How should we store avian faecal samples for microbiota analyses? Comparing efficacy and cost-effectiveness

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ABSTRACT

Analyses of bacterial DNA in faecal samples are becoming ever more common, yet we still do not know much about bird microbiomes. These challenges partly lie in the unique chemical nature of their faeces, and in the choice of sample storage method, which affects DNA preservation and the resulting microbiome composition. However, there is little information available on how best to preserve avian faeces for microbial analyses. This study evaluates five widely used methods for preserving nucleic acids and inferring microbiota profiles, for their relative efficacy, cost, and practicality. We tested the five methods (in-situ bead-beating with a TerraLyzer instrument, silica-bead desiccation, ethanol, refrigeration and RNA*later* buffer) on 50 fresh faecal samples collected from captive House sparrows (*Passer domesticus*). In line with other studies, we find that different storage methods lead to distinct bacterial profiles. Storage method had a large effect on community composition and the relative abundance of dominant phyla such as Firmicutes and Proteobacteria, with the most significant changes observed for refrigerated samples. Furthermore, differences in the abundance of aerobic or facultatively aerobic taxa, particularly in refrigerated samples and those stored in ethanol, puts limits on comparisons of bacterial communities across different storage methods. Finally, the methods that did not include in-situ bead-beating did not recover comparable levels of microbiota to the samples that were immediately processed and preserved using a TerraLyzer device. However, this method is also less practical and more expensive under field work circumstances. Our study is the most comprehensive analysis to date on how storage conditions affect subsequent molecular assays applied to avian faeces and provides guidance on cost and practicality of methods under field conditions.

Key words: Avian faeces, DNA preservation, gut microbiome, House sparrows.

1. Introduction

The gut microbiome is important for host health through its impacts on the immune system (Brisbin et al., 2008; Ruiz-Rodríguez et al., 2009b; Yang et al., 2012), digestion (Dewar et al., 2013; Godoy-vitorino et al., 2010; Ruiz-Rodríguez et al., 2009a), development (Barbosa et al., 2016; Teyssier et al., 2018; Torok et al., 2011; Videvall et al., 2019) and behaviour (Cryan and Dinan, 2012). While much research on the gut microbiome has focused on mammals, less is known about the causes and consequences of microbiome variation in birds. The applied value of studying avian microbiomes has long been realized in the poultry industry (Oakley et al., 2014). However, since the intimate interaction between hosts and their microbiota is thought to have wide-ranging effects on all aspects of host biology, there is tremendous potential for knowledge about the avian microbiome to contribute to research in avian ecology, evolution, and conservation (Hird, 2017; Trevelline et al., 2019).

A growing number of avian studies are capitalizing on this development and investigating interactions between host life-history traits, ecology, and the gut microbiota (Grond et al., 2018; Kohl, 2012; Teyssier et al., 2018; Trevelline et al., 2019; van Dongen et al., 2013; Videvall et al., 2019). Faecal sampling is commonly used for representing intestinal microbiota because it is non-invasive. Yet obtaining reliable molecular data from avian faeces is complicated by its chemical composition, as digestive excreta is mixed with urinary products such as uric acid that can degrade DNA or interfere with DNA extraction (Eriksson et al., 2017; Regnaut et al., 2006). The result is that DNA yields from avian faeces are typically low, making amplification difficult and pipelines more sensitive to contamination. The DNA degradation may also be influenced by exposure to ambient conditions, the presence of digested food items, and other natural degradation processes (Hájková et al., 2006). Thus, effective preservation methods are of critical importance. Moreover, faecal microbial communities will change over time with exposure to conditions outside the gut. Effective sampling and storage in the wild can be logistically difficult because methods such as freezing, are impractical under field conditions. Therefore, a key question for many ecological studies, is how to best store and preserve avian faecal samples for downstream molecular work as it affects sampling strategy, experimental design and study costs.

Most research on optimizing faecal microbiome protocols has focused on mammals and particularly humans, with much less work on birds and other vertebrates. Results are variable

and often contradictory. RNA*later* is frequently used to store faecal samples for microbiota analysis (Al et al., 2018; Broquet et al., 2007; Horng et al., 2018; Vlčková et al., 2012; Vogtmann et al., 2017), yet there is evidence that its performance decreases after a period of time at room temperature (Flores et al., 2015), and that the bacterial community profiles differ to those of frozen samples (Choo et al., 2015). Ethanol is also regularly used and has been shown to produce microbial profiles comparable to those obtained with RNA*later* (Vogtmann et al., 2017). However, some results when stored at 70% ethanol have shown higher species diversity compared to fresh samples (Horng et al., 2018) with particular disparity in bacterial counts of *Enterobacteriaceae* (Vlčková et al., 2012) and overall poor performance, showing an increase in relative abundance of certain taxa (Song *et al.* 2016). Previous methodological comparisons have suggested that refrigeration can be used as a practical alternative to freezing for storing faecal samples (Choo et al., 2015; Tedjo et al., 2015; Weese and Jalali, 2014), though Ott *et al.* (2004) showed significant changes in microbiota diversity in refrigerated samples over time, where the bacterial diversity reduced after 8 and 24 hours. Preserving samples at room temperature might be most practical , however the ability to accurately capture original microbial communities decreases rapidly within the first 24 hours at room temperature (Guo et al., 2016; Tedjo et al., 2015).

