Cloacal swabs are unreliable sources for estimating lower gastro-intestinal tract microbiota in chicken.

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Research Article

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Abstract

Background The gastrointestinal microbiota in chicken (*Gallus gallus domesticus*) has a central role in health and performance. The ceca are a vital site of functional activity, but assessing cecal microbiota in longitudinal studies remains a challenge. The cecal communities are broadly similar to large intestine samples. Cloacal sampling, due to their proximity to the large intestine, is an alternative, non-invasive method used for assaying and monitoring disease-causing agents, and maybe a viable option for longitudinal studies.

Results We collected paired cecal content, and cloacal swab samples from twenty randomly selected commercial broilers raised on two dietary treatments. The microbiota of each sample was assessed using 16S rRNA V4 hypervariable region sequencing on an Illumina MiSeq platform and analyzed using the MOTHUR pipeline. Analysis of fourteen paired samples resulted in 1603 OTU's assigned to 82 Families. Eleven families were shared between the cecal and cloacal samples, with seven and eleven families unique to cecal content and cloacal swabs, respectively. Paired t-test and Wilcoxon Signed-Rank test showed significant differences in the Chao1 index between the cecal content and cloacal swabs (p-value = 0.000845 and p-value = 0.001397, respectively). However, the Inverse Simpson species diversity estimator was not different using the Wilcoxon Signed-Rank test (p-value = 0.3258) and a paired t-test (p-value = 0.3864). β -diversity between the cloacal swabs and cecal microbiota also showed significant differences based on PERMANOVA (p-value = <0.001), HOMOVA (p-value <0.001), and Weighted Unifrac (WSig = <0.001) testing.

Conclusions Cloacal swabs do not approximate either the α or β diversity of cecal samples, based on a paired sample analysis. The high variability of cloacal microbiota has been reported previously, and this study provides additional evidence of the randomness of cloacal microbiota in contrast to cecal microbiota. Our findings indicate that cloacal samples are not suitable for longitudinal studies of gut microbiota patterns. High inter-individual variation of cloacal swab data warrants further assessment of their reliability as a targeted diagnostic method.

1 Background

Chicken (*Gallus gallus domesticus*) is the source of the most consumed animal protein globally at nearly twice the amount of pork and beef combined [1]. Because of this, there is a great emphasis on improving poultry health and performance [2–5]. Notably, the role of gut microbiota in improving performance [6–8], welfare [9], and health [4, 10–14], is a topic of intense interest. The gut microbiota is studied intensively in broilers; an NCBI PubMed Central search for "Poultry Gut Microbiota" yielded 2586 research articles within the last five years.

The gut microbiota, an ecological community of commensal and non-commensal microorganisms [15, 16], is found throughout the entire length of the broiler's gastrointestinal tract (GIT). Although most research concentrates on the organs within the lower sections: the small intestine (duodenum, jejunum,

and ileum), large intestine, cecum, and cloaca. The ceca, a pair of blind sacs, are especially important as the site of functional activity relevant to microbial communities and species studied in performance and health [7, 17]. The ceca retain nearly 10^{11} microbial cells per gram and are an important location for fluid resorption via the translocation of urea from the urodeum and the fermentation of carbohydrates [17–21]. As a consequence, the ceca are the most sampled gut segment in chicken gut microbiota studies [7, 22]. A standard experimental method of microbiota analysis in poultry involves the invasive sampling of the ceca, following euthanasia, which prevents longitudinal studies of the same experimental animals.

Cloacal (or vent) swabs are an alternative, non-invasive method used on domestic, migratory, or endangered bird species [23] where invasive sampling may not be permitted. Due to the non-invasive aspect, cloacal swabs are used frequently for assaying and monitoring agents such as *Salmonella* spp [23], Avian Influenza [24–26], Coccidiosis [27], and *Campylobacter coli* [28]. Importantly, these swabs were analyzed using real-time PCR or microorganism specific plating methods and not for total microbiota analysis. Therefore, their suitability of cloacal swabs for assessing gut microbiota is not apparent.

