


Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling

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Funding information

Stiftelsen Lunds Djurskyddsfond; Stiftelsen Lars Hiertas Minne; Stiftelsen Långmanska Kulturfonden; Knut och Alice Wallenbergs Stiftelse; Kungliga Fysiografiska Sällskapet i Lund; Helge Ax:son Johnsons Stiftelse; Vetenskapsrådet; Western Cape Government

Abstract

The gut microbiomes of birds and other animals are increasingly being studied in ecological and evolutionary contexts. Numerous studies on birds and reptiles have made inferences about gut microbiota using cloacal sampling; however, it is not known whether the bacterial community of the cloaca provides an accurate representation of the gut microbiome. We examined the accuracy with which cloacal swabs and faecal samples measure the microbiota in three different parts of the gastrointestinal tract (ileum, caecum, and colon) using a case study on juvenile ostriches, *Struthio camelus*, and high-throughput 16S rRNA sequencing. We found that faeces were significantly better than cloacal swabs in representing the bacterial community of the colon. Cloacal samples had a higher abundance of *Gammaproteobacteria* and fewer *Clostridia* relative to the gut and faecal samples. However, both faecal and cloacal samples were poor representatives of the microbial communities in the caecum and ileum. Furthermore, the accuracy of each sampling method in measuring the abundance of different bacterial taxa was highly variable: *Bacteroidetes* was the most highly correlated phylum between all three gut sections and both methods, whereas *Actinobacteria*, for example, was only strongly correlated between faecal and colon samples. Based on our results, we recommend sampling faeces, whenever possible, as this sample type provides the most accurate assessment of the colon microbiome. The fact that neither sampling technique accurately portrayed the bacterial community of the ileum nor the caecum illustrates the difficulty in noninvasively monitoring gut bacteria located further up in the gastrointestinal tract. These results have important implications for the interpretation of avian gut microbiome studies.

KEYWORDS

avian gut microbiome, cloaca, faeces, gastrointestinal tract, microbiota, sampling techniques

1 | INTRODUCTION

The community of bacteria harboured within the gastrointestinal tract of animals – “the gut microbiome” – has been established as an important determinant of host health and physiology (Sekirov, Russell, Antunes, & Finlay, 2010). Although research has largely focused on humans and model organisms, it is becoming increasingly

recognized that the gut microbiome may play an important role in a variety of ecological and evolutionary processes, as it has been associated with disease resistance, behaviour, mate selection, longevity and adaptation (Brooks, Kohl, Brucker, van Opstal, & Bordenstein, 2016; Ezenwa, Gerardo, Inouye, Medina, & Xavier, 2012; Koch & Schmid-Hempel, 2011; Muegge et al., 2011; Sharon et al., 2010; Smith et al., 2017). As a result, it is necessary that accurate methods

for monitoring the gut microbiome in ecologically relevant contexts are developed. To date, multiple studies have focused on the reliability of methods for storing and preserving samples, as well as techniques for processing data from high-throughput sequencing (see, e.g., Debelius et al., 2016; Song et al., 2016). However, it remains unclear whether different sampling techniques accurately represent the bacterial communities in different parts of the gastrointestinal tract.

A large number of studies investigating the gut microbiome of birds and reptiles have sampled bacteria from the cloaca (Allegretti et al., 2014; Barbosa et al., 2016; Bowman & Jacobson, 1980; Charreau, Pérez-Flores, Pérez-Juárez, Cedeño-Vázquez, & Rosas-Carmona, 2012; Cooper, Needham, & Lawrence, 1985; D'Aloia, Bailey, Samour, Naldo, & Howlett, 1996; Dewar, Arnould, Krause, Dann, & Smith, 2014; Dewar et al., 2013; Dickinson, Duck, Schwalbe, Jarchow, & Trueblood, 2001; van Dongen et al., 2013; Ganz et al., 2017; Hoar, Whiteside, Ward, Inglis, & Morck, 2007; Klomp et al., 2008; Kreisinger, Čížková, Kropáčková, & Albrecht, 2015; Lamberski, Hull, Fish, Beckmen, & Morishita, 2003; Lobato, Gerald, Melo, Doutrelant, & Covas, 2017; Lombardo et al., 1996; Lucas & Heeb, 2005; Martin, Gilman, & Weiss, 2010; Matson, Versteegh, van der Velde, & Tieleman, 2015; Maul, Gandhi, & Farris, 2005; Merkeviciene et al., 2017; Mills, Lombardo, & Thorpe, 1999; Moreno et al., 2003; Ruiz-Rodríguez, Lucas, Heeb, & Soler, 2009; Ruiz-Rodríguez, Soler, et al., 2009; Santoro, Hernández, & Caballero, 2006; Santos et al., 2012; Stanley, Geier, Chen, Hughes, & Moore, 2015; Stenkat, Krautwald-Junghanns, Schmitz Ornés, Eilers, & Schmidt, 2014; Xenoulis et al., 2010). Cloacal sampling is widely used because it is straightforward to perform, allows repeated sampling of individuals and affords the possibility of reliably obtaining samples from all individuals at the same time. This method can provide practical advantages over faecal sampling, which may be unreliable and difficult to establish the individual producing the sample and the time of defecation.

