RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Effects of early-life antibiotic administration on porcine respiratory microbiota and piglet immunity – **NPB #18-050**

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Industry Summary:
The impact of antibiotic treatments on the swine respiratory microbiota is poorly understood. It was hypothesized that antibiotic administration to piglets during the lactation period interferes with normal colonization patterns of the respiratory microbiota. It was also hypothesized that an impaired immune response play a role in antibiotic-mediated disturbances of the nasal microbiome. This study aimed, therefore, to characterize the impact of Tulathromycin administration, at either processing or weaning age, on the diversity of the nasal microbiota over a 56-day period (lactation and post-weaning phases). Additionally, this study also sought to determine host genetic variations associated with the immune response in the context of a hypothetical antibiotic-associated dysbiosis. To accomplish with the abovementioned objectives, three groups of piglets were followed up from birth to 56-day old. One group consisted of piglets that did not receive antibiotics. Two other groups received a single intramuscular injection of Tulathromycin at either 4 or 19 days of age, respectively. Nasal swabs were collected from sows post-farrowing and from piglets on days 4 (T1), 12 (T2), 19 (T3), 28 (T4) and 56 (T5) of age. In addition, a blood sample from all piglets was obtained to perform genome-wide association studies (GWAS). The results showed that the nasal microbiota was stable regardless of antibiotic treatment. However, the nasal bacterial diversity significantly increased with age, thus, significant changes in diversity were observed between pre-weaning and post-weaning time points. Although GWAS revealed single-nucleotide polymorphisms (SNPs) related to nasal microbiome diversity, these genetic variations could not be linked to the host immune response. In conclusion, the present results point out that the age and the transition to nursery/finisher sites may exert a greater influence over the nasal microbiome of piglets than early-life antibiotic treatments.

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Scientific Abstract:
The view on the use of antimicrobial in food animals has changed due to the increased knowledge on antibiotic resistances and their effect on the microbiota composition. Still, the impact of antibiotic treatments on the swine respiratory microbiota is poorly understood. To address this later and taking into account results from previous studies, it was hypothesized that antibiotic administration to piglets during the lactation period interferes with normal colonization patterns of the respiratory microbiota. It was also hypothesized that an impaired immune response play a role in antibiotic-mediated disturbances of the nasal microbiome. This study aimed, therefore, to characterize the impact of Tulathromycin administration, at either 4 or 19 days of age, on the diversity of the nasal microbiota over a 56-day period (lactation and post-weaning). Additionally, this study also sought to determine host genetic variations associated with the immune response in the context of a hypothetical antibiotic-associated dysbiosis.

To accomplish the abovementioned objectives, 85 piglets from litters of 10 different sows were randomly allocated to one of the following treatment groups. The control group 1 (n=29) consisted of piglets that did not receive antibiotics. Groups 2 (n=27) and 3 (n=29) received a single intramuscular injection of Tulathromycin, at either 4 or 19 days of age, respectively. Tulathromycin was administered at a dosage of 2.5 mg/kg, following manufacturer's recommendations. Nasal swabs were collected from sows post-farrowing and from piglets on days 4 (T1), 12 (T2), 19 (T3), 28 (T4) and 56 (T5) of age. In addition, a blood sample was obtained from peri-weaned piglets from all groups. NS collected through the study were used to assess nasal bacterial composition by amplifying the V3-V4 regions of 16S rRNA gene. Thus, sequences generated by Illumina MiSeq technology were processed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) workflow and microbiome analyses were conducted by means of R software. Genomic host DNA was separated from total DNA extracted from blood to perform genome-wide association studies (GWAS) among the genotypes of the sampled pigs and their nasal microbiota composition. Genotyping was conducted using 50K GeneSeek® Genomic Profiler porcine beadchip (Neogen Genomics).

Due to mortality across the study period, the number of sampled piglets within treatment groups differed between the assessed time points (T1 to T5). However, mortality was evenly distributed among groups. Overall, the nasal microbiota composition was dominated by Proteobacteria, Firmicutes and Actinobacteria whereas the predominant genera were *Moraxella*, *Actinobacillus* and *Rothia*. Based on diversity and composition analyses, the microbiome was not significantly different between treatment groups at any of the time points analyzed; nasal microbiota was stable regardless of antibiotic treatment. Remarkably, nasal microbiota diversity increased significantly with age, between pre-weaning (T1 to T3) and post-weaning (T4-T5) time points. Regardless to the treatment group, GWAS revealed a total of 4 single-nucleotide polymorphisms (SNPs) associated with the nasal microbiota diversity shortly after birth (T1). When considering the change in microbial composition over time, GWAS detected 2 associated SNPs. However, the candidate genes identified could not be related to piglet's immune response.

The obtained results could not validate the exposed hypothesis as the antibiotic administration to piglets during the lactation period did not interfere with normal colonization patterns of the nasal microbiota. Furthermore, the SNPs identified as possible genetic markers for nasal microbiota diversity did not show an apparent relationship with the host immune response. In conclusion, the present results point out that the age and the transition to nursery/finisher sites may exert a greater influence over the nasal microbiome of piglets than early-life antibiotic treatments.

Introduction:

The relationship and balance between microorganisms within the microbiome in health and disease is complex and remains poorly understood. However, there is growing evidence indicating the important role that microbiome diversity and composition play in regulation, elimination, and potentiation of infectious diseases [1–3]. The association between microbiome and outcome in infectious respiratory diseases in pigs has been studied mainly at gastrointestinal level [2,3]. Nevertheless, there is an increasing evidence that the respiratory microbiota modulates local immunity and sustains respiratory health [4]. In agreement, it has been shown that the microbial communities inhabiting the nasal cavities at weaning may influence the development of Glässer's disease later in life [5]. Importantly, microbial dysbiosis may appear secondarily to the use of antimicrobials and it can facilitate pathogen infections and enhance the tissue damage inflicted by pathogenic bacteria [6].

