Veterinary Microbiology 237 (2019) 108386

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Shifts in the nasal microbiota of swine in response to different dosing regimens of oxytetracycline administration

Kathy T. Mou[a,b], Heather K. Allen[b], David P. Alt[b], Julian Trachsel[b], Samantha J. Hau[a,b], Johann F. Coetzee[c], Devin B. Holman[d], Steven Kellner[b], Crystal L. Loving[b], Susan L. Brockmeier[b,*]

[a] Oak Ridge Institute for Science and Education, P.O. Box 117, Oak Ridge, TN, 37831, USA
[b] USDA, ARS, National Animal Disease Center, 1920 Dayton Ave., Ames, IA, 50010, USA
[c] Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, 228 Coles Hall, Manhattan, KS, 66506, USA
[d] Lacombe Research and Development Centre, Agriculture and Agri-Food Canada, 6000 C and E Trail, Lacombe, Alberta T4L 1W1, Canada

ARTICLE INFO

Keywords:
Swine
Nasal
Tonsil
Respiratory tract
Oxytetracycline
Post-weaning
Microbiota

ABSTRACT

The impacts of antibiotic treatment and dosing regimen of an antibiotic on the swine respiratory microbiota are poorly defined. To begin to address this, this study characterized the impact of oxytetracycline administration, given either parenterally or in feed, on the diversity of the nasal and tonsil microbiotas of post-weaned pigs over a two-week period. One group received a single intramuscular injection (IM) of oxytetracycline, the second was treated with oxytetracycline mixed in feed (IF), and the control group received non-medicated (NON) feed. Nasal samples were collected on days 0 (before start of treatment), 4, 7, 11, and 14. Tonsil tissue samples were collected from a subset of pigs selected for necropsy on days 4, 7, and 14. The results showed that the tonsil microbiota was stable regardless of antibiotic treatment. In contrast, the nasal bacterial diversity decreased for both oxytetracycline-treated groups compared to NON. The IF group also exhibited decreased diversity on more days than the IM group. The nasal bacterial community structures of the antibiotic treatment groups were significantly different from the NON group that persisted from day 4 until day 7 for the IM group, and up until day 11 for the IF group. This included relative increased abundances of *Actinobacillus* and *Streptococcus,* and relative decreased abundances of multiple commensal genera. The microbiota of the IF group was also more disturbed than the microbiota of the IM group, relative to NON. This study revealed that short-term exposure to broad-spectrum antibiotics like oxytetracycline can disturb the upper respiratory microbiota, and the dosing regimen has differential effects on the microbiota.

1. Introduction

In swine, antibiotics may be delivered by different dosing regimens for prophylaxis, metaphylaxis, or treatment of diseases. Studies have investigated collateral effects of antibiotics on the native gut microbiota, ranging from impaired "colonization resistance" against invading pathogens or opportunistic pathogens, decreased functional diversity of the microbiota, or general disruption of the homeostatic and complex network of the microbiota, all of which can increase the host's susceptibility to gastrointestinal diseases (Gresse et al., 2017; Holmes et al., 2012; Looft and Allen, 2012).

Although the focus of antibiotic effects on the microbiota of swine have primarily been on the gastrointestinal tract, the respiratory tract and its microbiota are also important areas of study as there is increasing evidence that the respiratory microbiota shapes local immunity and maintains respiratory health (Man et al., 2017). However, the effects of antibiotics used prophylactically or for the treatment of numerous economically significant respiratory diseases in the swine industry may have detrimental effects on the native microbial population (Karriker et al., 2012; Opriessnig et al., 2011; Post, 2012; USDA–APHIS–VS–CEAH–NAHMS, 2015). Dysbiosis of the microbiota as a result of antibiotic therapy has been shown to increase the risk of a diverse range of infections in swine and in humans (Gresse et al., 2017; Iizumi et al., 2017; Looft and Allen, 2012; Malik et al., 2018). In addition, a small number of studies have found different modes of antibiotic delivery can significantly influence the gut microbiota (Zhang et al., 2013) and respiratory microbiota (Zhao et al., 2014). Therefore, antibiotic stewardship is of the utmost importance in order to improve animal health outcomes while also preventing the selection of antimicrobial resistance.

* Corresponding author at: P.O. Box 70, 1920 Dayton Ave., Ames, IA 50010, USA.
E-mail address: susan.brockmeier@ars.usda.gov (S.L. Brockmeier).

https://doi.org/10.1016/j.vetmic.2019.108386
Received 6 May 2019; Received in revised form 26 July 2019; Accepted 7 August 2019
0378-1135/ Published by Elsevier B.V.

The impact of antibiotic treatment and dosing regimen of an antibiotic on the swine respiratory microbiota has been infrequently investigated. Presently, two studies have reported antibiotic-dependent shifts in the swine nasal microbiota composition (Correa-Fiz et al., 2019; Zeineldin et al., 2018). The nasal microbiota of animals treated with specific antibiotics, including oxytetracycline, recovered and returned to a composition similar to that observed at the start of the study (Zeineldin et al., 2018). Other antibiotics caused a continued deviation in composition from what was found at the start of the study through to the last sampling (Zeineldin et al., 2018). In another study, increased nasal bacterial diversity was observed after the removal of perinatal antimicrobial treatment, including increased relative abundances of beneficial bacteria, improved piglet health, and higher productivity in the nursery phase (Correa-Fiz et al., 2019). Another study showed a dramatic change in the piglet tonsil microbiota post-weaning, but could not attribute the degree to which the changes were due to in-feed antibiotic, diet, or the environment (Pena Cortes et al., 2018). Other reports have identified changes in diversity, with a number of bacterial taxa from respiratory microbial communities associated with specific phases of growth or influenced by management practices (Kernaghan et al., 2012; Lowe et al., 2011, 2012; Mann et al., 2015; Slifierz et al., 2015; Yue et al., 2011), or correlated with the presence of a specific pathogen (Correa-Fiz et al., 2016; Espinosa-Gongora et al., 2015, 2016; Siqueira et al., 2017; Weese et al., 2014).

The present study evaluated the effects of different dosing regimens of the antibiotic oxytetracycline on the respiratory (nasal and tonsil) microbiota in post-weaning pigs. Oxytetracycline is a broad-spectrum antibiotic used within the U.S. swine industry for the labeled therapeutic treatment of a number of swine bacterial diseases, including respiratory diseases caused by pathogens such as *Mycoplasma*, *Pasteurella*, and *Glässerella* in swine (Carlson and Fangman, 2000; National Center for Biotechnology Information, 2019). This tetracycline-family antibiotic was chosen because it can be administered to pigs via intramuscular injection (IM) or in feed (IF) (Karriker et al., 2012), enabling the evaluation of potential differential effects of dosing regimens on the respiratory microbial community. Results from this study revealed the nasal microbiota shifted in response to both dosing regimens of oxytetracycline administration, but the tonsil microbiota was stable regardless of which dosing regimen. In addition, in-feed oxytetracycline had a greater impact on the nasal microbiota than injected oxytetracycline, with changes in abundances of notable respiratory genera. This included increased abundances of *Actinobacillus* and *Streptococcus* and decreased abundances of known respiratory commensal genera.

