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### ► To cite this version:

Sarah Leclaire, Maxime Pineaux, Pierrick Blanchard, Joël White, Scott Hatch. Microbiota composition and diversity of multiple body sites vary with reproductive performance in a seabird. *Molecular Ecology*, In press, 10.1111/mec.16398 . hal-03770687

**HAL Id: hal-03770687**

**<https://cnrs.hal.science/hal-03770687>**

Submitted on 6 Sep 2022

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1 **Running head:** Microbiota and fitness in a seabird

2

3 **Microbiota composition and diversity of multiple body sites vary**  
4 **according to reproductive performance in a seabird**

5

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14

15 **Abstract**

16 The microbiota is suggested to be a fundamental contributor to host reproduction and survival, but  
17 associations between microbiota and fitness are rare, especially for wild animals. Here, we tested the  
18 association between microbiota and two proxies of breeding performance in multiple body sites of  
19 the black-legged kittiwake, a seabird species. First we found that, in females, non-breeders (i.e., birds  
20 that did not lay eggs) hosted different microbiota composition to that of breeders in neck and flank  
21 feathers, in the choanae, in the outer-bill and in the cloacae, but not in preen feathers and tracheae.  
22 These differences in microbiota might reflect variations in age or individual quality between breeders  
23 and non-breeders. Second, we found that better female breeders (i.e., with higher body condition,  
24 earlier laying date, heavier eggs, larger clutch, and higher hatching success) had lower abundance of  
25 several *Corynebacteriaceae* in cloaca than poorer female breeders, suggesting that these bacteria  
26 might be pathogenic. Third, in females, better breeders had different microbiota composition and  
27 lower microbiota diversity in feathers, especially in preen feathers. They had also reduced dispersion  
28 in microbiota composition across body sites. These results might suggest that good breeding females  
29 are able to control their feather microbiota - potentially through preen secretions - more tightly than  
30 poor breeding females. We did not find strong evidence for an association between reproductive  
31 outcome and microbiota in males. Our results are consistent with the hypothesis that natural  
32 variation in the microbiota is associated with differences in host fitness in wild animals, but the  
33 causal relationships remain to be investigated.

34

35 **Keywords:** Fitness; Black-legged kittiwake; Individual quality; Microbiota; Reproductive success;  
36 Bacteria; Feathers

37

## 38 **Introduction**

39 Host-associated bacterial communities are emerging as fundamental modulators of host phenotype  
40 (Clemente, Ursell, Parfrey, & Knight, 2012). The gut microbiota, for instance, can facilitate digestion,  
41 occupy the niche space of pathogens, prime the development of the immune system or influence  
42 learning and memory (McFall-Ngai et al., 2013). The microbiota is therefore likely to contribute to  
43 host reproduction and survival (Heil, Paccamonti, & Sones, 2019; Sison-Mangus, Mushegian, & Ebert,  
44 2014), and is, thus, increasingly hypothesized to play an important role in host evolution  
45 (Bordenstein & Theis, 2015; Moeller & Sanders, 2020; Simon, Marchesi, Mougel, & Selosse, 2019).  
46 However, in contrast to humans and several animal models (Barko, McMichael, Swanson, & Williams,  
47 2018; Raymann & Moran, 2018; Rosshart et al., 2017), the importance of the microbiota has not  
48 been well-established in wild animals. In natural populations, while a growing number of studies has  
49 associated microbiota with ecological or host traits (e.g. Leclaire et al., 2019; Mallott, Amato, Garber,  
50 & Malhi, 2018; Teyssier et al., 2018), direct associations between microbiota and proxies of fitness  
51 are rare (but see Ambrosini et al., 2019; Antwis, Edwards, Unwin, Walker, & Shultz, 2019; Stothart et  
52 al., 2021; Suzuki, 2017; Videvall et al., 2019).

53 Variations in fitness between hosts are expected to be associated with several characteristics  
54 of the microbiota. First, variations in fitness can stem from individual or environmental factors that  
55 displace sensitive bacterial taxa and thus produce deterministic changes in the composition of  
56 microbiota assemblage. For instance, age, immunogenetic characteristics, food availability and  
57 pollution levels can affect the ability to invest in reproduction (e.g., Ariyomo & Watt, 2012;  
58 Kowalczyk, Chiaradia, Preston, & Reina, 2014; Nol & Smith, 1987), and are often associated with  
59 variations in microbiota composition (Bolnick et al., 2014; Fontaine, Novarro, & Kohl, 2018; Jin, Wu,  
60 Zeng, & Fu, 2017). Second, variation in fitness may be related to heterogeneity in bacterial  
61 community, if it is associated with stressors that destabilise microbial community. This hypothesis  
62 lies within the framework of the "Anna Karenina principle", which suggests that dysbiotic individuals

63 have less stable microbiota, and thus vary more in microbial community composition, than eubiotic  
64 individuals (Ma, 2020; Zaneveld, McMinds, & Thurber, 2017). For instance, decreased fitness may be  
65 related to disruption in immunity (Eraud, Jacquet, & Faivre, 2009; Reaney & Knell, 2010; Uller,  
66 Isaksson, & Olsson, 2006), which may release the constraints exerted by immunity on microbiota  
67 (Belkaid & Harrison, 2017). This is expected to produce stochastic variations in microbiota  
68 composition and to result in high inter-individual variability in microbiota. Finally, although recently  
69 debated, it is widely assumed that more diversity in microbial community is better (Johnson &  
70 Burnet, 2016; Reese & Dunn, 2018). Therefore, individuals with higher fitness may be expected to  
71 have microbiota with different composition ( $\beta$ -diversity), lower heterogeneity ( $\beta$ -dispersion) and  
72 higher diversity ( $\alpha$ -diversity) compared to individuals with lower fitness.