Due to the ubiquity of cloacal swabbing, mainly for diagnostics, it is critical to determine if and how representative cloacal microbiota are of cecal microbiota. Cecal microbiota in chicken show broad similarities with lower large-intestinal microbiota [29], and the cloaca abuts the large-intestine. If cloacal microbiotas approximate the cecal microbiota, it would enable non-invasive longitudinal studies. On the other hand, if cloacal microbiota is not a reliable proxy for cecal microbiota occurrence and abundance, then its utility for assessing avian microbiota would be limited. To resolve the reciprocity of cecal and cloacal microbiotas, we used a paired sample approach to compare cecal and cloacal microbiota communities sampled from the same individuals. Based on previously published works about fecal microbiota, we hypothesized that cloacal microbiota is not representative of cecal microbiota from the same individuals. We used 16S rRNA sequence-based analysis of α and β diversity of the communities between the two sampling methods. Here we report that cloacal swabs are unreliable representatives of the presence-absence of taxa, as well as α and β diversity.

2 Materials And Methods

2.1 Study Design

Twenty fast-growth, high-yield commercial broilers were randomly sampled from a study conducted at the Texas A&M University Poultry Science Research Center in College Station, TX, USA. Samples taken represent two dietary treatments with two replicate pens, each pen containing five broilers, whose ages were thirty-three to thirty-six days of age. We raised Broilers under standard industry lighting conditions with ad libitum feed and water. The broilers in this study were raised on two diet treatments. Treatment 1 (T1) consisted of a Corn, Soybean Meal Protein (35%) diet, whereas treatment 2 (T2) consisted of a corn-based diet, with mixed protein sources (bone meal, corn gluten meal, fish meal, and soybean meal). Both diets were energetically equivalent. For each diet treatment, we raised birds in replicate pens. We included

diet treatments as a factor to assess the ability of cecal versus cloacal swabs to differentiate between dietary treatments.

2.2 Sample Collection

From each dietary treatment (T1, T2), we randomly sampled ten broilers. Within each diet treatment group, an equal number of individuals were sampled (five). We moved the randomly selected birds to a clean room for cloacal swab collection, euthanasia, and post-mortem sample collection from the ceca. For cloacal swabbing, we used a Puritan PurFlock Ultra Sterile mini-tip Flock swab with a sterile container (Puritan, ME, USA) to sample the cloacal microbiota from live birds following a modified protocol originally reported by Vo & Jedlicka [30]. First, the exterior surface of the cloaca was wiped with a cotton ball sprayed with 70% Ethanol. The PurFlock swab was gently inserted approximately 22 mm into the cloaca, a depth just beyond the length of the swab tip. The swab was rotated five times in slow clockwise motion around the cloaca, applying moderate pressure so that the swab-tip maintained contact. Additionally, we rolled the swab-tip so that the entire surface of the swab was coated with cloacal material. Following sample collection, the swab was inserted into the supplied sterile container, immediately placed on ice after collection, and transferred to a -80°C freezer until further processing.

After completing the cloacal swab sample collection, individual broilers were euthanized by CO₂ exposure, followed by cervical dislocation. The animal use protocol (AUP) and procedures employed were ethically reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University, reference AUP IACUC 2016-0064. We used sterile instruments for the post-mortem sample collection from the cecal content. We collected cecal content samples within thirty minutes of euthanizing the broiler. Approximately 2 g of the cecal content material was collected and immediately stored in a 1:5 ratio (w/v) of RNAlater (QIAGEN, Hilden, Germany). Tissue samples were stored at 4°C for twenty-four hours (following RNAlater storage protocol) and then moved to a -80°C freezer until further processing.