The use of antimicrobials to control bacterial diseases is a common practice in porcine production, and respiratory diseases represent one of its major usages [7,8]. In this line, the National Animal Health Monitoring System (NAHMS) reported respiratory diseases as the most common reason for using antimicrobials in the United States (US) swine production sites in 2016. Antibiotic administration to piglets during lactation is a regular practice to prevent and treat bacterial infections in newborn piglets [7]. Recent studies suggest an association between this early life administration of antibiotics and long-lasting detrimental effects on the pig's intestinal microbial community and functionality [9–11]. However, studies investigating whether the antibiotic treatment has an effect on the bacterial communities from the piglet's respiratory tract are scarce. To the author's knowledge, three studies have reported so far an antibiotic-dependent shift in the swine nasal microbiota composition [12–14]. These studies, however, assessed the effect of antibiotics after short time periods from administration (2 to 4 weeks). Thus, long-lasting effects of antibiotics in nasal microbiota communities have not been investigated.

Besides, studies have identified the optimal operating conditions in which the immune system-microbiota interactions allow the induction of protective responses to pathogens as well as the maintenance of regulatory pathways and tolerance to harmless antigens [15]. Likewise, the host immune system in turn also plays an essential role in maintaining the mutualistic nature of the host-microbial interactions [16]. Commensal bacteria are thus thought to be critical in shaping mucosal immune responses in both healthy and diseased animals. Moreover, host genetic variations are reported to modulate gut and upper airway microbial communities in humans [17,18]. In pigs, such significant links between microbiota and host inheritable traits have been identified only in the gut [19].

Objectives:

The overall hypothesis presented in this study was that antibiotic administration to piglets during the lactation period interferes with the colonization pattern of respiratory microbiota and impairs piglet immune development. Therefore the specific aims of this study were:

1. To characterize the composition of the nasal microbiota of piglets either treated or untreated with antibiotics prior to and at weaning.
2. To determine host genetic variations associated with impaired development of the immune system induced by antibiotic-mediated disturbances in porcine airway microbiome.

Materials & Methods:

Animals and study design

The study was carried out in a commercial sow farm in the Midwest of the US. Animal studies were performed under the approval of the corresponding Institutional Animal Care and Use Committee (IACUC). The study consisted of repeated pig samplings (5 time points) and evaluation of three experimental groups over a period of 56 days after farrowing. Eighty-five piglets from 10 different dams irrespective of parity from a farrowing week were allocated randomly within litter to one of the following experimental groups:

- Control (group 1) consisted of piglets that did not receive antibiotics during the suckling period (n=29).
- Processing treatment (group 2) consisted of piglets treated with injectable antibiotics at 4 days of age (n=27).
- Weaning treatment (group 3) consisted of piglets treated with injectable antibiotics at 19 days of age (n=29).

Antibiotic treatment was performed per farm routine practices using Tulathromycin (Draxxin® 25, Zoetis Inc.) injected intramuscularly as a single dose in the neck at a dosage of 2.5 mg/kg, following manufacturer's recommendations.

All piglets in the study were monitored for mortality. Nasal swab samples were obtained from sows post-farrowing and from piglets at 4, 12, 19, 28 and 56 days of age, which corresponded to the time points T1, T2, T3, T4 and T5, respectively. A blood sample was obtained from peri-weaned piglets from all groups. All samples were collected, processed and stored at -80ºC until used for DNA extraction.

DNA extraction and sequencing of the microbial 16S rRNA

Nasal swabs were thawed, vortexed vigorously and the total genomic DNA was extracted using MagAttract PowerMicrobiome DNA/RNA Kit (QIAGEN) on the automated work station (MagMAX™ Express 96, Applied Biosystems™) following manufacturer's instruction into 96-well plates. The library preparation was performed at the University of Minnesota Genomic Center (UMNGC). Sequencing of the V3-V4 regions of 16S rRNA gene was performed with Illumina MiSeq pair-end 2 x 300 bp technology following manufacturer's instructions (MS-102-2003 MiSeq® Reagent Kit v2, 500 cycles).

Sequence processing and taxonomy assignment from 16S rRNA datasets

Following sequencing, raw fastq data were retrieved from the MiSeq platform and processed using the Divisive Amplicon Denoising Algorithm 2 (DADA2), an open-source R package

(https://github.com/benjjneb/dada2), to estimate abundance of bacterial taxa in the 16S rRNA datasets collected from different study groups [20]. Filtered high-quality sequences were aligned and taxonomically classified using the SILVA dataset (https://www.arb-silva.de/). Contaminating archaeal, mitochondrial, and chloroplast sequences or sequences classified as unknown were removed from further analysis. Finally, amplicon sequence variants (ASV) were predicted from the obtained high-quality sequences, mapped to the sequence taxonomy file generated in DADA2 and converted to number of sequences to generate comparative taxonomy data for the datasets. The ASVs were identified in the samples that passed the quality-based filters through clustering sequences at 97% sequence homology. Thus, low abundance ASVs under 3% cutoff were removed prior to compositional analysis.

The alpha diversity, or the diversity of nasal microflora within a group across different time points, was measured using Shannon index [21]. The compositional similarity between samples from different experimental groups was assessed and compared to the pairwise taxonomic abundances from each group, against each other, and within the normalized datasets, using Bray-Curtis measure for estimation of beta diversity [22] followed by permutation-based multivariate analysis of variance (PERMANOVA). Computation of Bray-Curtis distances and PERMANOVA tests were carried out in R (3.6.0 version). The distance scores were then visualized using principal coordinate analysis (PCoA) plots to reveal the existing compositional segregations among samples. Lastly, an algorithm for discriminating high-dimensional biomarker of genomic features, linear discriminant analysis (LDA) effect size (LEfSe) was used to determine the differences in nasal microbiome at genus level among different time points.

