

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

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18

19 ABSTRACT

20

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

42

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

44

45 **1. Introduction**

46

47 The gut microbiome is important for host health through its impacts on the immune system
48 (Brisbin et al., 2008; Ruiz-Rodríguez et al., 2009b; Yang et al., 2012), digestion (Dewar et al.,
49 2013; Godoy-vitorino et al., 2010; Ruiz-Rodríguez et al., 2009a), development (Barbosa et al.,
50 2016; Teyssier et al., 2018; Torok et al., 2011; Videvall et al., 2019) and behaviour (Cryan and
51 Dinan, 2012). While much research on the gut microbiome has focused on mammals, less is
52 known about the causes and consequences of microbiome variation in birds. The applied value
53 of studying avian microbiomes has long been realized in the poultry industry (Oakley et al.,
54 2014). However, since the intimate interaction between hosts and their microbiota is thought
55 to have wide-ranging effects on all aspects of host biology, there is tremendous potential for
56 knowledge about the avian microbiome to contribute to research in avian ecology, evolution,
57 and conservation (Hird, 2017; Trevelline et al., 2019).

58

59 A growing number of avian studies are capitalizing on this development and investigating
60 interactions between host life-history traits, ecology, and the gut microbiota (Grond et al.,
61 2018; Kohl, 2012; Teyssier et al., 2018; Trevelline et al., 2019; van Dongen et al., 2013;
62 Videvall et al., 2019). Faecal sampling is commonly used for representing intestinal microbiota
63 because it is non-invasive. Yet obtaining reliable molecular data from avian faeces is
64 complicated by its chemical composition, as digestive excreta is mixed with urinary products
65 such as uric acid that can degrade DNA or interfere with DNA extraction (Eriksson et al., 2017;
66 Regnaut et al., 2006). The result is that DNA yields from avian faeces are typically low, making
67 amplification difficult and pipelines more sensitive to contamination. The DNA degradation
68 may also be influenced by exposure to ambient conditions, the presence of digested food items,
69 and other natural degradation processes (Hájková et al., 2006). Thus, effective preservation
70 methods are of critical importance. Moreover, faecal microbial communities will change over
71 time with exposure to conditions outside the gut. Effective sampling and storage in the wild
72 can be logistically difficult because methods such as freezing, are impractical under field
73 conditions. Therefore, a key question for many ecological studies, is how to best store and
74 preserve avian faecal samples for downstream molecular work as it affects sampling strategy,
75 experimental design and study costs.

76

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

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

96

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

111

112 **2. Methods**

113

114 2.1. Sampling

115 We collected fresh faecal samples from a population of captive House sparrows (*Passer*
116 *domesticus*) kept in large groups (100-200 birds per aviary) indoors at the Animal Research
117 facilities, Imperial College London. The house sparrows are descendants from wild birds that
118 have been kept captive since 2005 (see references for husbandry details; Girndt et al., 2018,
119 2017). A clear plastic sheet was placed on the aviary floor after morning feeding time and left
120 there for 180 minutes. Fifty faecal pellets were collected in total - ten biological replicates for
121 each of the five storage methods compared. We assume that each pellet belonged to a different
122 individual due to the large amount of birds in the aviaries. Some variation in pellet size is
123 expected. However, samples had a wet mass of close to 0.05g.

124

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

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

145

146 2.2. Nucleic acid extraction and DNA quantification

147 Total nucleic acids were isolated from all samples using the Quick-DNA Fecal/Soil Microbe
148 Miniprep Kit (Zymo Research), incorporating minor changes the protocol: samples from all
149 treatments, except the TerraLyzer, were processed in a bead-beater (Retsch MM 440) at 20Hz
150 for eight minutes and all of the supernatant was transferred into Zymo-Spin IV Spin Filters;
151 1000µl Faecal DNA Binding Buffer was used, instead of 1200µl as the protocol suggests; DNA
152 was finally eluted in 40µl rather than 100µl as the original protocol indicates, to maximize
153 DNA concentration. Eluted DNA was stored at 4°C for two weeks, and then at -20°C for a year
154 prior to shipping to the sequencing facility. Total nucleic acid concentration and DNA purity
155 were measured using spectrophotometry (ThermoFisher Scientific NanoDrop 2000); A_{260} was
156 used for the concentration calculation while the ratio A_{260}/A_{280} was used for estimating protein
157 contamination and A_{260}/A_{230} for DNA purity. Double stranded nucleic acid concentration was
158 measured using Fluorometry (ThermoFisher Scientific Qubit 2.0) with a dsDNA High-
159 Sensitivity Assay kit.

