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RESOURCE ARTICLE

Comparing invasive and noninvasive faecal sampling in wildlife microbiome studies: A case study on wild common cranes

Sondra Turjeman[1] | Sasha Pekarsky[1] | Ammon Corl[2] | Pauline L. Kamath[3] | Wayne M. Getz[4,5] | Rauri C. K. Bowie[2,6] | Yuri Markin[7] | Ran Nathan[1]

[1]Movement Ecology Laboratory, Department of Ecology, Evolution & Behavior, Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

[2]Museum of Vertebrate Zoology, University of California, Berkeley, California, USA

[3]School of Food and Agriculture, University of Maine, Orono, Maine, USA

[4]Department of Environmental Science, Policy & Management, University of California, Berkeley, California, USA

[5]School of Mathematical Sciences, University of KwaZulu-Natal, Durban, South Africa

[6]Department of Integrative Biology, University of California, Berkeley, California, USA

[7]Oksky State Reserve, pos., Brykin Bor, Spassky Raion, Ryazanskaya Oblast, Russia

Correspondence
Sondra Turjeman, Sasha Pekarsky and Ran Nathan, Movement Ecology Laboratory, Department of Ecology, Evolution, and Behavior, Alexander Silbertman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel.
Emails: sondra.turjeman@mail.huji.ac.il (S. T.); sasha.pekarsky@mail.huji.ac.il (S. P.) and ran.nathan@mail.huji.ac.il (R. N.)

Present address
Sondra Turjeman, Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel

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Abstract

In ecological and conservation studies, responsible researchers strive to obtain rich data while minimizing disturbance to wildlife and ecosystems. We assessed if samples collected noninvasively can be used for faecal microbiome research, comparing microbiota of noninvasively collected faecal samples to those collected from trapped common cranes at the same sites over the same periods. We found significant differences in faecal microbial composition (alpha and beta diversity), which likely did not result from noninvasive sample exposure to soil contaminants, as assessed by comparing bacterial oxygen use profiles. Differences might result from trapped birds' exposure to sedatives or stress. We conclude that if all samples are collected in the same manner, comparative analyses are valid, and noninvasive sampling may better represent host faecal microbiota because there are no trapping effects. Experiments with fresh and delayed sample collection can elucidate effects of environmental exposures on microbiota. Further, controlled tests of stressing or sedation may unravel how trapping affects wildlife microbiota.

KEYWORDS
avian microbiome, common crane, microbial community characterization, noninvasive sampling, stress, trapping

Sondra Turjeman and Sasha Pekarsky contributed equally.

1 | INTRODUCTION

Microbiome research in nonmodel species has proliferated in the past decade. A number of studies on diverse species have shown that endogenous microbiota can be used to assess host state (Peixoto et al., 2021). In most cases, studies are based on the faecal microbiome as extracting gut tissue is not realistic in the wild – animals may be harmed during biopsy collection, and aside from immediate risks, behaviours including movement and reproductive strategy, cannot be accurately associated with microbiota when invasive procedures are used. Accordingly, swab samples align less closely with gut microbiota than faecal samples, at least in birds (Videvall et al., 2018). Microbiota manipulation (faecal microbiota transplant), already a successful tool in treating some human maladies, is being examined in wildlife as a treatment option (Guo et al., 2020; Niederwerder, 2018). Studies have suggested the presence of a core microbiota that has coevolved with the host species (Risely, 2020) and have also revealed that much of a host's microbiota is relatively dynamic in composition, with shifts in response to environment, diet, and other changes in physiology and life-history. Thus, there are many potential applications of microbiota profiling in conservation ecology (Trevelline et al., 2019). Populations can be monitored for overall health (Peixoto et al., 2021) and for the presence/absence of specific pathogens (Choi et al., 2021), and compositional comparative analyses—or even interventions—of captive and wild populations' (Gibson et al., 2019; Oliveira et al., 2020) can be made prior to reintroductions. Recently, even reproductive health and potential breeding success of wild animals was assessed from microbiota profiling (Comizzoli & Power, 2019).