Single nucleotide polymorphism array genotyping and quantitative trait locus mapping

Genomic host DNA was separated from the total DNA extracted from blood samples using Gentra® Puregene® blood kit (QIAGEN). Genotyping was conducted using 50K GeneSeek® Genomic Profiler (GCP) porcine beadchip (Neogen Genomics). Quality control for genotypes was performed for the individuals with a call rate ≥ 95%. SNPs were selected with the following criteria: genotyping call rate ≥ 95%, P value of Chi-square test of Hardly-Weinberg equilibrium > 10^{-6} and minor allele frequency ≥ 5%. SNPs positions were updated according to the newest release from Ensembl (Sscrofa 10.2 genome version). Map information was adjusted based on the linkage disequilibrium (LD) decay for each chromosome. Only SNPs with reliable positions were used in this study. The missing genotypes were imputed by the software program FImpute 2.2 version [23].

Data and statistical analyses

The effect of treatments and time points on the alpha diversity and species richness was analyzed using one-way ANOVA, and Tukey's test was used to perform post-hoc comparisons. Further, the effect of treatments and time on the bacterial community composition was analyzed using PERMANOVA (adonis function, 99 permutations). The differentially abundant taxa driving the microbial shift between time points (T1 to T5) were determined by characterizing species indicator values or Indval. Tukey's test was used to identify changes in the relative abundance of these indicator taxa between time points and the significance was detected at $p < 0.05$.

Results:

Health status in the lactation and nursery phases

Overall, there was 27.1% piglet mortality in the entire study period and, among treatment groups, the mortality reported was 27.6%, 25.9% and 27.6% in group 1, group 2 and group 3, respectively. When litters were considered, piglet mortality ranged from 0% to 55% among the 10 sows from the study. Therefore, the number of piglets within an experimental group changed throughout the study and sampling time point. Table 1 shows how the sample size varied across the study period within each experimental group.

Sequencing of nasal swabs

With the aim of analyzing the effect of antibiotic treatment on the nasal microbiota, composition of the bacterial community was targeted by amplifying the V3-V4 regions of 16S rRNA gene. After quality filtering and demultiplexing, a total of 11,915,559 clean reads were obtained with an average of 20,722 assigned to 2500 different ASVs. Low abundance ASVs under 3% cutoffs were removed prior to compositional and LEfSe analysis. The sample reads were also normalized using cumulative sum scaling for downstream analysis.

Diversity and species richness from nasal microbiota

At phylum level, the nasal microbiota across all samples were assigned to Proteobacteria (42%), Firmicutes (33%), Actinobacteria (12%) and Bacteroidetes (9%). At genus level, the assignations were into *Moraxella* (14%), *Actinobacillus* (14%) and *Rothia* (12%). Overall, no significant differences were observed in microbiota diversity between groups at any time point, as indicated by the analysis of alpha diversity through Shannon diversity index (data not shown). Group 2 showed an increased bacterial diversity at 12 days of age (T2) when compared to the other groups 1 and 3 ($p < 0.05$; Figure 1). Irrespective to the treatment group, there was a significant difference in the microbial diversity as the piglets aged, from weaning (T4) to mid nursery (T5; $p < 0.05$; Figure 2). LEfSe algorithm on microbial abundances at phylum level across the time points studied (T1 to T5) and irrespective to the treatment received, is represented in Figure 3. Only the relative abundance of Bacteroidetes was significantly increased one week after weaning (T4; $p < 0.05$).

Using the Bray-Curtis distance matrix, the differential diversity (beta diversity) between samples, both in terms of presence/absence and abundance of taxa was identified (Figure 4). The dissimilarity matrices indicated that at pre-weaning, piglet nasal samples were more dissimilar compared to those from 28 and 56 days of age (T4 and T5, respectively). Additionally, PCoA analysis revealed significant segregations (clustering) among samples, indicating a time-dependent separation in nasal microbial community composition, and abundance. Thus, post-weaning samples (T4 and T5) diverged from all pre-weaning samples that clustered in a similar pattern irrespective to the treatment group. Furthermore, a close clustering of samples from littermates was observed at the first week of age (T1; Figure 5).

Thirteen phylotypes were identified at genus level as high-dimensional biomarkers at different days of sample collection (T1 to T5; Figure 6). Among these, seven genera, namely *Bergeyella*, *Moraxella*, *Streptococcus*, *Lactobacillus*, *Weissella*, *Aerococcus* and *Filobacterium* were significantly abundant at post-weaning (day 28 of age, T4; Figure 6).

SNPs array genotyping and QTL mapping

As changes in the microbiome composition over time were observed (Figure 3), QTL mapping was performed to investigate the genotypic effects associated with this phenotypic trait. Thus, the microbial diversity (Shannon diversity index) of nasal microflora at different time points was considered the phenotypic trait and its association with SNPs was performed. Manhattan plots were used to depict significant SNPs (Figures 7 and 8). GWAS were initially performed using antibiotic treatment groups as covariates and no significant associations were detected, probably due to the low number of analyzed individuals. Subsequently, GWAS were executed considering the change in the microbiome composition in all piglets over the consecutive time points as the phenotype.

At day 4 of age (T1), prior to the antibiotic administration, the nasal microbiome composition diversity was significantly associated with SNPs located on chromosome 2 (5,283,654 and 6,667,399 bp), 18 (22,658,506 bp) and X (130,717,707 bp) ($p < 0.05$; Figure 7). The candidate genes on chromosome 2 linked to the SNPs were KDM2A (lysine demethylase 2A) and EHBP1L1 (EH domain-binding protein 1-like 1) whereas SNPs on 18 and X chromosomes were linked to loci for hypothetical proteins. Considering the change in the nasal microbiota composition between pre-weaning and post-weaning time points, the significant SNPs were located on Chromosome 8 at 11,376,229 bp (BMP2K gene locus) and Chromosome 2 at 143,382,760 (hypothetical protein; $p<0.05$; Figure 8).