73         Most of the work on wild microbiota has focused on the gut. However, several studies have  
74 shown that other parts of the body are also inhabited by a multitude of microorganisms that are  
75 essential to the maintenance of physiological homeostasis (Dickson, Erb-Downward, Martinez, &  
76 Huffnagle, 2016; Grice & Segre, 2011). For instance, the microbiota of the reproductive tract can  
77 affect semen quality and pregnancy outcome and thus can have major impact on host fertility (Baud  
78 et al., 2019; Green, Zarek, & Catherino, 2015; reviewed in Rowe, Veerus, Trosvik, Buckling, & Pizzari,  
79 2020; Weng et al., 2014). The skin microbiota affects immune barrier response and epithelial  
80 integrity and is therefore an important modulator of skin health (Chen, Fischbach, & Belkaid, 2018).  
81 Accordingly, in bats and amphibians, skin bacteria have a role in the defence against fungal  
82 pathogens (Harris et al., 2009; Hoyt et al., 2015). In birds, feathers harbour feather-degrading  
83 bacteria, that can impair bird thermoregulation, flight and signalling and ultimately fitness (Burt Jr &  
84 Ichida, 1999; Gunderson, 2008; Leclaire, Pierret, Chatelain, & Gasparini, 2014), while the uropygial  
85 secretions (also called preen secretions) can harbour bacteria with antimicrobial properties (Martin-  
86 Vivaldi et al., 2010; Soler, Martín-Vivaldi, Peralta-Sánchez, & Ruiz-Rodríguez, 2010) that may be  
87 crucial for protecting the embryo from bacterial infection (Martín-Vivaldi et al., 2014). To better  
88 appreciate the role of the microbiota in the evolutionary biology of the host, there is therefore a

89 strong need for investigations on the relationship between fitness and bacterial communities  
90 harboured in various parts of the body in wild animals (Comizzoli & Power, 2019; Sarah M Hird, 2017;  
91 Ross, Rodrigues Hoffmann, & Neufeld, 2019).

92 Here we investigated associations between microbiota of multiple body sites and host  
93 reproductive outcome in the black-legged kittiwake (*Rissa tridactyla*). This colonial seagull is a  
94 sexually monogamous species (Helfenstein, Tirard, Danchin, & Wagner, 2004) with biparental care.  
95 Males compete for nest sites, participate in nest building, provide most of the food to females during  
96 egg formation and share incubation and parental care with their mates (Helfenstein, Wagner,  
97 Danchin, & Rossi, 2003; Leclaire, Helfenstein, Degeorges, Wagner, & Danchin, 2010). To characterize  
98 the reproductive outcome of individuals, we used two proxies: breeding status (breeders vs non-  
99 breeders) and an index of breeder reproductive performance that combines several traits associated  
100 with reproduction, including body condition, laying date, egg mass, clutch size and hatching success.  
101 Non-breeding may be an adaptive strategy to offset reproductive costs on survival (Cubaynes,  
102 Doherty, Schreiber, & Gimenez, 2011) and hence maximise lifetime reproductive success (Desprez,  
103 Gimenez, McMahon, Hindell, & Harcourt, 2018; Griffen, 2018). However, in kittiwakes, although the  
104 causes of non breeding are various (Cadiou, Monnat, & Danchin, 1994; Cam, Hines, Monnat, Nichols,  
105 & E., 1998; Danchin & Cam, 2002; Desprez, Pradel, Cam, Monnat, & Gimenez, 2011), non-breeders  
106 show lower survival probability than breeders (Cam et al., 1998), suggesting that skipping breeding is  
107 generally a determinant of fitness in this species, as found in common guillemots (*Uria aalge*) (Reed,  
108 Harris, & Wanless, 2015) and southern elephant seals (*Mirunga leonina*) (Desprez et al., 2018). We  
109 focused on microbiotas sampled during the pre-laying period (i.e., the copulation and nest-building  
110 period). To characterize the microbiota, we used high-throughput sequencing and focused on  
111 multiple body sites, including the cloaca, flank feathers, neck feathers, preen feathers (i.e., the small  
112 feathers that surround the duct pores of the preen gland and that are commonly saturated with  
113 preen gland secretions), upper respiratory tract (choana and trachea), and outer bill.

114

## 115 **Methods**

### 116 *Study site*

117 The study was carried out in 2016 and 2017 breeding seasons in a population of black-legged  
118 kittiwakes nesting on an abandoned U.S. Air Force radar tower on Middleton Island (59°26'N,  
119 146°20'W), Gulf of Alaska. Artificial nest sites created on the upper walls were observed from inside  
120 the tower through sliding one-way windows (Gill & Hatch, 2002). This enabled us to easily capture  
121 and monitor breeders. We captured birds during the pre-laying period (7 May 2016 - 12 June 2016  
122 and 29 April 2017 - 14 June 2017; mean  $\pm$  SE: 20  $\pm$  1 days before laying, range: 42 - 4 days before  
123 laying). Six birds were sampled both in 2016 and 2017. To reduce interdependency (Results S1), we  
124 decided to exclude their 2016 samples from the analyses. The sample size after filtering (see below  
125 for details about filtering) is given in Table 1 (total: 94 birds in 2016 and 67 birds in 2017). At capture,  
126 the microbiotas were sampled, birds were weighed to the nearest 5 g with a Pesola® scale, and head-  
127 bill length was measured to the nearest millimetre with a calliper. Studied birds were sexed based on  
128 molecular methods (Merkling et al., 2012) or sex-specific behaviours (copulation and courtship  
129 feeding) (Jodice, Lanctot, Gill, Roby, & Hatch, 2000).