While faecal microbiota profiling can be a powerful tool in humans and animals alike (Turjeman & Koren, 2021), trapping and handling animals poses challenges to conversation research. Often, animals are difficult to trap, efforts can be cost- and resource-prohibitive, and many species do not withstand the stress of trapping well (Beja-Pereira et al., 2009). Further, stress associated with trapping and handling can affect the microbiome (Collins & Bercik, 2009), even if it does not seem to visibly harm the target species, thus skewing findings. In other realms of conservation biology—population genetics, evaluation of mating systems, species surveys and counts—noninvasive sampling and environmental sampling combined with molecular tools have provided powerful means to study animals while keeping disturbances to a minimum (e.g., Russello et al., 2015). Here we assess the utility of noninvasive sampling in faecal microbiome studies by comparing the composition of faecal samples freshly defaecated from trapped common cranes (*Grus grus*) to noninvasively collected samples of different cranes collected in the same area. Despite faecal profiling's ability to only approximate the microbiota of the large intestine, this is currently the method most used in ecological and conservation studies (e.g., Pannoni et al., 2022) as well as in preclinical and clinical settings, and thus the faecal, rather than the gut, microbiota is the focus of our study. We detected non-negligible differences in the microbial composition of faeces collected using the two sampling methods, but we also found that the total number of represented taxa for each method were both high and similar, suggesting that if all samples are collected in the same noninvasive manner, comparative analyses between populations or across time can be performed. Of note, our noninvasive method is truly noninvasive – handing of animals is not required for any parts of the method unlike (Knutie & Gotanda, 2018) and (Pannoni et al., 2022) who collected faecal samples from trapped birds and elk, respectively.

2 | MATERIALS AND METHODS

2.1 | Sample collection

We obtained samples for this project as part of a wider study of common crane movement, foraging, and microbiome (Pekarsky et al., 2021). During the premigration period (early fall), cranes stage in western Russia (Ryazan area; 54°56′N, 41°02E) for several weeks before the onset of migration and feed mostly on residual grain left at fields from agricultural monocultures, thus providing them with a stable food resource (Pekarsky et al., 2021). We (Johnsgard, 1983; Leito et al., 2015) trapped 27 cranes during this period in 2017 using bait mixed with alpha-chlorolose, a routine oral sedation technique associated with low morbidity and mortality (Hartup et al., 2014). After trapping, birds were hooded, banded, GPS-tagged, and measured. Then hoods were removed, and birds were held under constant supervision until complete recovery from sedation. When they defaecated onto the ground (usually soon before recovering from sedation and flying away), we sampled the inner portion of the fresh faeces using sterile cotton swabs, stored it immediately in 95% EtOH at −20°C, and then transferred it to −80°C for long-term storage. The average time elapsed between trapping and sample collection was 405 min (range 39–1278 min, based on when GPS data-loggers recorded locations beyond the trapping site). In parallel, we collected 37 faecal samples from the ground following observations of birds defaecating in fields near the trapping site in the 2 weeks prior to and the 2 weeks during trapping. When a flock of cranes was observed, we scattered them by approaching the flock and then collected samples from individual droppings (>30 cm apart) as above. We visually examined samples to assess freshness by looking for moist and structurally intact droppings. We assume that samples were not from trapped birds, as GPS data suggested tagged birds were not present at the immediate site of sampling, and there were hundreds of birds in the staging area during this period, but the possibility cannot be ruled out completely. If we inadvertently sampled a subset of birds with both methods, we might observe slightly greater overlap in the two sampling-methods' microbiota profiles, but this should not confound results. Sampling efforts were spread throughout daylight hours for both methods. Post-sampling, samples were handled and stored identically.

2.2 | Microbial sample sequencing and seqpreprocessing

Sample extraction and processing for 16S rRNA metabarcoding is described in Pekarsky et al. (2021). Briefly, we PCR-amplified the V4 region of the 16S rRNA in triplicate for sequencing on an Illumina MiSeq (see Supporting Information text). In total, 64 samples were sequenced for this study. Data was processed in R version 4.1.1 to identify amplicon sequence variants (ASVs) as described in Pekarsky et al. (2021) following the protocols detailed in Callahan, Sankaran, et al. (2016). Briefly, the first 10 bases of each read were trimmed, and we filtered the reads to a maximum of two expected errors per read; reads with any N bases were excluded; and reads were truncated at the first base with a quality score ≤ 2. We used DADA2 (Callahan, McMurdie, et al., 2016) to infer ASVs from the pooled data across all samples and with substitution error rates estimated from a random subset of 40 samples from the sequencing lane. We then merged the forward and reverse reads (paired end reads) and excluded chimeric sequences. The SILVA taxonomy database (Glöckner et al., 2017; Pruesse et al., 2007; Quast et al., 2012) was used to annotate the ASVs using a SILVA version 132 training set. The sequence data was aligned with DECIPHER (Wright, 2015) and then a maximum likelihood phylogeny was inferred using phangorn (Schliep, 2011). Data was then merged into a phyloseq object (McMurdie & Holmes, 2013) for further analysis.