It is, however, not known if the microbiota of the cloaca provide an accurate reflection of the bacterial community in the gut and whether cloacal sampling is an accurate alternative to faecal sampling. From a theoretical perspective, there are reasons to believe that the bacterial community of the cloaca is not simply seeded with bacteria from faeces. The cloaca constitutes the single posterior opening for the digestive, reproductive and urinary tract in birds, reptiles, amphibians, sharks, rays and a few mammals and as such represents an important barrier to foreign bodies, including pathogens. For example, during copulation, many bird species engage in a so-called cloacal kiss, where they exchange not only sperm, but also cloacal microbes (Kulkarni & Heeb, 2007; White et al., 2010). In fact, the avian cloaca has a specialized immune organ, the bursa of Fabricius, that is involved in the development of B lymphocytes and antibody production (Warner & Szenberg, 1964), and enables contact between cloacal microbes and the lymphoid system (Schaffner et al., 1974). Furthermore, the cloacal mucosa likely constitutes an environment that is mostly aerobic compared to the anaerobic environment of the gastrointestinal lumen, as is the case for the mammalian rectum (Albenberg et al., 2014; De

Weirdt & Van de Wiele, 2015). Taken together, the proximity of the mucosal cloacal microbiome to both the external environment and host tissue, including secreted mucus with immune cells and antimicrobial molecules, likely results in a microbial environment different from that of the luminal gut, and potentially therefore structural differences in microbiota. Nevertheless, it is not uncommon that the bacterial composition of the cloaca in birds is assumed to be equivalent to that of faeces (Allegretti et al., 2014; Dewar et al., 2013, 2014; Stanley et al., 2015).

In line with the idea that the cloaca may accommodate different bacteria, two studies evaluating cloacal swabs and caecal samples in chickens found large differences in bacterial communities (Stanley et al., 2015; Zhang, Simon, Johnson, & Allen, 2017). It has been argued, however, that cloacal samples may still reflect the presence of the vast majority of caecal bacteria if they are sequenced deep enough (Stanley et al., 2015), and it is unclear whether faecal sampling would provide a more accurate picture. This question raises the issue of whether particular sampling techniques are superior at measuring specific groups of bacteria in the gut microbiome. For example, certain bacterial taxa may be more widely distributed along the gastrointestinal tract and hence easier to monitor, while other taxa may be confined to specific locations in the gut and thus not well represented by any sampling method. Uncovering what attributes of the gut microbiome different types of sampling methods are able to measure, and what they can infer about the microbial communities present in the different sections of the intestinal tract is essential to advance our understanding of host microbiomes.

In this study, we evaluate the accuracy of two commonly used microbiome sampling techniques for birds: cloacal swabs and faecal samples. We test the similarity of the cloacal and faecal microbiomes to three parts of the gastrointestinal tract: ileum, caecum and colon. For this purpose, 20 juvenile ostriches between four to six weeks old were used as a case study.

2 | MATERIALS AND METHODS

2.1 | Study species

We used the ostrich, *Struthio camelus*, kept under controlled conditions at the Western Cape Department of Agriculture's ostrich research facility in Oudtshoorn, South Africa. The samples in this study were obtained in 2014 from a total of 20 juveniles, which included ten individuals four weeks old and ten individuals six weeks old. Ostrich chicks can easily be maintained and handled in an experimental setting, and this specific age group is ideal in size and temperament for both faecal sampling and dissection, allowing us to efficiently retrieve all necessary samples in a standardized way. The chicks were housed and reared with their contemporaries in four separate groups in indoor pens in the same building, containing approximately 35–40 individuals in each group at the time of sampling. During the daytime, they had access to outside enclosures where they could peck freely in soil, and were given *ad libitum* access to fresh water and food.

2.2 | Sample collection

Faecal samples were collected from all chicks one day before scheduled euthanization and dissection, by placing sterile plasters over their cloaca and retrieving the collected fresh faeces approximately one hour later. Two to three chicks were randomly selected from each group for gut sampling, totalling ten individuals per sampling event, one taking place at four weeks of age and one at six weeks. Before dissection, the 20 randomly selected chicks were euthanized by a licensed veterinarian who severed the carotid artery. All procedures were approved by the Departmental Ethics Committee for Research on Animals (DECRA) of the Western Cape Department of Agriculture, reference number R13/90. During dissection, we collected four samples from each individual: cloacal swabs and samples from the ileum, caecum and colon. Cloacal samples were collected using sterile cotton swabs that were briefly moistened in phosphate-buffered saline (PBS), and the tip carefully inserted and rotated in the cloaca of the birds.

To minimize contamination between samples and individuals, a number of precautions were taken. Laboratory benches and surfaces were routinely sterilized with 70% ethanol, and equipment used during the dissection was first cleaned with hot water, then rinsed with 70% ethanol and subsequently placed in the open flame of a Bunsen burner between each sample collection. Control swabs were collected during both dissection events and during the faecal sampling. The control swabs followed the same initial procedure as the cloacal swabs (dipping sterile cotton swabs in PBS), but instead of sampling the bird, they were exposed to potential microbes in the air by waving the wet swab around in the dissection/sampling room. All samples were collected in plastic 2-ml microtubes (Sarstedt, cat no. 72.693) between 28 October and 12 November 2014 and stored at -20°C within two hours of collection. They were subsequently transported on ice to a laboratory and stored at -20°C .

2.3 | DNA isolation, library preparation and amplicon sequencing

We prepared sample slurries for all sample types with guidance from Flores, Henley, and Fierer (2012) and subsequently extracted DNA using the PowerSoil-htp 96 well soil DNA isolation kit (Mo Bio Laboratories, cat no. 12955-4) as recommended by the Earth Microbiome Project (www.earthmicrobiome.org) (for full details please see Supplementary Methods available online). Libraries for sequencing of the 16S rRNA V3 and V4 regions were prepared using the primers Bakt_341F and Bakt_805R (Herlemann et al., 2011) according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Part # 15044223 Rev.B). All samples in this study (Table S3) were sequenced in one 300-bp paired end run on an Illumina MiSeq platform at the DNA Sequencing Facility, Department of Biology, Lund University, Sweden. In a subsequent run, we sequenced blank samples and additional control samples that were collected during the trial for a related project. These control samples were not essential for this particular study, but were included to increase the number

of controls. As a result, a total of 117 different samples plus 54 sample replicates (see Supplementary Methods) were part of this study.