2.4 Sample Preparation and Nucleic Acid Isolation

Total genomic DNA (gDNA) was isolated from approximately 0.1 g of cecal content material using a QIAmp PowerFecal DNA Kit following the manufacturer's protocol (QIAGEN, Hilden, Germany). gDNA from the cloacal swabs was isolated following a modified extraction protocol using the DNAzol Reagent (Invitrogen, ThermoFisher Scientific, MA, USA). The modified protocol was as follows, briefly. First, we suspended the swab-tip in a 406µL solution containing 1X TBS, 0.01M EDTA, and 0.005% (w/v) Tween-20 (Sigma-Aldrich Corp, MO, USA) to release the material from the swab-tip. The suspension, along with the trimmed swab tip and 1.7 g of 1 mm zirconia beads (BioSpec Products, OK, USA) was placed in screw cap tubes and vortexed for ten seconds. Following vortexing, 1 mL of DNAzol was added to the swab-tip solution and homogenized for sixty seconds at 1500 rpm using a BioSpec Mini-Beadbeater-96 machine (BioSpec Products, OK, USA). The homogenized solution was left to rest for an additional ten minutes at room temperature to allow the DNAzol to lyse the phospholipid bilayer of the prokaryotes suspended in the solution. The DNA pellet was further purified and precipitated using 3M Sodium Acetate and 100%

ethanol. The purified pellet was washed with 70% ethanol a second time to remove any PCR inhibitors or residual chemicals left on the pellet. We suspended the DNA pellet in 100 µl to 450 µl of 8 mM NaOH and 2.3 µl 1M HEPES per 100 µl of NaOH for storage. Final gDNA concentrations and purity of all samples were measured on a NanoDrop n1000 (ThermoFisher Scientific, MA, USA) before 16S rRNA PCR amplification.

2.5 PCR, Library Preparation, and Sequencing for 16S rRNA Analysis

To amplify the 16S rRNA V4 hypervariable region, we performed a 50 µl PCR reaction consisting of 10 µl of template DNA (150 ng to 185 ng total), 13.8 µl Nuclease-free water, 0.6 µl (25 µM) forward primer (Hyb515F_rRNA: 5' - TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA - 3'), 0.6 µl (25 µM) reverse primers (Hyb806R-rRNA: 3' - TAATCTWTGGGVHCAATCAGGGACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-5') [14, 31], and 25 µl of NEBNext® High-fidelity 2X PCR Mastermix containing the hot-start Q5® High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA). Sequences of forward and reverse primers contain Illumina adapters, primer pad, and primer linker. We performed PCRs in triplicate (per sample) on an Eppendorf Mastercycler Pro (Eppendorf, Hamburg, Germany) with the following cycle conditions: initial single cycle denaturation step at 98°C for 30 s, twenty-five cycles at 98°C for 10 s, 65°C for 30 s, 72°C for 30 s, with a single cycle final extension step at 72°C for 5 minutes. A non-template negative control (Nuclease-free water used to prepare all solutions) and positive template control (cultured *E. coli*) were included in all PCR amplification reactions. All amplicon products were visualized on a 2% Agarose gel. No amplification bands were observed in the negative controls and were therefore not included in library preparation and sequencing. Before sequencing, amplicon products containing Illumina barcodes were cleaned using Ampure Magnetic beads (Beckman Coulter, CA, USA). Amplicon pools were barcoded and sequenced at the Genome Sequencing and Analysis Facility (GSAF, University of Texas, Austin, TX, USA). We sequenced the libraries on an Illumina MiSeq platform (Illumina, San Diego, CA, USA), running in 250 bp paired-end mode.

2.6 Bioinformatic Pipeline for Microbiota Evaluation

Resultant .fastq files from sequencing were processed using MOTHUR software v. 1.39.5 [32]. Briefly, paired-end reads were joined using the make.contigs command. We aligned the sequences to the SILVA database v. 132 [33] and removed chimeric sequences using the UCHIME program v. 4.2.40 [34]. Low abundance operational taxonomic Units (OTU's) and Singletons were removed from analysis with the split.abund command using cutoff = 1. Chloroplast, Mitochondria, Eukaryota, and other unknown sequences were removed from the dataset using the remove.lineage command. Total OTU's were then generated at the species level (0.03) and then classified using the classify.otu and dist.seqs commands, respectively. OTU tables and other output from MOTHUR were further analyzed on the R platform v. 3.6.2 [35] using the Phyloseq v. 1.28.0 [36] and Vegan v. 2.5.6 [37, 38] packages. We generated plots using the ggplot2 package v. 3.2.1 [39].