We used the package decontam (Davis et al., 2018) to identify and remove 27 contaminant ASVs (prevalence method, threshold: 0.5) based on three workflow negative controls and two PCR blank samples sequenced in the same run. We removed sequences that could not be assigned to a phylum and those classified as mitochondria or chloroplasts. We compared read depth between sampling methods using, as appropriate, *t*, Mann–Whitney *U*, and Kolmogorov–Smirnov tests. The median number of reads per individual across all samples following filtering was 14,397.5 (range: 5337–25,670).

2.3 | Comparison of microbiota

Analyses, unless otherwise specified, were performed using the phyloseq (McMurdie & Holmes, 2013) and microbiome (Lahti & Shetty, 2019) R packages. After examining rarefaction curves, we rarefied data to 8000 reads to optimize the trade-off between read depth and sample size (3 samples lost; final read depth 8,044; phyloseq, seed: 999). We compared alpha diversity of the microbiota in faecal samples from trapped birds to that of birds sampled noninvasively using Faith's phylogenetic diversity index (btools; Battaglia, 2021) and raw observed richness with Mann–Whitney *U* tests. Differences in community composition were measured using both weighted and unweighted UniFrac measures (phylogeny-based) and compared with PERMANOVAs (10,000 permutations) Bray-Curtis and Jaccard distance metrics were also examined, and results were consistent. We also examined differences in demographics (age, sex) of trapped birds using PERMANOVAs of UniFrac measures calculated only for the trapped group. Within-group dispersion was assessed with the vegan package (Oksanen et al., 2018); if dispersion is unequal, differences between groups, as identified by PERMANOVA, may result from individual dispersion patterns rather than the microbial composition differences. The *betadisper* function was used with 10,000 permutations; no bias correction was needed as sample sizes were sufficiently large and balanced (Anderson, 2006). Differentially abundant taxa were identified with ANCOM-BC (Lin & Peddada, 2020). We used a minimum prevalence filter of 10% and an FDR threshold of 0.05 when identifying significantly differentially abundant taxa. A heatmap based on significantly different genera with an absolute log-fold-change >0.58 was produced using pheatmap (Kolde, 2019). After identifying core microbiota for samples from each of the sampling methods, using a minimum threshold of 10% prevalence, we used eulerr (Larsson & Gustafsson, 2018) to generate a Venn diagram.

3 | RESULTS

Analyses were based on 61 faecal samples following rarefaction (775 ASVs lost, 28.3% of total ASVs), 25 samples from trapped birds and 36 noninvasive samples. There was no difference in mean sequencing depth or sequencing depth distribution between the two sampling methods prior to rarefaction (*t* test: $p = .17$; Kolmogorov Smirnov: $p = .13$; Figure 1), suggesting ASV loss between the groups was largely unbiased. Following rarefaction to 8,044 reads, comparison of alpha diversity using Faith's PD (Mann–Whitney: $p = .0009$) and observed richness (Mann–Whitney: $p = .0003$) revealed that faecal samples from trapped birds consistently had greater alpha diversity than those sampled noninvasively from free-foraging cranes (Figures 2a,b). There were significant differences in sample composition (phylogenetic beta diversity) of crane microbiota between the two methods, assessed with both weighted (PERMANOVA: $p < .0001$; Figure 2c) and unweighted (PERMANOVA: $p < .0001$; Figure 2d) UniFrac. Notably, community dispersion using the weighted measure was significantly greater among trapped birds (*betadisper*: $p = .034$; Figure 2c), but this pattern was not preserved with the unweighted metric (*betadisper*: $p = .29$; Figure 2d). The same pattern was observed for PERMANOVA and dispersion analyses of the Bray-Curtis (PERMANOVA: $p < .0001$, *betadisper:* $p < .0001$) and Jaccard (PERMANOVA: $p < .0001$, *betadisper:* $p = 3513$) distances.