To date, no studies have systematically investigated how to optimize sampling and storage of avian faeces for microbiota analysis, to maximize DNA quantity, quality, and cost-effectiveness. While much avian microbiome work has focused on commercially important species, such as chickens and turkeys (Waite and Taylor, 2015), the study of avian host-microbiota interactions is rapidly growing in ecology and evolutionary biology (Hird, 2017). In this field, microbiota research has covered a range of bird taxa (Lucas and Heeb, 2005; Risely et al., 2018; Videvall et al., 2019). Passerines represent over half of extant birds and are common subjects in field-based avian microbiome research. We therefore focus our methodological optimization on samples from a common passerine, the House sparrow (*Passer domesticus*) as model organism, representative of a large proportion of passerine research. Our aim is to compare five field-compatible sample storage methods (immediate bead-beating with a TerraLyzer instrument, silica-bead desiccation, ethanol, refrigeration and RNAlater), in terms of DNA extraction efficacy and the resultant composition of microbial communities derived. We then present our results in light of the cost and practicality of each method.

2. Methods

2.1. Sampling

We collected fresh faecal samples from a population of captive House sparrows (*Passer domesticus*) kept in large groups (100-200 birds per aviary) indoors at the Animal Research facilities, Imperial College London. The house sparrows are descendants from wild birds that have been kept captive since 2005 (see references for husbandry details; Girndt et al., 2018, 2017). A clear plastic sheet was placed on the aviary floor after morning feeding time and left there for 180 minutes. Fifty faecal pellets were collected in total - ten biological replicates for each of the five storage methods compared. We assume that each pellet belonged to a different individual due to the large amount of birds in the aviaries. Some variation in pellet size is expected. However, samples had a wet mass of close to 0.05g.

We tested the most commonly used methods for storing samples under field conditions: **(1)** Use of Zymo's Terralyzer device ('Terralyzer' treatment). Samples were immediately placed in Zymo BashingBead tubes (with 0.5 & 2mm beads) filled with 500µl of lysis solution, lysed with a TerraLyzer Cell Disruptor instrument (Zymo Research) for 10 seconds and transported to the lab for DNA extraction within one hour of collection. This method is expected to give the most accurate bacterial profiles as bacterial growth within samples is immediately interrupted and DNA is simultaneously stabilised. Therefore, for comparison purposes, this treatment was used as the reference throughout our analyses **(2)** Desiccation with silica beads ('Dry' treatment). Each sample was placed into a clean cryogenic vial which was then placed inside a plastic vial containing 1.0±0.2g of silica beads; CryoTube cryongenic vial caps were removed, the outer container shut and samples left to dry at room temperature and checked daily for the presence of mould (Regnaut et al., 2006); **(3)** Immediate submersion in 500µl 96% Ethanol ('Ethanol' treatment). Prior to DNA extraction, samples were placed onto filter paper to absorb most of the ethanol before adding lysis solution for the bead-beating process; **(4)** Transport back to the laboratory (within 3 hours of collection) on ice in a cool-box before refrigeration (4ºC) ('Refrigeration' treatment); **(5)** Immediate submersion in 500µl RNA*later* Stabilization Solution ('RNA*later*' treatment). For DNA isolation, prior to DNA extraction, samples were again dried on filter paper prior to homogenization in lysis buffer.

In all methods except the TerraLyzer treatment, samples were stored in their treatment method for one week prior DNA extraction.

2.2. Nucleic acid extraction and DNA quantification

Total nucleic acids were isolated from all samples using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research), incorporating minor changes the protocol: samples from all treatments, except the TerraLyzer, were processed in a bead-beater (Retsch MM 440) at 20Hz for eight minutes and all of the supernatant was transferred into Zymo-Spin IV Spin Filters; 1000µl Faecal DNA Binding Buffer was used, instead of 1200µl as the protocol suggests; DNA was finally eluted in 40µl rather than 100µl as the original protocol indicates, to maximize DNA concentration. Eluted DNA was stored at 4ºC for two weeks, and then at -20ºC for a year prior to shipping to the sequencing facility. Total nucleic acid concentration and DNA purity were measured using spectrophotometry (ThermoFisher Scientific NanoDrop 2000); A_{260} was used for the concentration calculation while the ratio $A_{260}/_{280}$ was used for estimating protein contamination and A_{260}/A_{230} for DNA purity. Double stranded nucleic acid concentration was measured using Fluorometry (ThermoFisher Scientific Qubit 2.0) with a dsDNA High-Sensitivity Assay kit.