We performed Principal coordinates Analyses (PCoA) based on the Bray-Curtis distance using the Phyloseq and ggplot2 packages. Rarefaction curves summary statistics and Q-Q plots of the Good's Coverage Index values are presented in supplementary materials (supplemental Fig. 1A and supplemental Fig. 1B). We compared family level microbial composition using relative abundance values after removing low abundance taxa (< 2%).

To assess whether OTU abundance structures are comparable between cloacal and cecal communities, we performed non-parametric tests on the relative abundance and cumulative distribution functions of the paired cecal and cloacal swab datasets. Q-Q plots were generated for each comparison to check for normality. We used the two-sample Kolmogorov-Smirnov (KS) test using the `ks.test` function in R to assess whether the microbiota populations (cecal vs. swabs) are from the same distribution. The two-sample ranked location-scale tests of Cucconi and Lepage were implemented using the `nonpar` package v. 0.1-2 [40] using the `cucconi.test` and `lepage.test` commands, respectively. The Cucconi test is a ranked test that assesses whether the locations and scales of the two population distributions are equal [41–43] while the Lepage test is a ranked location-scale test that combines the Ansari-Bradley test for scale and the Wilcoxon-Mann-Whitney test for location [44, 45].

2.8 Statistical Tests for α and β Diversity:

Two statistical tests were performed in R to evaluate the α and β diversity between sampling locations and amongst dietary treatments within each sampling location. We used the two-sample Wilcoxon Signed-Rank test to compare the Chao1 and Inverse Simpson (InvSimpson) α diversity measures.

To compare β diversity, we used the Permutational Multivariate Analysis of Variance (PERMANOVA) using the “Adonis” function of the `Vegan` package with 9999 permutations [46, 47]. In addition to PERMANOVA, we compared β diversity in MOTHUR using AMOVA and `unifrac.weighted` [48]. The weighted unifrac test was applied to investigate the probability that two or more communities have the same structure by chance. These three species-level non-parametric tests were computed using the Yule and Clayton measure of dissimilarity average phylogenetic distances [49]. The statistical significance of comparisons was assessed at $\alpha = 0.05$.

3 Results

3.1 Sampling Location Yields a Variability in Sequencing Depth

The raw data from sequencing generated a total of 560,935 reads, with an average of 20,033 reads per sample. Total read depth per sample was limited to an arbitrary minimum of 7,435 to ensure adequate read depth in any given sample [50], and thus was the cutoff for inclusion in further analysis. Out of the 40 libraries sequenced, we excluded six libraries as the to the read threshold. To retain the paired nature of our analysis, we, therefore, proceeded with fourteen paired samples for which both cloacal swab and cecal data was found. A summary of the complete information can be found in the supplementary tables

1 and 2. The cecal samples had an average of 22,968 reads (IQR 20,524 – 24,516 reads), whereas the cloacal swabs had an average of 17,099 reads (IQR 9,946 – 22,378 reads). The average Good's coverage was 99.64%, (SD 0.315%), showing that the retained datasets had adequate sequence coverage to sample OTUs. Summary statistics for both the cecal and cloacal datasets are given in Table 1.

Table 1

Descriptive Statistic	Cecal Content Reads	Cecal Content Good's Coverage	Cloacal Swab Reads	Cloacal Swab Good's Coverage
Sample Size	14	14	14	14
Minimum Reads	18428	99.63%	7435	98.98%
1st Quartile	20524	99.71%	9946	99.34%
Median	22926	99.74%	16608	99.66%
Mean	22968	99.73%	17099	99.55%
3rd Quartile	24516	99.74%	22378	99.77%
Maximum	29053	99.85%	31859	99.86%
IQR	3992	0.037%	12432	0.434%
Range	10525	0.21%	24424	0.88%

Table 1. Descriptive statistics of the sequencing data for the fourteen cecal content and fourteen cloacal swabs. Column titles are in bold font.