2.4 | Data processing

The 16S amplicon sequences were quality-controlled using FastQC (v. 0.11.5) (Andrews, 2010) together with MultiQC (Ewels, Magnusson, Lundin, & Källner, 2016). Primers were removed from the sequences using Trimmomatic (v. 0.35) (Bolger, Lohse, & Usadel, 2014), and the forward reads were retained for analyses. Quality filtering of the reads were executed using the script `multiple_split_libraries_fastq.py` from QIIME (v. 1.9.1) (Caporaso et al., 2010). All bases with a Phred score <25 at the 3' end of reads were trimmed, and samples were multiplexed into a single high-quality multi-fasta file.

Operational taxonomic units (OTUs) were assigned and clustered using Deblur (v. 1.0.0) (Amir et al., 2017). Deblur circumvents the problems surrounding clustering of OTUs at an arbitrarily threshold by obtaining single-nucleotide resolution OTUs (100% sequence identity) after correcting for Illumina sequencing errors. This approach results in exact sequence variants (ESVs), also called amplicon sequence variants (ASVs), oligotypes, zero-radius OTUs (ZOTUs), and sub-OTU (sOTUs). To avoid confusion, we chose to call these units OTUs, but the reader should be aware that they differ from the traditional 97% clustering approach as they provide more accurate estimates (Callahan, McMurdie, & Holmes, 2017). The minimum reads-option was set to 0 to disable filtering inside Deblur, and all sequences were trimmed to 220 bp. We used the biom table produced after both positive and negative filtering, which by default removes any reads which contain PhiX or adapter sequences, and only retains sequences matching known 16S sequences. This filtering step removed 230 reads matching primarily fungi and ostrich mitochondrial sequences. Additionally, PCR-originating chimeras were filtered from reads inside Deblur (Amir et al., 2017).

Taxonomic assignment of OTUs was performed using the GreenGenes database (DeSantis et al., 2006). We filtered all samples on a minimum read count of 1000 sequences, resulting in three of 171 samples being excluded (one ileum and two control samples). We further filtered all OTUs that only appeared in one sample, resulting in the removal of 8,846 OTUs, with 3,015 remaining. The samples with technical replicates (two control samples and seven individuals with replicates for all sample types; see Supplementary Methods) had the replicates merged within their respective sample type (i.e. ileum.rep1 + ileum.rep2) to increase the amount of valuable sequence information. The analyses were evaluated with both rarefied and nonrarefied data, which produced extremely similar and comparable results. We therefore present the results from the non-rarefied data in this study, as recommended by McMurdie and Holmes (2014).

2.5 | Data analyses

All analyses were performed in R (v. 3.3.2) (R Core Team 2017). We calculated alpha diversity using the Shannon measure with absolute

abundance of reads, and distance measures with the Bray–Curtis dissimilarity method on relative read abundances in phyloseq (v. 1.19.1) (McMurdie & Holmes, 2013). Differences between the microbiota of cloacal and faecal samples to the microbiota of each gut section were examined using permutational multivariate analysis of variances (PERMANOVA) on Bray–Curtis values using the Adonis function in vegan (v. 2.4-2) with 1000 permutations (Oksanen et al., 2017). To analyse if there were differences in the variance (dispersion) between sample types, we used the multivariate homogeneity of group dispersions test (betadisper) in vegan (Oksanen et al., 2017), followed by the Tukey's "honest significant difference" method. Blank and control samples showed highly dissimilar microbial composition to all other sample types (see Figures S1, S2 and S3) and were not included in any further analyses.

To evaluate bacterial abundances, we first filtered out all OTUs with <10 sequence reads and then, using DESeq2 (v. 1.14.1), counts were modelled with a local dispersion model and normalized per sample using the geometric mean (see the DESeq2 manual) (Love, Huber, & Anders, 2014). We examined the strength of the correlations between the abundance of bacteria (normalized OTU abundance), both at the level of phylum and class, in the three parts of the gut in relation to the abundances in both cloacal swabs and faecal samples. Two sets of correlations were performed, one where each data point represented the mean number of OTUs in that bacterial taxon averaged across the 20 individuals ("correlations across bacteria," n = number of OTUs per bacteria phylum or class) and one set of correlations where each data point represented the abundance of a bacterial taxon per individual ("correlations across individuals," n = 20). We used Spearman's rank-order correlation and tested the differences between correlations obtained for cloacal samples and those from faecal samples using cocor (v. 1.1-3) (Diedenhofen & Musch, 2015).

Differential abundances between sample types were subsequently tested in DESeq2 with a negative binomial Wald test using individual ID as factor and with the beta prior set to false (Love et al., 2014). The results for specific comparisons were extracted (e.g. faeces vs. ileum), and p-values were corrected with the Benjamini and Hochberg false discovery rate for multiple testing. OTUs were labelled significant if they had a corrected p-value (q-value) <0.01. Plots were made using ggplot2 (Wickham, 2009).

