be visualized in the PCoA plots of both the unweighted and weighted UniFrac distances (Fig. 2).

**Fig. 2.** PCoA of the (a) unweighted UniFrac distances and (b) weighted UniFrac distances for each treatment group. All samples from weanling (6 weeks), starting (9 weeks), growing (12 weeks), and finishing (19 weeks) are grouped together in each diet group (*n* = 24). The percent variation explained by each principal coordinate is indicated on the axes.
abundance of taxa between the antibiotic groups and the control group were temporal in nature. At weaning (6 weeks), there were significantly more bacterial sequences that were unclassified at the phylum level in the chlortetracycline-fed pigs. Tylosin-fed swine had significantly fewer sequences classified in the phylum Fibrobacteres. In addition, the relative abundance of sequences in the genus Coprococcus was significantly higher in the tylosin-fed pigs than in the control cohort.

Several changes in the relative abundance of taxa as a result of antibiotic administration were observed at the starting phase (9 weeks). At the phylum level, pigs treated with tylosin had a significantly lower proportion of Cyanobacteria and Fibrobacteres sequences than in the control group. WPS-2, a candidate phylum, was significantly enriched in chlortetracycline-fed swine. There were five genera that were differentially abundant at starting. In the tylosin-fed pigs, there was a significantly higher proportion of Streptococcus and Coprococcus compared with the control pigs. However, the relative abundance of Fibrobacter was significantly lower in the tylosin group. Also, at the starting phase, the proportion of SMB53 was significantly increased in pigs on the chlortetracycline diet, while Lactobacillus was significantly decreased (Table S2).

The sampling of growing phase pigs (12 weeks) revealed that tylosin-fed pigs had a significantly lower proportion of sequences in the Bacteroidetes phylum when compared with the antibiotic-free pigs. The tylosin group also had a reduced relative abundance of the genera Succinivibrio and Anaerovibrio. Members of the Verrucomicrobia phylum were increased in the tylosin cohort, as were the genera Coprococcus and Akkermansia. Notably, the relative abundance of Akkermansia was enriched almost 10-fold in the tylosin-supplemented pigs in comparison with the control pigs at the growing phase (Table S2).

At the finishing phase (19 weeks), the tylosin treatment significantly increased the proportion of sequences in the Cyanobacteria phylum. Chlortetracycline treatment reduced the relative abundance of Firmicutes while increasing the proportion of WPS-2 sequences (Table S2). Interestingly, archaeal sequences were also enriched in the feces of the chlortetracycline group in comparison with the control pigs (data not shown). No genus-level differences were detected at finishing, possibly reflecting the lower dosage of tylosin at this time (11 mg kg⁻¹ feed for tylosin).

The differences in OTU abundance were also calculated between the antibiotic-treated pigs and the control group. This analysis grouped all pigs in each treatment diet together (weaning to finishing) for comparisons. A total of 26 OTUs were differentially abundant between the tylosin-fed pigs and the control group (P < 0.05 FDR; Table S3). Eighteen of these OTUs were more abundant in the control pigs and the remaining 8 OTUs were more abundant in the tylosin cohort. Exactly, half of these OTUs were classified as members of the Bacteroidetes phylum. None of the OTUs were differentially abundant between the control and chlortetracycline-supplemented pigs (P > 0.05 FDR).

**Temporal changes in the swine fecal microbiota**

Temporal changes in the microbial communities of the pigs at each sampling time were evaluated using un-weighted and weighted UniFrac distances. A significant difference between sampling times was observed based on the anosim of unweighted UniFrac distances (P = 0.001). While the R-value (0.0983) was relatively small, the sucking (3 weeks) samples were clearly separated from the other sampling times based on the PCoA plot of the unweighted UniFrac distances (Fig. 3a). The anosim of the weighted UniFrac distances was also significant (P = 0.001), but the R-value (0.403) was much higher (Fig. 3b), indicating differences between sampling times are likely a result of alterations in the relative abundances of OTUs rather than the presence or absence of specific OTUs.

The α-diversity in the feces of pigs at each sampling period was also compared over the duration of the study using repeated measures ANOVA (Table S4). At sucking, the piglets had a significantly lower PD (whole tree) compared with all other sampling times (P < 0.0001) and a lower number of observed OTUs (P < 0.0001). The Shannon index in sucking samples was also significantly lower than the starting (9 weeks), growing (12 weeks), and finishing (19 weeks) samples (P < 0.05; Table S4).