3.2 Broad differences between cecal and cloacal microbiota members

Overall, the twenty-eight samples yielded 1603 OTU's assigned to 82 Families. The top three families based on relative abundance were Peptostreptococcaceae (12.623%, Phylum Firmicutes), Ruminococcaceae (10.802%, Phylum Firmicutes), and Lactobacillaceae (8.909%, Phylum Firmicutes). Twenty-two families were represented in the cloacal swab samples with eighteen families represented in the cecal content samples, as shown in Figs. 1 and 2, respectively. There were eleven families shared cecal and cloacal samples, with seven families (Atopobiaceae, Bacteria_unclassified, Bifidobacteriaceae, Clostridiales_unclassified, Clostridiales_vadinBB60_group (OTU 0045), Gastranaerophilales_fa (OTU 0010), and Helicobacteraceae (OTU 0004)) unique to cecal content and eleven families (Actinomycetaceae, Clostridiales_vadinBB60_group (OTU 0047), Corynebacteriaceae, Enterobacteriaceae, Enterococcaceae, Gastranaerophilales_fa (OTU 0013), Mollicutes_RF39_fa, Pasteurellaceae, Peptostreptococcaceae, and Planococcaceae) unique to cloacal swabs.

The fourteen cecal content samples yielded 1424 total OTU's from 54 total Families with Ruminococcaceae (23.062%, Phylum Firmicutes), Barnesiellaceae (12.700%, Phylum Bacteroidetes), and Rikenellaceae (7.841%, Phylum Bacteroidetes) as the top three most abundant families in the cecal content samples. The fourteen cloacal swab samples yielded 905 total OTU's from 79 total Families. The top three most abundant families in the cloacal swab samples do not seem uniform with regards to the distribution of families, nor is there an observable pattern. The top three most abundant families in the cloacal samples were Peptostreptococcaceae (12.912%, Phylum Firmicutes), Lactobacillaceae (12.059%, Phylum Firmicutes), and Barnesiellaceae (9.566%, Phylum Bacteroidetes). However, it is noteworthy that Peptostreptococcaceae is only present in four out of fourteen cloacal swab samples. These top three families found in cloacal data were present in all fourteen cecal content samples.

A PCoA (Fig. 3) was calculated to compare community member composition within the cecal content and cloacal swab methods. The cecal content samples cluster tightly together, whereas the cloacal swab samples show high variability while still encompassing the cecal content samples. This high variability is not surprising given the total number of families represented in the cloacal swabs. Overall, the ordination pattern of these paired samples shows broad-ranging differences between the two sampling approaches.

Finally, when comparing the cumulative distribution functions filtered for > 0% of the relative abundances between cecal and cloacal microbiotas, we found major differences in the structure of abundance. The KS ($D = 0.11745$, $p\text{-value} = 4.692e-07$) and Cucconi tests ($C = 298.959$, $p\text{-value} = 0$) were highly significant. The results of the Kolmogorov-Smirnoff and Cucconi tests on the cumulative distribution function further demonstrate that both the location and the scales of the cecal content and cloacal swab distributions of relative abundances are highly different.

3.3 Richness and diversity differences between cecal and cloacal samples

We compared microbial species richness and diversity of the cecal content and cloacal swabs using the Chao1 and Inverse Simpson estimators (Fig. 4). Both the Wilcoxon Signed-Rank test ($W = 4$, $p\text{-value} = 0.000845$) and paired t-test ($t = 4.042$, $p\text{-value} = 0.001397$) showed highly significant differences in the Chao1 index between the cecal content and cloacal swabs, with the highest richness observed in the cecal samples. A higher Chao1 value indicates a higher number of low abundance taxa, e.g., singletons [51, 52]. The higher value in cecal samples, suggests that more rare taxa were captured in cecal samples. However, the Inverse Simpson species diversity estimator was not different between cloacal and cecal samples based on a Wilcoxon Signed-Rank test ($W = 36$, $p\text{-value} = 0.3258$) and a paired t-test ($t = 0.89623$, $p\text{-value} = 0.3864$). Similar to the Chao1 findings, the cecal content had higher microbial diversity, compared to the cloacal swabs. As the Inverse Simpson index estimates the richness weighted by the proportional abundance of taxa present within samples, the non-significance suggests that the two types did not differ in their internal weighted abundances.