2. Materials and methods

2.1. Animal study design

Animal studies were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The animal experiments were reviewed and approved by USDA-National Animal Disease Center Animal Care and Use Committee. A total of 65 piglets were farrowed on-site for the study and weaned at two weeks of age. Between two and three weeks of age, animals were fed creep feed, which would be the same feed given to all animals during the study as appropriate to the specific treatment group. Three weeks after birth, piglets were randomly assigned to one of three rooms with each room representing a different treatment. Each room contained two pens that held 10–11 pigs per pen. Treatments were started on day 0 and the entire study period lasted for 14 days. Treatments were administered in the following manner based on the recommended therapeutic approach for treating swine respiratory diseases. One group consisted of 22 piglets that were given a formulated diet with the addition of oxytetracycline (Terramycin® 200, Phibro) at the labeled dose of 2.5 lb of *Terramycin* 200 per ton of feed for 7 days, which delivered oxytetracycline at approximately 10 mg/lb of body weight (in-feed or IF group). Animals in the IF group were then switched to antibiotic-free feed for the remainder of the study. A second group of 21 piglets was given a single intramuscular injection (IM) of oxytetracycline (Liquamycin® LA-200 ®, Zoetis) on day 0 at the recommended dose of 9 mg/lb based on the average group body weight (IM group). Animals in the IM group were fed antibiotic-free feed for the entire study. The third group of 22 piglets served as a control group and was given only non-medicated feed (NON group). Weights of the animals were taken on day 0 before treatments were administered, and on day of necropsy.

2.2. Tonsil tissue, nasal wash, and nasal swab sample collection

On days 4, 7, and 14, seven pigs from each group were euthanized and tonsil tissue was collected. Following similar tonsil tissue collection methodology as described previously (Lowe et al., 2012), a quarter of the tonsil of the soft palate was excised and minced on a sterile petri dish and stored at −80 °C until time for DNA extraction. Nasal wash samples were obtained from all pigs on day 0 (before start of treatment), and all remaining pigs on days 4, 7, 11, and 14. Five milliliters of 1X PBS was injected into the nostril with a needleless 5 ml syringe and effluent PBS was collected in a sterile flask. After the washes were collected, nasal passages were swabbed by inserting FLOQSwabs (Copan Flock Technologies, Brescia, Italy) into each nostril. Swabs were stored in the collected nasal washes and, once at the lab, swab and nasal wash were vortexed together before discarding the swab. The nasal wash sample was then centrifuged at 10,000xg and the supernatant was removed. The pellet was resuspended in 500 µl PBS and then stored at −80 °C until DNA extraction. The nasal cavity was sampled with both a nasal wash and swab to maximize sampling of the nasal microbiota.

A total of 220 nasal wash samples were collected by the end of the study. Sixty-five samples were collected on day 0, 65 samples on day 4, 44 on day 7, 23 on day 11, and 23 on day 14. A total of 65 tonsil tissue samples were collected, with 21 on each of days 4 and 7, and 23 on day 14.

2.3. HPLC/MS analysis of oxytetracycline concentrations in nasal wash samples

Nasal wash concentrations of oxytetracycline in collected samples were analysed at Iowa State University Veterinary Diagnostic Laboratory and the Iowa State University-Pharmacology Analytical Support Team (ISU-PhAST). Oxytetracycline concentrations were determined using high-pressure liquid chromatography (Agilent 1100 Pump, Column Compartment and Autosampler, Agilent Technologies, Santa Clara, CA) with mass spectrometry detection (LTQ Ion Trap, Thermo Scientific, San Jose, CA). Nasal wash samples, nasal wash spikes, nasal wash QC's, and blanks, 100 µl, were added with demeclocycline (at a concentration of 20 ng/mL) in 1.5 ml microcentrifuge tubes. The samples were vortexed for 5 s and centrifuged for 20 min at 2400 rpm prior to LC–MS analysis.

−For LC-MS analysis the injection volume was set to 15 µl. The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile at a flow rate of 0.275 ml/min. The mobile phase began at 5% B with a linear gradient to 95% B in 5.50 min, which was maintained for 1.75 min, followed by re-equilibration to 5% B. Separation was achieved with a HypersilGoldC18 column, 50 mm x 2.1 mm, 1.9 µm particles (Thermo Scientific, San Jose, CA) maintained at 50 °C. Oxytetracycline and demeclocycline eluted at 3.43 and 3.82 min, respectively. Full scan MS with wideband activation was used for analyte detection and three fragment ions were used for quantitation of each analyte species. The fragment ions for oxytetracycline were at 398, 408, and 426 m/z, while ions at 289, 430, and 431 m/z were characteristic of demeclocycline fragmentation. Sequences consisting of nasal wash blanks, calibration spikes, QC's, and

nasal wash samples were batch processed with a processing method developed in the Xcalibur software (Thermo Scientific, San Jose, CA). The processing method automatically identified and integrated each peak in each sample and calculated the calibration curve based on a weighted (1/X) linear fit. Nasal concentrations of oxytetracycline in unknown samples were calculated by the Xcalibur software based on the calibration curve. Results were then viewed in the Quan Browser portion of the Xcalibur software. Twelve calibration spikes were prepared in blank porcine nasal wash covering the concentration range of 0.1–400 ng/ml. Calibration curves exhibited a correlation coefficient (r^2) averaging 0.994. The limit of quantitation (LOQ) of the analysis was 1 ng/ml with a limit of detection (LOD) of 0.2 ng/ml.

Differences in nasal oxytetracycline concentrations between the NON group and either the IF or IM group was assessed using two-way analysis of variance (ANOVA) with repeated measures in GraphPad Prism (La Jolla, CA, USA). A *p*-value of 0.05 or less was considered significant.

2.4. DNA extraction, amplification, and sequencing

From the nasal and tonsil samples, bacterial DNA was extracted using the PowerMag Microbiome DNA/RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) on the Biomek FX^P Laboratory Automated Workstation (Beckman Coulter, Indianapolis, IN) following manufacturer's instructions into three 96-well plates. Preliminary analysis of extracted DNA from tonsil tissue samples showed a high presence of host DNA that interfered with the 16S rRNA gene amplification and analysis. Therefore, DNA samples were enriched for bacterial DNA using the NEBNext Microbiome DNA Enrichment Kit (New England BioLabs, Inc., Ipswich, MA) following manufacturer's instructions.

After DNA extraction and enrichment, the MiSeq Wet Lab SOP (Kozich et al., 2013) was used to prepare the hypervariable V4 region of 16S rRNA gene sequences for Illumina MiSeq sequencing platform: https://github.com/SchlossLab/MiSeq_WetLab_SOP/blob/master/MiSeq_WetLab_SOP_v4.md. The V4 region was analyzed because primers to the V1-V3 region of 16S rRNA gene amplified swine genomic DNA, which was relatively abundant in the nasal and tonsil DNA samples (data not published). To create a single final library for sequencing, 5 µl of each sample was pooled into a single pooled sample normalized to 0.4 nM. The pooled sample was submitted to the Genomics Facility in the Infectious Bacterial Diseases Research Unit at USDA-ARS-NADC in Ames, IA for preparation of 250bp paired-end sequencing on the MiSeq instrument (Illumina, San Diego, CA) using version 2 chemistry.