Visual examination of community composition at the phylum level supports our findings of differences between the microbiota of trapped and noninvasively sampled cranes (Figure 3a), and differential abundance analysis identified differentially abundant taxa, both at the phylum (Figure 3b) and genus (Figure 3c) levels. We examined the genus-level differences in light of both within-group relative abundance and within-group prevalence (Figures 3c,d, Table S1) and found that only four of the differentially abundant taxa had a relative abundance greater than 0.1%. Similarly, less than one third of significant genera had a within-group prevalence above 90%. In total, we identified 28 different phyla in our samples, of which, 10 (36%) differed

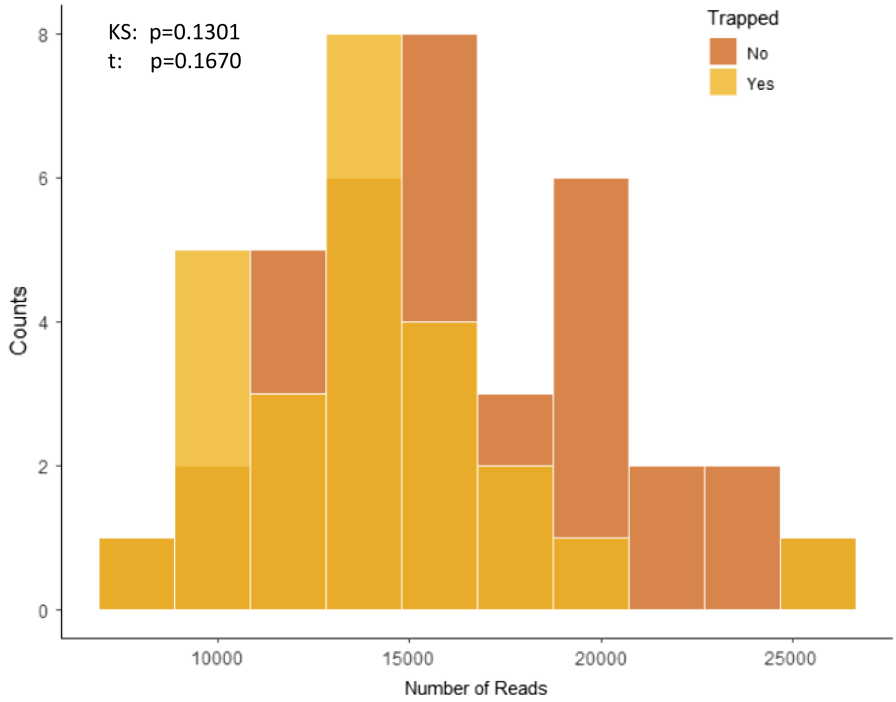


FIGURE 1 Sequencing depth for each sampling method. No differences in sequencing depth were found between samples collected from trapped birds (yellow) versus those collected noninvasively from free-ranging birds (orange).

between the groups in their relative abundances. Of the 444 genera identified, relative abundances for only 23 (6%) differed. When comparing a "core" of genera found in at least 10% of samples per group (a combined 182 genera), we found that ca. 60% overlapped between sampling methods, whereas c. 7% were only found in the noninvasive samples and ca. 32% were only found in trapped samples (Figure 3e). We also examined the effect of various minimum relative abundance thresholds (weighted UniFrac: 0.1%: $p < .0001$, 1% $p < .0001$) and ran analyses at the genus level (weighted UniFrac: $p < .0001$). In all cases, the different sampling methods resulted in significant differences in microbiota composition. Further, when examining the microbiota of the samples collected directly from trapped birds, we did not find evidence of age (weighted UniFrac: $p = .98$) or sex (weighted UniFrac: $p = .44$) stratification in the trapped birds' microbiota.

4 | DISCUSSION

Despite differences between the microbiota from trapped and noninvasively sampled cranes, our results indicated that both methods can be used as a source of microbial matter for 16S rRNA metabarcoding. We did not find differences in sampling depth across sample types. We found consistent differences, though, in bacterial richness and composition, with the samples from trapped birds exhibiting significantly higher richness and dispersion in overall composition when considering both bacterial lineages and their abundances. We thus conclude that the two sampling methods can yield different microbiota profiles for the same population.

Overall, 16S rRNA sequencing of the two sample types resulted in similar sequencing depths and a comparable number of ASVs (trapped: 1403, noninvasive: 1272). Of the prevalent genera (a subset of ASVs shared by >10% of birds), 60% were shared across the sampling methods, suggesting a core microbiota can be successfully identified using either method. This is impressive given the high intraindividual diversity previously reported for common cranes (Pekarsky et al., 2021). Similarly, most of the differentially abundant genera were low-abundance taxa, again suggesting that either method can be used to identify many of the most common core microbial taxa. Removing rare taxa and rerunning analyses did not change our results though, suggesting that while we can define a coarse core microbiota, the two sampling methods differ for some relatively common taxa (1% relative abundance, genus-level), which suggests that variation between the methods is not just due to random differences in sampling or sequencing of rare taxa.