160

161 *2.3 Microbiota characterization*

162 Bacterial communities were profiled by sequencing the V4-V5 region of 16S rRNA gene using
163 515F/926F “fusion primers” (Walters et al., 2015). Amplicons (~410 bp) were then sequenced
164 on a single 2x300-bp Illumina MiSeq sequencing run at the Integrated Microbiome Resource
165 (IMB) facility. The library preparation and sequencing protocol used is published in Comeau,
166 Douglas & Langille (2017).

167

168 *2.4. Bioinformatic processing*

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

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

182

183 2.5. Statistical analysis

184 DNA concentration and purity were compared across treatments using factorial ANOVAs. For
185 alpha diversity analyses, the effect of treatment on microbiota diversity was estimated using
186 the Shannon index calculated by the *breakaway* package (v4.6.8) (Willis and Bunge, 2015).
187 For beta diversity analyses, read counts were normalised using cumulative-sum scaling using
188 the *metagenomeSeq* package (v1.2) (Paulson et al., 2013). We calculated community
189 dissimilarity matrices (generalised UniFrac and Bray-Curtis dissimilarity) in the packages
190 *GUniFrac* (v1.1) and *vegan* (v2.5) (Chen et al., 2012; Dixon, 2003). These dissimilarity
191 matrices were then used in a permutational analysis of variance (PERMANOVA) to examine
192 how storage treatments affected community composition. We used the function *betadisper*
193 within package *vegan* (Anderson, 2001) to tests if differences in sample dispersion might
194 influence community composition differences among treatments. Finally, as most gut bacteria
195 are obligate or facultative anaerobes (von Martels et al., 2017), we also evaluated the effects
196 of different storage conditions on the ability to detect anaerobes and aerobes (see
197 Supplementary Information). This gives an insight on possible colonization and outgrowth of
198 aerobes after sample collection. All analyses were carried out in R (version 3.4.4, R Core Team,
199 2014).

200

201 2.6. Cost and practicality

202 Cost reflects price in US dollars of sample preservation, including the price of cryogenic vials,
203 buffers, ice and beads, and extra accessories (Table A2). Cost was calculated for projects of
204 100, 500 and 1000 samples. The cost of a TerraLyzer machine was excluded for project
205 expenses as all protocols require and instrument for bead-beating, the difference is whether this
206 is performed in the field (TerraLyzer) or in the laboratory (rest of the protocols). All prices
207 were estimated in March 2019 as displayed online, and do not include discounts for research
208 institutions. To assess the practicality of each method, we developed a time-effort index based
209 on convenience of a process under field conditions using 10 different criteria (Table 1). Each
210 index assigned to a treatment was plotted against cost.

211

212 **Table 1.** Practicality criteria developed for assessing storage methods for use in the field (top)
213 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 < $\pm 4^{\circ}\text{C}$)
iii.	Shelf-life	The method or one of its components has to be replaced every $\cong 7$ 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)

214

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

215

216 3. Results

217

218 3.1. DNA extraction assessment

219 In total 50 DNA extracts were obtained from 50 faecal pellets (~0.05g each). The mean nucleic
 220 acid concentration by spectrophotometry (NanoDrop) was 36ng/μl ±1 SE. TerraLyzer samples
 221 had the highest mean concentration (41ng/μl ±3 SE) while the refrigeration (4°C) method
 222 presented the lowest mean concentration (30ng/μl ±2 SE). As expected, double stranded DNA
 223 concentrations measured by Fluorometry (Qubit), were lower than the spectrophotometry
 224 (NanoDrop) measures (Table 2); the mean concentration was 0.22ng/μl ±0.01 SE, and DNA
 225 concentration was not significantly predicted by storage method ($F_{4,45} = 1.0$, $p = 0.133$). Average
 226 values for protein contamination in the samples ($A_{260/280}$) were outside the range of 1.8-2.0
 227 (1.29 ± 0.02 SE) regarded as indicative of low protein contaminant content (Table 2). Overall,
 228 the $A_{260/280}$ ratio was not significantly predicted by storage method ($F_{4,45} = 1.32$, $p = 0.275$); but
 229 ethanol had the highest protein contamination compared to TerraLyzer samples. DNA purity
 230 ratio ($A_{260/230}$) was below 1.8 in all samples (mean 0.24 ± 0.01 SE), possibly suggesting a high
 231 concentration of contaminants (Table 2); and it did not show significant differences with
 232 respect to treatment ($F_{4,45} = 0.77$, $p = 0.546$).