2.5. Sequence processing and taxonomy assignment

Following sequencing, raw fastq data were retrieved from the MiSeq platform and processed using mothur (version 1.39.5) and the MiSeq SOP as previously described (Kozich et al., 2013). Paired-end reads were aligned and poor-quality reads were discarded, including sequences that did not align with the V4 region of the 16S rRNA gene, were longer than 275bp, contained any ambiguous bases, or had homopolymeric tracts longer than 6bp. Chimeras were detected and removed using the VSEARCH algorithm within mothur. Singleton sequences, as well as sequences from chloroplasts, mitochondria, archaea, eukaryotes, and unknowns were removed. Sequences likely due to sequencing errors were also removed with the pre.cluster command to merge sequences that were within 2 mismatches of the sequence being considered. Singletons and samples with coverage less than 2000 sequences were removed to normalize data, which reduced the total sample size to 207 nasal samples and 35 tonsil samples (Table 1).

Sequences were assigned taxonomies at the genus level using classify.seqs with the default cutoff value of 80 and the SILVA database release 128 (https://www.arb-silva.de/) (Glöckner et al., 2017). Sequences were then clustered into operational taxonomic units (OTUs) at a dissimilarity level of 3% (0.03). This yielded a total of 420 OTUs in tonsil samples and 1587 OTUs in nasal samples. To normalize the number of sequences per sample for subsequent analyses, all samples were subsampled to 2000 sequences to account for uneven sequencing depth among samples (in particular the lower number of tonsil sequences compared to nasal sequences) and continuity of sampling.

Table 1
Number of nasal (A) and tonsil (B) samples from each treatment group (control (NON), in-feed (IF), injected (IM)) and time point used in subsequent analysis.

A.

	Day 0	Day 4	Day 7	Day 11	Day 14
NON	18	22	15	8	8
IF	19	20	14	7	7
IM	19	22	15	7	6

B.

	Day 4	Day 7	Day 14
NON	5	3	2
IF	5	3	3
IM	4	5	5

2.6. Nasal and tonsil microbiota data and statistical analyses

Data analysis and figure generation were done using R version 3.4.0, including the packages vegan, phyloseq, philentropy, tidyverse, and splitstackshape for various alpha- and beta-diversity measures of the samples from each treatment group (IF, IM, NON), day, and site (nasal, tonsil). Alpha-diversity metrics, including Shannon diversity index, were calculated to measure bacterial diversity of each treatment group within each sampled day and site. The wilcoxon rank sum test was used on pairwise comparisons of treatment groups' diversity indices to assess for any significant differences in diversity between treatment groups on a given day ($P < 0.05$).

Bray-Curtis dissimilarity was used to assess the effect of oxytetracycline treatment on the bacterial community structure within each sampled day and site. Permutational Multivariate Analysis of Variance using Distance Matrices (PERMANOVA) (adonis function) from the R vegan package (Oksanen et al., 2007) was used to determine the effect of time and treatment on the structure of the nasal and tonsil microbiota. The PERMANOVA included the effects of "day", "treatment," and "treatment x day" interaction with a significance threshold of $P < 0.05$ (9999 permutations).

For non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities, a wrapper function for vegan's metaMDS () was used. This function is available as part of the funfuns R package (https://github.com/Jtrachsel/funfuns) and generates a data frame containing NMDS coordinates for each sample, and centroids and standard error confidence ellipses for each group.

PERMANOVA pairwise comparisons of treatment groups using the pairwise.adonis function (R vegan package) function (Martinez Arbizu, 2019) were conducted to identify any significant differences in bacterial composition between groups on a given day for each site. Significance was set at $P < 0.05$.

To identify genera that were differentially abundant between groups, DESeq2 package version 1.16.1 (Anders and Huber, 2010; Love et al., 2014) was used. These contrasts were performed on OTU count data that was combined at the genus-level (i.e all OTUs classified as the same genus were combined) with Cook's cutoff outlier detection disabled. Contrasts were corrected for multiple comparisons using the Benjamini-Hochberg adjustment to limit the false discovery rate. The significance of each contrast's log2 fold change value was determined using Wald test, and contrasts with adjusted *p*-values ≤ 0.05 were considered significant. The null hypothesis was that abundance was not different between treatment groups on a given day in a specific tissue.

Differentially abundant genera that appeared for more than one consecutive day, were not differentially abundant in all three groups on day 0, and had relative abundances of over 2% at the genus-level were examined further. For less abundant genera displaying differential abundance, only genera from either group in the pairwise comparison that had at least 100 reads on at least one of the sampling days were considered informative.

2.7. Detection of Streptococcus suis in swine nasal samples using PCR

PCR was used to confirm the presence or absence of *S. suis*-specific DNA in nasal DNA samples from pigs of each treatment group using primers targeting the *gdh* gene and corresponding reaction conditions that have been described previously (MacInnes et al., 2008).

2.8. Data and code availability

Raw sequence data were deposited as FASTQ files in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) (Bioproject PRJNA518919). Code for data analysis is available at https://github.com/USDA-ARS-FSEPRU/FS1b

3. Results

3.1. Effect of oxytetracycline administration on the structure of the nasal and tonsil microbiota

To examine whether the nasal bacterial community structure was affected by oxytetracycline treatment and the type of dose regimen of oxytetracycline given within a given day, the PERMANOVA pairwise comparisons test (Martinez Arbizu, 2019) was performed using the treatment-by-day variable (Fig. 1). In addition, the PERMANOVA *F*-statistic was plotted against time (days 0–14) for visual comparison, with control set as the zero baseline (Fig. 2, Table 2).

On day 0, the IM group was given a single injection of oxytetracycline, and the nasal oxytetracycline levels in the IM group were not significantly different from the NON group on any of the days observed (Fig. 3). However, by days 4 and 7, the injected antibiotic led to changes in the IM group's nasal community that was significantly different from both the IF and the NON groups ($P < 0.05$, $0.10 < R^2 < 0.26$) (Fig. 1). Closer examination of the magnitude of change in IM group's nasal bacterial community structure relative to the NON group revealed increasing magnitude of changes in the IM group up until day 7 (Fig. 2). As for the IF group, its nasal oxytetracycline levels were significantly higher than the NON and IM groups on days 4 and 7 (Fig. 3). There were also significant differences in the IF group's nasal community relative to IM and NON groups on days 4 and 7 ($P < 0.05$, $0.16 < R^2 < 0.31$) (Fig. 1), and increasing magnitude of change in the IF nasal bacterial community structure relative to the NON group (Fig. 2).

By day 11, in-feed treatment had stopped and the nasal oxytetracycline levels were not significantly different among all three treatment groups (Fig. 3). However, the IF group's nasal bacterial community structure remained significantly different from the groups IM and NON ($P < 0.05$, $R^2 = 0.62$). The changes in the nasal bacterial community structure of the IF group relative to the NON group on day 11 (*F*-statistic) were also the largest observed on all the days sampled (Fig. 2). The IM group, on the other hand, had no significant differences in the nasal bacterial community structure compared to the NON group (Figs. 1 and 2).