We next considered the differentially abundant taxa to better understand potential effects of sampling methods on microbial community composition. Of the 23 differentially abundant genera, only seven were found in >90% of trapped bird samples and four were found in >90% of noninvasive samples; thus, the differentiating taxa are neither highly prevalent nor extremely conserved. Looking at the overall abundances of these taxa, the picture is similar: only three genera have a relative abundance >0.1%. Thus, most of the differentiating taxa appear to be somewhat rare. Rare taxa, though, can have important roles in the microbiota both in specific functionalities and in maintaining overall gut and microbial community homeostasis (Banerjee et al., 2018). We therefore conclude that our differential abundance analysis, together with the consistent differences in alpha- and beta-diversity, demonstrates that different sampling methodology may capture different microbiota. While we may be able to define common and even temporally stable (in cases of longitudinal sampling) core faecal microbiota, it could be difficult to reach conclusions regarding the ecological, functional, and host-adapted cores (Risely, 2020). Our findings contrast with those of a recent study examining trapped and caged small mammal microbiota

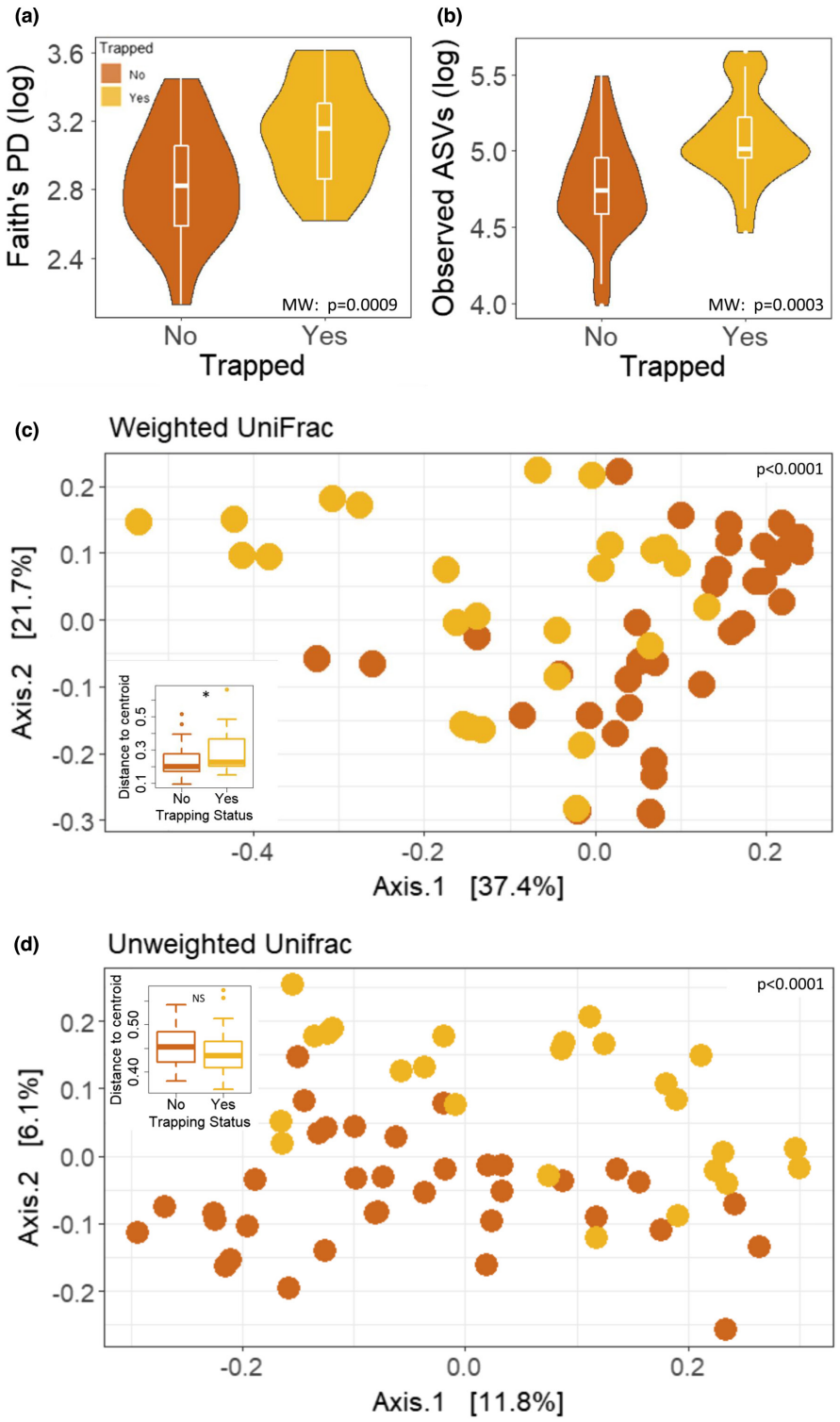
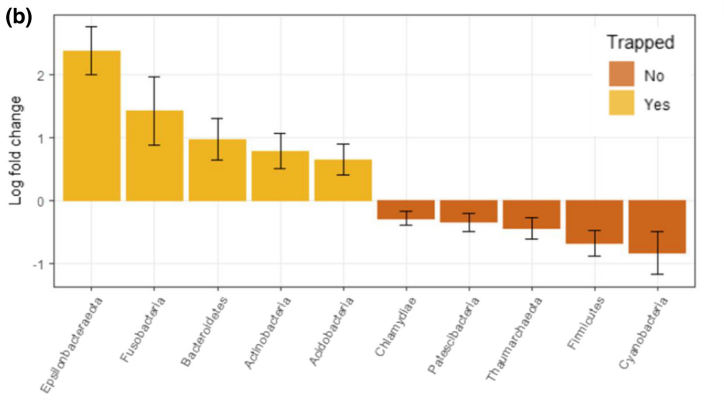


