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Assessing the impact of broiler genotype on cecal and tracheal microbiome composition using full-length 16S rRNA sequencing

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ABSTRACT

This study examined the tracheal and cecal microbiome composition across three broiler chicken genotypes including a heritage New Hampshire × Columbian cross (**NHC**) and modern Ross × Ross 308 (**R308**) and Ross YP × Ross 708 (**R708**) broilers, using full-length 16S rRNA sequencing. Birds were reared in floor pens, and at 56 d of age, cecal and tracheal samples were collected from 8 birds per genotype and subjected to DNA extraction followed by PCR amplification of full-length 16S rRNA. Obtained amplified PCR product was sequenced using MinION. A total of 1.8 million reads for tracheal samples and 1.2 million reads for ceca samples were obtained from 24 birds. Despite similar alpha diversity matrixes (Shannon, Simpson, Pielou's evenness, and Chao1) across genotypes in both tracheal and cecal samples, beta diversity analysis revealed significant differences in community composition. Tracheal and cecal microbiota varied significantly among genotypes, particularly NHC with the R308 and R708 groups. At the phylum level, Bacillota (Firmicutes) dominated both tracheal and cecal samples across genotypes. In the trachea, NHC and R708 birds exhibited high relative abundance of *Enterococcus cecorum*, while *Jeotgalicoccus meleagridis* dominated R308. Differential abundance analysis showed higher abundance of potentially beneficial bacteria such as *Limosilactobacillus pontis* and *Aerococcus viridans* in R308 and R708, while NHC birds had higher levels of species like *Merdibacter massiliensis* and *Agathobaculum butyriciproducens*. Cecal microbiome analysis revealed genotype-specific enrichment of species, with NHC birds showing higher abundance of potential pathogens like *Shigella boydii* and *Escherichia fergusonii* compared to R708. In contrast, R308 birds harboured more potentially beneficial taxa, including *Lactobacillus acidophilus* and *Limosilactobacillus vaginalis,* compared to R708. Pairwise comparisons further highlighted *Intestinibacter bartlettii* and other potentially beneficial microbes being significantly enriched in R308 over R708. Overall, while microbial richness remained consistent, significant genotype-associated differences in bacterial community structure and genotype-specific microbial abundance were observed, emphasizing the influence of host genetics on microbiota composition and potential implications for poultry health and performance.

Introduction

According to the USDA, over 9 billion broiler chickens are raised annually in the U.S., contributing significantly to national agricultural revenue (National Chicken Council, 2024). Intensive production systems has created challenges for maintaining the health and welfare of commercial poultry, especially in terms of controlling infectious diseases, foodborne pathogens, and promoting optimal growth. The respiratory and gastrointestinal tracts harbour dense, diverse, and functionally active microbial communities and these bacterial communities are capable of influencing bird health, pathogen colonization, and overall performance (Sun et al., 2022). Colonized microbiota plays an essential role in the host's immune maturation, metabolic function, and disease resistance (Cantarel et al., 2012; Cisek and Binek, 2014). In the respiratory tract, resident microbes contribute to mucosal immunity, maintain homeostasis, and prevent colonization of airborne pathogens (Man et al., 2017; Li et al., 2025). Meanwhile, in the gut, particularly the ceca, microbiota are key to digesting complex carbohydrates, synthesizing vitamins, and producing short-chain fatty acids (SCFAs) that fuel enterocytes and modulate inflammation (Maslowski and Mackay, 2011;

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Kamada et al., 2013; Sommer and Bäckhed, 2013; Silva et al., 2020; Liao et al., 2020; Portincasa et al., 2022; Wei et al., 2022). An imbalance or dysbiosis in either system has been linked to poor growth performance, increased susceptibility to infections, and greater economic losses in poultry production (Ducatelle et al., 2023; Kogut, 2022). Therefore, understanding microbial composition at species-level might help in the development of genotype-specific precision probiotics to precisely manipulate the microbiome to enhance the growth performance and reduce pathogen colonization.

Until recently, usage of short read producing second generation sequencing platforms for sequencing bacterial 16S rRNA spanning one or combination of hypervariable regions (such as V1/V2, V3/V4, V4 only, and V5/V6) was the common approach for investigating microbial composition (Walters et al., 2015; Stevens et al., 2023). Although the second-generation sequencing platforms like Illumina have the potential to produce high-throughput and higher sequencing accuracy, a major constraint is their ability to generate a partial sequence of 16S rRNA spanning only few hypervariable regions (Heikema et al., 2020; Stevens et al., 2023) which is only enough for accurately identifying microbial classification up to genus level (Heikema et al., 2020; Stevens et al., 2023). This prevented full characterization of microbial composition since the same genus can hold both commensal or beneficial bacterial species. The recent advancement and development of third-generation sequencing platforms, e.g. Oxford Nanopore, has facilitated the sequencing of full-length 16S rRNA and enhanced the ability to characterize the microbial composition at the species level of resolution (Johnson et al., 2019; Matsuo et al., 2021; Jeong et al., 2023; Esberg et al., 2024). The development of long-read sequencing technologies has solved the previous issues related to classifying microbes. Although high native error rates were a major limitation for the use of long-read sequencing in the past, upgrades in flow cell chemistry from R9.4.1 to R10.4 and base-calling software has increased the read-level accuracy, with a median of 99.2 % (Sanderson et al., 2024, Oxford Nanopore Sequencing Accuracy 2025). Therefore, in this study, we utilized the long-read sequencing platform Oxford Nanopore to analyze the microbial composition of the respiratory and gastrointestinal tract of broilers.