3 | RESULTS

3.1 | Overall microbiome composition in different sample types

We evaluated the overall pattern of the microbial community reflected by the two sampling techniques (cloacal swabs and faeces) and the three different sections of the avian gastrointestinal tract (Figure 1). The abundance of bacterial taxa in the microbiomes of the caecum, colon and faeces showed large overall similarities (Figure 1a,b,d), especially the faecal and colon samples, which closely clustered in the network plot (Figure 1a). These three sample types

also had the highest and most similar alpha diversity values (colon mean Shannon's diversity index $H = 4.47$, faeces $H = 4.25$, and caecum $H = 4.16$; Figure 1c). Bacteria from the classes *Clostridia* (phylum: *Firmicutes*) and *Bacteroidia* (phylum: *Bacteroidetes*) mainly dominated in the caecum, colon and faeces (Figure 1d). In contrast, the cloacal and ileum samples showed large overall dissimilarities in microbiota composition compared to the other samples types (Figure 1). The microbiome of the cloaca had significantly lower alpha diversity compared to the caecum, colon and faeces ($H = 3.40$, paired Wilcoxon signed rank test against caecum: $V = 37$, $p = .009$; against colon: $V = 4$, $p < .0001$, and against faeces: $V = 17$, $p = .0004$), and so did the ileum ($H = 2.50$, pairwise comparisons against caecum, colon and faeces: $V = 0$, $p < .0001$). The cloaca showed a distinct microbial community from all other samples at the class level with a high relative abundance of *Gammaproteobacteria* and *Bacilli*, and a lower abundance of *Clostridia* (Figure 1d). The ileum also showed higher abundance of *Bacilli* and lower abundance of *Clostridia*, but was overall dissimilar to all other samples with a high representation of *Betaproteobacteria* and few *Bacteroidia* (Figure 1d).

3.2 | Distances between the microbiomes of the cloaca and faeces to the gut sections

To evaluate overall microbiota dissimilarities between the two sample methods to the gut samples, we conducted multivariate analyses of variance (Adonis). All comparisons were highly significantly different from each other (PERMANOVA: $p < .001$), indicating that each sample type harbours a unique microbiome. This result was due to differences in mean distances between communities, not differences in variances, as there was no difference in dispersion between sample types (multivariate homogeneity test of group dispersions: adjusted $p > .152$). The two most similar sample types were the faeces and colon, which resulted in a low R^2 (.07), whereas the cloaca and colon were more dissimilar ($R^2 = .10$). Both sampling methods reflected greater dissimilarities to the gut sections further up in the gastrointestinal tract, with faecal samples being more distant to the caecum ($R^2 = .19$) and the ileum ($R^2 = .16$), as were cloacal samples (caecum: $R^2 = .14$, ileum: $R^2 = .15$).

To directly test how well cloacal swabs and faecal samples represented the microbiota in the gut, we calculated community Bray–Curtis distances between the faecal and cloacal samples to each of the three sections of the gut for each individual (Figure 2). Neither sampling technique was particularly good at measuring the microbiome of the ileum (cloacal mean distance = 0.87, faecal mean distance = 0.84, paired Wilcoxon signed rank test: $V = 136$, $p = .104$) or the caecum (cloacal mean distance = 0.82, faecal mean distance = 0.84, $V = 88$, $p = .546$). However, the distances between faecal and colon samples were significantly shorter than the distances between cloacal and colon samples (cloacal mean distance = 0.74, faecal mean distance = 0.63, $V = 164$, $p = .027$) (Figure 2).