130 All nest sites were checked twice daily (at 9:00 and 18:00) to record bird presence, and  
131 events such as egg laying and egg loss. When an egg was laid, it was weighed to the nearest 0.01 g  
132 with an electronic scale, and marked with nontoxic waterproof ink within 12 h of laying.

133

### 134 *Reproductive outcome*

135 We defined two indices of reproductive outcome. First, we considered breeding status, i.e. breeders  
136 vs. non-breeders. Individuals were considered non-breeders when they were regularly seen  
137 occupying a nesting site, but did not lay eggs. In our study, all non-breeders had mating partners and  
138 attempted to breed. In contrast, breeders built a nest and laid 1 or 2 eggs. All birds that were  
139 captured at the tower but never observed again were excluded from the analyses, as their breeding

140 status was unknown since they may have bred elsewhere on the island (see supplementary material  
141 for further details on the sample size).

142         Second, we focused on breeders, and defined an index of reproductive performance. We  
143 conducted a principal component analyses (PCA) on several life-history traits commonly related to  
144 individual reproductive performance: body condition, laying date, clutch size (one or two eggs), egg  
145 mass of the first-laid egg and hatching success. These measures are related to individual fitness in  
146 numerous bird species, including kittiwakes (e.g. Coulson & Porter, 1985; Moe, Langseth, Fyhn,  
147 Gabrielsen, & Bech, 2002; Verhulst & Nilsson, 2008). Body condition was estimated as the residuals  
148 of a linear regression between body mass and head-bill length performed within each sex. We used  
149 head-bill length, as it is known to correlate better with mass than other structural features in  
150 kittiwakes (Jodice et al., 2000). Clutch size was calculated as the number of eggs laid. Although two  
151 eggs is the typical clutch size in this population of kittiwakes (Gill & Hatch, 2002), 2016 and 2017  
152 were two very poor breeding seasons and an unusually high number of birds laid either no eggs or a  
153 single egg. In the studied population, the mean clutch size was < 1 egg in 2016 and 2017, while it was  
154 > 1.3 egg from 2000 to 2015 (SA Hatch, pers. obs.). Hatching success was calculated as the proportion  
155 of eggs that hatched. The egg of two breeders was not weighed, and these two breeders were thus  
156 excluded for the analyses on reproductive performance.

157         Breeder reproductive performance was described by the first two principal components of  
158 the PCA (hereafter referred as  $PC1_{RepPerf}$  and  $PC2_{RepPerf}$ ).  $PC1_{RepPerf}$  and  $PC2_{RepPerf}$  accounted for 35%  
159 and 24% of the variance observed among life-history traits, respectively. Individuals with higher  
160  $PC1_{RepPerf}$  were in higher body condition, laid earlier, had heavier eggs and larger clutches, and had  
161 higher hatching success (eigenvector: +0.54, -0.56, +0.60, +0.50 and +0.75). Individuals with higher  
162  $PC2_{RepPerf}$  laid earlier, but had lighter eggs and smaller clutches (eigenvector: -0.63, -0.60 and -0.54).  
163  $PC2_{RepPerf}$  was not associated with microbiota composition, dispersion or diversity in any body sites,  
164 and the results are thus not reported in the manuscript.

165



166 *Microbiota sampling*

167 Birds were caught by the leg using a metal hook and were manipulated with new clean latex gloves,  
168 which were changed between each capture. Feather microbiotas were collected from standardized  
169 positions of the neck and flank regions (Fig. S1), and from the preen tuft. For each of these body  
170 regions, we cut a few feathers using scissors and tweezers sterilized with 70% ethanol between each  
171 capture. Feathers were placed in sterile 2ml plastic tubes. Feathers were always held with tweezers.  
172 We detected similarity in feather microbiota within pairs (see result section below) and within  
173 individuals (see results S1 in supplementary material), as well as large differences between preen  
174 feathers and other feathers (see result section below), suggesting that potential transfers of bacteria  
175 between birds or within birds due to poor equipment sterilization or the gloves touching various bird  
176 body sites were low. We sampled outer bill, choanal and tracheal bacteria using sterile plain cotton  
177 swabs (Copan Italia, Brescia, Italy). We sampled cloacal bacteria by flushing the cloaca with 1 mL of  
178 sterile phosphate-buffered saline solution. This was performed by gently inserting the tip of a sterile  
179 needleless syringe 5 mm into the cloaca, injecting the saline solution and drawing it out again. We  
180 also collected control samples by collecting empty sterile tubes, blank swabs or by injecting saline  
181 solution directly into sterile tubes to control for any possible contamination coming from the  
182 manipulation or the material. All samples were stored at -20°C until molecular analyses. In 2017, all  
183 types of microbiota were sampled, while in 2016, only the cloacal microbiota was sampled. In  
184 addition, due to other experiments run during the 2017 breeding season, not all body sites were  
185 sampled on each single individual. This led to disparate numbers of samples among body sites (Table  
186 1).

187

188 *Molecular analysis of microbiota*

189 Because we originally wanted to study viruses in respiratory tracts, we used two different DNA  
190 extraction protocols. DNA was extracted from feather, outer bill and cloacal samples using the  
191 Qiagen® Blood and Tissue DNA Extraction kit and the standard protocol designed for purification of

192 total DNA for gram-positive bacteria (Qiagen, Venlo, Netherland, July 2006). In contrast, DNA was  
193 extracted from tracheal and choanal swabs using the Nucleospin RNA virus kit following the  
194 manufacturer's instructions (Macherey-Nagel, Düren, Germany).