Microbiota composition in the broiler might be influenced by various intrinsic confounding factors such as age, sex, genetics, anatomical location, as well as extrinsic factors such as rearing condition and environments, diet composition, water quality, and other management factors (Marietta et al., 2015; Wen et al., 2021; Chuwatthanakhajorn et al., 2023). Among these, genetic background can shape host physiology, immune response, and mucosal environments, which can potentially shape the microbial composition (Johnson et al., 2018; Marietta et al., 2015; Wen et al., 2021; Chuwatthanakhajorn et al., 2023). By leveraging the technological advancement on 16S rRNA sequencing, this study aimed to investigate the species-level diversity and abundance of microbiomes in the trachea and ceca of three genotypes of broilers: a heritage New Hampshire × Columbian cross (**NHC**) and modern Ross × Ross 308 (**R308**) and Ross YP × Ross 708 (**R708**) broilers.

Materials and methods

Experimental design, bird management, and sample collection

All the experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Auburn University (IACUC Approval No. 2024-5378). In this study, a total of 944 1-day-old male broiler chicks of three broiler genotypes: NHCR308, andR708 were obtained. The chicks were hatched in the Aviagen Hatchery in Albertville, AL, whereas fertilized eggs for NHC were sourced from flock maintained by University of Illinois and fertile eggs for R708 and R308 were sourced from Aviagen research farms in Albertville, AL and Spelderholt, Netherlands, respectively. The experiment was conducted in floor pens in the controlled environment poultry house at Charles Miller J. Poultry Research Center, Auburn University. The poultry house was equipped with an evaporative cooling system and negative air pressure fans, which allowed the temperature and humidity of the environmental house to be maintained. On placement temperature was set at 32°C, then reduced gradually to reach 21°C on day 21, which was maintained for the rest of the experiment. Experimental pens were bedded with white softwood (pine) shavings approximately 5 −10 cm in depth. The chicks were raised following the standard commercial guidelines and birds were provided with *ad libitum* access to feed and water. All three genotypes were fed the same diet across 4 phases including the starter (0 to 14 d), grower (14 to 28 d), finishers 1 (28 to 42 d), and finisher 2 (42 to 63 d) phases. Diets were formulated to nutrient and energy specifications recommended by Aviagen (2022). Starter diets were pelleted and crumbled, and all other feeds were fed as pellets. The genotype of birds served as the treatment with 8 replicate pens (each with an area of 2.32 m^2), which were arranged in a randomized complete block design. In each pen a total of 40 R308 and R708 were placed and for NHC 38 birds were placed in each sampling pens

At 56 d of age, 1 bird per pen (8 per genotype, 24 total) was randomly selected from each genotype for sample collection euthanized by CO_2 asphyxiation. The body weight of the sampled birds is listed as Supplementary Table 1. Whole ceca and trachea were collected from each sampled birds and samples were directly kept inside the sterile bag and stored in a −20°C freezer until further processing.

DNA extraction and amplification

The bacterial genomic DNA was extracted from the tracheal and cecal contents using QIAamp PowerFecal Pro DNA Kit using QIAGEN QIAcube Connect following the manufacturer's instructions. Before transferring samples to the QIAcube, both the tracheal and cecal content samples were homogenized using a Mini Bead Mill Homogenizer (VWR). For homogenization, approx. 250 mg of tracheal and cecal content samples were transferred into the tube containing beads (PowerBead Pro, Qiagen) and 800 µl of CD1 buffer and the prepared samples were homogenized in a bead mill for 3 min at speed level 5 (Mini Bead Mill Homogenizer, VWR). Thus, the prepared lysate was transferred to the QIAcube, and genomic DNA (gDNA) was extracted. The quantity and purity of the gDNA were assessed using NanoDrop (Thermo Scientific, USA).

The amplification of gDNA for full length 16S rRNA was conducted using Eppendorf Master cycler (Eppendorf, Enfield, CT) with thermocycler condition setup at 98°C for 30 sec, 35 cycles of 98°C for 10 s, 66°C for 20 s, 72°C for 90 s, and a final extension at 72°C for 5 min and 4°C hold. The hypervariable region V1-V9 of 16S rRNA was amplified using the primer pairs 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT). The PCR were performed in total volume of 25 µl, with a 12.5 µl Phusion® High-Fidelity PCR Master Mix (ThermoFisher Scientific), 1.25 µl of primers (each in final concentration 0.5 µM), and 2 µl (20 ng) of extracted gDNA and nuclease-free water. The resulting amplicons were then visualized on a 2 % agarose gel by electrophoresis. Thus, the obtained PCR products were purified using AMPure XP beads (Beckman Coulter, Brea, CA). The purified PCR products were quantified using NanoDrop (Thermo Scientific, USA) and subjected to library preparation.

Library preparation and read processing

The library was prepared using Native Barcoding Kit 96 V14 (SQK-NBD114.96) following the manufacturer's protocol. Thus, the prepared library was quantified using Qubit dsDNA high-sensitivity assay kit (Invitrogen), and a final library was loaded into the Flow cells (FLO-MIN114, R10.4) and sequenced using a portable Nanopore MinION device (MinION™ Mk1B DNA/RNA Sequencing Device, Oxford Nanopore Technologies, Oxford, UK). The generated raw reads were demultiplexed and base-called using MinKNOW v24.11.10 (Oxford Nanopore

Technologies, Oxford, UK) with higher accuracy, with a quality filter Q-score >10. Thus, the obtained fastq reads were further analyzed using wf-16 workflow of EPI2ME platform (Oxford Nanopore Technologies, Oxford, UK). The reads having base pair length ≤800 and ≥2000, as well as those aligning with host *Gallus gallus* genome (GCF_016699485.2), were filtered out. The remaining reads were mapped to NCBI 16S reference database using minimap2, with a minimum sequence identity and reference coverage threshold of 90 % for taxonomic classification.