KDM2A, EHBP1L1 and BMP2K are protein-coding genes that are ubiquitously expressed in different tissues of mammals. KDM2A encodes for a member of the F-box proteins, which play a role in epigenetic silencing [24]. EHBP1L1 encodes for a Rab effector protein that may play a role in vesicle trafficking and maintenance of apical plasma membrane [25]. BMP2K is the homolog of mouse BMP-2-inducible kinase. Bone morphogenic proteins (BMPs) play a key role in skeletal development and patterning [26].

Discussion:

The effect of antimicrobial treatments in the nasal microbiota of pigs has been already documented [12–14]. These recent studies have tested the effect on the swine nasal microbiota of different parental antibiotics [12], of the removal of perinatal antibiotics [5] and of different dosing regimens of an antibiotic [14]. In all cases, a decreased diversity and alterations in the nasal microbiota composition associated to antibiotic administrations were reported. In these previous studies, however, the effect of antibiotics was assessed after short time periods from administration (2 to 4 weeks). Thus, this study was posed to assess the effects of Tulathromycin on the nasal microbiome at different time points during a longer period, which comprised lactation and post-weaning phases. Oppositely to what has been reported before, here the nasal microbiota was stable regardless of antibiotic treatment. Therefore, Tulathromycin administered at either 4 or 19 days of life did not induce a shift in the swine nasal microbiota composition. This latter is, however, in line with results obtained from tonsilar microbiome, which did not change significantly as a result of antibiotic treatment [14]. These controversial results may be due to the fact that microbial communities are influenced by multiple factors as specific phases of growth, management practices, presence of other bacteria, etc. [4,5,27,28]. Altogether, it evidences the need to further investigate how antimicrobials may affect bacterial communities of the upper respiratory tract and how these alterations contribute to respiratory disease susceptibility.

Although the initial hypothesis of the study could not be proved, a significant increase of the nasal microbiota diversity with age of the animals was appraised. Hence, nasal microbiota diversity changed significantly between pre-weaning and post-weaning time points. Aging has been already significantly associated with an increased richness and diversity as well as with distinct changes to the core microbiota at both intestinal and respiratory levels [27]. Previously, an apparent nasal co-dominance between Proteobacteria and Firmicutes during early-life has been described [27]. Accordingly, we reported a predominance of both Proteobacteria and Firmicutes across all time points studied. After weaning, however, previous reports detected a shift in dominance towards nasal Proteobacteria. Indeed, this transition was marked by an increase in Gammaproteobacteria (particularly *Moraxella*) [27,28]. Here, we did not observe this particularly shift to a dominance of Proteobacteria at weaning, though the genera *Moraxella*, together with other genera, had a significantly higher abundance in post-weaning samples.

The role of the host genome in the modulation of the microbiota composition in pigs has been assessed at intestinal level [19]. However, this information at respiratory level lacks in the literature. Although host genetics appeared to have a minor impact in the microbiota compared with age, diet or the environment [29], 39 genes were suggested as candidates that may modulate the microbiota composition and manifest the association between host genome and gut microbiota in pigs [19]. By means of GWAS, we aimed to detect host genetic variations associated with impaired development of the immune system induced by antibiotic-mediated disturbances in nasal microbiome. Thus, the inclusion of the variability in the nasal microbiome composition to the host genome led to the identification of 6 significant associated SNPs. The genes associated to these SNPs are reported to be ubiquitously expressed in diverse tissues from mammals. Since the genetic variations detected could not be associated to any immunological trait, the role of the host immune system in the modulation of the nasal microbiota composition could not be stablished. Moreover, as antibiotic-mediated disturbances did not occur in our study, how these are related to an impaired immune system remain unknown.

In summary and under the conditions this study was developed, a single injection of Tulathromycin did not disturb the upper respiratory microbiota. Besides, our results suggest that aging is the most significant driver of diversity of the nasal microbiota, although management practices, may also play an important role.

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Table 1. Number of piglets sampled at each time point within each experimental group. Overall, there was 27.1% piglet mortality in the entire study period. Mortality was evenly distributed among groups.

Time point (days of life)	T1 (4)	T2 (12)	T3 (19)	T4 (28)	T5 (56)
Group 1	29	28	25	22	21
Group 2	27	24	23	20	20
Group 3	29	28	26	23	21
Total	85	80	74	65	62

Figure 1. Alpha diversity of pig nasal swab samples at 12 days of age (T2) within experimental groups. The lines inside boxes represent the mean Shannon diversity index and superscripts with different letters are significantly different from each other ($p < 0.05$). Group 1= negative control; Group 2= processing treatment; Group 3= weaning treatment.

