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Avian microbiome: From embryonic development to adulthood

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Avian microbiome: From embryonic development to adulthood

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Editorial: Avian microbiome: from embryonic development to adulthood

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Editorial on the Research Topic
Avian microbiome: from embryonic development to adulthood

Avian gastrointestinal and reproductive microbiota are composed of bacteria, fungi, viruses, and protists and characterized by commensal, symbiotic, and pathogenic relationships with the host. Microbial populations play an important role in modulating host growth and reproductive performance, including nutrient digestion, absorption, and utilization, metabolic and reproductive efficiency, pathogen exclusion, endocrine activity, and immune system development. In chickens, symbiotic relationships between the host and the microbiota have been characterized by nutrient exchange, modulation of the immune system, pathogen exclusion, and gastrointestinal tract (GIT) and reproductive physiology. Microbiota composition and function can be affected by many factors, including age, host genotype and sex, diet composition and form, feed additives such as antibiotics, probiotics, prebiotics, postbiotics, synbiotics, phytobiotics and bacteriophages, stress, and location in the GIT or reproductive tract.

Most microbiome research in avian species has been focused on the GIT of domestic poultry such as broilers, laying hens, and, to some extent, turkeys. The reproductive microbiota in domestic poultry, as well as both the intestinal and reproductive microbiota of wild birds, remain largely unknown. Moreover, most current microbiome research primarily focuses on compositional studies using 16S ribosomal RNA (rRNA), and sequencing and functional studies remain elusive. The goal of this Research Topic was to provide a comprehensive overview of the avian microbiome that includes studies addressing intestinal microbiomes in both domestic and wild birds, including compositional and functional studies.

Gastrointestinal microbiota composition varies by niche, and therefore studies characterizing avian GIT microbiome can be greatly affected by the experimental design choices and specific methodologies used. Weinroth et al. focused on standardizing microbiota an analysis protocol, specifically 16S rRNA sequencing, to examine common sampling practices in broiler chicken studies. Microbiota collected from the GIT was compared to those from cloacal swabs, and it was concluded that cloacal swabs are not

a good approximation of the actual internal community at other GIT locations. They also found that sample sizes over 7.6 birds increase new observed amplicon sequence variants by less than 1%, and that cecal pair mates provide adequate replication.

Microbiota may be modulated by many factors during development, as well as in mature birds. Roth et al. characterize the microbiota in GIT from the crop to the ceca in two different laying hens breeds that were fed different level of Ca- and P-supplemented diets. These minerals play a pivotal role in many physiological processes in birds, including homeostatic mechanisms involved in Ca and P utilization for eggshell deposition and bone remodeling in highly productive laying hens. They have found that supplementation of Ca and P at 20% below the recommendation level had only a minor effect on microbiota composition in the GIT in comparison to the effects of genetic background of the birds. Van Syoc et al. showed that metformin, a drug commonly used off-label for polycystic ovary syndrome that benefits metabolic and reproductive health, has the potential to modulate the microbiota of GIT in broiler breeder hens. Besides the modulatory effect on microbiota, metformin was shown to have beneficial effects on metabolism and reproduction in breeder hens by decreasing body weight and increasing egg production.

Bacterial composition of competitive exclusion products (CEP) and their efficacy in reducing *Salmonella* in poultry have been studied by Lee D. et al. They revealed that bacterial community composition of master stock or seeds, as well as CEP commercial lots, were not a good predictor of their potency in reducing *Salmonella* abundance. In a second paper, Lee et al. characterize the pioneer colonizers of the chicken GIT. They have shown that CEP administered at hatch positively effects intestinal morphology, including villi height, goblet cell production, and feed efficiency. Moreover, administration of CEP stabilized the ileal microbiota diversity and promoted *Clostridium* abundance. They also showed that *Bacteroides* may act as pioneer colonizers in chicks, facilitating successional colonization of anaerobic bacteria. Administration of defined CEP formulations have similar effects on ileal morphology but lower the abundance of *Lactobacillus*.

The dynamic changes in microbiota composition in ceca and litter from chicken raised in two different houses from hatch to pre-harvest were determined by Zwirzitz et al. The animal (ceca) and environmental (litter) bacterial communities underwent consistent changes over time and the changes were correlated with the differences in environmental factors such as humidity, temperature, and ammonia level between the houses. The Shterzer et al. paper focused on differences in GIT bacterial communities between modern and slow growing broiler breeder lines. Selection for growth and high meat yield resulted in changes to GIT microbiota, probably due two physiological changes of the intestinal mucosal layer. As the authors concluded, it is still unclear if the changes in microbiota are part of the mechanism affecting the growth or are secondary results of other physiological changes accelerating the growth.

Bacteria are the major component of microbiota, and their role in host health and growth has been extensively studied and is beginning to be understood; however, the role of the fungal population in the chicken GIT during microbiota development is not as well characterized. Temporal changes in the chicken mycobiota during the first 2 weeks post-hatch and due to delayed access to feed early post-hatch have been investigated by Davies et al. The authors show transient changes in mycobiota during post-hatch development in the GIT and determined that negative effects of delayed access to feed early post-hatch are not likely related to the changes in developmental pattern of the fungal population in the GIT.

The review by Jadhav et al. addresses the connection between microbes, short chain fatty acids (SCFA), serotonergic system, and behavior in avian species. The authors speculated that considering the nature of SCFA interactions and the conserved molecular and behavioral features of the serotonergic system, the chicken may be an emergent translational model for identifying underlying mechanisms of change within the GIT-microbiome-brain axis.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Metformin modulates the gut microbiome in broiler breeder hens

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Broiler breeder hens, the parent stock of commercial broiler chickens, are genetically selected for rapid growth. Due to a longer production period and the focus of genetic selection on superior carcass traits in their progeny, these hens have the propensity to gain excess adipose tissue and exhibit severe ovarian dysfunction, a phenotype that is similar to human polycystic ovary syndrome (PCOS). Metformin is an antihyperglycemic drug approved for type 2 diabetes that is prescribed off-label for PCOS with benefits on metabolic and reproductive health. An additional effect of metformin treatments in humans is modulation of gut microbiome composition, hypothesized to benefit glucose sensitivity and systemic inflammation. The effects of dietary metformin supplementation in broiler breeder hens have not been investigated, thus we hypothesized that dietary metformin supplementation would alter the gut microbiome of broiler breeder hens. Broiler breeder hens were supplemented with metformin at four different levels (0, 25, 50, and 75 mg/kg body weight) from 25 to 65 weeks of age, and a subset of hens (n = 8–10 per treatment group) was randomly selected to undergo longitudinal microbiome profiling with 16S rRNA sequencing. Metformin impacted the microbial community composition in 75 mg/kg metformin compared to controls (adjusted PERMANOVA p = 0.0006) and an additional dose-dependent difference was observed between 25 mg/kg and 75 mg/kg (adjusted PERMANOVA p = 0.001) and between 50 mg/kg and 75 mg/kg (adjusted PERMANOVA p = 0.001) but not between 25 mg/kg and 50 mg/kg (adjusted PERMANOVA p = 0.863). There were few differences in the microbiome attributed to hen age, and metformin supplementation did not alter alpha diversity. Bacteria that were identified as differentially relatively abundant between 75 mg/kg metformin treatment and the control, and between metformin doses, included *Ruminococcus* and members of the *Clostridia* family that have been previously identified in human trials of

PCOS. These results demonstrate that metformin impacts the microbiome of broiler breeder hens in a dose-dependent manner and several findings were consistent with PCOS in humans and with metformin treatment in type 2 diabetes. Metformin supplementation is a potentially promising option to improve gut health and reproductive efficiency in broiler breeder hens.

KEYWORDS

broiler breeder hens, gut microbiome, metformin, polycystic ovary syndrome (PCOS), poultry

Introduction

Broiler breeder chickens are the progenitors of broiler chickens which are raised for meat production. As such, broiler breeders are genetically selected for carcass traits, fast growth, and low feed conversion ratios. The rapid improvement of agricultural production in the 1900s resulted in a 400% increase in broiler growth from 1957 to 2005 (Zuidhof et al., 2014). Broiler chickens are harvested at 6 weeks of age, but broiler breeder hens have a projected production lifespan of 60 weeks or more. The combination of genetic selection for rapid muscle growth and a longer lifespan than their progeny has resulted in poor reproductive efficiency in broiler breeder hens including decreased egg production and lower fertility and hatchability of eggs (Yu et al., 1992). This phenotype of propensity to accrue excess adipose tissue and severe ovarian dysfunction resembles a condition in humans known as polycystic ovary syndrome (PCOS) (Johnson et al., 2009; Johnson, 2012). Improving reproductive efficiency in broiler breeder hens without feed restriction could improve animal welfare and production value (Decuypere et al., 2010).

Metformin is a synthetic biguanide that is the first-line treatment for type 2 diabetes mellitus (Association, 2011; Bosi, 2009; Federation, W.H.O.I.D, 2006). The known mechanisms of metformin are reduction of hepatic gluconeogenesis, decreased intestinal glucose absorption, and improved insulin sensitivity resulting in increased peripheral glucose uptake (Center for Drug Evaluation and Research, 1995). Furthermore, metformin is the most commonly off-label prescribed drug for the treatment of PCOS (Guan et al., 2020). Multiple systematic reviews have provided evidence that metformin treatment improves reproductive health in women with PCOS, including increased fertilization and pregnancy rates (Maniar et al., 2017), normalization of the endocrine profile, as well as a return to normal menstrual cyclicity (Velazquez et al., 1994; Morin-Papunen et al., 1998; Moghetti et al., 2000; van Santbrink et al., 2005; Xing et al., 2020). In addition to numerous physiologic effects, metformin changes the gut microbiome composition and diversity (Forslund et al., 2015; Wu et al., 2017; Elbere et al., 2020). Metformin is postulated to ameliorate gut microbial dysbiosis that is characteristic of obesity and type 2 diabetes mellitus, potentially shifting the microbiome towards a healthier state (Forslund et al., 2015). Polycystic ovary syndrome is associated with gut microbial dysbiosis, characterized by lower alpha diversity and different beta diversity compared to healthy women, decreased *Akkermensia* and *Ruminococcaceae*, and increased *Bacteroides* and *Escherichia/Shigella*, although the results across studies are varied (Yurtdaş and Akdevelioğlu, 2019). Gut microbial dysbiosis impairs the secretion of β-glucuronidase, an enzyme that deconjugates estrogen and enables binding to estrogen receptors, which in turn decreases circulating estrogen, contributing to reproductive dysfunction including PCOS (Baker et al., 2017). No previous studies have quantified the microbiome in metformin-treated women with PCOS (Rizk and Thackray, 2021), but it is likely that metformin improves symptoms associated with PCOS *via* multiple mechanisms, including improving gut microbial dysbiosis to rescue circulating estrogen levels and subsequent hormonal balance.

Although metformin's effects on the human gut microbiome have been well-studied, less is known about potential host-microbe-metformin interactions in poultry. Metformin treatment in broiler chicks at 600 mg/kg body weight per day decreased feed intake and body weight, presumably through increased glucagon secretion and appetite suppression (Ashwell and McMurtry, 2003). *In vitro* treatment of broiler breeder hen granulosa cells with metformin decreased the expression of genes related to steroidogenesis and decreased progesterone production, suggesting a potentially beneficial effect of metformin on the reproductive health of broiler breeder hens (Weaver and Ramachandran, 2020). Additionally, the gut microbiome of broiler breeder hens has not been well characterized compared to broiler chicks and laying hens (Kers et al., 2018). The few studies that have assessed the gut microbiome in broiler breeder hens are descriptive, and longitudinal temporal dynamics have not been characterized (Díaz-Sánchez et al., 2019; Trudeau et al., 2020).

To determine if metformin treatment alters the gut microbiome or improves reproductive efficiency in broiler breeder hens, we conducted a trial with four levels of metformin (0, 25, 50, and 75 mg/kg body weight) supplemented in the diet from 25 to 65 weeks of age. A subset of hens (n = 8–10/treatment group) was randomly selected for longitudinal profiling of the gut microbiome at 40, 50, and 60 weeks of age *via* high-throughput sequencing of the 16S

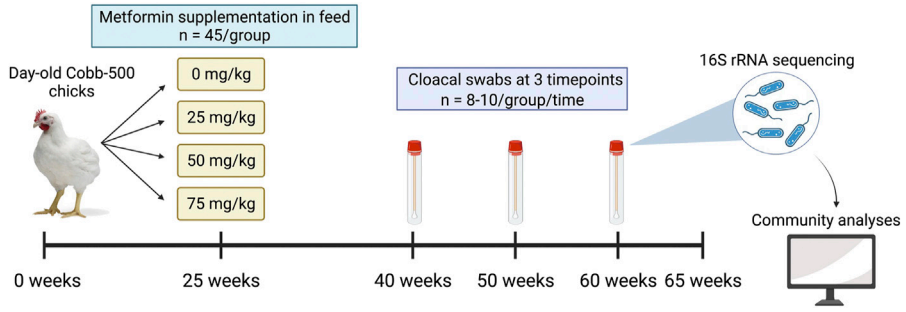

FIGURE 1
Schematic of experimental design. Broiler breeder hens were raised from day-old chicks and metformin was supplemented in feed at doses of 0, 25, 50, or 75 mg/kg at 25 weeks of age. A subset of hens (n = 8–10 per dose) was randomly selected for longitudinal profiling of the microbiome, and cloacal swabs were collected at 40, 50, and 60 weeks of age. The trial ended at 65 weeks of age.

rRNA gene V4 region. We hypothesized that metformin would modulate the gut microbiome, increase alpha diversity, and decrease the relative abundance of some gram-negative bacteria including *Akkermansia* and *Ruminococcaceae*. We expected these taxonomic changes to accompany improvement in egg laying frequency and production lifespan in the broiler breeder hens that may be driven by host-microbe-metformin interactions.

Materials and Methods

Animals and reagents

All animal procedures described herein were approved by Pennsylvania State University's Institutional Animal Care and Use Committee protocol number PRAMS200746656. A commercial strain of broiler breeder hens (Cobb 500) was maintained at the Poultry Education and Research Center at The Pennsylvania State University (University Park, PA, United States). The chickens were reared according to the Cobb 500 Breeder Management Guide and photo-stimulated beginning at 21 weeks of age. The length of light exposure was increased accordingly as they came into lay and birds were provided with a 16h light:8h dark (4:00 to 20:00) photoperiod for the duration of the study. The broiler breeder hens were moved from the rearing room at 22 weeks of age and randomly allocated to four experimental groups, n = 45 hens per treatment group. Broiler breeder hens were housed individually in battery cages and were feed-restricted according to the Cobb Breeder Management Guides and were provided with water *ad libitum*. Supplementation of metformin in the diet (0, 25, 50 or 75 mg/kg body weight; Midwest Veterinary Supply, Lakeville, MN, United States) began at 25 weeks of age and continued through the end of the study at 65 weeks of age. A subset of broiler breeder hens from each treatment group (n = 10) were weighed every 10 weeks to adjust the amount of metformin mixed into the feed according to their weight change over time.

Sample collection

A subset of hens (n = 8–10/treatment group) was randomly selected for longitudinal microbiome profiling and cloacal samples were collected at 40, 50, and 60 weeks of age (Figure 1). Sampling timepoints were chosen to coincide with the peak and subsequent decline in egg production. Birds were properly restrained on a breeding stool with chest facing down, and a sterile cotton swab was inserted into the cloaca and angled dorsally and to the right to avoid swabbing the oviduct. Swabs were swirled for 2–3 s, then placed into a sterile 2 ml centrifuge tube and stored on ice until returning to the laboratory, where samples were stored at −80°C until DNA extraction.

Sequencing library preparation

To optimize extraction yield, 1 ml sterile PBS buffer (pH 7.4) was added to cloacal swabs and homogenized at 20 Hz for 30 min (Bead Ruptor 96, Omni International, Kennesa GA). Swabs were removed and samples were centrifuged at 11,200 rpm for 30 min at 4°C, following which the supernatant was discarded and the pellet re-suspended in 300 µl sterile PBS (pH 7.4). The re-suspended samples were homogenized at 20 Hz for 30 min and stored at −80°C until extraction. High-throughput DNA extraction was performed in a Kingfisher instrument with the MagMAX CORE Nucleic Acid Purification Kit according to manufacturer instructions (Thermo Fisher Scientific, Austin,

TX, United States). Extracted DNA quantity and quality were assessed with a spectrophotometer (Nanodrop, Thermo Fisher Scientific Inc., Waltham, MA, United States). Negative and positive controls were extracted alongside the samples and carried through library preparation and sequencing. Genomic DNA was transported on dry ice to Novogene (Sacramento, CA, United States) for high-throughput sequencing of the hypervariable V4 region of the 16S rRNA gene. The sequencing platform was NovaSeq 6000, resulting in 250 × 250 bp paired-end reads.

Statistical analyses

Adapters were removed and ambiguous bases removed in cutadapt (Martin, 2013). Sequencing quality was visualized with fastQC and MultiQC (Andrews, 2010; Ewels et al., 2016). Quality trimming was performed with Trimmomatic to remove the 20 leading and 20 trailing base pairs, remove reads shorter than 100 bp, and truncate reads at average quality less than 20 in a 4 base pair sliding window (Bolger et al., 2014). Read statistics were collected with seqkit and further pre-processing was conducted in the dada2 R package (Callahan et al., 2016; Shen et al., 2016). Reads were dereplicated in dada2 and the learnErrors function was modified to accommodate binned Illumina quality scores from data generated in Novaseq instruments (see bash script in Data Availability Statement). Paired-end reads were merged and amplicon sequence variants (ASVs) were constructed, after which chimeras were removed with removeBimeraDenovo with the consensus method, and taxonomy was assigned to the genus level with the Silva database v138.1 (Quast et al., 2012). Putative contaminants (ASVs that appeared in negative controls or non-mock-community strains in the positive controls) were removed, non-bacterial ASVs or ASVs unassigned at the phylum level were removed, and ASVs with total relative abundance less than 1e-5 were removed. Negative and positive controls are further discussed in Supplementary Material.

All comparisons were made at the genus level. Statistical analyses comprised three hypothesis tests and subsequent correction for multiple comparisons to assess the longitudinal effects of metformin treatment. To determine an overall effect of metformin supplementation, the 75 mg/kg metformin treatment was compared to 0 mg/kg metformin. To profile the longitudinal effect of metformin, hen age was compared in the metformin-treated groups (combined). To detect a potential dose response, the three metformin doses (25, 50, and 75 mg/kg) were compared (hen age combined). These statistical comparisons were assessed in microbial alpha diversity, beta diversity, and differential relative abundance. Evenness (within-sample or alpha diversity) was calculated as Simpson's index in the phyloseq R package on filtered count data (McMurdie and Holmes, 2013). Outliers in Simpson's index were considered as greater/lesser than three times the standard deviation and one outlier was removed. Simpson's index was tested for normality using a diagnostic residual QQ-plot and residual histogram, and one-way ANOVA or *t*-test was performed. Significance was determined by comparing the raw *p* value to the critical alpha value calculated with Bonferonni's correction for three comparisons ($\alpha_{critical}$ = 0.05/3 = 0.01667). Post-hoc tests were conducted with Tukey's honest significant differences. Count data was transformed to center log-ratio (CLR) and visualized in a Principal coordinates analysis (PCA) with the microViz package (Gloor et al., 2017; Barnett et al., 2021). Beta diversity was assessed by permutational ANOVA (adonis test) with 999 permutations on Aitchison distances in the microViz package. Significance was determined as described above ($\alpha_{critical}$ = 0.05/3 = 0.01667). Differential relative abundance was assessed with linear models on log2-transformed total sum scaled data in the microViz R package,

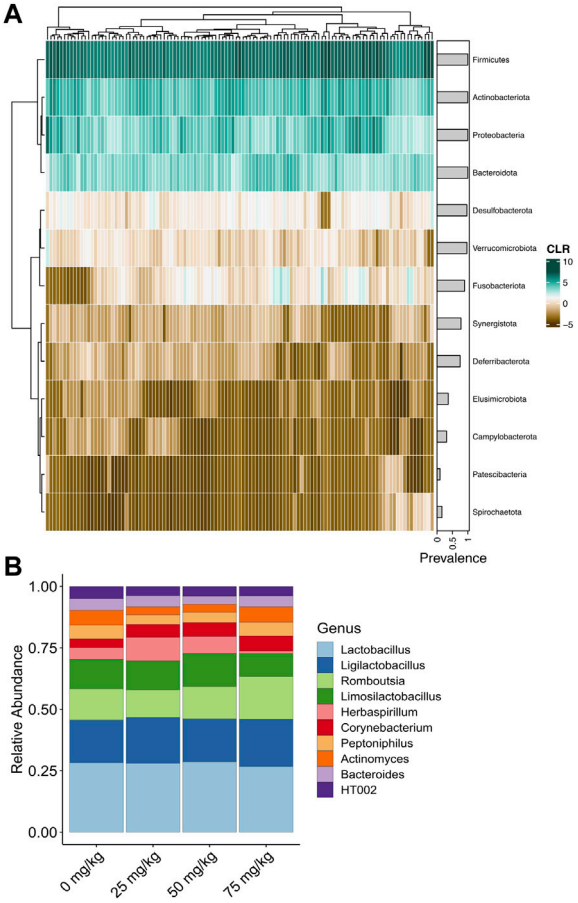

FIGURE 2
Relative abundance of bacteria detected in the cloaca of broiler breeder hens. **(A)** Heatmap of centered log-ratio relative abundance of bacterial phyla detected in the cloacal swabs of broiler breeder hens. Grey horizontal bars show the prevalence of each phylum. **(B)** Average relative abundance of the top 10 most abundant bacterial genera in each metformin dose.

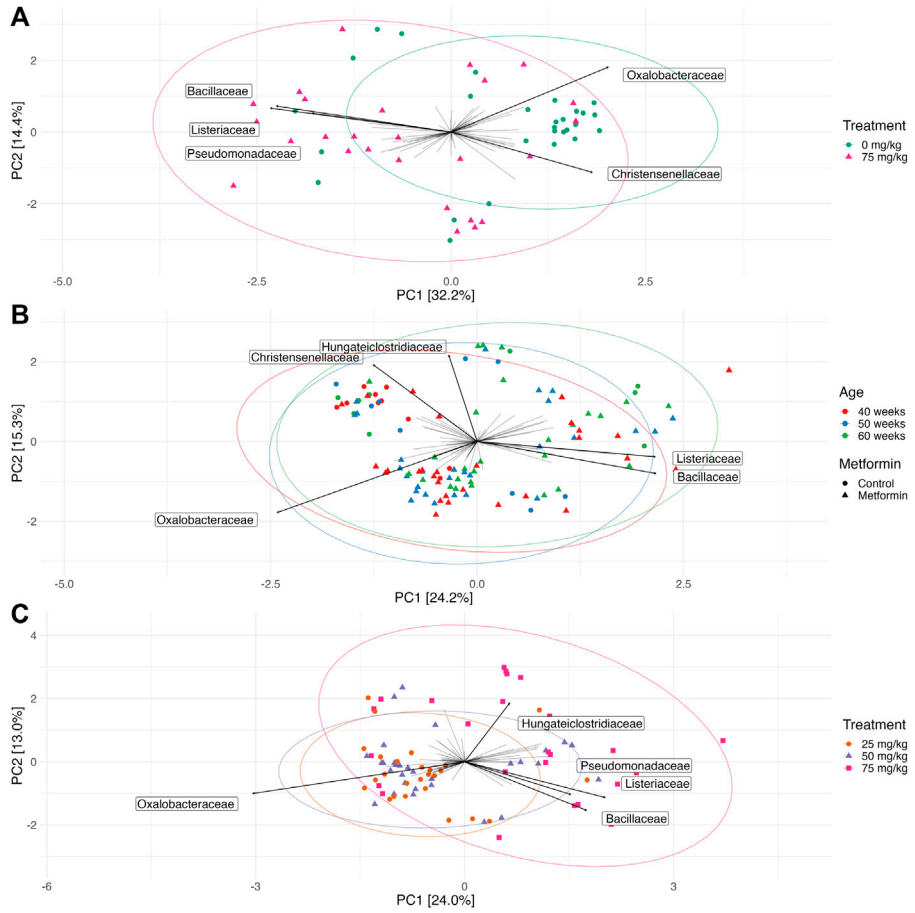

FIGURE 3
Microbial community composition is affected by metformin dose but not hen age. Principal coordinates analysis (PCA) of center log-transformed microbiome data at the family level showing the top five bacterial family loadings for each ordination. Ellipses represent 95% confidence intervals around the group centroid. **(A)** An overall metformin effect is observed by comparing the microbial community composition 0 mg/kg (purple circles) to 75 mg/kg (green triangles) metformin-treated hens. **(B)** Microbial community composition of broiler breeder hens by age. Colored points show hen age and shapes show metformin treatment (circle for control 0 mg/kg metformin; triangle for 25, 50, and 75 mg/kg metformin). **(C)** Metformin doses impact microbial community structure. (25 mg/kg metformin, green circles; 50 mg/kg metformin, blue triangles; 75 mg/kg metformin, purple squares).

and significance was determined as described above, correcting for 789 hypothesis tests (262–264 bacterial genera in each of three comparisons; $\alpha_{critical} = 0.05/789 = 6.33e^{-5}$). Visualizations were made with the microViz, ggplot2, or ggpubr R packages, or BioRender.com (Wickham, 2009; Kassambara, 2020; Barnett et al., 2021).

Results

Taxa summary

A total of 18,569,621 raw reads were filtered to 13,486,669 reads with an average of 91,126 reads per sample (Supplementary Table S1). The dada algorithm assigned 24,146 ASVs. After filtering and removing putative contaminants, 274 bacterial genera comprised the final dataset (Supplementary Material). The most abundant and prevalent phylum was Firmicutes, followed by Actinobacteriota, Proteobacteria, and Bacteriodota (Figure 2A; Supplementary Figure S3). The most abundant genera included *Lactobacillus*, *Ligilactobacillus*, *Romboutsia*, *Herbaspirillum*, and *Corynebacterium*, representing the Firmicutes, Proteobacteria, and Actinobacteriota phyla (Figure 2B).

Alpha diversity

Simpson's index was not affected by metformin treatment or hen age (Supplementary Table S2). There were no differences between 0 and 75 mg/kg metformin (*t*-test $t = 1.0683$, Bonferonni-adjusted $p =$

0.878), between 40, 50, or 60 weeks of age (one-way ANOVA $F_{2,82}$ = 2.168, Bonferonni-adjusted p = 0.368), or between metformin doses (one-way ANOVA $F_{2,82}$ = 1.19, Bonferonni-adjusted p = 0.927).

Beta diversity

Permutational ANOVA revealed a difference in microbial community structure between the control and 75 mg/kg metformin treatment (Bonferroni-adjusted p = 0.0015, r^2 = 0.06) (Figure 3A). Taxa PCA loadings at the family level indicated that *Oxalobacteraceae* and *Christensenellaceae* were associated with 0 mg/kg metformin while *Pseudomonadaceae*, *Listeriaceae*, and *Bacillaeceae* were associated with 75 mg/kg metformin treatment (Figure 3A). Hen age was not significant after correction for multiple comparisons (Bonferroni-adjusted p = 0.3192, r^2 = 0.032) (Figure 3B). Metformin treatment had a dose-dependent effect on microbial community structure (Bonferroni-corrected p = 0.0003, r^2 = 0.069) and pairwise comparisons showed differences between 25 mg/kg and 75 mg/kg metformin (pairwise adonis p = 0.001) and between 50 mg/kg and 75 mg/kg metformin (pairwise adonis p = 0.001), but not between 25 mg/kg and 50 mg/kg metformin (pairwise adonis p = 0.832) (Figure 3C). Taxa PCA loadings suggested that *Hungateiclostridiaceae*, *Pseudomonadaceae*, *Listeriaceae*, and *Bacillaceae* were associated with 75 mg/kg metformin while *Oxalobacteraceae* was associated with a small cluster of samples belonging to 25 mg/kg and 50 mg/kg metformin (Figure 3C). Metformin doses also differed in group dispersion (beta dispersion p = 0.00001) and post-hoc comparisons demonstrated a similar trend to PERMANOVA; there were differences in dispersion between 25 mg/kg and 75 mg/kg metformin (Tukey's post-hoc p = 0.0001) and between 50 mg/kg and 75 mg/kg metformin (Tukey's post-hoc p = 0.0006), but not between 25 mg/kg and 50 mg/kg metformin (Tukey's post-hoc p = 0.99) (Supplementary Figure S4). Hen age was not a significantly confounding factor for either metformin treatment or dose (Supplementary Figure S5A).

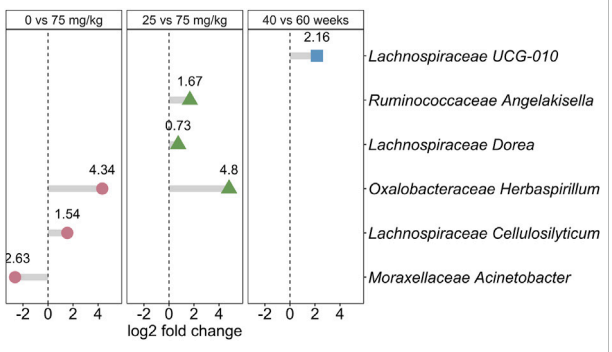

FIGURE 4
Metformin changes the relative abundance of bacterial genera. The effect size (log2 fold change) is shown for each bacterial genus, shown with the family name, that was significantly different between 0 mg/kg and 75 mg/kg metformin (pink circles; positive log2 fold change is more abundant in 75 mg/kg), between 25 and 75 mg/kg metformin (green triangles; positive log2 fold change in more abundant in 75 mg/kg), or between 40 and 60 weeks of age (blue squares; positive log fold change is more abundant at 60 weeks). The log2 fold change is shown above each point.

Differential relative abundance

The effects of metformin dose and hen age changed the relative abundance of bacterial genera (Figure 4; Supplementary Table S3). Two genera, *Oxalobacteraceae Herbaspirillum* and *Lachnospiraceae Cellulosilyticum*, were significantly more relatively abundant in 75 mg/kg than 0 mg/kg (log2 fold change 4.38 and 1.54, Bonferroni-adjusted p = 0.026 and 0.0072, respectively). *Moraxellaceae Acinetobacter* was significantly less abundant in 75 mg/kg compared to 0 mg/kg (log2 fold change −2.63, Bonferroni-adjusted p = 0.0038). A dose effect was observed in three genera, shown with the bacterial family, that were all significantly more abundant in 75 mg/kg compared to 25 mg/kg metformin; *Ruminococcaceae Angelakisella* (log2 fold change = 1.67, Bonferroni-adjusted p = 0.012), *Lachnospiraceae Dorea* (log2 fold change = 0.73, Bonferroni-adjusted p = 0.0337), and *Oxalobacteraeceae Herbaspirillum* (log2 fold change = 4.8, Bonferroni-adjusted p = 0.00047). *Herbaspirillum* comprised a larger proportional abundance than the other significant taxa, which were detected in very low abundance in the dataset (Supplementary Figure S6). Only one genus changed throughout the production lifespan of metformin-treated broiler breeder hens. *Lachnospiraceae UCG-010* was more abundant at 60 weeks compared to 40 weeks (log2 fold change 2.16, Bonferonni-adjusted p = 0.017). Hen age did not significantly confound the effects of metformin treatment or dosage (Supplementary Figure S5B).

Discussion

Metformin is an anti-hyperglycemic drug prescribed off-label for the treatment of human polycystic ovary syndrome (PCOS). Broiler breeder hens are genetically selected for fast growth and have reproductive dysfunction that can be phenotypically similar to PCOS. In this study, we supplemented metformin in the feed of broiler breeder hens and profiled the microbiome from 40 to 60 weeks of age to determine if metformin altered the gut microbiome. Metformin was supplemented in the diet at three doses (25, 50, and 75 mg/kg) in addition to a control treatment (0 mg/kg) and the cloacal microbiome was profiled with 16S rRNA sequencing at 40, 50, and 60 weeks of age. We hypothesized that metformin treatment would modulate the

gut microbiome, thereby increasing alpha diversity, and decreasing the relative abundance of some gram-negative species. We found that metformin affected the gut microbiome in a dose-dependent manner and there were few significant interactions with hen age.

In contrast with our hypothesis, the effect of metformin on gut microbiome alpha diversity (as measured by Simpson's index) was not significant. This is consistent with human studies on metformin, which have shown a slight reduction in alpha diversity in healthy people but no effect in type 2 diabetics (Elbere et al., 2020). Although we did not detect an effect of metformin on alpha diversity, there was an effect of metformin on beta diversity (composition of the gut microbiome). We detected significant differences in beta diversity between 0 and 75 mg/kg metformin, and a dose-dependent effect was observed between 25 mg/kg and 75 mg/kg and between 50 mg/kg and 75 mg/kg metformin. This is consistent with much of literature in human metformin treatment in type 2 diabetes, in which metformin exerts a strong effect on the gut microbiome composition as early as 24 h after initial treatment (Forslund et al., 2015; Wu et al., 2017; Sun et al., 2018; Elbere et al., 2020). There were no differences between 25 mg/kg and 50 mg/kg metformin, suggesting that the higher doses had a more noticeable effect on microbial composition. A few bacterial families that may have driven differences between 0 and 75 mg/kg metformin, indicated with PCA taxa loadings, included *Oxalobacteraceae*, *Pseudomonadacea* (Proteobacteria phylum), *Christensenellacea*, *Listeriaceae, and Bacillaceae* (Firmicutes phylum). We did not detect an effect of hen age on beta diversity, suggesting that hen age may not be a large driver of microbial community composition. Furthermore, hen age was not a significant variable in models comparing metformin treatment or dosage for beta diversity and differential relative abundance. Notably, broiler breeder hens have a much longer lifespan than their progeny, which are harvested at approximately 6 weeks, and longitudinal studies to characterize the microbiome are lacking; however, our results are consistent with a previous study that quantified the broiler breeder hen gut microbiome until 16 weeks of age and found that the microbiome stabilized after 3 weeks of age (Díaz-Sánchez et al., 2019).

Previous descriptive studies of the gut microbiome in broiler breeder hens were sampled from aggregated fecal samples collected from the pen but were in general agreement that the microbiome is dominated by Firmicutes, Actinobacteria, and Bacteroidetes (Díaz-Sánchez et al., 2019; Trudeau et al., 2020). We detected 1,270 ASVs and 271 genera in the cloacal swabs, which is similar to a meta-analysis that concluded 915 operational taxonomic units (OTUs) comprising 117 genera were present in the gut microbiome of broiler chickens (Clavijo and Flórez, 2018). Differential relative abundance tests revealed an effect of metformin on the relative abundance of only a few bacterial genera. Two bacterial genera were more relatively abundant in the 75 compared to 0 mg/kg metformin treatment, *Herbaspirillum* and *Cellulosilyticum*. *Cellulosilyticum* is a member of the *Clostridia* class of the Firmicutes phylum, which can be decreased in women with PCOS, but *Herbaspirillum* has not been previously associated with either metformin treatment or metabolic disease (Yurtdaş and Akdevelioğlu, 2019). *Acinetobacter*, a gram-negative coccobacillus of the *Moraxellaceae* family, was more abundant in 0 mg/kg compared to 75 mg/kg metformin. *Acinetobacter* is a potential pathogen and source of antibiotic resistance in humans and has been previously identified in fecal samples of broiler breeder hens, although it is unclear if it contributes to antibiotic resistance in poultry production (Munoz-Price and Weinstein, 2008; Karlsson et al., 2013; Trudeau et al., 2020).

A dose-dependent effect of metformin was observed in three genera that were all most abundant in 75 mg/kg compared to 25 mg/kg metformin; *Angelakisella*, *Dorea*, and *Herbaspirillum*. *Herbaspirillum* is a member of the *Oxalobacteraceae* family, which was a discriminating taxon loading in the PCA to distinguish both metformin dose and metformin compared to 0 mg/kg control. This suggests that *Oxalobacteraceae* may be strongly affected by metformin treatment with a dose-dependent effect. As neither the *Oxalobacteraceae* nor the genus *Herbaspirillum* have been previously associated with metformin treatment, this may be a species- or environment-specific finding. *Dorea*, a member of the *Lachnospiraceae* family, has been previously observed to be decreased in rodent models of PCOS in addition to associated with metformin treatment (Sun et al., 2018; Zhang et al., 2020; Rizk and Thackray, 2021). *Angelakisella* has not been previously associated with metformin or broiler breeder hens, but the family *Ruminococcaceae* is a well-established marker of dysbiosis that is consistently found to be depleted in irritable bowel disease, colorectal cancer, and human models of PCOS (Wirbel et al., 2019; Yurtdaş and Akdevelioğlu, 2019; Brüssow, 2020; Rizk and Thackray, 2021). Thus, some taxonomic findings seem to be species-specific while others are well-known players of metabolic disease and metformin treatment. While we hypothesized that metformin would affect the relative abundance of *Akkermansia*, such as has been documented in human studies, *Akkermansia* was not among the bacterial genera that was significantly changed by metformin treatment (Rodriguez et al., 2018). This may be because *Akkermansia* is not well documented in chicken microbiomes and may be more specific to the human gastrointestinal tract (Rychlik, 2020).

In addition to an impact on the gut microbiome, metformin supplementation at 75 mg/kg body weight was associated with a significant decrease in the body weight and accretion of abdominal adipose tissue, a normalization of the ovarian follicular hierarchy, a significant increase in the number of eggs laid/hen over the treatment period and an improved plasma endocrine profile of

reproductive hormones (Weaver and Ramachandran, 2022). A limitation of this study is that individual correlations between bacterial genera and production metrics were not possible since most production metrics were collected in aggregate. Overall, metformin treatment in broiler breeder hens impacted the gut microbiome composition (beta diversity) but not evenness (alpha diversity) in a dose-dependent manner, and several taxonomic findings were consistent with prior human studies. This suggests that the effects of obesity, PCOS, and metformin are not completely specific to the host species or environment, and that there may be direct effects of metformin on bacterial in the gastrointestinal tract.

We found that the microbial community composition in hens treated with higher doses of metformin (75 mg/kg body weight) were distinguished from lower doses (25 mg/kg and 50 mg/kg body weight). We observed that the gut microbiome did not change throughout the peak and decline of the production cycle, since there were few differences in alpha and beta diversity between 40, 50, and 60 weeks of age. Several bacterial genera were identified that were affected by metformin, including members of the *Clostridia* and *Ruminococcaceae* family which have been implicated in PCOS, type 2 diabetes, and metformin treatment (Karlsson et al., 2013; Wilkins et al., 2019; Rizk and Thackray, 2021). As metformin treatment also resulted in decreased body weight and increased egg production, we hypothesize that metformin-mediated modulation of the gut microbiome may contribute to beneficial shifts in metabolism and reproduction. Furthermore, given the dose effect we observed of only the 75 mg/kg metformin treatment, we postulate that a higher dose of metformin may be necessary to observe microbiome-mediated physiological effects. However, the novelty and relatively small size of this trial precludes drawing strong conclusion and husbandry recommendations. While future research is necessary to unravel the mechanisms underlaying host-microbe-metformin interactions, this study furthers our knowledge of the effects of metformin on the gut microbiome.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA826088, https://github.com/gandalab/broiler-breeder-metformin.

Ethics statement

The animal study was reviewed and approved by Pennsylvania State University's Institutional Animal Care and Use Committee.

Author contributions

EW and RR devised the feeding trial. ES and EG conducted sequencing. EVS, EG, and JS performed data analysis. All authors contributed to manuscript preparation and revision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1000144/full#supplementary-material

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The active core microbiota of two high-yielding laying hen breeds fed with different levels of calcium and phosphorus

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The nutrient availability and supplementation of dietary phosphorus (P) and calcium (Ca) in avian feed, especially in laying hens, plays a vital role in phytase degradation and mineral utilization during the laying phase. The required concentration of P and Ca peaks during the laying phase, and the direct interaction between Ca and P concentration shrinks the availability of both supplements in the feed. Our goal was to characterize the active microbiota of the entire gastrointestinal tract (GIT) (crop, gizzard, duodenum, ileum, caeca), including digesta- and mucosa-associated communities of two contrasting high-yielding breeds of laying hens (Lohmann Brown Classic, LB; Lohmann LSL-Classic, LSL) under different P and Ca supplementation levels. Statistical significances were observed for breed, GIT section, Ca, and the interaction of GIT section x breed, P x Ca, Ca x breed and P x Ca x breed ($p < 0.05$). A core microbiota of five species was detected in more than 97% of all samples. They were represented by an uncl. *Lactobacillus* (average relative abundance (av. abu.) 12.1%), *Lactobacillus helveticus* (av. abu. 10.8%), *Megamonas funiformis* (av. abu. 6.8%), *Ligilactobacillus salivarius* (av. abu. 4.5%), and an uncl. *Fusicatenibacter* (av. abu. 1.1%). Our findings indicated that Ca and P supplementation levels 20% below the recommendation have a minor effect on the microbiota compared to the strong impact of the bird's genetic background. Moreover, a core active microbiota across the GIT of two high-yielding laying hen breeds was revealed for the first time.

KEYWORDS

laying hens, core intestinal microbiota, functional prediction, active community, dietary treatment

Abbreviations: %, percentage; ASV, amplicon sequencing variant; av. abu, average relative abundance; Ca, calcium; Cae, caeca; Cr, crop; D, duodenum; g, gramm; G, gizzard; GIT, gastrointestinal tract; GS, gastrointestinal section; I, ileum; LB, Lohmann brown-classic; LDA, linear discriminant analysis; LSL, Lohmann LSL-classic; P, phosphorus; Br, breed; SCFA, short-chain fatty acid; uncl, unclassified.

Introduction

The laying hen gastrointestinal tract (GIT) microbiota consists of a complex community of diverse microorganisms. The host influences the composition of the microbial community, which may have effects on the immune system, nutrient digestion, and regulation of intestinal physiology (Stanley et al., 2014; Agus et al., 2018; Khan et al., 2020). Depending on the diet and nutrient supplementation, variations in microbial composition can be observed (Leeming et al., 2021). Moreover, it is essential to understand the interrelation between diet, microbiota, and host when investigating how they contribute to animal health.

Diets are formulated to fulfil the needs of the animals, and the specifically required nutrient concentrations are dependent on the host age, physiological status, and level of performance. Among required minerals, phosphorus (P) and calcium (Ca) are vital because of their function in avian biochemical pathways and bone and eggshell development (Selle et al., 2009). However, P supplements are costly and negatively impact the environment when accumulated in the excreta of the animals. This has stimulated research on hydrolysis of phytate, which is the main binding form of P in plants, in poultry's digestive tract and variation in the level of P supplementation (Rodehutscord et al., 2022). The influence of age, genotype and experimental design variations affect the results' comparability (Kebreab et al., 2009; Ahmadi and Rodehutscord, 2012; Deusch et al., 2015; Forgie et al., 2019). The Ca concentration of the feed is related to P, and in laying hens, the highest Ca requirement is during the laying period (Kebreab et al., 2009; Ahmadi and Rodehutscord, 2012). In this phase, the animal requirements must be fulfilled to maintain animal health and performance. Digested and undigested dietary compounds influence the microbial population in the GIT, which modifies the host intestinal integrity and improves pathogen resistance (Forgie et al., 2019). Moreover, there is a microbial distinction between mucosa and digesta samples (Deusch et al., 2015; Waite and Taylor, 2015). Mucosa samples of the gastrointestinal tract have shown higher microbial diversity than digesta samples (Borda-Molina et al., 2016). The complex microbial diversity in both sample types consists of hundreds of species across different phyla, inhibiting a clear understanding of GIT variations (Borda-Molina et al., 2016).

Little is known about the dynamics and influence of common active bacteria on the GIT of laying hens. Therefore, the microbiota's response to a specific challenge and environment by targeting the active community has to be reflected. Despite showing similar diversity to total communities, the microbial taxa composition is significantly different (Bastida et al., 2017). Shade and Handelsman (2012) defined that the core microbiome consists of shared microbial members within similar habitats and across complex microbial assemblages. Furthermore, a core microbiome is present and interacts in the entire GIT. In addition, transient or resident bacteria can be considered a core microbiome. It is an approach to understanding, adjusting, and optimizing microbial functions in individuals or complete ecosystems (Heumann-Kiesler et al., 2021; Hofmann et al., 2021). Knowledge about microbial changes across different GIT sections can help understand specific processes, e.g., food fermentation or predicting and controlling the microbiome (Giraffa, 2004; Stegen et al., 2018; Berg et al., 2020).

This study aimed to evaluate the impact of different concentrations of P and Ca on the active microbiota of the GIT (crop, gizzard, duodenum, ileum, caeca) of two high-yielding laying hen breeds and determine how the host genetic background and dietary changes influence the resident core microbiota.

Materials and methods

Sample collection, DNA extraction, and illumina library preparation

This research complements and extends recent publications (Sommerfeld et al., 2020; Heumann-Kiesler et al., 2021; Hofmann et al., 2021). Samples originated from an animal trial fully described by Sommerfeld et al. (2020). The study was approved by the Regierungspräsidium Tübingen (approval number HOH50/17 TE) and conducted following animal welfare regulations. Animals were housed at the University's Agricultural Experimental Station (Unterer Lindenhof, Eningen, Germany).

A total of 80 laying hens of the breeds Lohmann brown-classic (LB) and Lohmann LSL-classic (LSL) were used in this study. Upon the arrival of the hatchlings at the farm, birds were raised together under the same conditions (floor pens, deep litter bedding on wood shavings, and diets). At 27 weeks, ten hens per breed were allocated to four dietary treatments in a randomized design and kept individually in metabolism units. The individuals received water and feed for ad libitum consumption for 3 weeks. Soybean meal and corn-based diets were supplemented to reach a standard (5.3 g/kg dry matter (DM); P^+) or reduced (4.7 g/kg DM; P^-) P concentration and a standard (39.6 g/kg DM; Ca^+) or reduced (33.9 g/kg DM; Ca^-) Ca concentration. Diets ingredient compositions are fully described in Sommerfeld et al. (2020).

At 31 weeks of life, birds were stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 and sacrificed by decapitation. The crop (Cr), gizzard (G), duodenum (D), ileum (I) and caeca (Cae) were longitudinally opened, digesta was obtained with a sterile spoon, and after a cleaning step with sterile phosphate-buffered saline solution, the mucosa was collected by scratching it with a sterile glass slide. Collected samples were immediately stored in RNA later at −80°C until further analysis. RNA of a total of 800 samples were extracted using Trizol (Invitrogen Inc.,

Waltham, United States) according to the manufacturer's instructions with a preliminary step of bead beating (30 s, 5.5 m/s) in a FastPrep instrument (MP Biomedicals, Eschwege, Germany). RNA was quantified with Nanodrop (ThermoFisher Scientific, Waltham, United States) and stored at −80°C until further analysis. RNA samples were treated with the DNase kit (Invitrogen), and cDNA synthesis was performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).

Sequencing libraries were made according to the protocol described by Borda-Molina et al. (2020). All PCR reactions were done with PrimeSTAR® HS DNA Polymerase kit (TaKaRa, Beijing, China). The first two PCR were prepared in a total volume of 25 µl using 1 µl of cDNA template, 0.2 µM of each primer and 0.5 U Taq prime start HS DNA and the third PCR was set up in a total volume of 50 µl. An initial denaturation at 95°C for 3 min was followed by ten cycles (first and second PCR) or 20 cycles (third PCR) of denaturation at 98°C for 10 s, annealing at 55°C for 10 s and an extension at 72°C for 45 s and a final extension of 72°C for 2 min. PCR products were purified and standardized using SequalPrep Normalization Kit (Invitrogen Inc., Waltham, United States) and sequenced using 250 bp paired-end sequencing chemistry on Illumina Novaseq 6000.

Bioinformatics and statistical analysis

The bioinformatic analysis was performed with Mothur v1.44.3 (Schloss et al., 2009). Raw reads (forward and reverse fastq file) were assembled with make.contigs function. Reads with ambiguous bases, with homopolymers (>8) and longer than 354 bp were removed. A total of 678 samples passed this filtering and were used for downstream-analysis. Sequences were aligned to the silva.seed v1.38.1 (Quast et al., 2013). Chimeras were identified using vsearch (Rognes et al., 2016) and removed from the dataset. Sequences were classified using the Bayesian classifier and the Silva reference and taxonomy set silva.seed v1.38.1. The output was filtered to get the amplicon sequencing variants (ASVs) with a minimum of 50 reads across all samples resulting in 6179 ASVs. An average of 34.566 ± 17.567 reads were obtained per sample. The cut-off for bacterial taxonomy classification followed the recommendations of Yarza et al. (2014). Digesta and mucosa samples have been merged for further analysis per section and considered gastrointestinal tract sections. Sample reads were standardized, and a sample-similarity matrix based on the Bray-Curtis similarity coefficient (Bray and Curtis, 1957) was created using Primer6 (Clarke and Warwick, 2001). PERMANOVA routine was used to study the significant differences and interactions between groups and diets (Clarke and Warwick, 2001). Steel-Dwass test was performed to compare means of relative abundance data between genera and breed (Br), gastrointestinal tract section (GS), and Ca/P level combinations using JMP®Pro (Version 16.1 SAS Institute Inc., Cary, NC, 1989–2021). P-values based on ANOSIM results were adjusted using the Benjamin-Hochberg correction (FDR). The core microbiota across all samples was identified with the phyloseq and microbiome library in R v4.1 (McMurdie and Holmes, 2013; Lahti et al., 2017). ASV table, taxonomy information, and metadata were combined in a phyloseq file. Groups were subset according to the metadata (diet, GS and Br) to create a phyloseq file for each combination of the three factors. All phyloseq files of all groups were standardized by ASVs. The detection level of core members was set to 0.01% of abundance and a prevalence of 97% across all samples. The output ASV list was compared between all groups to determine the common ASVs, and venn diagrams were drawn with the InteractiVenn tool (Heberle et al., 2015).

The Shannon diversity index and richness were calculated using the phyloseq library in R v4.1. LDA scores were analyzed with microbiomeAnalyst (Chong et al., 2020). Data filter and normalization were set to default. P-values threshold was set to $p = 0.05$ and the FDR correction was applied. LEfSe-graphs were built with the build-in graph builder (Segata et al., 2011).

Functional prediction was performed in R with the latest version of Tax4Fun2 v1.1.5 (https://github.com/bwemheu/Tax4Fun2). Bacterial genomes detected on the microbiota dataset were downloaded from the NCBI database, and a reference database was created to improve functional accuracy. Functional predictions were then performed using the reference file and the ASV table of all samples. The threshold for clustering (uclast) was set to 100%, and the number of 16S rRNA copies were normalized and calculated for each ASV.

Results

Experiment evaluation

The overall microbiota consisted of 6179 ASVs, where 2272 ASVs were shared by all GIT sections, breeds, and dietary treatments. LSL samples shared 2868 and the LB 2970 (Figure 1). The number of unique ASVs varied from 61 to 284, depending on the breed and GIT section. Moreover, the breed comparison of each GIT section revealed that many ASVs were unique for each breed (Supplementary Figure S1).

According to the sequencing data, the microbiota of all samples consisted of Firmicutes (average relative abundance [av. abu]) of 84.5% in LSL and 76.7% in LB ($p < 0.05$), followed by Bacteriodetes, which was more abundant in LB (18.2%) in comparison to LSL (10.7%) ($p < 0.05$) (Supplementary Figure S2A). The most abundant genera were *Lactobacillus* (25.1% LSL; 17.4% LB), followed by uncl. Lactobacillaceae (21.2% LSL, 8.2% LB), uncl. Lachnospiraceae

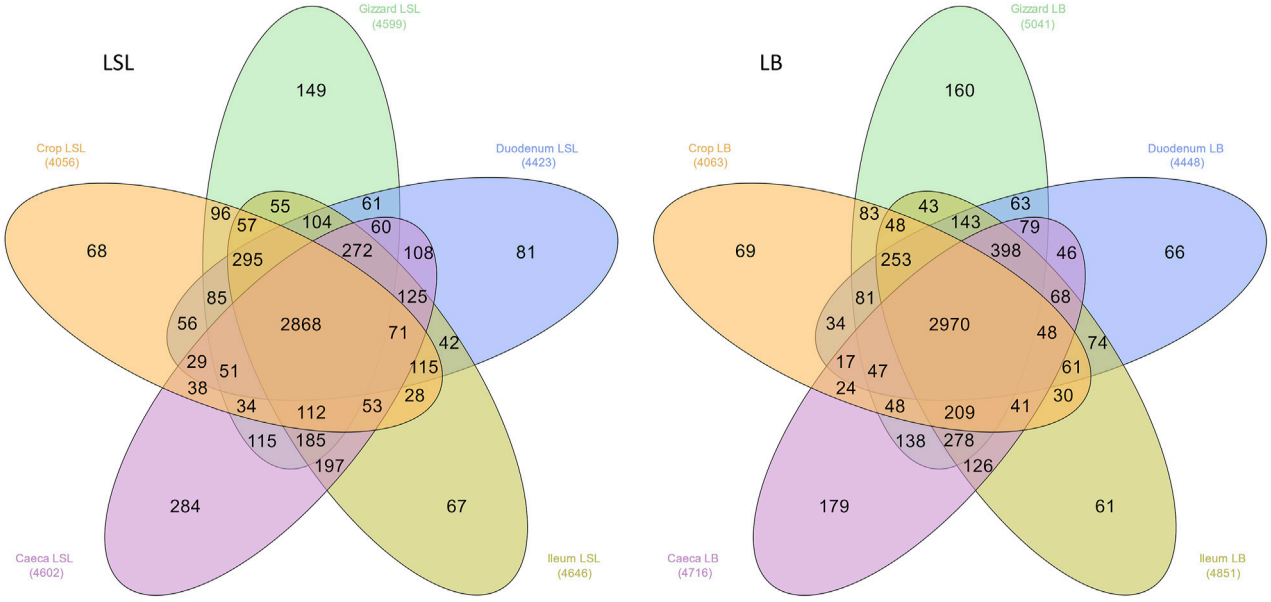

FIGURE 1
Distribution of the total number of ASVs among GIT sections across all samples in both breeds. The number in parenthesis is the observed number of ASVs in each group.