Statistical analysis

The taxonomic data obtained from the EPI2ME platform were analyzed using phyloseq v. 1.48.0 package (McMurdie and Holmes, 2013) and the vegan package v. 2.6-10 (Oksanen et al., 2025) in R software 4.4.2 (R Core Team, 2021). Contaminated taxon detected in the negative control were removed based on abundance of taxon present in the samples and negative control on normalized data. Samples having read below 20,000 were removed from further analysis. The alpha diversity matrices, Shannon, Simpson diversity index, Pielou Evenness, and Chao1 were calculated. The normality of each alpha diversity matrix

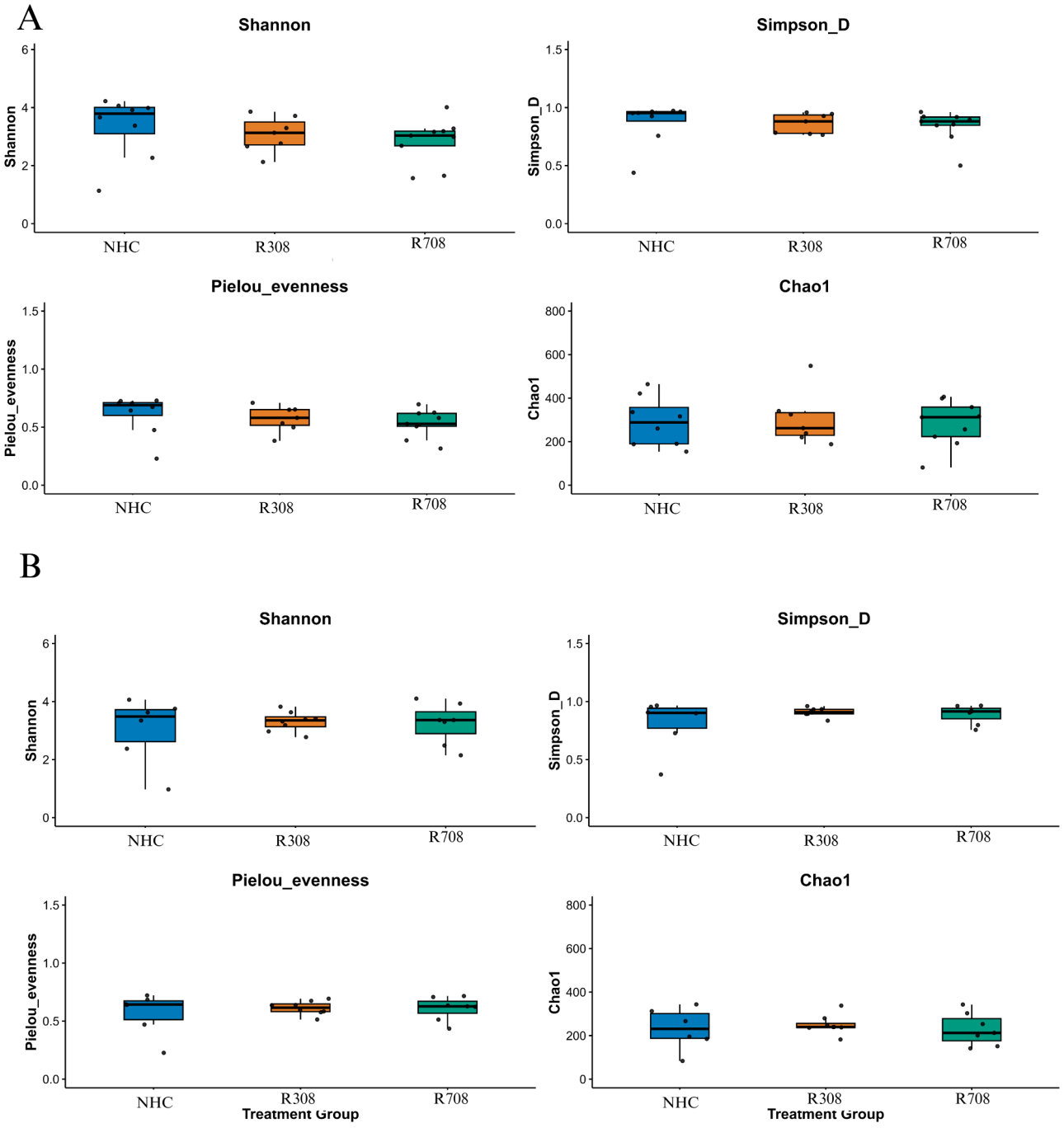

Fig. 1. Broiler genotype did not significantly alter tracheal and cecal alpha diversity.
The figure consists of eight box plots, presented in two panels (A) tracheal microbiome (B) cecal microbiome. Each panel includes four alpha diversity metrics -Shannon, Simpson, Pielou's evenness, and Chao1-across three treatments. Based on the Shapiro-Wilk test results either ANOVA or Kruskal-Wallis test was used to evaluate the overall difference.

was assessed using the Shapiro-Wilk test, and depending on the normality results, either ANOVA followed by Tukey's HSD or Kruskal-Wallis followed by Dunn's test with Benjamini-Hochberg correction was used.

To analyze the beta-diversity matrix, the read counts data were normalized using cumulative sum scaling using metagenomeSeq v. 1.46.0 (Paulson et al., 2013). The Bray-Curtis and Jaccard dissimilarity indices were calculated, and the differences in microbial composition between the birds' genotypes were tested using permutational analysis of variance (PERMANOVA) using the "adonis2" function and followed by pairwise comparison between the genotypes using the "pairwise adosin2" function (Oksanen et al., 2025). Additionally, homogeneity of sample dispersion was assessed using the "betadisper" function, followed by ANOVA and a Permutation test (Oksanen et al., 2025) The data was visualized using Principal Coordinate Analysis (PCoA).

Differential abundance analysis of tracheal and cecal microbiomes between the three genotypes of the birds was conducted using DESeq2 package v. 1.44.0 (Love et al., 2014). Differences in taxonomic counts were detected based on the Wald test with parametric fit and positive count normalization (sfType = "poscounts"). Adjusted p-value was obtained using the Benjamini-Hochberg method to control the false discovery rate (FDR).