195 To characterize the bacterial communities present in each sample, we performed 16S rRNA  
196 amplicon high-throughput sequencing. PCR amplifications were performed in 20 µl mixtures  
197 containing 2 µl of diluted DNA extract, 1 × AmpliTaq Gold™ 360 master mix (Applied Biosystem,  
198 Foster City, CA, USA), 0.5 µm of each primer and 3.2 µg of bovine serum albumin (Roche Diagnostics,  
199 Basel, Switzerland). PCR conditions consisted of an initial denaturation at 95°C for 10 min, followed  
200 by 30 cycles of denaturation (at 95°C for 30 s), annealing (at 57°C for 30 s) and elongation (at 72°C for  
201 90 s). A final elongation step was run at 72°C for 7 min. We used universal primers that specifically  
202 amplified the V5–V6 region (~295 bp in length) of the bacterial 16S rRNA gene (BACTB-F:  
203 GGATTAGATACCCTGGTAGT and BACTB-R: CACGACACGAGCTGACG) (Fliegerova et al., 2014). To  
204 discriminate between samples after sequencing, the 5' end of both forward and reverse primers  
205 included a combination of two different 8-bp tags. The PCR products were purified using the  
206 MiniElute™ PCR purification kit (Qiagen, Hilden, Germany), and then pooled (4 µl of each PCR  
207 product in the pool). Amplicons were sequenced with an Illumina MiSeq platform, using the 2 x  
208 300-bp protocol (Fasteris SA, Plan-les-Ouates, Switzerland). Samples were included in three Illumina  
209 runs: feather, bill and cloacal samples from 2017 were included in the first run, tracheal and oral  
210 microbiota were included in the second run, and cloacal samples from 2016 were included in the  
211 third run. We included sampling controls, extraction controls and PCR blank controls, as well as  
212 unused tag combinations, in the sequenced multiplexes to detect and withdraw potential  
213 contaminants (Salter et al., 2014).

214 We first analysed the sequence reads using the OBITools package (Boyer et al., 2016). Briefly,  
215 after assembly of paired-end reads, we assigned reads to their respective samples and excluded  
216 reads with low assembly scores or that contained ambiguous bases (i.e. "N"). We dereplicated strictly  
217 identical reads using the "obiuniq" algorithm (Boyer et al., 2016), and excluded sequences with fewer

218 than 10 reads. We then analysed the sequences using the FROGS pipeline (Escudié et al., 2018).  
219 Briefly, amplicons were clustered into operational taxonomic units (OTUs) using the "swarm" method  
220 and an aggregation distance of 3 (Mahé, Rognes, Quince, de Vargas, & Dunthorn, 2014), and  
221 chimeras were removed using VSEARCH (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). Taxonomic  
222 assignment was then performed using blast affiliation and the SILVA v138 database (Quast et al.,  
223 2012). Finally, we used the controls to remove potential contaminants from the data set. We  
224 considered a potential contaminant each OTU for which the mean number of reads across control  
225 samples was higher than the mean number of reads across non-control samples ( $n = 125$  OTUs). We  
226 also considered a contaminant each OTU for which the highest total number of reads per sample was  
227 in a control sample ( $n = 10$  additional OTUs). We excluded samples that had fewer than 500  
228 sequences ( $n = 19$  samples), and removed OTUs with fewer than 50 reads ( $n = 1818$  OTUs). We  
229 obtained mean  $\pm$  SE per sample: 14 328  $\pm$  584 sequences, 19 091  $\pm$  1 667 sequences, 16 177  $\pm$  833  
230 sequences, 19 522  $\pm$  2 556 sequences, 13 540  $\pm$  1 980 sequences, 21 279  $\pm$  2 137 sequences, 23 061  $\pm$   
231 1 440 sequences in cloacal, neck feather, flank feather, preen feather, outer-bill, tracheal and  
232 choanal samples respectively.

233 To analyse  $\beta$ -diversity, we used the "Phylogenetic Isometric Log-ratio Transform" (PhILR)  
234 which allows analysis of compositional data where the parts can be related through a phylogenetic  
235 tree (Silverman, Washburne, Mukherjee, & David, 2017). PhILR has been suggested to be a  
236 replacement for the weighted UniFrac distances when accounting for the compositional nature of  
237 the data is needed (Gloor, Macklaim, Pawlowsky-Glahn, & Egozcue, 2017). We calculated the relative  
238 OTU abundance per sample (also known as "total sum scaling" (TSS)) (McKnight et al., 2019). OTU  
239 sequences were aligned using Decipher (Wright, 2015). Then a maximum-likelihood tree was  
240 constructed using the package phangorn (Schliep, 2011). This tree was used as a reference from  
241 which the Euclidean distances were calculated on phylogenetic isometric log-ratio transformed OTU  
242 abundance (i.e., the PhILR transform) between samples (Egozcue, Pawlowsky-Glahn, Mateu-Figueras,

243 & Barcelo-Vidal, 2003; Gloor et al., 2017) using the package PhiLR (Silverman et al., 2017). In the  
244 PhiLR transform, we added a pseudocount of 0.1 reads.

245  $\alpha$ -diversity was measured as the weighted phylogenetic diversity (Faith's PD) based on the  
246 non-rooted phylogenetic tree and using the picante package (Kembel & Kembel, 2020). Faith's PD is  
247 the sum of total phylogenetic length of OTUs in each sample. Before  $\alpha$ -diversity analysis, we rarefied  
248 each sample to 5000 sequences. Results were similar when including or excluding the samples with <  
249 5000 sequences, and we report results when not excluding samples with < 5000 sequences. The  
250 number of reads per sample was lower in outer-bill than in other body sites. For the analysis of  $\alpha$ -  
251 diversity of outer-bill microbiota, we therefore set the rarefaction threshold to 1000 sequences.