FIGURE 2 Comparison of microbiota for each sampling method. Samples collected from trapped birds (yellow) had significantly higher diversity (a) and richness (b) compared to noninvasive samples (orange). Furthermore, the two methods resulted in significantly separated communities in the PCoA space when using both weighted (c) and unweighted (d) UniFrac metrics. Dispersion of the groups (insets) was significantly different (*) when using the weighted (c) but not unweighted (d) method, with trapped bird samples showing a more dispersed cluster of microbiota than noninvasive samples. Boxplots in violins: Boxes represent medians with first and third quartiles and whiskers represent maxima and minima.

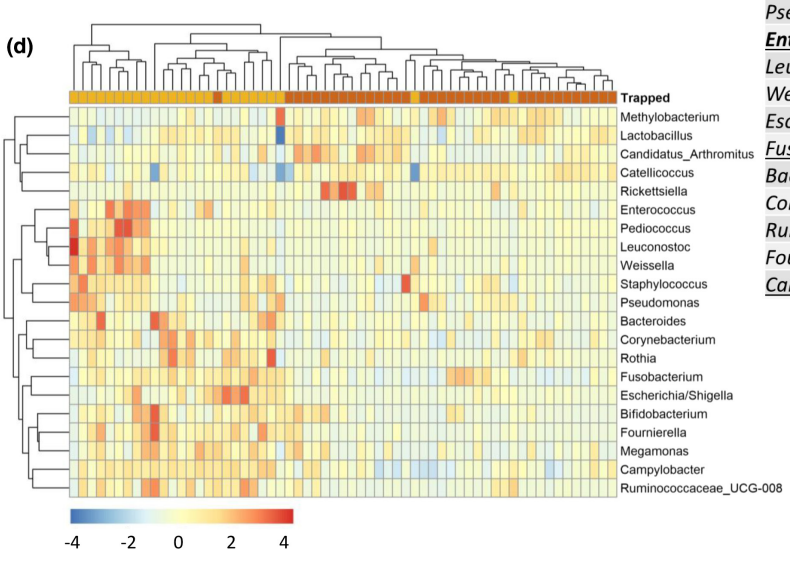
in a laboratory model, which concluded that despite some differences in specific taxa, sampled communities were relatively similar. Laboratory mice were euthanized either from their home cages (no treatment) or following exposure to stress via live trapping (16–18 h in a wooden live trap with food but no water) and caecal microbiota were compared. There were no differences in comparisons of alpha diversity or unconstrained comparisons of beta diversity between the groups (Čížková et al., 2021); this could be due to high stress thresholds of laboratory mice or sampling method.