Results

Overview of sequencing

A total of 1,816,570 reads for tracheal samples and 1,216,039 reads for the cecal samples were obtained for full-length 16S rRNA from 24 tracheal and cecal samples. For the tracheal samples, the number of reads ranged from 22,371 to 224,001, with a 75,690-mean number of reads per sample, with all samples $n = 8$ for all three genotypes NHC, R308, and R708 were retained for the final analysis For the cecal samples, reads ranged from 29,272 to 112,178, with 56,546 mean reads obtained, whereas in case of cecal samples for NHC ($n = 6$), R308 ($n = 8$), and R708 ($n = 7$) samples were retained for the analysis

Microbial diversity across broiler genotypes

To explore the tracheal microbiome diversity among the three broiler genotypes NHCR308, and R708, various alpha diversity indices were evaluated based on taxonomy data (Fig. 1A). The alpha diversity indices (Shannon, Simpson, Pielous evenness, and Chao1) results for tracheal microbiome between the three genotypes indicate that there were no significant differences in overall diversity, evenness, and richness in bacterial species populations. Similarly, the alpha diversity indices for cecal microbiome were evaluated based on taxonomic data (Fig. 1B). All four alpha diversity indices (Shannon, Simpson, Pielous Evenness, and Chao1) did not significantly differ between the three different genotypes of broilers.

The overall bacterial community differed significantly among the three broiler genotypes in both tracheal (Bray-Curtis $p = 0.005$, Jaccard $p = 0.052$) (Fig. 2A and 2B) and ceca (Bray-Curtis $p = 0.016$, Jaccard $p = 0.001$) (Fig. 2C and 2D) as determined by PERMANOVA based on Bray-Curtis and Jaccard distance metrics (Fig. 2). The pairwise comparison between the three broiler genotypes for the tracheal microbiome revealed that the tracheal microbiome of the NHC birds had a distinct microbial abundance compared to R308 and R708. As we observed, the difference in the pairwise comparison between the NHC vs R308 (Bray-Curtis $p = 0.015$, Jaccard $p = 0.343$), NHC vs R708 (Bray-Curtis $p = 0.002$, Jaccard $p = 0.001$), however there was no significant difference between R308 vs R708 (Bray-Curtis $p = 0.317$, Jaccard $p = 0.767$) (Fig. 2E and 2F). Similarly, the pairwise comparison of cecal microbiomes between the genotypes revealed that microbial abundance was different between the NHC compared to R308 (Bray-Curtis $p = 0.001$, Jaccard $p = 0.003$) but not with R708 (Bray-Curtis $p = 0.067$, Jaccard $p = 0.149$). Although there was no significant difference in bacterial community between R308 and R708 in the trachea, a significant difference in presence of species was observed in ceca between R308 vs. R708 as indicated by significant Jaccard index (Bray-Curtis $p = 0.508$, Jaccard $p = 0.002$) (Fig. 2E, 2F). Additionally, PERMDISP for both trachea (Bray-Curtis $p = 0.789$, Jaccard $p = 0.967$) and ceca (Bray-Curtis $p = 0.974$, Jaccard $p = 0.691$) were non-significant. This result indicates that the observed differences in the community composition were not due to dispersion within each group, the difference in beta diversity matrices specifically in pairwise comparison were actually due the variation in microbiome composition due to genotype of the broilers.

Microbiome composition across broiler genotypes

To understand the composition of tracheal microbiome among the three broiler genotypes, the bacterial abundance at the phylum and species level was analyzed. In the trachea of the broilers, the *Bacillota* (formally known as *Firmicutes*) was predominant phyla with abundance NHC (94.5 %), R308 (93.4 %), and R708 (85.3 %)., The second most dominant phyla was *Actinomycetota* phyla in NHC (4.1 %), R308 (4.3 %), whereas *Pseudomonadota* phyla was second most dominant phyla in R708 (10.1 %). The microbiome at the species level was dominated by the *Enterococcus cecorum* in NHC, whereas the dominant species for R308 and R708 was *Limosilactobacillus reuteri* (Fig. 3A). Additionally, DESeq2 analysis further revealed that at the species level, the pairwise comparison between the NHC vs. R308 and NHC vs R708 showed an increase in abundance of *Enterococcus faecalis, Streptococcus mitis, Limosilactobacillus pontis*, and *Aerococcus viridans* in R308 and R708 compared to NHC. Similarly, *Merdibacter massiliensis, Agathobaculum butyriciproducens, Lacrimispora amygdalina, Mediterraneibacter glycyrrhizinilyticus,* and *Blautia caecimuris* had increased abundance in NHC compared to R308 and R708. Additionally, while comparing the R308 vs. R708, *Fusobacterium polymorphum, Lachnoclostridium urinimassiliense, Mediterraneibacter faecis, Faecalicatena controrta,* and *Blautia wexlerae* had increased abundance in R308 and bacterial species like *Staphylococcus chromogenes, Streptococcus pluranimalium, Rhizobium aquaticum, Stenotrophomonas maltophilia, Acinetobacter radioresistens,* and *Nosocomiicoccus ampullae* had increased abundance in R708 (Fig. 4).