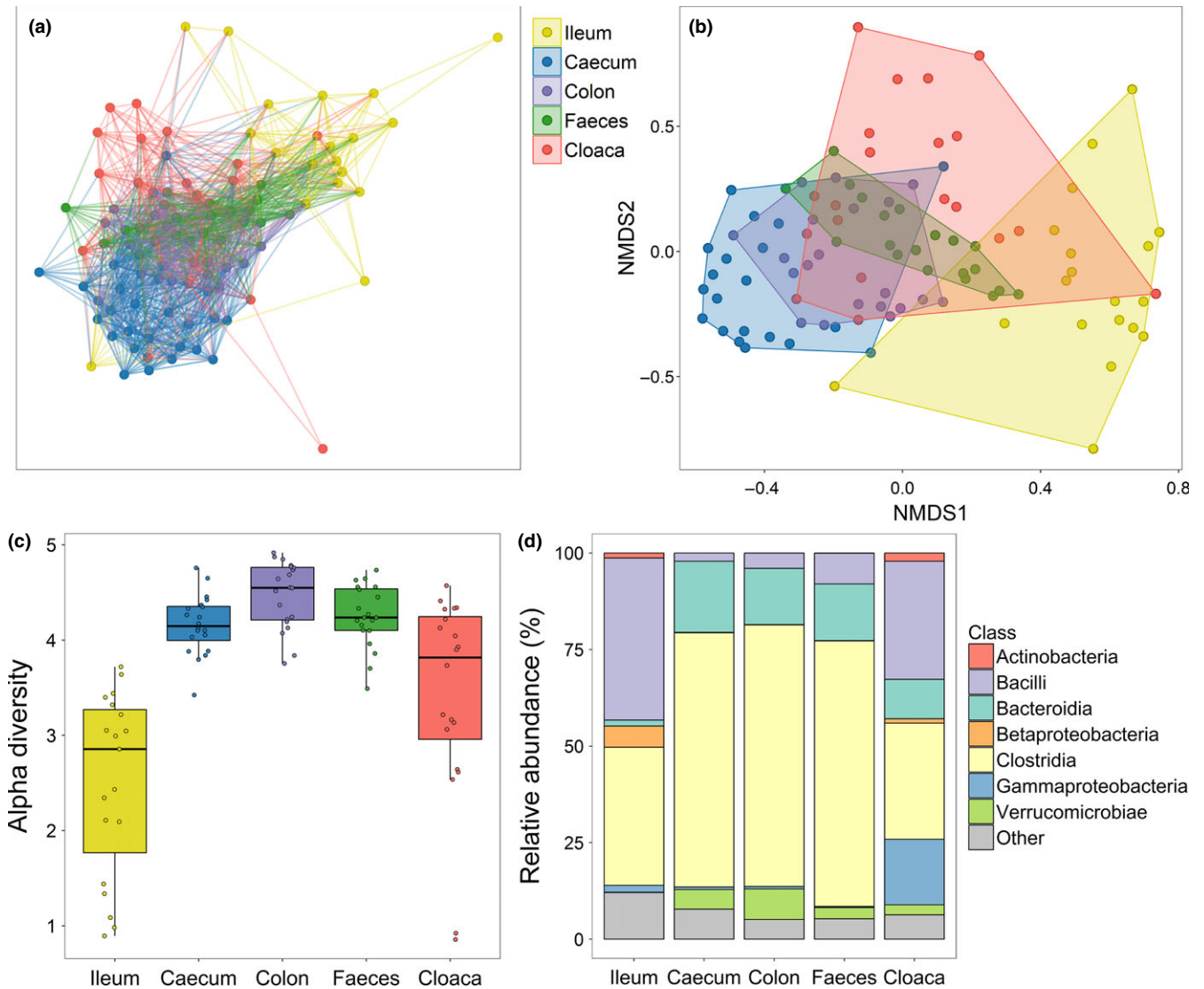


FIGURE 1 Overall microbiota similarities and differences between sample types. (a) Network of Bray–Curtis distances between samples, where colours indicate sample type and lines are drawn to nearest neighbours with a maximum distance of 0.85. (b) Nonmetric multidimensional scaling (NMDS) plot of Bray–Curtis distances between samples, with geometrical shapes encompassing the range of each sample type. (c) Boxplot of Shannon alpha diversity within sample types. (d) The relative abundance of all OTUs for each sample type

3.3 | Correlation of bacterial abundances in the cloaca and faeces with the gut sections

We further evaluated how accurately the sampling techniques represented the abundance of all OTUs in the gut sections and found that the Spearman correlations of both faecal and cloacal samples with the ileum and caecum samples were weak ($r_s = .05$ – $.27$; Figure 3). Conversely, the correlations with the colon were stronger, especially for the faecal samples ($r_s = .56$ vs. $r_s = .48$ for cloacal swabs; Figure 3). When analysing the abundances of different bacterial phyla separately, we again found that the correlations between the sampling methods and the ileum were weak for all six phyla ($r_s < .28$; Table S1). The phyla abundance correlations were stronger for the colon ($r_s = .25$ – $.80$; Table S1), but highly variable for the caecum ($r_s = -.13$ – $.63$; Table S1). Similar patterns of correlation were also

found when analysing abundances across different bacterial classes (Table S2). More specifically, the phylum *Bacteroidetes* had the strongest correlations between both sampling methods and each of the three gut sections (Table S1), and at a lower taxonomic level, the two classes *Bacteroidia* (phylum: *Bacteroidetes*) and *Coriobacteriia* (phylum: *Actinobacteria*) displayed strong correlations between each of the two sampling techniques to both the caecum and colon (Table S2). Overall, the correlations between faecal samples and cloacal swabs to the different parts of the gut were similar with a few exceptions. For example, the abundance of *Actinobacteria* in the colon and caecum appeared to be better represented in faeces, whereas the abundance of *Tenericutes* and *Betaproteobacteria* in the same intestinal regions appeared to be better represented in cloacal swabs.

In addition, we examined how well the abundances of different bacteria correlated between samples from the same host individuals

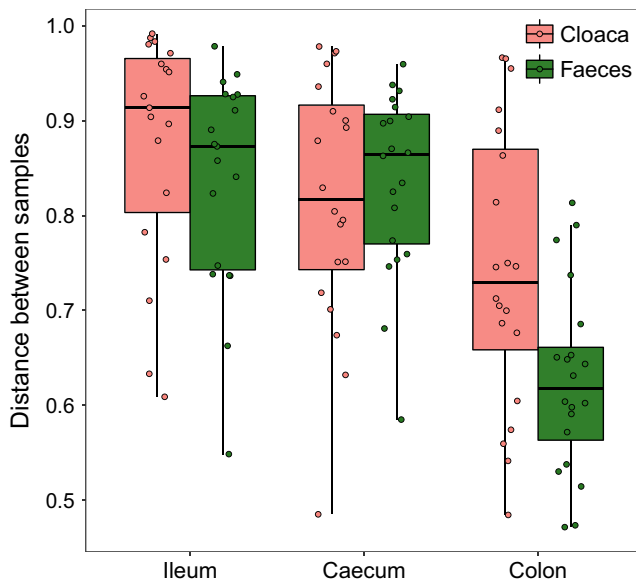


FIGURE 2 Bray-Curtis distances between the microbiota in cloaca and faecal samples compared to the microbiota in three parts of the gut (ileum, caecum, colon) within the same host individual. Higher distance measures indicate higher dissimilarity, where 1 = completely dissimilar bacterial community

(Figure S4). Overall OTU abundance in the ileum was weakly correlated with faeces ($r_s = .16$), but more strongly with cloacal swabs ($r_s = .49$). In contrast, individual faecal samples showed extremely high correlations to both the caecum ($r_s = .87$) and the colon

($r_s = .89$), whereas cloacal samples showed intermediate correlations to the caecum ($r_s = .51$) and colon ($r_s = .54$) (Figure S4).