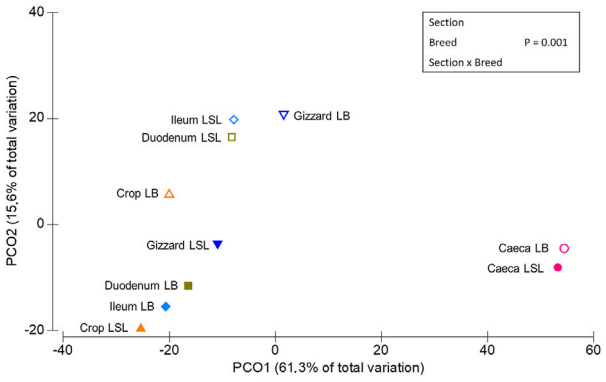

FIGURE 2
Multidimensional scaling of centroids showing the similarities among the sample types derived from sample combinations of GIT section x breed.

(10.8% LSL, 13.5% LB), and *Ligilactobacillus* (7.9% LSL, 12.5% LB). These genera reached an average relative abundance of more than 50% across all samples (Supplementary Figure S2B). Additionally, significant differences were found between breeds and GIT sections within the breeds (Supplementary Table S1).

PERMANOVA routine was used to study the overall significant differences and interactions between GIT sections, laying hen breeds, P and Ca supplementation. A statistical significance on ASV level was reached for each factor alone ($p < 0.03$) and the interactions between Br x GS, Br x Ca, P x Ca, P x Ca x Br ($p < 0.03$). A trend was observed for Br x P ($p = 0.09$) (Supplementary Table S2). The principal coordinates analysis plot revealed three clusters (Figure 2), one comprising the LSL samples of crop, gizzard, duodenum and ileum, another with those same samples but for the LB breed and a third one with the caeca samples of both breeds.

In crop samples, significant effects of the breed and Ca and a trend for the interactions of Br x Ca ($p < 0.08$) were observed. The gizzard, duodenum and ileum microbiota were significantly affected by the breed ($p < 0.05$). In the caeca, significant effects of the breed, P/Ca supplementation, the interactions of Br x Ca, Ca x Br, P x Ca x Br ($p < 0.03$) and a trend for P x Br were detected ($p < 0.08$). All significant interactions are provided in Supplementary Table S2.

Pairwise comparisons evaluating the Ca and P supplementation effects on the breed and GIT section, exhibited significant effects, depending on the GIT section. For an overview, see Supplementary Table S3. A significant difference was detected regarding P supplementation for LB caeca P^+ vs. P^- ($p < 0.01$). An effect of the Ca supplementation was observed in both breeds. In LB, a significant difference was identified in crop Ca^+ vs Ca^- ($p = 0.02$) and caeca Ca + vs Ca- ($p < 0.01$) was revealed. For LSL, significant differences were observed in caeca Ca^+ vs Ca^- ($p < 0.01$). However, the strongest effect was driven by the breed rather than GIT section, Ca or P supplementation levels. The breed effect is clearly shown in caeca samples (Supplementary Figure S3),

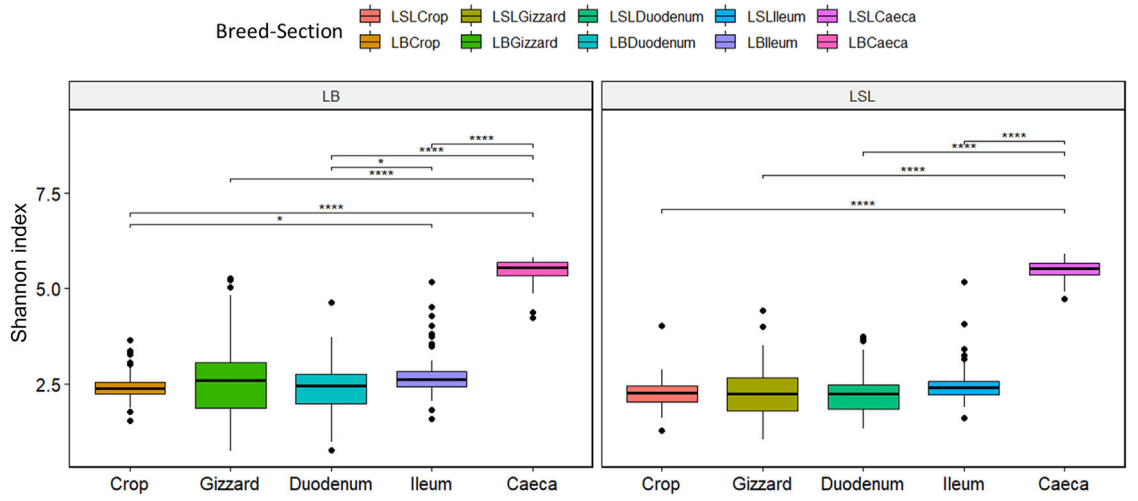

FIGURE 3
Boxplot of Shannon diversity index separated by the breed, section (color) and Ca/P combination of the diet (*$p < 0.02$; ****$p < 0.001$).

and all significant p-values are shown in Supplementary Table S3.

The LB showed significantly higher overall Shannon diversity (3.09) than LSL (2.93). A statistical significance between caeca and all GIT sections was observed for both breeds ($p < 0.05$). For the LB additional significances were observed between ileum and crop and ileum and duodenum. ($p < 0.03$) (Figure 3). Regarding the diet, the Shannon index differed depending on the GIT section and breed combination. Still, no statistical significance was observed between diets, with the highest index observed in caeca (Supplementary Figure S4).

Functional prediction

A total of 322 pathways and 7516 functions were assigned to the samples. Thirty KEGG pathways contributed to more than 50% of the total pathways across all samples and revealed significant differences between breeds and/or GIT sections of the same breed. These thirty KEGG pathways belonged to twelve second-level KEGG functional categories. The global/overview metabolism map was the most enriched function, followed by membrane transport metabolism and signal transduction. Significant effects in the caeca were observed for the breed and the interaction of Br x P ($p < 0.05$) (Supplementary Table S4). Two of the top 30 pathways [ko02010 (ABC transporters) and ko00190 (oxidative phosphorylation)] showed significant breed effects ($p < 0.05$). Despite the significance of breed × P interaction, only one inositol related individual function [K06607 (myo-inositol catabolism protein IolS)] showed differences in LSL (Supplementary Table S4). Regarding Ca supplementation and its effect on the caeca, a significant difference was detected for the myo-inositol catabolism protein IolS (K06607, $p = 0.01$) in LSL, and scyllo-inositol 2-dehydrogenase ($NADP^+$) (K22230, $p < 0.05$) in LSL and LB. In addition five other inositol related functions show breed effects (Supplementary Table S4).

Core Microbiota

A total of five ASVs were present in 97% of all samples (Figure 4). The core microbiota was represented by an uncl. *Lactobacillus* (ASV62, av. abu. 12.1%), *Megamonas funiformis* (ASV63, av. abu. 6.8%), *Ligilactobacillus salivarius* (ASV 137, av. abu. 4.5%), *Lactobacillus helveticus* (ASV197, av. abu. 10.8%) and uncl. *Fusicatenibacter* (ASV 561, av. abu. 1.1%). Except for the gizzard of LB and caeca of both breeds, the five bacteria accounted for 25%–71% of the total community (Supplementary Table S5). Uncl. *Lactobacillus* was more abundant in LSL compared to LB in all GIT sections (Supplementary Table S5). The highest abundance of *Megamonas funiformis* (ASV63) was observed in the crop of both breeds (Supplementary Table S5). *Ligilactobacillus salivarius* (ASV137) had the highest abundance in the crop and the lowest in the caeca. Furthermore, it was present in higher abundance in LB than LSL (Supplementary Table S5). Also, significant differences were shown between breeds in crop and between GIT sections within the breeds ($p < 0.05$, Supplementary Table S5). *Lactobacillus helveticus* (ASV197) was more abundant in all GIT sections of LSL, with the highest average relative abundance in the ileum, followed by duodenum and crop (Supplementary Table S5). Additionally, significant differences between breeds in all GIT sections ($p < 0.05$, Supplementary Table S5). Uncl. *Fusicatenibacter* (ASV561)

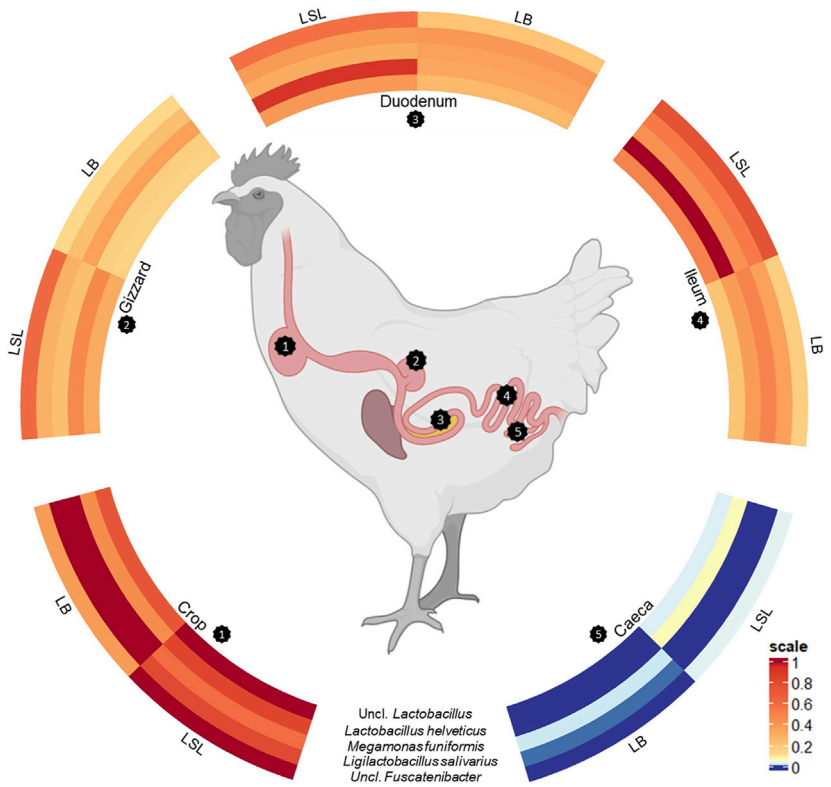

FIGURE 4
Scaled circulized heatmap of the five core microbiota separated by the GIT sections (crop, gizzard, duodenum, ileum, and caeca) and breed (LSL, LB).

was detected in very low abundances across the gastrointestinal tract (Supplementary Table S5). Moreover, significant differences existed between breeds and GIT sections within the breeds ($p < 0.05$, Supplementary Table S5).

The effect of P and Ca supplementation on the genera distribution and the core microbiome across the gastrointestinal tract

The Ca supplementation affected the microbial composition in LB crop ($p < 0.05$), and significant effects were found for the genus uncl. Lactobacillaceae and *Streptococcus* ($p < 0.01$) (Supplementary Figure S5). Further, the average relative abundance of uncl. Lactobacillaceae increased while *Streptococcus* decreased with Ca supplementation in the diet. Despite the higher diversity of the caeca, fewer differences at genus level were observed for Ca supplementation. Significant changes in LSL were observed for uncl. Bacteroides, uncl. Lachnospiraceae, *Ligilactobacillus* and *Megasphaera* in LB ($p < 0.10$) (Supplementary Figure S5). The average abundance of all genera increased by supplementing Ca except for uncl. Lachnospiraceae.

Significant shifts in the genera *Helicobacter,* uncl. Gammaproteobacteria, and uncl. Prevotellaceae and the trends for *Lachnoclostridium* and *Megasphaera* supported the significant P effect in LB caeca (Supplementary Figure S5). In addition, P supplementation increased the average abundance of uncl. Prevotellaceae, *Helicobacter,* and *Lachnoclostridium* while decreasing *Megasphaera* and uncl. Gammaproteobacteria.

LEfSe-analysis revealed the 25 most significant discriminant ASVs for breed and diet based on the average abundance across the factors combination (breed x diet). Even if no significance for those ASVs was revealed by comparing the dietary groups within the breeds, the average relative abundance changes across the breed x diet combinations. Eleven ASVs were assigned to a species (*Lactobacillus kitasatonis, Ligilactobacillus aviarius, Lactobacillus helveticus, Ligilactobacillus agilis, Megamonas funiformis, Bifidobacterium longum, Sutterella timonensis* and *Negativibacillus massiliensis*) and additional eight were assigned to a genus, the rest remained unclassified at lower taxonomic levels (Figure 5). Additionally, two ASVs belong to the core microbiota (ASV62, ASV197) and were more abundant in LSL

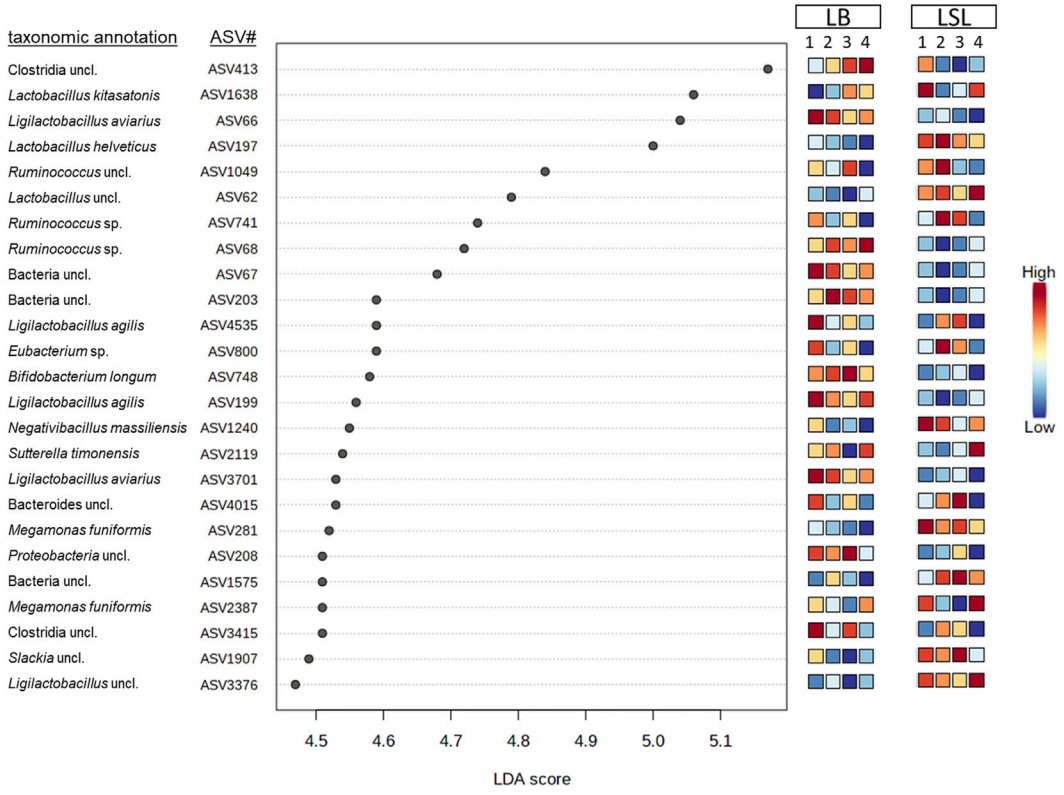

FIGURE 5
Discriminant analyses of the 25 most significant ASVs in caecal samples based on a LEfSe analysis showing the impact per diet (1: P^+Ca^+, 2: P^-Ca^-, 3: P^+Ca^-, 4: P^-Ca^+) and breed. The scale indicates the relative abundance in comparison to the average across the eight groups consisting of both breeds and the four diets.

compared to LB. Bacterial shifts were revealed across diets for each breed, either increasing or decreasing abundance and between the breeds, where some ASVs show higher relative abundance in one breed compared to the other. These results showed that the breed is the primary driver of microbial composition, followed by the GIT section and Ca/P supplementation.

Discussion

GIT microbiota in poultry is influenced by many exo- and endogenous factors such as animal age, stress, genotype, or diet (Wickramasuriya et al., 2022). Whereas the microbiome in broilers is extensively researched, knowledge about laying hens is scarce, especially the microbiota description along the whole GIT. Microbiota stimulates the immune system, contributes to host nutrition and pathogen inhibition, synthesizes amino acids and vitamins, and has a role in breaking down complex molecules and potential toxic feed components (Borda-Molina et al., 2016). Changes in microbiota composition, either by feed, disease or other external factors, can affect these functions; thus, its understanding and characterization are of primary importance. Therefore, this study aimed to identify differences in the active microbiota composition along the GIT including digesta and mucosa in two commercial breeds of laying hens fed diets with dietary Ca and P concentrations 20% below the recommended levels.

Among the factors studied in the present work, the breed had the most significant effect on the microbial community, leading to fluctuations in relative abundance on every taxonomic level across the complete GIT. Consistently, breed disparities have been reported in caecal samples of a recent study comparing Hy-Line W36 and Hy-Line Brown (Adhikari et al., 2020). Depending on the diet, such breed-related changes might be due to differences in body weight and average daily feed intake between breeds. Moreover, both breeds have different mechanisms regarding P absorption (Abudabos, 2012) and the significantly higher concentrations of inositol-6 phosphate and inositol-5 phosphate in LB gizzard and caeca (Sommerfeld et al., 2020) might be due to breed-dependent impacts of P, which results in changes in the GIT microbial community.

Previous studies have only characterized the microbiota of single sections of the GIT or feces and showed similar results at phylum and genus levels, as reported here (Stanley et al., 2012; Simon et al., 2016; Ding et al., 2017; Adhikari et al., 2020; Schreuder et al., 2020; Khan and Chousalkar, 2021; Su et al., 2021; Xiao et al., 2021). The use of different breeds also didn't affect the overall picture of the microbiota, being the main bacterial groups detected across all studied breeds (Ding et al., 2017; Huang et al., 2019; van der Eijk et al., 2019). There is still a discussion on whether richness in microbiome composition is positively (Stanley et al., 2012; Stanley et al., 2014; Yan et al., 2017) or negatively (Siegerstetter et al., 2017) correlated to animal health. The present study found the highest diversity in the caeca, followed by the duodenum and ileum, with statistical differences between breeds. The highest diversity in caeca is consistent with previous studies (Borda-Molina et al., 2016; Fu et al., 2018).

Besides the differences in diversity index, the animal breed affected phyla abundance and species distribution, which was previously reported in broilers (Paul et al., 2021). We detected fewer Firmicutes and higher levels of Bacteroidetes in LB than in LSL. Khan et al. (2021) reported that a lower abundance of Firmicutes in laying hens is associated with a decrease in certain bacteria, including *Peptostreptococcus* (Khan and Chousalkar, 2021) which is contrary to the recent study, where LB with lower abundances of Firmicutes compared to LSL showed no decrease in *Peptostreptococcus*. On the other hand, Bacteroidetes was significantly higher in LB and an increased abundance of Bacteroidetes has been associated with later stages of the laying phase, where the abundance of Firmicutes decreases and Bacteroidetes overtakes (Joat et al., 2021).

One of our aims was to identify the effect of lower supplementation of Ca and P in the GIT, because an insufficient supply of one or both minerals might reduce animal growth and bone mineralization due to interference with homeostasis (Shafey et al., 1990) and change the microbial community of the laying hens. Members of *Ligilactobacillus*, *Megasphaera*, Lachnospiraceae, Bacteroides, *Helicobacter*, Prevotellaceae, *Lachnoclostridium, Streptococcus* and Lactobacillaceae were affected by the diets. The relative abundance of Lachnospiraceae decreased with Ca supplementation, which might have a negative impact to gut health as members of Lachnospiraceae are related to the production of butyrate, crucial for the metabolism of the epithelial tissue (Biddle et al., 2013). The genus *Megasphaera* is known to be part of the SCFA production in the caeca of laying hens (Gan et al., 2020). In our study, the higher Ca supplementation was causing a decrease in this genus's abundance and might have reduced the SCFA production in LSL. *Ligilactobacillus* and other members of the family Lactobacillaceae are known colonizers of the GIT of laying hens (Forte et al., 2018). In this study, their prevalence changed depending on Ca and P supplementation, breed and GIT section. Members of these genera are usually associated with improved GIT health, productive performance and regulators of the immune system (de Cesare et al., 2017; Forte et al., 2018). In addition, *Streptococcus* is closely related to productive performance with negative correlations to feed conversion ratio (Gan et al., 2020). Higher levels of ASVs belonging to this genus were observed in LB hens supplemented with higher Ca levels and that had probably led to the reduced average daily feed intake under the same conditions in this breed (Sommerfeld et al., 2020). Moreover, in a companion study that used the same hens, P^- affected the immune system by increasing immune cell numbers and mitogen-induced response of innate and adaptive immune cells (Hofmann et al., 2021). In contrast, the relative abundance of potential pathogen *Helicobacter* increased with higher levels of P in the diet, which could have indicated some effect on the immune system (Fox, 1997; Miao et al., 2020); however, the numbers of T cells and $CD4^+$ increased in the same hens (Hofmann et al., 2021).

Most of the top 25 discriminant ASVs had higher relative abundances in LB compared to LSL, depending on the feature and the fed diet. Finally, the impact of the diet on the microbial composition showed that the offered diets were not challenging the laying hens GIT microbiota. Jing et al. (2018) reported that a reduction to 0.15% available P in the feed was not affecting growth, productive performance, and mRNA expression of P transporters in hens. It was assumed that a lower P and Ca supplementation might lead to functional shifts, as this was observed in a study with probiotic supplementation compared to a standard diet (Iqbal et al., 2021). But, the predicted functional pathways revealed no overall direct influence of P and Ca in the present study.

Previous studies in layers revealed that members of Lactobacillaceae, Bacteroidaceae, Lachnospiraceae, Ruminococcaceae, Veilonellaceae, Prevotellaceae, Clostridiaceae, Rickenellaceae, or Enterobacteriaceae account for the core microbiota (Videnska et al., 2014; Ngunjiri et al., 2019). However, none of the studies combined the information across the complete GIT or targeted the active microbiota. In the present study, five core bacteria were detected across 97% of the samples; uncl. *Lactobacillus*, *Megamonas funiformis*, *Ligilactobacillus salivarius*, *Lactobacillus helveticus* and uncl. *Fusicatenibacter*. Considering the high number of samples (n = 678) and the microbiota variation across the GIT, with common colonizers appearing or not in each GIT section digesta and mucosa, the likelihood of finding a core microbiota across all samples decreases (Johnson et al., 2018; Lee et al., 2019; Clavijo et al., 2022). In addition, the detection limit to classify a bacteria as a core member was set to its presence in more than 97% of the total sample number. This percentage is higher than the 50% coverage in Clavijo et al. (2022) and the 75% in Ngunjiri et al. (2019).

All core members are associated with animal health improvement and gut homeostasis. The genus *Lactobacillus* involves host-adapted lactic acid bacteria that colonize the digestive tract of humans and animals (Zheng et al., 2020) and is part of the core microbiome in the ileum and caeca of laying

hens (Videnska et al., 2014; Ngunjiri et al., 2019). A beneficial effect on egg size and weight induced by *Lactobacillus* cultures as probiotics was reported (Volf et al., 2021); however, in this study, LSL layers colonized with higher abundances of *Lactobacillus* had lighter egg weights (Sommerfeld et al., 2020). Previous studies have reported *M. funiformis* as a hydrogen consumer in laying hen's caecal microbiome (Zheng et al., 2020; Volf et al., 2021). It is a characteristic bacterium in adult hens (Volf et al., 2021) and accounted for the core microbiota in a recent broiler study (Clavijo et al., 2022). In our study, *M. funiformis* was found in higher abundance in crop, ileum, duodenum and gizzard samples and almost disappeared in the caeca, which is partially in contrast to the findings of Gan et al. (2020) as they observed the genus *Megamonas* in higher abundances in caeca. The genus *Megamonas* has been previously described in ducks and humans as an important fermenter of glucose into acetate and propionate, which provide health benefits to the host (Chevrot et al., 2008; Sakon et al., 2008). It can be postulated that *M. funiformis* fermented glucose mainly in the upper digestive sections and was displaced in the caeca by other SCFA-producing bacteria. Further, *L. salivarius* is commonly isolated from the intestine or faeces of birds and was part of the core microbiome in a recent laying hen study (Ngunjiri et al., 2019). Their response to food-borne pathogens by an antibacterial activity influences the host immune system and the microbial composition (Messaoudi et al., 2013). The LSL hens had a higher abundance of *L. salivarius,* and higher amounts of leukocytes, thrombocytes, monocytes, T cells, T helper cells, and cytotoxic T than LB (Hofmann et al., 2021), which might be a response of the host system to potential pathogens or a breed-dependent reaction to the housing conditions (Moe et al., 2010). *L. helveticus* is an early colonizer of the broiler GIT (de Cesare et al., 2017). Besides the function in pathogen reduction, this bacteria correlated positively with Ca absorption and bone metabolism *in vitro* (Narva et al., 2004). Overall, *L. helveticus* was less abundant in the crop than duodenum and ileum, with main differences between the GIT section of each breed, specifically in LSL. Moreover LSL might be more sensitive to stress, resulting in a more intense immune response and increased blood components (Hofmann et al., 2021) and the potential pathogen reduction and a decrease in stress-induced symptoms can be a breed-related effect. Uncl. *Fusicatenibacter* belongs to the family Lachnospiraceae and was previously associated with host GIT health (Biddle et al., 2013), and detected in the ileum and caeca of laying hens (van der Eijk et al., 2019) with a constant presence from day 1 to week 40 (Asakura et al., 2021). A recent study, using metagenomic analysis, showed several protologues for new candidatus *Fusicatenibacter* (Gilroy et al., 2021), this bacterial group was more abundant in crop and might be involved in the first steps of feed digestion together with *M. funiformis*. The taxonomic core microbiota are microorganisms of a dataset that are postulated to indicate inherent functional relationships with the host. They have the potential to be targeted for culturing and other omics analyses and can be used towards understanding the functional meaning of the core to the laying hen (Neu et al., 2021). The knowledge of the active core microbiota further develops hypotheses about their role within the microbiome.

For the first time, the current study presents data on the active microbiota associated with the whole GIT of two high-yielding laying hen breeds and the core active microorganisms detected in more than 97% of the samples. Significant differences in the microbiota composition were observed between the breeds which was unexpected to such an extent as hens were housed in the same stable, under the same conditions at the same time. Furthermore, we showed that a reduction of circa 20% of Ca and P concentration in the feed compared to the current standard had no effect on microbiota distribution and predicted functions.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB52942

Ethics statement

The animal study was reviewed and approved by the Regierungspräsidium Tübingen, Germany (approval number HOH50/17 TE).

Author contributions

MR, JS, and AC-S conceived and designed the experiments, TS and CR performed laboratory analysis, CR and AC-S analyzed the data and wrote the paper. All authors reviewed the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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16S rRNA gene-based assessment of common broiler chicken sampling methods: Evaluating intra-flock sample size, cecal pair similarity, and cloacal swab similarity to other alimentary tract locations

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16S rRNA gene sequencing for characterization of microbiomes has become more common in poultry research and can be used to both answer specific research questions and help inform experimental design choices. The objective of this study was to use 16S rRNA gene sequencing to examine common sampling practices in broiler chicken studies such as: the required number of birds selected from a flock to adequately capture microbiome diversity, the differences between cecal pairs within the same bird, and whether cloacal swabs are representative of other alimentary tract (AT) locations. To do this, nine market age broilers were euthanized and immediately sampled in ten AT locations: crop, gizzard, proventriculus, duodenum, jejunum, ileum, cecal samples from each pouch, colon, and cloacal swab. DNA was extracted and subjected to 16S rRNA gene amplification and sequencing. Each location within the broiler AT hosts distinct microbial communities. When each sampling location was considered, it was found that sampling after 2.8 birds (range 2–4) resulted in less than 10% new amplicon sequencing variants (ASV) being added while sampling after 7.6 birds (range 6–10) increases new observed ASVs by less than 1%. Additionally, when cecal pairs from the same bird were evaluated, it was found that cecal pair mates are an adequate replication if interested in the total cecal microbiome but may be less useful if a rare lineage is of interest. Furthermore, when compared to other AT locations, the cecal microbiome was enriched in Firmicutes and Bacteroides while several lineages, most notably Lactobacillus, were under-represented. Finally, when cloacal swabs were compared to other AT locations, community similarity exhibited a direct distance relationship, i.e., the more aborad samples were the more similar they were to the swab. These findings indicate that while cloacal swabs can approximate overall changes in microbiome composition, they are

not adequate for inferring changes to specific taxa in other parts of the AT tract—even those that are highly abundant within the microbial community. These data provide new insights guiding appropriate sample size selection within flocks and add to the consensus data regarding cecal pair similarity and destructive versus non-destructive sampling methods.

KEYWORDS

16S, microbiome, poultry (chicken), cecal microflora, cloacal swab

Introduction

Next generation sequencing in poultry science has seen wider implementation as a research tool as sequencing costs fall and bioinformatic tools become more accessible (Clavijo and Flórez, 2018; Weinroth et al., 2022). Specifically, 16S rRNA gene sequencing has been used to survey the microbiome of many poultry and related environments (Bucher et al., 2020; Marmion et al., 2021; Yaqoob et al., 2021). Using a culture-free approach to survey a microbial community allows for the identification of both culturable and non-culturable microorganisms. Inevitably, there are also limitations to this approach, such as the inability to distinguish between live and dead cells, the use of relative abundance data (as opposed to absolute numbers such as enumeration of bacterial colonies on a plate), and difficultly in identifying less abundant bacterial taxa (Mira Miralles et al., 2019; Weinroth et al., 2022).

Previous broiler microbiome work has addressed descriptions of diversity within ceca (Mancabelli et al., 2016), modulation of the microbiome as the result of antibiotic and probiotic treatments (Gao et al., 2017), changes associated with age (Oakley et al., 2014) and season (Oakley et al., 2018), as well as the description of other broiler related microbiomes such as litter (Bucher et al., 2020), feed, and meat (Marmion et al., 2021). In broiler sampling, the choice to use lethal (requiring euthanasia of the bird such as a cecal sample) or non-lethal sampling (such as a cloacal swab) is also of interest. 16S rRNA gene sequencing has also been used to understand this challenge, through assessing the validity of non-lethal sampling techniques as a proxy for other alimentary tract (AT) locations and comparisons of two paired ceca within one bird (Andreani et al., 2020; Williams and Athrey, 2020). As sequencing capabilities and our understanding of the broiler associated microbiome continue to grow, the validation of sound sampling practices is of paramount importance. Here, by surveying nine alimentary tract (AT) sites and cloacal swabs within the same nine broiler chickens from a single flock, we aimed to assess the suitability of common sampling practices. The objectives of this study were to use 16S rRNA gene sequencing to examine common sampling practices in broiler studies such as the number of birds needed from a flock to capture the microbiome diversity, the differences between the paired cecal communities within single birds, identification of bacterial lineages that are enriched in the ceca relative to other parts of the AT, and the validity of using cloacal swabs as proxies for inferring microbial communities at other gastrointestinal tract locations.

Materials and methods

Nine market age male broilers Cobb 500 were obtained full fed at flock termination from the University of Georgia Poultry Research Center, cooped, and transported to the US National Poultry Research Center (USNPRC) pilot processing plant where there they were individually euthanized following the USNPRC IACUC SOP#10 Euthanasia Methods approved for poultry (C. Electrocution of Poultry). Upon death, cloacal swabs were collected from each bird using a sterile PurFlock ultra regular tip double swabs (Puritan Medical Products, Guilford, ME) and kept on ice until storage at −20°C. For all birds the intact alimentary tract was excised and both the esophagus and vent were clamped to prevent ingesta leakage; all tracts were kept on ice until processing. For each tract, nine sampling locations were chosen in addition to the cloacal swab: 1) crop, 2) gizzard, 3) proventriculus, 4) duodenum, 5) jejunum, 6) ileum, (7–8) cecal samples from each pouch, and 9) large intestine (colon). For each sample, the outside was swabbed with alcohol and allowed to air dry. From there, a new sterile scalpel was used to open the location in the middle of the segment of interest cutting only one time as to not reintroduce outside contaminants. PurFlock double tip swabs were used to swab the inside of the tract with minimal pressure to capture the lumen microbiome. After sampling, swabs were immediately placed at −20°C until DNA extraction.

DNA was extracted from all samples in addition to two unused swabs to act as negative controls using a QIAGEN QICUBE HT with DNeasy 96 PowerSoil Pro QIAcube HT Kit following the manufacturer's protocol (QIAGEN, Hilden, Germany). DNA was quantified using a Quantus fluorometer (Promega Corporation, Madison, WI). DNA (≥200 ng) was shipped to Novogene Corporation (Beijing, China) for library preparation with the V4 515/804R 16S rRNA primers and sequencing (2 × 250 bp) on an Illumina HiSeq (Illumina, Inc., San Diego, CA, United States) to a target depth of 30,000 reads per sample for all non-negative controls.

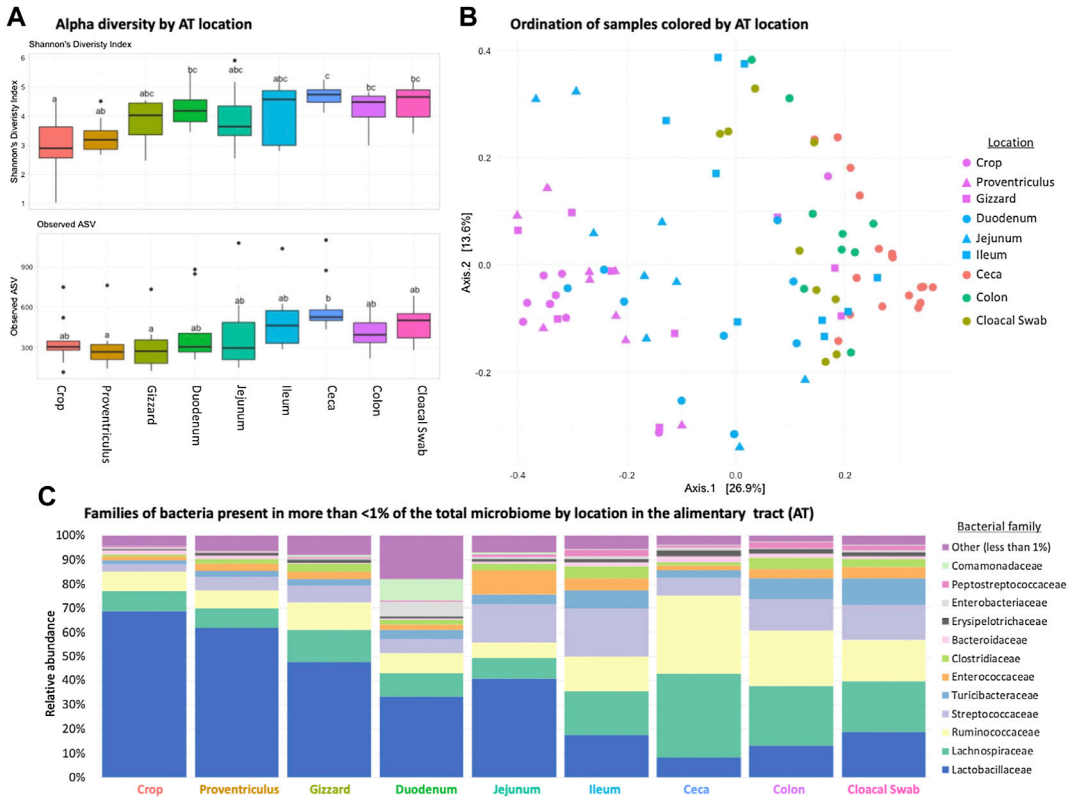

FIGURE 1
Differences in the broiler chicken alimentary tract (AT) by location. **(A)** Alpha diversity of AT locations in the birds; there were differences ($p < 0.05$) between locations. **(B)** Beta diversity differences of AT locations; locations were different (ANOSIM R = 0.6188, $p = 0.001$). **(C)** Stacked bar chart of relative abundance of more prominent bacterial classes found in the AT by location.

Demultiplexed samples were imported into QIIME2 v. 2021.8 (Bolyen et al., 2019) and amplicon sequencing variants (ASV) were assigned with DADA2 (Callahan et al., 2016) with the first 19 nucleotides of forward reads and the 20 lead nucleotides of the reversed reads trimmed as well as both reads truncation at 200. A phylogenetic tree was constructed with MAFFT v. 7 (Katoh and Standley, 2013) and FastTree2 (Price et al., 2010) while taxonomic classification was conducted using a Naïve Bayesian classifier pretrained using the 515-806R primers on the Greengenes database (DeSantis et al., 2006). Reads that were assigned to chloroplasts and mitochondria and those that did not have a kingdom classification were removed. Data was imported into R (4.0.2) using qiime2R (v0.99.6). Decontam (Davis et al., 2018) was used with the "combo" choice to remove contamination using fluorometer data and sequenced negative controls as well as the removal of two species of Lactobacillus that were high in negative controls but not known to be highly abundance in the chicken AT were removed (classified as *L. acidipiscis* and *L. helveticus*). The resulting ASV table was normalized using cumulative sum scaling (CSS) (Paulson et al., 2013).

Alpha diversity was measured as Shannon's diversity (Shannon, 1948) in phyloseq (v. 1.34.0) (McMurdie and Holmes, 2013). Beta diversity was assessed with PCoA using Bray-Curtis dissimilarity and compared with ANOSIM also in vegan (v. 2.5-7) (Oksanen et al., 2014). ANCOM (Mandal et al., 2015) was used to compare family level relative abundance differences between AT locations and specifically between cecal pairs using the QIIME2's composition plugin after the addition of a pseudo count. Family level differences between locations in the AT were compared with ordination and visualized with ggordiplots (0.4.1). Ordination of cecal pairs was conducted in the same way. Bray Curtis dissimilarity and weighted Unifrac distances were computed with phyloseq and visualized using ggplot2. Number of new and unique ASV were adapted from ROARY's create_pan_genome_plots R script (Page et al., 2015 Roary: rapid large-scale prokaryote pan genome analysis | Bioinformatics | Oxford Academic) and were visualized in ggplot2. Differential enrichment analysis was performed using a Wald Test ($p = 0.01$) implemented on DESeq2 (Love et al., 2014). Phylogenetic comparison of differentially enriched

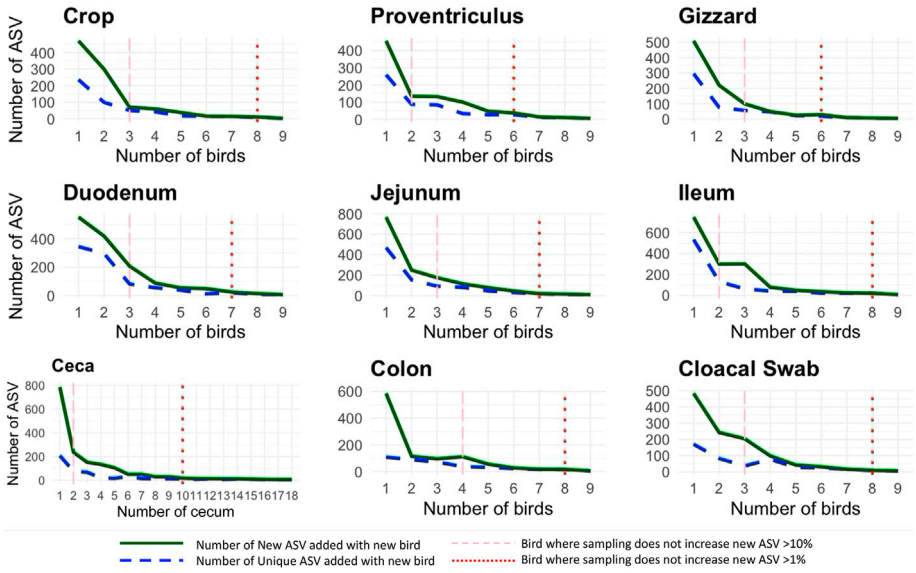

FIGURE 2
Number of new and unique amplicon sequence variants (ASVs) added when a new bird replication was added (faceted by AT location) with two different thresholds: the number of new ASVs increasing by <10% and <1%.

lineages was conducted using the phyloseq plot_tree() function. Enterobacteriaceae normalized read correlations were compared and visualized in ggpubr 0.4.0. Alpha was set at 0.05.

Results

Across all non-negative control samples, 7,282,584 reads were analyzed (average = 81,826, range 31,246–18,324) representing 45 phyla, 1,140 species, and 9,212 ASV.

Modulation of the microbiome throughout the chicken alimentary tract

Each AT location sampled had a unique microbiome composition, with differences in alpha and beta diversity as well as the relative abundance of bacterial families (Figures 1A–C). Alpha diversity was lower ($p < 0.05$) in the orad portions of the AT and was numerically highest in the ceca (Figure 1A). When beta diversity was compared there were also differences according to AT location (ANOSIM R = 0.6188, $p = 0.001$, Figure 1B). Within all AT locations, Firmicutes were the most abundant phyla present with *Lactobacillaceae*, *Lachnospiraceae*, and *Ruminococcacceae* dominating family-level relative abundance (Figure 1C). Throughout the AT, *Lactobacillaceae* and *Lachnospiraceae* decreased in relative abundance from crop to colon.

Within flock clonality to determine appropriate sample size

Throughout the AT, similarity of each location across birds was high as specific sites were similar in relative abundance of bacteria. When each AT location was assessed for the number of new ASVs added with the addition of a new bird as well as the number of unique ASV (those specific to just that bird), the same diminishing returns were found across all sample locations (Figure 2). When two different parameters were evaluated (the number of new ASVs increasing by <10% and <1%), it was found that at 2.8 birds (range 2–4) subsequent addition of birds resulted in less than 10% new ASV being added while 7.6 birds (range 6–10) resulted in additional samples increasing by less than 1% new ASVs. The proventriculus and gizzard required the least number of samples to reach the 1% threshold with six birds, the duodenum and jejunum required seven birds, the crop, ileum, colon, and cloacal swab need eight and finally, the ceca required 10 cecum pouches.

Within bird cecal pairs similarity

When cecal pairs among broilers were compared to each other from an ordination and composition standpoint (Figure 3), pairs were found to be similar. An average of 95.33% (range 79.33%–98.09%, Figure 3A) of the relative abundance of reads was present in both cecum within one bird. On the other hand, when unique ASVs were considered,

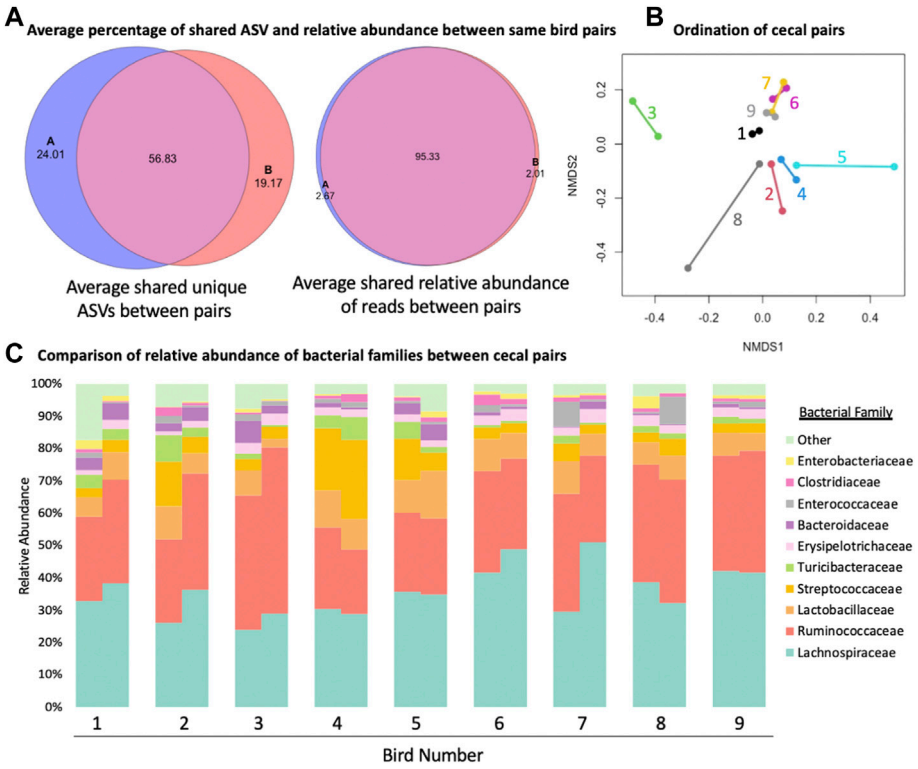

FIGURE 3
Comparison of similarity of the two cecal pouches within the same bird. **(A)** The average number of shared amplicon sequence variants (ASVs) between the two cecal pouches of the same bird and the average shared relative abundance between of reads between the two cecal pouches of the same bird. **(B)** Bet diversity of ordination of cecal pairs colored and connected by pair mate. **(C)** Staked taxonomic bar plot of relative abundance of bacterial families between cecal pairs from the same bird.

the average number that was shared fell to 56.83% (range 23.07%–69.18%, Figure 3A). When beta diversity was considered, in most cases the nearest neighbour was the matching cecal pair mate (Figure 3B), though there was little variation among ceca pairs between birds, presumably due to all samples originating from the same flock. Finally, when family level differences were compared, some ceca pairs were more similar to their mates than other pairs (Figure 3C) but overall pairs were similar to their mate. There were not families that were consistently significantly different between cecal pairs, meaning that variation was more likely individual bird driven and not reflective of a biological difference between cecal pouches.

Differentially enriched cecal taxa

A pooled differential abundance test model was used to compare significant (Wald Test, $p = 0.01$, Figure 4A) lineage enrichments in all cecal samples relative to all non-cecal samples. Results show 30 differentially enriched lineages, specifically, 19 and 11 lineages were cecal depleted and enriched, respectively (Figure 4B). Seven of the eleven cecal-enriched lineages belong to the bacterial order Clostridiales and include members of the following genera: Oscillospira, Ruminococcus, Butyricicoccus, Subdoligranulum, and Faecalibacterium. Other cecal-enriched lineages were classified as members of the Bacteroidales and Coriobacteriales orders. The most common cecal depleted lineages were predominantly classified as *Lactobacillus* spp. but also include members of the Actinomycetales, Bacillales, Burkholderiales, and Clostridiales orders.

Cloacal swab representation of alimentary tract

Cloacal swab microbiomes were compared to nine AT location microbiomes to understand if swabs are a good predictor of the microbial community of these locations. Both Bray-Curtis dissimilarly and weighted Unifrac distances were compared at each location to the cloacal swab of the same bird. The general trend was that there was a shorter distance (more similarity) between cloacal

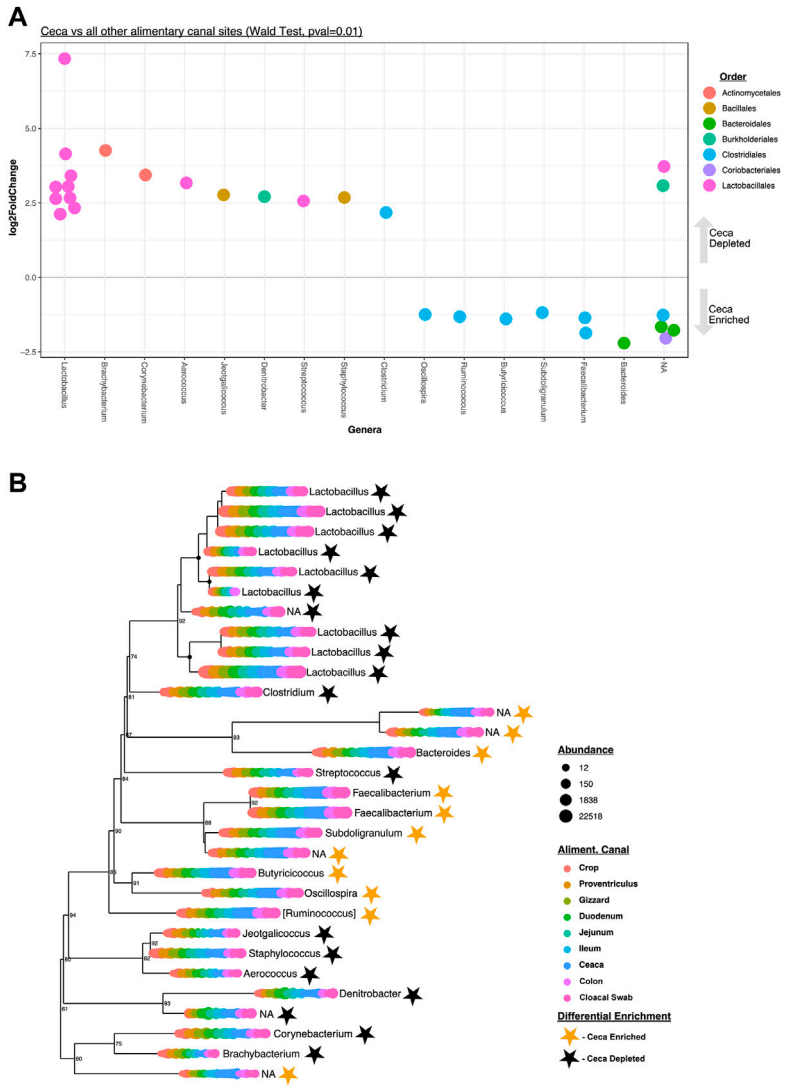

FIGURE 4
Differentially enriched cecal taxa. **(A)** Significantly (Wald Test, Pval = 0.01) differentially enriched cecal taxa (n = 30). Each circle represents an ASV that was either significantly enriched (negative LogFold2 values, n = 11) or depleted (positive LogFold2 values, n = 19) in cecal samples. Colored code depicts Order-level taxonomy and columnar arrangement represents Genus-level taxonomy assignments. **(B)** Phylogenetic tree of the 30 differentially enriched taxa identified depicted in **(A)**. The circles at each leaf represent the normalized abundance of each taxon at each color-coded AT site. The genus-level taxonomy assignment of each differentially enriched ASV is also shown. Stars represent the status of each taxon in the tree as either enriched (golden) or depleted (black) in cecal samples relative to all other samples.

swabs and other sampling types as the sampling was more aborad in the AT (Figure 5). Normalized *Enterobacteriaceae* read counts were compared between each AT location to the number of normalized *Enterobacteriaceae* read counts in each cloacal swab. The only significant correlation between a AT location and cloacal swabs was the adjacent colon (p = 0.035, R = 0.70, Figure 6).

Distance-decay analysis showed significant relationships between community similarity and physical distance between samples for all but one bird (Figure 7).

Discussion

When the microbiome of the AT described here was compared to other studies there was a high level of congruency. Past studies have also described *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* as the most predominant phyla in the AT (Wei et al., 2013; Waite and Taylor, 2014). Other studies have also described different AT locations within the same birds to have distinct microbiomes (Han et al., 2016; Saxena et al., 2016) as well

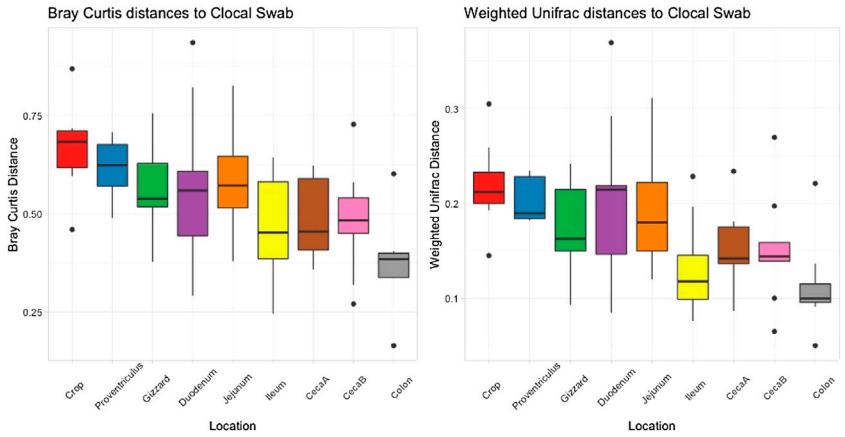

FIGURE 5
Bray Curtis dissimilarly and weighted unifrac distances between the cloacal swab and other AT location in individual birds averaged.

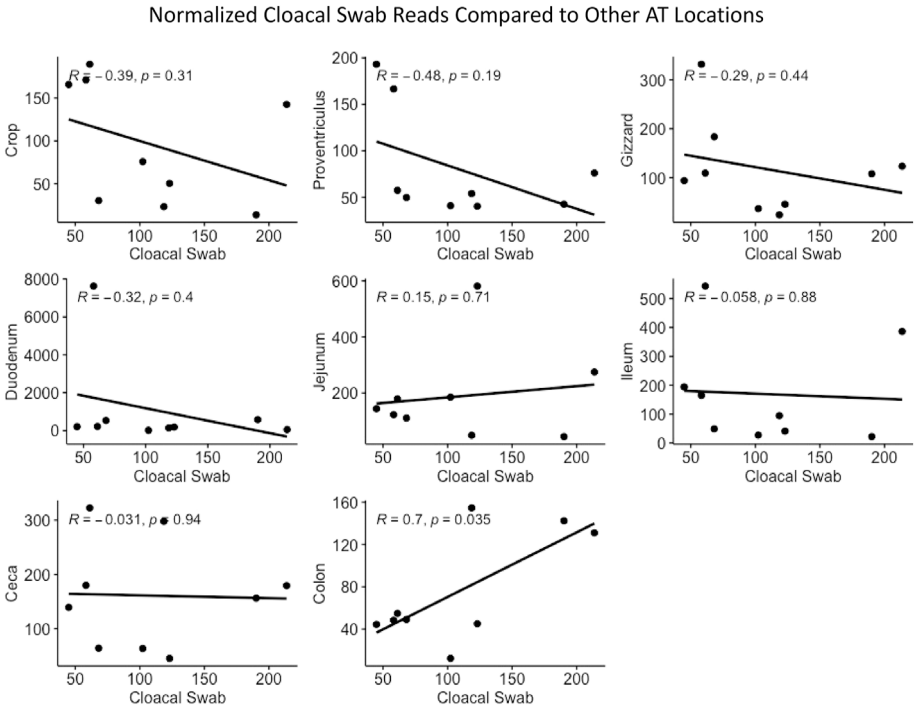

FIGURE 6
Correlation between normalized Enterobacteriaceae read numbers found in the cloacal swab and other AT locations.

as reviews that have highlighted the differing predominant phyla within different AT locations (Yeoman et al., 2012; Feye et al., 2020). However, this data set is unique in the sense that there are 10 locations within the same birds that were sampled from a single flock, allowing for an understanding of the modulation of the microbiome according to location in the AT.