Taxonomic-based analysis yielded a number of significant differences in the relative abundance of taxa between sucking and all other sampling times. Among the most abundant genera (> 1% of sequences), the proportion of Prevotella, Treponema, Lactobacillus, Succinivibrio, SMB53, Ruminococcus, Roseburia, Streptococcus, and Coprococcus were all significantly reduced at sucking (Table S5). The proportion of archaeal sequences in the sucking fecal samples was also significantly lower than all other sampling times (P < 0.05), with the exception of weaning (data not shown). The phyla Cyanobacteria and Spirochaetes both accounted for significantly fewer sequences in sucking samples in comparison to every other sampling time.

Suckling samples also had significantly greater proportions of the genera Bacteroides, Clostridium, Butyricimonas, Oscillospira, Desulfovibrio, and Parabacteroides (Table S5). At the phylum level, Proteobacteria were significantly enriched in sucking samples (P < 0.05). Interestingly,
suckling samples had a significantly higher proportion of Enterobacteriaceae, a bacterial family from the phylum Proteobacteria, class Gammaproteobacteria, order Enterobacterales that includes pathogens such as Salmonella and Escherichia coli. The percentage of Enterobacteriaceae sequences at suckling was 3.1 ± 0.80% (SEM), while all subsequent sampling periods had <1% (data not shown).

When OTU abundance was compared between suckling (3 weeks) and weanling (6 weeks) samples, a total of 215 OTUs were observed to be differentially abundant \((P < 0.05\) FDR; Table S6). Meanwhile, there were no significant differences in the abundance of OTUs between the weaning and starting (9 weeks) periods \((P > 0.05\) FDR; data not shown). There were four differentially abundant OTUs between starting and growing and three OTUs between growing and finishing \((P < 0.05\) FDR; data not shown).

Samples taken at the growing phase (12 weeks) had a greater relative abundance of Bacteroidetes when compared with every other time period (Table S5). Along with this increase in Bacteroidetes, pigs at the growing phase had significantly lower proportions of Firmicutes than every other sampling time with the exception of suckling (Table S5).

### Discussion

We evaluated the effect of the continuous administration of subtherapeutic concentrations of tylosin and chlortetracycline on the fecal microbiota of swine. We monitored changes in the fecal microbiota for the entire duration of the swine production cycle using high throughput sequencing. To our knowledge, this is also the first time that HTS has been used to compare the gut microbiota of the suckling vs. weanling piglet. Our results demonstrate that tylosin causes shifts in the relative abundance of specific taxa and OTUs and that the microbiota of suckling piglets is significantly different from that of post-weaning swine.

The large majority (> 70%) of sequences were classified as either Firmicutes or Bacteroidetes, a finding that is in agreement with several other studies. Similarly, as in previous reports, Prevotella was the most abundant genus in the present study (Kim et al., 2011, 2012; Looft et al., 2012). The microbial diversity of the fecal microbiota as measured with the Shannon index was similar to the values previously reported by Kim et al. (2011) of 5.74–6.17. Our finding that the core microbiota of all samples constitutes a minority of the OTUs (22.2%), but a majority of the sequences (80.4%) is congruent with the observation of Kim et al. (2012) that the core OTUs (6.88–7.68%) of total OTUs) encompasses 82.4–85.4% of the sequences.

We hypothesized that antibiotics added to the swine diet would decrease diversity and alter the gut microbiota. While diversity was not affected according to several α-diversity metrics (Table S1), the addition of tylosin to the swine diet shifted the overall microbial community structure when OTU abundance was taken into account using the weighted UniFrac and PCoA (Fig. 2b). This indicates that tylosin changed the proportion of specific taxa in the swine gut microbiota rather than altering community membership. This observation is in accordance with the work of Kim et al. (2012) who reported that the changes in the gut microbiota of commercially raised swine in response to 40 mg kg−1 feed of tylosin (from 10 to 22 weeks), occurred only at certain times and in specific genera and OTUs. These authors also did not record any significant differences in α-diversity indices, including the Shannon index and observed OTUs, between tylosin and no-tylosin pigs (Kim et al., 2012). Poole et al. (2013) also found no significant effect on...
α-diversity when pigs were fed 50 mg kg⁻¹ feed of chlortetracycline for 28 days beginning 3 weeks postweaning. In contrast, Looft et al. (2014b) observed significant decreases in total OTUs and the Shannon index in the early period (up to 4 days) of administration of carbadox at 50 mg kg⁻¹ feed in 6-weeks-old piglets. Also of note, these authors found that changes observed in Prevotella were relative rather than absolute (Looft et al., 2014b).