2.3 Microbiota characterization

Bacterial communities were profiled by sequencing the V4-V5 region of 16S rRNA gene using 515F/926F "fusion primers" (Walters et al., 2015). Amplicons (~410 bp) were then sequenced on a single 2x300-bp Illumina MiSeq sequencing run at the Integrated Microbiome Resource (IMB) facility. The library preparation and sequencing protocol used is published in Comeau, Douglas & Langille (2017).

2.4. Bioinformatic processing

Sequence data was processed using the R package *DADA2* (v1.8) (Callahan et al., 2016) to infer amplicon sequence variants (ASVs) (Callahan et al., 2017). First, sequence trimming and quality filtering parameters were chosen and ASVs inferred, then chimeras were removed and taxonomy assigned using the Silva reference database (v128) (Supplementary Information). After the final ASV table was created, taxonomic filtering steps were performed in package *Phyloseq* (v1.22) (McMurdie and Holmes, 2013). We removed taxa assigned as chloroplasts because they are non-informative taxa within this analysis. Abundance filtering was also performed for beta diversity analyses, in that taxa present in less than 5% samples were removed from the dataset, to limit the potential influence of contaminants or sequencing artefacts. The R package *iNEXT* (v2.0) (Hsieh et al., 2016) was used to create sampling completeness curves and decide cut-off parameters for low quality samples. ASV richness

plateaued by approximately 1000 reads, such that any samples with read counts below this threshold were excluded.

2.5. Statistical analysis

DNA concentration and purity were compared across treatments using factorial ANOVAs. For alpha diversity analyses, the effect of treatment on microbiota diversity was estimated using the Shannon index calculated by the *breakaway* package (v4.6.8) (Willis and Bunge, 2015). For beta diversity analyses, read counts were normalised using cumulative-sum scaling using the *metagenomeSeq* package (v1.2) (Paulson et al., 2013). We calculated community dissimilarity matrices (generalised UniFrac and Bray-Curtis dissimilarity) in the packages *GUniFrac* (v1.1) and *vegan* (v2.5) (Chen et al., 2012; Dixon, 2003). These dissimilarity matrices were then used in a permutational analysis of variance (PERMANOVA) to examine how storage treatments affected community composition. We used the function *betadisper* within package *vegan* (Anderson, 2001) to tests if differences in sample dispersion might influence community composition differences among treatments. Finally, as most gut bacteria are obligate or facultative anaerobes (von Martels et al., 2017), we also evaluated the effects of different storage conditions on the ability to detect anaerobes and aerobes (see Supplementary Information). This gives an insight on possible colonization and outgrowth of aerobes after sample collection. All analyses were carried out in R (version 3.4.4, R Core Team, 2014).

2.6. Cost and practicality

Cost reflects price in US dollars of sample preservation, including the price of cryogenic vials, buffers, ice and beads, and extra accessories (Table A2). Cost was calculated for projects of 100, 500 and 1000 samples. The cost of a TerraLyzer machine was excluded for project expenses as all protocols require and instrument for bead-beating, the difference is whether this is performed in the field (TerraLyzer) or in the laboratory (rest of the protocols). All prices were estimated in March 2019 as displayed online, and do not include discounts for research institutions. To assess the practicality of each method, we developed a time-effort index based on convenience of a process under field conditions using 10 different criteria (Table 1). Each index assigned to a treatment was plotted against cost.

Table 1. Practicality criteria developed for assessing storage methods for use in the field (top) and scoring system assessed by single sample for the practicality index (below).

	Criterion	Description
i.	Size	The equipment is large or heavy to carry and may require the use of additional boxes for transportation
ii.	Temperature sensitivity	The method is sensitive to temperature and has to be kept in stable environment (fluctuations < ±4°C)
iii.	Shelf-life	The method or one of its components has to be replaced every ≅7 days
iv.	Monitoring	The method requires frequent monitoring of external conditions such as temperature and humidity (check samples at least once a day)
v.	Sample reorganization	The method requires moving a sample between tubes/buffers or reagents from its original storing tube.
vi.	Workforce required	The method requires the presence of more than one person to help with the storage of a sample
vii.	Electricity	The method involves machinery which requires access to electricity or needs to be charged
viii.	Leak or spillage	The method involves liquid buffers/reagents which can spill or leak onto other equipment or samples
ix.	Travel restrictions	The method includes components which may be restricted when traveling (liquids for air travel, dry ice, high concentrations of ethanol, lithium-ion batteries, etc.)
x.	Time from source to storage	Time taken from sample collection to completion of storage (≥10 seconds per sample)