On day 14, the IM group's bacterial community structure relative to NON group remained similar (Figs. 1 and 2). Although the IF group's bacterial community structure returned to a state that approached that of the NON group, the community structure of the IF group relative to the NON group approached significant difference ($P = 0.071$, $R^2 = 0.15$; Fig. 2). In addition, after day 0, the degree of change of the IF group's nasal bacterial community structure (*F*-statistic) was consistently larger than IM group up until the end of the study, with the

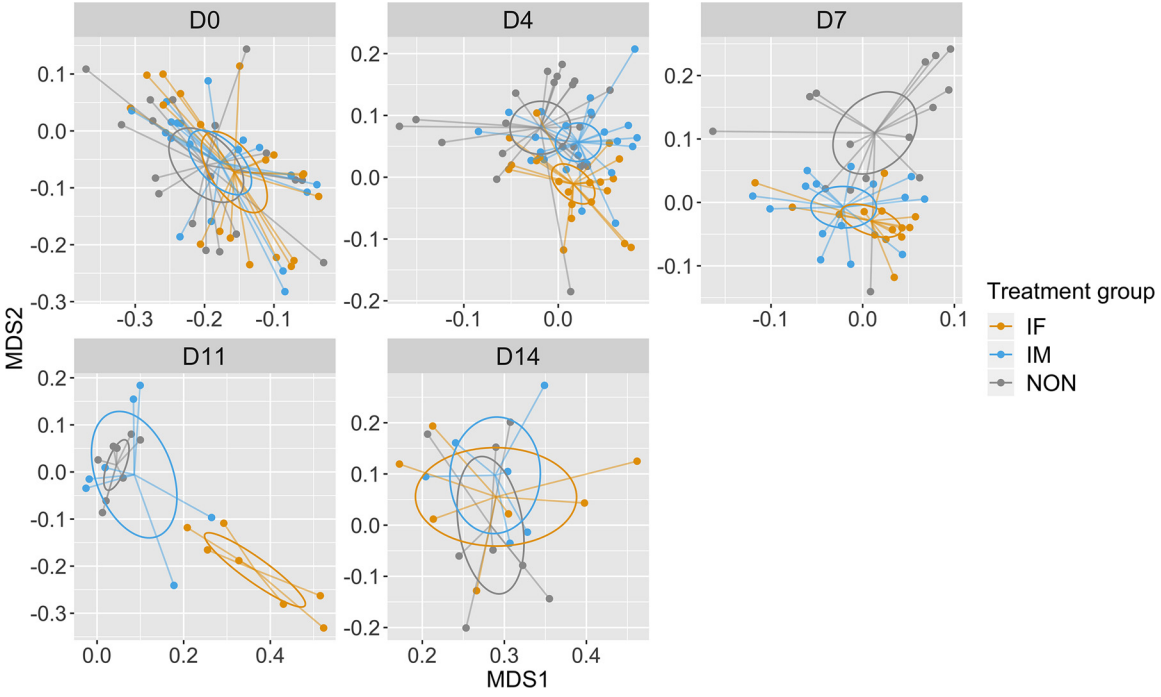

Fig. 1. Nasal bacterial community structure in IF (orange) and IM (blue) groups compared to NON (gray) group over a 14-day period. Non-metric multidimensional scaling (NMDS) ordinations of nasal 16S rRNA gene-based Bray-Curtis dissimilarities calculated from rarefied OTU abundance data (clustered at 97% similarity). Each point represents a sample. Closer distances between samples indicate more similarity in microbial compositions between samples. Samples are linked to the treatment group centroid by segments and the standard error of the treatment group is depicted with an ellipse. The top of each panel refers to the day sampled (D0 = day 0, D4 = day 4, D7 = day 7, D11 = day 11, D14 = day 14). Within each day are the three treatment groups: NON (control) = gray, IF (in-feed) = **orange,**IM (injected) = blue.

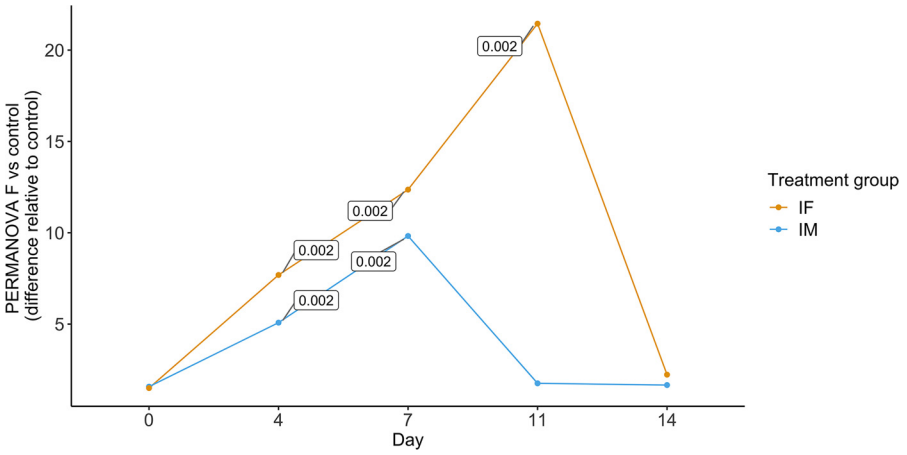

Fig. 2. PERMANOVA pseudo F statistic over the 14-day period of the nasal bacterial community analyses of IF (orange) and IM (blue) groups relative to NON group. Each point represents the PERMANOVA test statistic (F: intergroup dissimilarity/intragroup dissimilarity) of each antibiotic treatment group over time (days 0 to 14), with NON group (control) set as the zero baseline. Only where bacterial community structure of the treatment group is significantly different from NON group are *p*-values shown. IF (in-feed) = **orange,**IM (injected) = blue.

largest difference of *F*-statistics between the two antibiotic groups on day 11.

In summary, the nasal bacterial community structure changed significantly in response to both dose regimens of oxytetracycline over the course of the study. In addition, the nasal microbiota of the IF group continued to change even after oxytetracycline administration had stopped and oxytetracycline levels were not significantly different from the levels in the NON group. This is an indication that the transition of the IF group's nasal microbiota to a state similar to the non-medicated group requires a longer time period when exposed to in-feed oxytetracycline as opposed to injected oxytetracycline. As for tonsil samples, PERMANOVA showed no significant differences among all three groups on any of the days sampled (Table 2).

To further understand the observed shifts in the nasal bacterial community structure among treatment groups over time, the variables treatment, day, and treatment-by-day were tested with the adonis function (Oksanen et al., 2007). All three variables had significant effects ($P < 0.001$) on the variance observed in the nasal communities. Time (day) had a larger effect ($R^2 = 0.27$) than treatment ($R^2 = 0.04$) and there was also a significant treatment x day interaction ($R^2 = 0.11$). This indicated that the 'day' variable was the strongest contributor to the variation observed in the nasal bacterial community structure, followed by 'treatment per day' and, lastly, 'treatment'. None of these variables had significantly affected the tonsil microbial community structure (data not shown). No differences in weight gain were observed between the treatment groups as well (data not shown).

3.2. Effect of oxytetracycline dosing regimen on alpha diversity measures

Underlying the differences in beta-diversity described above, analyses of the alpha diversity of the nasal microbiota showed clear differences in bacterial diversity (Fig. 4) and membership (Fig. 5A) among antibiotic-treated groups and NON. The nasal microbiota of the three treatment groups had similar Shannon diversity index values on day 0 (means ranging between 2.4–2.6; Fig. 4). This was also evident when looking at the percent relative abundances of genera found in each treatment group on day 0 (Fig. 5A). However, there were significant differences in diversity on days 4, 7, and 11 ($p < 0.05$). Specifically, on day 4, the nasal microbiota of the IF group showed significantly lower diversity than that from the NON group. This day was also when nasal oxytetracycline levels in the IF group were significantly higher than both the IM and NON groups (Fig. 3).