233 **Table 2. DNA concentration, protein contamination and purity of house sparrow faecal**
 234 **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

235

236 3.2 Microbiota profiles

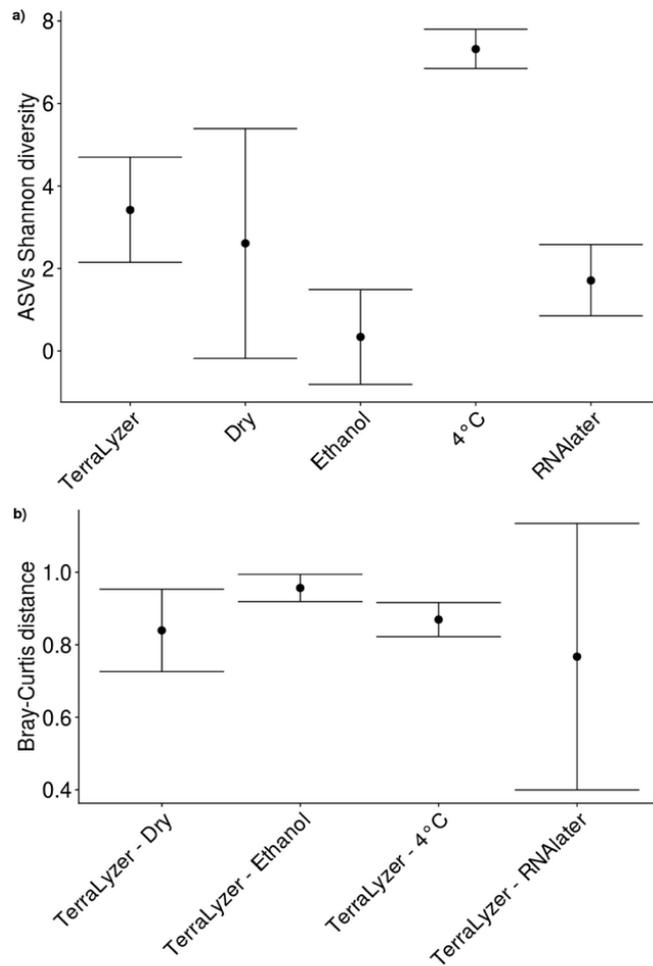
237 Only 38 of 50 samples (76%) were included in 16S rRNA microbiota profiling. Of these, 17
238 (45%) satisfied quality filtering parameters during the bioinformatic pipeline (100%
239 TerraLyzer, 71% Dry, 33% ethanol, 43% ice and 100% *RNAlater*). A total of 851,284
240 sequence reads were obtained following quality filtering, comprising $22,402 \pm 5,748$ SE raw
241 reads per sample. Read count was not significantly predicted by treatment (Kruskal-Wallis chi-
242 squared= 7.22, df= 4, p= 0.124).

243

244 All treatments differed in Shannon diversity compared to the TerraLyzer treatment, though
245 the direction varied (estimated $\sigma^2_u = 17.15$, p= 0.00), except for the samples stored
246 dried (p= 0.18), though these samples also presented the highest variability in diversity (Fig.
247 1a).