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Score	Description

0	Not practical. Six or more of the criteria are met
1	Borderline practicality. Five of the criteria are met
2	Satisfactory practicality. Three or four of the criteria are met
3	Practical. Meets up to two of the criteria

3. Results

3.1. DNA extraction assessment

In total 50 DNA extracts were obtained from 50 faecal pellets (~0.05g each). The mean nucleic acid concentration by spectrophotometry (NanoDrop) was 36ng/µl ±1 SE. TerraLyzer samples had the highest mean concentration (41ng/µl ±3 SE) while the refrigeration (4°C) method presented the lowest mean concentration (30ng/µl ±2 SE). As expected, double stranded DNA concentrations measured by Fluorometry (Qubit), were lower than the spectrophotometry (NanoDrop) measures (Table 2); the mean concentration was 0.22ng/µl ±0.01 SE, and DNA concentration was not significantly predicted by storage method ($F_{4,45}$= 1.0, p= 0.133). Average values for protein contamination in the samples ($A_{260}/_{280}$) were outside the range of 1.8-2.0 (1.29±0.02 SE) regarded as indicative of low protein contaminant content (Table 2). Overall, the $A_{260}/_{280}$ ratio was not significantly predicted by storage method ($F_{4,45}$= 1.32, p= 0.275); but ethanol had the highest protein contamination compared to TerraLyzer samples. DNA purity ratio ($A_{260}/_{230}$) was below 1.8 in all samples (mean 0.24±0.01 SE), possibly suggesting a high concentration of contaminants (Table 2); and it did not show significant differences with respect to treatment ($F_{4,45}$= 0.77, p= 0.546).

Table 2. DNA concentration, protein contamination and purity of house sparrow faecal sample DNA extractions for each method tested. Mean±SE is shown in all cases.

Treatment	DNA conc. (Spectrophotometry, ng/µl)	dsDNA conc. (Fluorometry, ng/µl)	Protein contamination ($A_{260}/_{280}$)	DNA purity ($A_{260}/_{230}$)
TerraLyzer	41 ±3	0.24 ±0.01	1.22 ±0.05	0.27 ±0.03
Dry	37 ±2	0.23 ±0.02	1.32 ±0.01	0.26 ±0.02
Ethanol	34 ±3	0.18 ±0.02	1.39 ±0.08	0.25 ±0.04
4°C	30 ±3	0.95 ±0.70	1.29 ±0.05	0.21 ±0.02
RNAlater	38 ±3	0.20 ±0.02	1.25 ±0.04	0.20 ±0.03

3.2 Microbiota profiles

Only 38 of 50 samples (76%) were included in 16S rRNA microbiota profiling. Of these, 17 (45%) satisfied quality filtering parameters during the bioinformatic pipeline (100% TerraLyzer, 71% Dry, 33% ethanol, 43% ice and 100% RNA*later*). A total of 851,284 sequence reads were obtained following quality filtering, comprising 22,402 ±5,748 SE raw reads per sample. Read count was not significantly predicted by treatment (Kruskal-Wallis chi-squared= 7.22, df= 4, p= 0.124).

All treatments differed in Shannon diversity compared to the TerraLyzer treatment, though the direction varied (estimated sigma^2_u= 17.15, p= 0.00), except for the samples stored dried (p= 0.18), though these samples also presented the highest variability in diversity (Fig. 1a).

Overall, treatment had a strong and significant effect on microbial community composition (PERMANOVA on weighted UniFrac, $F_{4,16}$= 2.74, R^2= 0.47, p= 0.007), and we didn't find different levels of dispersion within treatment (betadisper, $F_{4,12}$= 0.50, p= 0.73; Fig. A1). The treatment that had the most similar community composition to TerraLyzer on average was RNA*later*, however samples from this treatment, also had the highest variation in community composition (Fig. 1a); the storage method that produced an average composition most distinct from that of the TerraLyzer was ethanol with a mean Bray-Curtis distance of 0.95 (Fig. 1b).