252

### 253 *Statistics*

254 We performed all statistical analyses using the R statistical software (R Core Team, 2017). To analyze  
255 differences in microbiota composition between breeding status (breeder vs. non-breeder) or breeder  
256 performance ( $PC1_{RepPerf}$ ), we performed PERMANOVAs ("Adonis2" function in the VEGAN package in  
257 R, with 5000 permutations and the "margin" option), based on the PhiLR transform distance matrix.  
258 In the models, breeding status and date of sampling, or  $PC1_{RepPerf}$  and the difference between laying  
259 date and capture date, were included as predictors. To account for the non-independence of the two  
260 members of a pair (see result section below), we carried out PERMANOVAs within each sex. The two  
261 members of a breeding pair were never both sampled for tracheal or choanal microbiota, but  
262 tracheal and choanal microbiotas of females were sampled later during the breeding season than  
263 those of males (Kruskal-Wallis test:  $\chi^2_1 = 10.42$ ,  $P = 0.001$  and  $\chi^2_1 = 9.92$ ,  $P = 0.002$ ). Therefore, to  
264 avoid confounding the sex and date effects, we carried out PERMANOVAs within each sex for  
265 tracheal and choanal microbiota also. We did not test for associations between tracheal and choanal  
266 microbiota composition and  $PC1_{RepPerf}$  as the sample size within each sex was very low (Table 1).  
267 Cloacal samples collected in 2016 were analysed in a different Illumina run compared to the cloacal

268 samples collected in 2017. The year effect is therefore confounded with a sequencing run effect, and  
269 in the analysis of cloacal microbiota, we constrained the permutation within years of sampling.

270 To predict the OTUs that were best associated with breeding status and breeding  
271 performance, we used two different procedures. First, we used correlation indices with the point  
272 biserial coefficient of association (multipatt procedures in the indicpecies package in R) (De Cáceres  
273 & Legendre, 2009) to determine a small set of OTUs that best characterize breeding status or  
274 breeding performance. The multipatt procedure cannot associate OTU to a continuous variable.  
275 Therefore, PC1<sub>RepPerf</sub> was split into two categories of equal sample size. In the multipatt analyses, we  
276 did not adjust for multiple comparisons, as the purpose of these analyses was exploratory in order to  
277 generate, rather than confirm, hypotheses (Bender & Lange, 2001; Ranstam, 2019). We also used the  
278 selbal procedure, which compares the average abundance of two groups of microbial species (selbal  
279 package in R) (Rivera-Pinto et al., 2018). In the multipatt and selbal procedures, we considered OTUs  
280 that were present in at least 10 % of the individuals and whose the mean relative abundance was >  
281 0.025 % across samples. Correlation indices and Selbal cannot be compared as they rely on two  
282 different concepts. In correlation indices, microbial signature is defined as a combination of  
283 individual taxa, while in selbal, it is defined as a compositional balance between two groups of taxa.

284 To determine whether  $\alpha$ -diversity varied with breeding status or breeder performance, we  
285 used linear mixed models with reproductive outcome (breeding status or PC1<sub>RepPerf</sub>), sex, date  
286 (sampling date or the difference between laying date and sampling date), year (for the analysis on  
287 cloacal microbiota only) and the interaction between reproductive outcome and sex as fixed effects  
288 and we included nest identity as a random factor. For tracheal and choanal microbiota, we used  
289 linear models. Normality of the residuals was checked using Shapiro-Wilk normality tests.  
290 Homogeneity of variance was checked visually (for continuous variables) or using Levene's tests (for  
291 dichotomous variables).  $\alpha$ -diversity in cloacal microbiota was square-root transformed in the  
292 analyses of breeding success.

293 Dispersion in microbiota composition was calculated as the average distance to group  
294 centroids using the "betadisper" function in the VEGAN package. We studied both inter-individual  
295 dispersion within each body site and intra-individual dispersion across body sites. For the calculation  
296 of inter-individual dispersion, groups were defined according to breeding status or hatching success  
297 (i.e., a categorical variable related to  $PC1_{RepPerf}$ ). The association between inter-individual dispersion  
298 and breeding status or hatching success was tested using permutation tests ("permutest" function in  
299 the VEGAN package in R). These inter-individual dispersion analyses were also needed to judge the  
300 adequacy of PERMANOVA results, as PERMANOVA tests may confound location and dispersion  
301 effects, especially for unbalanced designs (Anderson & Walsh, 2013).

302 For the calculation of intra-individual dispersion across body sites, each group was defined as  
303 a bird individual. Because not all body sites were sampled on each single individual, we restricted the  
304 intra-individual dispersion analyses on individuals for which cloaca, flank feathers, neck feathers,  
305 preen feathers and outer-bill were sampled (n = 36 and 21 individuals for the analysis on breeding  
306 status and reproductive performance respectively). We did not include the tracheal or choanal  
307 samples, as it would drop the sample size to fewer than 9 individuals. The association between intra-  
308 individual dispersion and breeding status or  $PC1_{RepPerf}$  was tested using Kruskal-Wallis tests or  
309 Spearman correlation tests.