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

256



257

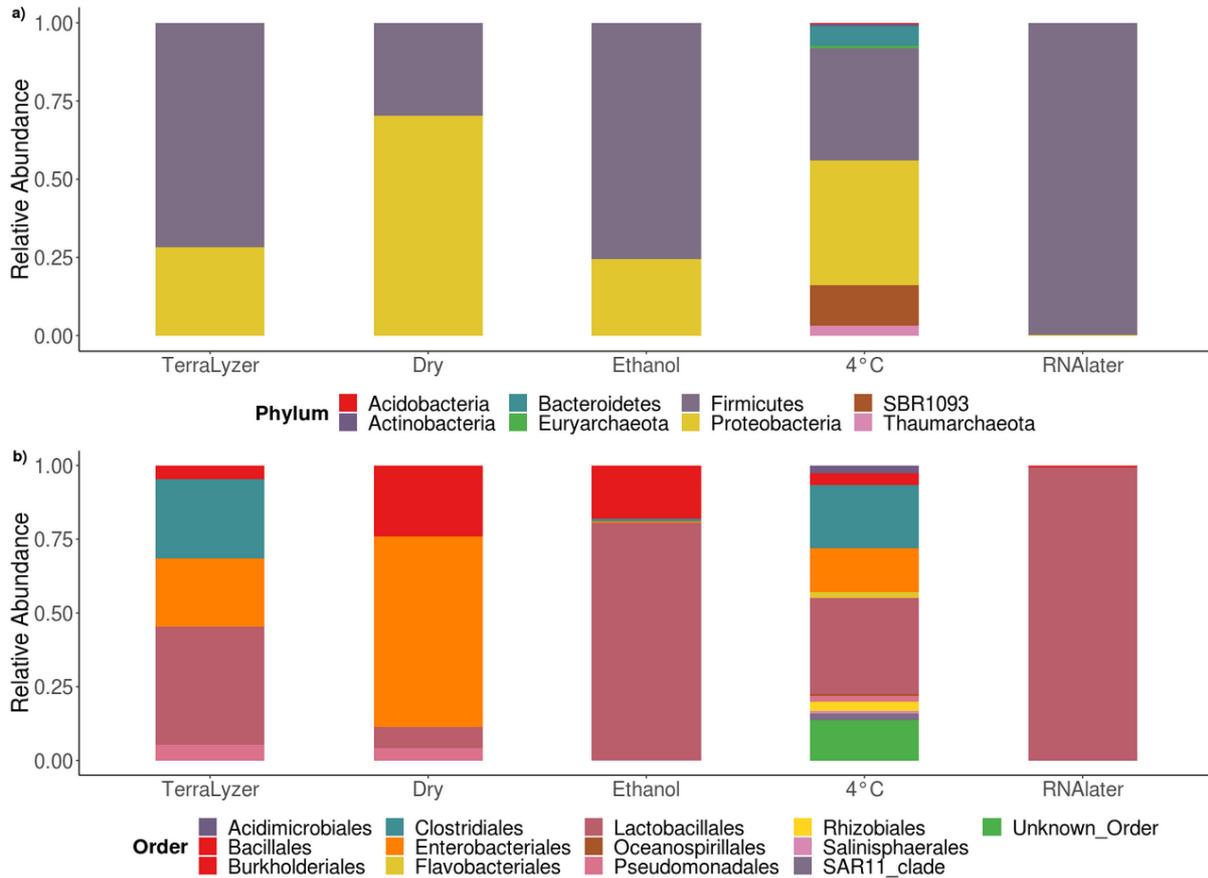
258 **Figure 1.** Microbial community diversity and composition differences for the five tested
 259 treatments. a) Estimated Shannon diversity of ASVs for each of the five treatments. Points
 260 and error bars indicate mean diversity estimates and confidence intervals respectively.
 261 (b) Bray-Curtis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

264

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

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

275
 276



277
 278 **Figure 2.** Relative abundance of bacterial (a) phyla and (b) orders, across the five treatments.
 279 For clarity only taxa with >5% relative abundance are plotted.

280
 281 A total of 101 ASVs were identified to genus level and included in the analysis of respiration
 282 type/aerotolerance (Table A1). The proportion of detected genera that were either obligate or
 283 facultative anaerobes (expected in the gut) was similar in TerraLyzer, refrigerator and
 284 RNAlater treatments. However, refrigeration revealed proportionally more aerobic genera than
 285 the other treatments. Samples stored dried and in ethanol presented substantially lower relative
 286 abundance of obligate anaerobic genera compared to facultatively aerobic bacteria (Table 3).
 287 This result suggests that storage methods may differ in the extent to which they allow
 288 aerotolerant or aerobic bacteria to multiply post-collection.