3.4 | Differences in abundance of specific OTUs in the cloaca and faeces versus the gut sections

Finally, we analysed whether specific OTUs were more or less abundant when using either of the two sampling techniques by testing for significant differences ($q < 0.01$) in OTU abundance in the cloacal and faecal samples compared to the three gut sections (Figure 4; Tables S4–S9). Consistent with our previous analyses, we found the highest number of significantly different OTUs when comparing the ileum to both the faecal ($n = 307$) and cloacal samples ($n = 250$), followed by the comparisons with the caecum (144 significant OTUs for faeces vs. 123 for cloacal swabs). The colon showed the least differences in abundance to both sampling methods, but the cloacal samples had twice as many significant OTUs ($n = 64$) compared to faecal samples ($n = 32$), indicating substantially more differences between cloaca-colon than faeces-colon (Tables S8–S9).

We further examined the taxa that showed significantly different abundances across the six sample comparisons. Relative to the ileum, a large number of OTUs in the class *Clostridia* were significantly more abundant in both faeces and the cloaca (Figure 4; Tables S4–S5). The most significant *Clostridia* families included *Ruminococcaceae*, *Lachnospiraceae*, *Clostridiaceae* and *Christensenellaceae* (Tables S4–S5). The *Enterobacteriaceae* (*Gammaproteobacteria*), the *Verrucomicrobiaceae* (*Verrucomicrobiae*) and several families within the

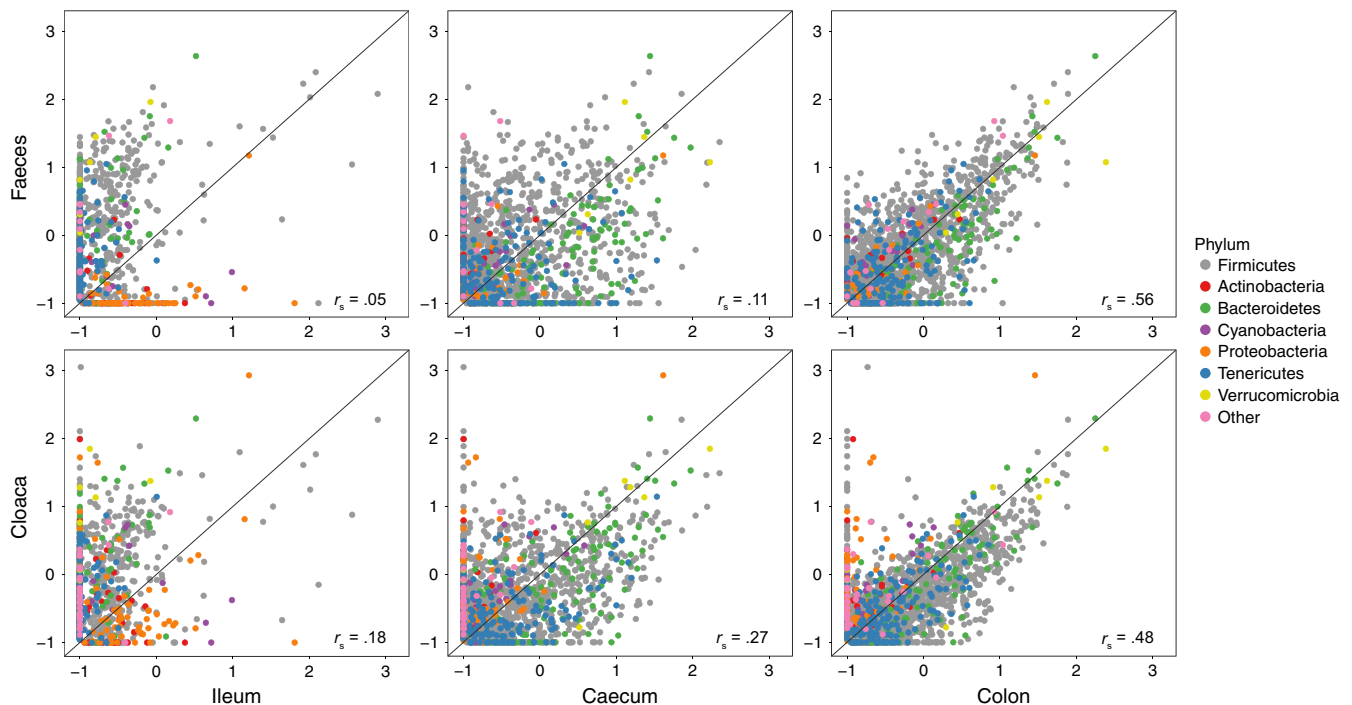


FIGURE 3 Correlations in OTU abundances between each of two sampling methods (cloaca and faeces) compared to three sections of the gut (ileum, caecum and colon). OTU abundances were normalized according to the DESeq2 method and log-transformed (+0.1) for graphical purposes. The straight line shows the 1:1 relationship between the samples, and the r_s values display the Spearman's rank correlation across all OTUs