A previous study examining faecal samples exposed to natural weather conditions for 0–6 days found decreased anaerobes and increased facultative aerobic and aerobic taxa (Menke et al., 2015). In our study, however, samples were collected within several hours of defaecation in both data collection methods. Therefore, the decrease in alpha diversity among noninvasive samples does not support a hypothesis of increased environmental contamination. Further, even if anaerobic bacteria are not viable in noninvasive samples, their DNA will still be present in sequencing results (degradation of the DNA



(c)

Genus	W	p-val	q-val
Williamsia	**-3.7574**	**<0.0001**	**<0.0001**
Catellicoccus*	-3.2443	0.0011	0.0136
Paenisporosarcina	-3.2088	0.0013	0.0136
Methylobacterium	-2.7218	0.0063	0.0430
Rickettsiella	-2.7151	0.0065	0.0430
Candidatus_Arthromitus	-2.7148	0.0065	0.0430
Lactobacillus*	-2.6948	0.0069	0.0431
Bryobacter	1.8274	<0.0001	<0.0001
Arthrobacter	2.0513	<0.0001	<0.0001
Pediococcus	2.6328	<0.0001	<0.0001
Bifidobacterium	2.7491	0.0060	0.0430
Staphylococcus	2.8682	0.0042	0.0343
Megamonas	2.8700	0.0042	0.0343
Rothia	2.9375	0.0034	0.0311
Pseudomonas	3.0323	0.0025	0.0243
Enterococcus	3.2386	0.0012	0.0136
Leuconostoc	3.3311	0.0009	0.0121
Weissella	3.4366	0.0006	0.0093
Escherichia/Shigella	3.4808	0.0005	0.0090
Fusobacterium	3.7620	0.0002	0.0034
Bacteroides	3.8452	0.0001	0.0034
Corynebacterium	3.9280	0.0001	0.0030
Ruminococcaceae_UCG-008	4.4330	<0.0001	0.0004
Fournierella	5.3984	<0.0001	<0.0001
*Campylobacter**	5.9159	<0.0001	<0.0001



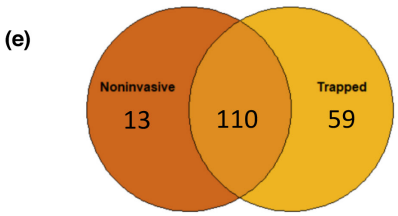


FIGURE 3 Differentially abundant taxa. We examined differentially abundant taxa between the microbiota of trapped (yellow) and noninvasively sampled free-ranging (orange) cranes at the phylum (a, b) and genus (c) levels. Individual bar plots (a) suggest sampling method-specific differences at the phylum level, confirmed by ANCOM-BC (b). A positive log-fold-change denotes bacterial taxa relatively increased among trapped samples and a negative log-fold-change denotes those relatively increased in noninvasive samples. Only significant taxa following FDR correction are shown. At the genus level (c), we found 23 significant taxa. A positive W denotes the abundance of genera that were increased in the trapped group, and a negative W denotes the abundance of genera that were increased in the noninvasive group. Genera in bold are found in at least 90% of the noninvasive samples and those underlined are found in at least 90% of trapped bird samples. *denotes mean relative abundance of >0.1%. A heat map of the significant taxa (d) shows clustering of the noninvasive samples together based on differentially abundant genera. The colour bar denotes normalized, standardized abundances (from ANCOM-BC). Only genera with a log-fold-change >0.58 are included. In total, we found (e) 110 shared genera when using a minimum threshold of 10% (for a given sampling method) and 13 unique genera for the noninvasive samples versus 59 unique genera for the trapped samples.

in the short post-defaecation window is minimal) such that the argument of a trade-off between anaerobes and contaminants is not valid her. This is also supported by our examination of oxygen-use profiles of the two sampling methods' prevalent genera (>10%; downloaded from https://bacdive.dsmz.de/) which did not reveal increased aerobes in the noninvasive microbiomes (see Table S2 for method and comparison). To further assess if differences in the noninvasive samples' microbiota were derived from extended exposure to aerobic conditions or contaminants, we manually examined functionality of the differentially abundant genera (Figure 3c) overexpressed in noninvasive samples. *Catellicoccus* is a facultative anaerobe that has previously been found in gull faeces (Koskey et al., 2014). Similarly, *Methylobacterium* and *Candidatus arthromitus* have also been found in birds (Escallón et al., 2019) and other animals (Snel et al., 1995), though the former is also found in soil and water and could be a contaminant. *Paenisporosarcina* is an aerobic genus previously isolated from soil and *Williamsia* is also an opportunistic aerobe. Interestingly, *Lactobacillus*, an anaerobic genus, was enriched in the noninvasive samples, though there were more lactic acid bacteria and anaerobes among the significantly differentially enriched genera of the trapped samples. In contrast, we did find two genera overrepresented in the trapped samples, *Bryobacter* and *Arthrobacter*, that are typically found in soils. Together, we conclude that contamination is likely not the main driver of differentiation, especially considering all samples (trapped and invasive) were collected off the ground from the middle of the droppings.