310 To determine whether microbial composition varied with body sites (i.e., cloaca, flank  
311 feathers, neck feathers, outer bill, preen feathers, trachea and choanae) and bird identity, we  
312 performed PERMANOVAs, with body site, bird identity, and date as predictors. We restricted the  
313 analyses to the 2017 samples. We did not include sex in the models as PERMANOVAs carried out  
314 within body sites (see above) did not show significant differences in microbiota composition between  
315 males and females (see result section below). Pairwise post-hoc PERMANOVAs with Bonferroni  
316 correction were carried out to verify that all body sites were different from one another. To  
317 determine the OTUs that were preferentially associated with each body site, we used correlation  
318 indices with duleg option = FALSE to consider group combinations (De Cáceres, Legendre, & Moretti,

319 2010). We restricted these correlation index analyses on OTUs whose mean abundance was at least >  
320 5 % in one of the body sites (n = 15 OTUs). We

321 Because the two members of a pair share the same nesting environment, preen each others,  
322 share food and copulate, microbial composition may be more similar within pairs than between  
323 pairs. For each body site, we therefore tested similarity in microbiota composition between the two  
324 members of a pair using PERMANOVAs with nest identity and date as predictors. We constrained the  
325 PERMANOVA permutations within breeding status. We restricted the analyses on pairs in which both  
326 members were captured. Similarity between mates in tracheal and choanal microbiota could not be  
327 tested as the two members of a pairs were never both captured. To test differences between males  
328 and females in microbiota composition, we carried out PERMANOVAs with sex and date as fixed  
329 effects and constrained the permutations within each nest.

330

## 331 **Results**

### 332 *Association between microbiota and breeding status*

333 The composition of cloacal microbiota varied with breeding status in females ( $F_{1,79} = 1.74$ ,  $R^2 = 0.02$ ,  $P$   
334  $= 0.025$ ; Fig. 1a), and was marginally associated with breeding status in males ( $F_{1,66} = 1.58$ ,  $R^2 = 0.02$ ,  
335  $P = 0.053$ ; Fig. S2a). Correlation indices did not identify many OTUs as being characteristics of non-  
336 breeders or breeders in cloaca (Table S1) and the discrimination value of selbal is relatively low (Fig.  
337 S3), suggesting that the cloacal microbiota of breeders and non-breeders cannot be well-  
338 discriminated using a few OTUs only.

339 The composition of flank feather microbiota and neck feather microbiota varied with  
340 breeding status in females ( $F_{1,19} = 1.89$ ,  $R^2 = 0.08$ ,  $P = 0.004$  and  $F_{1,19} = 1.62$ ,  $R^2 = 0.08$ ,  $P = 0.018$ ; Figs.  
341 1b and 1c), but not in males ( $F_{1,28} = 1.17$ ,  $R^2 = 0.04$ ,  $P = 0.18$  and  $F_{1,27} = 1.18$ ,  $R^2 = 0.04$ ,  $P = 0.13$ ; Figs.  
342 2b and 2c). Correlation indices showed that several of the OTUs best representing the differences  
343 between breeders and non-breeders in females belonged to the families *Lachnospiraceae* and  
344 *Salinisphaeraceae* and were enriched in non-breeders (Table S1). To determine whether, more

345 generally, breeding status was associated with higher relative abundance of *Lachnospiraceae* and  
346 *Salinisphaeraceae*, we summed the relative abundance of all *Lachnospiraceae* (n = 16 OTUs) or  
347 *Salinisphaeraceae* OTUs (n = 12 OTUs) within each sample and carried out Kruskal-Wallis tests. We  
348 found that, compared to breeding females, non-breeding females had higher abundance of  
349 *Lachnospiraceae* in flank feathers ( $\chi^2_1 = 12.67$ ,  $P < 0.001$ ; Fig. 2a) and had marginally higher  
350 abundance of *Lachnospiraceae* in neck feathers ( $\chi^2_1 = 3.43$ ,  $P = 0.067$ ; Fig. 2a). Non-breeding females  
351 had also higher abundance of *Salinisphaeraceae* in flank and neck feathers ( $\chi^2_1 = 6.26$ ,  $P = 0.012$  and  
352  $\chi^2_1 = 4.36$ ,  $P = 0.037$  respectively; Fig. 2b).

353 The composition of outer-bill and choanal microbiota varied also with breeding status in  
354 females ( $F_{1,16} = 1.72$ ,  $R^2 = 0.10$ ,  $P = 0.013$  and  $F_{1,10} = 1.89$ ,  $R^2 = 0.14$ ,  $P = 0.035$ ; Figs. 1d and 1e), but  
355 not in males ( $F_{1,26} = 0.87$ ,  $R^2 = 0.03$ ,  $P = 0.69$  and  $F_{1,17} = 0.85$ ,  $R^2 = 0.05$ ,  $P = 0.71$ ; Figs. S2d and S2e).  
356 Indicator value showed that the some of the OTUs best representing the differences between  
357 breeders and non-breeders in the outer-bill microbiota of females belonged to the genus  
358 *Salinisphaera* (enriched in non-breeders; Table S1). Indicator value showed that the OTUs best  
359 representing the differences between breeders and non-breeders in choanal microbiota belonged to  
360 diverse families (Table S1), but interestingly, both indicator value and selbal identified  
361 *Pasteurellaceae* OTUs as being enriched in non-breeders compared to breeders (Table S1). However,  
362 in females, inter-individual dispersion in outer-bill and choanal microbiota was higher in non-  
363 breeders than breeders (see below; Figs. 1d and 1e). These differences in dispersion might thus have  
364 contributed to the significant differences obtained in PERMANOVAs (Anderson & Walsh, 2013),  
365 especially for outer-bill whose the sample size was not balanced for (6 non-breeding females and 12  
366 breeding females). Although plots (Figs. 1d and 1e) suggest that both composition (group centroid)  
367 and dispersion were different between breeding and non-breeding females, further studies including  
368 larger sample size are required to ascertain the effect of breeding status on outer-bill and choanal  
369 microbiota composition in females.