The cecal microbiome analysis of three broiler genotypes at the phylum level indicates that cecal microbiome was also dominated by the *Bacillota* (formally known as *Firmicutes*) with abundance NHC (89.05 %), R308 (95.36 %), and R708 (93.26 %). Second most dominant phyla was *Actinomycetota* in NHC (5.36 %), R308 (4.22 %), whereas in case of R708 *Pseudomonadota* was second most dominant phyla with abundance of 4.22 %. The bacterial species *Romboutsia ilealis* dominated the cecal microbiome of R308 and R708, whereas NHC was dominated by *Faecalibacterium hattorii* (Fig. 3B). The difference in abundance analysis using DESeq2 at the species level of the cecal microbiome among the broiler genotypes shows variation between some specific species between the genotypes. Compared to NHC, in both R308 and R708, bacterial species like *Lactobacillus crispatus, Blautia glucerasea, Romboutsia ilealis, Romboutsia timonensis,* and *Turicibacter sanguinis* had increased abundance. Opportunistic pathogenic bacterial species like *Shigella boydii, Escherichia fergusonii, Staphylococcus cohnii and Staphylococcus ureilyticus* had increased abundance in cecal microbial communities of the NHC birds compared to R308. Additionally, some commensal or beneficial bacterial species, such as *Lactobacillus acidophilus, Lactobacillus gallinarum, Limosilactobacillus pontis, Limosilactobacillus vaginalis, Limosilactobacillus portuensis, and Limosilactobacillus urinaemulieris,* were found to have increased abundance in R308 compared to NHC birds. Whereas only one bacterial species, *Enterococcus faecium,* was increased in abundance inNHC genotype compared to R708 genotype. While comparing the differentially abundant bacterial species between the R308 and R708, potentially beneficial bacterial species such as *Limosilactobacillus vaginalis, Limosilactobacillus pontis, Limosilactobacillus portuensis, Limosilactobacillus urinaemulieris,* and *Intestinibacter bartlettii* had

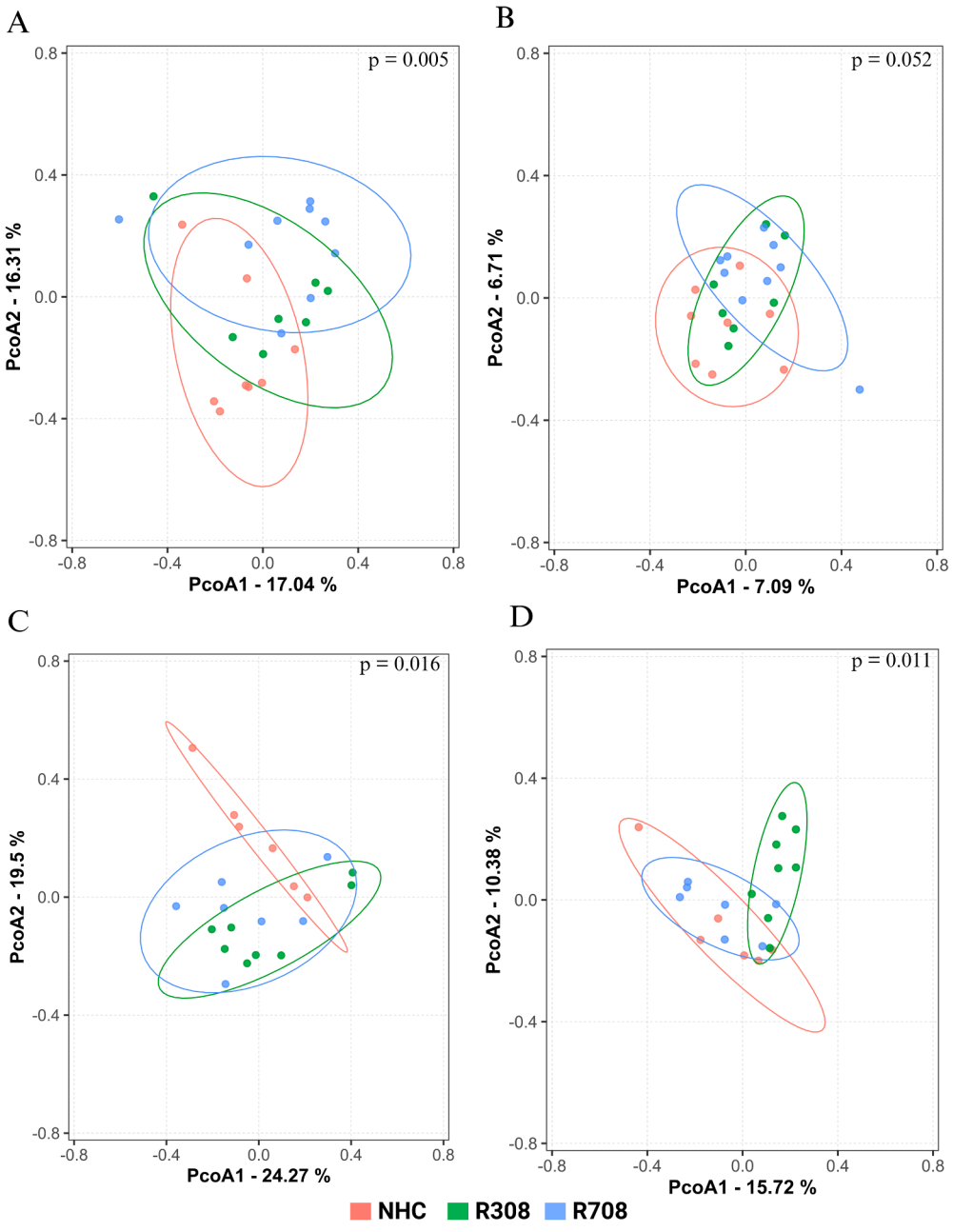

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Fig. 2. Broiler genotype significantly altered the tracheal and cecal microbiome composition.
(A-D) Principal Coordination Analysis (PCoA) plots illustrate beta diversity patterns of microbial communities from the trachea and ceca of three broiler genotype NHC (Pink), R308 (Green), and R708 (Blue), based on the Bray-Curtis and Jaccard distance matrix. The statistical difference in community composition were evaluated using PERMANOVA and p-value is listed at the top of each figure. (A) Bray-Curtis Trachea (B) Jaccard Trachea (C) Bray-Curtis Ceca (D) Jaccard Ceca. (E) Pairwise Bray-Curtis p-values between genotypes, summarized in a matrix table. P-values correspond to trachea are inside green boxes and ceca are inside blue boxes. (F) Pairwise Jaccard dissimilarity P-values between genotypes, summarized in a matrix table. P-values correspond to trachea are inside green boxes and ceca are inside blue boxes.