Because soil contamination did not appear to be a main driver of microbiota differences, we considered a second source of microbiota differentiation: stress associated with trapping and handling. In our case, specifically, birds were not only exposed to stress, but also to a sedative. While we cannot easily differentiate between these two external pressures' effects on the microbiome, both can bias microbiota profiling: Stress effects on microbiota have been examined in a number of animal species (Noguera et al., 2018; Stothart et al., 2016), and anaesthesia in mice was also found to have rapid (4 h) effects on microbiota composition (Serbanescu et al., 2019). Similarly, sedated birds may defaecate less frequently, which may affect microbiota composition (Vandeputte et al., 2016). Of note, none of the taxa overrepresented in our trapped crane samples were enriched in yellow legged gulls (*Larus michahellis*) experimentally implanted with corticosterone. Rather, several, *Pseudonomas* and *Campylobacter*, enriched in our trapped samples, were underrepresented in the corticosterone-implanted birds (Noguera et al., 2018). Simiarly, *Ruminococcaceae*, enriched in our trapped samples, is typically associated with reduced stress. Their abundance may be an immediate effect of alpha-chlorolose used in trapping. We did not find a correlation between phylogenetic alpha diversity (Faith PD) or phylogenetic beta diversity and time until departure from the trapping site ($p > .05$) which can serve as a proxy for time between sedation and defaecation/sample collection, but differences in recovery time could have caused the increased dispersion observed in the trapped birds' samples compared to the noninvasive ones.

Limitations of our study include small sample size and lack of dedicated environmental control samples. Controlled experiments with captive animals—collecting faeces via swab, fresh following excretion, and at various increments following defaecation—should shed light on how samples change post-defaecation, though some studies suggest that short delays in freezing are not critical (Al et al., 2018; Bassis et al., 2017). Careful environmental sampling (soil, air) in conjunction with noninvasive sampling may also allow researchers to filter taxa that proliferated during the time to collection. These methods could also be combined with findings from controlled stressing or sedating experiments in wild or semi-wild settings towards better understanding how stress affects the microbiota of nonmodel organisms.

In summary, we found significant differences among faecal microbial communities revealed by sampling trapped versus non-trapped common cranes in the same sites over the same temporal periods. Some differences may be associated with soil contamination, but others seem to reflect effects of trapping. While sedation might be a species-specific practice in wildlife research, trapping has been broadly applied, and potential effects of trapping (with and without sedation) on microbiota composition should be considered. Further, in some cases, trapping is cost prohibitive (Sutherland et al., 2004) or can endanger fitness, especially problematic for rare or endangered species (Blomberg et al., 2018; Dennis & Shah, 2012; Spotswood et al., 2012). Importantly, our results suggest that the potential contamination of noninvasive samples might not be substantial, supporting expanded use in conservation ecology. Noninvasive sampling will promote animal welfare while allowing for sampling of a microbiome that presumably more closely represents the faecal microbiome of the host under typical conditions. When all noninvasive samples are collected in the same manner, we believe that comparative microbiota analyses and monitoring will be valid and can be an effective tool in comparative analyses, health surveys, and pathogen tracking of wild species. Our results serve as a proof of concept for truly noninvasive fieldwork.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://www.ncbi.nlm.nih.gov/sra/PRJNA578383.

DATA AVAILABILITY STATEMENT

The sequence data generated as part of this study have been deposited in the Sequence Read Archive (BioProject ID: PRJNA578383; https://www.ncbi.nlm.nih.gov/sra/PRJNA578383) along with associated metadata (Turjeman & Koren, 2021). Codes (Appendix S2) and phyloseq object (Appendix S3) are included as Supporting Information.

ORCID

Sondra Turjeman https://orcid.org/0000-0002-5224-1215
Ammon Corl https://orcid.org/0000-0002-5156-4047
Pauline L. Kamath https://orcid.org/0000-0002-6458-4514
Rauri C. K. Bowie https://orcid.org/0000-0001-8328-6021
Ran Nathan https://orcid.org/0000-0002-5733-6715

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