Past microbiome work on broilers has described differences between different flocks were smaller than the variation as a result of the age of the birds or the location

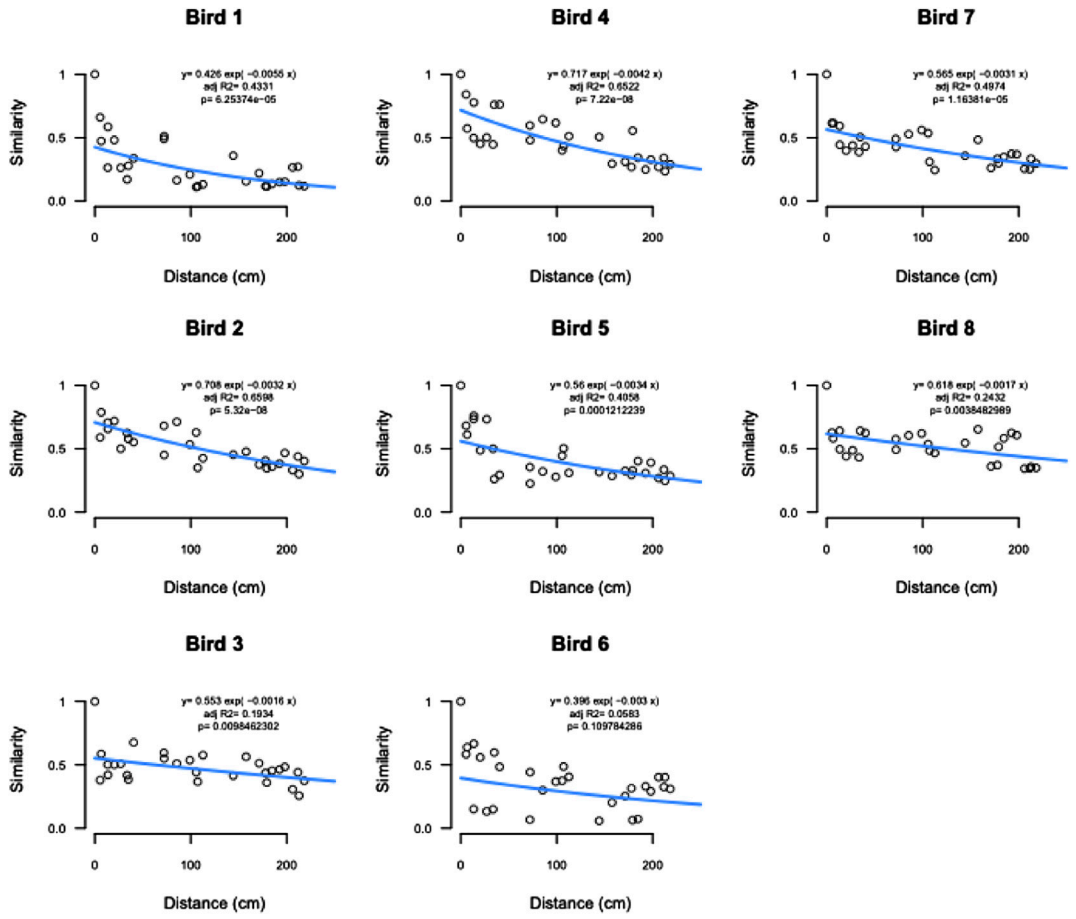

FIGURE 7
Distance decay analysis by bird showing significant relationships between community similarity and physical distance between samples for all but bird 6.

sampled in the AT (Johnson et al., 2018). In terms of determining appropriate sample size within a flock, multivariant microbiome studies have proven to be more challenging, with current methods limited to specific tests, variable types, or experimental designs (Kelly et al., 2015; Mattiello et al., 2016). The data provided here estimates the amount of new data that could be gained by sampling additional birds across each AT location. Across all locations in the AT, there was a rapid decrease in the addition of new information with the addition of replicates, demonstrating a gain of no more than 10% new ASVs after the third bird and less than 1% after the eighth bird in most cases and it the case of the ceca the 10th bird. Depending on the research question, these findings can be used as a starting point to determine appropriate sample numbers within one flock. Caution must be used when classifying a collection of birds as one flock, as even small variation can result in different microbiomes. For example, even flocks that are grown in the same house but in different rooms can result in different AT microbiomes (Schokker et al., 2021).

The important biological role of the ceca from a nutritional standpoint and the ability to find many disease-causing microbes in this AT segment make it a common sampling location for broiler studies (Clench and Mathias, 1995; Clavijo and Flórez, 2018). In some studies, contents from both ceca are combined and analyzed as one sample, while in others, the pouches are considered to be replicates and different analysis are conducted on them with the bird as the experimental unit. Past work comparing the two pouches from the same bird using 16S rRNA sequencing did not find significant differences in alpha or beta diversity and noted short distances between pairs when comparing beta diversity (Stanley et al., 2015). This work agrees with the current study which demonstrated high congruency between pairs within the same bird. While the overall microbiome composition was not different between pairs, unique ASVs were found in both pouches. This paired with the fact that in terms of relative

abundance more than 95% of the reads were shared, indicates that treating different cecal pouches as replicates may be appropriate with viewing the microbiome as a whole but not when looking for rare or low occurring ASV (due to the fact the while relative abundance was high, unique ASV overlap was much lower, around 56%).

Cecal-enriched lineages detected from our pooled flock comparison (e.g., *Bacteroides*, *Faecalibacterium*, *Butyricicoccus*, *Ruminococcus*, etc.) are also commonly reported from the cecal communities of commercial chickens (Ramírez et al., 2020). This suggest that the ceca are highly specialized anaerobic niches where thermodynamic constrains result in the high degree of community convergence observed across studies. The unique environment present in these organs, hosting the bulk of GI track fermentations, suggests that cecal-enriched lineages may play important roles in the avian-microbe symbiosis and, potentially, host energetic harvest. Overall, our work shows individual cecum pouch comparisons as robust community replicates to its mate and helps to define the ceca as unique and highly understudied symbiosis-relevant microbial AT environments.

The final comparisons that were made was the similarity of different AT locations to a cloacal swab and distance-decay analyses of microbial community similarity compared to physical distance between samples. We report that cloacal swabs exhibited a direct distance relationship, i.e., the more aboral samples were the more similar they were to the swab and, thus, best represent colon samples. However, we also show that this relationship may not extend to individual lineages. Cloacal swabs and feces have been used as a proxy for internal samples given their ease of access, ability to be used as a repeated measure, and non-destructive nature. There is a growing body of work on using non-lethal sampling as a proxy for sampling that requires bird euthanasia. In two studies that compared fecal to cecal swabs, Stanley et al. (2015) found that the prominent bacteria phyla in the microbiome were present in both location, with more rare OTU not found in both locations (although this comparison was not done between individual bird but instead a comparison of AT sites across a flock birds). Oakley and Kogut (2016) found distinct communities between the fecal and cecal microbiomes when compared within a bird. When cloacal swabs were evaluated, Andreani et al. (2020) found that while over 99% of the abundance of reads was captured with a cloacal swab, rare OTUs were not (but the high abundance captured in both sample types may be an effect of comparing pooled samples as opposed to individual birds). In a separate analysis in their study, they did find that the cecal samples from individual birds did have a moderate correlation to their corresponding cloacal swabs. Finally, while one study (Williams and Athrey, 2020) came to the conclusion that cloacal swabs are not an appropriate measure of the AT, this study only focused on differences in alpha and beta diversity and did not look into any correlations specific to the sample types.

Our work builds on past studies and here only compares AT locations to cloacal swabs within the same bird. Overall, it was found that the more aborad a sample was taken in the AT, the more similar it was to the cloacal swab from that bird. That is to say, the crop was the poorest approximation for the cloacal swab and the colon was the closest. Based on our findings that alpha and beta diversity were distinct between sites within AT locations and community similarity was significantly correlated with physical proximity of samples, we conclude that cloacal swabs are not a good approximation of the actual internal community at other AT locations. This conclusion is further supported by the fact that normalized read counts for *Enterobacteriaceae* between cloacal swabs and other AT locations were not significantly correlated. From a practical standpoint, this means that while cloacal and fecal swabs can be used as a loose approximation of overall microbiome shifts, it should not be used to infer changes to specific phyla—even those that are highly abundant within the microbial community.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA844895.

Ethics statement

The animal study was reviewed and approved by the USNPRC IACUC.

Author contributions

Conceptualization, all authors; sample collection and lab work, MW and RB; analysis MW, BO, and GR; figure preparation MW, BO, and GR; writing—original draft preparation, MW, BO, and GR; writing—review and editing, all authors.; all authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Connecting gut microbiomes and short chain fatty acids with the serotonergic system and behavior in *Gallus gallus* and other avian species

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The chicken gastrointestinal tract has a diverse microbial community. There is increasing evidence for how this gut microbiome affects specific molecular pathways and the overall physiology, nervous system and behavior of the chicken host organism due to a growing number of studies investigating conditions such as host diet, antibiotics, probiotics, and germ-free and germ-reduced models. Systems-level investigations have revealed a network of microbiome-related interactions between the gut and state of health and behavior in chickens and other animals. While some microbial symbionts are crucial for maintaining stability and normal host physiology, there can also be dysbiosis, disruptions to nutrient flow, and other outcomes of dysregulation and disease. Likewise, alteration of the gut microbiome is found for chickens exhibiting differences in feather pecking (FP) behavior and this alteration is suspected to be responsible for behavioral change. In chickens and other organisms, serotonin is a chief neuromodulator that links gut microbes to the host brain as microbes modulate the serotonin secreted by the host's own intestinal enterochromaffin cells which can stimulate the central nervous system via the vagus nerve. A substantial part of the serotonergic network is conserved across birds and mammals. Broader investigations of multiple species and subsequent cross-comparisons may help to explore general functionality of this ancient system and its increasingly apparent central role in the gut-brain axis of vertebrates. Dysfunctional behavioral phenotypes from the serotonergic system moreover occur in both birds and mammals with, for example, FP in chickens and depression in humans. Recent studies of the intestine as a major site of serotonin synthesis have been identifying routes by which gut microbial metabolites regulate the chicken serotonergic system. This review in particular highlights the influence of gut microbial metabolite short chain fatty acids (SCFAs) on the serotonergic system. The role of SCFAs in physiological and brain disorders may be considerable because of their ability to cross intestinal as well as the blood-brain barriers, leading to influences on the serotonergic system via binding to receptors and epigenetic modulations. Examinations of these mechanisms may translate into a more general

understanding of serotonergic system development within chickens and other avians.

KEYWORDS

gut microbiome, gut-brain axis, short chain fatty acids, serotonin, chicken, avians, behavior, translational science

Introduction to the chicken gut-microbiome-brain axis

Chickens are an important source of food in the human diet worldwide, and the poultry industry is one of the fastest-growing fields in agriculture (Nkukwana, 2019). Ongoing studies surrounding chicken husbandry and physiology have generated substantial amounts of knowledge regarding the chicken gut-microbiome-brain axis. The diverse chicken gut microbiome, for instance, is now known to have strong effects on the feed conversion ratio impacting growth and health (Stanley et al., 2012), early stages of immune system development (Schokker et al., 2017), resistance to enteric pathogens (Feng et al., 2010), and behavior (Kraimi et al., 2019a).

There are multiple routes to how host physiology and molecular processes interact with different gut microbial varieties and associated microbial metabolites. Management practices like overcrowding in cages, high temperature, and rough transportation all of which exert stress on chickens (Virden and Kidd, 2009; Sanchez-Casanova et al., 2019). These stressors in chicken affect gut microbial community composition. This is evident by studies involving external environmental stressors and studies administering corticosterone (Calefi et al., 2016; Noguera et al., 2018; Zaytsoff et al., 2020). The changes in the gut microbiome composition may be induced by the CNS via the sympathoadrenal system and the hypothalamic-pituitary-adrenal (HPA) axis (Villageliũ and Lyte, 2017). Decades of research have shown the effect of these stressors on host serotonin synthesis (Chaouloff et al., 1999). Other neurochemicals along with serotonin have been documented in the broiler chicken intestinal track with their levels being altered during a stressed condition (Dennis, 2009; Lyte et al., 2022). The systemic circulation of neurochemicals in chickens has been found to affect general physiology (Denbow et al., 1983; Chapman et al., 2008) and the immune system (Borsoi et al., 2015), as well as the gut and growth of different bacterial species including pathogens (Lyte and Ernst, 1992; Bailey et al., 1999; Freestone et al., 2008; Truccollo et al., 2020; Lyte et al., 2021a). Gut microbiota are furthermore known to produce and stimulate host neurotransmitter synthesis, with these effects found to ultimately influence host physiology and behavior (Beaver and Wostmann, 1962; Reigstad et al., 2015; Van Staaveren et al., 2021). Such a bidirectional relationship between microbiomes and neurochemistry was recently demonstrated in a Japanese quail model where management stress response led to changes in microbial composition, with the effect of gut microbes on tissue serotonin concentration outside the gut being also observed (Lyte et al., 2021b).

Serotonin levels in the gut are influenced by gut microbes as has been demonstrated by pioneering studies comparing conventional and germ-free chick models (Phillips et al., 1961; Beaver and Wostmann, 1962). Serotonin is a major neurotransmitter regulating aggression in chicken (Dennis, 2009). Chickens may cope with stress by exhibiting aggressive behavior such as, for example, aggressive FP (Cheng and Muir, 2007; Van Staaveren and Harlander, 2020). FP birds harm not only themselves but also other birds by pecking and pulling their feathers leads to decreased performance of birds and loss to the poultry industry (Jensen et al., 2005). Several studies also indicate a regulatory role of gut microbes in the gut-brain axis that includes probiotic modulations that mitigate aggressive behavior in birds (Abdel-Azeem, 2013; Cheng et al., 2019; Mindus et al., 2021). Dietary modulations of gut microbiota have been found to overall improve chicken behavior and overall health (Dixon and Nicol, 2008; Pan and Yu, 2014). The gut microbial modulation could therefore have considerable value with respect to common challenges with chicken health and husbandry. Beyond discovering effects of microbiomes on chickens, a translational objective is to evaluate whether advantages of chickens as a model organism and underlying mechanisms of the chicken gut-microbiome-brain axis would help to inform understanding and investigation of the gut-microbiome-brain axis in humans. For instance, with humans, stress and diet substantially alter gut microbial ecosystems with varying impacts on human health (Singh et al., 2017; Gubert et al., 2020). Common mechanisms surrounding the gut-brain axis in humans and chickens involve the serotonergic system being modulated by conditions of stress (Leonard, 2005). The impact of gut microbes on the serotonergic system and behavior have been closely linked with phenotypes of FP in chicken and depression in humans (Cheng et al., 2019; Huang and Wu, 2021).

For various animals, including chickens and humans, serotonin is mainly synthesized by serotonergic neurons in brain and intestinal enterochromaffin cells (Parent, 1981). The large portion of serotonin in the body is produced by intestinal enterochromaffin cells and production is stimulated by gut microbial metabolites like SCFAs (Gershon, 2013; Reigstad et al., 2015). Some of the more abundant microbial SCFAs, butyrate and acetate, induce dramatic shifts of expression for the rate-limiting enzyme, Tryptophan hydroxylase 1 (Tph1), which is associated with mucosal serotonin synthesis by

intestinal enterochromaffin cells (Côté et al., 2003; Reigstad et al., 2015). This review provides a report and synthesis of current molecular and physiological findings surrounding how the serotonergic system and behavior relate to gut microbiota and SCFAs in chickens. This review, in addition, critically evaluates the use of chicken as an animal model that may help influence and guide the study of the gut-microbiome-brain axis in humans as would relate to SCFAs and the serotonergic system.

Chicken gut microbiota and potential function

The gastrointestinal (GI) tract of chicken is inhabited by a complex and dynamic microbial community that is established during hatching and initial period of exposure to the environment, stabilizing later in life. Chickens hatched within hatcheries receive microbes from environmental flora (Stanley et al., 2013; Volf et al., 2021). This microbiome undergoes dramatic changes, overall expanding throughout the life of a chicken, leading to an adult chicken GI tract having trillions of bacteria, representing more than 600 bacterial species (Apajalahti et al., 2004; Apajalahti & Kettunen, 2006; Borda-Molina et al., 2018). Similar to what has been found for human gut microbiota, analyses of broiler and layer chicken gut microbiota have identified *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* as the more abundant phyla. Other phyla, such as *Actinobacteria*, while less abundant, are consistently found as well (Qin et al., 2010; Li et al., 2014; Tong et al., 2017; Mandal et al., 2020).

In the chicken GI tract, the cecum is a major anatomical location with higher microbial diversity and metabolism (Sergeant et al., 2014; Polansky et al., 2016). Recent metagenomic analysis of the chicken cecum has identified 42 novel genera, 40 of which are of the taxonomic class *Clostridia* which is observed in high abundance in the ceca. More prevalent taxonomic orders within the *Clostridia* class are *Oscillospirales* and *Lachnospirales* (Glendinning et al., 2020). At the family level, the cecum encompasses *Clostridiaceae*, *Bacteroidaceae*, *Lactobacillaceae*, and SCFA butyrate producing *Lachnospiraceae* families (Witzig et al., 2015). Analyses of the chicken microbiome found in the cecum have helped to identify new gut microbes and unravel their functionality. For instance, it has revealed those microbes having genetic material that encodes polysaccharide and numerous oligosaccharide-degrading enzymes. The degrading of polysaccharides occurs in large part due to lineages belonging to the taxonomic classes *Actinobacteria*, *Clostridia,* and *Bacteroidia*. Genes involved in SCFA (acetate and butyrate) production have furthermore been identified, with most of these genes and their associated functions occurring for lineages that belong to the *Firmicutes* and *Bacteroidetes* phyla (Sergeant et al., 2014).

The metabolic capacity and overall colonization pattern of gut microbes lead to various health benefits and behavioral outcomes for chicken and other avian species. The main source of carbon and energy for the microbes in the lower intestine comes from undigested complex dietary carbohydrates and starch (Cummings and Macfarlane, 1991). Moreover, plant-based poultry diets have a large amount of non-starch polysaccharides (NSP) (Józefiak et al., 2004; Raza et al., 2019). Fermentation of undigested food by gut microbes in the cecum and colon produces SCFAs (van der Wielen et al., 2000), which benefit the host by providing a source of energy, stimulating gut epithelial cell proliferation, and by lowering the colon pH to help prevent secondary bile production (Sakata, 1997). Some beneficial gut microbes are known to protect the intestine against colonization by pathogenic bacteria such as *Salmonella* spp. (Nurmi and Rantala, 1973). In addition, gut bacteria produce and sometimes metabolize various neurochemicals like serotonin, essential amino acids like tryptophan, vitamins, and antimicrobial compounds (Jeurissen et al., 2002; Yanofsky, 2007; Lyte, 2011; Kogut, 2019). Much of the same has been generally found for humans (Rowland et al., 2018). Gut bacteria have an overall regulatory impact on the gut-brain axis leading to behavioral changes as well (Cryan and Dinan, 2012; Arneth, 2018). A dietary study of great tits, being provided an insect diet versus a seed diet, showed compositional change in the gut microbiome occurring in parallel to reduced problem-solving skills for birds fed the insect diet (Davidson et al., 2020).

Effect of gut microbiota on cognition and behavior

Domestic chickens are the most common and widely used species of poultry in agriculture and are a domesticated breed of red junglefowl (*G. gallus*) (Siegel et al., 1992; Yamashita et al., 1994). Despite many effects of selective breeding, domestic chickens retain cognitive and behavioral similarity to their ancestors. Both wild and domestic chickens follow a similar social structure and behavior of interaction within their populations and have complex cognitive ability, along with emotional and communicative behavior (Appleby et al., 2004). Hens and chicks are in the center of a domestic chicken community whereas roosters live independently and protect hen and chicks in the group. Chickens communicate information regarding territory, mating, nesting, distress, danger or fear, contentment, and food discovery with the help of 30 distinct vocalizations (Appleby et al., 2004). Findings regarding fear response show complex emotional behavior which is accompanied by physiological reactions like fever that can also be found with humans (Cabanac and Aizawa, 2000).

TABLE 1 Studies investigating effects of gut microbiota interventions on bird behavior.

Study details	Bird species	Behavioral outcome	Findings	References
Ingesion of *L. rhamnosus*	White Leghorn, laying hens; Selected HFP, LFP lines	Reduced stress induced FP	Increased T cell population of spleen and the cecal tonsils Limited cecal microbial dysbiosis	Mindus et al. (2021)
FMT during early life from aged donor	Healthy commercial broilers	FM from adult chickens improves fearfulness in chicks	FMT administration might improve the physiology and behavior of chickens	Yan et al. (2021)
Early life FMT from HFP or LFP adults	White Leghorn birds Selected HFP, LFP lines	FMT influenced FP behavior; Homologous FMT resulted in reduced fearfulness	FMT had immediate and long-term effects on behavior and immune characteristics and peripheral serotonin	van der Eijk et al. (2020)
FM transfer from 13 weeks old adult female quails in GF Chicks	Japanese quails from quail line selected for high (E+) and low (E−) emotional reactivity	GM from (E−) quails in GF chicks reduced emotional reactivity in early life	Change in the GM composition in treatment groups associated with behavioral modification	Kraimi et al. (2019)
GF quails compared to quails with FM from adult female quails	Japanese quails	GF quails showed reduced emotional reactivity compared to quails with gut microbiota	Absence of gut microbiota reduces emotional reactivity in Japanese quails with no effect on growth	Kraimi et al. (2018)
Effect of heat stress and *or Clostridium perfringens* infection	Broiler chickens	*C. perfringens* infection decreased the frequency of feeding, walking, FP and standing; Increased the frequency of SB behavior	Showed links among degree of intestinal lesions, behavioral outcomes, brain activity, and serum levels of corticosterone	Calefi et al. (2016)
Administration of probiotic spores of *Bacillus amyloliquefaciens*	Turkey poults	Probiotics administration increased the feeding frequency and decreased distress call and aggressive behaviors		Abdel-Azeem (2013)

CR, cage rearing; FR, free-range; FMT, fecal microbiota transplantation; FM, fecal microbiota; FP, feather pecking; SB, sickness behavior; GF, germ free; GM, gut microbiota; HFP, high feather pecking; LFP, low feather pecking; SCFAs, short chain fatty acids.

For how gut microbiomes and their metabolic products connect dynamics within the gut to the brain, resulting in effects on behavior, this is being studied as an applied area of research that may considerably improve our understanding of human health and animal behavior and wellness. It has indeed been possible to adjust the microbiome toward positive behavioral outcomes with, for example, supplementation with *Lactobacillus rhamnosus* between 19 and 26 weeks of age being found to reduce FP in chickens (Mindus et al., 2021) (Table 1). Gut microbial composition changes have shown the potential to aid mammals in their adaptation to stress as well (Boonstra, 2005). Biomedical findings arising mainly from studies on humans and mice have found gut microbial-derived products like neurotransmitters, SCFAs, indoles, bile acids, choline metabolites, lactate, and vitamins to have general effects across animal host physiology (Krautkramer et al., 2021). Broad-ranging impacts between microbiomes and behavior have been found in chickens, quail, and turkey (Table 1). A recent study of Japanese quail has demonstrated how emotional reactivity can be influenced by gut microbiota transfers that alter taxa of the *Firmicutes* phylum (Kraimi et al., 2018; Kraimi et al., 2019b). Changes in abundance for the *Firmicutes* phylum have also been associated with stress, anxiety, or depression (Bailey et al., 2011; Jiang et al., 2015). In a similar study in turkey, probiotic administration has been found to reduce distress calls and agonistic behavior in birds (Abdel-Azeem, 2013). On the contrary, the prolonged deprivation of natural bird behaviors like foraging, nesting, perching, and dust-bathing is believed to affect brain function and lower gut microbial diversity (Chen et al., 2019).

There have been some initial studies on the association of gut microbial metabolites with chicken behavior. A study conducted by Meyer et al. (2013) investigated differences in gut microbial metabolites in high and low FP chickens. The study analyzed gut metabolites like biogenic amines, SCFAs, ammonia, and lactate. Total SCFAs were elevated in high FP birds due to the utilization of ingested feathers by cecal microbes (Meyer et al., 2013). While chicken gut microbial composition is increasingly studied for microbial diversity and microbial modulations that influence poultry production (Grond et al., 2018), there remains a dearth of metabolomic and functional studies illustrating the effect of microbial metabolites on host physiology and behavior. As shown in Table 1, not all studies evaluate for behavioral outcomes along with both microbial and metabolite-related outcomes. These studies also varied in terms of ages studied, with some only lasting for a few weeks (Abdel-Azeem, 2013; Calefi et al., 2016; Kraimi et al., 2018) and others continuing for two or more months (van der Eijk, et al., 2020; Mindus et al., 2021; Yan et al., 2021). Future studies are needed to evaluate dynamics across potentially interconnected microbial and metabolite-related outcomes.

The serotonergic system

Serotonin is an important neurotransmitter that connects the gut-brain axis and exists ubiquitously across diverse biological systems, including for vertebrates, invertebrates, and some plants (phytoserotonin) (Smith, 1971). Central serotonin has been

found to regulate temperature (Freeman, 1979), appetite, sleep, and energy metabolism (Lv and Liu, 2017; Hillman et al., 1980). Serotonin is also associated with cognition and behavior across the animal kingdom (Bacqué -Cazenave et al., 2020), which makes the serotonin system a potential target for treating behavioral problems (Nishizawa et al., 1997).

Peripheral serotonin acts as hormone and improves nutrient absorption, and regulates GI motility, pancreatic secretion and peristaltic reflex (Martin et al., 1993; Li et al., 2001). It participates in multiple physiological functions through the diverse receptors it binds to, including vasoconstriction and dilation (Rapport et al., 1949), adipogenesis in white adipose tissue (WAT), muscle, and liver glucose uptake (Namkung et al., 2015). Serotonin modulates insulin secretion and the immune system (Cataldo Bascunan et al., 2019). Within the intestine, serotonin acts as a pro-inflammatory as well as anti-inflammatory signaling molecule (Bischoff et al., 2009). Pro-inflammatory signaling is studied in serotonin transporter-knockout mice which exacerbates experimental GI inflammatory disease through activating 5-HT7 receptors expressed by dendritic cells (Bischoff et al., 2009; Kim et al., 2013). However, serotonin is also involved in anti-inflammatory signaling via epithelial 5-HT4 receptor activation, reducing colon inflammation in mice (Spohn et al., 2016).

Central and peripheral serotonin system

Central serotonergic neurons are located in dorsal raphe and median raphe nuclei that are present in the midline of the brainstem (Puelles et al., 2018; Fujita et al., 2022). These neurons occupy most central nervous system regions with their projections (Reiner, 2001; Matragrano et al., 2012; García-González et al., 2017). As has been found in humans, chickens and other animals, serotonin is synthesized from its precursor tryptophan by the rate-limiting enzyme tryptophan hydroxylase 2 (Tph2) in the serotonergic neurons of the brain (Böhm et al., 1979; Fujita et al., 2022; Sako et al., 1986), while peripheral serotonin is synthesized by its isoform Tph1 (Walther et al., 2003). Cofactors (Fe^{2+}), co-substrates (O_2 and BH_4) and stress hormones are also activators of Tph (i.e., Tph1 or Tph2). Sustained tryptophan hydroxylase activity influences the firing rate of serotonergic neurons (Maximino, 2012). Furthermore, tryptophan is an essential amino acid derived from the diet. Tph converts L-tryptophan into 5-hydroxytryptophan (5-HTP) which transforms into serotonin, 5-hydroxytryptamine (5-HT), by the action of aromatic L-amino acid decarboxylase (Leathwood, 1987). Serotonin has a very short half-life in the brain (Brodie and Reid, 1968). Active serotonin gets transported to the synaptic space while inactive serotonin is metabolized in and outside the cell. The enzyme monoamine oxidase A (MAO-A), located in the outer mitochondrial membrane of the neuron, deaminates or metabolizes 5-HT into 5-hydroxy-indol-acetaldehyde, which is then oxidized into urinary metabolite 5-hydroxy-indole-acetic acid (5-HIAA), a urinary marker of serotonin synthesis (Kuhn and Hasegawa, 2020). Disruptions to this 5-HT metabolism, mainly as regards 5-HIAA, is associated with aggressive behavior in mammals as well as birds (Coccaro et al., 2010; Kops et al., 2013). In the brain, high tryptophan levels increase the production of serotonin (Fernstrom and Wurtman, 1971). The brain receives peripheral tryptophan through active transportation across the blood-brain barrier, where tryptophan has to compete with tyrosine and other branched-chain amino acids for transport (Fernstrom and Fernstrom, 1995; Fernstrom and Fernstrom, 2007).

In the case of serotonergic transmission, synthesized neuronal serotonin is released from presynaptic neurons into the synaptic space through vesicle transport. Upon release, these molecules bind to serotonin receptors in the postsynaptic membrane and transmit signals to different brain projection areas (Millan et al., 2008). The excess serotonin in the synaptic space is bound to by the serotonin reuptake transport (SERT) membrane protein of presynaptic neurons (Krause et al., 2017). After reuptake in the raphe neuron, inactive serotonin is degraded by monoamine oxidase (MAO) (Borue et al., 2007). Binding of synaptic as well as peripheral serotonin to receptors modulate the central and peripheral function of serotonergic neurons and thus influence behavior. There are 14 serotonin receptor proteins identified in mammals and in poultry birds with varying distributions in the brain as well as peripheral regions (Banerjee et al., 2007; Stępińska et al., 2015).

Presence of serotonin in chicken GI track has been known for decades (Phillips et al., 1961), as has been known how enterochromaffin cells are distributed throughout the avian gut (Rawdon 1984). Apart from enterochromaffin cells, peripheral serotonin is synthesized by serotonergic neurons from the enteric nervous system (ENS) (Neuhuber and Worl, 2018). Out of these sites, enterochromaffin cells in the gut synthesize most of total body serotonin. A recent study providing concentration of neurotransmitters in the GI track of broiler chicken reported serotonin and 5-HIAA levels in tissue as well as luminal content at varying bird ages (Lyte et al., 2022). The tissue serotonin levels in jejunum, ileum, and cecum are higher than the luminal content levels at varying ages. Moreover, the luminal serotonin levels at jejunum, ileum, and cecum regions are not age dependent. This may indicate increased synthesis of serotonin in these regions.

Blood thrombocytes in birds store the serotonin produced (Maurer-Spurej, 2005), and the level of serotonin in the blood is strongly dependent upon its synthesis in the gut (Meyer et al., 1973). Upon release into the gut wall, serotonin acts as a luminal signal transducer to the central nervous system via intrinsic and extrinsic primary afferent neurons (vagal afferent neurons) of enteric nervous system (Li et al., 2000; Gershon and Tack, 2007). These afferent neurons receive and transmit physical as well as chemical stimuli to CNS initiated by enterochromaffin cells and immune cells. The enteric nervous system is an intrinsic system of the GI track. It is composed of neurons and glial cells that

innervate the intestine and regulate GI motility, absorption, and fluid secretion (Doyle et al., 2004). Non-neuronal serotonin activates intrinsic primary afferent neurons of ENS through 5-HT1P receptor and mediates gut peristaltic and secretory reflexes, while the activation of the 5-HT3 receptor of extrinsic nerves communicates distress and other signals to the CNS (Gershon and Tack, 2007). Serotonin released outside the gut epithelium also activate the 5-HT4 receptor in the ENS and induce neuroprotective and neurogenerative effect (Liu et al., 2009). Serotonin produced by serotonergic neurons in the ENS influences gut motility and development of enteric neurons, and serotonin furthermore modulates the immune system (Neuhuber and Worl, 2018). However, there is less knowledge about functioning of these receptors in avian species (Stępińska et al., 2015).

Similarity between the avian and mammalian serotonin system

Serotonin is an ancient and highly conserved biomolecule in the vertebrate species found to be localized in the raphe system and reticular nucleus (Challet et al., 1996; Hay-Schmidt, 2000). The serotonin system, including serotonin, 5-HT receptor structure and function, and serotonin transporter, is well-conserved across diverse vertebrates (Bubak et al., 2020). Distribution of serotonin in vertebrate brains has been studied decades ago and is found to coincide with expectations of phylogeny. A comparative study of serotonin and catecholamines distribution by Bogdanski et al. (1963) found occurrence of these amines in mammals and lower vertebrates, including fish and birds. In vertebrates, serotonin exhibits inhibitory action on aggressive behavior as has been observed across diverse animals. Autoradiography of neurotransmitter receptors in a brain basal ganglion in pigeon, rat and human brain have shown similarity in distribution. This includes the 5-HT1B receptor subtype in the globus pallidus (GP) region of basal ganglia which regulates the release of neurotransmitters including serotonin (Dietl and Palacios, 1988; Sari, 2004).

Anatomical structure of the serotonergic system is similar across different vertebrates, but levels of molecular expression and physiologic development do vary. A study reported the serotonin to catecholamine ratio to be 1.1:1 in rats while a 2: 1 ratio has been reported in birds (Bogdanski et al., 1963). The anatomical distribution of monoamine-producing neurons in the avian brain has shown this cell population to occur in the hypothalamus (located below the thalamus) and lateral presence in tegmentum (the ventral part of the midbrain). Similar lateralization is also observed in mammals (Fuxe and Ljunggren, 1965; Dubé and Parent, 1981). Immunohistochemical and immunohistofluorescence techniques have been used to study distribution of serotonin fibers and terminals in pigeon brains and have found similarity in pattern as compared to mammals. Similar to the mammals in birds, serotonergic neurons in the midbrain tegmentum have shown descending projections towards the spinal cord whereas ascending projections towards prosencephalon (the future forebrain/ cerebrum). The projection size is greater however in mammals than in birds (Challet et al., 1996).

Gut microbes in serotonergic system development in avians and mammals

Diverse gut microbes acquired since birth influence neural pathways and CNS signaling, thus contributing to an organism's systems-level development. This specific influence has been studied with various germ-free (GF) animal models (Smith, 2015). Developmental effect of gut microbiota on serotonergic system has been studied in a GF mouse model where chronic absence of microflora elevates striatal 5-HT turnover (Heijtz et al., 2011). Similar results have confirmed this in another study where, observed elevated hippocampal 5-HT and 5-HIAA levels did not change after restoring microbiota in later life. GF animals also exhibit abnormally reduced levels of anxiety which can be restored on GI microbiota transfer. This suggests a crucial role of intestinal microbes in influencing the central serotonin system (Clarke et al., 2013).

Gut microbiota are also known to play an important role in immune system and endocrine system development which are essential elements of CNS signaling. A recent GF study of mice has highlighted the impact of gut microbes on microglial cell maturation and activation where absence of microbes leads to microglial defects affecting innate immune response. This study found, in particular, microbial SCFAs to be a regulator of microglial homeostasis (Erny et al., 2015). Microglial cells have been recently studied as well for their interaction with serotonin and have had reported effects contributing to brain maturation (D'Andrea et al., 2020; Kolodziejczak et al., 2015). Another GF mouse study has shown gut microbes to influence adult ENS maturation through release of serotonin which further activates 5-HT4 receptor in ENS associated with adult neurogenesis and neuroprotection. The study demonstrated the difference in ENS anatomy in GF and with microbiota transfer models influencing intestinal function (De Vadder et al., 2018).

In the case of chickens, Beaver and Wostmann (1962) studied the influence of gut microbes on intestinal serotonin synthesis and observed reduced intestinal 5-HT levels in conventional chicken compared to germ free model. The influence of gut microbes on serotonin system development has been studied in the context of FP behavior whereas the serotonergic pathway is suspected to contribute to FP. The influence of gut microbiota on the serotonergic system and bird behavior has been studied by early life microbiota transplantation in hens selected for high and low FP. The investigation after 15 weeks of treatment observed

variation in peripheral serotonin levels in low FP lines (Van der Eijk et al., 2020). There is another investigation on central serotonin turnover in 28 days-old chicks. Lower serotonin turnover was found for high FP chicks, but this study did not observe an influence of gut microbes (Van Hierden et al., 2002). The regulatory influence of gut microbes on peripheral serotonin system has been established in birds, mice, rats, and humans as well, including for instances specific to disease (Phillips et al., 1962; Böhm et al., 1979; Uribe et al., 1994; Wikoff et al., 2009; Yano et al., 2015; Kelly et al., 2016; Sampson et al., 2016).

Microbiota and microbial metabolites affecting the serotonergic system

Food animals, along with humans, have diversity in their intestinal microbiota that is mainly influenced by the surrounding environment and diets and thus share common microbes. These microbes and their hosts have a close relationship surrounding how metabolism occurs for mutualistic or detrimental benefit, depending on the microbial metabolic activity happening in which part of the host gut (Apajalahti, 2005). Different studies have highlighted some influence of gut microbes and their metabolites on the host's serotonergic system through tryptophan metabolism, serotonin metabolism, and the kynurenine and indole pathway. Among these metabolites, microbial degradation and fermentation product SCFAs are major metabolites produced in the hind gut of avian species (Józefiak et al., 2004). SCFAs have been considered for maintaining gut health of poultry (Liu et al., 2021). The rapid absorption of SCFAs in the hind gut (Ruppin et al., 1980), the association of SCFAs with the BBB (Gerhart et al., 1997; Li et al., 2016), the neuroimmunoendocrine regulatory function of SCFAs (Wikoff et al., 2009; Clarke et al., 2013; Matsumoto et al., 2013) and the neuroprotective effect of SCFAs (Kim et al., 2007) indicate SCFAs to be metabolites important to study for the serotonergic system and overall body.

Short chain fatty acids

SCFAs, also called volatile fatty acids, provide substantial amounts of energy, commonly fulfilling about 10% of human caloric needs and about 8% of the caloric needs of chicken (Annison et al., 1968). SCFAs in addition modulate the physiology and behavior of animals in various ways. Major SCFAs include acetate (C2), propionate (C3), and butyrate (C4) which are produced in animals through the fermentation of various complex carbohydrates such as dietary fibers, resistant starch, and endogenous substance-like mucins (Annison et al., 1968; Langhout and Schutte, 1996; Józefiak et al., 2004; Sun et al., 2021). The proportion of acetate, propionate, butyrate in the colons of herbivorous animal species ranges from 75:15:10 to 40:40:20 (Bergman, 1990). The cecum is the primary site of microbial fermentation in chickens (Marounek et al., 1999). This is evident by the germ free birds cecum having traces of SCFAs compared to conventional bird however, similar quantities of acetate were found in the peripheral blood of conventional and germ-free birds that demonstrate endogenous source of SCFAs, rather than microbial origin (Annison et al., 1968; Høverstad and Midtvedt, 1986). SCFAs production is advantageous to the host as it is known to improve gut health via maintaining intestinal barrier integrity and immune homeostasis (Furuse et al., 1991; Hu and Guo, 2007; Sunkara et al., 2012; Liu et al., 2021). SCFAs also have been found to inhibit growth of *Salmonella* (Van Immerseel et al., 2003), promote the body weight of broiler chickens (Leeson et al., 2005), and modulate inflammation and oxidative stress (Li et al., 2017). A germ-free mice study highlighted the role of butyrate in improving blood-brain barrier integrity which ensures controlled exchange of biological substances essential for brain activities (Braniste et al., 2014). SCFAs are produced by many bacteria through the glycolytic pathway but there are some varieties, such as *Bifidobacterium* spp., that can produce SCFAs via the pentose phosphate pathway (Macfarlane and Macfarlane, 2003; Cronin et al., 2011). Several bacterial varieties from the *Firmicutes* phylum include butyrate producing *Ruminococcaceae*, *Lachnospiraceae,* and clostridial varieties. *Bacteroides* and *Bifidobacterium* spp. are involved in acetate production. Table 2 shows some of the important studies that have detailed SCFAs with chicken gut bacteria.

Short chain fatty acids and the serotonergic system

SCFAs produced in the gut lumen (undissociated form) diffuse through colonocytes or (dissociated form) transported by monocarboxylated transporters such as monocarboxylated transporter 1 (MCT1, a type of pH-dependent hydrogen-coupled monocarboxylated transporter) and sodium-coupled monocarboxylate transport (SMCT1) (Ritzhaupt et al., 1998) (Figure 1). These SCFAs are metabolized by colonocytes for energy production while unutilized SCFAs undergo hepatic portal circulation (Bloemen et al., 2009). From there, SCFAs are taken up by hepatocytes where they are metabolized for energy or utilized for biosynthesis. Thus, a small portion of SCFAs enters peripheral circulation. In circulation SCFAs interact with different host proteins that include G protein-coupled receptors (GPR41, GPR43, GPR109A) on different tissues (Müller et al., 2019). SCFAs (mainly butyrate) in gut lumen stimulate Tph1 expression in enterochromaffin cells. This then leads to increased production of 5-HT by the enterochromaffin cells (Reigstad et al., 2015). Butyrate elevates Tph1 expression through a butyrate inducible zinc finger transcription factor ZBP-89 (Essien et al., 2013). SCFAs in

TABLE 2 Selected studies of SCFA-producing gut microbes in chickens.

Bird sp. and region of isolation	Type of SCFA	Gut microbes identified	References
Broiler chicken cecal-4 weeks old	Butyrate	*Butyricicoccus pullicaecorum* (a *Firmicutes* clostridial cluster IV)	Eeckhaut et al. (2008)
Broiler chicken cecal 6 weeks old	Butyrate	*Faecalibacterium prausnitzii*	Meimandipour et al. (2010)
Broiler chicken cecal 4 weeks old	Butyrate	Isolates of clostridial cluster IV related to *Flavonifractor plautii, Pseudoflavonifractor capillosus, Subdoligranulum variabile, Eubacterium desmolans* and *Butyricicoccus pullicaecorum,* cluster XIVa isolates related to *Anaerostipes caccae, Eubacterium hallii, Clostridium populeti* and *Anaerostipes butyraticus,* cluster XVI related *Eubacterium tortuosum, Eubacterium cylindroides, Streptococcus pleomorphus*	Eeckhaut et al. (2011)
Broiler chickens, ileal mucosa, 3 weeks old	Butyrate propionate	Related to *Enterococcus cecorum* (butyrate) *Butyrivibrio, Coprococcus* (butyrate) *Paludibacter* (propionate)	Shang et al. (2018)
White leghorn chicken caeca	Butyrate	*Megasphaerastantonii* sp. Nov. from genus *Megasphaera*	Maki & Looft (2018)
Cobb 500 broiler chicken, ileal, cecal, 6 weeks	Butyrate	*Ruminococcus, Anaerostipes,* and *Lachnospiraceae*	Jacquier et al. (2019)
Layer chickens, cecal, 8, 20, 50 weeks old	Butyrate Propionate Acetate	Genus *Alistipes* (*Bacteroidetes*) 8 weeks- *Anaerostipes* (butyrate), *Bacteroides thetaiotaomicron* (acetate, propionate) 20 &50 weeks—*Phascolarctobacterium* (propionate) 20 weeks—genus *Bifidobacterium* (acetate)	Sun et al. (2021)

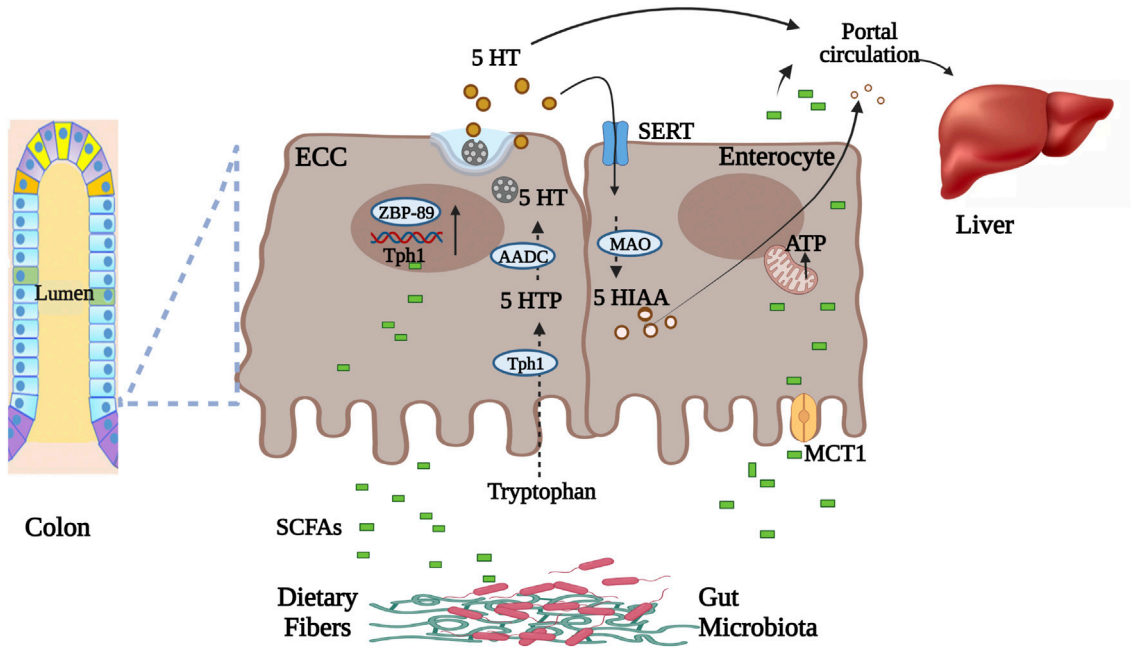

FIGURE 1
Microbial metabolite SCFAs transportation and role in gut serotonin production. Undissociated form of SCFAs in gut lumen diffuse through enterocytes while dissociated is transported through MCT1 into the circulation. Intestinal enterochromaffin cells synthesize serotonin from tryptophan using Tph1 enzyme. SCFAs in gut lumen stimulate Tph1 expression via zinc finger transcription factor. Secreted serotonin, before entering circulation, is either utilized in the liver or metabolized by enterocytes to 5-HIAA. Part of luminal SCFAs is utilized for energy production by enterocytes. Abbreviations: Enterochromaffin cells (ECC), serotonin (5-HT), zinc finger transcription factor (ZBP-89), tryptophan hydroxylase 1 (Tph1), 5-hydroxytryptophan (5-HTP), amino acid decarboxylase (AADC), monocarboxylated transporter 1 (MCT1), serotonin reuptake transporter (SERT), monoamine oxidase (MAO), hydroxyindoleacetic acid (5-HIAA), short Chain Fatty Acids (SCFAs) (Ritzhaupt et al., 1998; Bloemen et al., 2009; Essien et al., 2013; Reigstad et al., 2015). Figure created with BioRender.com.

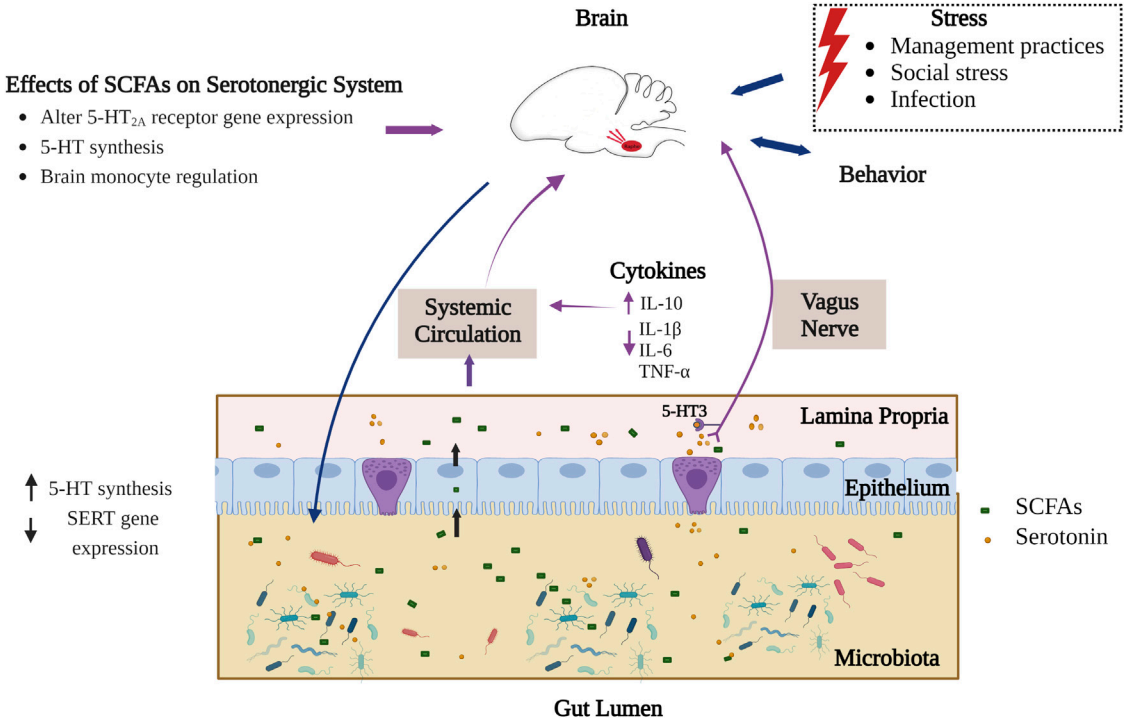

FIGURE 2
Interaction of SCFAs and serotonergic system in the gut-brain axis. Part of the SCFAs produced in gut lumen interact with the central serotonergic system directly (crossing intestinal and blood-brain barrier) by epigenetic modulation and via activating extrinsic primary afferent vagus nerve (interaction of SCFAs through FFAR3). Serotonin is synthesized by both enterochromaffin cells of the gut epithelium and by gut microbiota. SCFAs also stimulate intestinal serotonin synthesis whereas extracellular serotonin binds to 5-HT3 receptors on afferent vagus nerve and communicates signals to the CNS. On the other hand, different external stressors affect gut microbial composition in birds through the HPA axis and influences production of microbial metabolites like SCFAs. The blue arrows indicate established connection in birds while the violet arrows indicate connections known to occur for some animals but not yet identified in birds (Liu et al., 2012; Sealy and Chalkley, 1978; Yamawaki et al., 2012; Huuskonen et al., 2004; Cook et al., 2021; Gill et al., 2013; Essien et al., 2013; Calefi et al., 2016; Noguera et al., 2018; Gershon and Tack, 2007; Meyer et al., 2012). Figure created with BioRender.com.

colonocytes, through varying signaling pathways, influences inflammation by inhibiting NFkB transcription factor), cellular differentiation and proliferation essential for maintaining intestinal homeostasis (Venegas et al., 2019).

The mechanism through which circulatory SCFAs influence the serotonergic system is not fully elucidated and has mainly been investigated with respect to human and mouse models. Considering the very short half-life of SCFAs (such as has been found for butyrate in the bloodstream due to uptake by peripheral tissues), there may be only a minimal concentration of SCFAs that reach the brain when crossing the blood-brain barrier (BBB) (Cummings et al., 1987; Daniel et al., 1989; Mitchell et al., 2011). Within the brain, SCFAs affect brain functioning through direct interactions with G protein-coupled receptors (GPCR) like FFAR2 and FFAR3 (varieties of free fatty acid receptors) (Figure 2). These GPCRs are found in both CNS and peripheral system and are most dense in peripheral organs (Lagerström et al., 2006; Meslin et al., 2015). SCFAs also communicate with the brain via the afferent vagus nerve, leading to the activation of neurons in the CNS area (De Vadder et al., 2014). However, the type of interaction of SCFAs with the vagus nerve, being direct or indirect, is unknown. A study of the vagus nerve FFAR3 knockout mice model showed that SCFAs receptor FFAR3 on the vagus nerve is essential to regulate feeding behavior in animals (Cook et al., 2021). The presence of FFAR3 in the vagus nerve and its influence on feeding behavior may indicate the possibility of SCFA mediated signaling to the central serotonergic system. Additionally, FFAR3 plays an important role in propionate-mediated signals to peripheral and CNS areas for intestinal gluconeogenesis and enhanced noradrenaline secretion by sympathetic neurons respectively (Kimura et al., 2011; De Vadder et al., 2014). Synaptic levels of both neurotransmitters noradrenaline and serotonin are responsible for depressive behavior (Thor et al., 2007). More investigation is overall needed to reveal interactions of SCFAs with the serotonergic system, and the

degree to which these interactions may be present and consistent across different varieties of animals, including birds.

Another way by which SCFAs affect the serotonergic system is in their regulation of tryptophan synthesis. As stated earlier, tryptophan is the only precursor for serotonin biosynthesis and its circulating levels depend on dietary intake and gut bacterial tryptophan metabolism (Fernstrom and Wurtman, 1971). Most of the free tryptophan in blood is utilized by the kynurenine (KYN) pathway. Remaining tryptophan has to pass through the BBB for central serotonin synthesis (Peters, 1991). The systemic level of tryptophan is closely linked with inflammation. As proinflammatory cytokines can induce metabolic enzymes like indoleamine 2,3-dioxygenase (IDO) and Tryptophan-2,3-dioxygenase (TDO) involved in KYN synthesis from tryptophan metabolism (Wirleitner et al., 2003; Hestad et al., 2017). Thus, systemic inflammation can limit availability of tryptophan for serotonin synthesis. However, SCFAs in systemic circulation are known to lower the proinflammatory cytokines (TNF-α, IL-1β, IL-6) and elevate anti-inflammatory and regulatory cytokines such as IL-10 which may indirectly increase availability of tryptophan for serotonin synthesis by balancing the cytokines (Liu et al., 2012; Piazzon et al., 2016).