To assess whether cecal or cloacal swabs captured differences between dietary treatments, we performed richness and diversity analyses, comparing the two diets. Figure 5A shows comparisons within

cecal data, and Fig. 5B shows cloacal swab comparisons. The Wilcoxon Signed-Rank test on the Chao1 estimator returned non-significant p-values for the cecal content treatments ($W = 10$, $p\text{-value} = 0.1119$) and cloacal swab treatments ($W = 12$, $p\text{-value} = 0.1898$). The Chao1 values were higher for T1 than T2 in both the cecal content and cloacal swab methods. Overall, neither the cecal nor the cloacal swab microbiotas differed between the dietary treatments based on the Chao1 index.

On the other hand, the Inverse Simpson index was not different based on cecal samples ($W = 24$, $p\text{-value} = 0.8981$), but the cloacal swab data showed significant differences between diets ($W = 6$, $p\text{-value} = 0.02897$). In summary, the cloacal samples showed significant differences between dietary treatments despite the absence of such distinction when comparing cecal microbiotas.

3.4 Cloacal swabs do not reflect the community structure inferred from cecal samples

The PERMANOVA analysis showed significant difference ($F. \text{Model} = 4.903$, $R^2 = 0.15866$, $p\text{-value} = < 0.001$) in the centroids and dispersion between cecal and cloacal microbiota. This difference of community structure was further supported with significant results from the HOMOVA ($B\text{Value} = 3.07143$, $p\text{-value} < 0.001$) and Weighted Unifrac ($W\text{Score} = 0.664192$, $W\text{Sig} = < 0.001$) tests. On the whole, the results from the PERMANOVA, HOMOVA, and Weighted Unifrac tests all show that the microbiota communities inferred from cloacal swabs are different from cecal microbiota.

Next, we investigated whether differences in the diet treatments (T1 and T2) elicit differences in communities (β diversity) inferred using cecal versus cloacal samples. We found that neither sampling method detected differences in β diversity between the diets. The results from PERMANOVA showed that the cecal content was not different between dietary treatments ($F. \text{Model} = 1.4155$, $R^2 = 0.10551$, $p\text{-value} = 0.1455$). The cloacal swabs also revealed the same information ($F. \text{Model} = 1.065$, $R^2 = 0.08148$, $p\text{-value} = 0.3523$). HOMOVA and AMOVA both yielded the same information, suggesting that between dietary treatments, the differences in variances ($B\text{Value} = 0.518171$, $p\text{-value} = 0.125$) or the genetic diversity of communities ($B\text{Value} = 0.024201$, $p\text{-value} = 0.492$) were not different. In contrast, the Weighted Unifrac comparing the cecal microbiota between dietary treatments was significant ($W\text{Score} = 0.584437$, $W\text{Sig} < 0.001$), as was the comparison of cloacal microbiotas between diets ($W\text{Score} = 0.77311$, $W\text{Sig} < 0.001$). However, in this instance, it is difficult to determine how the high variability among cloacal samples influence the comparisons of β diversity between dietary treatments.

Further comparison of the dietary treatments within each method and between methods using the KS, Lepage, and Cucconi tests yielded no significant differences (Table 2). We found non-significant results using the same three tests for the comparison of dietary treatments within each sampling method. These results are expected as we found no major difference in the structure and abundances between the treatments using either cecal content and cloacal swabs.

Table 2

Statistical testing results for geometric mean distributions of dietary treatments within and between methods

Comparison	KS Test	Lepage Test	Cucconi Test
Cecal Content T1 vs. Cecal Content T2	D = 0.3348; p-value = 0.306	L = 2.777; p-value = 0.2555	C = 1.394; p-value = 0.25
Cloacal Swab T1 vs. Cloacal Swab T2	D = 0.3375; p-value = 0.2152	L = 3.02; p-value = 0.2215	C = 1.584; p-value = 0.194

Table 2. Summary of the statistical test used in comparing the geometric mean distributions. Geometric means of relative abundance were calculated and filtered for relative abundances > 2%. Specific dietary comparisons are in the first column, with the statistical tests in the remaining columns. Each cell contains the respective test statistic and p-value for that comparison.