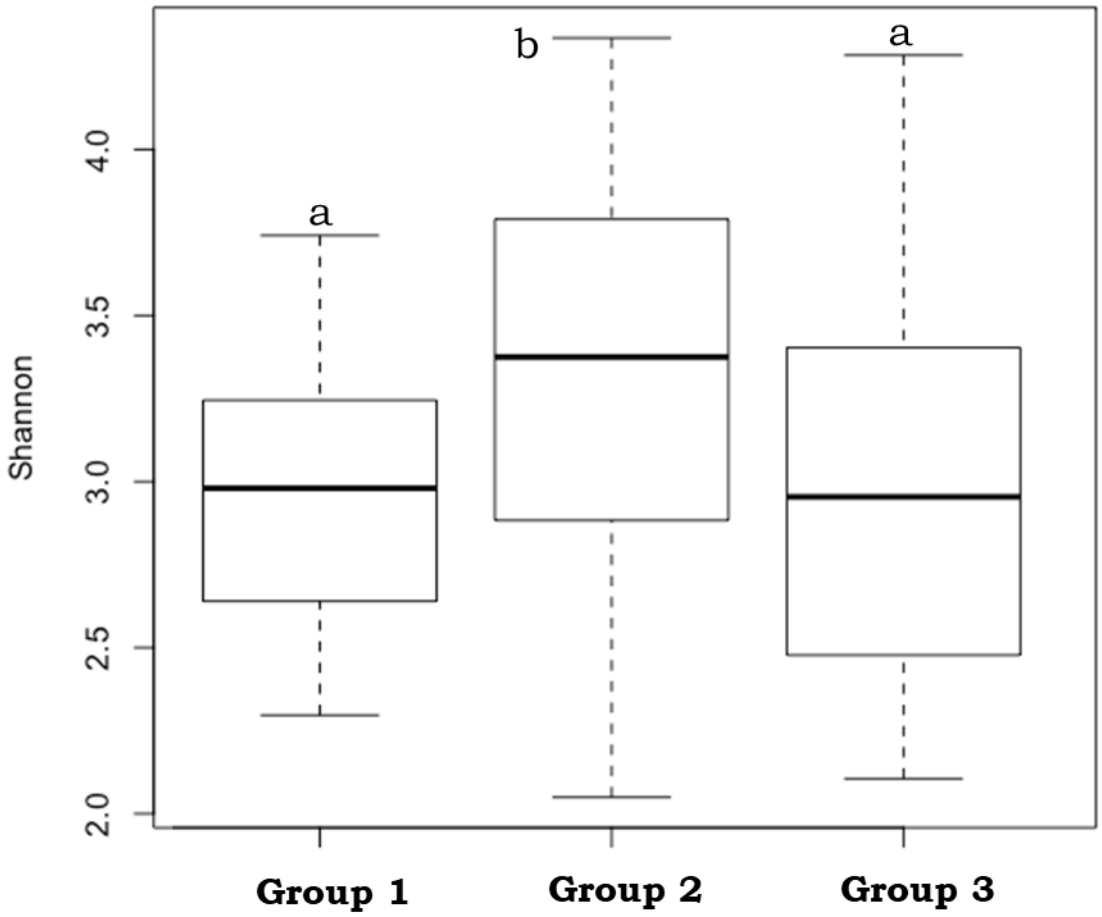
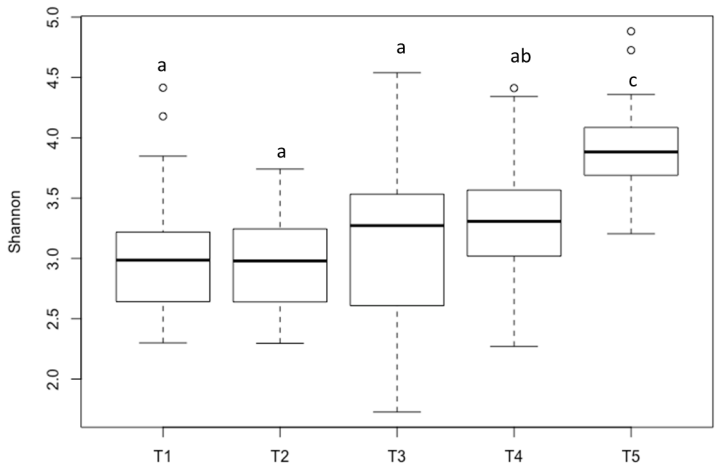


Figure 2. Alpha diversity of pig nasal swab samples at different time point across the study within experimental groups. Comparisons of Shannon diversity index scores are shown for group 1 (A), group 2 (B) and group 3 (C). The lines inside boxes represent the mean and superscripts with different letters are significantly different from each other ($p < 0.05$).

A



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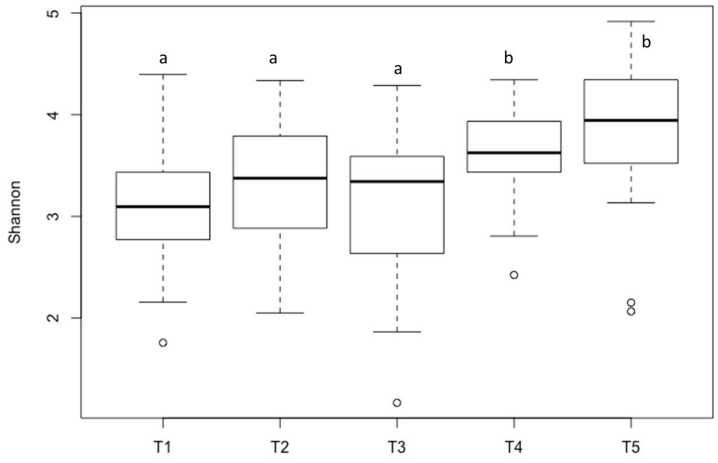

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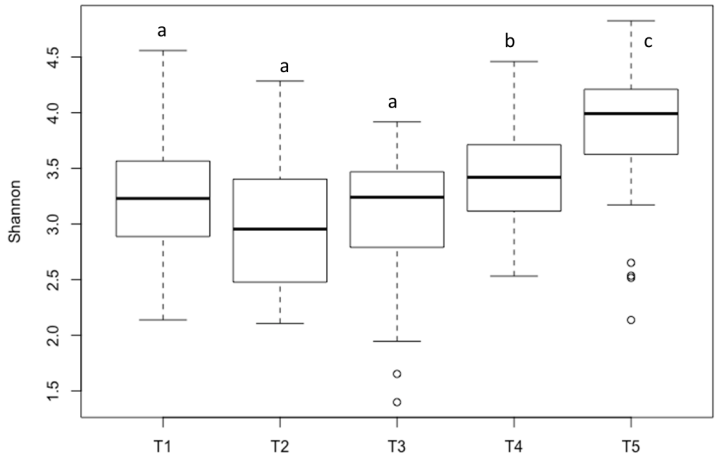

Figure 3. Phylum level nasal microbial composition of piglets at different time points. The majority of the bacterial species identified in all nasal samples belonged to the phylum Proteobacteria (green), Firmicutes (yellow), followed by Actinobacteria (purple) and Bacteroidetes (red) irrespective of the treatment group. The relative abundance of Bacteroidetes was significantly increased one week after weaning (T4; $p < 0.05$).