Short chain fatty acids and histone deacetylase-mediated epigenetic modulation

SCFAs contribute to epigenetic modulation through interaction with histone deacetylases (HDACs) in the brain (Figure 2), however this research has mainly been carried out in mammals. HDACs are crucial in histone deacetylation, which limits the accessibility of genetic material to transcription by compacting chromatin and thus plays an essential role in gene expression (Turner, 2000). HDACs and their regulation are essential for brain development and are studied for neuropsychiatric diseases (Volmar and Wahlestedt, 2015). SCFAs such as butyrate can inhibit HDAC, leading to hyperacetylation resulting in increased accessibility of genes for transcription (Sealy and Chalkley, 1978; Chriett et al., 2019). Monoaminergic neurons, including serotonergic and neuropeptidergic neurons in the brain hypothalamus, express HDACs that deacetylate nuclear as well as cytoplasmic proteins (Takase et al., 2013). Inhibitory effects of butyrate on HDACs have been investigated for serotonin receptor 5-HT2A which are densely present in CNS and high in the cerebral cortex. A gene expression study in sodium butyrate-administered rats has shown downregulation of the 5-HT2A receptor potentially due to inhibitory action of butyrate on HDAC leading to an antidepressant outcome in rats (Yamawaki et al., 2012). Another *in vivo* study on intestinal epithelial cells has further implicated SCFAs with epigenetic change and has shown there to be an inhibitory role of butyrate on HDAC2 that regulates SERT gene expression. Intestinal SERT is essential in maintaining extracellular serotonin levels (Gill et al., 2013). SCFAs have in addition been investigated for brain histone crotonylation as an epigenetic modification that involves transfer of a crotonyl group to lysine residues which influences the gene expression (Tweedie-Cullen et al., 2012), but the functional role of this crotonylation is still unknown (Fellows et al., 2018).

Short chain fatty acids and neuroinflammation

An understanding of neuroinflammation and the role of short-chain fatty acids in chickens awaits further study. A general understanding would for now involve dynamics as reported for other types of organisms. Butyrate in particular has been found to improve CNS neuroinflammation in mice models induced by lipopolysaccharides (LPS) (Wang et al., 2018; Yamawaki et al., 2018). Neuroinflammation is characterized by activating microglial cells (immune cells of CNS) that follow the elevation of proinflammatory cytokines like IL-6 and TNF-α. At the same time, cytokines and their signaling pathways affect serotonin synthesis and metabolism (Jeon and Kim, 2017). Butyrate can improve circumstances of neuroinflammation through suppression of NF-κB activation and through its aforementioned role in HDAC inhibition, overall controlling the number of microglia cells and astrocytes as has been found in both *in vitro* and *in vivo* models (Huuskonen et al., 2004). These neuroprotective effects of butyrate are observed to enhance memory and restore cognitive functions in mice after systemic or local administration of sodium butyrate (Ferrante et al., 2003; Govindarajan et al., 2011). SCFAs also play a crucial role in immune cell maturation and differentiation. In particular, it has been proposed that SCFAs might regulate brain monocytes such as Ly6Chi, which has been proposed to be essential for hippocampal neurogenesis and memory retention. These monocytes are important for maintaining brain homeostasis (Möhle et al., 2016).

Discussion

Research on the gut-brain axis has been increasingly extensive in the last decade, stemming from its importance in health and disease, and in maintaining physiological homeostasis. This axis is proving to be particularly important to neurodevelopment and neuropsychiatric disorders. The advancement and availability of sequencing technology has led to a plethora of studies investigating how the gut microbiome plays a major role in the gut-brain axis. The dynamic across this axis regarding the effect gut microbial composition with conditions of the brain has been shown to be influenced by multiple factors, including diet, age, and stress.

Chicken microbiome studies include mostly 16S rRNA gene amplicon sequencing-based studies, but there have been some metagenomics approaches as well (Gilroy et al., 2021). Microbial compositional results of similar chicken breeds have shown variation that can be attributed to experimental protocol or differences between individual chickens (Borda-Molina et al., 2018). Most chicken gut microbiome studies of the gut-brain axis are limited to gut microbial modulations that do not identify underlying mechanisms, such as those possibly involving metabolites. Further research regarding chicken gut microbial metabolites is needed to elevate our knowledge to a level comparable to studies of humans and other common animal models such as mice.

Both for agribusiness and translational objectives, further investigations of the chicken gut-brain-microbiome axis would be well-warranted. Previous studies in chicken have shown bird behavior relating to broad-ranging differences in gut microbiota (Meyer et al., 2013b; Ji et al., 2019). Current findings suggest that some of this dynamic can be circular. Gut microbes potentially influence the serotonergic system and FP behavior in chickens (de Haas and van der Eijk, 2018). Conversely however, feather ingestion also by itself alters gut composition and SCFAs production (Meyer et al., 2012). For how FP continues to pose economic and animal welfare problems, investigating gut microbial metabolites' effect on the serotonergic system and chicken behavior such as FP and vice versa would be essential for identifying exact mechanisms and associated interventions.

In the case of the translational potential of gut-brain axis research, animal models have helped to reveal the connection between gut microbes and their metabolites with brain neural processes and functioning. Microbiome, behavioral, serotonin and other physiologic indicators implicate similar dynamics across these two different organisms. Compared to chickens, while some other animal models have helped illuminate methodologies and general findings of gut-brain axis dynamics, their translational value can be limited. The germ-free mouse model has enriched gut-brain axis research, showing for instance that cognitive deficits that can be restored on microbiota transplantation (Luczynski et al., 2016). Current clinical beneficial effects of microbiota transplantation have been limited to treating irritable bowel syndrome (IBS). The possible reasoning behind this limited translational impact thus far may relate to the constrained range of animal models that have been utilized. The detachment of laboratory mice from the natural environment means that these models lack the environmental exposure similar to humans and thus lack gut microbial diversity (Masopust et al., 2017). By comparison, chickens can be readily studied in outdoor and indoor environments through commonly available agricultural enclosures. Past research on avian cognitive neuroscience has furthermore found that the avian brain can be used to understand human cognition despite significant physiological and genetic differences (Rose, 2000; Clayton and Emery, 2015). Domestication of chicken by humans and similarity in the microbial community at higher taxonomic levels supports logistics and relevance for how chickens as a model animal can be used to investigate the gut-brain axis with the hope of high translational efficiency (Kohl, 2012). The similarity in microbial community and complexity facilitates further development and calibration of underlying biotechnological and analytical methodologies needed for robust examinations of microbiomes. Finally, as is the case with other vertebrates, the chicken GI tract may be considered to enclose diverse microbiota and their metabolites, with some of these metabolites being modulators of birds' behavior. Gut microbial metabolites SCFAs stimulate enteric serotonin synthesis and are responsible for maintaining gut health. SCFAs affect brain functioning through direct interaction via HDAC-mediated epigenetic modulation and immune signaling. A challenge remains however with most of these studies being from mice models. Further mechanistic and longitudinal studies in chickens would help validate the likely consistency by which these mechanisms dynamics could be considered across animals in general, including humans. There is overall joint benefit for how further research into SCFAs within chickens helps to advance chickens as a model animal to be considered further for translational and applied gut-brain axis studies, as would both help tackle complex, multifaceted neuropsychiatric disorders in humans and investigate conditions of health and behavior of chickens in agricultural contexts.

Conclusion

Previous research studies in avian species have shown that experimental manipulation of gut microbiota has an impact on bird behavior. There is a wide range of behaviors that are influenced in birds that includes FP which is considered important for poultry welfare. However, there are fewer studies in birds investigating exact mechanisms that drive the gut-microbiome-brain axis. Chicken gut microbiota have a high abundance of *Bacteroidetes* and *Firmicutes* phyla which includes most of those bacterial genera that produce SCFAs. SCFAs and serotonin are important mediators of the gut-microbiome-brain axis with, for an instance, the influence of SCFAs on peripheral as well as central serotonergic systems and the potential association of serotonin with FP behavior in birds. Chicken gut microbial metabolites like SCFAs and their effects on the serotonergic system remain an essential area for further inquiry needed to understand behavioral outcomes in birds. Considering the nature of SCFAs interactions and the conserved molecular and behavioral attributes of the serotonergic system, poultry chicken may be an emergent translational model for identifying underlying mechanisms of change within the gut-microbiome-brain axis.

Author contributions

YF, JH, and SH conceived the general idea for the overall review area of study. VJ conceived specific ideas for the review and took the lead in writing the manuscript. YF, JH, and SH provided editing and critical feedback. All authors read and approved the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Temporal dynamics of the chicken mycobiome

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The microbiome is an integral part of chicken health and can affect immunity, nutrient utilization, and performance. The role of bacterial microbiota members in host health is relatively well established, but less attention has been paid to fungal members of the gastrointestinal tract (GIT) community. However, human studies indicate that fungi play a critical role in health. Here, we described fungal communities, or mycobiomes, in both the lumen and mucosa of the chicken ileum and cecum from hatch through 14 days of age. We also assessed the effects of delayed access to feed immediately post-hatch (PH) on mycobiome composition, as PH feed delay is commonly associated with poor health performance. Chicken mycobiomes in each of the populations were distinct and changed over time. All mycobiomes were dominated by *Gibberella*, but *Aspergillus*, *Cladosporium*, *Sarocladium*, *Meyerozyma*, and *Penicillium* were also abundant. Relative abundances of some taxa differed significantly over time. In the cecal and ileal lumens, *Penicillium* was present in extremely low quantities or absent during days one and two and then increased over time. *Meyerozyma* and *Wickerhamomyces* also increased over time in luminal sites. In contrast, several highly abundant unclassified fungi decreased after days one and two, highlighting the need for improved understanding of fungal gut biology. Mycobiomes from chicks fed during the first 2 days PH *versus* those not fed during the first 2 days did not significantly differ, except during days one and two. Similarities observed among mycobiomes of fed and unfed chicks at later timepoints suggest that delays in PH feeding do not have long lasting effects on mycobiome composition. Together, these results provide a foundation for future mycobiome studies, and suggest that negative health and production impacts of delayed feeding are not likely related to the development of fungal populations in the GIT.

KEYWORDS

chicken, broiler, fungi, mycobiome, microbiome temporal chicken mycobiome

Introduction

The gastrointestinal (GI) microbiome is a complex and diverse group of microorganisms including bacteria, archaea, fungi, viruses, and protists. The bacterial members (bacteriome) are the most abundant microbial group in the microbiome and have been investigated in detail through 16S-based sequencing. The bacteriome has been shown to play a critical role in host health through its role in nutrition, immune system development, metabolism, and pathogen control (Chambers and Gong, 2011; Oakley et al., 2014; Pan and Yu, 2014; Stanley et al., 2014; Clavijo and Flórez, 2018). The fungal members (mycobiome) are considered part of the "rare biosphere" based on their numerical inferiority in the gut microbiome (Huffnagle and Noverr, 2013). Investigations of the mycobiome through high-throughput sequencing technologies have lagged behind bacteriome studies due to difficulties in isolating DNA from fungal cells, primer design complexities, and database inaccuracies and missing data (Huffnagle and Noverr, 2013; Arfken et al., 2022). However, recent progress in the mycobiome field demonstrates that fungi play a vital role in host health through fungal-bacterial interactions and fungal-host interactions (Mason Katie et al., 2012; Iliev and Leonardi, 2017; Sam et al., 2017; Tso et al., 2018; Leonardi et al., 2022).

Interactions in the gut are complex and often mediated by diverse metabolites released by both the microbes and the host. These metabolic interactions are vital in biological processes including digestion and health (Iliev and Leonardi, 2017; Mims et al., 2021; dos Santos et al., 2020; Kim et al., 2017; Banani et al., 2016; Curbete and Salgado, 2016; Hallen-Adams and Suhr, 2017). Data on the mycobiome in poultry health is limited and most studies have been culture-based with limited organs investigated to date (Shokri et al., 2011; Hume et al., 2012; Byrd et al., 2017; Sokół et al., 2018; Cafarchia et al., 2019). Recently, the GI tract of the chicken was investigated with ITS2-based Illumina sequencing and temporal and spatial changes in the mycobiota were demonstrated (Robinson et al., 2022). The dominant fungal taxa identified in the GI tract was *Fusarium pseudonygamai* regardless of age of broiler chickens (Robinson et al., 2022). Given the potential impact of the gut fungal communities on chicken health, it is important to investigate the mycobiome as a target of dietary manipulation to enhance animal performance and disease resistance.

In commercial broiler production, newly hatched chicks are often deprived of feed for up to 72 h (Careghi et al., 2005; Mitchell, 2009; van de Ven et al., 2009; Willemsen et al., 2010; de Jong et al., 2017) due variable hatch times (24–48 h), sexing and sorting, vaccination, and transport from the hatchery to farms (Careghi et al., 2005). This post-hatch (PH) delay in access to feed is associated with poor health performance including reduced weight and growth rate (Bigot et al., 2003; Careghi et al., 2005), altered GI development (de Jong et al., 2017), and decreased nutrient utilization (Shinde Tamboli et al., 2018) and breast muscle development (Bigot et al., 2003; Powell et al., 2016). We showed previously that PH delay induces changes in some microbial gut taxa (Proszkowiec-Weglarz et al., 2022), as well as changes in the expression of genes involved in lipogenesis (Richards et al., 2010), cecal development (Qu et al., 2021), calcium and potassium transporters (Proszkowiec-Weglarz et al., 2019), carbohydrate and amino acid utilization (Payne et al., 2019), and the functioning of small intestine gut barrier and tight junction related genes (Proszkowiec-Weglarz et al., 2020). Here, we investigated the cecal and ileal mycobiomes in newly hatched chicks with or without delayed access to feed through 14 days of age.

Methods

Animals and experimental protocols

All animal experiments were approved by the USDA-ARS-BARC Institutional Animal Care and Use Committee. Samples in these studies were obtained concurrently with a previously published study (Proszkowiec-Weglarz et al., 2022) and all animal and experimental procedures were performed as in that study. In brief, 250 fertile Ross 708 broiler chicken eggs were acquired from Perdue Hatchery (Hurlock, MD) and incubated as described previously at USDA-ARS (Proszkowiec-Weglarz et al., 2019; Proszkowiec-Weglarz et al., 2020). Birds were hatched during a 486–496 h window of incubation. Three batches of hatchlings were removed within 180–240 min of occlusion, and randomly assigned among experimental groups (Table 1) such that each battery pen included birds from each batch (14–15 hatchlings per battery pen total). Battery-brooders were heated and equipped with two nipple drinkers and one feeder. Gender of chicks was determined at sampling time and equal proportions of male and female chicks were included in the study. In order to mimic PH feed delay in commercial hatchery operations, hatchlings were randomly divided into two treatment groups (n = 6 battery pens for each treatment), the "fed" group, which received feed immediately upon entry to the pen, and the "unfed" group which did not receive feed during the first 48 h. After 48 h, both groups have equal access to feed. Feed was a commercial type of corn-soybean meal-based starter diet (Proszkowiec-Weglarz et al., 2019; Proszkowiec-Weglarz et al., 2020).

Tissue sampling

Birds were sampled at days 1 (24 h), 2 (48 h), 3 (72 h), 4 (96 h), 6 (144 h), 8 (192 h), 10 (240 h), 12 (288 h) and 14 (336 h) after the start of feeding. Sampling times were based on prior data (Richards et al., 2010) and adjusted to obtain a comprehensive coverage of the 2 week period PH. Starting at 24 h PH, one chick per pen was randomly selected and sacrificed by cervical dislocation.

TABLE 1 Experimental design indicating all factors tested in this study.

Number of Samples (n)	Day PH	Site of Collection							
		IL_L		IL_M		CE_L		CE_M	
		fed	unfed	fed	unfed	fed	unfed	fed	unfed
	1	5	3	5	5	5	5	5	5
	2	6	1	6	4	5	5	6	4
	3	6	5	5	5	5	5	5	5
	4	5	5	6	4	5	5	6	4
	6	5	5	5	5	5	5	5	5
	8	5	5	5	5	5	5	5	5
	10	5	5	6	6	6	6	6	6
	12	5	5	6	6	6	6	6	6
	14	5	5	6	6	6	6	6	6

Organ contents and epithelial scrapings were collected from the ileum (from Meckel's diverticulum to ileocecal junction) and the middle of the ceca to represent luminal (L) and mucosal (M) samples, respectively. Samples were snap-frozen in liquid nitrogen and stored at −80°C until fungal DNA isolation. Samples from a single experimental group, day 2-unfed-ileum lumen, were removed from all statistical analyses due to insufficient sample size (n = 1).

DNA extraction and sequencing

DNA was extracted from 374 samples as previously described (Arfken et al., 2020). Briefly, DNA was extracted from the ileum and cecum (200 mg of content or 100 mg of scrapings) with the DNeasy PowerSoil kit (Qiagen, Valencia, CA) utilizing a QIAcube instrument (Qiagen) according to the manufacturer's protocol. DNA concentration and quality were assessed by NanoDrop (ThermoFisher Scientific, Waltsham, MA) and a Tapestation System (Agilent Technologies, Santa Clara, CA, respectively. The ITS region was sequenced utilizing primers ITS3 (5′ GCATCGATG AAGAACGCAGC 3′) and ITS4 (5′ TCCTCCGCTTATTGATAT GC 3′) with the Illumina adaptor sequence added to the 5′ end. ITS regions were sequenced with the Illumina MiSeq Sequencing platform, generating 300 bp paired-end reads, respectively.

Fungal ITS processing

Sequences with an average quality score less than Q15 across four bases or more were removed using the sliding window option in Trimmomatic 0.38 (Bolger et al., 2014). Reads were then imported into QIIME2 version 2021.11 for further analysis. Cutadapt was used to remove forward and reverse primers from paired reads (Martin, 2011). Amplicon sequence variants (ASVs) were identified using the dada2 plug-in. The QIIME2 formatted UNITE fungal ITS database version 8.3 (clustered at 99%) (Kõljalg et al., 2013) was downloaded and imported into QIIME2. Taxonomic classifications were assigned to ASVs using a naïve bayes classifier trained on the UNITE database. Rarefaction curves were plotted in QIIME2, and a threshold of 10,000 reads was selected as the minimum sequencing depth for each sample. Alpha diversity metrics including the number of ASVs, Pielou's Evenness, and the Shannon index were calculated within QIIME2 on rarefied data.

Statistical analyses

Statistical calculations were performed in R (R Core Team, 2022). Feature tables and alpha diversity values were exported from QIIME2 and imported into R using the package qiime2R. Alpha diversity metrics were tested for normality and homogeneity of variances using the Shapiro-Wilke test implemented using the shapiro.test function within the stats package (R Core Team, 2022) and Levene's Test implemented using the levene Test function in the car package (Fox and Weisberg, 2019). Data were transformed using the Box-Cox transformation, function boxcox, within the package MASS (Venables and Ripley, 2002) when data were not normally distributed. Alpha diversity metrics were compared across time (days 1–14), treatment (fed *versus* unfed), and site (cecal lumen, cecal mucosa, ileal lumen, ileal

TABLE 2 ANOVA results from alpha diversity analyses.

Model term	# ASVs	Evenness	Shannon index
Days	1.92E-7 ***	1.19E-10 ***	0.0001 ***
Treatment	0.0306 *	0.0009 ***	0.0274 *
Site	8.2E-7 ***	<2.00E-16 ***	<2e-16 ***
Days x Treatment	NA	0.022 *	0.11
Days x Site	3.84E-9 ***	0.0031 **	0.0050 **
Treatment x Site	0.0140 *	0.132	NA

NA, term not included in final model.
Signif. codes: $p < .0001$ '***' , $p < 0.001$,'**' $p < 0.01$, '*' $p < 0.05$.

mucosa), as well as across all interactions (time x treatment, time x site, site x treatment, and time x treatment x site) using the aov function within the stats package (R Core Team, 2022). Posthoc pairwise testing was performed using the TukeyHSD function within the stats package (R Core Team, 2022).

Multiple functions in vegan (Oksanen et al., 2022) were used to interrogate changes in beta-diversity across samples with respect to time, treatment, and site, as well as interactions between the factors as defined above. Bray-Curtis dissimilarity matrices were constructed using vegdist (Oksanen et al., 2022) on rarefied data, and principle coordinates analysis (PCoA) was performed on Bray Curtis matrices using the cmdscale function in the stats package (R Core Team, 2022). The adonis (Oksanen et al., 2022) function in conjunction with adonis.pair (package: EcolUtils (Salazar, 2022)) were used to test for differences among group centroids, and betadisper (Oksanen et al., 2022) and permutest (Oksanen et al., 2022) were used to test for homogeneity of multivariate dispersions across time, treatment, and site.

Changes in relative abundances within each site and across time ("early", days 1–4, and "late", days 6–14) and treatment were assessed using Maaslin2 with default parameters (Mallick et al., 2021). In brief, data were log transformed, normalized using total sum scaling (TSS), standardized using the z-score, and modeled with a linear model. Taxa were assumed to be differentially abundant if q-values <0.2, as recommended by the software. ASVs present in less than 1% of samples were removed prior to analysis to minimize spurious results. All data in the manuscript are plotted with ggplot2 (Wickham, 2016).

Results

Alpha diversity

Whether a chick was fed or not during the first 2 days PH did not have a large effect on evenness, richness (as measured by the number of ASVs), or the Shannon index (Supplementary Figures S1–S3). The main effects for treatment (fed vs. unfed) were significant across all three metrics (Table 2, Shannon: p = 0.0274, Evenness: p = 0.0009, # ASVs: p = 0.0306), and treatment x site was significant in the number of ASVs (Table 2, p = 0.0117). However, only a single pairwise comparison within a site was significant; a greater number of ASVs were detected in the ileal lumen in fed than unfed chicks (p = 0.0140) (Figure 1). Evenness differed among fed and unfed chicks during days 1 and 2 but this was not statistically significant (Supplementary Figure S2).

The number of ASVs, Shannon Index, and evenness differed significantly across time, but only in the cecum. In the cecal lumen, significantly fewer ASVs were identified during days 1–4 than in days 8–14 (Supplementary Figure S4; Supplementary Table S1). In the cecal mucosa and lumen, evenness tended to decrease from days 1–8, and then increase from days 8–14. However, in pairwise calculations, in the cecal lumen only day 1 was significantly different than day 8, and in the cecal mucosa, days 1–4 were significantly greater than day 8, and day 1 was greater than day 10 (Supplementary Figure S5; Supplementary Table S2). Shannon diversity showed a similar trend to evenness in the cecal mucosa, with days 1–3 significantly greater than day 8 (Supplementary Figure S6; Supplementary Table S3). All three alpha diversity metrics were significantly different across sites (Shannon: $p < 2 \times 10^{-16}$, Evenness: $p < 2 \times 10^{-16}$, # ASVs: $p = 8.2 \times 10^{-7}$), with evenness, richness, and Shannon higher in the mucosal communities than the luminal ones (Figure 2).

Beta diversity

Overall community structure differed across time (p = 0.001), site (p = 0.001), site x time (p = 0.001), site x treatment (p = 0.005), and site x time x treatment (p = 0.009) (number of permutations = 999). Overall community structure was not significantly different among fed and unfed chicks within each organ (Figure 3), except during days 1 and 2 where fed and unfed communities in the cecum lumen and day 1 in the ileal lumen clustered independently (Supplementary Figures S7, S8). This pattern was not observed in the cecal and ileal mucosa (Supplementary Figures S9, S10). In contrast, communities differed significantly from each other across all sites (p = 0.001, Figure 4). Similarly, differences were observed across time (Figure 5, p = 0.001). In the cecal lumen, most days were significantly different than all other days, while in the cecal mucosa, days 1–2 differed from all other days and days 3–4 differed from a few other days (Supplementary Table S4). In the ileal lumen, the majority of differences occurred between day 1 and all other days, while in the ileal mucosa, days 1 and 2 differed from all others (Supplementary Table S4).

Differential relative abundance

All communities were dominated by *Gibberella* (63% in cecum, 38% in ileum) and unidentified Fungi (12% in cecum, 46% in ileum), but additional genera *Aspergillus, Penicillium, Sarocladium,*

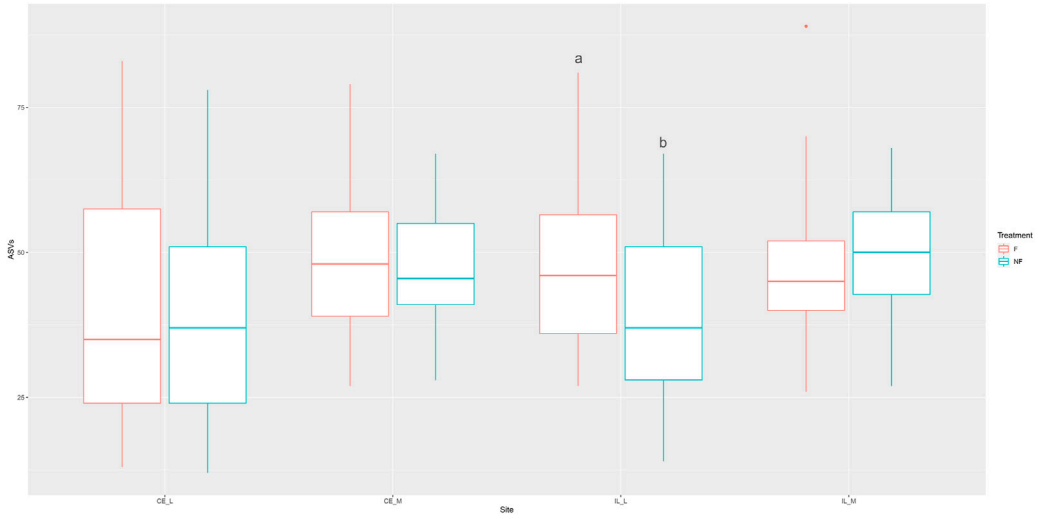

FIGURE 1
Boxplot of the number of ASVs observed across all four sites (CE_L = cecal lumen, CE_M = cecal mucosa, IL_L = ileal lumen, IL_M = ileal mucosa) in fed and unfed chicks. The number of ASVs was not significantly different at any site except for the ileal lumen, where a greater number of ASVs was observed in fed compared to unfed chicks. Box represents the interquartile range, with the lower edge of box = 25% percentile, upper edge of box = 75% percentile, the midline = the median, and upper whisker = the largest value within 1.5 times the interquartile range above 75% percentile, lower whisker = the largest value within 1.5 times the interquartile range below 25% percentile. Dots represent outliers. Means not sharing any letter are significantly different by the Tukey-test, alpha = 0.05.

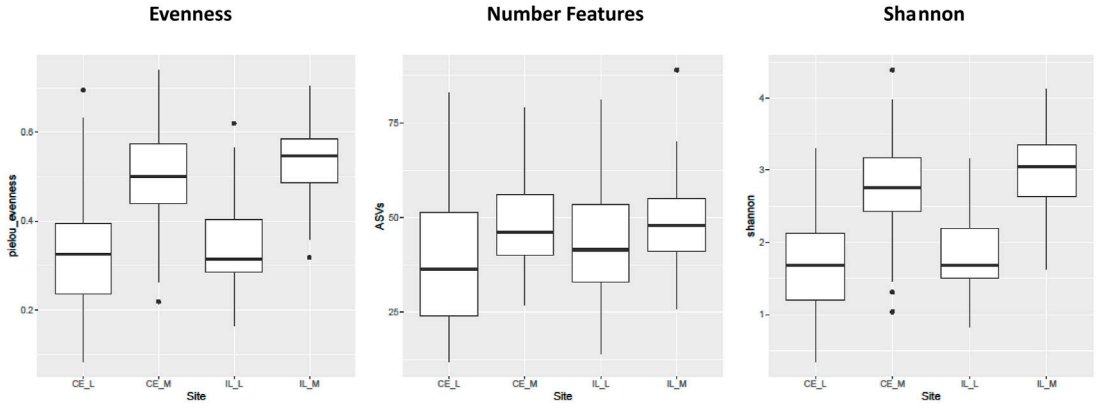

FIGURE 2
Boxplots of all the main effects of all three alpha diversity metrics across sites. Alpha diversity is greater in luminal (CE_L, IL_L) than mucosal (IL_M, IL_M) samples. Plot definitions as in Figure 1.

Cladosporium, and *Meyerozyma* were also abundant among all samples (Figure 6). At each site, we compared relative abundances of genera between days 1–4 ("early") and days 6–14 ("late"). In the cecal and ileal lumen, a greater number of genera (19 and 17, respectively) were detected as differentially abundant over time compared to the mucosal samples, where the relative abundances of only three genera changed in the cecal mucosa. No differentially abundant taxa were found in the ileal mucosa (Table 3). In the cecal lumen, 18 genera including *Wallemia, Meyerozyma*, *Penicillium*, and *Pyxidiophora* (adjusted *p*-values = 0.002, 0.021, 0.022, 0.045, respectively) increased in relative abundance over time (Figure 7), and unknown genera decreased (Table 3). In the ileal lumen, *Coniochaeta* levels decreased over time while that of 16 genera increased including *Clavispora, Suhomyces, Dipodascus*, and *Fusarium*. In the cecal mucosa, *Gibberella* increased over time while unknown genera decreased. Few taxa were observed to be

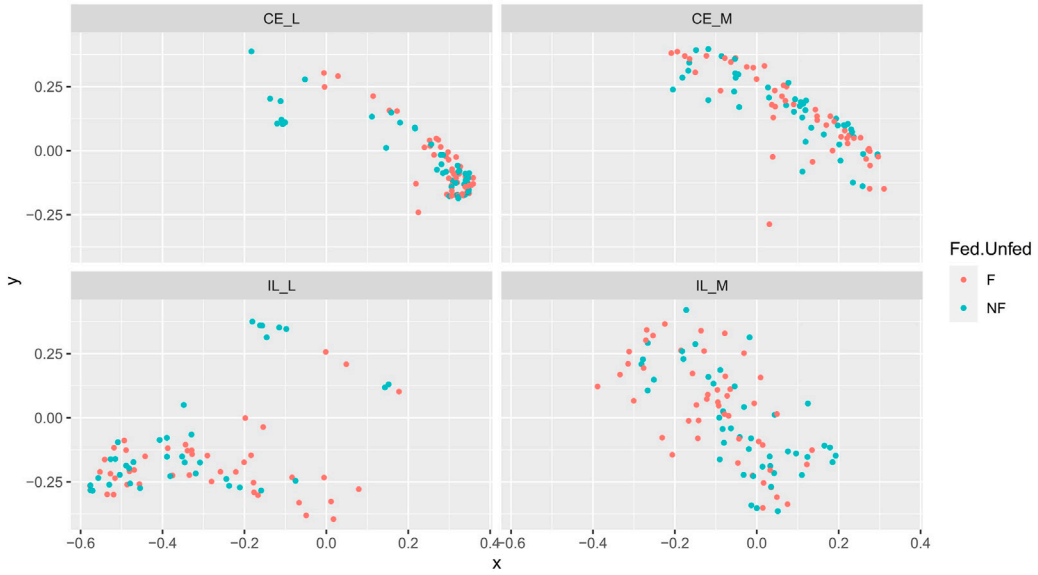

FIGURE 3
Comparison of beta-diversities. Principal coordinates analysis of Bray-Curtis matrices of fed and unfed samples at each site. Community structure was not significantly different among fed and unfed chicks at any site.

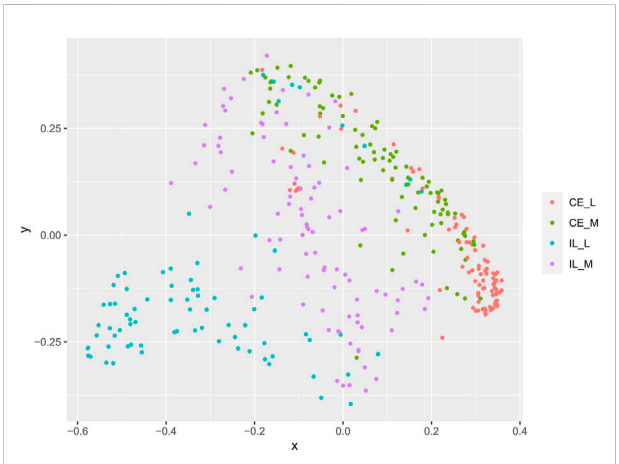

FIGURE 4
Comparison of beta-diversities. Principal coordinates analysis of Bray-Curtis matrices at each site. Community structure at all sites differed from all other sites.

differentially abundant in fed *versus* unfed chicks. However, unknown genera were more abundant in the fed than in the unfed samples in both the cecal lumen and mucosa.

Discussion

The mycobiome is becoming increasingly recognized as a critical component of the gut microbiota, with multiple roles in host health including interactions with the immune system, alteration of metabolism, the reduction or exclusion of pathogens, and digestion (Cui et al., 2013; Iliev and Leonardi, 2017; Iliev and Cadwell, 2021). However, few studies have addressed fungal populations in chickens, and the majority utilized culture-based techniques which may not provide a complete inventory of fungal taxa (Shokri et al., 2011; Hume et al., 2012; Byrd et al., 2017; Subramanya et al., 2017; Sokół et al., 2018; Cafarchia et al., 2019). Here, we use next-generation sequencing to investigate developmental changes in the mycobiome in both mucosal and luminal portions of the ileum and cecum and determine whether delays in PH access to feed affect fungal gut communities.

In total, we identified 88 unique fungal genera across 374 samples. *Gibberella* and unidentified Fungi dominated all samples, with *Gibberella* present in higher proportion in the cecum (63%) than in the ileum (38%). Two prior mycobiome studies in chicks which utilized ITS2 and Illumina sequencing identified a similar number of genera, 125 (Robinson et al., 2020), and 81 (Robinson et al., 2022), and identified *Microascus* and *Fusarium pseudonygamai* as the dominant genera, respectively. *Fusaria* is the anamorph of *Gibberella*, thus our findings coincide with these results somewhat. However, unidentified Fungi were also prevalent in our samples, as well as *Aspergillus, Cladosporium, Sarocladium, Meyerozyma*, and *Penicillium*. Of these, only *Aspergillus* was among the most abundant genera in prior studies (Robinson et al., 2022). Although few mycobiome studies have been performed in chickens, a meta-analysis of human mycobiome studies also indicated that only a small

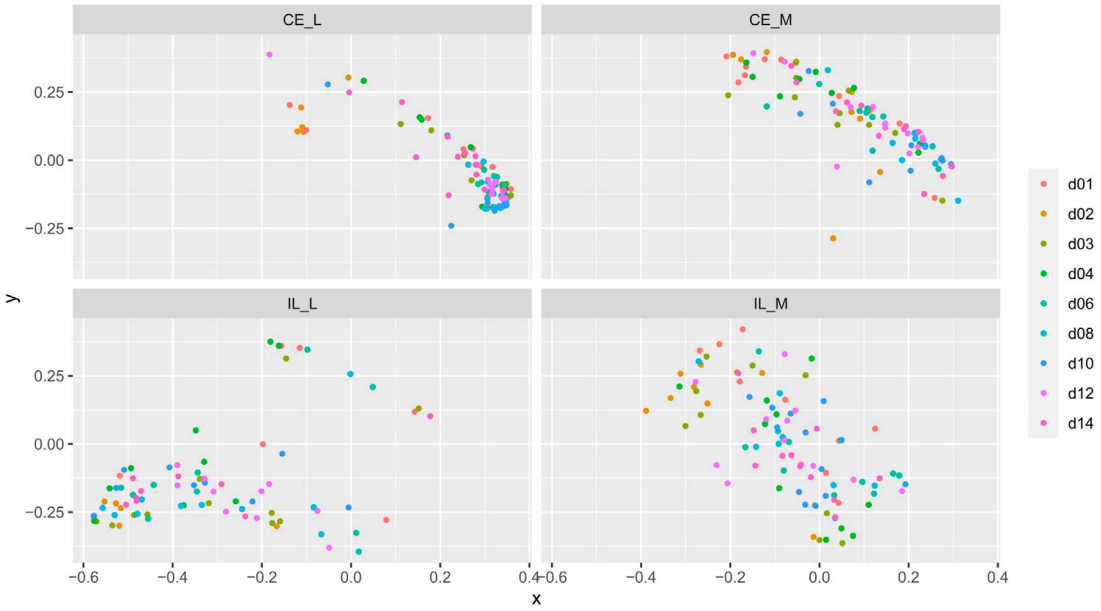

FIGURE 5
Comparison of beta-diversities. Principal coordinates analysis of Bray-Curtis matrices of each day within each site. Community structures significantly differed across time.

proportion of identified gut fungi (15/267 species) were common across studies (Hallen-Adams and Suhr, 2017).

The variable taxonomic composition of the mycobiome can be attributed to the fact that few fungal taxa are suited for permanent colonization of the gut, and are instead transient (Hallen-Adams and Suhr, 2017). Populations are highly influenced by food and the environment as strains enter the gut through ingestion (Penders et al., 2006; Dollive et al., 2013; David et al., 2014; Hallen-Adams and Suhr, 2017; Mims et al., 2021; Robinson et al., 2022). In commercially produced chickens, the majority of mycobiome members present in the early PH days match those present in the hatchery environment, feed, and bedding (Robinson et al., 2022). Further, the most highly abundant taxa are most frequently associated with environmental habitats. *Gibberella* is a plant pathogen (Desjardins, 2003), *Aspergillus* spp. are environmental saprobes (Pangging et al., 2022), *Cladosporium* is ubiquitous among various organic materials (Salvatore et al., 2021) and *Penicillium* is often associated with food (Yadav et al., 2018).

Due to the critical role of diet and the environment in shaping fungal gut communities, we asked whether delayed PH access to feed affects the development of the chick mycobiome. To mimic conditions in commercial production facilities, we formed two groups, "unfed" which received no feed during the first 2 days PH, and "fed" which received feed upon entry to the battery pen. By day 3, both groups had unlimited access to feed. In the luminal samples of both the cecum and ileum, beta-diversity analyses showed a significantly different community structure in fed and unfed samples during days 1 and 2, but not during later days (Supplementary Figures S7, S8). In the cecal lumen at day 1, fed samples consisted mainly of *Gibberella* (83%), while unfed communities were composed of *Incrucipulum* (32%), *Cladosporium* (25.4%), *Coniochaeta* (14%), and unidentified Fungi (11%). *Incrucipulum* and *Coniochaeta* are environmental taxa, with *Incrucipulum* often associated with fallen leaves or twigs (Tochihara and Hosoya, 2019), and *Coniochaeta* found on diverse substrates including soil, plants, butter, and feces (Si et al., 2021). At day 2 *Gibberella* remained low (4%), *Incrucipulum* dropped to less than 1%, and *Coniochaeta*, unidentified Fungi, and *Cladosporium* comprised the majority of the remainder of the samples. By day 3, *Gibberella* dominated both fed and unfed samples (84.9% and 69.1%, respectively), and remained the dominant genus through day 14 (fed, 75% and unfed, 74%). In the ileal lumen, fed samples were dominated by *Gibberella* and unidentified Fungi through all days, but unfed samples on day 1 also contained *Purpureocillium* (21%), a ubiquitous environmental saprobe found in soil, air, and plant matter (Luangsa-ard et al., 2011), and at day 2, *Coniochaeta* became dominant (60%). It should be noted that only a single sample comprised the day 2, ileal lumen, unfed experimental group. This sample is described here but excluded from all statistical analyses. Similar to the cecal lumen, by day 3, *Gibberella* and unidentified Fungi dominated all ileal lumen samples, regardless of whether they received feed immediately PH. Thus, our findings are in line with those prior showing that the mycobiome is greatly influenced by diet (David et al., 2014; Hallen-Adams and Suhr, 2017).

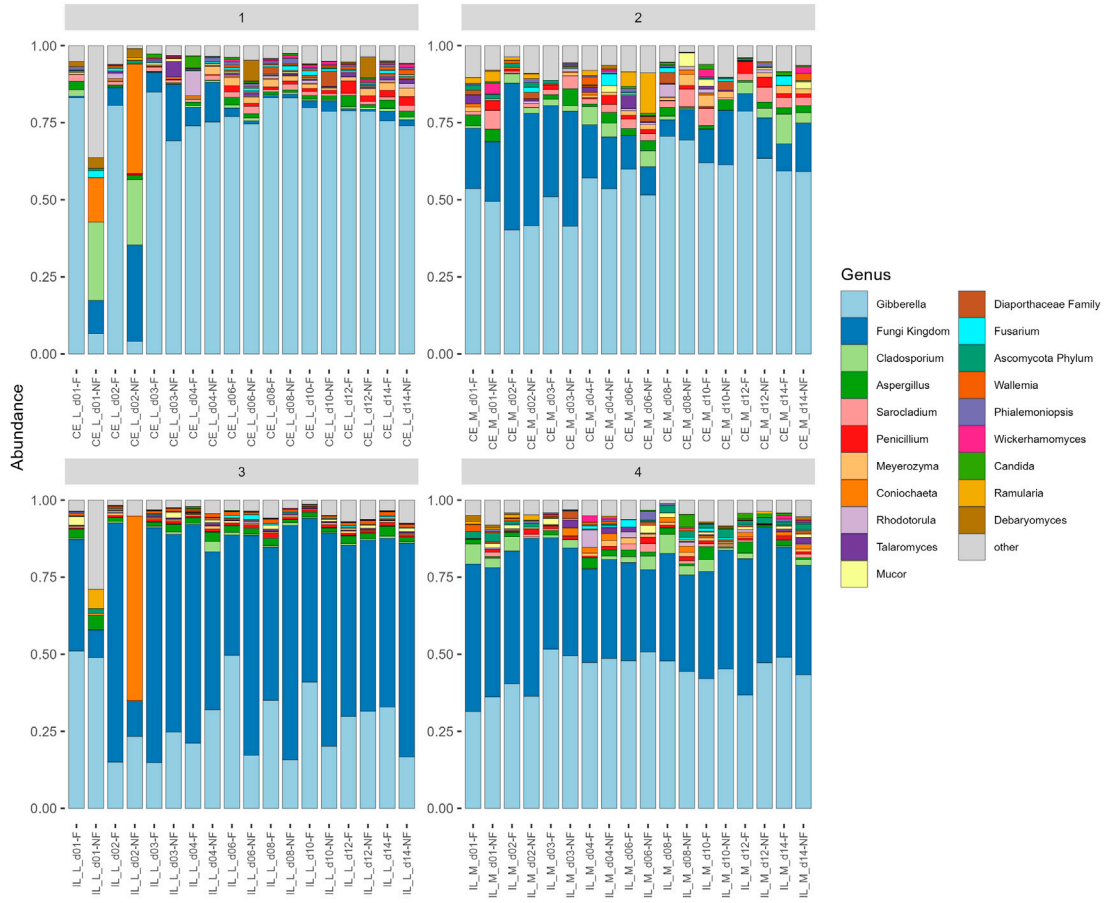

FIGURE 6
Barplot of average relative abundances of each sample group at each site. Only the 20 most abundant taxa are shown, all other are combined into a category "other." *Gibberella* and unidentified Fungi dominate all samples, but the proportion of each varies.

Alpha diversity metrics did not differ significantly across fed and unfed samples, except for the number of ASVs, which was greater in the ileal lumen in fed than in unfed chicks (Figure 1; Supplementary Figure S1). In the cecal lumen, a greater number of ASVs was also identified in fed *versus* unfed chicks during six of the 9 days tested but was not statistically significant. It is possible that with a greater sample size, statistical significance would be obtained. It is unclear why an increased number of taxa was observed among fed chicks across all time points when PCoA on Bray-Curtis matrices did not show segregation of fed and unfed groups at days past day 2. One explanation is that lowly abundant taxa contribute to the observed differences in ASVs, as Bray-Curtis values are highly influenced by the most abundant taxa. Therefore, it is possible that the withholding of feed during days 1–2 does result in a slight lowering of the number of total taxa present in the ileal lumen through day 14, but likely only among taxa which are present in extremely low abundances.

In contrast, there were no differences in alpha or beta-diversity among fed and unfed samples in the mucosal communities (Supplementary Figures S8, S9). Mucus is a dense layer of polysaccharides and proteins which provides a distinct niche for microbial colonization (Sonnenburg et al., 2004). Microbial residents of the mucosa are often long-term residents of the gut which can withstand variable pH, body temperatures (105–106 in the chicken), low oxygen, and other challenges of the host environment (Hallen-Adams and Suhr, 2017). Luminal communities may also contain taxa capable of surviving in the gut, however, the lumen is also home to numerous environmental taxa which are transient. Thus, mucosal and luminal communities often differ in both bacterial (Chen et al., 2012) and fungal composition (Qiu et al., 2015). Microhabitats may also form within either mucosal or luminal regions which are influenced by the presence of local species and may also affect the spatial organization of taxa within the gut (Eckstein et al., 2020). In our samples, we did not observe a difference in the fed and unfed

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TABLE 3 List of genera which are differentially abundant across time and treatment. For each genus, the model coefficient, standard error, *p*-values, FDR corrected *p*-values (q-values) are also listed.

Genus	Metadata	Coef	Stderr	Oval	Rival	Site
Wallemia	earlylate	2.164	0.491	0.000	0.002	CE_L
Meyerozyma	earlylate	1.552	0.434	0.001	0.021	CE_L
Penicillium	earlylate	1.720	0.499	0.001	0.022	CE_L
Pyxidiophora	earlylate	1.352	0.432	0.002	0.045	CE_L
Kurtzmaniella	earlylate	0.825	0.276	0.004	0.056	CE_L
Unknown	earlylate	−1.477	0.506	0.004	0.058	CE_L
Candida	earlylate	1.691	0.639	0.010	0.093	CE_L
Scopulariopsis	earlylate	1.130	0.425	0.009	0.093	CE_L
Talaromyces	earlylate	1.728	0.669	0.011	0.099	CE_L
Issatchenkia	earlylate	0.979	0.388	0.013	0.104	CE_L
Fusarium	earlylate	0.888	0.403	0.030	0.180	CE_L
Kodamoea	earlylate	0.581	0.256	0.026	0.180	CE_L
Trichosporon	earlylate	0.809	0.365	0.029	0.180	CE_L
Asperpillus	earlylate	1.073	0.499	0.034	0.189	CE_L
Acremonium	earlylate	0.618	0.298	0.041	0.210	CE_L
Wickerhomomyces	earlylate	0.896	0.437	0.043	0.210	CE_L
Rhodotorulo	earlylate	1.242	0.629	0.051	0.235	CE_L
Xeromyces	earlylate	0.682	0.350	0.054	0.235	CE_L
Unknown	treatment	−0.962	0.507	0.061	0.244	CE_L
Xerochrysium	earlylate	0.592	0.314	0.063	0.244	CE_L
Unknown	earlylate	−1.040	0.184	0.000	0.000	CE_M
Gibberello	earlylate	0.285	0.097	0.004	0.058	CE_M
Unknown	treatment	−0.440	0.184	0.019	0.176	CE_M
Dipodoscus	earlylate	1.748	0.667	0.010	0.158	IL_L
Clavispora	earlylate	1.016	0.367	0.007	0.158	IL_L
Soccharomycetales unidentified	earlylate	0.981	0.366	0.009	0.158	IL_L
Talaromyces	earlylate	0.760	0.359	0.037	0.219	IL_L
Meyerozyma	earlylate	0.856	0.410	0.039	0.219	IL_L
Issatchenkia	earlylate	0.833	0.385	0.033	0.219	IL_L
Suhomyces	earlylate	0.906	0.441	0.043	0.219	IL_L
Coniochaeta	earlylate	−0.836	0.368	0.026	0.219	IL_L
Wallemia	earlylate	1.107	0.481	0.024	0.219	IL_L
Filobasidium	earlylate	0.875	0.439	0.049	0.226	IL_L
Penicillium	earlylate	0.532	0.277	0.058	0.242	IL_L
none detected						IL_M

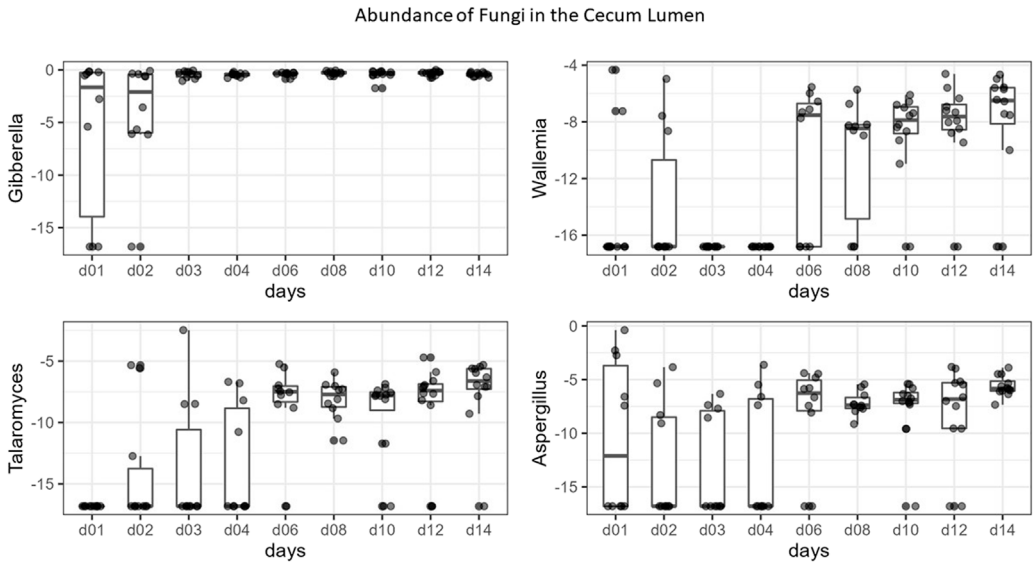

FIGURE 7
Boxplots of the top differentially abundant genera in the cecal lumen across time. Y-axis values are the log base two of relative abundance of each genus, X-axis = days.

mucosal samples, suggesting that ingested taxa do not establish in the mucosa.

In agreement with diversity analyses, at all four sites examined, only two unknown genera were detected to be differentially abundant among fed *versus* unfed chicks, and q-values were only marginally significant (Table 3). Overall, the deprivation of feed during the first 2 days of a chick's life does not appear to result in substantial long-term changes in either the luminal or mucosal chick mycobiome.

In contrast, at each site, the composition of the mycobiome as measured by beta diversity changed significantly throughout development from hatch through day 14 (Figure 5). A similar pattern was observed in a prior study which examined the luminal mycobiome of broiler chickens across the duodenum, ileum, cecum, and colon on days 3, 7, 14, 21, 27, 35, and 42 (Robinson et al., 2022). Robinson et al. (2022) also observed patterns of alpha diversity which are similar to those in this study including a significant increase in the number of ASVs across days 3, 7, and 14 in the cecal lumen, and no changes in any alpha diversity metric in the ileum. However, they found an increase in Shannon diversity and evenness across day 3, 7, and 14 in the cecal lumen while our data showed a decrease (Robinson 2022). In the ileum, the lack of changes in alpha diversity coupled with significant changes in beta diversity suggest that taxonomic changes drive differences in beta diversity. However, no taxa were detected to be differentially abundant in the ileum mucosa. This may be due to the fact that differential abundance analysis was conducted on combined days 1–4 (early) *versus* days 6–14 (late), and taxa whose abundances may differ between only two individual days may not have been detected.

Discordance among studies could be caused by methodological or biological differences, or both. For example, Robinson (2022) used male Cobb birds whereas we used a mix of female and male Ross 708 birds, and the mycobiome may vary by gender (Strati et al., 2016) and host genotype (Wu et al., 2021). Robinson (2022) used birds raised in floor pens on shavings whereas we raised birds in battery pens. Furthermore, DNA isolation kits differed among the studies as did the bioinformatic workflows including the choice of database.

Bacterial communities in the gut generally increase in diversity and become more stable over time (Agans et al., 2011; Danzeisen et al., 2013; Oakley and Kogut, 2016; Dill-McFarland et al., 2017). In contrast, mycobiome alpha diversity values are generally high at hatch and then decrease over time (Strati et al., 2016; Arfken et al., 2020). However, trends are often variable during the earliest stages of development following hatch (Ward Tonya et al., 2018; Arfken et al., 2020), and our data support this. For example, the number of ASVs plotted at each timepoint (Supplementary Figure S8), shows that trends across sites vary by age. Thus, at least some proportion of the discrepancies across studies can be attributed to variability in the mycobiome during early development, as well as methodological differences.

Changes in taxonomic composition were also observed across development at all sites except for the ileal mucosa (Table 3; Figure 6). In the cecal lumen, 19 genera were

detected as differentially abundant, 15 were detected in the ileal lumen, and 3 in the cecal mucosa. In the cecal lumen, the largest increases in abundance were noted among *Wallemia, Meyerozyma, Penicillium, Pyxidiophora*, and *Kurtzmaniella*, while total unidentified Fungi decreased (Figure 7), but in the cecal mucosa, only *Gibberella* increased over time and total unidentified Fungi decreased (Table 3). In the ileal lumen, *Clavispora, Suhomyces, Dipodascus*, and *Fusarium* increased while *Coniochaeta* decreased. The fact that a greater number of taxa changed over time in luminal samples than in mucosal ones supports the idea that mucosal communities may be less susceptible to changes in diet and/or the environment, and that luminal taxa are more transient.

It is well established that gut microbial composition shifts during early development (Ballou et al., 2016; Moore and Townsend, 2019), and the microbiome plays a key role in maturation of the immune, metabolic, and hormone development in young animals (Stiemsma and Michels, 2018). However, less information is available regarding the mycobiome. Due to the large influence of diet and the environment on fungal communities, the mycobiome is often more variable than bacterial gut communities (Nash et al., 2017; Ward Tonya et al., 2018). Still, a small number of key fungi consistently dominate the neonatal and infant gut (Fujimura et al., 2016), as well as that of chicks (Robinson et al., 2022). Since fungi play a critical role in disease and the establishment of bacterial populations (Stiemsma and Michels, 2018), further studies are needed to establish the role of the mycobiome in early development.

Last, our analyses also indicate that mycobiome communities differ across locations in the GI. Beta diversity analyses show that all sites (cecal lumen, cecal mucosa, ileal lumen, and ileal mucosa) differed significantly from each other (Figure 4). In addition to *Gibberella* and unidentified Fungi, *Aspergillus* and *Cladosporium* were among the most abundant taxa at all sites, along with *Meyerozyma* and *Sarocladium* at both cecal sites, *Wallemia*, *Penicillium*, and *Mucor* in the ileal lumen, and unidentified genera within the Ascomycota Phylum, *Coniochaeta*, and *Rhodotorula* in the ileal mucosa. Many rare taxa also differed across sites (Supplementary Table S5). The independent clustering of fungal communities at different locations along the GI is supported by prior studies (Robinson et al., 2020; Robinson et al., 2022). However, trends of alpha-diversity in this study differed slightly from recent studies. Robinson (2022) found that at day 42, alpha diversity metrics differed significantly along the GI, and values at each site roughly followed a bell shaped curve with the lowest diversity reported in the crop, then increasing in the ventriculus, peaking in the duodenum and jejunum, and then lowering through the ileum, cecum, and colon (Robinson et al., 2022). In a prior study at day 28, general trends suggested alpha diversity was higher in the upper GI than in the lower GI (Robinson et al., 2020). In our analysis, which included data from days 1–14, alpha diversity metrics in the cecum and ileal lumens were not significantly different from each other (Figure 2). As discussed above in regard to temporal patterns of alpha-diversity, discrepancies in alpha diversity trends across sites are also variable just after hatch (Supplementary Figure S11) or may be due to methodological differences.