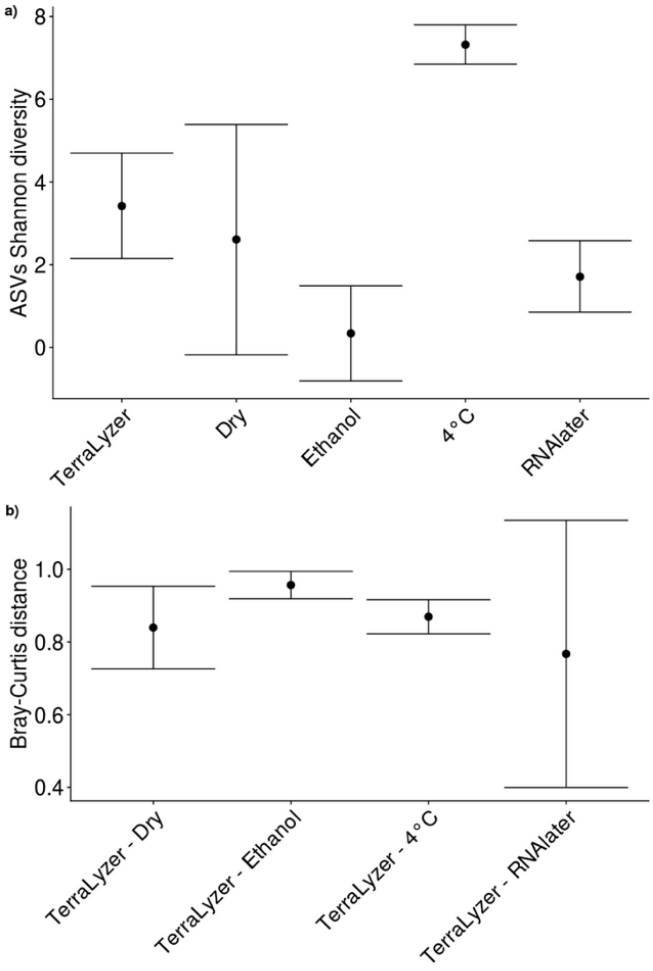

Figure 1. Microbial community diversity and composition differences for the five tested treatments. a) Estimated Shannon diversity of ASVs for each of the five treatments. Points and error bars indicate mean diversity estimates and confidence intervals respectively. (b) Bray-Curtis distance in community composition between samples in the TerraLyzer treatment and those analysed with other treatments. Points and error bars in both plots indicate means and standard deviations for each comparison, respectively.

Across all storage conditions, the dominant phyla detected were Firmicutes and Proteobacteria, but the ratio of relative abundance between these two differed significantly among treatments (Kruskal-Wallis chi-squared= 141.47, df= 4, p= 0.00). A pairwise Wilcoxon rank sum test was applied to detect differences of relative abundance of the eight most abundant phyla among treatments; the greatest differences between the Terralyzer samples and the rest, were seen for refrigerated samples (pairwise Wilcoxon test p= 0.00) with a considerably higher proportion of Bacteroidetes, SBR1093, Thaumarchaeota and Actinobacteria (Fig. 2a). Also, refrigerated samples had higher relative abundances of *Flavobacteriales* (2%), *Rhizobiales* (3%),

Salinisphaerales (0.7%), SAR11_clade (2.5%) and from other unassigned orders (13%), compared to the rest of the treatments (Fig. 2b).