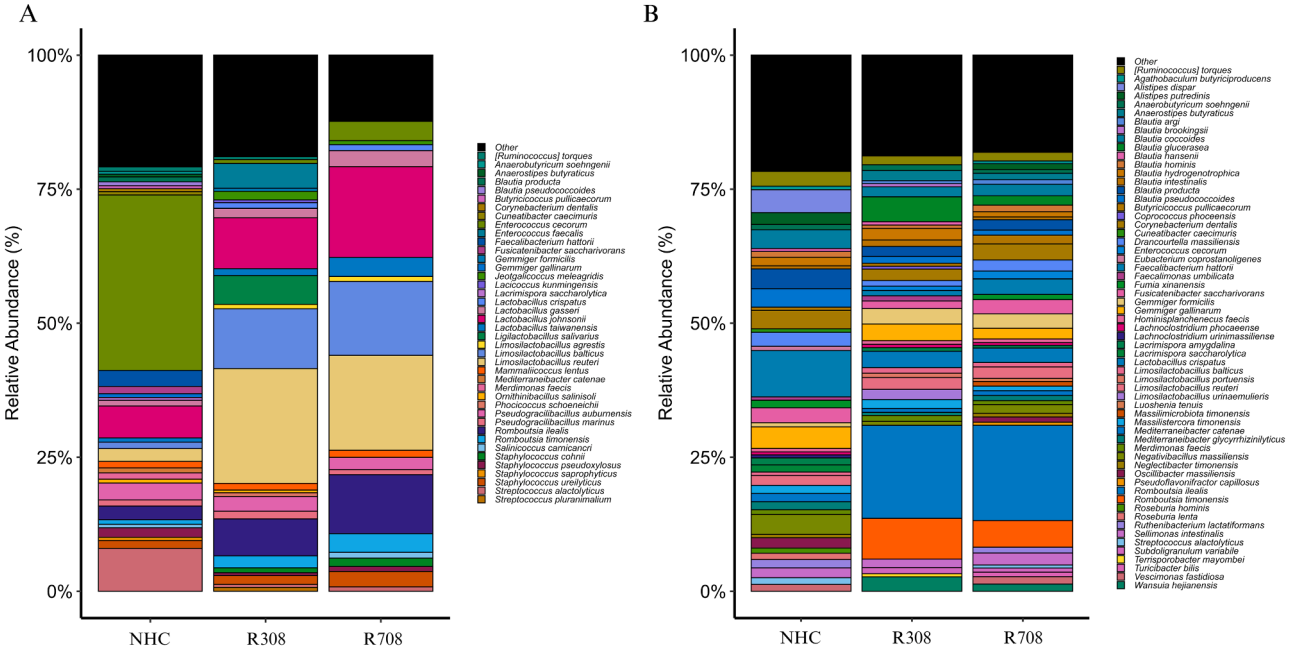

Fig. 3. Species-level relative abundance of the tracheal and cecal microbiome in three different broiler genotypes.
Relative abundance of bacterial species in tracheal microbiome (A) and cecal microbiome (B). The stacked bar graph plots represent bacterial species with relative abundance ≥ 0.05 and bacterial species having relative abundance ≤ 0.05 grouped as Other. The taxonomic classification was performed at species level using NCBI 16S rRNA database using wf-16S workflow of EPI2ME platform.

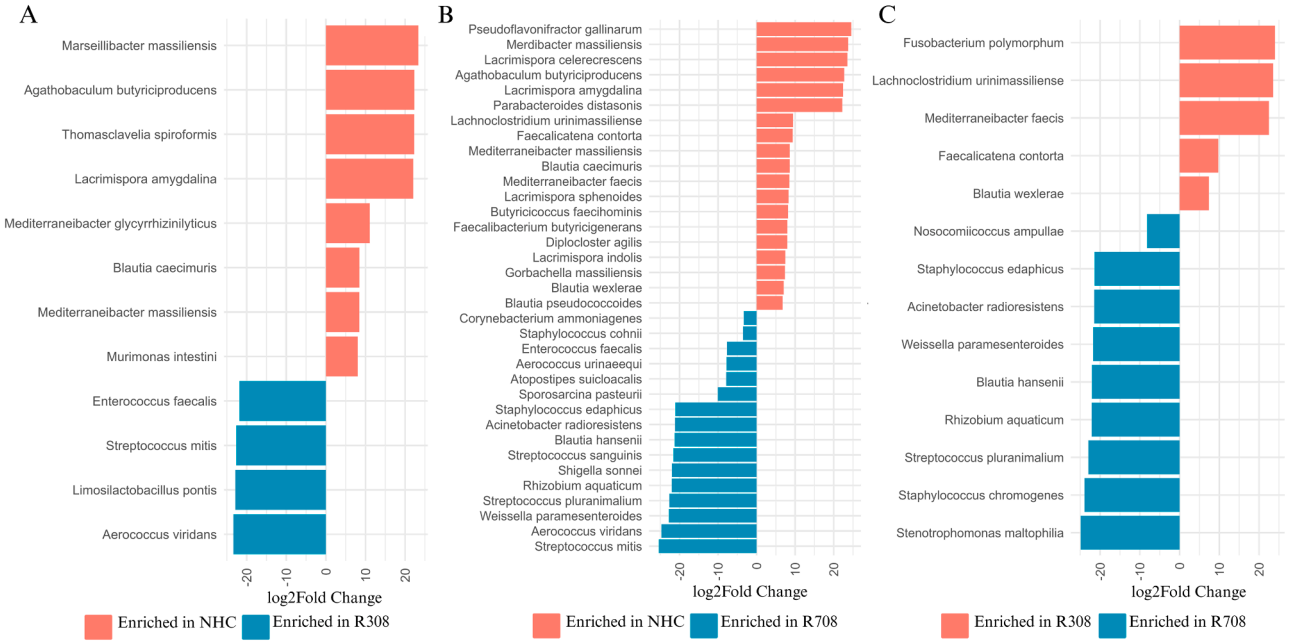

Fig. 4. Differential abundance of bacterial species in trachea between broiler genotype via DESeq2.
Plots display log2 fold-change values. (A) Comparison between NH vs. R308. (B) Comparison between NH vs. R708. (C) Comparison between R308 vs. R708.

significantly higher abundance in R308 compared to R708 and only two bacterial species, *Merdibacter massiliensis* and *Alistipes putredinis,* had increased abundance in R708 compared to R308 (Fig. 5).