Although we did not find differences in alpha diversity values between the cecum and ileum luminal communities, we did find that the number of ASVs, evenness, and the Shannon index values were greater in the mucosal communities than in the luminal ones (Figure 2). In humans, mucosal communities are less diverse than luminal ones (Leonardi et al., 2022), and it is unclear why our data shows the opposite trend. Again, it is possible that trends fluctuate during early development, and later stabilize. Regardless, to our knowledge, this is the first study of mucosal fungal communities in the GI of chickens. Mucosal communities play a critical role in health due to their close association with the host (Luan et al., 2015; Huseyin et al., 2017). Thus, understanding changes in the mucosal mycobiome alongside those which occur in the lumen are integral to understanding and ultimately manipulating host health.

Together, our results indicate that the chick mycobiome is a dynamic component of the gut microbiome which changes throughout development and across sites in the GI tract. We did not find that withholding feed during the first 2 days of life leads to long term consequences in mycobiome composition, suggesting that the developing mycobiome does not play a role in the negative health consequences observed with PH delays in feeding. However, it is likely that the developing mycobiome does play a role in other aspects of chicken health as has been shown in other organisms (Cui et al., 2013; Iliev and Leonardi, 2017; Iliev and Cadwell, 2021). Research to develop fungal probiotics and other therapeutics in chickens is ongoing (Saleh et al., 2014; Sugiharto et al., 2017), and this study provides a foundation by which to advance such efforts and ultimately improve the health, wellbeing, and productivity of chickens.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA779402.

Ethics statement

The animal study was reviewed and approved by the USDA-ARS-BARC Institutional Animal Care and Use Committee.

Author contributions

MP-W conceived, planned, and performed the experiments and assisted with writing the manuscript, KS assisted with experimental design and writing the manuscript, CD assisted with data analyses and writing the manuscript, AA assisted with experimental design and data analyses, JF performed scientific experiments, ND assisted with the first draft of bioinformatic analyses, AC performed lab analyses, library construction and sequencing, and assisted with writing , LS performed animal work, lab analyses, and assisted with writing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1057810/full#supplementary-material

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Bacterial composition of a competitive exclusion product and its correlation with product efficacy at reducing *Salmonella* in poultry

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The mature intestinal microbiome is a formidable barrier to pathogen colonization. Day-old chicks seeded with cecal contents of adult hens are resistant to colonization with *Salmonella*, the basis of competitive exclusion. Competitive exclusion products can include individual microbes but are commonly undefined intestinal communities taken from adult animals and in commercial production is amplified in fermentator and sold commercially in freeze dried lots. While superior to single and multiple species probiotics, reducing *Salmonella* colonization by multiple logs, undefined products have limited acceptance because of their uncharacterized status. In this study, the bacterial composition of the master stock, preproduction seed stocks and commercial lots of a poultry competitive exclusion product, was defined by 16S rRNA sequence analysis, targeting the 16S rRNA variable region (V1-V3). The samples contained a diversity of genera (22–52 distinct genera) however, the commercial lots displayed less diversity compared to the seeds and the master stock. Community composition varied between seeds and the master stock and was not a good predictor of potency, in terms of $\log_{10}$ reduction in *Salmonella* abundance. While there was significant correlation in composition between seeds and their commercial lots, this too was a not a good predictor of potency. There was linear correlation between unclassified *Actinobacteria, Peptococcus,* and unclassified Erysipelotrichaceae, and *Salmonella* abundance (r^2 > .75) for commercial seeds. However, upon review of the literature, these three genera were not consistently observed across studies or between trials that examined the correlation between intestinal community composition and *Salmonella* prevalence or abundance.

KEYWORDS

avian, microbiome, *Salmonella*, exclusion, competition

Introduction

Many studies have shown that probiotics or direct-fed microbials can reduce intestinal disease in humans and animals (Gómez-Gallego et al., 2016; Kim et al., 2019; Lai et al., 2019; Renaud et al., 2019; Jakubczyk et al., 2020; Lucey et al., 2021; Xu et al., 2021). This is particularly important in animal production where production costs are directly impacted by intestinal health. The composition of the normal intestinal microbial community plays an important role in animal health and performance through its effect on gut morphology, nutrition, pathogenesis of intestinal disease and the immune response (Hooper et al., 2000). The term competitive exclusion (CE), introduced by Nurmi et al., 1973 is used to describe the process by which

beneficial bacteria exclude *Salmonella* from the intestine (Nurmi et al., 1992).Formulations containing beneficial commensal bacteria have been marketed in some countries as probiotics, competitive exclusion formulations, or direct-fed microbials (Food and Agriculture Organization of the United Nations, 2016; Szott et al., 2022). There has been considerable interest in utilizing competitive exclusion to exclude select pathogens, especially *Salmonella*, from the gastrointestinal tract of food animals in order to reduce foodborne transmission to humans (Stern et al., 2001; Szott et al., 2022). Competitive exclusion formulations produced from the intestinal content of healthy chickens have been shown to be very effective at eliminating or reducing the prevalence of *Salmonella* in broiler chicken flocks and on carcasses (Hirn et al., 1992; Corrier et al., 1998; Stern et al., 2001).

The intestinal tract is a novel ecosystem that contains a community of bacteria rivaling the diversity of any other ecosystem on the planet. The density and number of bacteria in the community is higher than the number of host cells. Culture-based studies have suggested that intestinal microbial community is composed primarily of obligate anaerobes (Franks et al., 1998). The predominant cultivatable bacteria present in the chicken ceca are obligate anaerobes at a density of 10^{11} cells per gram of contents (Barnes et al., 1972) including at least 38 different types of anaerobic bacteria within the chicken cecum (Salanitro et al., 1974a; Barnes, 1979) with more than 200 total bacterial strains identified (Mead, 1989). However only 10%–60% of the bacteria visualized microscopically were cultured indicating a rich community of uncharacterized organisms (Barnes et al., 1972; Salanitro et al., 1974b; Barnes, 1979; Mead, 1989).

Using the DNA sequences of the small subunit ribosome genes present in a bacterial community (16S rRNA clone libraries), the composition of the intestinal community has been evaluated for many animals with very surprising results (Tannock, 1999). Commonly cultured organisms, such as *E. coli*, have been found to be a minor component of the intestine and novel uncultured organisms have been found to be the most abundant. Applications of molecular ecological profiling on poultry intestinal communities have concurred somewhat with the culture-based studies in that the chicken intestinal communities are primarily composed of Gram-positive bacteria related to mid and low G + C genera such as *Clostridia* and *Lactobacillus* (Gong et al., 2002; Lan et al., 2002; Lu et al., 2003; Zhu and Joerger, 2003). While lactobacilli are frequent in the small intestine, *Clostridia* are abundant throughout the intestinal tract of healthy chickens. The cecal intestinal microbial community is dominated by atypical and novel *Clostridia,* some of which have high G + C genomes (Apajalahti et al., 2001; Lu et al., 2003; Zhu and Joerger, 2003). The DNA sequences indicate that these normal flora *Clostridia* are not closely related to pathogenic *Clostridium* (such as *perfringens*) and they do not appear to be pathogenic themselves. Only recently have the members of the order *Clostridiales* been isolated from the chicken cecum and characterized by whole genome sequencing (Medvecky et al., 2018). Approximately half of the chicken gut anaerobes (n = 69) were *Clostridiales*; consisting of four families (*Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae*, and *Ruminococcaceae*) and, at least, 12 distinct genera (*Anaerofilum, Anaeromassillibacillus, Anaerotruncus, Blautia, Butyricicoccus, Clostridium, Drancourtella, Eubacterium, Faecalibacterium, Flavonifractor, Gemmiger*, and *Pseudoflavonifractor*). Thirty-six of these isolates show <97% or 95% 16S identity to *Clostridia* and clostridial species, respectively, in public databases and represent new clostridial genera and species awaiting taxonomic classification or reclassification. Because of their fermentative metabolism and ability to produce short chain fatty acids (SCFAs) (Medvecky et al., 2018), these organisms appear to be important players in developing an exclusive community that reduces the competition or behavior of pathogens.

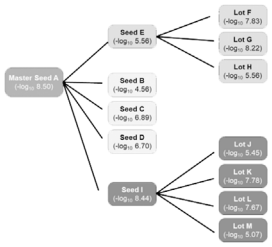

FIGURE 1
Source of competitive exclusion seeds and commercial lots. (–log10: Reduction in *Salmonella* abundance).

To explore the genomic features of complex microbial communities, a culture-independent 16S rRNA amplicon sequencing approach has become practical and cost effective due to the advent of high throughput sequencing, a method allowing simultaneous sequencing of hundreds of thousands of individual DNA strands (Shang et al., 2018). The analysis is performed by comparing each 16S rRNA gene sequence to all others that have been detected within the community in order to determine the frequency of occurrence. The abundant organisms are frequently detected while the genes of less abundant organisms are rarely detected. However, if enough sequences are analyzed the method can reveal the presence of rare organisms. The sequence analysis can be performed at the strain level (99% DNA similarity), species level (97% DNA similarity), genus level (95% DNA similarity), and group/family level (90%). While commercial exclusion products are effective at reducing *Salmonella* colonization in poultry, their market distribution is limited by regulatory restrictions on undefined microbial products. Using a 16S rRNA-based approach, we have characterized the composition of several batches of a competitive exclusion product, marketed for controlling *Salmonella* in poultry, and used this information to determine if a particular organism or an assemblage of organisms correlated with product efficacy.

Materials and methods

Characterization of master seed and seed batches of a competitive exclusion product

Commercial samples were shipped in sealed plastic bags, in the same packaging sent to customers. Thirteen samples, with potency results supplied by manufacturer were received frozen and were kept frozen until processing. All samples were opened within biosafety cabinet, previously treated with ultraviolet light and 10% bleach, and transferred to microfuge tubes for DNA extraction. Samples consisted of: master seed (sample A), two seed batches with potency <6 $\log_{10}$ reduction of *Salmonella* abundance (samples E and B), three seed batches with potency >6 $\log_{10}$ reduction (samples C, D, I), three commercial batches with potency <6 $\log_{10}$ reduction (samples H, J, M), and four commercial batches with potency >6 $\log_{10}$ reduction (samples F, G, K, L). Figure 1 shows the derivation of seeds and commercial lots from the

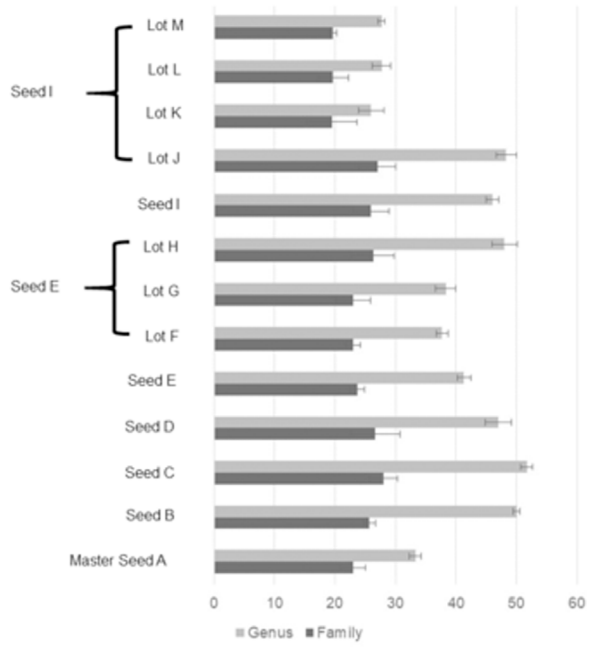

FIGURE 2
Number of different taxa in 16S rRNA sequences from master seed A, seed stocks (B–E, I) and commercial lots (F–H, J–M) at family (90% similarity) and genus (95% similarity) level. The error bars estimate sequence analysis error detected by sequencing the same sample three times.

master seed (Sample A) and their potency (-$\log_{10}$ reduction in *Salmonella* abundance). *Salmonella* reduction data was provided by the manufacturer for seeds and commercial lots; this data is used in their quality control evaluation of product efficacy. Commercial lots were released for sale if they reduced *Salmonella* colonization by at least 5 $\log_{10}$ compared to the untreated control.

Preparation of bacterial 16S rRNA amplicons for sequencing

DNA was extracted using a MoBio Soil DNA extraction kit (MoBio Laboratories, Carlsbad California, United States) as previously described (Lu et al., 2003). The DNA quality was evaluated by gel electrophoresis with 1 kb ladder molecular weight standards. The DNA was quantified using Nanodrop (Thermo Scientific, Wilmington Delaware, United States). Barcoded PCR primers (Supplementary Tables S1, S2) targeting the 16S rRNA gene variable regions V1-V3 was used to amplify sample DNA (Hamady et al., 2008). Positive and negative, no template controls were included in the 16S PCRs, using *Salmonella* Typhimurium SR11 genomic DNA or molecular grade dH_2O, respectively as template. All PCR reactions were set up in a PCR clean hood, in a confined room, separate from where DNA was extracted in one room and thermocyclers, housed in a physically-separate room. PCR clean hood was previously treated with ultraviolet light and surfaces were wiped down with 10% bleach. Separate set of pipettors are kept in the PCR clean hood, they are only used to set up PCR reactions, and never leave this set up area. Barrier tips are used to dispense PCR reagents and template to prevent contamination of the pipette barrels. No amplicon was observed for the negative, no template control. PCR was performed as described by Garcia et al. (2011). In order to standardize the method and to reduce PCR amplification error, 200 ng of sample DNA was used in each reaction and PCR was run for 20 cycles. Three separate PCR reactions were produced and pooled for sequencing, for each sample. Triplicate trials for each sample were done resulting in 135 independent PCR reactions. *Clostridium perfringens* ATCC 13124 DNA was used as positive control for amplification and sequencing, and a control lacking template was used to detect reagent contamination.

PCR reactions were run on agarose gels to evaluate quantity and quality of amplicon. Any sample which showed a low amount of DNA was repeated. PCR amplicons were excised from gel, purified using Qiagen Gel extraction kit (Qiagen Inc., Valencia California, United States) and pooled for each sample. Agencourt AMP pure XP kit (Beckman Coulter, Indianapolis, IN, United States) was used to further clean up and concentrate amplicon from gel extractions for 454 pyrosequencing. PCR amplicons were quantified using Nanodrop and their concentration standardized by adjusting to 10 ng/μl. Any sample which contained less than 10 ng/μl was repeated in order to produce the necessary amount of DNA for standardization. Samples were submitted to the Georgia Genomics and Bioinformatics Core (University of Georgia, Athens, GA, United States) for pyrosequencing using 454 protocols established by Roche Inc. (Branford Connecticut, United States) which manufactures the instrument and reagents.

Bioinformatics and statistical analyses

The sequence analysis pipeline was performed using Roche and Mothur software (Schloss et al., 2009). Sequences were sorted by barcodes in order to organize by sample, trimmed based on size (≤500 bp) and quality to remove those sequences with gaps or ambiguous base calls. Sequences were aligned in order to detect and remove chimeras produced from PCR artifact.

The bacterial diversity was calculated comparing the similarity of the sequences and those deposited at Ribosomal Project Database (http://rdp.cme.msu.edu/) using the RDP6 database. The bacterial composition based on genus (95% similarity) and family (90% similarity) was generated in order to determine the phylogenetic composition of each sample. Sørensen's coefficient of similarity (QS) was used to quantify the similarity of samples (Sorenson, 1948). QS = 2C/A + B where A and B are the number of species in samples A and B, respectively, and C is the number of species shared by the two samples. Sørensen's coefficient ranges from 0–1. Simple correlation was used to statistically verify the similarity of sample composition. Chi-squared test was used in order to test differences in the proportion of the genus observed among the samples. Linear regression was adopted to determine the correlation between the bacterial genus and the levels of *Salmonella* reduction reported for commercial lots or seeds. Analysis of covariance was used to identify the effect of the seed on the commercial product. Statistic tests were performed using SAS software.

Screening samples for *Salmonella*

γ-Proteobacterial 16S sequences do not exhibit enough sequence diversity to reliably identify *Salmonella* and differentiate it from closely related member species. Therefore, some sequences may be erroneously reported as genus: *Salmonella* by Mothur, using the RDP6 database. In order to determine if *Salmonella* was present in commercial samples, a diagnostic PCR, targeting a *Salmonella*-specific locus, was applied. Fifty ng of sample DNA was used in PCR reactions, as described by Liu et al., 2002 (Liu et al., 2002) using *invA* primers. The samples were screened by gel electrophoresis using a 1.5% agarose gel containing ethidium bromide in order to visually detect amplicons of the expected size (450 bp). *Salmonella* Typhimurium genomic DNA served as a positive amplification control. A no template control was included to identify PCR contamination (false positive). The no template control was consistently negative in these *Salmonella* PCR screens.

Results

Sequencing quality control and culling anomalous sequences, ambiguities and chimeras from final sequence dataset

725,293 total sequences were obtained from the pyrosequencing reactions (Supplementary Table S3). However, after elimination of anomalous long sequences, homopolymers, and ambiguous bases, 703,522 sequences were subjected to chimera analysis for additional quality control. Chimeras are PCR artifacts that erroneously increase sample diversity and alter composition (Ley et al., 2008). After these quality control procedures, 332,559 sequences were of sufficient quality for compositional and statistical analysis. The distribution of sequences among the samples varied from 1,913–64,816 (Supplementary Table S3). The reads were therefore normalized to run data analysis for correlation, chi-square test, linear regression and covariance.

16S-rRNA based compositional analysis of CE seeds and commercial lots

The number of families detected varied from 15 to 28 and the number of genera from 22 to 52 for each sample (Figure 2). The seeds tended to contain the largest number of genera (mean = 45) with commercial lots containing the fewest (mean = 34). There was no correlation between the number of genera or families with *Salmonella* reduction. The bacterial composition is presented at the phyla (Figure 3), family (Figure 4; Table 1) and genus (Table 2) level. Unclassified bacteria, organisms that have yet been assigned a phylum, made up the smallest proportion, while *Firmicutes* was the dominant phylum, throughout CE samples, with the order *Clostridia* representing the most abundant group (58%–95.5%) within the phylum Firmicutes. Twelve different taxonomic families of *Clostridia* were detected in CE product, indicating that there was high diversity within this order. Firmicutes and the minor proteobacteria were the two phyla present in the master seed. Other phyla were identified in seeds and commercial lots, varying in their proportion, and included phyla Bacteroidetes, Fusobacteria, and Actinobacteria. While CE seed E had the highest proportion of Bacteroidetes (>30%), the proportion of this phyla in commercial lots derived from this seed was low. The same was observed for CE seed I, with regards to the 2nd major phyla, Proteobacteria (~10%) and abundance of this phyla in resulting commercial lots were sporadic and low. *Clostridiaceae*, *Veillonellaceae*, and *Lactobacillaceae*, were the major families present in the master seed; their proportion in seeds and commercial lots varied. The proportion of these three families in the seeds was not predictive of their

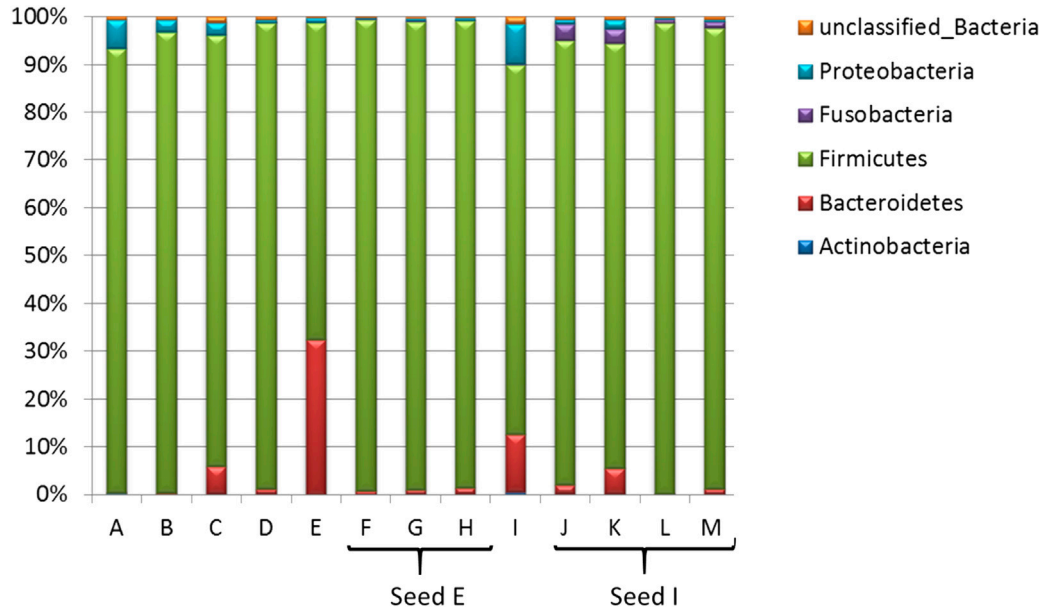

FIGURE 3
Bacterial composition of competitive exclusion master seed, seed stocks and derived commercial lots at the phyla level.

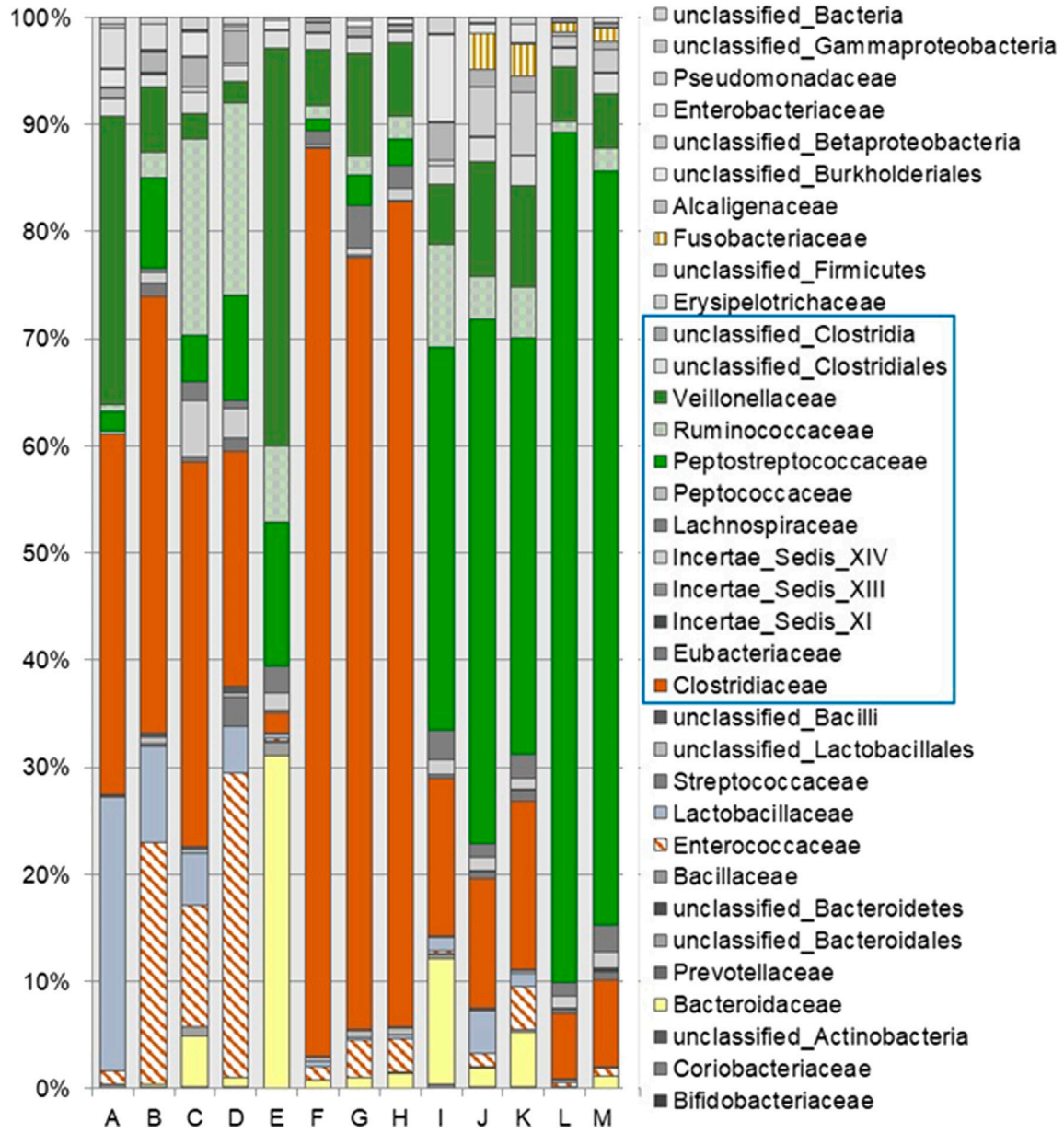

FIGURE 4
Bacterial composition of competitive exclusion master seed, seed stocks and derived commercial lots at the family level. The most abundant organisms are shown in **bold**, families belonging to *Clostridia* are denoted by the blue box.

abundance in the resulting, commercial lots. Bacteria belonging to the families *Clostridiaceae, Bacteroidaceae, Lactobacillaceae, Enterococcaceae, Peptostreptococcaceae,* and *Veillonellaceae* comprised more than 20% of any one sample. The seeds tended to contain higher proportions of *Lactobacillaceae* and *Enterococcaceae* while the commercial lots tended to have higher levels of *Peptostreptococcaceae*. Seed I, which was a progenitor seed stock for commercial lots J–M had a highest proportion of *Peptostreptococcaceae*, of the seed stocks. This seed stock proved the most efficacious of the seeds at reducing *Salmonella*. The resulting commercial lots, generated from this seed stock, also had a significantly high proportion of this bacterial family.

γ-Proteobacteria were detected among all of the samples and in a number of samples the Mothur software reported *Salmonella* among the genera detected. γ-Proteobacterial 16S sequences, and particularly the *Enterobacteriaceae* family, do not exhibit enough sequence diversity to be reliably used to report *Salmonella* because other genera within this group have highly similar 16S sequences. A diagnostic PCR was used to screen commercial samples for *Salmonella*. This PCR test has been used extensively to screen clinical and environmental samples for the presence of *Salmonella* (Liu et al., 2002). None of the samples gave a positive reaction therefore the erroneously classified sequences were corrected in the report to reflect their identity as an unknown *Enterobacteriaceae* species.

TABLE 1 Proportions of bacterial orders or families detected in competitive exclusion seeds and commercial lots.

Order or Family	Master Seed (A), Seed (B-E, I,), or Commercial Lots (F-H, J-M)												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Bifidobacteriaceae	0.02	0.03	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00
Coriobacteriaceae	0.29	0.16	0.11	0.14	0.00	0.01	0.05	0.03	0.37	0.11	0.05	0.06	0.10
unclassified *Actinobacteria*	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Bacteroidaceae	0.00	0.21	4.73	0.92	31.34	0.82	0.99	1.48	11.81	1.80	7.40	0.16	1.12
Prevotellaceae	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.03	0.00	0.01	0.00	0.00
Unclassified *Prevotellaceae*	0.00	0.00	0.89	0.02	1.30	0.01	0.04	0.07	0.33	0.06	0.11	0.00	0.03
Unclassified *Bacteroidales*	0.03	0.00	0.07	0.00	0.11	0.00	0.01	0.01	0.13	0.01	0.02	0.00	0.00
Bacillaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified *Bacillales*	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Enterococcaceae	1.17	22.44	11.28	28.47	0.22	1.20	3.47	3.25	0.35	1.32	4.48	0.31	0.77
Lactobacillaceae	25.68	8.84	4.88	4.42	0.33	0.46	0.31	0.39	1.20	3.93	1.22	0.28	0.03
Streptococcaceae	0.07	0.31	0.10	2.69	0.00	0.00	0.00	0.01	0.01	0.00	0.01	0.00	0.00
Unclassified *Lactobacillales* 1	0.12	0.60	0.25	0.37	0.13	0.46	0.62	0.64	0.04	0.16	0.26	0.06	0.07
Unclassified *Lactobacillales* 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Unclassified *Lactobacillales* 3	0.11	0.42	0.29	0.59	0.03	0.09	0.16	0.11	0.06	0.11	0.16	0.03	0.03
Clostridiaceae	34.04	40.68	35.72	21.75	1.85	84.71	71.15	76.20	14.71	12.08	16.27	6.02	8.09
Eubacteriaceae	0.00	1.27	0.50	1.26	0.30	0.15	0.18	0.19	0.33	0.68	0.88	0.47	0.70
Incertae Sedis XI	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.09	0.12	0.28
Incertae Sedis XIII	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00
Incertae Sedis XIV	0.20	0.91	5.31	2.79	1.56	0.25	0.58	1.06	1.37	1.33	1.15	1.00	1.53
Lachnospiraceae	0.00	0.35	1.76	0.74	2.47	1.30	4.18	2.27	2.68	1.26	1.91	1.28	2.44
Peptococcaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Peptostreptococcaceae	1.85	8.58	4.36	9.75	13.16	1.04	2.82	2.44	35.85	49.06	29.13	79.05	70.47
Ruminococcaceae	0.62	2.36	18.30	18.02	6.84	1.29	1.81	2.09	9.57	3.95	5.72	1.12	2.06
Veillonellaceae	26.29	6.15	2.38	1.94	37.42	5.17	10.04	7.24	5.58	10.57	13.81	5.14	5.06
Unclassified *Clostridiales* 1	1.61	1.20	1.90	1.52	1.57	1.51	1.57	1.03	1.72	2.24	2.78	1.87	1.88
Unclassified *Clostridiales* 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Unclassified *Clostridiales* 3	0.10	0.05	0.04	0.05	0.01	0.05	0.10	0.05	0.07	0.13	0.09	0.12	0.10
Erysipelotrichaceae	0.01	0.14	0.49	0.24	0.04	0.07	0.09	0.05	0.44	4.62	5.16	1.03	2.16
Unclassified Bacilli	0.86	1.97	2.78	2.95	0.14	0.81	0.85	0.57	3.39	1.54	2.15	0.47	0.70
Fusobacteriaceae	0.00	0.00	0.00	0.00	0.00	0.10	0.04	0.06	0.03	3.41	4.19	0.90	1.39
Alcaligenaceae	0.12	0.00	0.15	0.02	0.04	0.03	0.03	0.03	0.19	0.02	0.13	0.00	0.03
Unclassified β-*Proteobacteria* 1	1.63	0.13	2.20	0.37	0.80	0.34	0.59	0.53	8.05	0.84	1.96	0.16	0.28
Unclassified β-*Proteobacteria* 2	0.10	0.01	0.15	0.19	0.02	0.00	0.00	0.01	0.05	0.00	0.02	0.00	0.03
Enterobacteriaceae	3.87	2.51	0.03	0.03	0.00	0.00	0.00	0.01	0.00	0.08	0.10	0.16	0.00
Pseudomonadaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.07
Unclassified γ-*Proteobacteria* 1	0.42	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Unclassified γ-*Proteobacteria* 2	0.07	0.01	0.10	0.05	0.02	0.01	0.00	0.01	0.07	0.00	0.02	0.00	0.00

Heat map scale (low to high)

0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.11	0.13	0.92	1.26	1.94	2.15	2.68
3.39	4.19	5.16	7.40	9.57	11.81	13.16	14.71	18.30	21.75	31.34	35.72			

Consistency in bacterial composition of commercial lots with their seeds is not a good predictor of product efficacy

Table 3 presents the similarity between the community composition of the master seed, at the family and genus level, and the seeds produced from it. Statistical analysis, using simple correlation, varied across seeds compared to the master (Family: .719–.272; Genus .641–.078). Similarity with the master seed was not a good predictor of efficacy as the seed most similar to the master and with a high correlation value (Seed B) had the lowest *Salmonella* $\log_{10}$ reduction compared to one of the power seeds, with regards to community similarity (Seed I; Table 3). While individual seeds produced commercial lots with similar community composition at the family and genus level, this was not a good predictor as to which lots would be expected to have the greatest reduction of *Salmonella* counts (Table 4). Even comparing commercial lots against themselves, varied in their ability to reduce *Salmonella* abundance, was not a significant predictor of product efficacy. However, community similarity tended to reflect commercial lots origins with seed stocks used to generate CE product (Table 5).

Identification of genera within seeds and commercial lots that correlated with reduction of *Salmonella* colonization in chickens

Linear regression was performed to determine if a particular organism correlated with *Salmonella* reduction. In the seeds, unclassified *Actinobacteria, Peptococcus,* and unclassified *Erysipelotrichaceae* correlated with product efficacy ($\log_{10}$ *Salmonella* reduction) at r^2 greater than 75% (Table 6, Supplementary Tables S4, S5). There was a linear correlation among these organisms with *Salmonella* reduction in the seeds (Figure 5); specifically, an antagonistic relationship for unclassified *Erysipelotrichaceae* and *Peptococcus versus* a facultative relationship with *Actinobacteria*. In contrast, linear regression did not detect an

organism correlating significantly with efficacy in the commercial lots themselves (Supplementary Table S5), including the afore mentioned genera. However, because all of the commercial products produced at least a 5 $\log_{10}$ reduction in *Salmonella* colonization, it may be difficult to detect correlation using this method. Furthermore, *Salmonella* reduction may not be due to the actions of a particular organism but due to the combined metabolic activity of organisms which produce an exclusive community.

Discussion

The host must maintain surveillance over the composition of the microbiota and exhibit control over the abundance and behavior of members that affect the host-microbe homeostasis. Therefore transfer of microbial communities from parent to offspring may have evolved to initially establish ecological health within the lumen of host mucosal systems and reduce susceptibility to mucosal disease (Neish, 2009). A number of studies have demonstrated that administration of complex microbial communities can reduce the ability of *Salmonella* to colonize young animals; these have been extensively reviewed (Nurmi et al., 1992; Nisbet, 1998; Becker, 2005) including those specifically addressing poultry production (Ferreira et al., 2003). Complex microbial communities, such as those used in competitive exclusion, have been shown in multiple independent studies to be effective in reducing *Salmonella* in poultry (Cameron et al., 1996; Cameron et al., 1997; Deruyttere et al., 1997; Guillot et al., 1997; Guerra-Garcia Miranda, 2000; Stephan, 2000; Sisak et al., 2001; Nakamura et al., 2002; Ferreira et al., 2003). Products that consist of single bacteria, such as *Lactobacillus* and *Bacillus*, are less efficacious (Nurmi et al., 1992). However the complex microbial communities are difficult to characterize using classical bacteriological methods (Hume et al., 1996).

The competitive exclusion product contains a diversity of microbial species, many of which are not consistently present in preproduction seeds or commercial lots. In fact, there are some taxa shared between seeds or lots that were not detected in the master by our methods. *Peptostreptococcus*, abundant in commercial lots, was not detected in the Master Seed, but had to be present to seed these lots and suggests limits of this 16S rRNA gene sequencing approach to detect minor taxa. While certain taxa were identified that seemed to correlate with *Salmonella* abundance, they are not consistently observed across studies (Azcarate-Peril et al., 2018; Ma et al., 2020; Leyva-Diaz et al., 2021; Pedroso et al., 2021). While others have identified a similar correlation between prevalence or abundance of certain taxa with *Salmonella* colonization in poultry, these taxa are also not uniformly present in other studies or even between trials, in the same study (Videnska et al., 2013; Azcarate-Peril et al., 2018; Ma et al., 2020; Mon et al., 2020; Pedroso et al., 2021). The bacterial composition of this competitive exclusion product was not reflected in the cecal community composition in animals administered the commercial product (Pedroso et al., 2016). This may be due, in part, to the microbial succession that occurs in the maturation of the intestinal microbiome (Lu et al., 2003). However, the CE product does contain members that appear in an intestinal compartment, ileum or cecum, at some point in the intestine's development (Lu et al., 2003). Community diversity appears to be key in pathogen exclusion (Pedroso et al., 2021) in that it is not as important who is present as what the community does "collectively" to exclude *Salmonella*. Others have also noted the importance of community diversity in pathogen exclusion (van Elsas et al., 2012; Antharam et al., 2013; Lone et al., 2013; Stanley et al., 2014; Xu et al., 2014; Zhang et al., 2015; Chopyk et al., 2016). This diversity provides resilience needed to return to homeostasis following some perturbation to the system (Weimer, 2015). As long as there are sufficient members present that collectively perform a specific function, the system is maintained; in this case microbiome's ability to maintain a barrier to pathogen colonization. Therefore, seeding the chick with the adult intestinal microbiome, provides it with sufficient members, collectively capable of excluding *Salmonella* while its own microbiome develops as the animal matures.

It is unclear how the products exclude pathogens but it seems unlikely that composition is only one characteristic responsible for their protective effects. The possible mechanisms of action of competitive exclusion formulations have been previously reviewed (Nurmi et al., 1992; Nisbet, 1998; Tuomola et al., 2001; Becker, 2005). Bacteria comprising exclusive communities may: produce bactericidal molecules that damage the cellular integrity of pathogenic bacteria; decrease the growth rate of pathogens by providing competition for nutrients or produce molecules that inhibit processes involved in cell division; produce molecules that reduce expression of, or function of, factors involved in colonization; cause or enhance predation; or physically occupy or modify the ecological niche targeted by the pathogen. Multiple mechanisms are likely responsible for pathogen exclusion. This would explain why diversity is key; why no one specie(s) was consistently associated with *Salmonella* exclusion in this study. *Salmonella* is metabolically versatile in that it can utilize some metabolites (ethanolamine, propanediol, etc.) that few other community members can. A metabolic gene(s) involved in competition for substrate A may be distributed across a diversity of bacterial species, where any one, member species could compete for substrate A. This may explain why no one bacterial species has been consistently associated with reduced *Salmonella* abundance. While an intestinal member species may be able to compete with *Salmonella* for one substrate or metabolite, *Salmonella* could turn to another, and another substrate enabling persistent colonization even with low energy substrates. Only organisms with similar metabolic potential could outcompete *Salmonella*, especially another *Salmonella* (Cheng et al., 2015). Therefore, if competition were the mechanism of competitive exclusion, it would take a broad array of member species for the community to outcompete *Salmonella* for all the substrates and metabolites present in the different portions of the intestine.

Similarly, antagonism may be at the heart of competitive exclusion. The antibacterial activity of a competitive bacterial species may be attributed to several factors (Alakomi et al., 2005). For example, the mechanism of antibacterial activity of exclusive lactobacilli might be due to a synergistic action of lactic acid and bacteriocins. Lactate acts as a permeabilizer of the outer membrane of Gram-negative bacteria, increasing their susceptibility to antimicrobial molecules (Fayol-Messaoudi et al., 2005). Lactate also affects the intestinal pH which may affect the surface structures and metabolism of *Salmonella* (Foster et al., 1994). Other bacterial metabolites, such as acetate, propionate and butyrate, may also contribute to community exclusion of some bacterial species because the undissociated organic acids freely diffuses across the bacterial membrane, lowering the cytoplasmic pH and uncoupling electron transport (Ricke, 2003). The intestinal tract contains high levels of these volatile short chain fatty acids (SCFA) which are produced from the breakdown of complex carbohydrates by anaerobes such as the *Clostridia, Bacteroides,*

TABLE 2 Proportion of genera detected in competitive exclusion seeds and commercial lots.

Genus	Master Seed (A), Seed (B-E, I,), or Commercial Lots (F-H, J-M)												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Bifidobacterium	0.020	0.027	0.005	0.004	0.000	0.003	0.014	0.008	0.000	0.004	0.000	0.000	0.000
Collinsella	0.098	0.049	0.029	0.054	0.000	0.003	0.013	0.015	0.111	0.040	0.011	0.031	0.000
Olsenella	0.000	0.009	0.007	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Slackia	0	0.007	0.005	0.012	0	0	0.009	0.005	0.136	0.018	0	0.031	0.035
Unclassified *Actinobacteria* 1	0.196	0.092	0.068	0.074	0.003	0.003	0.027	0.014	0.123	0.057	0.034	0.000	0.070
Unclassified *Actinobacteria* 2	0.010	0.004	0.002	0.004	0.003	0.000	0.000	0.001	0.000	0.018	0.000	0.000	0.000
Bacteroides	0.000	0.208	4.730	0.923	31.34	0.825	0.992	1.480	11.80	1.796	7.398	0.156	1.116
Unclassified *Bacteroidales* 1	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.031	0.004	0.011	0.000	0.000
Unclassified *Bacteroidales* 2	0.000	0.000	0.893	0.023	1.299	0.013	0.040	0.072	0.327	0.062	0.114	0.000	0.035
Unclassified *Bacteroidales* 3	0.029	0.000	0.070	0.000	0.113	0.003	0.014	0.008	0.130	0.009	0.023	0.000	0.000
Bacillus	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000
Exiguobacterium	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Unclassified *Bacillales*	0.010	0.000	0.000	0.000	0.000	0.000	0.009	0.003	0.000	0.000	0.000	0.000	0.000
Enterococcus	1.174	22.18	11.18	28.28	0.203	1.158	3.337	3.174	0.351	1.275	4.402	0.280	0.732
Unclassified *Enterococcaceae*	0.000	0.251	0.097	0.182	0.015	0.044	0.130	0.076	0.000	0.048	0.008	0.031	0.035
Lactobacillus	25.61	8.809	4.851	4.377	0.325	0.460	0.301	0.383	1.178	3.910	1.212	0.280	0.035
Pediococcus	0.000	0.000	0.000	0.000	0.006	0.000	0.004	0.005	0.000	0.000	0.000	0.000	0.000
Unclassified *Lactobacillaceae*	0.068	0.034	0.032	0.039	0.003	0.000	0.000	0.003	0.018	0.018	0.011	0.000	0.000
Streptococcus	0.069	0.306	0.099	2.689	0.000	0.000	0.000	0.009	0.012	0.000	0.011	0.000	0.000
Unclassified *Lactobacillales* 1	0.117	0.597	0.254	0.372	0.125	0.456	0.620	0.645	0.043	0.163	0.263	0.062	0.070
Unclassified *Lactobacillales* 2	0.000	0.000	0.002	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000
Unclassified *Lactobacillales* 3	0.108	0.418	0.288	0.590	0.027	0.085	0.162	0.106	0.062	0.110	0.160	0.031	0.035
Anaerobacter	0.049	0.031	0.027	0.023	0.000	0.003	0.000	0.003	0.031	0.004	0.011	0.000	0.000
Clostridium	22.14	29.77	18.60	14.38	1.740	33.67	27.35	29.68	4.889	4.280	6.529	2.743	3.034
Sarcina	0.000	0.000	0.017	0.023	0.000	0.205	0.224	0.194	0.000	0.013	0.034	0.000	0.035
Unclassified *Clostridiaceae*	11.86	10.88	17.08	7.318	0.113	50.83	43.57	46.32	9.790	7.784	9.697	3.273	5.021
Acetobacterium	0.000	0.054	0.027	0.078	0.030	0.022	0.009	0.012	0.037	0.044	0.092	0.000	0.035
Eubacterium	0.000	0.016	0.002	0.004	0.000	0.000	0.000	0.002	0.006	0.004	0.011	0.000	0.000
Unclassified *Eubacteriaceae*	0.000	1.199	0.474	1.183	0.268	0.132	0.171	0.173	0.284	0.627	0.778	0.468	0.662
Sporanaerobacter	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.092	0.123	0.244
Unclassified bacteria	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.035
Unclassified Incertae Sedis XI	0.000	0.000	0.002	0.004	0.000	0.000	0.000	0.000	0.037	0.004	0.000	0.000	0.000
Blautia	0.196	0.913	5.314	2.794	1.564	0.255	0.584	1.062	1.375	1.333	1.155	0.998	1.534
Coprococcus	0.000	0.007	0.010	0.000	0.072	0.000	0.000	0.003	0.012	0.018	0.023	0.000	0.070
Dorea	0.000	0.036	0.039	0.023	0.134	0.022	0.031	0.017	0.284	0.035	0.126	0.094	0.000
Roseburia	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Unclassified *Lachnospiraceae* 1	0.000	0.300	1.680	0.640	2.166	1.253	4.051	2.200	2.343	1.196	1.761	1.153	2.336
Unclassified *Lachnospiraceae* 2	0.000	0.004	0.027	0.074	0.101	0.022	0.094	0.046	0.043	0.013	0.000	0.031	0.035
Peptococcus	0.000	0.000	0.002	0.004	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000
Peptostreptococcus	0.000	0.036	4.300	9.141	13.14	1.039	2.816	2.103	35.65	48.98	29.11	78.96	70.43
Sporacetigenium	1.840	8.219	0.048	0.427	0.012	0.003	0.000	0.321	0.142	0.040	0.000	0.094	0.000
Unclassified *Peptostreptococcaceae* 1	0.010	0.318	0.010	0.178	0.006	0.000	0.000	0.012	0.062	0.031	0.011	0.000	0.035
Unclassified *Peptostreptococcaceae* 2	0.000	0.004	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000
Butyricicoccus	0.001	0.040	0.029	0.039	0.006	0.000	0.000	0.002	0.031	0.040	0.011	0.000	0.000
Oscillibacter	0.000	1.005	14.27	16.00	2.136	0.381	0.503	0.977	7.928	3.190	3.739	0.623	1.220
Sporobacter	0.049	0.011	0.271	0.047	0.066	0.016	0.076	0.022	0.056	0.044	0.046	0.000	0.000
Subdoligranulum	0.000	0.011	0.012	0.004	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000
Unclassified *Ruminococcaceae* 1	0.558	1.103	3.363	1.587	4.603	0.608	1.141	1.038	1.523	0.627	1.681	0.499	0.802
Unclassified *Ruminococcaceae* 2	0.000	0.188	0.360	0.341	0.030	0.286	0.094	0.052	0.025	0.053	0.240	0.000	0.035
Anaeroglobus	0.049	0.002	0.002	0.012	0.006	0.016	0.018	0.022	0.012	0.022	0.011	0.000	0.000
Dialister	0.303	0.072	0.164	0.062	0.039	0.164	0.189	0.157	0.117	0.287	0.332	0.031	0.000
Megamonas	5.353	3.672	0.474	0.016	35.41	0.116	0.153	0.460	0.351	3.071	2.047	0.343	0.384
Megasphaera	1.282	0.094	0.090	0.105	0.164	0.374	1.127	0.881	0.234	0.353	0.549	0.156	0.105
Phascolarctobacterium	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000
Veillonella	0.000	0.000	0.000	0.000	0.000	0.003	0.049	0.171	0.000	0.000	0.000	0.000	0.000
Unclassified *Clostridiales* 1	19.31	2.309	1.648	1.746	1.805	4.498	8.506	5.548	4.858	6.840	10.87	4.614	4.568
Unclassified *Clostridiales* 2	1.6146	1.1971	1.897	1.5171	1.5671	1.5139	1.5718	1.026	1.72	2.2416	2.7787	1.8703	1.8828
Unclassified *Clostridiales* 3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.011	0.000	0.000
Unclassified *Clostridiales* 4	0.098	0.049	0.041	0.050	0.009	0.054	0.103	0.048	0.068	0.132	0.092	0.125	0.105
Coprobacillus	0.010	0.074	0.269	0.132	0.036	0.063	0.076	0.034	0.086	3.954	4.483	0.842	1.883
Unclassified *Erysipelotrichaceae* 1	0.000	0.063	0.223	0.112	0.009	0.009	0.009	0.012	0.358	0.671	0.675	0.187	0.279
Unclassified *Erysipelotrichaceae* 2	0.861	1.974	2.780	2.949	0.140	0.812	0.849	0.566	3.391	1.536	2.150	0.468	0.697
Fusobacterium	0.000	0.000	0.000	0.004	0	0.101	0.045	0.059	0.031	3.402	4.174	0.904	1.290
Unclassified *Fusobacteriaceae*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.011	0.000	0.105
Sutterella	0.117	0.002	0.150	0.023	0.039	0.025	0.031	0.031	0.185	0.022	0.126	0.000	0.035
Unclassified β-*Proteobacteria* 1	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000
Unclassified β-*Proteobacteria* 2	1.634	0.132	2.202	0.369	0.801	0.343	0.588	0.526	8.051	0.843	1.955	0.156	0.279
Unclassified β-*Proteobacteria* 3	0.098	0.013	0.150	0.186	0.024	0.000	0.004	0.006	0.049	0.004	0.023	0.000	0.035
Citrobacter	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Enterobacter	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Escherichia	3.640	2.452	0.010	0.012	0.003	0.003	0	0.002	0	0.071	0.103	0	0
Unclassified *Enterobacteriaceae* 1	0.078	0.002	0.010	0.008	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.094	0.000
Unclassified *Enterobacteriaceae* 2	0.147	0.042	0.010	0.008	0.000	0.000	0.000	0.003	0.000	0.013	0.000	0.062	0.000
Pseudomonas	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.070
Unclassified γ-*Proteobacteria* 1	0.421	0.027	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000
Unclassified γ-*Proteobacteria* 2	0.069	0.013	0.099	0.054	0.018	0.010	0.004	0.006	0.074	0.004	0.023	0.000	0.000
Unclassified-γ-*Proteobacteria* 3	0.705	0.656	1.202	0.675	0.238	0.091	0.287	0.187	1.517	0.587	0.675	0.187	0.558

Heat map scale (low to high)

0.000	0.001	0.002	0.003	0.004	0.005	0.006	0.007	0.008	0.009	0.011	0.012	0.013	0.016	0.017
0.022	0.031	0.039	0.040	0.778	0.913	1.202	1.648	2.202	2.780	3.363	4.300	4.730	4.851	5.314
7.318	9.141	14.380	16.000	17.08	18.6	29.11								

and *Bifidobacterium*. SCFAs have inhibitory effects on *Salmonella* colonization of the gastrointestinal tract (Bohnhoff et al., 1964); and can modulate expression of *Salmonella* invasion genes (Durant et al., 1999; Durant et al., 2000a; Durant et al., 2000b). In addition, bile salt deconjugation by *Clostridia*, to form cholate and deoxycholate, can also synergistically inhibit *Salmonella* invasion (Ducarmon et al., 2019). The culmination of all of these factors are likely at play in pathogen exclusion requiring collective species metabolic activity.

Community diversity appears to be important in providing an ecosystem with multi-functionality and redundancy of function

TABLE 3 Similarity in bacterial composition of the master seed A with seeds B, C, D, E, or I.

Seeds	Log_{10} reduction[a]	Family similarity coefficient	Family correlation[b, c]	Genus similarity coefficient	Genus correlation[1,2]
B	−4.56	.893	.719	.750	.641
C	−6.89	.847	.624	.727	.541
D	−6.70	.678	.390	.722	.320
E	−5.56	.630	.360	.721	.078
I	−8.44	.750	.272	.696	.153

[a]Reduction in *Salmonella* abundance.
[b]Pearson's correlation coefficient.
[c]Not significant.

TABLE 4 Similarity in bacterial composition between seeds, exhibiting high (Seed I) or low (Seed E) *Salmonella* reduction, with their commercial lots.

Seeds	Log_{10} reduction[a]	Family similarity coefficient	Family correlation[b,c]	Genus similarity coefficient	Genus correlation[1,2]
I	−8.44				
J	−5.45	.857	.916	.796	.922
K	−7.78	.885	.921	.849	.933
L	−7.67	.784	.873	.690	.889
M	−5.07	.852	.888	.731	.901
E	−5.56				
F	−7.83	.889	.038	.835	−.001
G	−8.22	.873	.103	.826	.013
H	−5.56	.881	.073	.857	.017

[a]Reduction in *Salmonella* abundance.
[b]Pearson's correlation coefficient.
[c]Not significant.

TABLE 5 Similarity in the bacterial composition of commercial lots produced from seeds E (F–H) and I (J–M) as defined by Sørensen's coefficient and simple correlation ( ).

Family / Genus	F (−7.83)[a]	G (−8·22)[a]	H (−5.56)[a]	J (−5.45)[a]	K (−7.78)[a]	L (−7.67)[a]	M (−5.07)[a]
F (−7.83)[a]		.945 (.995)	.915 (.999)	.885 (.215)	.847 (.431)	.857 (.058)	.746 (.094)
G (−8.22)[a]	0.899 (.992)		.933 (.998)	.871 (.250)	.833 (.478)	.800 (.085)	.868 (.122)
H (−5.56)[a]	.843 (.998)	.854 (.997)		.879 (.238)	.844 (.460)	.778 (.077)	.807 (.114)
J (−5.45)[a]	.808 (.169)	.781 (.213)	.864 (.193)		.879 (.926)	.786 (.970)	.847 (.978)
K (−7.78)[a]	.825 (.340)	.796 (.400)	.847 (.371)	.885 (.936)		.815 (.824)	.842 (.845)
L (−7.67)[a]	.769 (.050)	.759 (.090)	.717 (.071)	.702 (.980)	.667 (.870)		.894 (.999)
M (−5.07)[a]	.660 (.079)	.800 (.119)	.735 (.100)	.800 (.986)	.774 (.886)	.757 (.999)	

[a]–log_{10}–: Reduction in *Salmonella* abundance. Family comparison is highlighted in light gray. *p*-values for Pearson's correlation coefficient were >.05.

(Delgado-Baquerizo et al., 2016a; Delgado-Baquerizo et al., 2016b). Diversity maintains function, even when a perturbation is introduced (Isobe et al., 2020). Most juveniles obtain their microbiomes from the parent (Ferretti et al., 2018; Kubasova et al., 2019; Zhu et al., 2021; Chen et al., 2022). The adult microbiome provides the young with pioneer colonizers important in intestinal development, immune function and pathogen exclusion. In poultry production, juveniles are separated from the adult hen, prior to hatch. Seeding chicks with a competitive exclusion product provides an effective barrier to pathogen colonization, that would be otherwise absent in this production environment.

TABLE 6 Genera shared with the master competitive exclusion stock with seeds as determined by chi-square test.