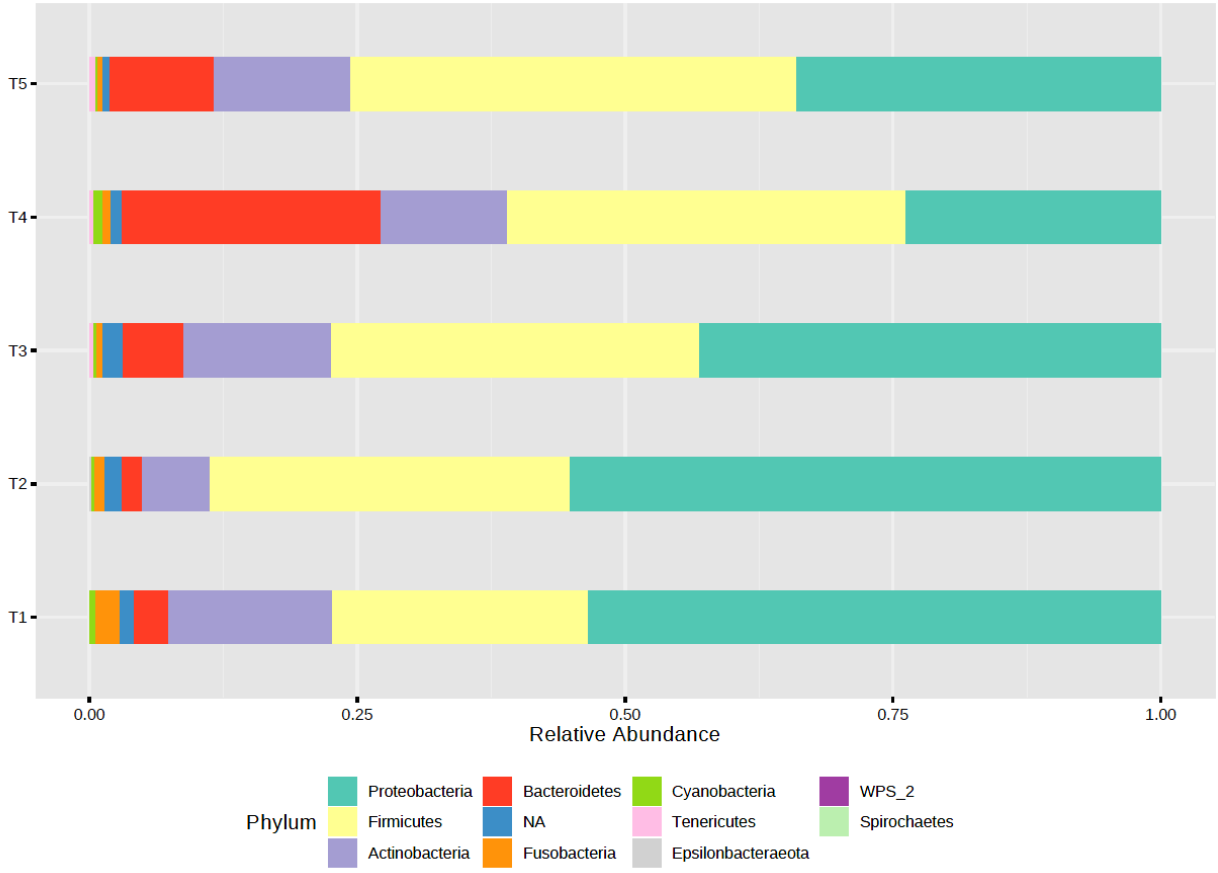

Figure 4. Bray-Curtis matrix for beta diversity index. Non-metric multidimensional scaling of Bray-Curtis distances between nasal samples based on microbial abundances. PCoPoints are colored by time point (T1 to T5) to which the samples belonged. The shape of each point indicates the treatment group. G1= negative control; G2= processing treatment; G3= weaning treatment.