On day 7, the nasal microbiota from both the IM and IF groups had significantly lower diversity than that from the NON group. The decreased diversity in the antibiotic-treated groups compared to the NON group could also be attributed to the higher relative abundance of *Actinobacillus* and lower relative abundance of other genera in both antibiotic-treated groups (Fig. 5A).

On day 11, diversity of IF was still significantly lower than NON even after oxytetracycline levels in the IF group were no longer significantly higher than the levels measured in the NON and IM groups. In addition, IM showed significantly higher diversity than IF on the same day. This is evident by the larger proportion of *Pseudomonas* and less relative abundance of other genera in the nasal microbiota of the IF group relative to that of the NON and IM groups (Fig. 5A). By day 14, the diversity of all three groups were no longer significantly different from one another.

In general, both dosing regimens of oxytetracycline led to decreased nasal bacterial diversity compared to control during the course of treatment and after treatment had ended. The nasal microbiota of the IF group was also less diverse than that of the NON group on more days than IM was to NON. However, by the end of the study, the diversity of the antibiotic-treated groups had returned to levels comparable with NON. Tonsil bacterial diversity and membership were relatively stable among all three groups regardless of which oxytetracycline dosing regimen (Fig. 5B).

Table 2
PERMANOVA tests of associations of nasal and tonsil bacterial community structures between control group and either in-feed (IF) or injected (IM) oxytetracycline treatment group on a given day.

	Nasal						Tonsil					
	IF			IM			IF			IM		
Day	F model	R^2	p-value	F model	R^2	p-value	F model	R^2	p-value	F model	R^2	p-value
0	1.4984	0.0411	0.192	1.5758	0.0431	0.192	NA	–	–	–	–	–
4	7.6890	0.1612	0.003	5.0797	0.1079	0.003	0.7373	0.0844	0.832	0.9141	0.1155	0.832
7	12.3623	0.3141	0.003	9.8223	0.2597	0.003	1.1542	0.2239	0.832	1.9651	0.2467	0.832
11	21.4453	0.6226	0.003	1.7583	0.1191	0.071	NA	–	–	–	–	–
14	2.2313	0.1465	0.071	1.6627	0.1217	0.161	1.0427	0.2579	0.832	0.9257	0.1562	0.832

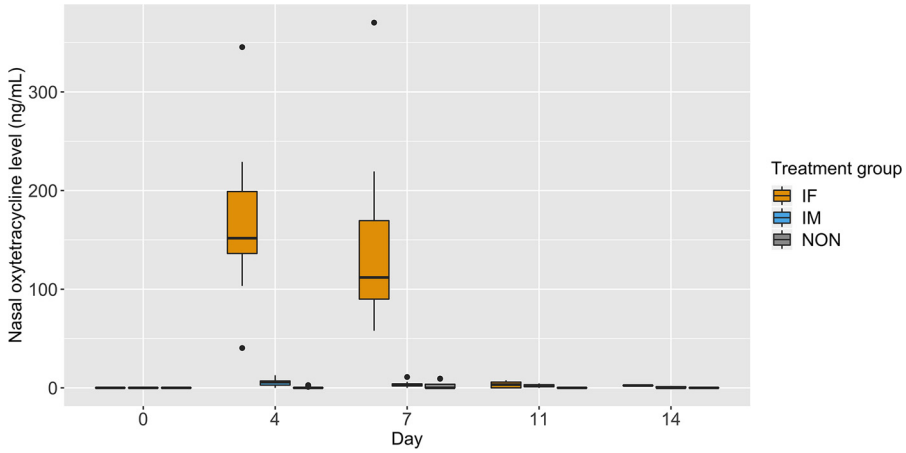

Fig. 3. Oxytetracycline levels detected in nasal wash samples over the 14-day period for each treatment group. The treatment groups are: NON (control) = gray, IF (in-feed) = **orange,**IM (injected) = blue.

3.3. Differentially abundant genera at the nasal site in response to dosing regimen of oxytetracycline

Several members of the swine nasal microbiota showed significant differences in relative abundance in the IF group compared to the NON group (Fig. 6A). Typical swine nasal genus *Lactobacillus* had relatively decreased abundances in the nasal microbiota of the IF group compared to that of the NON group on days 4 and 7 (Fig. 6A). *Lachnospiraceae_unclassified* increased in relative abundance on days 4 and 7, but then decreased on day 11 compared to NON (Fig. 6A). In contrast, *Acinetobacter*, showed a decreased relative abundance on day 7 and then was significantly more abundant than NON on day 11 (Fig. 6A). *Blautia* had significantly decreased relative abundance in the IF group compared to the NON group on days 4, 7, and 11 (Fig. 6A).

The oxytetracycline treatment also seemed to affect the relative abundance of two important respiratory genera in the nasal microbiota: *Actinobacillus* and *Streptococcus*. These two genera are well known in swine for the pathogenic species within these genera (*Actinobacillus suis, Actinobacillus pleuropneumoniae*, and *Streptococcus suis*). DESeq2 analysis showed a significantly greater relative abundance of *Actinobacillus* in the nasal microbiota of the IF and IM groups on days 4 and 7 compared to that of the NON group (Fig. 6AB). After treatment had ended (days 11 and 14), the relative abundances of both genera were not significantly different between NON and either treatment group. The nasal microbiota of the IM group showed a decreased relative abundance of *Streptococcus* relative to that of the NON group on day 4 (Fig. 6B). However, on day 7, the nasal microbiotas of both the IM and IF groups harbored a significantly higher relative abundance of *Streptococcus* than the NON group (Fig. 6AB). In addition, PCR confirmed the presence of *S. suis* in the nasal samples of pigs from these three groups on days 4 and 7 (data not shown).

In summary, there was a significant decrease in nasal bacterial diversity as well as shifts in the nasal bacterial composition after either oxytetracycline dosing regimen compared to the NON group. These shifts led to significant changes in the relative abundances of several notable genera of interest. Results also showed the in-feed route of oxytetracycline had a significantly higher impact on the nasal microbiota composition than the parenteral route.