289

290 **Table 3. Relative abundance (%) of bacterial genera classified by their cellular**
 291 **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
RNAlater	1.5	36.4	61.8	0.3

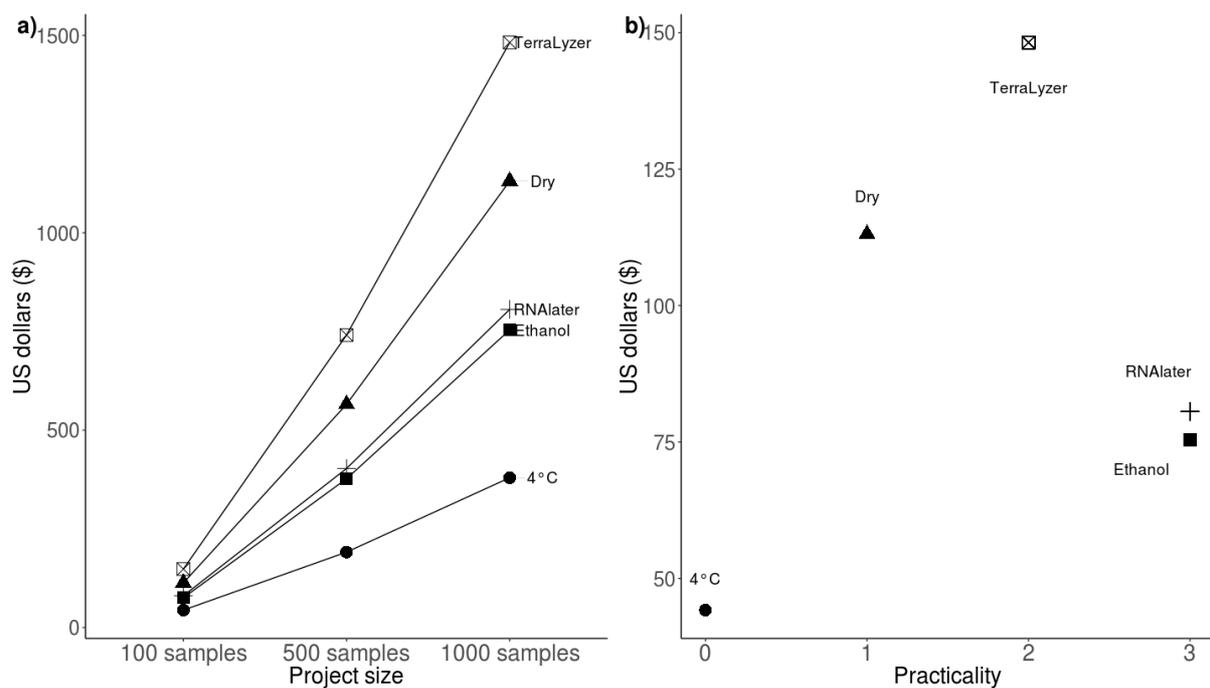
*0.09% rounding error in Dry treatment

292
293

3.3 Cost and practicality

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

304



305

306 **Figure 4.** Cost and practicality of five sample storage treatments. **a)** Total costs of projects
307 using different number of samples: 100, 500, 1000. **b)** Practicality and costs for a 100-sample
308 project. In the practicality index, “0” is the least practical treatment, and “3” the most
309 practical.
310

311 **4. Discussion**

312
313 Results of this study show that faecal sample storage method affects the microbial community
314 detected in downstream analysis. Three major findings derive from the current study. First,
315 microbial composition is determined by storage method; relative abundances of certain phyla
316 change across treatments, especially on refrigerated and ethanol samples; this could be driven
317 by the differentiated proportion of aerobes and anaerobes, indicating selective detection rates.
318 Second, the efficiency on faecal DNA quality (concentration and purity) is not determined by
319 the storage of faeces, and it does not reflect microbiome composition results. Third, treatments
320 that include the use of *RNAlater* and ethanol meet important criteria such as being low-cost
321 and are highly practical under field conditions, however they do not necessarily reliably store
322 the microbial composition of house sparrow faeces. Together, these results suggest that
323 knowing the caveats associated with each storage method are crucial during design, analyses
324 and interpretation of avian microbial results.
325

326 The evidence here confirms that each treatment alters microbial communities by affecting the
327 relative abundances in great magnitude; thus, care should be taken when comparing values
328 across studies using different protocols, especially when incorporating metrics such as Shannon
329 index. The most abundant phyla across all samples were Proteobacteria and Firmicutes, which
330 is consistent with what was previously reported for House sparrows (Kohl et al., 2019; Mirón
331 et al., 2014); however, we found higher relative abundance of Proteobacteria in samples stored
332 dried. This result suggests that consideration should be given to differences in abundance at
333 certain taxonomic levels that have undergone this type of storage, particularly those involving
334 Proteobacteria and Actinobacteria which are able to grow at a range of temperatures (Weese
335 and Jalali, 2014).
336