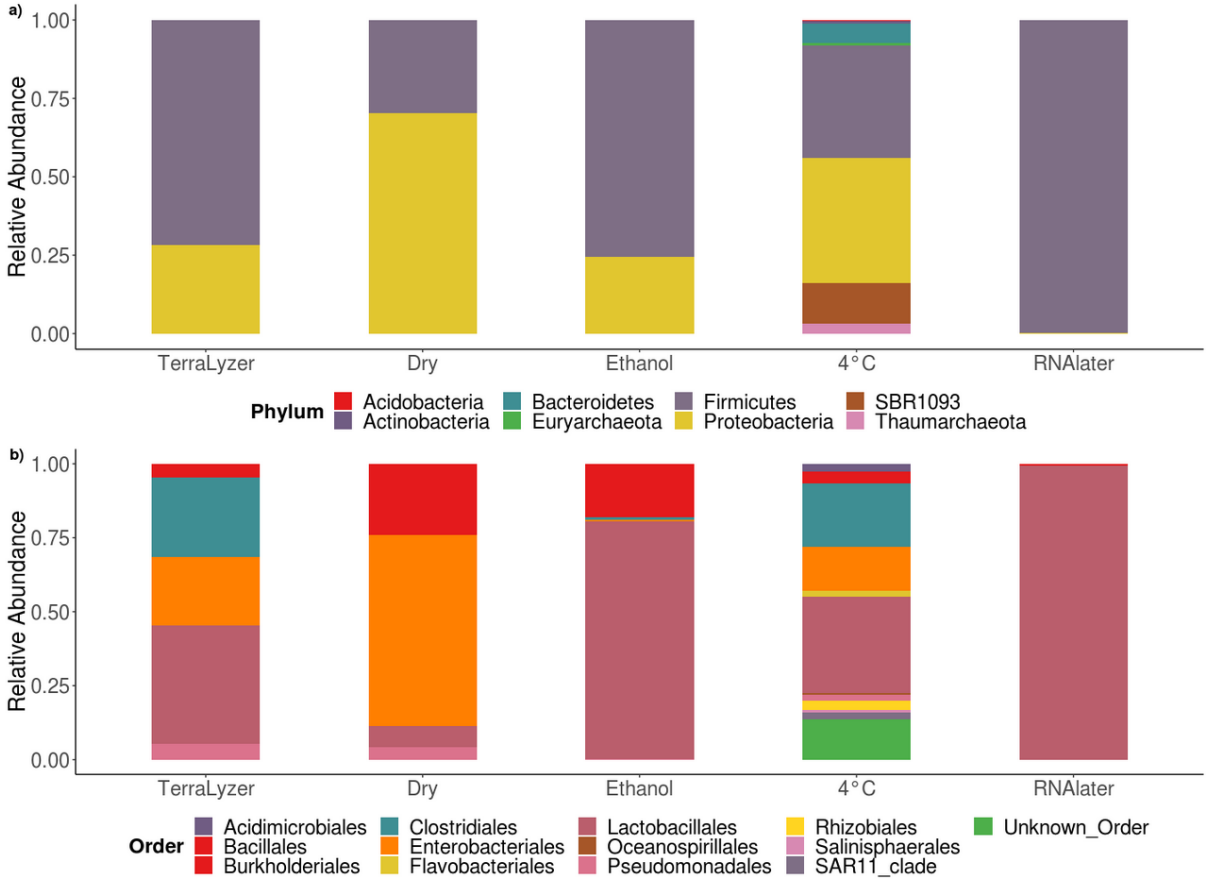

Figure 2. Relative abundance of bacterial (a) phyla and (b) orders, across the five treatments. For clarity only taxa with >5% relative abundance are plotted.

A total of 101 ASVs were identified to genus level and included in the analysis of respiration type/aerotolerance (Table A1). The proportion of detected genera that were either obligate or facultative anaerobes (expected in the gut) was similar in TerraLyzer, refrigerator and RNA*later* treatments. However, refrigeration revealed proportionally more aerobic genera than the other treatments. Samples stored dried and in ethanol presented substantially lower relative abundance of obligate anaerobic genera compared to facultatively aerobic bacteria (Table 3). This result suggests that storage methods may differ in the extent to which they allow aerotolerant or aerobic bacteria to multiply post-collection.

Table 3. Relative abundance (%) of bacterial genera classified by their cellular respiration, found in different sample storage conditions.

	Aerobic	Anaerobic	Facultative	Unclassified
TerraLyzer	1.5	35.5	62.8	0.2
Dry*	0.0	3.2	96.6	0.1
Ethanol	1.6	5.6	92.6	0.2
4°C	3.1	28.3	51.8	16.8
RNA*later*	1.5	36.4	61.8	0.3

*0.09% rounding error in Dry treatment

3.3 Cost and practicality

According to the cost analysis ethanol is the cheapest method per sample ($0.75 USD) and the use of ice with additional refrigeration to keep samples refrigerated at 4°C is the most expensive method per sample ($8.16 USD, Table A2), but as the size of the project increases, refrigeration becomes the cheapest method ($379.6 USD for 1000 samples), and the use of TerraLyzer (in situ bead-beating) method the most expensive ($1482 USD for 1000 samples; Fig. 4a). If the practicality of using each method in the field is analysed together with the cost of a 100-sample project, then the methods with the best price-practicality ratio are ethanol and RNA*later*. The refrigeration method is the most affordable storage method, however, is also the least practical to perform in field work conditions (Fig. 4b).