4. Discussion

The differential effects of antibiotic administration and dosing regimen of an antibiotic on the swine upper respiratory microbiota have not been well characterized. Recent studies have examined the effects of different parenteral antibiotics on the swine nasal microbiota (Zeineldin et al., 2018) or the effects of the removal of perinatal antimicrobial treatment on the swine nasal microbiota (Correa-Fiz et al., 2019). Other studies have described the nasal or tonsil microbiota from

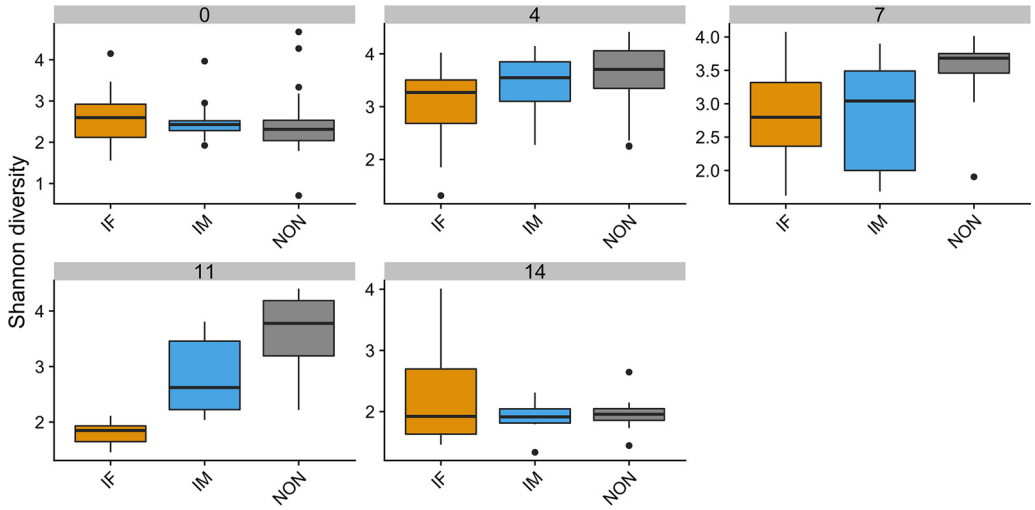

Fig. 4. Observed Shannon diversity of bacteria at the nasal site for each treatment group sampled over the 14-day period. Each panel, from left to right, top to bottom refer to days 0, 4, 7, 11, and 14. Treatment groups are listed below each box plot, and include NON (control) = gray, IF (in-feed) = **orange,**IM (injected) = blue.

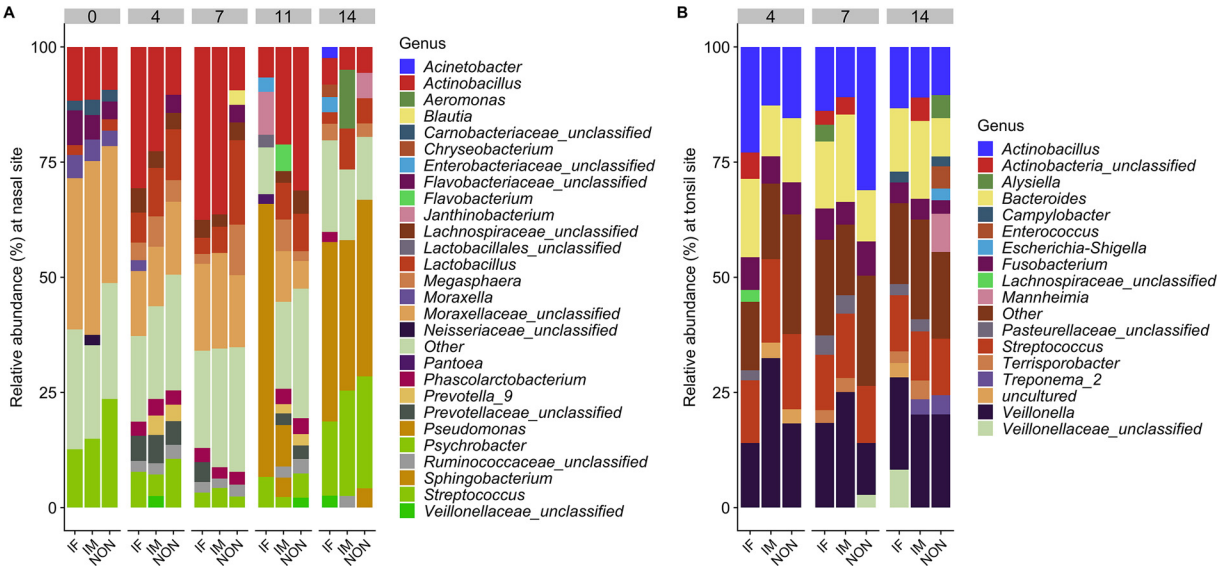

Fig. 5. Relative abundance of bacteria observed at the nasal (A) and tonsil (B) sites over the 14-day period within each treatment group. Within each day (days are listed at the top of the bar plots), each bar plot shows the percentages of total genera found in each treatment group. Each genus is shown with the exception of genera with less than 2% abundance, in which case they were grouped in the "Other" category. The treatment groups are: NON (control) = gray, IF (in-feed) = orange, IM (injected) = blue.

healthy animals of different ages or animals on farms with known disease status (Correa-Fiz et al., 2016; Espinosa-Gongora et al., 2016; Kernaghan et al., 2012; Lowe et al., 2011, 2012; Mann et al., 2015; Pena Cortes et al., 2018; Slifierz et al., 2015; Weese et al., 2014; Yue et al., 2011). This was the first study to compare the impact of two dosing regimens of an antibiotic on the nasal and tonsil microbiota. The results revealed a dynamic nasal bacterial community that was altered by either dosing regimen of oxytetracycline. The alterations included decreased diversity and specific changes in bacterial composition that were significantly different from the nasal microbiota of pigs not given antibiotics. As for the tonsil, regardless of the type of dosing regimen of oxytetracycline, the microbiota did not change significantly in this study as a result of antibiotic treatment.

In-feed oxytetracycline, in particular, was shown to have greater impact on the nasal microbiota than injected oxytetracycline when compared with the NON group. The magnitude of change in bacterial community structure as a result of in-feed antibiotic administration was much higher than injected oxytetracycline from days 4 to 11. In terms of the length of impact, the nasal bacterial community structure of the IF group was significantly different from the NON group up until day 11. By day 14, the measure of difference in the nasal bacterial community structure between IF group and NON group trended towards, but did not reach, significance ($P = 0.071$). The bacterial community structure of the IM group was significantly different from NON up until day 7, but not through day 11, suggesting the nasal microbiota can recover to a non-medicated state quicker when exposed to intramuscular administration of oxytetracycline than to in-feed administration. Zeineldin et al. also found that parenteral oxytetracycline, unlike some of the other parenteral antibiotics tested, did not have a lasting effect on the nasal microbiota composition of older-aged animals where the microbiota is more stable (Zeineldin et al., 2018). The effects of these other parenteral antibiotics on the nasal microbiota persisted until the last sampling day (day 14) (Zeineldin et al., 2018). This suggests that the type of dosing regimen of an antibiotic could impact the nasal microbiota for a longer amount of time even after treatment had stopped (e.g. in-feed oxytetracycline as observed in our study, or the specific antibiotics used as observed by Zeineldin et al.).