370 The composition of tracheal and preen feather microbiota did not vary with breeding status  
371 in males and females (trachea:  $F_{1,18} = 1.34$ ,  $R^2 = 0.07$ ,  $P = 0.15$  and  $F_{1,11} = 1.31$ ,  $R^2 = 0.11$ ,  $P = 0.12$ ;  
372 preen feathers:  $F_{1,20} = 1.15$ ,  $R^2 = 0.05$ ,  $P = 0.19$  and  $F_{1,19} = 1.18$ ,  $R^2 = 0.06$ ,  $P = 0.15$ ).

373  $\alpha$ -diversity did not vary with breeding status in any of the microbiota types (all  $P > 0.14$ ). In  
374 flank feathers however,  $\alpha$ -diversity was marginally associated with breeding status in interaction with  
375 sex ( $F_{1,27} = 3.92$ ,  $P = 0.058$ ). However, when considering each sex separately,  $\alpha$ -diversity was  
376 significantly different between non-breeders and breeders neither in males (mean  $\pm$  SE:  $47.5 \pm 3.4$  vs.  
377  $52.7 \pm 2.4$  respectively;  $F_{1,28} = 1.74$ ,  $P = 0.20$ ), nor in females (mean  $\pm$  SE:  $54.8 \pm 2.0$  vs.  $49.4 \pm 3.6$   
378 respectively;  $F_{1,19} = 0.69$ ,  $P = 0.42$ ).

379 In females, inter-individual dispersion in outer-bill and choanal microbiota was higher in non-  
380 breeders than breeders ( $F_{1,16} = 7.81$ ,  $P = 0.013$  and  $F_{1,11} = 7.43$ ,  $P = 0.016$ ; Figs. 1d and 1e). Inter-  
381 individual dispersion was also found to be higher in breeding males than in non-breeding males in  
382 preen feather microbiota ( $F_{1,20} = 6.55$ ,  $P = 0.016$ ). Intra-individual dispersion in microbiota across  
383 body sites was not different between breeders and non-breeders either in males or in females ( $\chi^2_1 =$   
384  $2.15$ ,  $P = 0.14$ ,  $n = 20$  and  $\chi^2_1 = 2.31$ ,  $P = 0.13$ ,  $n = 16$ ).

385

### 386 *Association between microbiota and breeding performance*

387 The composition of cloacal microbiota varied with  $PC1_{RepPerf}$  in females ( $F_{1,36} = 2.18$ ,  $R^2 = 0.06$ ,  $P =$   
388  $0.004$ ; Fig. 3) but not in males ( $F_{1,34} = 0.73$ ,  $R^2 = 0.02$ ,  $P = 0.98$ ). Correlation indices showed that the  
389 majority of the OTUs that best characterized  $PC1_{RepPerf}$  belonged to the family *Corynebacteriaceae*  
390 and were enriched in the cloacae of females with low  $PC1_{RepPerf}$  (Fig. 3 and Table S2). Selbal analysis  
391 identified a balance of OTU\_73 (*Clostridia*) and OTU\_320 (*Corynebacteriaceae*) vs. OTU\_12  
392 (*Psychrobacter*) as being associated with  $PC1_{RepPerf}$  in female cloaca (Table S2 and Fig. S4a).

393 The composition of preen feather microbiota varied with  $PC1_{RepPerf}$  in females ( $F_{1,12} = 1.61$ ,  $R^2$   
394  $= 0.12$ ,  $P = 0.018$ ), while the composition of neck and flank feather microbiota was marginally

395 associated with PC1<sub>RepPerf</sub> in females ( $F_{1,10} = 1.37$ ,  $R^2 = 0.11$ ,  $P = 0.066$  and  $F_{1,11} = 1.50$ ,  $R^2 = 0.11$ ,  $P =$   
396  $0.053$ ). The composition of preen, neck and flank feather microbiota did not vary with PC1<sub>RepPerf</sub> in  
397 males ( $F_{1,11} = 1.01$ ,  $R^2 = 0.08$ ,  $P = 0.38$ ,  $F_{1,16} = 0.95$ ,  $R^2 = 0.05$ ,  $P = 0.57$  and  $F_{1,16} = 0.84$ ,  $R^2 = 0.04$ ,  $P =$   
398  $0.80$  respectively). Among the OTUs that best correlated with PC1<sub>RepPerf</sub> in the preen feathers of  
399 females, both indicator value and selbal analyses showed that one OTU (OTU\_123; *Porphyromonas*)  
400 was enriched with low PC1<sub>RepPerf</sub> (Table S2 and Figs. S4d and S5). The composition of outer bill did not  
401 vary with PC1<sub>RepPerf</sub> (all  $P > 0.69$ ).

402 In none of the body sites, inter-individual dispersion in microbiota composition varied with  
403 hatching success (all  $P > 0.10$ ). Intra-individual dispersion in microbiota decreased with PC1<sub>RepPerf</sub> in  
404 females ( $S = 216$ ,  $r^2 = -0.80$ ,  $P = 0.014$ ,  $n = 9$ ; Fig. 4), while it did not vary with PC1<sub>RepPerf</sub> in males ( $S =$   
405  $392$ ,  $r^2 = -0.37$ ,  $P = 0.24$ ,  $n = 12$ ; Fig. 4).