Discussion

This study provides a comprehensive assessment of species-level microbiome composition in the trachea and ceca of three genetically distinct broiler chicken genotypes, heritage NHC, R308, and R708, using full-length 16S rRNA gene sequencing via Oxford Nanopore Technology (ONT). Our findings reveal that although alpha diversity metrics did not differ significantly across genotypes, beta diversity and differential abundance analyses demonstrated clear genotype-specific microbial community patterns. This suggests that host genetics could potentially influence the composition of microbial communities in both the respiratory and gastrointestinal tracts. This aligns with previous studies in both avian and mammalian systems, indicating that genetic background can influence the colonization potential, immune signaling, and microbiome–host interactions within distinct mucosal environments (Zhao et al., 2013; Fan et al., 2020; Tůmová et al., 2021). Different chicken genotypes are associated with distinct cecal microbial communities, each with unique structural and functional characteristics (Zhao et al., 2013). Specific genetic loci, particularly those related to intestinal barrier integrity, have been identified as key factors influencing microbial composition in the gut (Zhao et al., 2013). Similar physiological processes might hold for the tracheal microbial composition too. Previous studies have documented that the host genetic background including distinction between fast and slow growing strains, high and low egg producing chickens, could influence the microbiome composition in GI tract of birds (Sun et al., 2018; Wen et al., 2021; Marcato et al., 2023; Wu et al., 2025).

One of the primary functions of the mucosal lining of the trachea is to protect it from colonization by pathogens. At the species level in the trachea, *Enterococcus cecorum* was dominant in NHC. Similarly, Johnson et al. (2018) reported microbial ecology at the genus level reported that *Enterococcus spp.* as core members of the broiler tracheal microbiome. This pattern highlights the genotypic specificity in microbial niche occupation. While comparing the tracheal microbiome of NHC, both R308 and R708 had shared increased abundance of commensal bacterial species, *Streptococcus mitis*, and *Limosilactobacillus pontis*, suggesting similar mucosal environments or immune tolerance mechanisms that allow for such colonization in R308 and R708. *Streptococcus mitis*, part of the mitis group of *Streptococci*, is primarily known as an inhabitant of the oral microbiome and is characterized by its facultative commensal behaviour (Balsalobre et al., 2006). Whereas *Limosilactobacillus pontis*, a lactic acid bacterium frequently isolated from the broiler chicken gut, is associated with probiotic functions and a stabilizing effect on intestinal microbial communities (Christofoli et al., 2020; Liu et al., 2021; Lei et al., 2024). Additionally, some opportunistic pathogens, such as *Enterococcus faecalis,* were increased in abundance in R308 and R708. Some strains of *E. faecalis* are opportunistic pathogens and might have been transferred from the gut-lung axis (Tian et al., 2023). Also, this species of bacteria has been isolated from the pneumonic lungs of humans (Tian et al., 2023). Additionally, two opportunistic pathogens, such as *Shigella sonnei, Staphylococcus cohnii,* had increased abundance in R708. Furthermore, we observed that the NHC birds trachea had higher abundance of *Faecalibacterium butyricigenerans, Butyricicoccus faecihominis,* and *Agathobaculum butyriciproducens, Blautia caecimuris* although these bacterial species roles in the respiratory tract of birds is unknow, but based on the human research, these bacterial species were found to be involved in the butyrate production and anti-inflammatory roles (Trivedi and Barve, 2020; Liu et al., 2021; Zou et al., 2021; Wang et al., 2022). Furthermore, many of these bacteria enriched in NHC trachea are typical gut microbes, suggesting that they may have translocated from gastrointestinal tract to trachea.

In ceca, several beneficial microbes such as *Lactobacillus acidophilus, Limosilactobacillus pontis*, and *Limosilactobacillus vaginalis,* were enriched in R308 birds compared to NHC, indicating potential probiotic advantages in this genotype that could enhance gut health, nutrient utilization, feed conversion, suppress harmful bacteria, and enhance immune responses (Forte et al., 2018; Royan and Navidshad, 2024). Additionally, the increased abundance of *Intestinibacter bartlettii,* SCFAs producing bacteria, formerly known as *Clostridium bartlettii,* in R308 birds compared to NHC, further supports the idea that gut microbiota is genotype specific. Previous studies have demonstrated that dietary supplementation of butyrate enhances broiler growth performance and gut