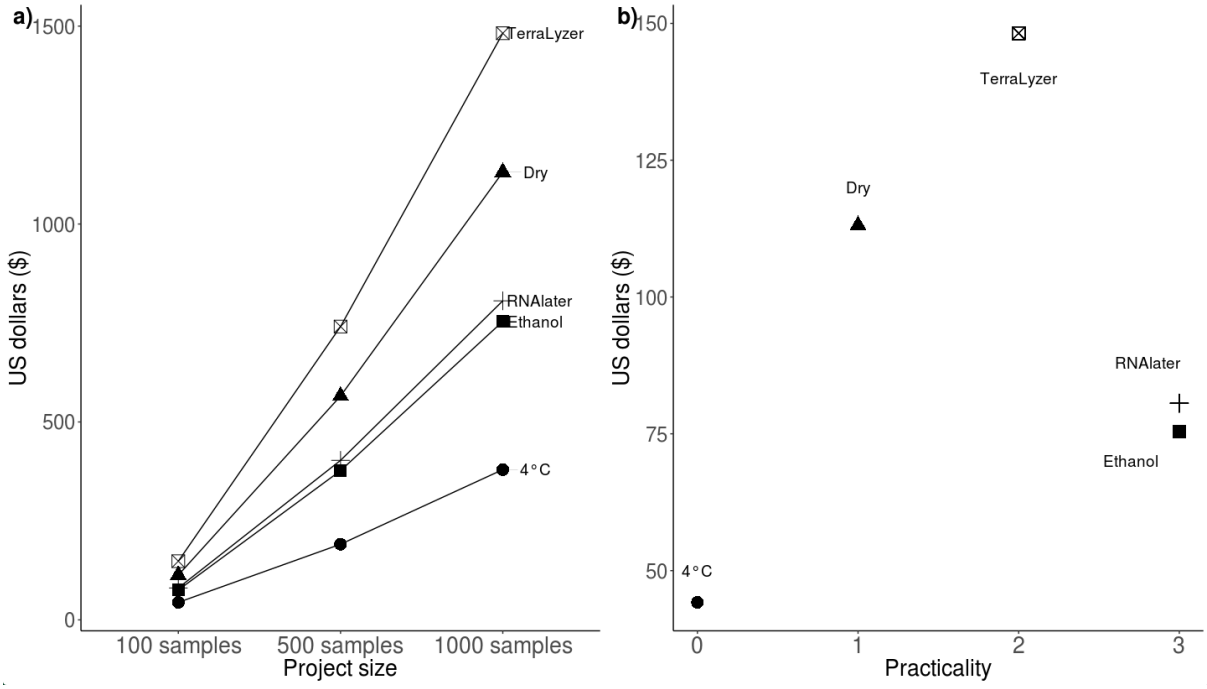

Figure 4. Cost and practicality of five sample storage treatments. **a)** Total costs of projects using different number of samples: 100, 500, 1000. **b)** Practicality and costs for a 100-sample project. In the practicality index, "0" is the least practical treatment, and "3" the most practical.

4. Discussion

Results of this study show that faecal sample storage method affects the microbial community detected in downstream analysis. Three major findings derive from the current study. First, microbial composition is determined by storage method; relative abundances of certain phyla change across treatments, especially on refrigerated and ethanol samples; this could be driven by the differentiated proportion of aerobes and anaerobes, indicating selective detection rates. Second, the efficiency on faecal DNA quality (concentration and purity) is not determined by the storage of faeces, and it does not reflect microbiome composition results. Third, treatments that include the use of RNA*later* and ethanol meet important criteria such as being low-cost and are highly practical under field conditions, however they do not necessarily reliably store the microbial composition of house sparrow faeces. Together, these results suggest that knowing the caveats associated with each storage method are crucial during design, analyses and interpretation of avian microbial results.

The evidence here confirms that each treatment alters microbial communities by affecting the relative abundances in great magnitude; thus, care should be taken when comparing values across studies using different protocols, especially when incorporating metrics such as Shannon index. The most abundant phyla across all samples were Proteobacteria and Firmicutes, which is consistent with what was previously reported for House sparrows (Kohl et al., 2019; Mirón et al., 2014); however, we found higher relative abundance of Proteobacteria in samples stored dried. This result suggests that consideration should be given to differences in abundance at certain taxonomic levels that have undergone this type of storage, particularly those involving Proteobacteria and Actinobacteria which are able to grow at a range of temperatures (Weese and Jalali, 2014).

Furthermore, changes observed on microbial abundances at order level, particularly from the ones stored at chilled temperature can be attributed to oxygen exposure resulting in bacterial degradation (Ott et al., 2004). The ability to detect total aerobes and anaerobes from different

storage conditions can be used as a proxy of the global effect on storage methods (Fouhy et al., 2015); we found that a greater proportion of aerobes were recovered following refrigeration, suggesting that oxygen-tolerant bacteria are thriving after collection, driving biases on the community composition. We also found that the levels of total anaerobic and facultative bacteria in RNA*later* samples were similar to the ones detected in samples processed using the TerraLyzer, which suggests that immediate submersion in buffer solution following collection enables the recovery of comparable types of microbiome. Remarkably, the recovery rates of taxonomic groups in RNA*later* are not comparable to those found in samples processed by the TerraLyzer.

Encouragingly, inter-individual variations were smaller than variation between methods, suggesting consistency in sampling within each method applied; therefore, as long as the same preservation method is used across a study, unbiased comparisons can be made between samples. Having said this, there will always be methodologic or biologic related biases as established by Hallmaier-Wacker *et al.* (2018) and (Pollock et al., 2018); this highlights the need for proper validation and standardization for each sample type and the use of blank control samples, to assess the limitations in protocols and datasets.