We detected changes in the relative abundance of taxa at several taxonomic levels and at various sampling times as a result of tylosin supplementation (Table S2). Interestingly, these alterations were observed less often in weaning (6 weeks) samples, particularly when compared with starting (9 weeks) and growing (12 weeks) samples. Pigs were weaned at 24 days and started immediately on their experimental diets. It may be that tylosin takes time to cause observable changes in the gut microbiota. In contrast, we reported a rapid increase in tylosin resistance in anaerobic bacteria in a previous report using these same samples, although the concentration of total anaerobes remained unchanged (Holman & Chenier, 2013).

Of particular interest is the finding of a decreased proportion of Bacteroidetes at growing (12 weeks) in the tylosin group compared with the control pigs. A decrease in the relative abundance of Bacteroidetes has been associated with weight gain in swine (Guo et al., 2008), although in the present study the tylosin group did not exhibit any differences in weight gain compared with the other diet groups (Holman & Chenier, 2013). Similarly, Looft et al. (2012) also described a decrease in the abundance of Bacteroidetes in swine exposed to ASP250, a mixture of 100 mg kg⁻¹ feed of chlortetracycline, 100 mg kg⁻¹ feed of sulfamethazine, and 50 mg kg⁻¹ feed of penicillin. Furthermore, these authors noted a decrease in the genus Succinivibrio in antibiotic-treated pigs, a result that was also observed in the current study using tylosin.

Given the lower dose of chlortetracycline (5.5 mg kg⁻¹ feed) used in comparison with tylosin (44–11 mg kg⁻¹ feed), it was not unexpected that fewer changes were seen in the microbiota of chlortetracycline-supplemented pig samples. In the latter pigs, the overall community structure did not appear markedly different from the control samples when visualized using PCoA plots of either weighted or unweighted UniFrac distances and none of the OTUs were differentially abundant when compared with the control group. However, some alterations were evident in the chlortetracycline group, most notably a significantly reduced proportion of Firmicutes at finishing (19 weeks). The relative abundance of Lactobacillus sequences also decreased with chlortetracycline-supplementation at starting (9 weeks) (Table S2).

It should be noted that most of the shifts in the relative abundance of individual taxa due to either antibiotic were temporary rather than permanent. The fact that tylosin was progressively halved from weaning to starting to growing may have played a role or it could be due to the resiliency of the gut microbiota to long-term changes. Kim et al. (2012) suggested that the development of the ‘mature’ gut microbiota in swine is accelerated by the addition of tylosin, although the gut microbiota of untreated pigs eventually reaches this state as well. Therefore, it may be that once this climax community is attained, the gut microbiota is increasingly more resistant to dietary perturbations, including antibiotics (Carney-Hinkle et al., 2013). In addition, despite using the maximum dosage of tylosin and chlortetracycline allowed in Canada for growth promotion in swine, we did not detect any differences in growth rate between diet groups (Holman & Chenier, 2013). Pigs that exhibit an increase in growth rate in response to antibiotic supplementation may have changes in their gut microbiota that are different from the current study. Unfortunately, the growth rate of the pigs is rarely reported in studies examining the effect of antibiotic supplementation on the swine gut microbiota (Kim et al., 2012; Looft et al., 2012, 2014a; Poole et al., 2013).