4 Discussion

In this study, we showed that the microbiota identified from cloacal swabs are not representative of the cecal microbiota, and therefore not a suitable approach to sampling the microbial communities of the lower gastrointestinal tract. This result was highly surprising, given that the cecal and large intestine microbiotas are alike by week five in chicken [29]. Not only were the cloacal communities limited in their resemblance to cecal communities, the patterns of presence-absence as inferred by richness estimates were also significantly different. These findings suggest that there is a high degree of stochasticity to taxa sampled from the cloaca. Our results show similarities to the findings of Videvall et al. [53], who compared cloacal swabs and fecal samples in the ostrich (*Struthio camelus*) to analyze the lower GIT microbiota community and demonstrated the inaccuracy of fecal and cloacal swabs to portray the microbiota communities of the lower GIT organs. The broad-ranging differences between cloacal and cecal microbiota mirror the patterns seen with fecal microbiota in chicken. Fecal samples show qualitative similarities with quantitative differences compared to GIT [27, 54]. Hieke et al. [14] also showed fecal samples are not representative of cecal communities in young layer-type chicken.

High variability of cloacal microbiota

While the factors influencing fecal microbiota differences from cecal communities (external conditions, environmental microbiota) are expected, the cloacal swab dissimilarities and variability are more surprising. It is not clear if the cloaca of chicken is colonized, unlike other parts of the GIT. While numerous surveys of cloacal microbiota exist in the literature, in wild birds, the cloacal microbiota is often the only locus for characterizing gut microbiota as euthanasia may not be an option. However, our results show that the taxonomic composition and community profiles obtained from cloacal swabs can be highly random, with little consensus even when collected under controlled conditions. The high-interindividual differences in cloacal microbiomes were also reported for barn swallows [55]. Barn

swallows have different social structures and sex-based behavioral differences and make direct comparisons with chicken difficult, but the poor reproducibility of cloacal microbiota is, nonetheless, a notable similarity. We found lower richness and diversity of microbial taxa in the cloaca, compared to the cecal microbiota. Van Veelen et al. [56] showed lower richness and diversity of cloacal microbiota but surmised that top-down regulation by the host's genetics drives this pattern. However, our data showing significantly higher richness in the ceca, suggests that host genetics are not driving lower richness or diversity in the cloaca. The variability of cloacal swab data was revealed only in contrast with the paired cecal datasets.

On the other hand, Hird et al. [57] found that cloacal microbiomes differed among species of ducks, and by Influenza infection status. In this case, the interspecies differences may be driving the resolution of differences among species. Furthermore, as they did not characterize cecal microbiota, it is not possible to determine how the cloacal data compared to cecal data. Our analysis leads us to advocate extreme caution when inferring lower GIT microbiota patterns from cloacal swabs of birds.

Cloacal swabs are used routinely to assess infection status in domesticated, pet, and wild bird species [58–60]. In the majority of these cases, targeted assays (RT-PCR) used swab samples for the detection of pathogenic species. In these cases, the sensitivity of the assays provides valuable information for treatment or containment of pathogens, especially in poultry operations. While our data show high variability in the representation of taxa in cloacal samples, the sensitivity of RT-PCR approaches may allow lower detection thresholds. However, the reciprocity of taxon representation with cloacal 16S rRNA sequencing and targeted PCR methods needs to be established experimentally.