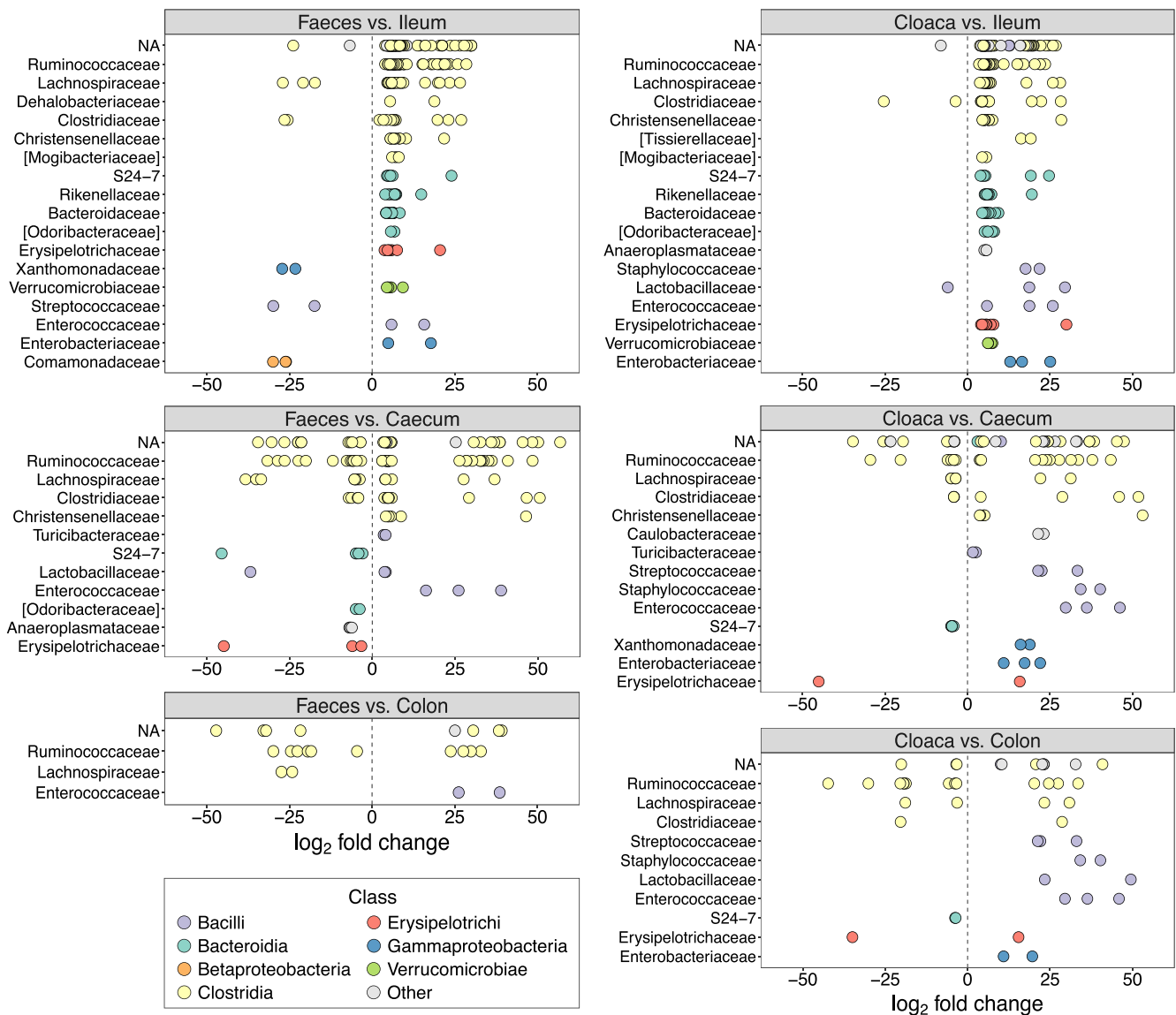


FIGURE 4 OTUs that were significantly different in abundance in the sampling methods (faeces and cloaca) relative to three parts of the gut (ileum, caecum and colon). The y-axes show taxonomic families, and all OTUs have been coloured within their respective class. Positive \log_2 fold changes signify increased OTU abundance in either faeces (left column) or cloacal swabs (right column), and negative \log_2 fold changes display increased abundance in one of the gut sections (ileum, caecum or colon). Families with only a single significant OTU are not shown; the complete data set can be found at Tables S4–S9. Family names in brackets are proposed taxonomies by Greengenes. NA = OTUs without family classification

Bacteroidia were also significantly more abundant in both faeces and the cloaca compared to the ileum. When comparing sampling methods against the caecum, *Clostridia* showed significantly different abundances in both directions (Figure 4). The caecum showed, however, a significantly higher abundance of *Bacteroidia* relative to both the cloaca and faeces, with one exception: an OTU within the *Rikenellaceae*, which was completely absent in the caecum samples but present in both sampling methods. Furthermore, the cloaca had more *Proteobacteria* OTUs that were significantly different ($n = 19$) than faeces ($n = 2$) in the comparison with the caecum, and all but one *Epsilonproteobacteria* were more abundant in the cloacal samples (Figure 4; Tables S6–S7). The last comparison between the colon

and faeces only resulted in 13 significantly different bacterial families within five phyla, while the difference between the colon and cloaca was much larger with a greater phylogenetic breadth, representing 28 significantly different families from 11 phyla (Figure 4; Tables S8–S9).

4 | DISCUSSION

Measuring the gut microbiome of birds and other animals is becoming increasingly important for ecologists and evolutionary biologists due to its potential implications for host fitness. Numerous studies

sample either the cloaca or faeces of birds as a proxy for estimating the bacterial community in the gut. However, it has remained untested whether cloacal or faecal sampling constitute accurate ways of measuring avian gut bacteria. In this study, we examined the microbiota of cloacal swabs and faeces and compared them to the microbiota in three different sections of the gastrointestinal tract in ostriches. We found that cloacal swabs were less accurate at representing the microbiome of the colon compared to faecal samples, which provided more similarities in community composition and abundances of bacteria. Nevertheless, neither faeces nor cloacal swabs could accurately portray the bacterial communities of the ileum and the caecum. In a related study, we additionally found that cloacal swabs had substantially lower repeatability ($r_s = .39$) compared to faecal samples ($r_s = .72$) (Videvall, Strandh, Engelbrecht, Cloete, & Cornwallis, 2017). These results have important implications for the interpretation of bird gut microbiomes, and we hope they will aid researchers in the planning of future studies.