In terms of temporal changes, the suckling piglets (3 weeks) had a significantly different fecal microbiota in comparison with subsequent sampling times (Fig. 3; Tables S5 and S6). One of the more notable observations was that suckling piglets had a significantly greater proportion of Enterobacteriaceae, a family that includes potentially pathogenic bacteria such as E. coli and Salmonella (Schierack et al., 2007). In agreement with this finding, culture-based studies have reported decreasing numbers of Enterobacteriaceae 11 days postweaning (Piper et al., 2006). Similarly, another group of potentially pathogenic bacteria, Campylobacter, was significantly enriched in the feces of suckling piglets. Culture-based methods have found a high prevalence of Campylobacter spp. in piglets shortly after birth (Weijtens et al., 1997). At the same time, Lactobacillus, a genus associated with beneficial health effects in swine and other mammals (Daly et al., 2014) was reduced in the current study. The change in the relative abundance of Bacteroides from 8.44% at suckling to 1.99% at weanling was also particularly striking. Bacteroides is a genus of Gram-negative obligate anaerobes that, along with Lactobacillus, comprise a relatively large fraction of the culturable bacteria of the swine gut microbiota and are among the major fermenters of aromatic amino acids (Moore et al., 1987; Gaskins, 2001).
current study from suckling to weaning (Lalles et al., 2007). Physiological changes in the piglet gut also occur during the transition from suckling to weaning. For example, the pH of the cecum has been reported to be higher in suckling piglets vs. weanling piglets (Snoeck et al., 2004) and the large intestine is relatively larger in the postweaning period (Kelly et al., 1991). The immune system of suckling piglets is also relatively immature compared to that of postweaned pigs, with suckling piglets relying heavily on maternal antibodies for protection (Stokes et al., 2004). Following weaning, maternal antibodies wane and the piglet’s immune system must learn to tolerate commensal microorganisms and harmless antigens and yet react appropriately to pathogens. As a result, until the piglet’s immune system reaches a mature-state, changes in the gut microbiota are expected (Bailey et al., 2005). The rapidly changing gut ecosystem during the suckling to weaning transition may also explain why recent studies of antibiotic growth promoters have indicated a positive effect on growth rate only at this period (Holt et al., 2011).

Fewer major alterations were also observed from weaning through to finishing and it appears that once established following weaning, the swine gut microbiota undergoes more subtle changes over time. This was clear when OTU abundance was compared between sampling times. While a total of 215 OTUs were differentially abundant from suckling (3 weeks) to weaning (6 weeks), there were no significant differences in OTU abundance between weanling and starting (9 weeks). Studies using DGGE have demonstrated temporal variation in the gut microbiota of piglets up to 4 weeks following birth with increasing stability beyond this period (Thompson et al., 2008).

The relative abundance of archaeal sequences decreased over time, although the overall proportion remained relatively low at < 1% of total sequences, with the exception of suckling where piglets carried a higher percentage of archaeal sequences (1.2%). This finding is in accordance with previous estimates of archaeal abundance in the swine gut microbiota (Lamendella et al., 2011). Archaeal diversity was also relatively low, with only one previously cultured genus of *Archaea* identified. This genus, Methanobrevibacter, is comprised of methanogens commonly found in swine feces (Mao et al., 2011; Su et al., 2014).

**Conclusions**

Understanding how and when the swine gut microbiota changes in response to antibiotics will aid in the development of alternatives to antibiotics. In the current study, the administration of tylosin at subtherapeutic concentrations resulted in changes in the fecal microbiota that were identifiable at the phylum through genus levels. Chlortetracycline had relatively minor effects in comparison but alterations were noticeable in specific taxa. The swine gut microbiota also demonstrated considerable resilience to antibiotic perturbation as most changes to the relative abundance of specific taxa were temporary. Suckling piglets had a microbial community which was very different from that of the postweaning phase and once established, the gut microbiota of postweaning swine was significantly more stable in terms of community membership. Therefore, dietary manipulations beginning around the weaning period are likely to have the most impact on the swine gut microbiota.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Microbial diversity and abundance estimates of the control, chlortetracycline-, and tylosin-supplemented pigs.

**Table S2.** Effect of tylosin and chlortetracycline on the relative proportion (percentage ± SD of the mean) of the most abundant phyla and genera (> 0.1% of taxa) in decreasing order of abundance at various production phases (excluding suckling) when either the tylosin or chlortetracycline group was significantly different (P < 0.05) from the control (n = 6).

**Table S3.** Differentially abundant OTUs between the tylosin-supplemented pigs and the control group (n = 24).

**Table S4.** Diversity and abundance estimates at each sampling time (± SD of the mean).

**Table S5.** Effect of sampling time on the relative proportion (percentage ± SD of the mean) of the most abundant phyla and genera (> 1% of taxa) in decreasing order.

**Table S6.** Differentially abundant OTUs between suckling and weanling piglets (n = 18).