Resolution of microbial community differences between diets

In our analysis of microbiota between the two dietary treatments, we found that neither cecal nor cloacal samples were able to differentiate between diets. Both these sample types appear to be equivalent in their inability to differentiate between diets. However, we emphasize that this equivalency exists aside from the fact that the cecal and cloacal communities were highly dissimilar. Also, while the cecal samples were similar due to the overlapping distributions between diets, the similarity of cloacal swabs is driven by the high variability across all cloacal samples (Fig. 5B). Additionally, the housing environment, rather than dietary protein source, is known to be a more significant factor driving cecal microbiota differences. Hubert et al. [61] reported that birds raised in the same housing environment, regardless of dietary protein source, had similar cecal microbiota. In this present study, all the chickens were raised in the same barn (across replicate pens), where they were provided with the same bedding material and water source. Therefore, the high variability among cloacal samples, all collected in a controlled environment, represents, in our opinion, the high variability inherent to cloacal samples.

5 Conclusions

In this study, we showed that cloacal swabs do not faithfully approximate either the α and β diversity of cecal samples, based on paired samples. Therefore, cloacal swabs are unsuitable for assessing lower

GIT microbiota in birds. While the high variability of cloacal microbiota has been reported previously, our study provides experimental evidence to capture the randomness of cloacal microbiota, concerning the consistency of cecal samples. One of the consequences of our finding is that cloacal samples, akin to fecal samples, are not suitable or reliable for longitudinal studies of gut microbiota patterns in birds. Finally, the high inter-individual variation of cloacal swab data warrants an experimental assessment of their reliability for targeted diagnostic methods.

6 Declarations

6.1 Ethics approval and consent to participate

No human subjects, human material, or human data were used for this study. The animal use protocol (AUP), experiment procedures employed, and animal euthanasia methods were ethically reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University, reference AUP IACUC 2016-0064.

6.2 Consent for publication

Not applicable.

6.3 Availability of data and material

All the data and metadata files have been deposited on Figshare and can be accessed at [10.6084/m9.figshare.11819655](https://doi.org/10.6084/m9.figshare.11819655).

6.4 Competing Interests

The authors declare that they have no competing interests.

6.5 Funding

Not applicable.

6.6 Authors Contributions

The experiment was conceived and designed by TW and GA. Samples were collected by TW. Wet lab work was performed by TW. TW and GA performed the data analysis. TW and GA wrote the manuscript. All authors read and approved the final manuscript.

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Abbreviations

AUP Animal Use Protocol

bp base pair

CO₂	Carbon Dioxide
EDTA	Ethylenediaminetetraacetic Acid
g	grams
gDNA	Genomic DNA
GIT	Gastrointestinal Tract
IACUC	Institutional Animal Care and Use Committee
InvSimpson	Inverse Simpson α-diversity index
IQR	Interquartile Range
KS	Kolmogorov-Smirnov Test
mL	milliliter
mM	millimolar
mm	millimeter
NaOH	Sodium Hydroxide
OTU	Operational Taxonomic Unit
PCoA	Principal Components Analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
PCR	Polymerase Chain Reaction
rpm	Rotations per minute
RT	Room Temperature
SD	Standard Deviation
T1	Dietary Treatment 1
T2	Dietary Treatment 2
TBS	Tris Buffered Saline

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Figures

Figure 1

The relative abundance of families observed for each cloacal swab samples that have ≥ 7435 reads; $n = 14$. The vertical black line is a visual separator so that treatment 1 samples are on the left, and treatment 2 is on the right.

Figure 2

The relative abundance of families observed for each cecal content samples that have ≥ 7435 reads; $n=14$. The vertical black line is a visual separator so that treatment 1 samples are on the left, and treatment 2 is on the right.

Figure 3

Principal Component Analysis (PCoA) comparing the cecal content and cloacal swab testing methods. PCoA plot is based on the Curtis-Bray distances and showed that the cecal samples cluster tightly together, while the cloacal swab samples show high variability. The 95% confidence level for the specified comparison is depicted by the respective transparent colored areas.

Figure 4

Boxplots of the observed, Chao1, and Inverse Simpson (InvSimp) α diversity indices for the comparison of cecal content and cloacal swab samples.

Figure 5

Figure 5A. Boxplot of the Observed and Inverse Simpson (InvSimpson) measurements for alpha diversity of cecal content samples by Treatment. Figure 5B. Boxplot of the Observed and Inverse Simpson (InvSimpson) measurements for alpha diversity of cloacal swab samples by Treatment.

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