In-feed oxytetracycline administration had a greater impact on the nasal bacterial diversity than did intramuscular administration, resulting in lower nasal bacterial diversity in the IF group compared to the NON group. This is expected as the rooting behavior of pigs increased exposure of the nasal cavity to the feed containing oxytetracycline in contrast to parenteral oxytetracycline. This increased exposure to in-feed oxytetracycline was evident based on the significantly

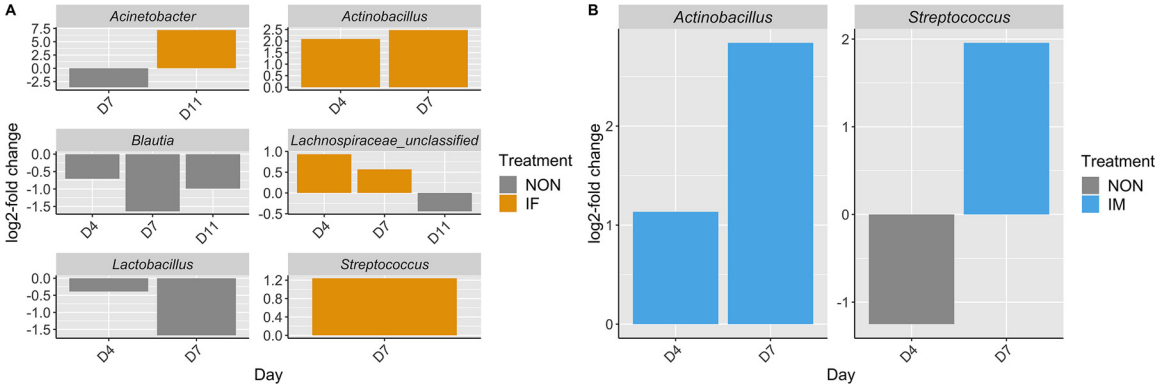

Fig. 6. Select genera with significant differential abundances in the nasal cavity in the IF relative to NON (A) and IM relative to NON (B). Differentially abundant genera between groups were determined using the DESeq2 package. The x-axis represents the day (D4 = day 4, D7 = day 7, etc.), y-axis displays the $\log_2$-fold changes of the genera, and corresponding genus is listed above each panel. NON (control) = gray, IF (in-feed) = **orange,**IM (injected) = blue.

higher levels of oxytetracycline that were detected in the nasal wash samples of the pigs from the IF group compared to the NON and IM groups. Low nasal bacterial diversity resulting from antibiotic treatment has also been observed in a study comparing pigs fed a liquid diet containing antibiotics against pigs fed conventional solid feed that were not given antibiotics (Weese et al., 2014). Additionally, lower nasal bacterial diversity in pigs has been associated with outbreaks of Glässer's disease (Correa-Fiz et al., 2016). Further research is needed to evaluate whether decreased nasal bacterial diversity in swine due to antibiotic treatment is a potential risk factor for respiratory diseases.

A closer examination of the overall changes in the nasal bacterial membership by either dosing regimen of oxytetracycline revealed changes in relative abundances of specific genera that contribute to the porcine respiratory disease complex. This study observed significantly higher abundances of the genera *Streptococcus* and *Actinobacillus* in the nasal microbiota of the IF and IM groups as compared to the NON group. Increased relative abundances of these genera has also been seen in the nasal microbiota of pigs from farms experiencing Glässer's disease outbreaks when compared with farms that have no history of Glässer's disease (Correa-Fiz et al., 2016). Interestingly, the removal of antimicrobial treatment, as demonstrated in a recent study, resulted in decreased relative abundance of *Streptococcus* (Correa-Fiz et al., 2019). While *Streptococcus* species are common respiratory commensal organisms, this genus also harbors an important swine respiratory pathogen, *S. suis* (Gottschalk, 2012). The presence of *S. suis* in the nasal samples and the observed relatively higher abundances of *Streptococcus* and *Actinobacillus* genera after antibiotic administration signal the need for further investigations to examine what consequences antibiotic perturbation of the nasal microbiota can have on these notable respiratory organisms, characterizing these organisms at the strain level, and how they can impact the respiratory health of the pig.

We also identified a decrease in the relative abundance of genera in the IF group relative to NON that were previously associated with the microbiota of healthy animals (Correa-Fiz et al., 2016; Slifierz et al., 2015). *Lactobacillus* and *Blautia* were significantly relatively less abundant in IF compared to NON. Both genera are core members of the nasal microbiota in post-weaned pigs (Slifierz et al., 2015). On the other hand, we observed higher relative abundances of *Lachnospiraceae_unclassified* in the IF group compared to NON during antibiotic administration. Interestingly, *Lachnospiraceae* family and *Lactobacillus* showed relatively higher levels in farms with no history of disease than in farms with Glässer's disease (Correa-Fiz et al., 2016). The removal of perinatal antimicrobial treatment also showed a relatively higher abundance of *Lactobacillus* in the piglet nasal microbiota (Correa-Fiz et al., 2019). The changing abundances of these healthy farm-associated genera after oxytetracycline treatment require further studies to assess whether there are potential detrimental effects to the host's microbiota development and increased susceptibility to diseases.

The abundances of some genera affected by oxytetracycline treatment have also been previously correlated with the carriage status of *Staphylococcus aureus* or methicillin-resistant *S. aureus* (MRSA) in pigs (Espinosa-Gongora et al., 2016). In our study, we found decreased relative abundance of the genus *Acinetobacter* in the IF group compared to the NON group. A similar trend was identified in *S. aureus* carrier pigs, which showed lower abundances of these genera than in *S. aureus* non-carrier pigs (Espinosa-Gongora et al., 2016). A decreased relative abundance of *Lactobacillus* was also found in the nasal microbiota of the IF group. This genus has been noted as an indicator for MRSA-negative status in pigs (Espinosa-Gongora et al., 2016). Taken together, the relative abundances of *Acinetobacter* and *Lactobacillus* genera associated with in-feed oxytetracycline treatment, and their known correlation with *S. aureus* carriage from previous studies, require further characterization.

The findings from this study help open an unexplored area of what effects broad-spectrum antibiotics like oxytetracycline and the specific dosing regimens of an antibiotic have on the diversity and composition of the upper respiratory tract microbiota. And while this study identified numerous genera affected by dosing regimens of oxytetracycline, caution should be taken when interpreting these results due to experimental limitations of this study. For instance, though sampling of the nasal region in all three treatment groups were performed in the same way, the physical disruption of the nasal mucosa from repeated sampling of the nasal passages could affect the observed nasal microbiota response to oxytetracycline. In addition, the absence of appropriate negative control samples (for example, the PBS used for nasal washes) in the analysis prevent differentiation of background contamination from the antibiotic induced changes and variation observed in the microbiota. With these in mind, additional studies are needed to further assess how oxytetracycline administration affects the roles of these bacterial members in maintaining respiratory health and modulating disease susceptibility.

In conclusion, we discovered that both dosing regimens of oxytetracycline caused a reduction in nasal bacterial diversity and changed the bacterial community structure relative to pigs that were not administered antibiotics. Moreover, the in-feed oxytetracycline resulted in greater changes to the nasal bacterial community than parenteral oxytetracycline. This research raises new questions surrounding the impact of exposure to broad-spectrum antibiotics, like oxytetracycline, and the dosing regimens of such antibiotics on the network of interactions between the upper respiratory tract microbiota and the animal and will contribute to our understanding of how these changes impact animal health and development.

Acknowledgements

We are deeply grateful for the animal caretakers at the NADC, and Jennifer Jones for assistance in preparing samples for sequencing. We thank Cassandra Wattenburger, Damarius S. Fleming, Jennifer Chang, and Darrell Bayles for computational support. We also appreciate helpful discussions with Nicole Ricker and Martha Mulks. This research used resources provided by the SCINet project of the USDA Agricultural Research Service, ARS project number 0500-00093-001-00-D. This research was also supported in part by an appointment to the Agricultural Research Service (ARS) Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy (DOE) and the U.S. Department of Agriculture (USDA). ORISE is managed by ORAU under DOE contract number DE-SC0014664. All opinions expressed in this paper are the author's and do not necessarily reflect the policies and views of USDA, ARS, DOE, or ORAU/ORISE. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S Department of Agriculture. USDA is an equal opportunity provider and employer.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108386.