Genera	Master seed A ($-\log_{10}$ 8.50) vs.	Master seed A ($-\log_{10}$ 8.50) vs.	Master seed A ($-\log_{10}$ 8.50) vs.	Master seed A ($-\log_{10}$ 8.50) vs.	Master seed A ($-\log_{10}$ 8.50) vs.
	Seed B ($-\log_{10}$ 4.56)	Seed E ($-\log_{10}$ 5.56)	Seed D ($-\log_{10}$ 6.70)	Seed C ($-\log_{10}$ 6.89)	Seed I ($-\log_{10}$ 8.44)
Bacteroides	ns	.001	ns	.03	.001
Enterococcus	.001	ns	.001	.001	ns
Lactobacillus	ns	.001	.001	.001	.001
Anaerobacter	ns	.001	ns	ns	.001
Sarcina	ns	.001	ns	ns	.001
unclassified *Clostridiaceae*	.002	ns	ns	ns	ns
Blautia	ns	ns	ns	.03	ns
Peptostreptococcus	ns	.001	.001	ns	.001
Megamonas	ns	.001	.02	.04	.04
Veillonella	.001	.001	.001	.001	.001

$-\log_{10}$: Reduction in *Salmonella* abundance. Ns-not significant. Not statistically significant association of genus (n = 68), in comparison of seed stocks with the master: *Bifidobacterium*; *Collinsella*; *Olsenella*; *Slackia*; unclassified *Actinobacteria* 1,2; unclassified *Bacteroidales* 1–3; *Bacillus*; *Exiguobacterium*; unclassified *Bacillales*; unclassified *Enterococcaceae*; *Pediococcus*; unclassified *Lactobacillaceae*; *Streptococcus*; unclassified *Lactobacillales* 1–3; *Clostridium*; *Acetobacterium*; *Eubacterium*; unclassified *Eubacteriaceae*; *Sporanaerobacter*; unclassified bacteria; unclassified Incertae Sedis XI; *Coprococcus*; *Dorea*; *Roseburia*; unclassified *Lachnospiraceae* 1,2; *Peptococcus*; *Sporacetigenium*; unclassified *Peptostreptococcaceae* 1,2; *Butyricicoccus*; *Oscillibacter*; *Sporobacter*; *Subdoligranulum*; unclassified *Ruminococcaceae* 1,2; *Anaeroglobus*; *Dialister*; *Megasphaera*; *Phascolarctobacterium*; unclassified *Clostridiales* 1–4; *Coprobacillus*; unclassified *Erysipelotrichaceae* 1,2; *Fusobacterium*; unclassified *Fusobacteriaceae*; *Sutterella*; unclassified β-*Proteobacteria* 1–3; *Citrobacter*; *Enterobacter*; *Escherichia*; unclassified *Enterobacteriaceae* 1,2; *Pseudomonas*; unclassified γ-*Proteobacteria* 1–3.

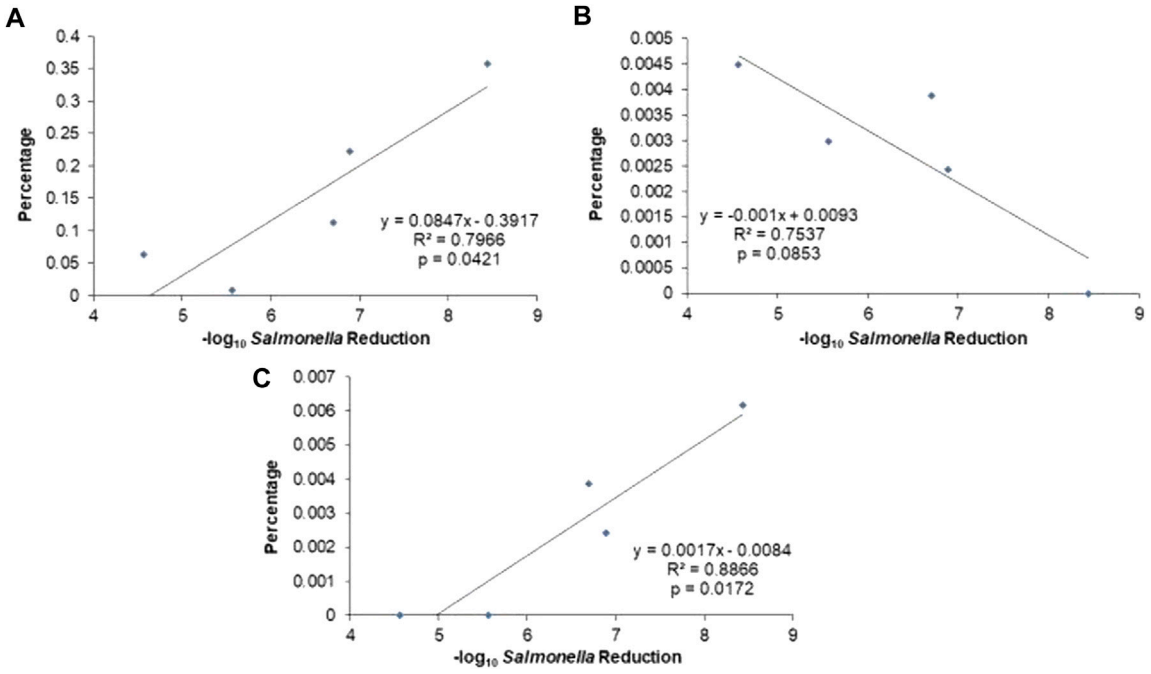

FIGURE 5
Linear correlation between genus abundance and –log10 reduction in *Salmonella* by competitive exclusion seeds B, C, D, E, and I. **(A)** Unclassified *Erysipelotrichaceae 1*. **(B)** Unclassified *Actinobacteria 2*. **(C)** *Peptococcus*. *Salmonella* reduction data was provided by the manufacturer for seeds and commercial lots.

Conclusion

Pathogen exclusion may be a combination of competition and antagonism. In either case, a bacterial census is not likely to identify the magic bullet, the single organism, responsible for excluding *Salmonella* from a mixed community as exists in the chicken intestine. The key to understanding competitive exclusion will come from comparing communities that permit and exclude *Salmonella* and associated transcriptome, proteome, or metabolome evidence of competition and antagonism (García et al., 2017). Regulatory agencies are starting to come around to commercial acceptance of these fecal communities for treating or preventing bacterial infections, as evident from US Food and Drug Administration's approval of fecal microbiota product for the treatment of *Clostridium difficile* infections (Pharmaceutical Technology Editors, 2022).

Data availability statement

16S sequence data is available through MG-RAST, metagenomics analysis server (mg-rast.org). The individual data sets can be accessed through https://www.mg-rast.org/linkin.cgi?project = followed by mgp40703 (Commercial Lot M), mgp40704 (Commercial Lot L), mgp4075 (Commercial Lot K), mgp40706 (Commercial Lot J), mgp40707 (Competitive Exclusion Seed I), mgp40708 (Commercial Lot H), mgp40709 (Commercial Lot G), mgp40710 (Commercial Lot F), mgp40711 (Competitive Exclusion Seed E), mgp40712 (Competitive Exclusion Seed D), mgp40713 (Competitive Exclusion Seed C), mgp40714 (Competitive Exclusion B) and mgp40715 (Competitive Exclusion Master Seed A). Therefore, to access Master Seed A, the link is https://www.mg-rast.org/linkin.cgi?project=mgp40715.

Author contributions

ML was responsible for funding acquisition, conceptualization, writing, review, and editing. AP performed the experiments, formal analysis and data curation described in this article. AP and JM were involved in the writing, review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CC declared a shared parent affiliation with the author AP to the handling editor at the time of the review.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1043383/full#supplementary-material

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Temporal dynamics of the cecal and litter microbiome of chickens raised in two separate broiler houses

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In this study, we investigated the dynamics of the ceca and litter microbiome of chickens from post-hatch through pre-harvest. To achieve this, six hundred one-day old Cobb 500 broiler chicks were raised on floor pens for 49 days in two separate houses. We performed short-read and full-length sequencing of the bacterial 16S rRNA gene present in the meconium and in cecal and litter samples collected over the duration of the study. In addition, we determined the antimicrobial resistance (AMR) phenotype of *Escherichia coli* and *Enterococcus* spp. isolated from the meconium and the ceca of 49-day old chickens. We monitored the relative humidity, temperature, and ammonia in each house daily and the pH and moisture of litter samples weekly. The overall microbial community structure of the ceca and litter consistently changed throughout the course of the grow-out and correlated with some of the environmental parameters measured ($p < 0.05$). We found that the ceca and litter microbiome were similar in the two houses at the beginning of the experiment, but over time, the microbial community separated and differed between the houses. When we compared the environmental parameters in the two houses, we found no significant differences in the first half of the growth cycle (day 0–21), but morning temperature, morning humidity, and ammonia significantly differed ($p < 0.05$) between the two houses from day 22–49. Lastly, the prevalence of AMR in cecal *E. coli* isolates differed from meconium isolates ($p < 0.001$), while the AMR phenotype of cecal *Enterococcus* isolates differed between houses ($p < 0.05$).

KEYWORDS

broiler chickens, microbiome, antimicrobial resistance (AMR), pre-harvest, environmental condition

Introduction

Broiler house environment is one of the most important management factors that has been shown to significantly affect broiler performance, welfare, and health (Winn and Godfrey, 1967; Deaton et al., 1978; Weaver and Meijerhof, 1991; Jones et al., 2005; Bessei, 2006; Wei et al., 2015; Baracho et al., 2018; Nassem and King, 2018). Temperature and relative humidity of a broiler house are interconnected factors that affect litter moisture and emitted ammonia (Ritz et al., 2005). Together, these environmental parameters have been shown to influence broiler growth, feed conversion efficiency, disease etiology, occurrence of pathogens and in some cases, mortality (Miles et al., 2004; Ritz et al., 2004; Bessei, 2006; De Jong et al., 2014; Wei et al., 2015; Baracho et al., 2018). The results from these earlier studies served as the framework for broiler management/husbandry guidelines used by the poultry industry (Donald, 2010; Vantress, 2013). Therefore, there is sufficient data supporting the importance of proper environmental management.

Contrastingly, there is limited data on how changes in environmental factors affect the microbiome of broiler chickens. The few studies that have investigated the role of the environment have focused on exposing broilers to an environmental stressor e.g., temperature or ammonia (Wang et al., 2018; Zhou et al., 2021c; Han et al., 2021; Yang et al., 2021; Emami et al., 2022). Broiler chickens exposed to temperature levels that induce heat stress harbored a different bacterial community structure in the ceca compared to non-stressed control chickens (Shi et al., 2019; Liu et al., 2022). Similarly, exposing broilers to 25–35 ppm of ammonia was reported to alter the microbiota of the trachea (Zhou et al., 2021b). Changes in litter moisture and pH have been shown to perturb the microbiome of litter and affect the survival of bacterial pathogens including *Salmonella* (Lovanh et al., 2007; Payne et al., 2007; Chinivasagam et al., 2012; Dunlop et al., 2016; Bucher et al., 2020). Kers et al. (2019) showed that the microbial diversity in the ceca of broilers was influenced by the type of house and resulted in significant variability in the interventions tested.

Other studies have focused on the litter and its interaction with the gastro-intestinal tract (GIT) microbiome of broiler chickens and the occurrence of pathogens (Cressman et al., 2010; Roll et al., 2011; Roberts et al., 2013; Wang et al., 2016). For instance, broilers raised on fresh litter were shown to harbor a different microbiome compared to chicks raised on reused litter (Cressman et al., 2010; Wang et al., 2016; Oladeinde et al., 2022). Our research group (Oladeinde et al., 2022) and others (Fanelli et al., 1970; Corrier et al., 1992) have also reported that chickens grown on reused litter are less likely to be colonized by *Salmonella* than chickens on fresh litter. Taken together, these studies support the hypothesis that changes in environmental conditions during grow-out will affect the microbiome in the GIT and litter of chickens raised.

Therefore, our objectives for this study were 3-fold: i) determine the temporal changes in the GIT and litter microbiome of broiler chickens from post-hatch to pre-harvest ii) determine environmental parameters that correlated with changes in the microbiome of broiler chickens and iii) evaluate if changes in the microbiome and environment resulted in bacterial strain-level changes in antimicrobial resistance (AMR) phenotype. Our results revealed that the overall microbial community structure of the ceca and litter consistently changed throughout the course of the grow-out and that these changes correlated with some of the environmental parameters measured in the two different houses. We found no significant differences in environmental parameters between the houses in the first half of the grow-out (day 0–21), but morning temperature, morning humidity, and ammonia significantly differed between houses from day 22–49. The AMR phenotype of cecal *Escherichia coli* isolates differed from the meconium isolates, while the AMR phenotype of cecal *Enterococcus* isolates differed between the houses.

Materials and methods

Study design

Six hundred 1-day old Cobb 500 broiler chicks were raised in two separate houses (H1 and H2) for 49 days (Figure 1) both located at the experimental farm of the University of Georgia (33.907101 N 83.380368 W). Before chick placement, each house was cleaned-out and steamed. Broiler chicks were raised in floor pens (12 pens/house, 25 chicks/pen) measuring 1.84 m (length) L by 1.16 m width, and fresh pine shavings were used as the bedding material (Figure 1). Broiler chickens were given water and feed *ad libitum* and were raised antibiotic-free on starter (days 0–15), grower (days 15–29), and finisher (days 29–49) feeds (feed was synthesized by the University of Georgia's Poultry Research Center's feed mill). On day 49, feeders were removed from 6 pens in each house for 8 h before all chickens were euthanized. Husbandry and management followed commercial broiler chicken industry guidelines. Chicken mortality was recorded daily while body weights were measured on day 0, 14, 28, 42 and 49. Additionally, we used Portacool evaporative fans (Port-A-Cool, L.L.C., Center, TX; model PAC2K24HPVS) to reduce the air temperature when the house temperature was above 85°F. Broiler chickens were euthanized as approved by the University of Georgia Office of Animal Care and Use under Animal Use Protocol (A2018 05–013-R1) before cecal sampling and at the completion of the study. The study was conducted from 11 July 2019–29 August 2019.

Determination of environmental parameters

Litter moisture was determined gravimetrically while litter pH was determined using a Thermo-Scientific Orion probe (ThermoFisher Scientific) as described before (Johnson et al., 2021). Broiler house ammonia levels were monitored by attaching ammonia dosimeter tubes (Gastec Corporation) to metal chains hung at about 37 cm ± 5.4 cm from the litter floor of three pens from each house (Johnson et al., 2021). Readings on ammonia tubes were recorded ~7.5 h after installation and performed once a week. The pen used for monitoring ammonia changed weekly for each house. The temperature and relative humidity reading inside each house was recorded from thermostats (Temperature, Johnson Controls, Inc., Milwaukee, WI, model: A419 Temperature Control with NEMA 4X, Enclosure and A99 Temperature Sensor; Humidity, AcuRite Lake Geneva, WI, model: AcuRite® indoor digital thermometer and

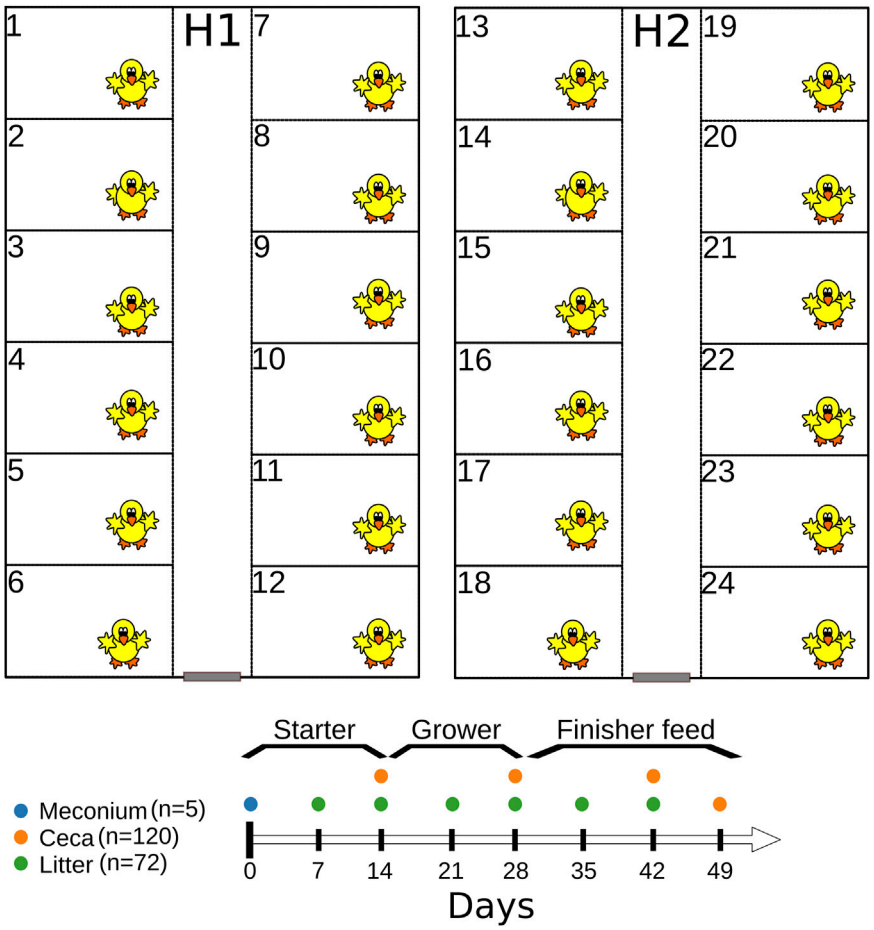

FIGURE 1
Graphical representation of the experimental design.

hygrometer 00609SBLA) installed in each house at the same time in the mornings (7 a.m.–10:00 a.m.) and afternoons/evenings (5 p.m.–9 p.m.). Litter moisture, pH and ammonia levels were measured weekly, while temperature and humidity were measured daily, resulting in different sample sizes for these indicators.

Meconium, cecal and litter sampling

Chick pads used for transporting 1-day old broiler chicks from the hatchery were used for meconium (the dark greenish-yellow first droppings of a chick) recovery. In addition, chick pads ($n = 5$) that were not used for chick transportation were included as controls. Each chick pad ($n = 5$) was transferred into a 1-gallon Whirl Pak bag and 500 mL of buffered peptone water (BPW) was added. Afterwards, the bag was shaken by hand for 2 min and incubated for 1 h at 37°C. After incubation, 200 mL aliquots of the mixture were transferred to 250 mL Nalgene bottles and centrifuged at 4,600 g for 10 min. Thereafter the supernatant was decanted, and the pellet was resuspended in an equal volume of Luria Bertani (LB) broth containing 60% glycerol (final glycerol concentration was 30%), vortexed and saved in cryovials at −80°C. Pellets saved in LB glycerol were used for 16S rRNA gene sequencing and the retrospective isolation of *E. coli* and *Enterococcus* spp.

Cecal contents were collected from chickens ($n = 120$) on days 14, 28, 42, and 49 (Figure 1). Briefly, floor pens were given numbers at the start of the experiment (1–24), and six odd- or even-numbered pens/house were randomly sampled on day 14 and 28. For example, on day 14, we selected one chicken each from six odd-numbered pens from each house ($n = 12$ per sampling day), while on day 28, chickens were selected from six even-numbered pens from each house ($n = 12$). On day 42 and 49, two chickens were selected from each pen from each house ($n = 48$ for each sampling day). The weight of individual chickens was measured before the ceca were removed from the eviscera. Thereafter, the ceca were stomached for 60 s after the addition of 3 × volume to the weight (vol/wt) of BPW. Cecal contents were resuspended in an equal volume of LB broth containing 60% glycerol, vortexed and saved in cryovials at −80°C. Cecal contents saved in LB glycerol were used for 16S rRNA gene sequencing and the retrospective isolation of *E. coli* and *Enterococcus* spp. from day 49 samples.

Litter samples were collected from floor pens on days 7, 14, 21, 28, 35 and 42 (Figure 1). Litter was collected as grab samples from

seven locations in each pen, including the corners and under the waterers (Johnson et al., 2021). On each sampling day, litter was collected from six odd- or even-numbered pens per house (Johnson et al., 2021) ($n = 72$). Litter samples were mixed thoroughly by hand in the Whirl Pak bags and processed as described previously (Johnson et al., 2021). Briefly, 10 g of litter were placed in a Nalgene bottle containing acid-washed glass beads covering the bottom of bottle (S800242, ThermoFisher Scientific), and 50 mL of 1 × phosphate-buffered saline (ThermoFisher Scientific) was added. Sample bottles were mixed on an automatic wrist shaker (Boekel Scientific) at 450 rpm for 10 min and allowed to rest upright for 5 min after shaking (Johnson et al., 2021). Five milliliter of the eluate was transferred into LB broth containing 60% glycerol, vortexed and saved in cryovials at −80°C. Eluate saved in LB glycerol was used for 16S rRNA gene sequencing.

DNA extraction and sequencing

DNA was extracted from 250 µL of LB glycerol containing either meconium, cecal contents or litter eluate using the Qiagen DNeasy PowerLyzer Powersoil kit (Qiagen Inc., MD, United States) according to manufacturer instructions. In addition, DNA was extracted from 13 negative controls (5 Chick pad paper and 8 DEPC-treated H_2O samples). Amplicon sequencing libraries for all samples were generated as previously described (Allen et al., 2016). Briefly, the V4 hypervariable region of the bacterial 16S rRNA gene was PCR amplified and sequenced using the paired-end (250 × 2) method on the Illumina MiSeq platform. Additionally, 62 samples (24 cecal, 24 litter, 5 meconium, 5 chick pad paper, and 4 negative control samples) were sequenced on the Pacbio Sequel II platform to get the full-length of the 16S rRNA gene for better species classification. Preparation and sequencing of full-length (V1–V9) 16S rRNA gene libraries were done by the sequencing core center of University of Georgia (Athens, GA, United States) as described previously (Schloss et al., 2016). Raw sequence reads are available under NCBI accession number PRJNA699167.

16S rRNA sequence processing and data analysis

Raw sequence reads obtained from the Illumina Miseq were processed in R using the DADA2 package (version 1.14) (Callahan et al., 2016). Only reads with a maximum number of expected errors lower than or equal to 2 were retained. In addition, reads were truncated where the phred quality score dropped below 30. Chimeras were identified and removed using the consensus method and the remaining reads were annotated to the SILVA database release 138 with a minimum bootstrap threshold of 50 (Quast et al., 2013). Additionally, full-length 16S rRNA gene sequences generated on the Pacbio Sequel II were processed in the SMRT Link software package version 8.0. The circular consensus reads (ccs) were determined with a minimum predicted accuracy of 0.99 and the minimum number of passes set to 3. After demultiplexing, the ccs were further processed with DADA2 (version 1.14) to obtain high quality amplicons with single-nucleotide resolution as previously described (Callahan et al., 2019). Same as the Illumina reads, the full-length 16S rRNA gene sequences were annotated to the SILVA database 138. Hereafter, the annotated Pacbio reads were used to create a custom formatted database that was utilized as a reference for the Illumina reads that were generated from the same samples. Iterating the species taxonomy assignment of the Illumina reads to the custom database and adding this information to the taxonomy table improved species classification rate by 35%. Amplicon sequence variants (ASVs) with less than 5 sequences in total were removed from the dataset before decontamination. Contaminant sequences were identified from extracted negative controls with the R package decontam and the probability threshold set to 0.5. After contaminant removal, samples with less than 1,000 sequences were removed. The average sequence depth per sample was 23,088.38, ranging from 1,769 to 93,023 sequences.

In-depth microbial community analysis was performed in the R environment using the packages "phyloseq", "Ampvis2", "vegan", and "MaAsLin2". Alpha diversity indices were calculated with a dataset rarefied to the smallest sample size. Values of alpha diversity indices were checked for normal distribution by visually assessing qqplots and histograms and by calculating the Shapiro-Wilk normality test. The groups that were not normally distributed were compared using the Wilcoxon Signed Rank test. A non-metric multidimensional scaling ordination based on Bray-Curtis distances was performed to calculate changes in microbial beta diversity. In addition, a permutational multivariate analysis of variance (PERMANOVA) was performed to assess the influence of experimental factors on the microbial community in ceca and litter samples. Prior to this analysis, ASV's that are not present in more than 0.1% relative abundance in any sample have been removed. ASVs were considered part of the core microbiome with a relative abundance cutoff above 0.01% and a prevalence cutoff above 80% of the samples. Temporal microbial shifts and differences of ASVs between houses were computed using MaAsLin2. Only associations for ASVs with a minimum prevalence of 10% and a minimum relative abundance of 1% were calculated. For temporal microbial shifts the variables "Pen" and "House" were set as random effects, while for differences between houses only "Pen" was set as a random effect. Benjamini–Hochberg procedure was applied as a correction method for computing the q-values.

Retrospective isolation of *E. coli* and *Enterococcus* spp. from meconium and cecal samples

One hundred microliters of meconium samples ($n = 5$) and 10 µL of cecal samples ($n = 48$) previously saved in LB glycerol at −80°C were vortexed and spread plated onto CHROMagar™ ECC (DRG International, Inc., Springfield, NJ). CHROMagar™ ECC was incubated for 18 h–24 h at 37°C and 5 isolated blue-green colonies typical of *E. coli* were subcultured for isolation to a fresh CHROMagar™ ECC and incubated as above. For *Enterococcus* spp. isolation, 100 µL of meconium and cecal samples was spread plated onto mEnterococcus agars (Neogen, Lansing, MI). mEnterococcus agar was incubated for 48 h at 37°C and 5 pink

to dark red colonies indicative of *Enterococcus* spp. were re-struck for isolation to a fresh mEnterococcus agar and incubated as indicated.

After isolation of *E. coli* and *Enterococcus* spp. on selective agar, all isolated colonies were subcultured to Tryptic Soy Agar with 5% sheep blood (BAP) agar (Remel, Lenexa, KS), incubated 18 h at 37°C and then re-struck to BAP. Isolate identification was confirmed using qPCR on a CFX96 Touch Real-Time System (Bio-Rad, Hercules CA). Primers (Ludwig and Schleifer, 2000; Jackson et al., 2004; Chern et al., 2011) were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Supplementary Table S1. Reaction mixtures (20 µL) for all assays contained 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 600 nM (each) primers, and 4 µL of isolate whole cell template (1 colony in 100 µL nuclease-free water; boiled for 10 min). Thermal conditions for all assays except individual *Enterococcus* spp. were initial denaturation at 98°C for 3 min followed by 40 cycles of denaturation at 95°C for 15 s and an annealing/extending step at 60°C for 30 s before melting from 65°C to 95°C at 0.5°C increments. The *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus hirae* species assays were adapted from Jackson et al. (2004) using the cycling conditions above but decreasing the annealing temperature to 55°C. Melt curves were visually inspected to ensure standards and samples had peaks at the same temperature and no secondary peaks were formed.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations for isolates were determined by broth microdilution using the Sensititre™ semiautomated antimicrobial susceptibility system (Thermo Fisher Scientific, Waltham, MA). Using the National Antimicrobial Resistance Monitoring System (NARMS) protocol (US FDA, 2019) *E. coli* isolated from meconium (n = 25) and ceca (n = 96) were tested using the CMV4AGNF panel while *Enterococcus* spp. isolated from meconium (n = 25) and ceca (n = 90) were tested using the CMV3AGPF panel. Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines when available (CLSI, 2019); otherwise, breakpoints established by NARMS were used (US FDA, 2019).

Heatmaps were generated using the pheatmap v1.0.12 package in R. A distance matrix was generated using the jaccard metric *via* the vegdist function from the Vegan v2.6 package. Optimal number of clusters was identified using the silhouette method implemented by the fviz_nbclust function from the factoextra v1.0.7 package. Hclust () from the stats v3.6.2 package was then utilized to perform hierarchical clustering under the "complete" method using the determined optimal number of clusters. All analyses were done in R v4.0.4 utilizing RStudio v1.2.1106.

Statistical analyses

The measured environmental parameters were tested for normal distribution by calculation of the Shapiro–Wilk normality test and visually as histograms and Q-Q plots. Normal distributed parameters were compared using a student t-tests and not normal distributed parameters were compared using a pairwise Wilcoxon signed rank test. p values were corrected with the Benjamini–Hochberg method. Wilcoxon rank sum test was performed to determine if there were significant differences between sample type (meconium vs. ceca) and houses (house 1 vs. 2) in the number of antibiotic drug classes and antibiotic drugs *E. coli* and *Enterococcus* isolates were resistant to. p values were corrected with the Benjamini–Hochberg method. Statistical comparisons were performed using R v4.2.0 using the stats v3.6.2 package.

Results

Microbial diversity of litter and ceca increased throughout grow-out

Meconium samples showed the lowest species richness of all sample types with an average of 44.4 amplicon sequence variants (ASVs). In comparison, ceca and litter samples harbored an average of 85.0 ASVs and 142.3, respectively on day 7 and 14. The number of observed ASVs increased significantly over the course of the study, reaching 258.6 ASVs in cecal (day 49) and 180.6 ASVs in litter (day 42) samples (Figure 2). The Shannon and Inverse Simpson indices of alpha diversity showed that the diversity of the ceca and litter microbiome increased from the start to end of the grow-out. Furthermore, chickens raised in house 2 had higher cecal alpha diversity (Observed, p = 0.026; Shannon, p = 0.041; Simpson, p = 0.13) than chickens in house 1 at day 28, while chickens in house 1 had higher alpha diversity (Observed, $p < 0.001$; Shannon, $p < 0.001$; Simpson, $p < 0.001$) at day 49 (Supplementary Figure S1). Similarly, litter from house 2 had higher alpha diversity than house 1 at day 28, while litter from house 1 had higher alpha diversity than house 2 at day 49, however, these differences were not statistically significant (Supplementary Figure S1).

Environmental factors, chicken weight, mortality and the bacterial community of the ceca and litter differed between houses

We found that chicken body weights differed between houses at the end of the grow-out (Supplementary Figure S2A). The average weight of 1-day old chicks was 43.04 ± 0.73 g and there was no significant difference ($p > 0.05$) in weight between chicks placed in house 1 compared to house 2. However, at the end of the grow-out, chickens in house 1 (average = 3,375.58 ± 482 g) weighed more than chickens in house 2 (average = 3,035.42 ± 349 g) ($p < 0.01$). Furthermore, chickens in house 2 experienced higher premature mortality (~6%) than chickens in house 1 (~3%) (Supplementary Figure S2B).

The overall bacterial community structure changed throughout the course of the grow-out in both cecal and litter samples and the changes correlated with several environmental parameters (Figure 3). Litter moisture, litter pH, house temperature, house humidity, and house NH_3 levels were factors that explained bacterial community heterogeneity in litter samples, while in cecal samples, only house temperature was found to correlate with changes in

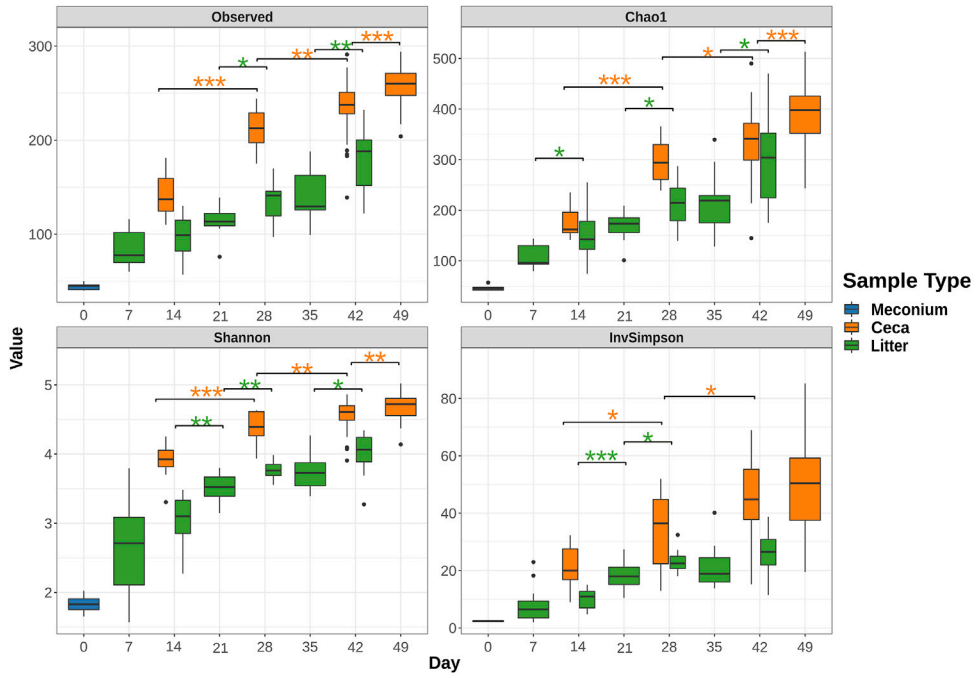

FIGURE 2
Change of alpha diversity indices from rarefied 16S rRNA gene libraries over time. Boxes indicate the interquartile range (75th to 25th) of the data. The median value is shown as a line within the box. Whiskers extend to the most extreme value within 1.5 * interquartile range and dots represent outliers. Only significant changes are shown with asterisks: *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. The color of asterisks shows pairwise comparisons between ceca (orange) or litter (green) samples.

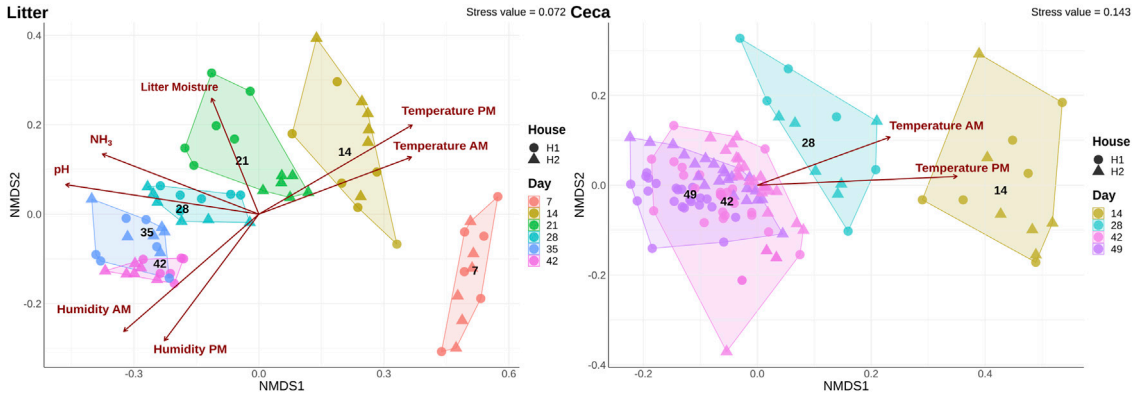

FIGURE 3
Shifts of microbial community composition in cecal and litter samples. Beta diversity was calculated using a non-metric multidimensional scaling (NMDS) ordination of 16S rRNA gene libraries based on Bray-Curtis distances. Colors show samples obtained on different days and the shape displays samples from different houses. Only significant ($p < 0.05$) environmental variables were fitted onto the ordination.

community structure. Additional factors that explained the variation in bacterial community composition were sample type, day, and house (Table 1). Since the house was determined as a significant factor affecting bacterial community heterogeneity, we calculated analysis of similarities (ANOSIM) tests for each individual day and sample type.

The ceca and litter microbiome were similar in the two houses at the beginning of the experiment, but over time the bacterial community separated and differed between the houses (Table 2). Therefore, we compared the environmental parameters that were monitored in the two houses (Supplementary Figure S3). No significant differences were observed in the first half of the grow-out (day 0–21), but morning house temperature and humidity, and NH_3 levels varied between the two houses throughout the second half of the grow-out (day 22–49) (Supplementary Table S2).

TABLE 1 Results from a PERMANOVA test for the influence of sampling groups.

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr (>F)
Sample type	1	15.974	15.974	83.601	0.247	<0.001***
Day/age	6	12.844	2.141	11.203	0.199	<0.001***
House	1	0.924	0.924	4.835	0.014	<0.001***
Pen	22	4.344	0.197	1.033	0.067	0.344,931
Residuals	160	30.572	0.191		0.473	
Total	190	64.657			1	

Df = Degrees of Freedom, SumsOfSqs = Sum of Squares, MeanSqs = Mean Squares, F. Model = Pseudo-F, R2 = coefficient of determination, Pr (>F) = *p*-value.

TABLE 2 Results from an analysis of similarities (ANOSIM) test between the two different houses.

	Day/age	R-value	*p*-value
Litter	7	0.0130	0.3779
	14	0.3278	0.0022
	21	0.7037	0.0020
	28	0.3463	0.0154
	35	0.5907	0.0026
	42	0.6981	0.0032
Ceca	14	0.0167	0.3747
	28	0.2815	0.0224
	42	0.2877	0.0002
	49	0.3216	0.0002

Morning temperature ($p < 0.01$) and NH_3 levels ($p < 0.05$) were significantly higher in house 2 (H2), while morning humidity ($p < 0.05$) was higher in house 1 (H1).

To identify individual ASVs that contributed to the observed difference in microbial community composition between the houses, we performed a multivariable association analysis using MaAsLin2. In congruence with the results of the ANOSIM test, no ASVs were different in relative abundance on day 7 in litter samples, but from day 14 onwards, several ASVs were significantly more or less abundant in H1 compared to H2 (Figure 4A). Similarly, no ASVs were different at the beginning of grow-out in ceca samples, but several ASVs were different on day 42 and 49 (Figure 4B).

Microbial community profiles of the meconium, ceca, and litter

Six different phyla were detected in the meconium, 12 phyla in the ceca, and 15 phyla in litter samples (Figure 5). The community profiles differed between the 3 sample types. For example,

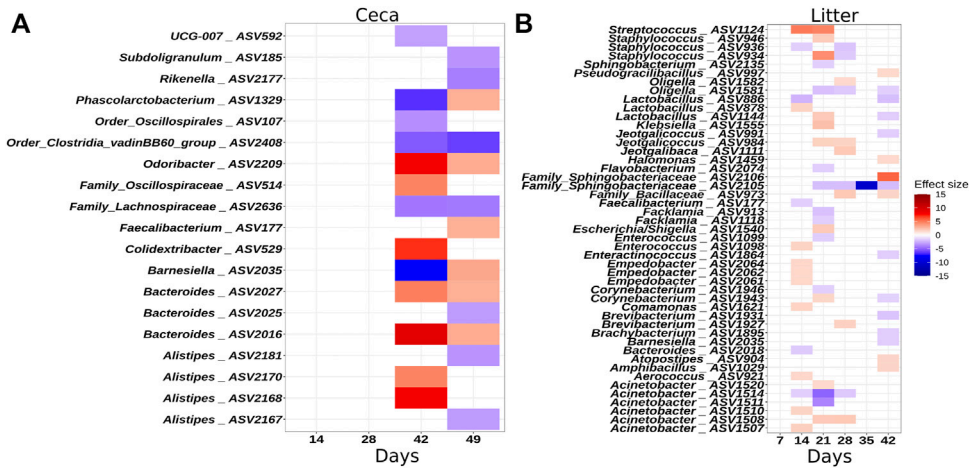

FIGURE 4
Heatmaps illustrating ASVs that were significantly differentially abundant between houses. **(A)** Ceca samples. **(B)** Litter samples. The effect size depicts the negative log of the q-value multiplied by the sign of the coefficient. A positive effect size denotes higher abundance in house 2. A negative effect size denotes higher abundance in house 1. Taxonomy of the ASVs is indicated at the genus levels or at the lowest rank that could be assigned confidently (Bootstrap support above 50). Only significant changes are shown.

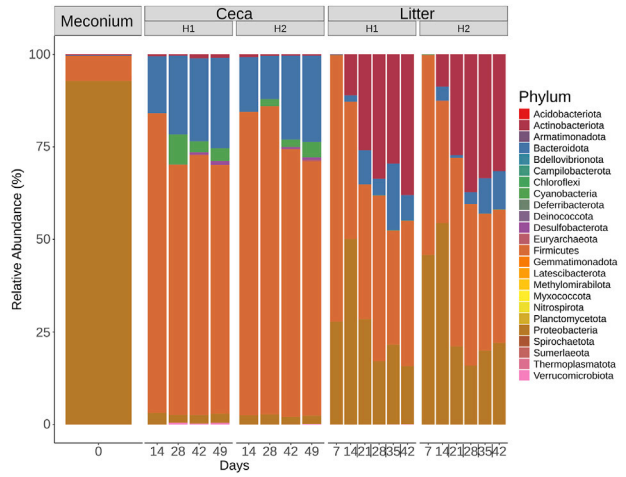

FIGURE 5
Phylum-level classification of 16S rRNA gene sequence reads. Samples were grouped by days and split by sample type (Meconium, Ceca, Litter). Relative abundance was normalized by total sum scaling and the mean for each sample group is depicted.

meconium samples were dominated by Proteobacteria, Firmicutes and the less abundant phyla Actinobacteriota, Bacteroidota, Deinococcota, and Verrucomicrobiota. Cecal samples were composed of Firmicutes and Bacteroidota and to a lesser extent of Cyanobacteria, Proteobacteria, and other phyla with a mean relative abundance of less than 1%. The composition of the microbial communities in litter samples was different. Here, Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidota were the dominant phyla throughout the grow-out. Interestingly, Actinobacteriota increased from 0.4% relative abundance on day 7%–34.81% relative abundance on day 42 in litter samples. Similarly, Bacteroidota were found at low levels at the beginning and at higher levels at the end of the grow-out.

On a finer taxonomic resolution, meconium samples were dominated by two ASVs classified as *Escherichia/Shigella* and *Enterococcus* (Figure 6). Both ASVs were also highly abundant in litter samples at the beginning of the grow-out, but their abundance decreased over time. The most abundant bacteria were different between cecal and litter samples. For example, *Barnesiella*, *Phascolarctobacterium*, *Faecalibacterium*, *Bacteroides*, and *Alistipes* were found in high numbers in cecal samples, but not in litter samples. Highly abundant ASVs in litter samples were classified as *Corynebacterium*, *Lactobacillus*, *Luteimonas*, and *Klebsiella*.

Eighty ASVs were part of the core microbiome in both houses (Supplementary Figure S4). The core ASVs in cecal samples were classified as Bacilli, Bacteroidia, and Clostridia while in litter samples they were identified as Bacilli, Clostridia and Actinobacteria (Supplementary Table S3). Furthermore, we found 59 ASVs to differ between house 1 and house 2. For example, 23 ASVs were exclusively part of the core microbiome of the cecal samples of house 1, but not of house 2 and 21 ASVs were part of the core microbiome

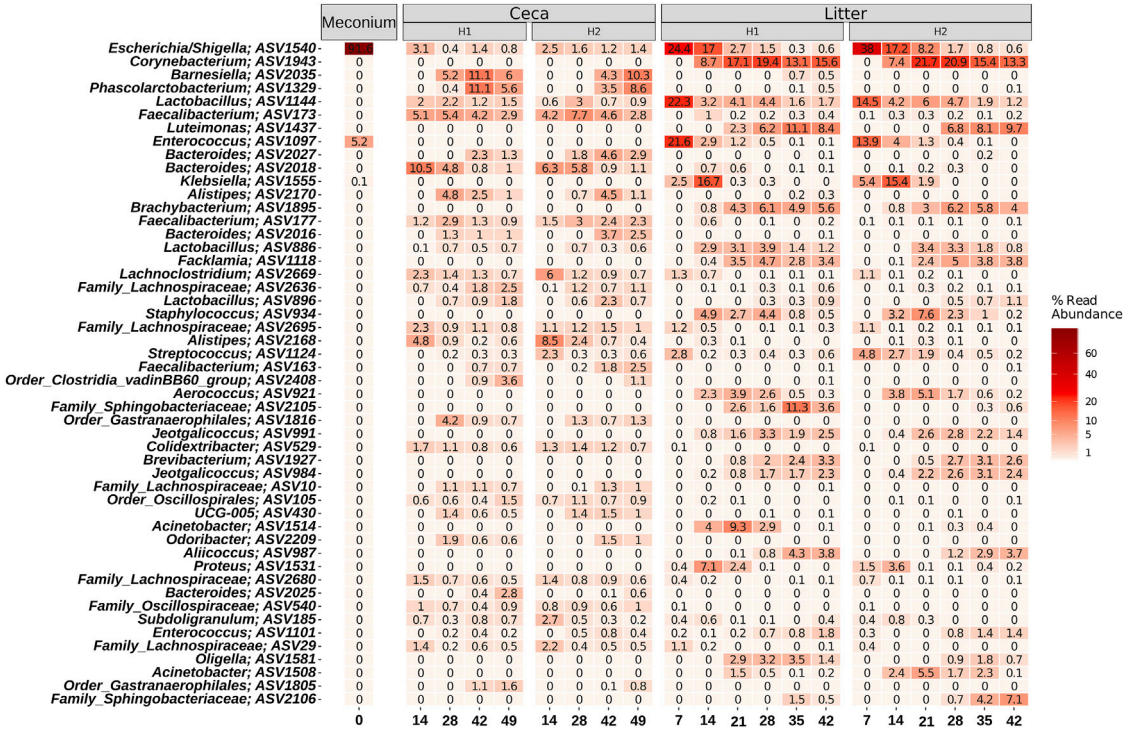

FIGURE 6
Heatmap showing the relative abundance of the 50 most abundant ASVs. Samples were grouped by days and split by sample type (Meconium, Ceca, Litter). Relative abundance was normalized by total sum scaling and the mean for each sample group is depicted. Taxonomy of the ASVs is indicated at the genus levels or at the lowest rank that could be assigned confidently (Bootstrap support above 50).

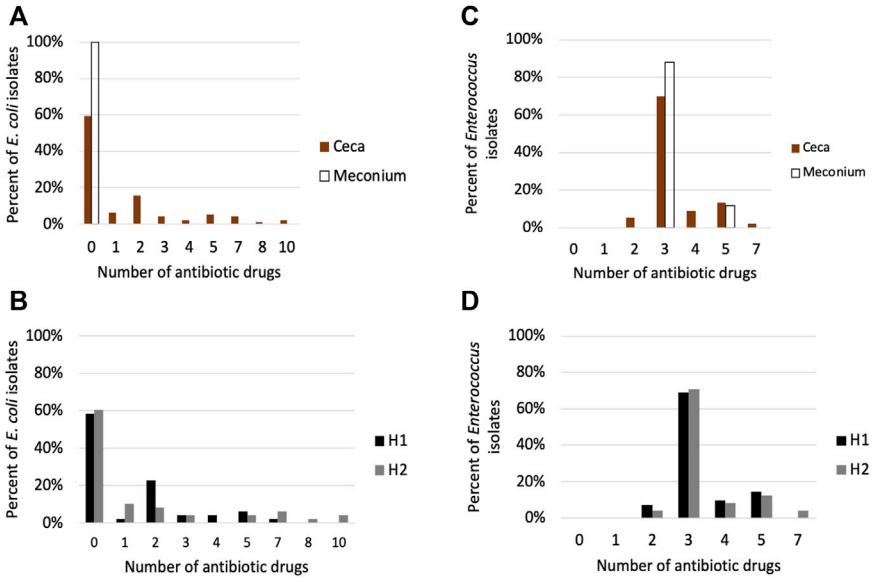

FIGURE 7
Antimicrobial susceptibility testing of (E) *coli* and *Enterococcus* isolates. **(A)** Percentage of *E. coli* isolates in the meconium (n = 25) and ceca (n = 96) that are resistant to 0–10 antibiotics. **(B)** Percentage of cecal *E. coli* isolates from house 1 (n = 48) and house 2 (n = 48) that are resistant to 0–10 antibiotic drugs **(C)** Percentage of *Enterococcus* isolates in the meconium (n = 25) and ceca (n = 90) that are resistant to 0–10 antibiotics **(D)** Percentage of cecal *Enterococcus* isolates from house 1 (n = 42) and house 2 (n = 48) that are resistant to 0–10 antibiotic drugs.

of cecal samples of house 2, but not of house 1. In litter samples, only 6 and 9 ASVs were part of the core microbiome in house 1 and 2, respectively (Supplementary Figure S4). The core ASVs found in the ceca of chickens from house 1 were associated with 15 families while the core ASVs from chickens in house 2 were classified into 7 families (Supplementary Table S4). The core ASVs in the litter of chickens from house 1 were classified into 3 families while ASVs in the litter from house 2 were grouped into 8 families.

We also determined the abundance of 16S rRNA gene reads that were associated with *Salmonella* or *Campylobacter* to see if there are differences between houses in the occurrence and abundance of food-borne pathogens. *Salmonella* was detected in the meconium and litter but not in the ceca and no reads were found for *Campylobacter*. For house 1, *Salmonella* was detected only in litter samples from day 14, while in house 2, it was found in litter samples from day 7, 14, 21, and 35 (Supplementary Table S5).

Temporal microbiome changes in the ceca and litter

Temporal shifts for individual microbiota members were separately determined for cecal and litter samples. In total, the relative abundance of 37 ASVs changed significantly over time in cecal samples (Supplementary Figure S5). Some genera comprised ASVs with diverging abundances. For example, *Alistipes* ASV 2168 was reduced over time, while others (ASVs 2,170, 2,173, 2,181) were significantly higher at later time points. Similarly, some ASVs of the genus *Bacteroides* increased while another ASV of this genus decreased. The *Escherichia/Shigella* ASV 1540 which was highly abundant in meconium samples decreased significantly over time in both cecal and litter samples but persisted until the end of the grow-out. Similarly, *Enterococcus* ASV 1097 was highly abundant in meconium samples, decreased but persisted in litter samples, but not in cecal samples. Overall, 5 ASVs decreased and 33 ASVs increased significantly throughout the course of the grow-out in litter samples (Supplementary Figure S6). Interestingly, ASV 1555, classified as *Klebsiella*, was the only ASV that showed an initial increase (from day 7 to day 14), before a subsequent decrease in abundance (day 7 compared to days 21, 28, 35, 42). Other ASVs that showed a reduction in abundance were associated with *Streptococcus*, *Lactobacillus*, and *Enterococcus*, while ASVs from the genera *Staphylococcus, Jeotgalicoccus, Facklamia, Brevibacterium, Corynebacterium, Brachybacterium,* and *Aerococcus* increased over time.

On day 49 feed was withdrawn from half of the chickens (n = 24) for 8 h to determine whether feed withdrawal affected the cecal microbiome of broiler chickens. Our analysis revealed that there was no significant difference ($p > 0.05$) in the cecal bacterial community structure between the feed and feed withdrawal group (Supplementary Figure S7).

AMR phenotype of *E. coli* and *Enterococcus* isolates

Although microbiome analysis informs us on the composition and relative abundance of bacterial species in a sample it lacks the resolution needed to infer strain level phenotypic differences. To get some insight on the phenotypic differences between bacterial strains from this study, we performed antimicrobial susceptibility testing on *E. coli* (n = 121) and *Enterococcus* strains (n = 115) recovered at the beginning (meconium samples) and end of the grow-out (day 49 cecal samples). We focused on antimicrobial resistance (AMR) because it is strongly correlated with the horizontal acquisition of antibiotic resistance genes and mutational resistance (Martinez and

Baquero, 2000; Von Wintersdorff et al., 2016; Bortolaia et al., 2020). We used *E. coli and Enterococcus* because they had the highest relative abundance in the meconium of one day old chicks.

All isolates were confirmed to be either *E. coli* or *Enterococcus* spp. using qPCR (Supplementary Table S1). The AMR phenotype of cecal *E. coli* isolates differed from the meconium isolates ($p < 0.001$). Meconium *E. coli* isolates were susceptible to all antibiotic drugs tested, while 40.6% of cecal isolates were resistant to 1–10 antibiotics belonging to 1–7 drug classes (Figure 7A). The most common resistance found were to tetracycline ($n = 32$), ampicillin, ($n = 21$), streptomycin ($n = 22$) and nalidixic acid ($n = 15$) (Supplementary Figure S8). There was no significant difference between the houses in the number of drug classes or drugs *E. coli* isolates were resistant to ($p > 0.05$), however, 6 of the 7 isolates that were resistant to 7–10 antibiotics were from cecal samples from house 2 (Figure 7B; Supplementary Figure S8).

Eighty-nine percent of *Enterococcus* isolates were *E. faecalis*, while *E. hirae* represented 2% of the isolates. We could not determine the species of *Enterococcus* for 11 isolates. There was no significant difference in AMR phenotype between meconium and cecal *Enterococcus* isolates ($p > 0.05$) (Figure 7C). *E. faecalis* isolates (101 of 102) were resistant to lincomycin, Synercid and tetracycline. Seventeen *E. faecalis* isolates displayed additional resistance to tylosin and erythromycin (Supplementary Figure S9). The two *E. hirae* isolates were resistant to lincomycin and tetracycline. Cecal *Enterococcus* isolates from house 1 differed from house 2 in the number of antibiotic drug classes they were resistant to ($p < 0.05$) (Figure 7D).

Discussion

Early studies on the microbiome of the gastrointestinal tract of broiler chickens revealed that the core bacterial phyla in the GIT of broilers includes Firmicutes, Bacteriodota, Proteobacteria and Actinobacteriota (Oakley et al., 2014b; Borda-Molina et al., 2018; Feye et al., 2020) and that as chickens grow a successional change in bacterial composition and abundance is expected (Oakley et al., 2014a; Jurburg et al., 2019; Zhou Q. et al., 2021). Likewise, spatial differences in microbiome composition have been replicated across studies (Oakley et al., 2014b; Oakley and Kogut, 2016; Zhou Q. et al., 2021; Weinroth et al., 2022), and the microbiome diversity of the GIT and litter have been shown to be different (Cressman et al., 2010; Danzeisen et al., 2015; Wang et al., 2016). In this study, we present results that support some of these findings and provide new data on the environmental factors that are correlated with a change in the microbiome of broiler chickens. We found key differences in the relative abundance of the four core phyla between sample types. In the meconium Proteobacteria was the phylum with highest abundance, while Firmicutes dominated the ceca and litter microbiome. This difference in phyla abundance is expected since the environmental selective pressures present in each ecosystem are dissimilar.