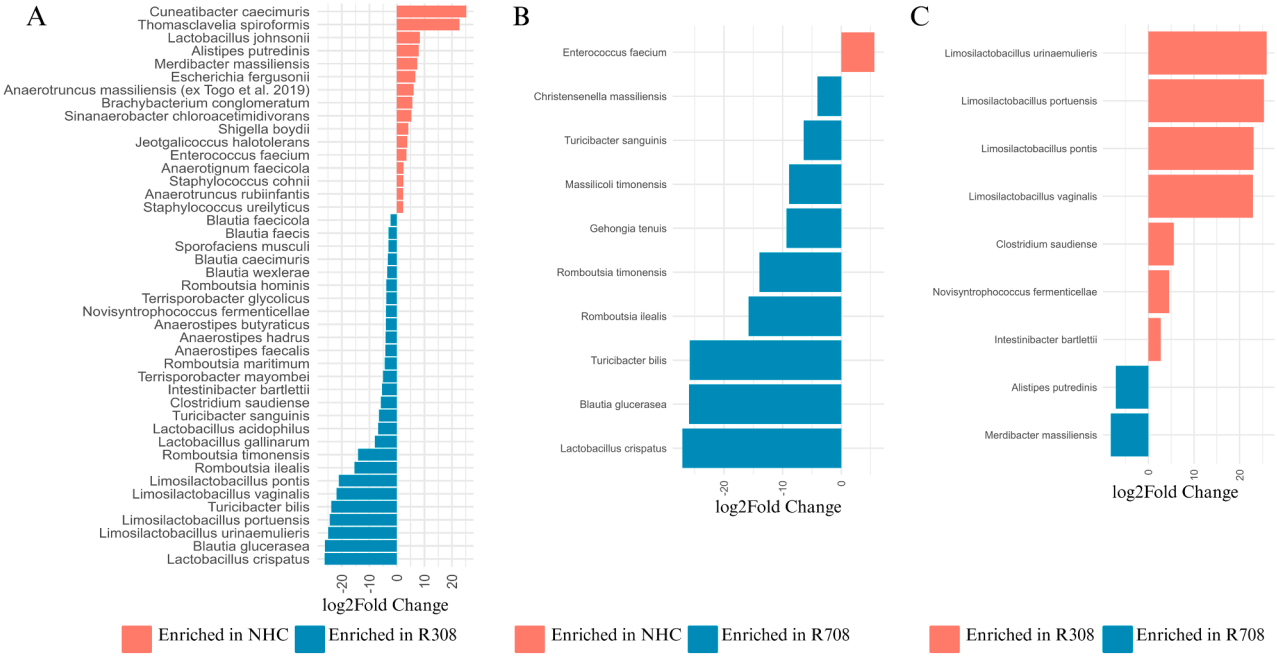

Fig. 5. Differential abundance of bacterial species in ceca between broiler genotype via DESeq2.
Plots display log2 fold-change values. (A) Comparison between NH vs. R308. (B) Comparison between NH vs. R708. (C) Comparison between R308 vs. R708.

health (Elnesr et al., 2020; Lan et al., 2020; Zhao et al., 2022). Conversely, the presence of potential pathogens such as *Shigella boydii* and *Escherichia fergusonii* in the ceca of NHC birds raises concerns about genotype-specific risks for gut dysbiosis or zoonotic pathogen carriage. In poultry, *Shigella boydii* is a harmful bacterium responsible for intestinal infections (Shi et al., 2014), and *Escherichia fergusonii* is an emerging pathogen linked to multiple infections and is concerning due to its carriage of antimicrobial resistance genes (Simmons et al., 2014). Along with some pathogenic some potentially beneficial bacterial such as *Enterococcus faecium* were increased in abundance in NHC compared to both R308 and R708, as supplementation of probiotic strains of *Enterococcus faecium* in chicken diet, improved body weight, reduced mortality, as well as reduced the invasion of *Salmonella enterica* into spleen, reduced *Salmonella* load from 6.8 CFU/g to 3.9 CFU/g, and increased level of butyric acid production in the intestine (Khalifa and Ibrahim, 2023). In contrast to R708, R308 showed increased abundance of several potentially beneficial bacterial species in ceca, such as *Limosilactobacillus urinaemulieris, Limosilactobacillus portuensis, Limosilactobacillus pontis, Limosilactobacillus vaginalis,* these bacteria have been associated with fiber fermentation and SCFAs production (Węsierska et al., 2025; Ksiezarek et al., 2022).

Although, current study utilizes the full-length 16S rRNA sequencing to characterize the microbial composition of the broilers, accurately distinguishing beneficial bacteria from the opportunistic pathogens, and pathogenic species of bacteria remains challenging due to the complex nature of host-microbial interaction and distinct microbial functional roles in different anatomical locations and animal species. The genotype-specific microbiome compositional variation observed in this study might also be influenced by maternal antibodies and microbiome composition, which were not considered in this study. Additional limitation includes, we used EPI2ME platform for taxonomic assessment and performed contaminant removal in R. Additionally, compared to short read 16S sequencing V3-V4, long-read Oxford Nanopore approach provides more accurate species level classification. However, short 16S rRNA (V3-V4) sequencing remains cost effective approach and more widely used, whereas shotgun metagenomics sequencing offers deeper functional insights with much higher cost. Using Nanopore sequencing could provide the promising balance between the cost and species-level classification, which might be increasingly practical along with the improvements in sequencing platforms read accuracy.

Conclusion

In conclusion, this study demonstrates that broiler genotype significantly impacts the composition and abundance of both tracheal and cecal microbiota at the species level. The microbiome composition of two modern broiler genotypes, R308 and R708, were more closely related in both trachea and ceca compared to the heritage genotype NHC. The modern fast growing broiler genotypes R308 and R708 cecal microbiome had enriched several beneficial microbes of *Lactobacillus* genus compared to heritage slow growing genotype NHC. These finding highlights the potential of using advanced sequencing technology to enhance our understanding regarding poultry microbiome and its relationship with host genotype and physiology. Furthermore, this study is one of the few studies to utilize the long-read Oxford Nanopore for sequencing full-length 16S rRNA for microbiome profiling in poultry. Therefore, using long-read sequencing approach, future research could achieve more widespread species-level resolution, which may help identify beneficial microbes that support poultry health or detect potential pathogens, ultimately informing targeted interventions and improved flock heath.

Data Availability: The raw sequences will be made available on reasonable request for secondary use.

Declaration of AI: Codes used for the data analysis and data visualization were optimized using AI-tools.

CRediT authorship contribution statement

Sabin Poudel: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Farazi Abinash Rahman:** Methodology, Data curation. **Erasmo Alexander Flores Granados:** Methodology, Data curation. **Yagya Adhikari:** Writing – review & editing, Data curation. **Muhammad Naeem:** Writing – review & editing, Methodology. **Samuel J. Rochell:** Project administration, Methodology, Funding acquisition, Conceptualization. **Dianna Bourassa:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Disclosures

Authors declare no Conflict of Interest for this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.106072.

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