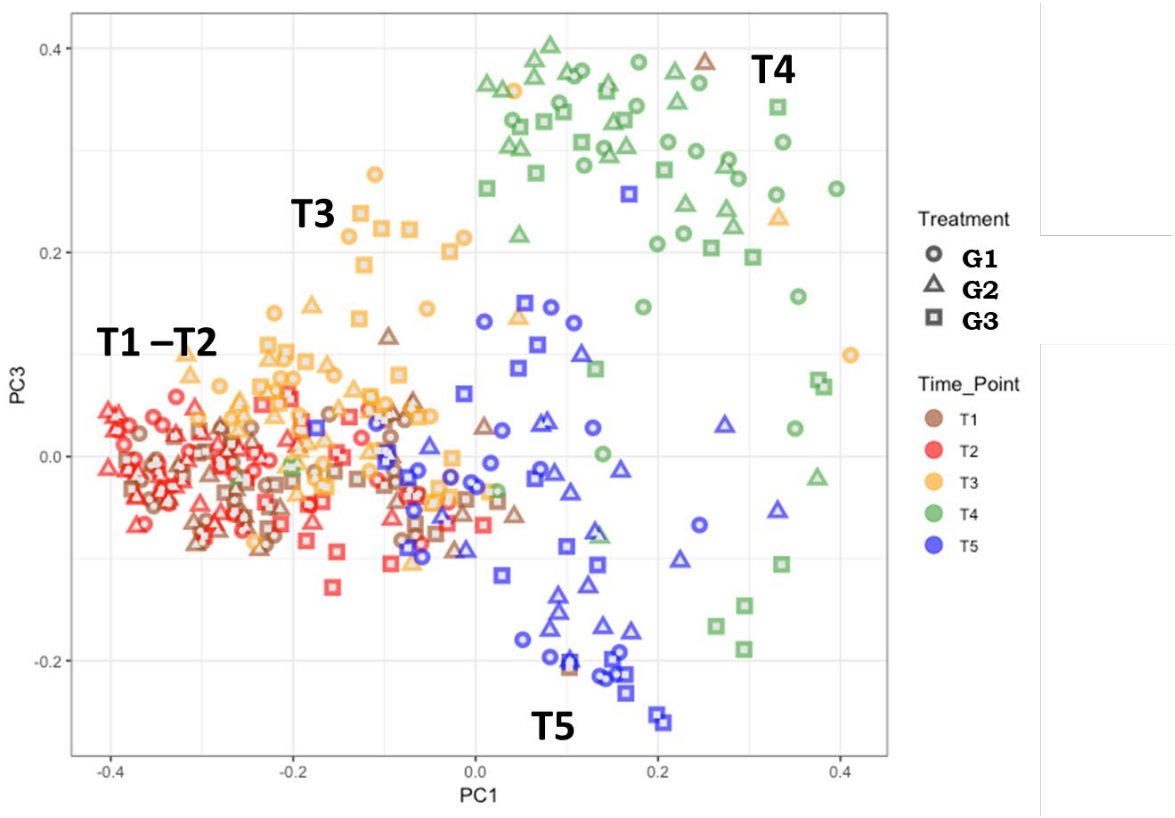

Figure 5. Bray-Curtis matrix for beta diversity index. Non-metric multidimensional scaling of Bray-Curtis distances between nasal samples from day 4 of life (T1) based on microbial abundances. PCoPoints are colored by litter (sow 1 to sow 10) to which the samples belonged. The shape of each point indicates the treatment group. G1= negative control; G2= processing treatment; G3= weaning treatment.

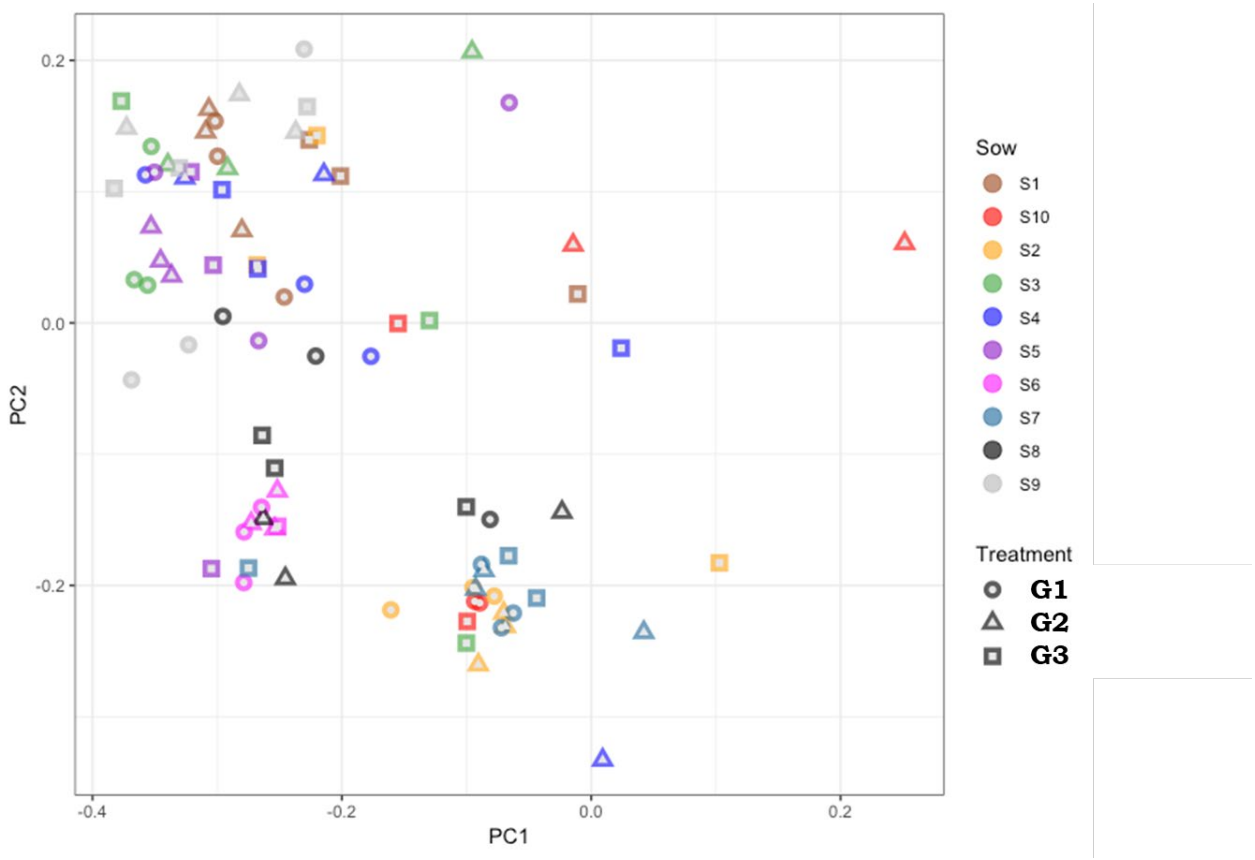

Figure 6. Differences in phylotypes of nasal microbiota corresponding to different time points. Linear discriminant analysis (LDA) scores computed for differentially abundant taxa among time points. The enriched taxa in different time points are shown in different colors. A higher LDA score value indicates the importance of that taxa in separating time points regarding microbial abundances.