Fertilized eggs to be hatched are incubated under warm temperatures (99–102°F) and are kept in clean/disinfected environments (Berrang et al., 2000; French, 2009; Archer and Cartwright, 2012; Wales and Davies, 2020). Therefore, the hatchery environment may select for bacterial species that can survive elevated temperatures and exposure to disinfectants. These surviving bacterial population would be the first colonizers of the GIT broiler chicks. Here, we found that members of the genera *Escherichia/Shigella* and *Enterococcus* were the main bacterial species in the meconium (the dark yellowish-green first droppings of a chick), of one-day old chicks. Jurburg et al. (2019) also reported that *Escherichia/Shigella* and *Streptococcus* were the major taxa found in fecal samples of one-day old broiler chicks. Similarly; Cárdenas-Rey et al. (2022), showed that *Escherichia/Shigella* was in high abundance in the ceca of one-day old broiler chicks (relative abundance of 37.3% ± 24.0%). Together, these results suggest that the bacterial taxa found in the meconium of day-one old chicks in the study were under selection in the hatchery.

After chicks were placed on pen floors in the broiler house, they were trained on how to drink from nipple waterers, and they began pecking at litter and feed. Therefore, it is plausible that they ingested microbes attached to physical, biological, and environmental matrices in the broiler house. Upon entry into the GIT, bacterial populations ingested are challenged with several selection pressures in the upper and lower GIT, including acidic pH, low oxygen levels, competition from resident microbiota and the chicken host immune responses. The cecum is part of the lower GIT that carries the highest bacterial densities, has the longest residence time of digesta, and is an important site for urea recycling and water regulation (Clench and Mathias, 1995; Oladeinde et al., 2019). In addition, bacterial populations in the ceca experience low redox potential that can lead to an increase in the abundance of obligate anaerobic bacteria and a reduction in aerobes or facultative anaerobes (Rinttilä and Apajalahti, 2013).

In this study, we found that members of the phylum Bacteriodota increased in abundance in the ceca (Figure 5). For instance, ASVs classified as *Barnesiella* and *Phascolarctobacterium* increased in the ceca from <1% relative abundance on day 14 to > 3.5% on days 42 and 49 (Supplementary Figures S5, S6). Members of these two bacterial taxa are obligate anaerobes that play a crucial role in the breakdown of carbohydrates and the production of short chain fatty acids (Weiss et al., 2014; Ikeyama et al., 2020). Contrastingly, we saw a significant decrease in the abundance of ASVs of facultative anaerobes such as *Escherichia/Shigella* and *Enterococcus* that were the dominant taxa in the meconium (Supplementary Figures S5, S6). Although, oxygen levels may have influenced the observed temporal changes in the abundance of the different taxa in ceca, other factors such as changes in diet at different chicken ages (i.e., starter diet from age 0–15, grower diet from age 15–29, and finisher diet from age 29–49) have been reported to affect microbial successional changes in the GIT of broiler chickens (Pan and Yu, 2014; Schokker et al., 2021). In fact, we found the age of chickens to be a significant factor that affected the bacterial beta-diversity in the ceca (Figure 3; Table 1).

The litter is a complex environment that is composed of decaying plant-based bedding material, feces, urine, feathers, and other broiler-sourced material. Furthermore, litter is exposed to broiler house environmental conditions such as temperature and relative humidity that are known to affect the physico-chemical characteristics of litter including litter moisture/water activity, pH, and ammonia. These environmental factors have been shown to affect the microbial community in litter (Ritz et al., 2005). Also, the bacterial population in litter are challenged with higher oxygen

levels compared to the cecal microbiome. Therefore, it is not surprising that the relative abundance of strict anaerobes such as *Barnesiella* and *Phascolarctobacterium* decreased in litter, while aerobes (*Brachybacterium, Brevibacterium Corynebacterium* and *Luteimonas*) and facultative anaerobes (*Aerococcus, Facklamia, and Staphylococcus)* increased. These bacterial taxa displaced *Enterococcus* and *Escherichia/Shigella* in litter starting from day 14 and 21, respectively.

The majority of the ASVs that increased in the litter belonged to phylum Actinobacteria (Figure 5). Actinobacteria are known for their capability to biodegrade complex biopolymers and produce antimicrobials and bioactive substances (Vaijayanthi et al., 2016; Binda et al., 2018; van der Heul et al., 2018). For example, *Corynebacterium urealyticum* produces urease that catalyzes the hydrolysis of urea into carbon dioxide and ammonia (Salem et al., 2015; Gutierrez and Schneider, 2022), while some *Brevibacterium* spp. can efficiently degrade ammonia (Kim et al., 2013; Forquin and Weimer, 2014). In the current study, the relative abundance of *Corynebacterium* peaked between 21–28 days and coincided with the period that the highest broiler house ammonia levels were recorded (Supplementary Figure S3). Like the ceca, we also found that the day/age of broilers significantly affected the bacterial beta-diversity in the litter.

Broiler house environmental parameters (temperature, relative humidity, and ammonia) differed significantly between houses from day 22–day 49 of grow-out. House 2 had higher temperatures and higher ammonia in the mornings, while house 1 had higher relative humidity. Notably, these environmental changes coincided with a higher mortality and lower body weight of chickens in house 2. Likewise, we saw differences in the alpha and beta diversity of the ceca and litter microbiome between houses around this period (Table 2). For example, the alpha diversity of cecal samples decreased from day 28 to day 42 in house 2 but not in house 1, which suggests a perturbed gut microbiome (Pickard et al., 2017) (Supplementary Figure S1). Furthermore, we found core ASVs specific to the ceca and litter of each house (Supplementary Table S4). Elevated temperatures, atmospheric ammonia and relative humidity have been shown to increase mortality and reduce feed efficiency, body weight and feed intake in broilers (Weaver and Meijerhof, 1991; Miles et al., 2004; Vale et al., 2010; Wang et al., 2010; Alloui et al., 2011; Wasti et al., 2020). Heat stress and high ammonia have also been linked to a change in the microbiome of chickens (Shi et al., 2019; Zhou et al., 2021b; Liu et al., 2022). Taken together, these results suggest that there is a link between environmental conditions, an imbalance of the gut microbiome and poor broiler performance.

It is not clear why this difference in environmental conditions appeared after day 21 but we observed that the outside temperature from day 1–21 (average highs: 93.22 ± 3.54°F; range: 86–99°F) was higher than day 22–49 (average highs: 91.3 ± 4.74°F; range: 79–97°F) (https://www.ncdc.noaa.gov/cdo-web/; weather station: USW00013873, Athens Ben Epps Airport, Georgia, United States, 33.94773,-83.32736). The climate and weather outside can significantly influence broiler house environmental parameters and proper ventilation is one of the best management practices recommended to ensure that house conditions are optimal for broiler welfare and health. In this study, we used Portacool fans during hot weather days to reduce the temperature inside the house. Therefore, one or both variables (i.e., hot weather and use of Portacool fans) may have contributed to the differences in environmental conditions between houses. It is not clear whether the differences in environmental conditions caused changes in the microbiome, or if they just coincided. Nonetheless, we did observe not only changes in relative abundances, but also differences in the core microbiome between the two houses.

For instance, the ceca of chickens from house 2 harbored four core ASVs classified as Order_Clostridia_vadinBB60_group (Supplementary Table S4). This group of bacteria are not well classified, and little is known about their metabolism or role in the microbiota (Richards et al., 2019). Zhou et al. (2017) reported an increase in the abundance of order_Clostridia_vadinBB60_group in the ceca of chickens infected with *Eimeria tenella*. Also, we have shown that members of the Order_Clostridia_vadinBB60_group increased in abundance in the ceca and litter of broiler chicks infected with *Salmonella* Heidelberg and raised on fresh pine shavings (Oladeinde et al., 2022). Furthermore, three core ASVs found in the litter of chickens from house 1 but not in the litter from house 2 were classified as *Lactobacillus* including *L. johnsonii* (Supplementary Table S4). *Lactobacillus* spp. are regarded as safe and beneficial microbes and have been extensively employed in the development of probiotics (Zhang et al., 2018). Additionally, we found *Salmonella* 16S rRNA gene reads in the litter from house 2 during four sampling time points while *Salmonella* was detected only in day 14 litter samples from house 1 (Supplementary Table S5). The lack of *Salmonella* 16S rRNA gene reads in cecal samples was unexpected and suggest that *Salmonella* was in low abundance and/or in a non-viable state in the litter. Bacterial cell viability and inoculum concentration could affect the rate *Salmonella* colonizes the GIT of chickens. Taken together, these results suggest that the environment and the microbiome in house 1 was different from house 2.

Lastly, we found that the prevalence of AMR in *E. coli* isolates differed between meconium and ceca. Horizontal gene transfer is the main mechanism bacteria acquires AMR genes (Von Wintersdorff et al., 2016). *E. coli* isolates recovered from the meconium were susceptible to all antibiotics tested, while ~41% of cecal *E. coli* isolates were resistant to at least 1 antibiotic (Figure 7A). Contrastingly, there was no significant difference in AMR prevalence between meconium and cecal *Enterococcus* isolates suggesting that limited HGT of AMR occurred in *Enterococcus* isolates. All *E. faecalis* isolates from the meconium (n = 23) and 99% of cecal isolates (n = 78) were resistant to lincomycin, Synercid (quinupristin/dalfopristin) and tetracycline and only three cecal *E. faecalis* isolates displayed resistance to additional antibiotics that were not seen in meconium isolates. It is possible that *E. coli* and *Enterococcus* isolates selected from the meconium are not representative of all AMR phenotypes present in one-day old broilers.

In conclusion, this study showed that the microbiome of the ceca and litter of broiler chickens changed over time. Furthermore, differences in microbiome between houses were correlated with changes in house environmental parameters. However, since our study has no repeatability and environmental conditions were not controlled, additional studies are necessary to investigate whether this is generally true, or it is specific only to the broiler houses in this study. Therefore, it is crucial that animal studies pay close attention to environmental differences between houses/barns/cages as this can

potentially be a source of confounders and introduce variability in experimental outcomes.

Data availability statement

The datasets presented in this study can be found in online repositories. 16S rRNA gene sequences are publicly available under NCBI accession no: PRJNA699167.

Ethics statement

The animal study was reviewed and approved by University of Georgia Office of Animal Care and Use under Animal Use.

Author contributions

AO and SA designed the study. JJ, GZ, BZ, AO, MM, AG, JF, and LF performed live broiler chicken studies. BZ and MM performed DNA extractions. TL made 16S rRNA gene libraries and sequencing, while BZ performed 16S rRNA bacterial community analysis and interpretation. JT, JL, and DC performed bacteriological analyses and antibiotic susceptibility testing. JL performed qPCR. BZ and RW performed statistical analysis. AO, BZ, JJ, RW, and JL drafted the manuscript, which was reviewed and edited by all authors. AO, SA, and ZA contributed resources and supervised the study.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor BO declared a past collaboration with the author SA.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1083192/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Change of alpha diversity indices from rarefied 16S rRNA gene libraries between houses. Dots represent values from individual days, lines represent loess curves of the moving average, and shaded areas indicate the confidence interval (0.95).

SUPPLEMENTARY FIGURE S2
Chicken body weight **(A)** and cumulative mortality **(B)** throughout the course of the study.

SUPPLEMENTARY FIGURE S3
Measurement of broiler house and litter environmental parameters. Boxes indicate the interquartile range (from 75 to 25th) of the data. Whiskers extend to the most extreme value within 1.5 times interquartile range and dots represent outliers beyond that range. Only significant changes are shown with asterisks: *: $p \leq 0.05$, **:$p \leq 0.01$. House temperature and relative humidity were measured daily, while NH3, pH and moisture were measured weekly from pooled litter from six pens per house.

SUPPLEMENTARY FIGURE S4
Venn diagram showing the number of core and non-core ASVs found in the ceca and litter of broilers.

SUPPLEMENTARY FIGURE 5
Heatmap illustrating temporal microbial shifts in cecal samples. Changes in microbial abundance were computed between day 7 and each of the other days separately. The effect size depicts the negative log of the q-value multiplied by the sign of the coefficient. Taxonomy of the ASVs is indicated at the genus levels or at the lowest rank that could be assigned confidently (Bootstrap support above 50). Only significant changes are shown.

SUPPLEMENTARY FIGURE S6
Heatmap illustrating temporal microbial shifts in litter samples. Changes in microbial abundance were computed between day 7 and each of the other days separately. The effect size depicts the negative log of the q-value multiplied by the sign of the coefficient. Taxonomy of the ASVs is indicated at the genus levels or at the lowest rank that could be assigned confidently (Bootstrap support above 50). Only significant changes are shown.

SUPPLEMENTARY FIGURE S7
Principal component analysis of bacterial communities from cecal samples on day 49. Points indicate samples from chickens raised in house H1 and triangles show samples from chickens raised in house H2. Samples from chickens with or without feed withdrawal are illustrated in blue and red respectively.

SUPPLEMENTARY FIGURE S8
Heatmap of the antimicrobial resistance phenotype of E. coli isolates. Each row represents one isolate tested for susceptibility against the CMV4AGNF panel. Columns on left hand side represent the isolate associated metadata. Number of antibiotics reflects the total number of antibiotics an isolate was resistant to on the panel and number of drug classes refers to the

enumeration of antibiotics an isolate is resistant to on the panel of the same drug class. AMP, Ampicillin; TET, Tetracycline; GEN, Gentamicin; SOX, Sulfizoxazole; FOX, Cefoxitin; AMC, Amoxicillin–Clavulanic Acid; AXO, Ceftriaxone; SXT, Trimethoprim– Sulfamethoxazole; AZM, Azithromycin; CHL, Chloramphenicol; NAL, Nalidixic acid; STR, Streptomycin.

SUPPLEMENTARY FIGURE S9
Heatmap of the antimicrobial resistance phenotype of Enterococcus Isolates. Each row represents one isolate tested for susceptibility against the CMV3AGPF panel. Columns on left hand side represent the isolate associated metadata. Number of antibiotics reflects the total number of antibiotics an isolate was resistant to on the panel and number of drug classes refers to the enumeration of antibiotics an isolate is resistant to on the panel of the same drug class. LIN, Lincomycin; SYN, Synercid (Quinupristin/ Dalfopristin); TET, Tetracycline; ERY, Erythromycin; TYL, Tylosin; GEN, Gentamicin; KAN, Kanamycin; CIP, Ciprofloxacin; NIT, Nitrofurantoin.

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Differences in gut bacterial community composition between modern and slower-growing broiler breeder lines: Implications of growth selection on microbiome composition

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In the last century broiler chicken lines have undergone an extensive breeding regime aimed primarily at growth and high meat yield. It is not known if breeding has also resulted in a change to the broiler breeder's associated gut microbiota. Here we compared the gut microbiota of 37-week-old commercial Cobb breeding dams with dams from a broiler Legacy line which has not undergone selection since 1986. The dams from both lines were kept together in the same shed under the same management protocol from day of hatch to avoid additional confounders. We chose this age to allow significant bacterial exchange, thus avoiding exposure dependent artifacts and so that we could compare dams at the same developmental state of adulthood and peak laying performance. Significant differences in the composition of the cecum bacterial communities were found. Bacteria of the genus *Akkermansia*, implicated in mucin degradation and associated with host metabolic health, accounted for 4.98% ± 5.04% of the Cobb cecum community, but were mostly absent from the ceca of the Legacy line dams. Inversely, Legacy dams had higher levels of Clostridiales, Lactobacillales and Aeromonadales. These results show that breeding has resulted in a change in the gut microbiota composition, likely by changing the physiological conditions in the mucosa. It remains unclear if changes in gut microbiota composition are a part of the mechanism affecting growth or are a secondary result of other physiological changes accelerating growth. Therefore, the identification of these changes opens the door to further targeted research.

KEYWORDS

akkermansia, gut microbiome, broiler breeders, breeding program, genetics microbiome interaction

Introduction

Breeding programs primarily targeting growth and high meat yield have successfully transformed broiler lines in the last decades by substantially increasing growth (Zuidhof et al., 2014). While many physiological effects of breeding programs are known, such as changes to metabolism and the intestinal tract, including an increase of surface area (Mitchell and Smith, 1991; Zuidhof et al., 2014; Tallentire et al., 2016), it remains unclear if the gut microbiota has been modulated by breeding programs. The aim of this study is to address this point by comparing the cecum microbiota of a current modern commercial breeding dam line and a legacy broiler line, which has not undergone any selection since 1986 (Yair et al., 2017; Ben-Gigi et al., 2021).

It can be hypothesized that the gut microbiota is likely to be affected by physiological changes introduced in the host through breeding programs. These changes can include differences in retention time, affecting microbial clearance (Rougière and Carré, 2010). Differences in mucin expression levels can also affect the gut microbiota since bacteria use mucin as a binding site or as a nutrient (Cheled-Shoval et al., 2014). Other factors include reduced nutritional availability due to changes in host absorption (Croom et al., 1999; Schmidt et al., 2009), and changes in the regulation of components of the immune system, such as changes in secretion of antimicrobial peptides or IgA into the gut lumen (Qureshi and Havenstein, 1994; Schokker et al., 2015).

The gut microbiota can affect host growth. For example, different gut microbial communities can induce host obesity (John and Mullin, 2016) but can also reduce weight (von Schwartzenberg et al., 2021). One mechanism by which gut bacteria can positively affect growth is by converting indigestible fibers into short-chain fatty acids which the host can absorb and utilize (Krajmalnik-Brown et al., 2012). However, gut bacteria are also potential competitors and can reduce nutrient availability for the host (Romano et al., 2017). Finally, the gut microbiota can also affect the maturation and development of the host's intestinal tract, thereby affecting its ability to utilize the feed (Hutsko et al., 2016; Dougherty et al., 2020). Thus, theoretically, there are multiple mechanisms by which modulation of gut microbiota composition during breeding programs may have supported or opposed the target of fast growth and higher meat yield.

Previous studies have examined the relationship between broiler lines and gut microbiota composition. A comparison of the cecal (Richards et al., 2019) and ileal (Richards-Rios et al., 2020) microbiomes of three fast growing commercial broiler lines up to day 42 revealed differences only on the day of hatch and day 3 of life. Thus, while some differences were observed early on, possibly because of different chick sources, the composition over time converged, likely because the chicks were raised together and exchanged gut microbes. This implies that fast growing commercial broiler lines are similar in their interaction with their gut microbiota at least until the age of 42 days. In comparison, studies comparing fast growing broiler lines to a historic line, or a line selected for slow growth, were able to identify differences in ileal and fecal bacterial communities. Lumpkins et al. (2010) identified composition differences between the ileal bacterial community of a historic line and two commercial modern broiler lines in the first 35 days of life, and Zhao et al. (2013) identified multiple composition differences in fecal samples at the age of 245 days of two divergent lines, selected for 54 generations for high or low body weight. Finally, a recent study comparing the ileal microbiota of four different lines of fast and slow growing broilers including the ancestral Jungle Fowl at the age of 56 days found unique signatures for the different lines and predicted microbiota functions (Emami et al., 2022). Here we extend these studies by comparing the cecum bacterial community of breeder dams from a commercial broiler line and a Legacy line. As the cecum is the site of bacterial fermentation in chickens, any relevant effect of the gut community on poultry growth would likely occur at the cecum. Furthermore, differences in adult breeders might be easier to detect because the microbiota has stabilized, they might reveal physiological differences that are relevant to younger birds, and they might affect the fertility as well as egg laying efficiency of breeders, including the nutrients deposited into the egg.

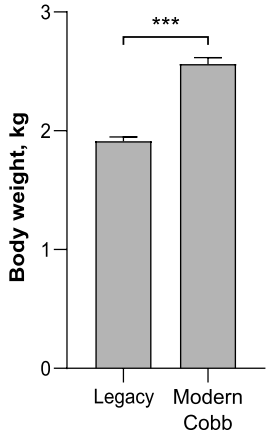

FIGURE 1
Body weight of Legacy line and Cobb line individuals. Data are presented as mean ± SEM; n = 10; Welch's t-test; ***$p < 0.0001$.

Thus, to examine the interaction of genetics and microbiota composition in the context of breeding programs, we raised modern Cobb breeder dams alongside dams from a Legacy line, which was kept as a relaxed line (without selection) from 1986 (Yair et al., 2017; Ben-Gigi et al., 2021) and compared their cecum bacterial communities.

Materials and methods

Genetic lines

All animal trials were conducted in accordance with the guidelines of the National Council for Animal Experimentation and were subjected to approval by the Hebrew University of Jerusalem's Ethics committee, approval No. AG-19-15897-3.

Two genetic lines were utilized: Cobb—the current Cobb breeder line, and Legacy—a local Israeli broiler line which has not been under selection pressure since 1986 (Yair et al., 2017; Ben-Gigi et al., 2021).

Growth conditions and confounder avoidance

Eggs from both lines were incubated and hatched on site. Sixty-two Cobb breeders and 84 Legacy breeders were kept in the same shed, under the same conditions and handled by the same individuals from hatch and

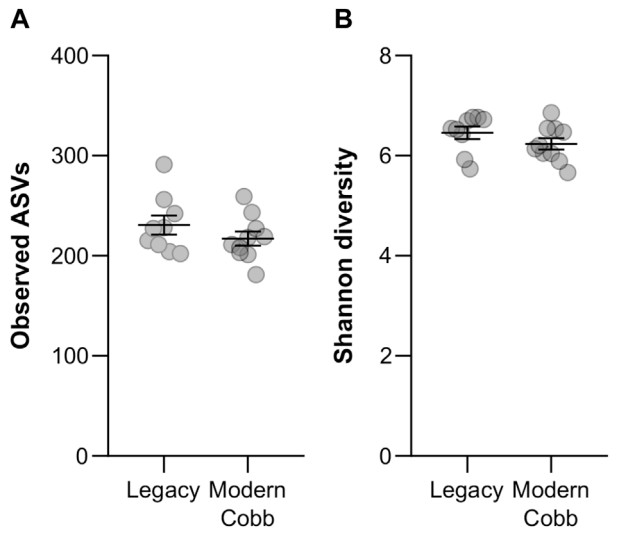

FIGURE 2
Alpha-diversity measures. Richness **(A)** and Shannon diversity **(B)** of Legacy line and Cobb line individuals. Data are presented as mean ± SEM; Legacy n = 9, Cobb n = 10; Mann-Whitney test; $p > 0.05$.

throughout the experiment. Birds were placed in individual cages (45 × 45 cm) at 6 weeks of age. All the birds were raised according to the same breeder management protocol (Cobb-Vantress, 2018), including the same feed. During the production stage, birds were fed once a day in the morning according to the feeding tables in the management protocol. At the age of 24 weeks the birds were transferred to cages in an open shed and were exposed to 16 h of light per day. Eggs were collected manually twice a day, and individual laying was monitored. Sampling was done at the age of 37 weeks, after both lines have reached their peak laying state and were still producing at high levels (Supplementary Figure S1). At the age of 37 weeks, it is assumed that there was ample time for microbial exchange between animals to offset any differences in initial exposure. Furthermore, by waiting until adulthood and peak laying status, we were ensuring that differences between the birds are not an artifact of different effective physiological age.

Sample collection

At age 37 weeks, ten animals of each line were randomly selected, weighed and then euthanized by cervical dislocation. Cecum samples were removed, their contents were emptied out into 5 mL of sterile PBS and flash frozen with liquid nitrogen. All samples were stored at −20°C until processing. One sample of a Legacy dam was contaminated during the sampling procedure, and therefore was removed from the microbiota analyses.

Sampling of other broiler and broiler breeder sources

To determine the presence of *Akkermansia* in other broilers, four more sources of modern broilers and broiler breeders were sampled. Broiler source #1—ceca of six Ross breed broilers were sampled from a commercial farm at age 32 days; Broiler source #2—ceca of five Ross breed broilers were sampled from a commercial farm at age 34 days; Broiler breeders source #1—ceca of five Ross breed broiler breeders were sampled from the experimental farm in the Hebrew University of Jerusalem's Faculty of Agriculture at age 55 weeks (Shterzer et al., 2022); Broiler breeders source #2—ceca of five Ross breed broiler breeders were sampled from a commercial farm at age 56 weeks. All ceca were sampled as mentioned above.

DNA extraction

DNA was extracted by mixing 700 µL of sample with 700 µL of Tris-saturated phenol and 100 µL of 10% SDS. The mixture was disrupted with 0.1 mm glass beads followed by phenol-chloroform extraction, as described previously (Stevenson and Weimer, 2007). Briefly, the aqueous phase was extracted twice with phenol, then twice with a phenol-chloroform mixture (1:1) and finally twice with chloroform. DNA was subsequently precipitated with isopropanol and suspended in double distilled water.

16S rRNA gene sequencing

16S rRNA gene library was prepared and sequenced according to the Earth Microbiome Project protocol (Thompson et al., 2017) using V4 primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT). 250 bp paired-end sequencing was carried out on an Illumina Miseq platform using a V2 reagent kit by Hylabs (Rehovot, Israel). Sequence processing and taxonomy assignment were performed using Quantitative Insights Into Microbial Ecology (QIIME2) version 2020.11.1 (Bolyen et al., 2019) as described previously (Shterzer et al., 2020). Briefly, amplicon sequence variants (ASVs) were determined with Dada 2 plugin version 2020.11.1 (Callahan et al., 2016) using the denoise-paired method, which filters out reads with estimated number of errors >2. All reads were truncated at position 200; otherwise, default parameters were used. After denoising, a total of 313,070 reads were retained, with 16,477 ± 3,945 reads per sample (min—9,691; max—28,241). ASVs with under five reads were discarded and all samples were normalized to 4,000 reads per sample with the feature-table plugin using the rarefy method (Weiss et al., 2017). Taxonomy was assigned using a naive-bayes classifier (Pedregosa et al., 2011) trained on the Greengenes database (McDonald et al., 2012). All ASVs with the taxonomic assignment of "Bacteria" were compared to the NT database using BLAST (Altschul et al., 1990) and removed if they were 100% identical to *Gallus* mitochondrion.

Statistical analysis

ANCOM analysis was implemented using QIIME2 (Mandal et al., 2015) to identify differential abundance of phylogenetic groups in all levels (phylum, class, order, family, genus and species). To that end, the ASV feature table was collapsed at different taxonomic levels and ANCOM analysis was performed on the collapsed tables, as well as on the original ASV-level table. To test the significance of the differences in microbiome

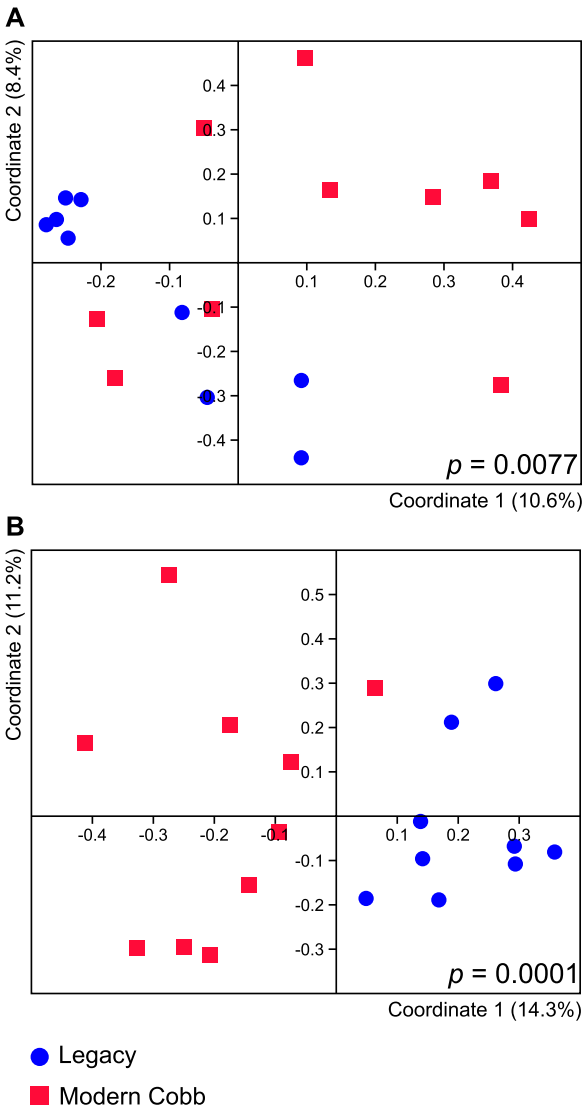

FIGURE 3
Cecal microbiome differences between Legacy line and Cobb line individuals. PCoA analysis of Legacy line and Cobb line using Jaccard **(A)** and Bray-Curtis **(B)** metrics. ANOSIM *p* values for each metric are indicated in the figure. Legacy $n = 9$, Cobb $n = 10$.

composition between the lines, ANOSIM test was performed using Past 4.05 (Hammer et al., 2001). Otherwise, all statistical tests (Welch's *t*-test, Mann-Whitney and Spearman's rank correlation) were performed with GraphPad Prism 8.0.0 (GraphPad Software, San Diego California United States of America, www.graphpad.com).

Results

Weight comparison between the Cobb and Legacy breeder dams

While marketing age poultry of modern and Legacy lines are very different in size, breeders are kept on a strict diet as to avoid obesity which will negatively affect their laying ability (Zuidhof et al., 2017). To quantify the weight differences between breeders of both lines we weighed the birds before sampling their microbiota. Indeed, on week 37 Cobb dams were 34% heavier than Legacy dams (Welch's *t*-test $p < 0.0001$; Figure 1).

Diversity analysis of the microbiome of Cobb and Legacy dams

To compare the gut community profile of the two lines, we collected samples of cecum contents and performed 16S rRNA gene sequencing to characterize the bacterial community. A comparison of the number of observed ASVs between Cobb and Legacy dams showed similar richness in the cecum communities (Mann-Whitney test $p > 0.05$; Figure 2A). An analysis of Shannon diversity, also integrating evenness measures, revealed the same trend (Mann-Whitney test $p > 0.05$; Figure 2B). Thus, regarding alpha-diversity measures of richness and evenness the cecum communities of the two lines are similar.

Dissimilarity analysis of the microbiome of Cobb and Legacy dams

Dissimilarity analysis utilizing Jaccard index showed a significant difference in the cecum communities of Cobb and Legacy dams (ANOSIM $p = 0.0077$; Figure 3A). A similar analysis utilizing Bray-Curtis index showed a difference with a greater statistical significance (ANOSIM $p = 0.0001$; Figure 3B). As Jaccard index is based solely on presence and absence of specific ASVs, whereas Bray-Curtis also integrates relative abundance data, this implies that differences between the cecum communities of Cobb and Legacy dams are based on both the ability of specific strains to colonize the different lines as well as their ability to grow to large numbers and perhaps compete with other parts of the microbial community.

Composition analysis of the cecum microbiome of Cobb and Legacy dams

An analysis of the cecum communities at the order level revealed, as expected, that the two most abundant orders in the cecum samples were Bacteroidales and Clostridiales for both dam lines (Figure 4). An analysis of differential abundance using ANCOM (Mandal et al., 2015) identified differences between Cobb and Legacy dams in all phylogenetic levels from phylum down, all of them belonging to the lineage of the genus *Akkermansia* (phylum Verrucomicrobia, class Verrucomicrobiae, order Verrucomicrobiales, family Verrucomicrobiaceae, and genus *Akkermansia*; Supplementary Data Sheet S1). *Akkermansia* was the only member in the Verrucomicrobiales order present in our dataset and accounted for 4.98% ± 5.04% of the microbiota in Cobb dams, while in Legacy dams it was absent in all but one individual (in which it had a relative abundance of 0.22%). This order was also significantly different by Mann-Whitney test ($p = 0.0002$). Other significantly different bacterial orders identified by Mann-Whitney test are Clostridiales ($p = 0.0133$), Lactobacillales ($p = 0.0172$) and Aeromonadales ($p =$

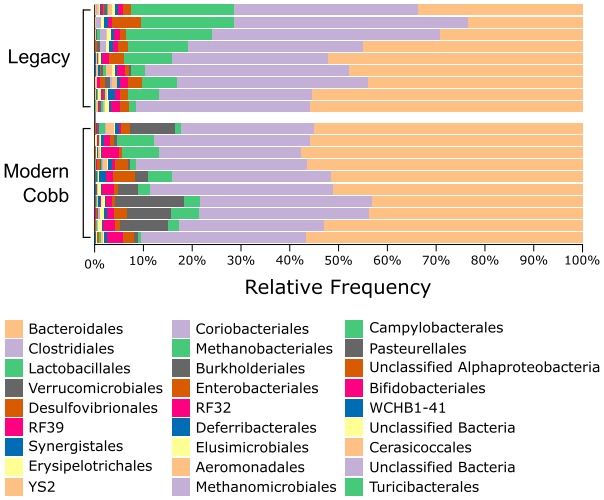

FIGURE 4
Taxonomic composition of Legacy line and Cobb line Cecal microbiome at the Order level.

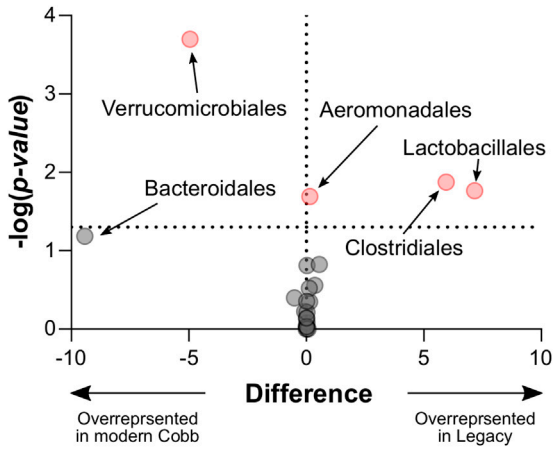

FIGURE 5
Volcano plot of Mann-Whitney results for all taxonomic orders. Significantly different orders are marked red. Legacy n = 9, Cobb n = 10.

0.0204), which are higher in Legacy dams (Figure 5; Figure 6). *Akkermansia* levels were significantly negatively correlated to Lactobacillales and Aeromonadales levels, and Lactobacillales levels were also found to be negatively correlated to Bacteroidales levels (Table 1).

Akkermansia incidence in sampled chicken communities

To understand the relevance of *Akkermansia* levels in Cobb dams, we studied the incidence of the genus *Akkermansia* in the ceca of other groups of chickens from a modern broiler line sampled over the last few years (Figure 7). *Akkermansia* was found in two other groups of adult broiler breeders that we previously sampled. In two groups of younger slaughter aged broilers, *Akkermansia* was not represented, i.e., no reads of ASVs annotated as *Akkermansia* were identified. Thus, adults of modern lines were colonized by *Akkermansia*, while adults of the Legacy line and younger modern broilers were not.

Discussion

To determine if breeding programs also modulated the gut microbiota, dams from the current Cobb commercial line, which targets high growth and meat yield, were compared with dams from a Legacy line that had not undergone targeted selection since 1986. Indeed, in the 35 years that have passed, the primarily growth targeted breeding program had a substantial effect on chicken size even under a feed restricted diet. This change in total weight is likely accompanied with multiple physiological changes that can affect gut microbiota community composition (Mitchell and Smith, 1991; Zuidhof et al., 2014; Tallentire et al., 2016).

To avoid confounding factors, we sampled Cobb and Legacy breeder dams that were housed together from day of hatch. This way, all dams were subjected to the exact same environmental conditions, including temperature, exposure to pathogens, feed (Havenstein et al., 2003), and handlers. When sampling age-matched animals that grow at different rates, any identified differences might be a result of the different developmental states. We avoided this confounder by sampling at an age when both breeds have reached peak laying performance and are well into adulthood. Last, by allowing the two lines to grow together for 37 weeks, we have allowed enough time to pass for multiple microbial cross contamination events between birds to occur. By removing these confounders, we have ensured that identified differences in microbiota composition are likely to be a result of gut environment differences due to differences in genetics.

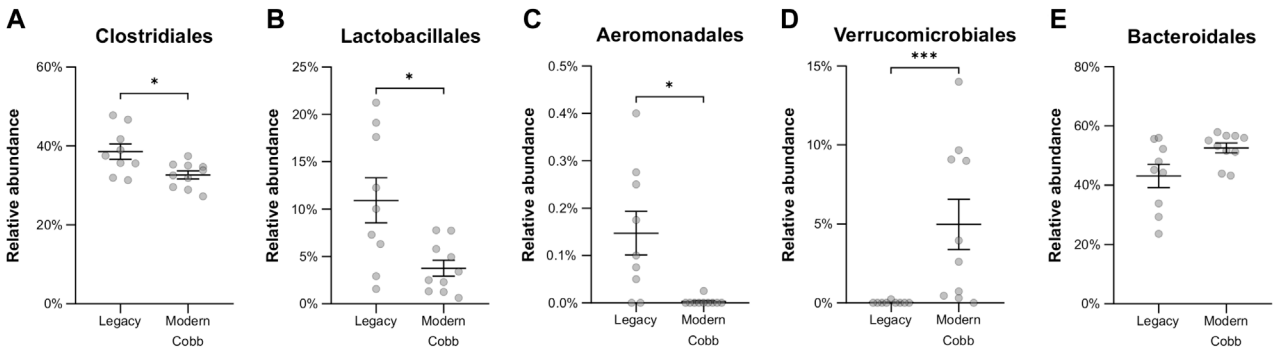

FIGURE 6
Relative abundance of orders that were significantly different between the Legacy and Cobb lines. **(A)** Clostridiales **(B)** Lactobacillales **(C)** Aeromonadales **(D)** Verrucomicrobiales **(E)** Bacteroidales. Data are presented as mean ± SEM. Legacy n = 9, Cobb n = 10; Mann-Whitney test; *$p \leq 0.05$, ***$p \leq 0.001$.

TABLE 1 Spearman Correlation between phylogenetic groups.

Group 1	Group 2	*p-value*	r value
Akkermansia	Lactobacillales	0.0039	−0.6287
	Aeromonadales	0.0302	−0.4976
	Clostrediales	0.1199	−0.3691
Bacteroidales	Lactobacillales	0.0124	−0.5614

The effects of breeding programs targeting growth on host physiology are still being assessed. Apart from the positive effects, including growth itself, a number of negative effects have been identified, including skeletal defects, metabolic disorders and altered immune function (reviewed by Zuidhof et al., 2014). Our results show a change in the cecum community composition between a current modern breeding line and the Legacy line. These results add to a previous report showing differences in the ileal bacterial community of a historic line and two commercial modern broiler lines (Lumpkins et al., 2010), and another report showing unique bacterial signatures for four different fast and slow growing broilers, including the ancestral Jungle Fowl (Emami et al., 2022). Thus, it can be concluded that genetic changes introduced during the breeding program resulted in a change in the gut bacterial community. These results raise an interesting question: are these genetic and physiologic differences between the two lines a direct result of a breeding program aimed at fast growth and meat yield, or did they happen by chance? The most prominent difference in the cecum community was that *Akkermansia* genus was a relatively high abundance member in the Cobb dams, while in the Legacy dams it was mostly absent (Figure 6). Interestingly, a high prevalence of *Akkermansia* was also found in Ross breeders (Figure 7). Assuming the breeding programs that gave rise to the current Cobb and Ross breeds are independent, these results might imply that *Akkermansia* in breeders is associated with fast growth and high meat yield phenotypes. Further research is required to establish this association.

Akkermansia bacteria are interesting as they are also found in humans and are studied as a future probiotic strain (Naito et al., 2018). These bacteria are mucin degraders and have been inversely correlated with metabolic disease in humans and mice (Everard et al., 2013; Naito et al., 2018). It was also correlated with high feed efficiency in layer chickens (Yan et al., 2017). Other bacterial orders which are different between Cobb and Legacy dams include Clostridiales, Lactobacillales, and Aeromonadales, which are more abundant in the ceca of Legacy dams. Higher levels of Lactobacillales in Legacy dams may result in reduced pH levels in the cecum, as these bacteria produce lactic acid which reduces the environment's pH (O'Hanlon et al., 2013). This environmental change may inhibit pH-sensitive *Akkermansia* bacteria (Van Herreweghen et al., 2017). This is supported by the negative correlation observed between Lactobacillales and *Akkermansia* levels. Last, we identified a relatively large variation in Lactobacillales levels in the Legacy breed (Figure 6). In an attempt to explain this variability, we also noted a large variability in Bacteroidales levels (Figure 6). Indeed, we found a negative correlation between the two (Table 1). This negative correlation was previously observed, and it was suggested that these groups have an overlapping ecological niche based on their encoded carbohydrate utilizing functions (Ma et al., 2020).

Our results show specifically that *Akkermansia* bacteria colonized Cobb but not Legacy dams. One hypothesis for this difference is that breeding programs select not only for host genetics but also for specific bacteria. If some bacteria are vertically transmitted between generations, perhaps by surviving in or on the egg and colonizing the chicks (Ding et al., 2017; Lee et al., 2019; Shterzer et al., 2020) genetic drift processes in these bacteria could result in divergent strains that are specifically adapted to the selected chicken line. Indeed, it is known that different mouse strains harbor different gut microbial communities (Jacobson et al., 2018). However, most of the bacterial composition between Cobb and Legacy dams was similar, implying this was not true for most bacterial strains. Intentional exposure of newly hatched chicks to *Akkermansia* resulted in colonization at high levels (Kubasova et al., 2019). However, *Akkermansia* were not found in young individuals sampled from other sites. It could be expected that if *Akkermansia* bacteria were common to Cobb because they were carried on or in eggs, they would flourish by marketing age. Therefore, the

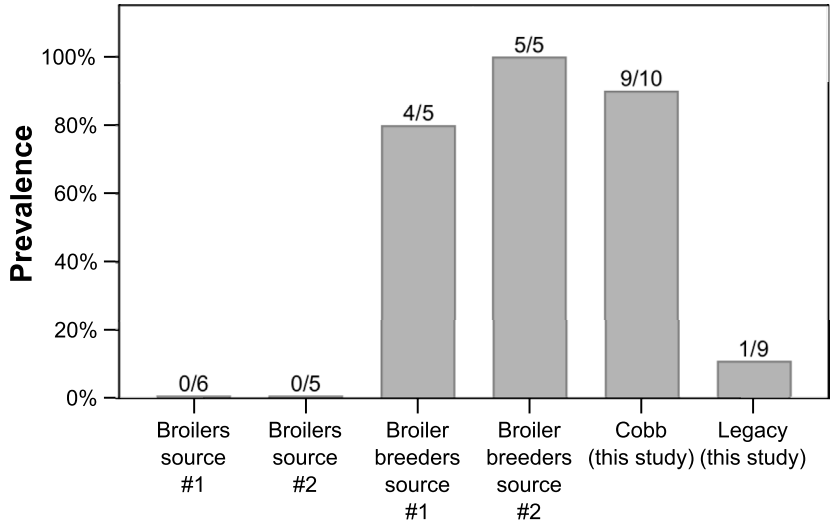

FIGURE 7
Prevalence of *Akkermansia* in previously sampled datasets. Data are presented as percentage, and the number of birds with *Akkermansia* out of total birds in each dataset is indicated above the bars.

differences we identified between Cobb and Legacy dams are not likely dependent on the origin facility or on vertical transmission, but on genetic differences between the lines. Moreover, any differences originating from origin facility likely disappeared through cross contamination, because both lines were housed in the same shed since day of hatch. This suggests the difference in *Akkermansia* colonization between Cobb and Legacy dams stems from physiological differences affecting the gut environment.

The identification of *Akkermansia* as differentially abundant between the two lines is of interest also because this group of organisms are known to degrade mucin. Theoretically, at least two options exist to explain this difference. One is that some of the genetic changes which have occurred during the growth directed breeding program regulate mucin secretion levels and/or composition. In this case, *Akkermansia* organisms might better colonize the industrial Cobb line because they find more suitable mucin in the cecum, which they can degrade and utilize as a nutrient source. The other option is that in the less feed-efficient Legacy dams, more nutrients pass the small intestine into the cecum, allowing the creation of a bacterial community which utilizes diverse nutrient sources. In comparison, the feed-efficient Cobb dams absorb most of the feed derived nutrients in the small intestine, leaving less feed derived nutrients to reach the cecum. In such an environment, mucin degraders feeding off cecum produced mucins will be more successful. In this scenario, host mucin genetics are likely not changed; rather genetic changes that affect feed-efficiency indirectly affect cecum composition. However, other mechanisms might also affect colonization of *Akkermansia* and other bacteria. For example, differences in metabolism causing changes in body temperature may affect colonization success (Tallentire et al., 2016). Thus, further research is needed to determine whether mucin levels or composition are indeed involved in the differential levels of *Akkermansia* between slow- and fast-growing lines.

The contribution of the gut microbiota to the mechanism of action of breeding programs is unknown. Our results show that cecum microbiota composition is different between the two groups of dams. As the cecum microbial community contributes to the digestion of nutritional fibers found in the feed that the chicken cannot digest by itself (Józefiak et al., 2004), it is possible that these differences contribute to the high feed efficiency of modern commercial breeds. However, it should be noted that we have identified these differences in mature dams and not in younger poultry, which are the main target of breeding programs. Furthermore, our results show that *Akkermansia*, which specifically colonize modern breeder dams, do not colonize modern broilers at least until marketing age. Therefore, it is unclear if the identified differences in the cecum bacterial community contribute to the fast growth of modern poultry lines. We have also shown that a major difference is that *Akkermansia* organisms colonize Cobb but not Legacy dams. Indeed, *Akkermansia muciniphila* has been correlated with better feed efficiency in layers (Yan et al., 2017). On the other hand, exposure of newly hatched chicks to *A. muciniphila* seems to have a minimal impact on growth (Zhu et al., 2020).

The identification of higher levels of *Akkermansia* in Cobb dams raises an interesting question: does *Akkermansia* cause Cobb dams to divert energy from egg to mucin production? If this is the case, strategies that will limit *Akkermansia* colonization might improve egg production.

Conclusion

In this study, we compared the composition of the gut microbiota of the current Cobb commercial breeder line and a Legacy line which has not undergone selection for 35 years. We were able to identify differences in the cecal bacterial community that were the result of genetic changes brought about by the broilers breeding programs. Specifically, Bacteria of the genus *Akkermansia* implicated in mucin degradation and associated with host metabolic health were a

prominent member of the Cobb breeders' cecum community, but were mostly absent from Legacy line dams. Inversely, Legacy dams had higher levels of Clostridiales, Lactobacillales and Aeromonadales. While we do not know if these differences also contribute to the fast growth of the current commercial line, by identifying these bacteria, we can now specifically target them for further study.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA899345, PRJNA936484, PRJNA885292.

Ethics statement

The animal study was reviewed and approved by the Hebrew University of Jerusalem's Ethics committee, approval No. AG-19-15897-3.

Author contributions

EM and SD designed the study. All authors contributed to performing the study. EM, NS, and SD wrote the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Pioneer colonizers: Bacteria that alter the chicken intestinal morphology and development of the microbiota

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Microbes commonly administered to chickens facilitate development of a beneficial microbiome that improves gut function, feed conversion and reduces pathogen colonization. Competitive exclusion products, derived from the cecal contents of hens and shown to reduce *Salmonella* colonization in chicks, possess important pioneer-colonizing bacteria needed for proper intestinal development and animal growth. We hypothesized that inoculation of these pioneer-colonizing bacteria to day of hatch chicks would enhance the development of their intestinal anatomy and microbiome. A competitive exclusion product was administered to broiler chickens, in their drinking water, at day of hatch, and its impact on intestinal morphometrics, intestinal microbiome, and production parameters, was assessed relative to a control, no treatment group. 16S rRNA gene, terminal restriction fragment length polymorphism (T-RFLP) was used to assess ileal community composition. The competitive exclusion product, administered on day of hatch, increased villus height, villus height/width ratio and goblet cell production ~1.25-fold and expression of enterocyte sugar transporters 1.25 to 1.5-fold in chickens at 3 days of age, compared to the control group. As a next step, chicks were inoculated with a defined formulation, containing *Bacteroidia* and *Clostridia* representing pioneer-colonizing bacteria of the two major bacterial phyla present in the competitive exclusion product. The defined formulation, containing both groups of bacteria, were shown, dependent on age, to improve villus height (jejunum: 1.14 to 1.46-fold; ileum: 1.17-fold), goblet cell numbers (ileum 1.32 to 2.51-fold), and feed efficiency (1.18-fold, day 1) while decreasing *Lactobacillus* ileal abundance by one-third to half in birds at 16 and 42 days of age, respectively; compared to the phosphate buffered saline treatment group. Therefore, specific probiotic formulations containing pioneer colonizing species can provide benefits in intestinal development, feed efficiency and body weight gain.

KEYWORDS

microbiome, performance, feed efficiency, intestinal development, anaerobes

Introduction

The microbiome has been shown to serve as an effective barrier to pathogen colonization or pathogenic behavior in numerous examples while the mechanisms underlying pathogen exclusion remains elusive (Nurmi and Rantala, 1973; Berg, 1980; Faure et al., 1984). Approximately 50 years ago, Nurmi demonstrated that chicks seeded with the cecal microbiome from adult birds were resistant to *Salmonella* colonization (Nurmi and Rantala, 1973) and termed the phenomena "competitive exclusion". Since this discovery, numerous groups have investigated single or multiple microbial species as probiotics to replace the effectiveness of growth-promoting antibiotics (Vuong et al., 2016) or suppress other harmful microorganisms (Fukata et al., 1991; Hofacre et al., 1998a). However, no defined consortium has been quite as effective at pathogen exclusion as Nurmi's approach using the cecal microbiome. Competitive exclusion has since been commercialized; amplifying cecal bacteria from an original seed stock and distributing lyophilized cultures with >5 log10 *Salmonella* reduction to customers, marketed as a *Salmonella* exclusion product for poultry (Lee et al., 2023). Hofacre et al. demonstrated that administration of this competitive exclusion product could also reduce the severity of necrotic enteritis in poultry (Hofacre et al., 1998a). The study, which was replicated in 2019 with conditions intended to increase the severity of disease (Hofacre et al., 2019), illustrated an important new concept in disease control. The chicks were given one dose at day of hatch, then challenged with three sequential high oral doses of an avian pathogenic *C. perfringens* isolate 3 weeks later. The findings suggested a paradigm shift because the principle of competitive exclusion was inadequate to explain the chicks' resistance to repeated doses of a billion pathogenic, toxigenic *Clostridium perfringens* cells administered orally for 3 days in a row 3 weeks after receiving the intestinal bioproducts. In fact, a subsequent study showed that one dose of the intestinal bioproduct, Aviguard®, performed as well as continuously feeding bacitracin or virginiamycin to prevent necrotic enteritis (Hofacre et al., 1998b). These findings indicated that competitive exclusion products could alter the intestinal environment leading to greater resistance to enteropathogens.

Understanding the microbiome, a consortium of microbes found in and on animal and plant species, offers a new perspective on eukaryotic development. An understanding of the nutritional and physiological contributions of the monogastric vertebrate intestinal microbiome is still emerging. The role of the microbiome in disease, physiology or development is often inferred from studies using "germ-free" subjects *versus* "conventionally-raised" litter mates. Monogastrics can be raised "germ-free" (gnotobiotic) (Pleasants, 1959; Meyer et al., 1964) however, gnotobiotic mice, pigs and rats exhibit reduced growth and weight gain compared to conventionally raised litter mates (Dymsza et al., 1965; Waxler and Drees, 1972; Al-Asmakh and Zadjali, 2015). Gnotobiotic animals are also more susceptible to enteric infections which makes them an excellent model for studying enteric pathogens (Sprinz et al., 1961; Eaton et al., 2008; Reeves et al., 2012). Augmentation with certain bacterial species has been shown have a profound effect on the intestinal physiology, growth, and disease resistance of gnotobiotic animals (Dymsza et al., 1965; Shirkey et al., 2006; Mahowald et al., 2009; Cheled-Shoval et al., 2014; Greig et al., 2018; Yin et al., 2022). Furthermore, the microbiome composition can have a profound impact on animal weight gain as evident when gnotobiotic mice receive fecal transplants from obese mice (Turnbaugh et al., 2006).

In comparisons with other germ-free animal models, chickens possessing an intestinal microbiota were believed to be at a growth disadvantage except when grown on vitamin-deficient diets (Coates et al., 1968) or those with high fiber/low metabolizable energy (Muramatsu et al., 1991). Gnotobiotic-chickens produce fewer goblet cells, more sulfated mucins (Cheled-Shoval et al., 2014), shorter villi, and lower crypt depth, compared to conventionally-raised birds (Cowieson, 2022). However, weight gain and feed conversion improved when chickens were raised with antimicrobial-amended feed but this was not observed in germ free animals (Lev and Forbes, 1959). Furthermore, it was most pronounced in birds raised in heavily contaminated environments indicating that the growth disadvantage was likely the result of pathogens. Therefore, in commercial poultry production, antibiotics such as virginiamycin and bacitracin improved growth performance on farms with high stocking density or poor hygienic conditions or practices (Eyssen and de Somer, 1963). Because this improvement was believed to be due to the suppression of pathogenic intestinal bacterial species such as *C. perfringens*, it led to the widespread use of antibiotics, in the U.S., as a prophylactic to prevent necrotic enteritis in poultry. This practice has been in decline since the European ban of growth-promoting antibiotics (Casewell et al., 2003) and the movement in the U.S. towards antibiotic free production (Diaz-Sanchez et al., 2015).

The maternal intestinal microbiome is an important source of organisms for the progeny and many studies have shown that the initial microbiome seeding is crucial for health of the progeny (Neu and Rushing, 2011; Koleva et al., 2015; Chen et al., 2018; Kubasova et al., 2019a; Klein-Jöbstl et al., 2019; Treichel et al., 2019; Yu et al., 2019). The modern poultry production system, in order to increase productivity and reduce disease transmission, eliminated the physical presence of the progenitors during the incubation and hatching process thereby interrupting the transfer of bacteria from hen to chick. As a result, newly hatched chicks do not have access to a diverse maternal microbiome are easily seeded with environmental microbes (Pedroso et al., 2015) and these organisms would not be expected to provide beneficial effects in early intestinal development or pathogen exclusion. Yet, there is a consistent and predictable microbial succession within the chicken intestine, beginning with oxygen-consuming streptococci and γ-proteobacteria at 4 days of age, followed by their displacement with the obligate anaerobic *Clostridia* (Lu et al., 2003; Jurburg et al., 2019). However, if chicks are presented early with cecal microbiome, they develop a stable community resistant to pathogen colonization (Ramírez et al., 2020). Because the microbiome affects animal physiology (Cheled-Shoval et al., 2014; Heath-Heckman et al., 2014; Kremer et al., 2014; McFall-Ngai, 2014), this would be especially evident in early intestinal development as the host responds to early pioneer colonizers (Shao et al., 2014; De Maesschalck et al., 2015; Gourbeyre et al., 2015).