References

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol. 11, R106.

Carlson, M.S., Fangman, T.J., 2000. Antibiotics and Other Additives for Swine: Food Safety Considerations. University of Missouri Extension.

Correa-Fiz, F., Fraile, L., Aragon, V., 2016. Piglet nasal microbiota at weaning may influence the development of Glässer's disease during the rearing period. BMC Genomics 17, 404.

Correa-Fiz, F., Gonçalves dos Santos, J.M., Illas, F., Aragon, V., 2019. Antimicrobial removal on piglets promotes health and higher bacterial diversity in the nasal microbiota. Sci. Rep. 9, 6545.

Espinosa-Gongora, C., Dahl, J., Elvstrøm, A., van Wamel, W.J., Guardabassi, L., 2015. Individual predisposition to *Staphylococcus aureus* colonization in pigs on the basis of

quantification, carriage dynamics, and serological profiles. Appl. Environ. Microbiol. 81, 1251–1256.
Espinosa-Gongora, C., Larsen, N., Schønning, K., Fredholm, M., Guardabassi, L., 2016. Differential analysis of the nasal microbiome of pig carriers or non-carriers of *Staphylococcus aureus*. PLoS One 11, e0160331.
Glöckner, F.O., Yilmaz, P., Quast, C., Gerken, J., Beccati, A., Ciuprina, A., Bruns, G., Yarza, P., Peplies, J., Westram, R., Ludwig, W., 2017. 25 years of serving the community with ribosomal RNA gene reference databases and tools. J. Biotechnol. 261, 169–176.
Gottschalk, M., 2012. Streptococcosis. In: Zimmerman, J.J., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. John Wiley & Sons, Inc., Ames, IA, pp. 841–855.
Gresse, R., Chaucheyras-Durand, F., Fleury, M.A., Van de Wiele, T., Forano, E., Blanquet-Diot, S., 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends Microbiol. 25, 851–873.
Holmes, E., Li, J.V., Marchesi, J.R., Nicholson, J.K., 2012. Gut microbiota composition and activity in relation to host metabolic phenotype and disease risk. Cell Metab. 16, 559–564.
Iizumi, T., Battaglia, T., Ruiz, V., Perez Perez, G.I., 2017. Gut microbiome and antibiotics. Arch. Med. Res. 48, 727–734.
Karriker, L.A., Coetzee, J., Friendship, R.M., Prescott, J.F., 2012. Drug pharmacology, therapy, and prophylaxis. In: Zimmerman, J.J., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. John Wiley & Sons, Inc., Ames, IA, pp. 106–118.
Kernaghan, S., Bujold, A.R., MacInnes, J.I., 2012. The microbiome of the soft palate of swine. Anim. Health Res. Rev. 13, 110–120.
Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120.
Looft, T., Allen, H.K., 2012. Collateral effects of antibiotics on mammalian gut microbiomes. Gut Microbes 3, 463–467.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.
Lowe, B.A., Marsh, T.L., Isaacs-Cosgrove, N., Kirkwood, R.N., Kiupel, M., Mulks, M.H., 2011. Microbial communities in the tonsils of healthy pigs. Vet. Microbiol. 147, 346–357.
Lowe, B.A., Marsh, T.L., Isaacs-Cosgrove, N., Kirkwood, R.N., Kiupel, M., Mulks, M.H., 2012. Defining the "core microbiome" of the microbial communities in the tonsils of healthy pigs. BMC Microbiol. 12, 20.
MacInnes, J.I., Gottschalk, M., Lone, A.G., Metcalf, D.S., Ojha, S., Rosendal, T., Watson, S.B., Friendship, R.M., 2008. Prevalence of *Actinobacillus pleuropneumoniae, Actinobacillus suis, Haemophilus parasuis, Pasteurella multocida*, and *Streptococcus suis* in representative Ontario swine herds. Can. J. Vet. Res. 72, 242–248.
Malik, U., Armstrong, D., Ashworth, M., Dregan, A., L'Esperance, V., McDonnell, L., Molokhia, M., White, P., 2018. Association between prior antibiotic therapy and subsequent risk of community-acquired infections: a systematic review. J. Antimicrob. Chemother. 73, 287–296.
Man, W.H., de Steenhuijsen Piters, W.A.A., Bogaert, D., 2017. The microbiota of the respiratory tract: gatekeeper to respiratory health. Nat. Rev. Microbiol. 15, 259.
Mann, E., Pinior, B., Wetzels, S.U., Metzler-Zebeli, B.U., Wagner, M., Schmitz-Esser, S., 2015. The metabolically active bacterial microbiome of tonsils and mandibular lymph nodes of slaughter pigs. Front. Microbiol. 6.
Martinez Arbizu, P., 2019. pairwiseAdonis: Pairwise Multilevel Comparison Using Adonis. R package version 0.3.
National Center for Biotechnology Information, 2019. Oxytetracycline (CID = 54675779) (PubChem Compound Database).
Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J., Suggests, M., 2007. The vegan package. Community Ecology Package 631–637.
Opriessnig, T., Giménez-Lirola, L.G., Halbur, P.G., 2011. Polymicrobial respiratory disease in pigs. Anim. Health Res. Rev. 12, 133–148.
Pena Cortes, L.C., LeVeque, R.M., Funk, J., Marsh, T.L., Mulks, M.H., 2018. Development of the tonsillar microbiome in pigs from newborn through weaning. BMC Microbiol. 18, 35.
Post, K.E., 2012. Overview of bacteria. In: Zimmerman, J.J., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. John Wiley & Sons, Inc., Ames, IA, pp. 649–652.
Siqueira, F.M., Pérez-Wohlfeil, E., Carvalho, F.M., Trelles, O., Schrank, I.S., Vasconcelos, A.T.R., Zaha, A., 2017. Microbiome overview in swine lungs. PLoS One 12, e0181503.
Slifierz, M.J., Friendship, R.M., Weese, J.S., 2015. Longitudinal study of the early-life fecal and nasal microbiotas of the domestic pig. BMC Microbiol. 15, 184.
USDA–APHIS–VS–CEAH–NAHMS, 2015. Swine 2012 Part I: Baseline Reference of Swine Health and Management in the United States, 2012. USDA–APHIS–VS–CEAH–NAHMS, ed. .
Weese, J.S., Slifierz, M., Jalali, M., Friendship, R., 2014. Evaluation of the nasal microbiota in slaughter-age pigs and the impact on nasal methicillin-resistant *Staphylococcus aureus* (MRSA) carriage. BMC Vet. Res. 10, 69.
Yue, M., Bei, W., Chen, H., 2011. Nasal Bacterial Microbiome: Probing a Healthy Porcine Family (Nature Precedings).
Zeineldin, M., Aldridge, B., Blair, B., Kancer, K., Lowe, J., 2018. Microbial shifts in the swine nasal microbiota in response to parenteral antimicrobial administration. Microb. Pathog. 121, 210–217.
Zhang, L., Huang, Y., Zhou, Y., Buckley, T., Wang, H.H., 2013. Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota. Antimicrob. Agents Chemother. 57, 3659–3666.
Zhao, J., Murray, S., Lipuma, J.J., 2014. Modeling the impact of antibiotic exposure on human microbiota. Sci. Rep. 4, 4345.