Going forward, numerous studies have suggested that inadequate storage can result in reduced DNA quantity and quality and addressing this issue will ensure effective and accurate genotyping (Murphy et al., 2007; Soto-Calderón et al., 2009). However, this study shows that adequate storing protocols are not enough to achieve high quality avian gut microbiome profiles. Faecal extracts are characterized by low DNA concentration and high degradation (Dai et al., 2015; Demay et al., 2013), and sparrow samples analysed here are no exception. Avian DNA concentrations and purity are consistently lower compared to those reported for mammal faeces DNA (Bubb et al., 2011; Costa et al., 2017; Horng et al., 2018). This suggests that further studies should focus on the implementation of methodologies that improve DNA recovery from avian faeces beyond sampling optimization.

These analyses represent the first attempt to test how storage methods of bird faeces affect microbiome research. We are still in search of the best methodologies, however the sole focus on the storage protocol will not resolve other difficulties associated with working with avian faeces, such as high uric acid content. Until then, other factors can be taken into account such

as cost and practicality under field conditions. The present study allows to choose the affordability of the equipment and reagents used for each protocol.

The use of the TerraLyzer has not been widespread, however, we showed the use of such an instrument to be useful a preliminary bead-beating step to break tissues in the field and increase optimal storage. Such a device is easy to use as it ensures a good bead-motion. In particular, the TerraLyzer becomes cost-effective when used for multiple eDNA studies under field conditions. Applying the two-step silica desiccation method has demonstrated to be useful on recovering microbiome communities similar to those on control samples (Bhagavatula and Singh, 2006), nevertheless this method requires special attention and extra care when handling and monitoring the samples, and climatic variables should also be considered when working in humid and hot environments. Freezing is not possible under field conditions, unless there is access to electricity or liquid nitrogen. This study substituted it by placing the samples on ice and refrigerating them and, similarly, to freezing the samples, the practicality of this method was low. The treatment that involves the use of a buffer (RNA*later*) has the best cost/practicality ratio, as does the use of ethanol, however careful attention must be paid to these methods as they might be underrepresenting the original microbial community.

5. Conclusions

The results shown provide guidelines to aid researchers embarking a microbial project on wild bird populations. We further advise other to perform a pilot study to determine which storage approach is optimal for them, as this will be dependant not only on their objectives, but also on the practicality and cost-efficiency of each approach. The optimization of the sampling protocols should take into account the environments from which samples will be collected, the length of time the sample will be in storage for, and the size of the project. Importantly, we show that regardless of the method chosen, consistency of storage within project is a prime practice to achieve replicable and reliable results for microbial ecology.

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Appendix A. Supplementary Information

Methodology for bioinformatics

The first step for the bioinformatics followed a pipeline from the R package *DADA2* used to evaluate the quality and size of raw reads, this enabled to choose the cut-off parameters for the trimming and filtering of the sequences; we used the standard filtering parameters and 78% of the sequences survived this step. Next, an error model was calculated for the specific dataset, then, to reduce computational time, we dereplicated the sequences by eliminating redundant comparisons and allocating abundances of each "unique sequence". Amplicon sequence variants (ASVs) were then inferred and spurious ones were further reduced by overlapping reads, this step removes substitution and indel errors, but not chimeras, therefore, a simple phase on identifying and removing chimeras was applied. At this point was possible to classify sequence variants taxonomically.

Methodology for determining bacteria respiration type

We selected the lowest taxonomic level –Genus- in order to have the highest resolution on the identity of each taxa. We created a search strategy for each taxon to find the respiration type: we used Google Scholar, PubMed and the book *"The Prokaryotes. prokaryotic Biology and Symbiotic Associations"* (Rosenberg et al., 2013). Once the respiration type was identified, each ASV was labelled with either aerobic, anaerobic, facultative or not-identified. This labeller allowed to know the relative abundances of each type of respiration found in each treatment (Table A1). In the cases where no information was found for a specific Genus, the taxon was not considered for the analysis. A total 101 ASV were included for this part of the analysis.

Table A2. Material costs (USD*) per sample for each sample storage treatment

Treatment	Tubes		Medium		Extras		Total
TerraLyzer	*BashingBead tubes (0.5/2mm)*	0.54	*Lysis solution*	0.93	-	0.0	1.48
RNA*later*	*1.5 ml CryoTubes*	0.36	*RNAlater*	0.44	-	0.0	0.80
Ethanol	*1.5 ml CryoTubes*	0.36	*Ethanol (90%)*	0.39	-	0.0	0.75

Refrigeration (4°C)	*1.5 ml CryoTubes*	0.36	*Ice*	1.3	*Cool box*	6.5	8.16
Desiccation (dry)	*1.5 ml CryoTubes*	0.36	*Silica beads*	0.35	*Plastic vials*	0.46	1.13

*Prices to March 2019.

Figure A1. Differences in the faecal microbiota between treatments. Bray-Curtis distances between the faecal bacterial communities. Mean and s.e.m. values are plotted.