The different sections of the gastrointestinal tract were associated with spatial heterogeneity in their bacterial composition, which is largely expected given their different physiological functions. The ileum is the final part of the small intestine and has a primary role of absorbing nutrients from food while maintaining a neutral pH. In our study, the ileal microbiome had the lowest alpha diversity, which is consistent with other studies investigating the small intestine of birds and reptiles (Bjerrum et al., 2006; Danzeisen et al., 2015; Kohl et al., 2017). It also had the highest relative abundance of *Bacilli* and *Betaproteobacteria* compared to the other sample types. The second sample site of the gastrointestinal tract, the caecum, provides important functions by breaking down plant and fibrous material, and birds typically have two caeca, located between the small and large intestines. Although the caecal samples in our study were dissimilar to other sample types, they clustered most closely with samples from the colon, at least at higher taxonomic levels. Both of these intestinal regions had high abundances of *Clostridia* and *Bacteroidia*. In comparison with both faecal and cloacal samples, the caecum had a significantly higher abundance of several *Bacteroidetes*, similar to previous research on the chicken caecum (Stanley et al., 2015). The final part of the intestinal tract, the colon, has a primary function to absorb water and salt from ingested material. The colon samples in our study had the highest alpha diversity of all sample types and the strongest taxa correlations to both sampling methods, although significantly higher to faeces than to the cloaca.

The similarities of both the cloacal and faecal microbiota to that of the gut increased the further down the gastrointestinal tract we sampled, as perhaps expected given the proximity to the cloaca. Nevertheless, there was substantial variation in how well bacterial abundances in different parts of the gut correlated with faecal and cloacal samples across different taxonomic groups, both when examining across individual OTUs, and across individual hosts. This variation does not appear to be simply explained by differences in the total abundance of different bacteria (e.g. more abundant bacteria might be more widely distributed in the gut and

so more strongly correlated across samples), as some classes of bacteria had high numbers of OTUs, but were poorly correlated and vice versa (Table S2). The strength of correlations between different sample types may potentially reflect the fact that different bacteria vary markedly in the environmental conditions they can tolerate, and hence the breadth of their spatial distribution in the gut. The causes underlying this variation require further investigation, but by presenting effect sizes of the strength of associations we hope to provide useful information on which bacteria can reliably be monitored in different locations of the gastrointestinal tract (Tables S1–S2).

A common goal of microbiome studies, particularly in ecological contexts, is to understand how gut bacteria relate to phenotypic variation. Because it is not feasible to collect intestinal samples in wild birds without highly invasive techniques, faecal or cloacal samples are often the only options, especially if repeated sampling is required. Our results suggest that the bacterial communities in the upper and middle gastrointestinal tract are distinct from those recovered by noninvasive sampling methods, and as such, any inferences made about the gut microbiome and its relationship to phenotypic variation may only be possible for processes occurring in the colon. Further studies are needed to investigate if the results of this study hold true across the avian phylogeny in ecologically and physiologically different bird species, and in other animals with cloacae, such as frogs, lizards and egg-laying mammals. Most mammals possess a rectum instead of a cloaca, which differs in both function and physiology, and rectal swabs are therefore likely to differ substantially to cloacal swabs in the degree to which they are useful for monitoring gut microbiomes. The current evidence as to whether rectal swabs constitute a representative sampling method of the gut microbiome of mammals is conflicting and suffers from low sample sizes, thus warranting additional evaluation (Alfano et al., 2015; Bassis et al., 2017; Budding et al., 2014).

In conclusion, for gut microbiome sampling of birds, we recommend faecal samples whenever possible, as this sampling procedure best captured the bacterial community of the colon.

ACKNOWLEDGEMENTS

We are grateful to all the staff at the Oudtshoorn Research Farm, Western Cape Government and Naomi Serfontein, Maud Bonato and Julian Melgar for assisting during the collection of samples. Adriaan Olivier, Klein Karoo International, generously provided instructions on dissection and performed euthanization of chicks. Thomas Johansson performed the sequencing, and Paul McMurdie, Michiel Op De Beeck, Se Jin Song and Amnon Amir provided valuable advice on initial analyses. This research was funded by research grants from the Helge Ax:son Johnson Foundation, the Långmanska Cultural Foundation, the Lund Animal Protection Foundation, the Lars Hierta Memorial Foundation and the Royal Physiographic Society of Lund to E.V., by a Wallenberg Academy Fellowship and Swedish Research Council (VR) grant to C.K.C. and by the Western Cape Government. Conflict of interest: none declared.

AUTHOR CONTRIBUTIONS

E.V. and C.K.C. planned the study design. S.C. provided animal facilities and A.E. supervised the experiment. A.E., C.K.C. and E.V. collected the samples. M.S. planned and performed the laboratory work. E.V. performed the analyses and wrote the paper with assistance of C.K.C. All authors reviewed and approved of the final manuscript.

DATA ACCESSIBILITY

Supporting information has been made available online. Sequences have been uploaded to the European Nucleotide Archive (ENA) under Accession no.: PRJEB22640.

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SUPPORTING INFORMATION

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How to cite this article: Videvall E, Strandh M, Engelbrecht A, Cloete S, Cornwallis CK. Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. *Mol Ecol Resour*. 2017;00:1–11. <https://doi.org/10.1111/1755-0998.12744>