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

340 storage conditions can be used as a proxy of the global effect on storage methods (Fouhy et al.,
341 2015); we found that a greater proportion of aerobes were recovered following refrigeration,
342 suggesting that oxygen-tolerant bacteria are thriving after collection, driving biases on the
343 community composition. We also found that the levels of total anaerobic and facultative
344 bacteria in RNA_{later} samples were similar to the ones detected in samples processed using the
345 TerraLyzer, which suggests that immediate submersion in buffer solution following collection
346 enables the recovery of comparable types of microbiome. Remarkably, the recovery rates of
347 taxonomic groups in RNA_{later} are not comparable to those found in samples processed by the
348 TerraLyzer.

349

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

357

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

368

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

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

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

389

390 **5. Conclusions**

391

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

400

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632

633 **Appendix A. Supplementary Information**

634

635 *Methodology for bioinformatics*

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

646

647 *Methodology for determining bacteria respiration type*

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

657

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

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

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

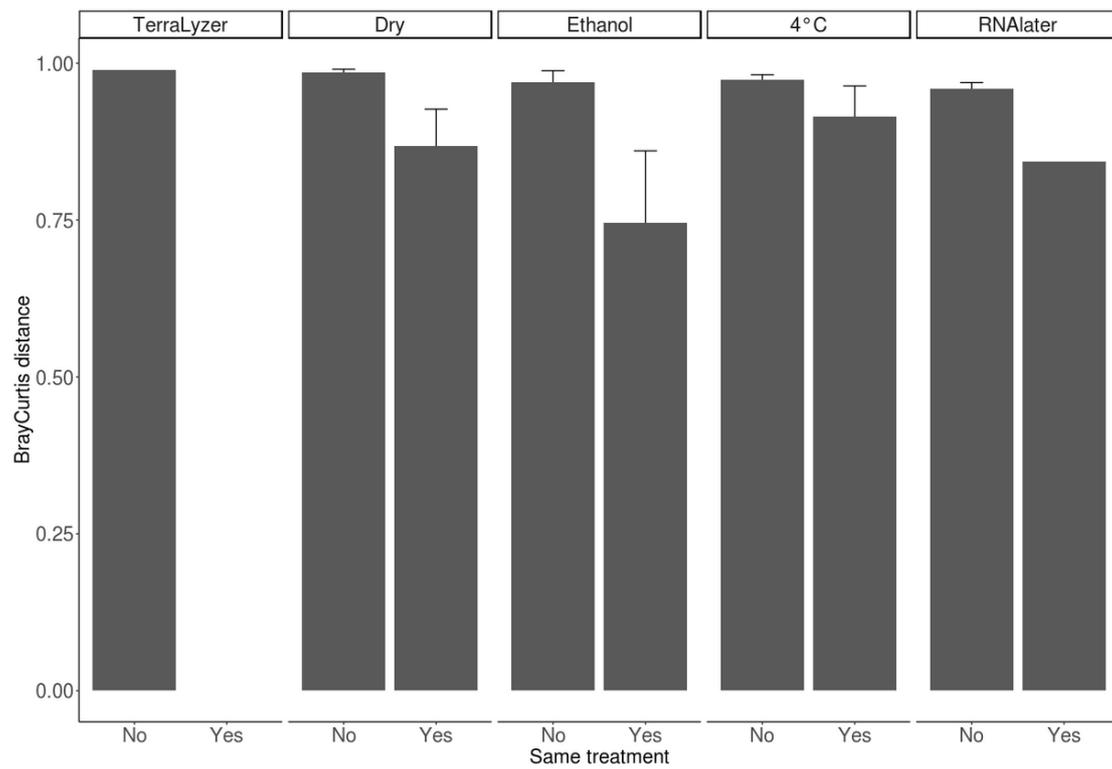
*Prices to March 2019.

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661 **Figure A1. Differences in the faecal microbiota between treatments.** Bray-Curtis distances

662 between the faecal bacterial communities. Mean and s.e.m. values are plotted.



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