The ecological concept of pioneer colonizers is well established and has been shown to be crucial in augmenting development of a functionally diverse intestinal microbiome. In 2005, Backhed et al. conceptualized the process by which pioneer colonizers coevolve

with their animal hosts and influence the intestinal environment from a nutritional and anatomical standpoint (Backhed et al., 2005). Using gnotobiotic mice, they demonstrated that the developmental deficiencies associated with the absence of an intestinal microbiome, could be fully mitigated by administering a single *Bacteroides thetaiotaomicron* species. Subsequent studies illustrated that stem cell differentiation was stimulated by bacterial metabolites from utilization of host intestinal mucin (Sommer and Backhed, 2013). These findings indicated that probiotic formulations, containing pioneer colonizers from the intestinal microbiota of mature chickens, may accelerate intestinal development and improve performance in newly hatched chicks.

In this study, we treated day-of-hatch broiler chickens with a competitive exclusion product and investigated the impact of the treatments on intestinal community structure, as measured by 16 S rRNA gene terminal restriction fragment length polymorphism (T-RFLP), intestinal morphometrics (villus height, villus height to width ratio, goblet cell numbers), body weight gain and feed conversion ratios; compared to the no treatment, control group. As a second step, we selected specific species from chicken ceca, that represent the two dominant phyla in the competitive exclusion product Aviguard® (Lee et al., 2023), and administered different formulations, consisting of these cecal organism, or PBS to newly hatched chicks in order to determine their effects on production performance, intestinal physiology and changes to the intestinal microbiome, relative to the PBS treatment group. Similar morphometric improvements to chicken gut function could be obtained with a two to five species, probiotic cocktail, consisting of obligate anaerobic pioneer colonizers, than a competitive exclusion product that consists of 20–50 distinct genera (Lee et al., 2023).

Materials and methods

16S rRNA gene analysis of intestinal communities from chickens receiving a competitive exclusion product, probiotic formulation, or PBS

In order to recover bacteria from the commercial competitive exclusion product, the bacterial cells were rehydrated by incubation for 10 min in saline solution and recovered by centrifugation. Chicken intestines were collected from the various treatments and time points outlined below. The bacterial fraction was recovered from the intestinal contents through multiple rounds of differential centrifugation as described previously (Apajalahti et al., 1998; Lu et al., 2003). DNA was extracted using Mo Bio kit (Mo Bio Laboratories Inc., Solana Beach, CA), beating cell suspensions at 6,000 rpm for 20 min (Lu et al., 2008). The bacterial communities were assayed by 16 S rRNA terminal restriction fragment length polymorphism (T-RFLP) analysis, using a sequence-based database, as previously described (Lu et al., 2006; Lu et al., 2008). universal 16S rRNA primers 8F labeled with 5′FAM (carboxyfluorescein-N-hydroxysuccinimide ester-dimethyl sulfoxide) and unlabeled 1429R were used to amplify community DNA (Lu et al., 2008). Three separate 18-cycle PCR reactions were performed for each DNA sample and pooled for T-RFLP analysis. No DNA template was included with PCR, as a negative control. No amplicons were ever observed for this negative control. Amplicons were digested with restriction enzyme *Hae*III (New England BioLabs; Ipswich, MA) and analyzed by electrophoresis on ABI PRISM 310 DNA sequencer (PE Biosystems; Foster City, CA). For each sample, only peak areas and peak heights over a threshold of 50 units, above background were analyzed by manually aligning fragments to size standards; and only DNA fragments between 35 and 525 bp were examined. T-RFL peaks were identified by comparison to a 16S rRNA gene database, of Insilco *Hae*III patterns, from previously published clone libraries (Lu et al., 2003).

The relative abundance of bacterial species or phylotypes detected by T-RFLP was determined by calculating the ratio between the areas of each peak and the total areas of all peaks within one sample (Lukow et al., 2000); mean ratios of three analyses were converted to percentages. The Shannon diversity information index (Shannon and Wiener, 1963) was used to evaluate the diversity of the bacterial communities. The diversity indices were analyzed using analysis of variance (SAS, 2008) to determine differences between the intestinal communities from birds given Aviguard® or nothing (no treatment control group).

Isolation of pioneer colonizing bacteria from the chicken intestine

Aviguard®, consists predominantly of obligate anaerobes (Pedroso et al., 2015; Lee et al., 2023), belonging to *Clostridia* and *Bacteroidia* orders and because it improved intestinal morphometrics in young birds, we sought to isolate and identify pioneer colonizing species that could supplant this competitive exclusion product. Anaerobic, pioneer colonizing bacterial were isolated from the ceca of commercial broiler chicken carcasses obtained from a local processing plant. The cecal contents of three chickens were squeezed into pre-reduced serum bottles and serially diluted with 20 ml of phosphate-buffered saline (PBS) in within an anaerobic chamber containing 90% N_2 and 10% H_2. The suspensions were plated on rumen fluid-glucose-cellobiose plus peptone (RGCAP)-10, RGCAP-30, 10% modified rumen fluid medium (M98-5), and rich medium (RM) (ATCC Medium 1,341; 20 g glucose, 10 g yeast extract, 2 g K_2HPO_4, 15 g agar per 1L dH2O) agar (Kelley, 1983), and incubated under 95% N_2 and 5% H_2 for 5 days at 41°C. Isolated colonies were characterized by 16 S rDNA sequencing as previously described (Lu et al., 2003). Selected isolates of *Escherichia coli*, *Parabacteroides distasonis*, *Bacteroides salyersiae*, *Phocaeicola dorei* and *Romboutsia lituseburensis* ATCC 25759 were grown on RGCAP-10 agar under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) for 5 days. Colonies were harvested and resuspended in pre-reduced saline solution to reach the concentration of 10^9 CFU/ml.

Obligate anaerobes were isolated by culture and identified by 16S rRNA amplicon sequencing. Subculture yielded obligate anaerobes belonging to the order *Bacteroidia* which were identified as *P. distasonis*, *B. salyersiae*, and *P. dorei* (formerly, *Bacteroides dorei*). Partial sequence of their genomes revealed polysaccharide utilization loci and associated glycosyl hydrolases (Grondin et al., 2017) characteristic of the *Bacteroidia*. DNA

TABLE 1 Cecal bacteria used to formulate probiotics described in this study.

Group	Species	Features	Identity[i]	Source
Bacteroidia	*Parabacteroides distasonis*	*susC,D* polysaccharide utilization loci[b], associated glycosyl hydrolases (11), core saccharidases[c] and additional enzymes annotated as: arabinogalactan endo-1,4-β-galactosidase, α–mannosidase, α-glucosidase, *ß*-glucanase 2), α-1,6-mannanase, mannan endo-1,4-β-mannosidase; conjugative transposon; respiratory hydrogenases and cytochromes; propionate metabolism[d]; acetyl-CoA hydrolase	16S, 23 S rRNA, *gyrA*, *susC,D* loci, Supplemental Files[e]	Chicken cecum[f]; RGCAP-30 medium[g]
	Bacteroides salyersiae	*susC,D* polysaccharide utilization loci[b], associated glycosyl hydrolases, core saccharidases[c] and additional enzymes annotated as: arabinosidase, α-glucosidase, rhamnogalacturonan lyase, α–mannosidase pectate lyase, α-1,6-mannanase, α-glucosidase; propionate metabolism[d] including propionyl-CoA carboxylase, butyrate metabolism[h]	23 S rRNA, *susC,D* loci, ATP synthase subunit, Supplemental Files[e]	Chicken cecum[f]; RGCAP-30 medium[g]
	Phocaeicola dorei	*susC,D* polysaccharide utilization loci[b], associated glycosyl hydrolases, core saccharidases[c] and additional enzymes annotated as: arabinosidase, arabinogalactan endo-1,4-β-galactosidase, rhamnogalacturonan lyase, pectate lyase; propionate metabolism[d] including propionyl-CoA carboxylase, butyrate metabolism[h], acetyl-CoA hydrolase	23 S rRNA, *susC,D* loci, ATP synthase subunit, Supplemental Files[e]	Chicken cecum[f]; RGCAP-30 medium[g]
Clostridia	*Romboutsia lituseburensis*[a]	Glycosyl or glycoside hydrolases (17) including: α or *ß*-glucosidase, *ß*-galactosidase, α-mannosidase; butyrate metabolism[h], lactate dehydrogenase, formate dehydrogenase, acetyl-CoA decarbonylase/synthase complex, and Fe-Fe hydrogenases; ethanolamine utilization; vitamin B12 synthesis; flagella/motility; sporulation	NA	ATCC 25759
γ-Proteobacteria	*Escherichia coli*	Aerobic/anaerobic respiration including hydrogenases associated with H_2 consumption; enzymes and transporters associated with di- and mono-saccharide metabolism	16S, 23 S rRNA, *gyrA*, respiratory hydrogenases *hyfJ*, *hybF*, nitrate reductase *napF*; Supplemental Files[e]	Chicken cecum[f]

[a]Features inferred from the annotated genome for *Romboutsia lituseburensis* DSM, 297 (NCBI, RefSeq: NZ_FNGW00000000.1).
[b]*susC,D*: signature polysaccharide transport proteins commonly associated with polysaccharide utilization in *Bacteroidia* (Grondin et al., 2017). Other genes associated with these loci include: glycosyl hydrolases and regulatory genes: membrane sensor, alternate sigma factor and anti-sigma factor. These same ancillary genes have also been reported as part of polysaccharide utilization loci in other *Bacteroidia* genomes (Grondin et al., 2017).
[c]Fucosidase, glucoamylase, α-amylase, neopullulanase (*susA*), sialidase, arabinofuranosidase, polygalacturonase, *ß*-galactosidase, *ß*-glucosidase, α-1,2 mannosidase, *ß*-mannosidase, *ß*-hexosaminidase, α-rhamnosidase, maltodextrin glucosidase, *ß*-xylosidase.
[d]Methylmalonyl-CoA, mutase, methylmalonyl-CoA decarboxylase, methylmalonyl-CoA, epimerase.
[e]"Supplemental Excel Files: *Clostridia Bacteroidia* Probiotic Formulation Sample one and 2.
[f]Bacteria were isolated from cecal contents of chicken carcasses collected at a poultry processing plant.
[g]Reference: (Kelley, 1983).
[h]Phosphotransbutyrylase, butyrate kinase.
[i]Identity confirmed at nucleotide level by BLAST (Altschul et al., 1990) with 100% query coverage and 98%–100% identity.

sequence of these genes, 16S rRNA, and other housekeeping genes confirmed their identity to the species level (BLAST scores: ≥98% nucleotide identity; 100% coverage) (Table 1). The *Bacteroidia* genomes exhibited several annotated genes for acetate and propionate metabolism. *Bacteroides salyersiae* and *P. dorei* also possessed genes annotated as phosphotransbutyrylase and butyrate kinase, responsible for butyrate production. These genes were absent in a search of the isolated *P. distasonis'* genome as well as a search of published, annotated *P. distasonis* genomes, including a specified BLAST search, at the amino acid level. While these *Bacteroidia* contained core carbohydrate-active enzymes (CAZymes), there were differences in the distribution of other CAZymes among these isolates. Because of the variances in carbohydrate and fermentation metabolism, it was decided to include multiple species as part of a *Bacteroidia* cocktail to administer to birds.

As *R. lituseburensis* was an abundant phylotype in birds fed the competitive exclusion product Aviguard® or other feed additives (Lu et al., 2008), an *R. lituseburensis* isolate was purchased from the American Type Culture Collection (ATCC 25759) to be included in this study. In addition, an *E. coli* isolated from the chicken intestinal samples, served as a γ-proteobacteria pioneer for establishing the anaerobic environment needed for seeding chicks with obligate anaerobes (Espey, 2013).

Pools of isolates were created by mixing equal volumes of suspensions. Three pools of probiotic cultures were prepared for administration to day of hatch chicks and consisted of the following formulations; probiotic cocktail 1: *P. distasonis*, *B. salyersiae*, and *P. dorei*, and *E. coli*; probiotic cocktail 2: *R. lituseburensis* and *E. coli*; and probiotic cocktail three containing *P. distasonis*, *B. salyersiae*, *P. dorei*, *R. lituseburensis and E. coli*. Glycerol (15%) was added to aliquots of each probiotic formulation and stored at −80°C.

Birds treated with competitive exclusion product

For assessment of the effects of the commercial competitive exclusion product (Aviguard®, Lallemand, Montreal Canada), 120 one-day-old commercial Ross-Cobb hybrid broiler chicks

were raised in two groups of 60 on sawdust bedding. Both groups were fed a commercial corn-soy bean meal diet devoid of antimicrobials. Chicks in one group were administered the commercial competitive exclusion product, Aviguard® in their drinking water on the day of hatch, as per the manufacturer's instructions, while the other group just received standard drinking water, no product. Birds were sacrificed, by cervical dislocation, at 3, 7, 14, 21, 28, and 49 days of age and intestines were collected. The ileal contents were collected and processed as previously described for 16S rRNA gene TRFLP analysis. Intestinal morphometrics and glucose transporter gene expression were performed on intestines collected from birds at 3 days of age, as described below.

Birds treated with probiotic cocktails

Eight hundred and 40 day of hatch chicks (Cobb 500) were divided into four treatments of three replications each containing 70 chicks. Chicks were orally inoculated with 50 µl of 1×10^8 *Bacteroidia* cocktail (*P. distasonis*, *B. salyersiae*, and *P. dorei*) with *E. coli, R. lituseburensis* with *E. coli,* and *Bacteroidia* cocktail with *R. lituseburensis and E. coli.* The control group received 50 µl sterile PBS. Chickens were fed a corn-soy bean meal diet free of antimicrobials (Table 1). Birds were sacrificed by cervical dislocation 3 hours following oral administration with probiotic formulation or PBS and at days 1, 2, 3, 7, 16 and 42 days and intestines were collected.

Intestinal histology and morphometrics

Following inoculation with the competitive exclusion product Aviguard® (n = 60), probiotic formulation (3 different formulations, 210 birds per treatment) or PBS (n = 210), chickens were sacrificed at 3 hours after inoculation, or at time points described above and the small intestines were collected. A no treatment group (n = 60) was included with the competitive exclusion trial. The middle portion of the jejunum and ileum from 4 birds per experimental unit were excised, fixed in 10% formalin, embedded in paraffin and cut in five um thick sections. Three intact, well-oriented villi were selected in eight replicates for each intestinal cross section, totaling 24 villus height and width measurements for each intestinal sample and 288 measurements per treatment. In addition, intestinal sections were stained using Mayer's Mucicarmine (Val-Bernal et al., 1999) and the number of goblet cells counted. Morphological indices were determined using a light microscope and a ×16 magnification lens. Images were analysis using the Image-Pro Plus Version 3.0 software (Media Cybernetics, Silver spring, MD). Expression of glucose transporters were measured by reverse-transcriptase (RT) qPCR according to method described by Gilbert et al. (2007).

The jejunum and ileum from four animals per treatment group (three probiotic cocktails and PBS control) were collected, measured, flushed using deionized water, and the empty weight recorded. Relative intestinal weight (grams/kg of body weight) and relative intestinal lengths (mm/kg of body weight) were determined.

Animal performance

Body weight and feed intake were recorded and body weight gain and gain: feed were calculated. At the occurrence of mortality, feed intake was adjusted based on bird days on feed. At 42 days of age, fifteen chickens per pen were randomly selected and wing-banded and fasted overnight. Birds were weighed individually, slaughtered, eviscerated, and carcasses were chilled for 12 h. The yield was obtained for the entire carcass, and parts.

Whole genome sequencing and genomic analyses of *Parabacteroides distasonis, Bacteroides salyersiae, Phocaeicola dorei, Escherichia coli* and *Romboutsia lituseburensis* probiotic cocktail

Two samples were thawed on ice, centrifuged at 10,000xg at 4°C for 15 min, and DNA was extracted from the bacterial pellet using Promega Wizard® Genomic DNA extraction kit (Madison, WI), with an added lysozyme treatment, as described by the manufacturer. DNA was submitted to Georgia Genomics and Bioinformatics Core for sequencing using Illumina sequencing (San Diego, CA). FastQC and FastQ/A were used to clean raw sequence reads of adapters and low-quality sequences (Patel and Jain, 2012; Afgan et al., 2018). SPADES sequence alignment tool was used to assemble processed pair-end Illumina reads (Bankevich et al., 2012). Assembled sequence files were uploaded and annotated in the Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008). Species identity of individual contigs (≥17 kb) was determined by Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) at nucleotide level (≥98% identity). Identity of species, within this probiotic formulation, was confirmed by BLAST for sequences annotated as: "small ribosomal subunit RNA" (16 S rRNA); "large ribosomal subunit RNA (23S rRNA); gene annotated as "SusC", "SusD", polysaccharide utilization genes commonly present in the *Bacteroidia* (Grondin et al., 2017); or housekeeping genes listed in Table 1 (≥98% identity, 100% coverage). The *Bacteroidia* are adept at metabolizing complex carbohydrates, whether its indigestible fiber from the animal's diet or mucin, and producing volatile fatty acid from said metabolism for its host. As multiple *Bacteroidia* species were identified, a more detailed genomic analysis was performed to determine which isolates to include in the probiotic formulation that had the broadest repertoire of carbohydrate metabolism. Carbohydrate-active enzymes (CAZymes) were identified among the annotated sequences through a word search for genes annotated as "Sus", "glycosyl hydrolases", "amylase", "pullanase" or "idase"; and species identity and enzyme confirmation was determined by BLAST at the nucleotide and amino acid level, respectively. CAZymes identified had motifs consistent with these enzymes at the amino acid level. In *Bacteroidia*, CAZymes are often associated with Polysaccharide Utilization Loci (PUL) denoted by polysaccharide transporters Sus (Grondin et al., 2017). Several loci were identified with Sus minus any genes annotated as some CAZyme. Genes annotated as "hypothetical protein" were identified as CAZyme *via* BLASTX search of annotated bacterial genomes. Fermentation enzymes were identified by similar word search of

TABLE 2 Ileal morphology of broiler chicks at 3 days of age that were administered an intestinal bioproduct Aviguard®.

	Villus height (µm)	Height/width ratio	Crypt area (µm²)	Mucosal layer width (µm)	Numbers of goblet cells per villus
Control	362.73[b]	2.58[b]	6,735.60[a]	141.94[a]	8.24[b]
Aviguard®	455.92[a]	3.52[a]	6,946.10[a]	155.53[a]	10.96[a]

Different superscripts within each column indicate significant differences, $p < 0.05$.

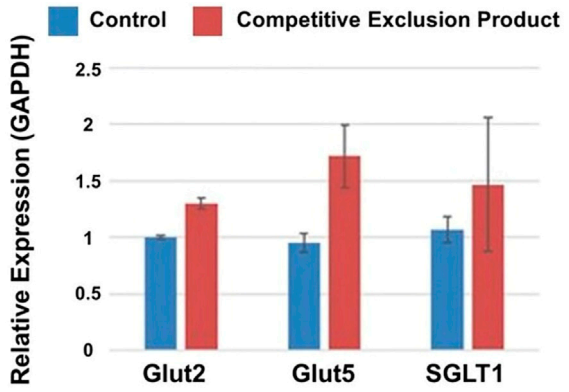

FIGURE 1
Relative expression of glucose transporters Glut2, Glut5 and SGLT1 ($p < 0.05$) in the small intestine of 3 d old broiler chickens receiving a competitive exclusion product (Aviguard®) or no treatment (control) on the day of hatch. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method. GAPDH was used to normalize gene expression for targeted genes.

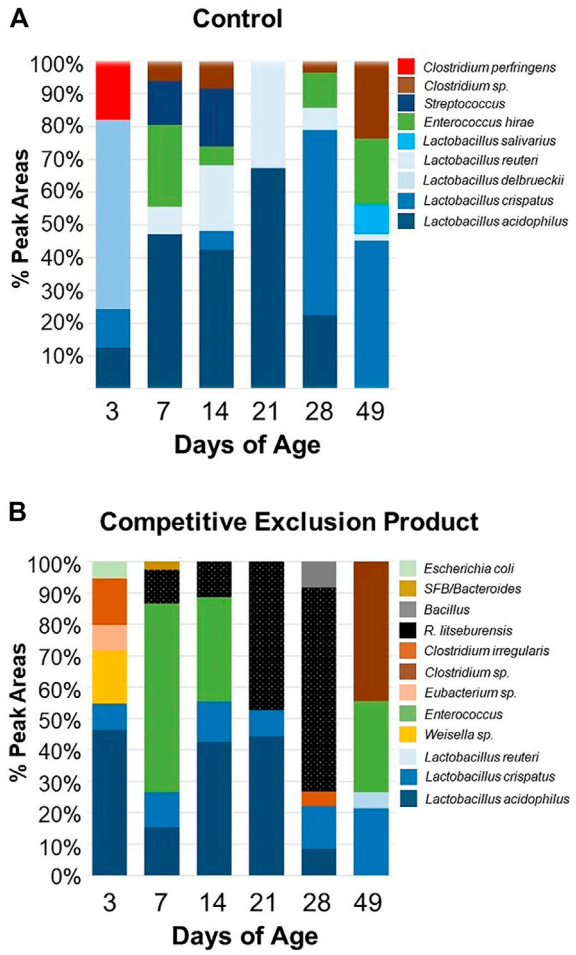

FIGURE 2
Composition of the ileal bacterial community of chicks administered Aviguard® (panel **(B)**) or no treatment (control, Panel **(A)** on day of hatch as determined by 16S rRNA T-RFLP at 3, 7, 14, 21, 28 and 49 days of age.

gene annotations for enzymes listed in Table 1. Their identity and species assignment were determined by BLAST search at the amino acid and nucleotide level, respectively.

Statistical analysis

Performance and intestinal measurements were subjected to Analysis of Variance (ANOVA) procedure for completely randomized design using the general linear model procedure of SAS (SAS, 2008). Statistical significance of differences among treatments was assessed using the least significant difference test (Steel and Torrie, 1980). A probability level of $p < 0.05$ was used to determine statistical significance. The Standard Error of Measure (SEM) was calculated from the standard deviation of all values divided by the square-root of the sample size.

Results

Competitive exclusion product improves intestinal morphology and enterocyte function

While competitive exclusion products have been shown effective at pathogen exclusion, can this microbial consortium, of chicken intestinal origin, also effectively modulate intestinal morphology and function? To address this question, birds were either administered the competitive exclusion product, Aviguard® at day of hatch, in their drinking water or not (untreated, control group). Administration of Aviguard® improved intestinal morphology, increasing villus height, height/width ratio; and percentage of goblet cells per villus 1.26 to 1.36-fold, $p < 0.05$ (Table 2), compared to untreated birds. Furthermore, increased expression 1.25 to 1.5-fold of the enterocyte transporters, GLUT2, GLUT5, and

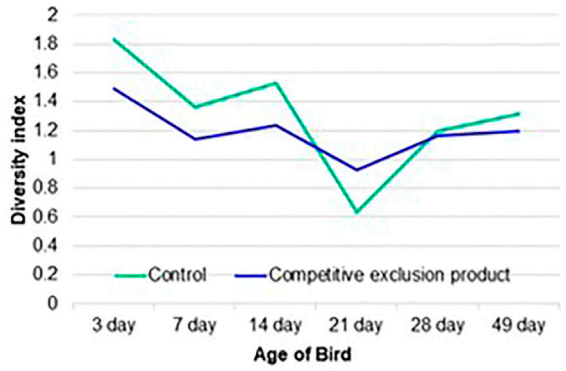

FIGURE 3
Shannon's H diversity index of samples collected from the ileal bacterial community of chicken from the control (no treatment) or birds administered a competitive exclusion product (Aviguard®) at day of hatch. Samples were collected at 3, 7, 14, 21, 28 and 49 days of age.

SGLT1 was exhibited in the ileum compared to control group in 3-day-old broiler chickens (Figure 1; $p < 0.05$).

Competitive exclusion product stabilizes community diversity and promotes clostridia abundance in the chicken ileum

Figure 2 and Figure 3 illustrate the composition and successions of bacteria in the microbiome in response to administration of Aviguard® to birds at day of hatch. There were differences in the succession of bacterial phylotypes over the 49-day period between the control and birds administered the competitive exclusion product, Aviguard® (Supplementary Figure S1). There were also significant differences in the distribution of phylotypes between the control and birds administered the competitive exclusion product, especially evident were differences in *Lactobacillus crispatus* and *R. lituseburensis* (*Clostridia*) abundance. This was most pronounced in birds at 21 days of age and older (Figure 2). *Lactobacillus* species were the most abundant group in untreated birds (Figure 2, Supplementary Figure S1), while the abundance of other species varied. However, administration of Aviguard® produced an ileal bacterial community in which the *Clostridia* were abundant at 3 days of age while *Enterococcus* phylotypes represented 60% of total phylotypes on day 7 (Figure 2, Supplementary Figure S1). But SFB/*Bacteroides* phylotypes emerged with *R. lituseburensis* day 7 with *Romboutsia* becoming the most abundant ileal species by day 28 representing 70% of the total phylotypes for the treatment group. This observation suggested that *Bacteroides* may act as a pioneer colonizer in chicks enabling successional colonization of poultry anaerobic bacteria.

The Shannon diversity index indicated that age-related instability in the diversity of the ileal communities could be reduced by Aviguard® (Figure 3). There were significant differences between the control and treatment groups at all ages analyzed ($p < 0.05$). At 21 days of age, there was a distinct reduction in diversity which was most pronounced for the control group. Aviguard® administration lowered diversity but provided stability compared to the dramatic shifts in control birds.

Pioneer colonizers promote intestinal function and growth performance

Newly hatched chicks inoculated with *R. lituseburensis and E. coli cocktail* had the greatest (15.8 vs 14.7 g; $p < 0.05$), body weight gain 24 h following its administration (Table 3), relative to the PBS control. Similarly, the feed: gain ratio was improved 1.18-fold relative to PBS control (1,408 vs. 1,198 kg/g; $p < 0.05$) in chicks receiving the *P. distasonis*, *B. salyersiae*, *P. dorei*, *E. coli* and *R. lituseburensis* cocktail. In addition, *R. lituseburensis* and *E. coli* cocktail or the *P. distasonis*, *B. salyersiae*, *P. dorei*, *E. coli* and *R. lituseburensis* cocktail produced a higher body weight gain at 16 days of age (438 or 421 vs. 411; $p < 0.05$). Probiotic cocktails consisting of *R. lituseburensis* and *E. coli* or *P. distasonis*, *B. salyersiae*, *P. dorei*, *E. coli* and *R. lituseburensis* reduced body weight gains by 4% at the end of the rearing period, compared to birds administered PBS or the *P. distasonis*, *B. salyersiae*, *P. dorei*, and *E. coli* cocktail. However, birds administered *R. lituseburensis* and *E. coli* cocktail had higher carcass yield (Table 4; 76.1% vs 73.7%, $p < 0.05$). No differences were observed on the legs, thighs, wings and breast yield for either

TABLE 3 Body weight gain and feed efficiency of birds inoculated with *Parabacteroides distasonis*, *Bacteroides salyersiae*, *Phocaeicola dorei*, and *Escherichia coli* (Cocktail 1); *Romboutsia lituseburensis* and *E. coli* (Cocktail 2); *P. distasonis*, *B. salyersiae*, *P. dorei*, *E. coli* and *R. lituseburensis* (Cocktail 3); or PBS.

	Body weight gain g)						Gain: Feed (g/kg)					
Treatment	1 d	2 d	3 d	7 d	16 d	42 d	1 d	2 d	3 d	7 d	16 d	42 d
PBS	14.8^{b}	13.9	16.5	101	411^{b}	2551^{a}	1189^{c}	1,086	929	864	740	573
Cocktail 1	15.0^{b}	13.4	16.7	103	429^{a,b}	2527^{a}	1225^{b,c}	999	916	871	787	580
Cocktail 2	15.7^{a}	13.1	16.7	105	438^{a}	2448^{b}	1372^{a,b}	1,137	933	861	777	570
Cocktail 3	14.8^{b}	13.6	16.4	103	421^{a}	2427^{b}	1408^{a}	1,063	948	852	760	577
SEM	0.19	0.22	0.26	1.16	5.49	26.99	47.82	66.78	54.58	19.54	13.58	4.84

$^{a-c}$Means within a column and parameter with no common superscript differ significantly ($p < 0.05$).

TABLE 4 Carcass yield (%) of chickens at 42 d old that were administered *Parabacteroides distasonis*, *Bacteroides salyersiae*, *Phocaeicola dorei*, and *Escherichia coli* (Cocktail 1); *Romboutsia lituseburensis* and *E. coli* (Cocktail 2); *P. distasonis*, *B. salyersiae*, *P. dorei*, *E. coli* and *R. lituseburensis* (Cocktail 3); or PBS.

Treatment	Carcass	Legs	Thighs	Wings	Breast
PBS	73.7[b]	14.4	17.2	12.7	21.4
Cocktail 1	74.2[b]	14.5	18.2	11.8	21.4
Cocktail 2	76.1[a]	14.2	18.2	10.9	21.2
Cocktail 3	74.2[b]	14.0	17.7	11.3	21.0
SEM	0.49	0.14	0.36	0.62	0.61

[a,b]Means within a column with no common superscript differ significantly ($p < 0.05$).

TABLE 5 Relative intestinal weight and length of the jejunum of chickens administered *Parabacteroides distasonis*, *Bacteroides salyersiae*, *Phocaeicola dorei*, and *Escherichia coli* (Cocktail 1); *Romboutsia lituseburensis* and *E. coli* (Cocktail 2); *P. distasonis*, *B. salyersiae*, *P. dorei*, *E. coli* and *R. lituseburensis* (Cocktail 3); or PBS.

Treatment	0 d	1 d	2 d	3 d	7 d	16 d	42 d
	Relative weight (g/kg of BW)						
PBS	10.0[b]	20.5	22.3[b]	27.6	29.3	22.9	9.8[a]
Cocktail 1	14.4[a]	21.9	26.6[a]	27.0	29.2	21.8	9.3[a]
Cocktail 2	9.8[b]	19.4	22.1[b]	29.5	31.1	22.4	8.0[b]
Cocktail 3	12.6[ab]	20.0	22.7[b]	25.6	28.3	20.2	8.9[ab]
SEM	0.93	1.12	1.09	1.42	1.15	0.86	0.43
Relative length (cm/kg of BW)							
PBS	318	321	303	309	181	89	27
Cocktail 1	375	342	316	306	188	83	28
Cocktail 2	336	308	315	311	193	83	28
Cocktail 3	339	322	321	293	179	84	27
SEM	17.05	14.04	11.22	11.55	6.42	2.98	1.32

[a-c] Means within a column with no common superscript differ significantly ($p < 0.05$).
[a]3 h following administration of probiotic.

TABLE 6 Jejunal and ileal villi height of chickens administered *Parabacteroides distasonis*, *Bacteroides salyersiae*, *Phocaeicola dorei*, and *Escherichia coli* (Cocktail 1); *Romboutsia lituseburensis* and *E. coli* (Cocktail 2); *P. distasonis*, *B. salyersiae*, *P. dorei*, *E. coli* and *R. lituseburensis* (Cocktail 3); or PBS.

Treatment	0 d	1 d	2 d	3 d	7 d	16 d	42 d
	Jejunum (μm)						
PBS	149[b]	205	246[ab]	287[a]	409[b]	580[b]	692
Cocktail 1	136[b]	218	198[c]	242[b]	422[b]	654[a]	708
Cocktail 2	124[b]	187	226[bc]	245[b]	441[ab]	645[a]	732
Cocktail 3	218[a]	221	264[a]	272[ab]	467[a]	668[a]	694
SEM	12.3	9.4	15.2	10.9	13.6	23.2	23.2
Ileum (μm)							
PBS	115[a]	183[ab]	182	223	260[a]	314	379[c]
Cocktail 1	72[b]	180[ab]	187	214	238[b]	320	423[b]
Cocktail 2	122[a]	168[b]	192	222	232[b]	304	529[a]
Cocktail 3	139[a]	190[a]	207	222	239[b]	311	443[a]
SEM	8.9	6.2	19.8	10.3	6.5	16.9	14.2

[a-c] Means within a column with no common superscript differ significantly ($p < 0.05$).
[a]3 h following administration of probiotic.

probiotic formulations or PBS control therefore the weight gains were likely tied to changes in intestinal development.

During the first week, changes in the intestinal development were observed in response to the probiotics administered to day of hatch chicks. Birds administered the *P. distasonis*, *B. salyersiae*, *P. dorei*, and *E. coli* cocktail had a higher relative jejunal weight, 1.28 to 1.44-fold increase, just 3 h following administration compared to chicks receiving *R. lituseburensis* and *E. coli* cocktail or the PBS control, respectively (Table 5). At 2 days of age, the group that received *P. distasonis*, *B. salyersiae*, *P. dorei*, and *E. coli* cocktail had a relative jejunal weight, ~1.2-fold greater than the control or the other probiotic formulations. The relative weight of the jejunum was significantly decreased by 18% for birds administered *R. lituseburensis* and *E. coli* cocktail in comparison to the PBS control at 42 days ($p < 0.05$). There were no significant differences in jejunal length for either probiotic administration compared to the control. The probiotics also did not impact the relative weight or length of the ileum (Supplementary Table S2).

The *P. distasonis*, *B. salyersiae*, *P. dorei*, *E. coli* and *R. lituseburensis* cocktail induced longer jejunal villi just 3 h following administration (Table 6; Supplementary Figure S2), and continued to increase villus height at 7 and 16 days of age, compared to the PBS control (1.46, 1.14, and 1.15-fold increase, respectively; $p < 0.05$). However, the villus height was shorter in birds at 2 and 3 days of age, for *R. lituseburensis* and *E. coli* cocktail or *P. distasonis*, *B. salyersiae*, *P. dorei*, and *E. coli* cocktail compared to the PBS control (~20% decrease; $p < 0.05$). By 42 days of age, there were no significant differences in jejunal villus height for either group. The probiotic formulations did not seem to elicit enhancement of villi height in the ileum as seen in the jejunum until birds were 42 days of age. At this time point, all three formulations increased villi height compared to the PBS control with *R. lituseburensis* and *E. coli* cocktail or *P. distasonis*, *B. salyersiae*, *P. dorei*, *E. coli* and *R. lituseburensis* having the most pronounced effect on villus height (1.39 and 1.16-fold increase, respectively; $p < 0.05$). At earlier time points, the probiotics appeared to reduce ileal villus height, compared to the control group, 3 h (*P. distasonis*, *B. salyersiae*, *P. dorei*, and *E. coli* cocktail; 40% decrease; $p < 0.05$) following probiotic administration; and at day 7, all three probiotic formulations reduced villus height ~20% relative to the PBS control ($p < 0.05$).

The *P. distasonis*, *B. salyersiae*, *P. dorei*, *R. lituseburensis* and *E. coli* cocktail significantly increased 1.3 to 2.5-fold the number of goblet cells in the ileum in newly hatched chicks, just 3 h following its administration, and at day 2 in the ileum, respectively (Table 7; $p < 0.05$). The *P. distasonis*, *B. salyersiae*, *P. dorei*, and *E. coli* cocktail increased goblet cell numbers 1.5-fold at day 3 and the *R.*

TABLE 7 Goblet cells number per jejunum and ileal villus length of chickens inoculated *Parabacteroides distasonis, Bacteroides salyersiae, Phocaeicola dorei,* and *Escherichia coli* (Cocktail 1); *Romboutsia lituseburensis* and *E. coli* (Cocktail 2); *P. distasonis, B. salyersiae, P. dorei, E. coli* and *R. lituseburensis* (Cocktail 3); or PBS.

Treatment	0 d[+]	1 d	2 d	3 d	7 d	16 d	42 d
	Jejunum (number of goblet cells per villus length (μm))*						
PBS	0.1824 ± 0.0161	0.1251 ± 0.0139^{a,b}	0.1108 ± 0.0201	0.0889 + 0.0105^{a}	0.1048 ± 0.0094^{a}	0.0796 ± 0.0087	0.2429 ± 0.0390^{a}
Cocktail 1	0.1561 ± 0.0169	0.0969 ± 0.0077^{a}	0.0888 ± 0.0137	0.0733 + 0.0095^{a,b}	0.1187 ± 0.0092^{a}	0.0893 ± 0.0097	0.1804 ± 0.0202^{a,b}
Cocktail 2	0.2005 ± 0.0145	0.1941 ± 0.0269^{b}	0.0941 ± 0.0053	0.0694 + 0.0145^{a,b}	0.0723 ± 0.0059^{b}	0.0777 ± 0.0071	0.1750 ± 0.0172^{a,b}
Cocktail 3	0.1963 + 0.0096	0.1223 ± 0.0136^{a,b}	0.0990 ± 0.0054	0.0638 + 0.0041^{b}	0.0835 ± 0.0125^{a,c}	0.0931 ± 0.0087	0.1443 ± 0.0032^{b}
Treatment	Ileum (number of goblet cells per villus length (μm))*						
PBS	0.1918 + 0.0097^{a}	0.1054 ± 0.0125	0.0707 ± 0.0071^{a,b}	0.0810 + 0.0066^{a}	0.1365 ± 0.0167^{a,c}	0.1172 ± 0.0211^{a,b}	0.1939 ± 0.0210
Cocktail 1	0.2024 + 0.0162^{a,b}	0.1155 ± 0.0115	0.0553 ± 0.0105^{a}	**0.1223 ± 0.0162^{b}**	0.1030 + 0.0135^{a}	0.0775 ± 0.0076^{a,b}	**0.2796 ± 0.0273^{a,c}**
Cocktail 2	0.2018 + 0.0081^{a,b}	0.1083 ± 0.0142	0.0871 ± 0.0116^{b}	0.0690 ± 0.0070^{a,c}	**0.1512 + 0.0118^{b,c}**	0.1045 ± 0.0094^{a,c}	**0.3387 ± 0.0175^{b,c,d}**
Cocktail 3	**0.2536 + 0.0270^{b}**	0.1060 ± 0.0129	**0.1777 ± 0.0209^{c}**	0.0988 + 0.0136^{a,b,d}	0.1383 ± 0.0172^{a,c}	0.0930 ± 0.0075^{a,c}	**0.2901 ± 0.0256^{a,d}**

$^{a-d}$Means within a column and parameter with no common superscript differ significantly ($p < 0.05$). $^{+}$3 h following administration of probiotic. *As villus length increases with, goblet cell numbers per villus was normalized against villus length. **Bold** denote statistically significant increase in goblet cells in response to probiotic *versus* the control. Bold values significantly different ($p < 0.05$) from PBS control.

lituseburensis and *E. coli* cocktail improved goblet cells numbers at day 7 relative to the PBS control ($p < 0.05$). All probiotic formulations increased ~1.5-fold goblet cells per villus at 42 days (Table 7), in the ileum, however, a significant decrease was observed in the proportion of goblet cells in the jejunum with probiotic formulations *R. lituseburensis* and *E. coli* (30% reduction, day 7), or *P. distasonis*, *B. salyersiae*, *P. dorei*, *R. lituseburensis* and *E. coli* cocktail (28% and 40% reductions on days 3 and 42, respectively) relative to PBS control (Table 7; $p < 0.05$).

P. distasonis, Bacteroides salyersiae, P. dorei, Romboutsia lituseburensis and Escherichia coli cocktail lower *Lactobacillus* abundance in the chicken ileum

The probiotic cocktails were shown to modify the intestinal microbiota of birds compared to PBS control (Figure 4). Similar to Aviguard® treatment, the probiotic formulations affected the *Lactobacillus* population in the intestine. With the exception of day 3, *P. distasonis*, *B. salyersiae*, *P. dorei*, *R. lituseburensis and E. coli* cocktail reduced ileal *Lactobacillus* abundance 23%–60% compared to the PBS control. However, other probiotic formulations increased *Lactobacillus* abundance, depending on the intestinal segment (jejunum vs. ileum) or day of age.

Discussion

Poultry feed has diversified to vegetarian options and use of non-traditional ingredients that result in additional supplementation with amino acids and vitamins that enhance animal growth, physiology and performance (Alagawany et al., 2020). Gone are antibiotics once used to promote animal growth and prevent disease; replaced by probiotics, prebiotics, organic acids or essential oils. Some of these same feed additives have been shown to be comparable to growth-promoting antibiotics in improving intestinal development, animal growth, and pathogen exclusion or control (Gadde et al., 2017; Ricke, 2021; Abd El-Hack et al., 2022). These additives have been shown to alter the chicken gastrointestinal microbiome (Dittoe et al., 2018; Khan et al., 2020; Ali et al., 2021). The challenge now is piecing out their mechanism of action.

Poultry producers seek to imprint desirable attributes such as optimal feed conversion, disease and pathogen resistance, onto recipient hatchlings. Many studies have shown that a complex microbiota prohibits the establishment of harmful pathogens and fosters beneficial microbes that reduce inflammation, promote healing, improve feed efficiency and promote growth (van der Waaij et al., 1972; Candela et al., 2008; Fukuda et al., 2011; McNulty et al., 2011). Based on this concept, early intestinal colonization is essential to intestinal development, feed conversion and animal growth. Pioneer colonizers, as probiotics, offer an approach to ensure a mature and stable microbiome for newly hatched chicks.

Aviguard®, a commercially available competitive exclusion product, has been shown in multiple studies to improve disease resistance in broilers (Hofacre et al., 1998a; Hofacre et al., 1998b; Hofacre et al., 2019). In our current study it also altered the microbiome of chicks and improved development of the small

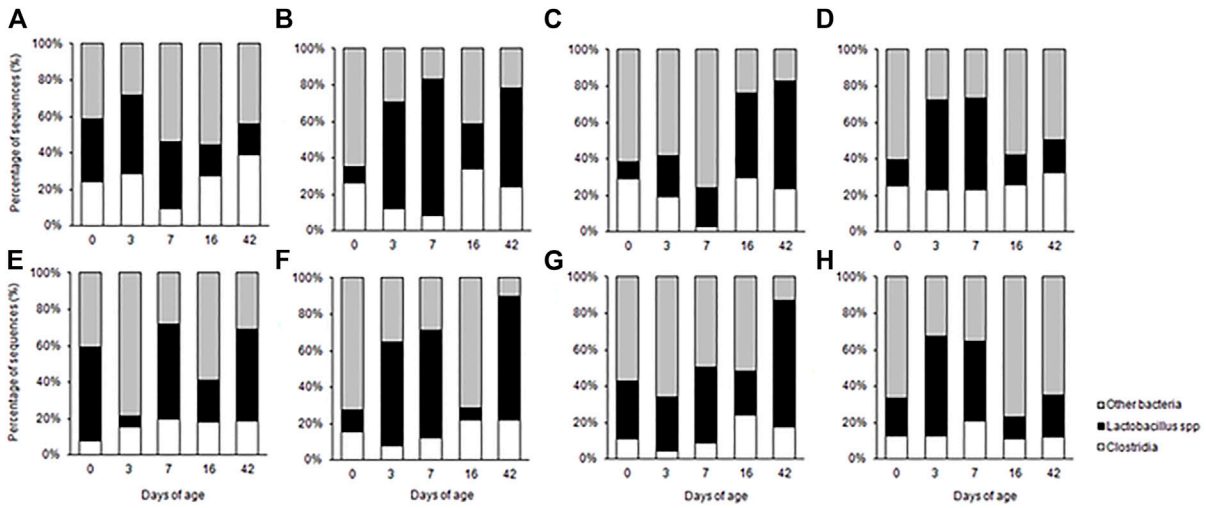

FIGURE 4
Composition of the small intestine bacterial community of chicks administered pioneer colonizers on day of hatch as determined by 16 S rRNA T-RFLP from samples collected from the jejunum **(A–D)** and ileum **(E–H)** of chickens from the *phosphate buffered saline* control group **(A, E)**, *Romboutsia lituseburense* and *Escherichia coli* cocktail **(B, F)**, *Parabacteroides distasonis*, *Bacteroides salyersiae*, *Phocaeicola dorei*, and *Escherichia coli* cocktail **(C, G)**, *P. distasonis*, *B. salyersiae*, *P. dorei*, *R. lituseburense* and *E. coli* cocktail **(D, H)**.

intestine. The *Lactobacillus* population of the jejunum and ileum was more quickly replaced with intestinal anaerobes and the diversity of the ileal community was more stable indicating that the previously reported community successions could be altered (Lu et al., 2003). A more stable intestinal community structure in chicks at 3 weeks of age is important because this is a critical time of vulnerability for intestinal health (Hofacre et al., 1998a). Compositionally, the competitive exclusion product contained abundant intestinal member species, as potential pioneer colonizers, with sufficient diversity to induce intestinal development and animal growth (Flint et al., 2015; Kettle et al., 2015; Pedroso et al., 2015).

The most abundant bacterial phyla in the small intestine, following administration of the competitive exclusion product Aviguard®, were the phyla *Bacteroidetes* and *Firmicutes*, and specifically, with regard to the latter phyla, *Clostridia* was the dominant order. The impact of the intestinal microbiota on host physiology is being intensively studied and it is becoming increasingly clear that the intestine does not function or develop properly in the absence of its resident microbiota (Dubos et al., 1968; Smith et al., 2007; Sommer and Backhed, 2013). While *Clostridia* and *Bacteroidia* have fundamental differences in polysaccharide utilization and feeding strategies, they are similar in their reliance on carbohydrates for metabolism. *Bacteroidetes* harvest mucus glycans, a nutrient generated by its animal host (Koropatkin et al., 2012) but *Clostridia* are also known for their ability to harvest energy from indigestible fiber. The *Bacteroidia* and *Clostridia* species, examined in this study possessed many CAZymes for liberating sugars from mucin and indigestible fibers. In addition, the *Bacteroidia* have been shown to influence the carbohydrate composition of the intestinal glycome by liberating fucose by hydrolysis of mucin and the byproducts of fucose fermentation stimulate stem cell development (Bry et al., 1996). Fucose has been shown to be a terminal carbohydrate in the chicken's intestinal glycome indicating that *Bacteroides* may also function as a pioneer colonizer in birds (Alroy et al., 1989; Madrid et al., 1989; Bryk et al., 1999). In addition, the species used in this study, *B. salyersiae*, *P. dorei*, and *R. lituseburensis,* possess fermentation enzymes and pathways for producing butyrate. While *P. distasonis* lacked these enzymes, it did possess enzymes necessary for producing propionate and several of the isolates also had acetyl-CoA hydrolases involved in acetate production.

Therefore, these probiotic isolates produce volatile fatty acids (VFA) that can be metabolized by the host animal. Metabolically, members of the order *Clostridia* and *Bacteroidia* cooperate rather than compete for the same nutrients in the intestine (Mahowald et al., 2009). This cooperation has the added benefit of increasing the VFA butyrate, which benefits their animal host in a number of ways including stimulating stem cell differentiation and reducing expression of inflammatory cytokines (Mahowald et al., 2009). Both *Clostridia* and *Bacteroidia* produce a variety of VFA, as end-products of fermentation, that can alter the composition of the microbiome and affect intestinal physiology (Segain et al., 2000; Pryde et al., 2002; Atarashi et al., 2013; Cockburn et al., 2015). Butyrate stimulates butyrate transporters in the host intestinal cells (Mahowald et al., 2009), dampens inflammation (Vieira et al., 2012), promotes intestinal integrity (Peng et al., 2009) and healing of intestinal damage (Butzner et al., 1996). In contrast, use of proteobacteria such as *E. coli* and *Citrobacter*, as pioneer colonizers in chicks elicited an intestinal inflammatory state that may lead to reduced intestinal health (Wilson et al., 2020; Chasser et al., 2021a; Chasser et al., 2021b).

The *P. distasonis* isolate used in this study also possessed a Vitamin B12 dependent ethanolamine utilization locus and vitamin B12 transporters that would allow it to compete with proteobacteria such as *Salmonella* and other intestinal bacteria for ethanolamine (Thiennimitr et al., 2011; Anderson et al., 2015; Kaval and Garsin, 2018). Furthermore, these *Clostridia* and *Bacteroidia* species may have elicited an indirect improvement of feed conversion by suppression of the *Lactobacillus* population. The lactobacilli are auxotrophs, deficient in their ability to synthesize up to eight different amino acids, vitamins and important co-factors (Makarova et al., 2006; Cai et al., 2009). While they are capable of fermenting a large repertoire of carbohydrates, they do not possess the enzymes to acquire these sugars from mucin (Makarova et al., 2006; Cai et al., 2009). Therefore, *Lactobacillus* is in competition with its host for free sugars, peptides and amino acids while the strict anaerobes such as *Clostridia* and *Bacteroidia* focus on utilizing mucin. Under feed restriction or a diet with low digestibility such as a wheat vs corn-soy diet, the composition of the small intestinal microbiome may have a significant impact on feed conversion and weight gain because of this competition (Torok et al., 2008; Metzler-Zebeli et al., 2019). In fact, a negative correlation between *Lactobacillus* abundance in the ileum and total body weight gain has been shown under feed restriction (Metzler-Zebeli et al., 2019). Low body weight birds tend to also have microbiome dominated by lactic acid bacteria (Zhao et al., 2013).

This is not to say the lactobacilli do not perform important functions for its animal host including dampening inflammation (Chen et al., 2012; Gong et al., 2020; Šefcová et al., 2020) or pathogen exclusion (Chen et al., 2012; Gong et al., 2020). However the mechanism of action of growth-promoting antibiotics may not only be due to suppression of pathogens (Arakawa and Oe, 1975), but the streptogramin (Lamb et al., 1999), glycopeptide (Chow and Cheng, 1988) and bacitracin (Toscano and Storm, 1982) antibiotics have broad activity against lactobacilli. In fact, antibiotic growth promoters suppress *Lactobacillus*, reduce community diversity and favor *Clostridia* in the ileum, similar to results observed with the competitive exclusion product used in this study (Lu et al., 2008). Therefore it is not surprising that growth promoting antibiotics profoundly affect microbiome composition and diversity (Lu et al., 2008). And while the growth-promoting properties of antibiotics and probiotics might be attributed to control of intestinal pathogens such as *C. perfringens*, it is also likely that their true impact is from enhancing intestinal development, modulating metabolism of the microbiome, and allowing the animal to better compete for nutrients liberated in the small intestine.

The *Bacteroidia* contain foundational genera, *Bacteroides* and *Parabacteroides* transmitted from the adult hen to its progeny, when hens are reared with their chicks (Kubasova et al., 2019a). The *Bacteroidetes* become the dominant phyla by day 18, for gnotobiotic chicks seeded with the intestinal microbiome from feral chickens (Thomas et al., 2019) and members of this phyla can stably colonize the cecum of chicks administered a complex cocktail containing this phyla, *Firmicutes* and *Proteobacteria* (Kubasova et al., 2019b). *Bacteroidia* member species have been shown to exclude certain pathogens from the chicken gastrointestinal tract (Kubasova et al., 2019b; Papouskova et al., 2023).

While we observed significant improvement to intestinal development and animal performance with our five-member probiotic formulation, this does not imply that this probiotic performs all the same functions as the competitive exclusion product examined in this study. While variable in composition, this product is consistent at reducing *Salmonella* colonization in chicks (Lee et al., 2023) and has been shown to be effective at controlling other enteropathogens (Hofacre et al., 1998a; Hofacre et al., 2019). This competitive exclusion product is a complex consortium, of chicken cecal origin, that consists of 20–50 distinct genera. While Kubasova et al. demonstrated significant *Salmonella* exclusion with an eight-member probiotic formulation, including *P. distasonis* (Kubasova et al., 2019b), it is not known whether this same formulation can exclude other enteropathogens or has growth promoting properties. Perhaps, it requires sufficient community diversity to outcompete pathogens, promote intestinal development and function, and repair any perturbation to gut function brought about by disease. Microbiome diversity is important in pathogen exclusion (Pedroso et al., 2021) and restoring homeostasis following any perturbation to the gastrointestinal tract (Weimer, 2015).

Conclusion

In addition to excluding pathogens, competitive exclusion product contains foundational bacterial species to promote intestinal function, development and animal growth. Intestinal pioneering colonizers selected from chicken ceca, based on their prominence in competitive exclusion product and consisting of five-member intestinal species, was comparable to a competitive exclusion product in improving intestinal morphology and animal performance. The balance of proteobacter, lactobacilli and anaerobic intestinal member species is critical to a healthy microbiome that promotes intestinal development, feed efficiency and animal growth (Foo et al., 2017). In the past, growth-promoting antibiotics provided this balance. Now, as the poultry industry has moved towards antibiotic free production, defined intestinal bioproducts are needed to stimulate intestinal development and function, support lower feed conversion rates and improved body weight gains, and maintain a healthy balance in the intestinal microbiota.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by University of Georgia Institutional Animal Care and Use Committee.

Author contributions

ML was responsible for funding acquisition, conceptualization, writing, review and editing. AP, BL, and YC performed the experiments and formal analysis described in this article. JM performed metagenome analyses and data curation. ML and JM were involved in the writing, review and editing.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1139321/full#supplementary-material

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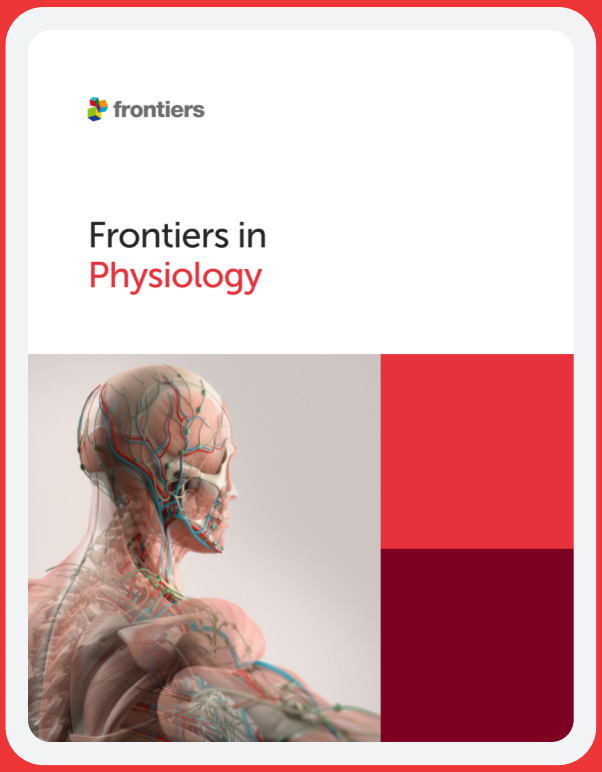

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