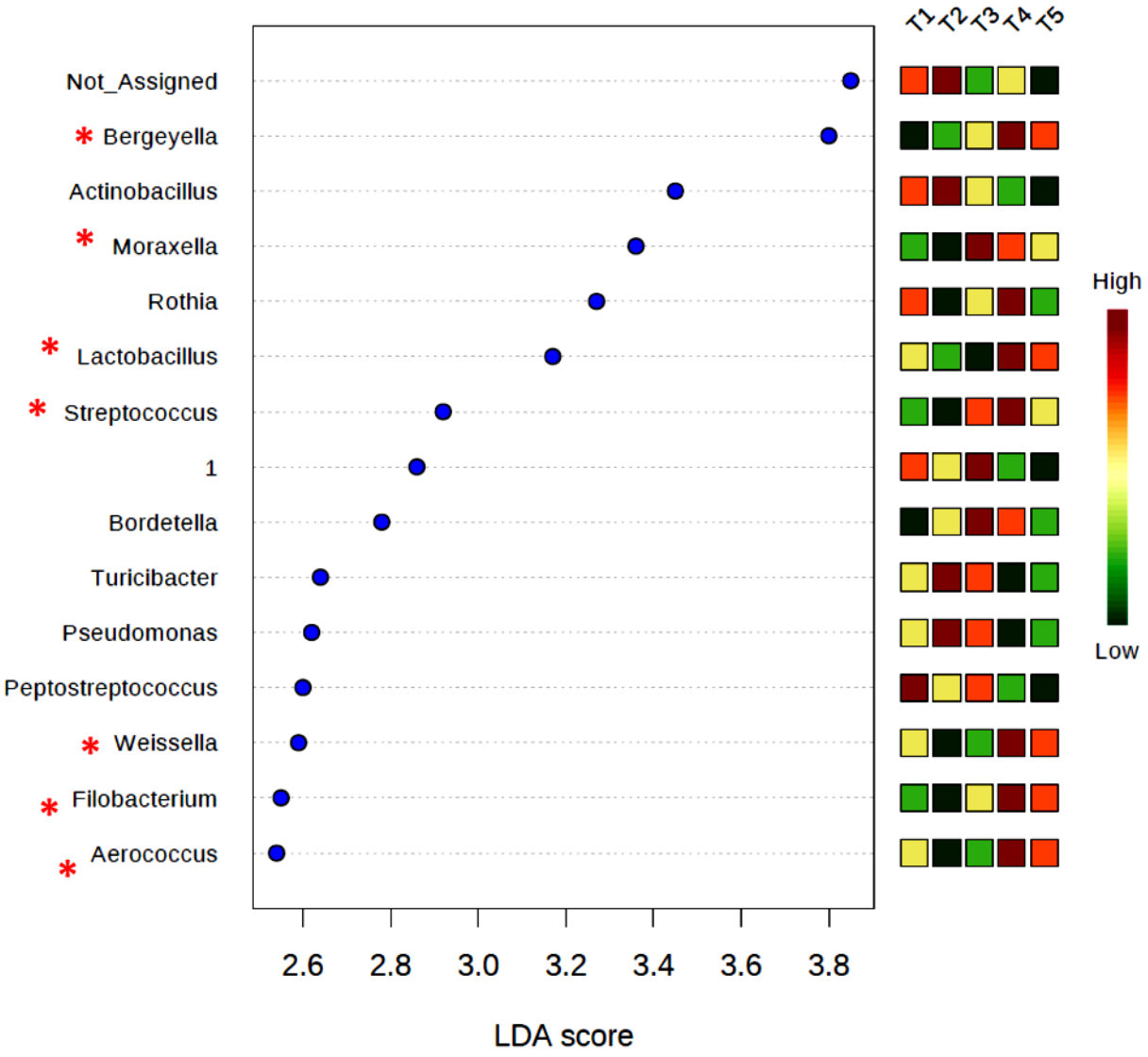

Figure 7. Genome-wide association studies for the variability in the nasal microbiome composition observed among piglets at 4 days of life (T1). Each point represents a tested SNP, displayed by chromosomal position (x-axis). The y-axis shows–log10 (P-value) for each SNP. Both the red and blue lines correspond to Bonferroni-corrected genome-wide significance thresholds of significance. Dots above the red line are considered significant SNPs.

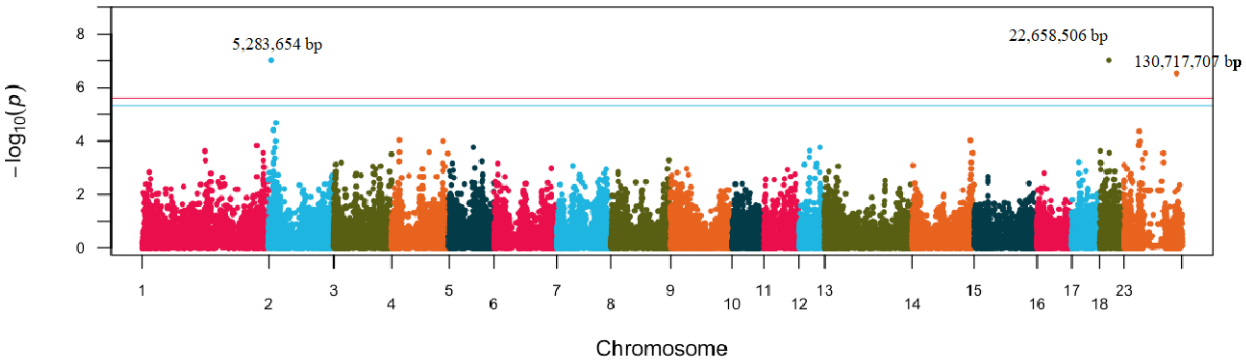

Figure 8. Genome-wide association studies for the variability in the nasal microbiome composition observed among piglets over the consecutive time points (T1-T5). Each point represents a tested SNP, displayed by chromosomal position (x-axis). The y-axis shows–log10 (P-value) for each SNP. Both the red and blue lines correspond to Bonferroni-corrected genome-wide significance thresholds of significance. Dots above the red line are considered significant SNPs.