406  $\alpha$ -diversity of preen feather microbiota decreased with PC1<sub>RepPerf</sub> in both sexes ( $F_{1,25} = 11.48$ ,  
407  $P = 0.002$ ; sex \* PC1<sub>RepPerf</sub>:  $F_{1,23} = 1.16$ ,  $P = 0.29$ ; Fig. 5a).  $\alpha$ -diversity of flank feathers decreased with  
408 PC1<sub>RepPerf</sub> in females ( $F_{1,1} = 7.13$ ,  $P = 0.020$ ; Fig. 5b), while it decreased marginally with PC1<sub>RepPerf</sub> in  
409 males ( $F_{1,1} = 3.70$ ,  $P = 0.073$ ; sex\*PC1<sub>RepPerf</sub>:  $F_{1,10} = 7.42$ ,  $P = 0.021$ ; Fig. 5b).  $\alpha$ -diversity of neck feather,  
410 outer-bill and cloacal microbiota did not vary with PC1<sub>RepPerf</sub> in males or females (all  $P > 0.26$ ).

411

#### 412 *Body-site specific microbiota and sex-variation*

413 Composition of the microbiota varied with body sites ( $F_{6,247} = 27.97$ ,  $R^2 = 0.30$ ,  $P < 0.001$ ; all pairwise  
414 comparisons:  $P < 0.0004$ , with Bonferonni-corrected  $\alpha = 0.002$ ; Fig. 6). When considering the OTUs  
415 with mean relative abundance  $> 5\%$  in at least one body site, correlation indices showed that cloacal  
416 microbiota was characterized by high levels of two *Firmicutes*: OTU\_1 (genus: *Catelliboccus*) and  
417 OTU\_3 (genus: *Lactobacillus*) (Fig. 6). Cloacal microbiota, neck feather and flank feather microbiota  
418 were characterized by high levels of the *Fusobacteriota* OTU\_16 (genus: *Cetobacterium*). Flank  
419 feather, neck feather and outer-bill microbiotas were characterized by high levels of the

420 proteobacterium OTU\_12 (genus: *Psychrobacter*), the actinobacterium OTU\_21 (genus: *Arthrobacter*)  
421 and the firmicute OTU\_24. Preen feather microbiota was characterized by high levels of the  
422 actinobacterium OTU\_10 (genus: *Rothia*), while tracheal microbiota was characterized by high levels  
423 of the proteobacterium OTU\_6 (genus: *Pasteurella*) and the *Bacteroidota* OTU\_20 (genus:  
424 *Porphyromonas*) and choanal microbiota by high levels of the proteobacterium OTU\_7 (genus:  
425 *Cardiobacterium*). Preen feather, tracheal and choanal microbiotas were all characterized by high  
426 levels of the *Bacteroidota* OTU\_2 (genus: *Ornithobacterium*), and the *Proteobacteria* OTU\_4 (genus:  
427 *Cardiobacterium*). Choanal and preen feather microbiotas were characterized by high levels of the  
428 *Bacteroidota* OTU\_5 (genus: *Flavobacterium*) and the *Bacteroidota* OTU\_11 (genus: *Riemerella*).

429         Despite overall differences among body sites, bacterial composition of the different body  
430 sites was more similar within than between individuals ( $F_{66,247} = 1.72$ ,  $R^2 = 0.20$ ,  $P < 0.001$ ). Results  
431 were similar when restricting the analyses to the 13 individuals for which we had samples from 6 or 7  
432 different body sites ( $F_{12,64} = 1.83$ ,  $R^2 = 0.15$ ,  $P < 0.001$ ; Fig. S6). In addition, we detected similarity  
433 between mates in flank, neck and preen feather microbiota and in cloacal microbiota ( $F_{1,18} = 1.361$   $R^2$   
434  $= 0.53$ ,  $P < 0.001$ ,  $F_{1,16} = 1.36$ ,  $R^2 = 0.55$ ,  $P < 0.001$ , Fig. S7,  $F_{1,16} = 1.16$ ,  $R^2 = 0.52$ ,  $P = 0.026$  and  $F_{1,35} =$   
435  $1.32$ ,  $R^2 = 0.56$ ,  $P = 0.002$  respectively). In contrast, we did not find similarity between mates in outer-  
436 bill microbiota ( $F_{1,15} = 0.97$ ,  $R^2 = 0.47$ ,  $P = 0.65$ ).

437         Microbiota composition varied with sex in cloaca ( $F_{1,149} = 1.12$ ,  $R^2 = 0.01$ ,  $P = 0.048$ ), but not  
438 in other body sites (all  $P > 0.10$ , except in preen feathers where sex was marginally significant:  $F_{1,41} =$   
439  $1.14$ ,  $R^2 = 0.03$ ,  $P = 0.058$ ). Indicator values showed that OTU\_31 (genus *Coynebacterium*), OTU\_94  
440 (family *Hungateiclostridiaceae*), OTU\_71 (genus *Parvimonas*) and OTU\_129 (genus *Atopobium*) were  
441 enriched in female cloaca compared to male cloaca.

442

## 443 **Discussion**

444 Using high-throughput sequencing, we investigated the association between fitness-related traits  
445 and microbiota in black-legged kittiwakes. We found that the microbiota of several body sites prior

446 to laying was associated with breeding status (breeders vs. non-breeders) and reproductive  
447 performance in females.

448

#### 449 *Association between microbiota and breeding status*

450 In females, non-breeders hosted different microbiota to that of breeders in neck and flank feathers,  
451 and in the outer-bill, the choanae and the cloaca. In kittiwakes, the causes of skipping breeding are  
452 various and may include young age (Cadiou et al., 1994), low number of previous breeding episodes  
453 (Desprez et al., 2011), recent dispersal (Danchin & Cam, 2002), poor individual quality (Cam et al.,  
454 1998) and high pollutant levels (Tartu et al., 2013). In our study, non-breeding females were younger  
455 than breeding females (mean  $\pm$  SE: 5.5  $\pm$  0.5 years old vs. 7.4  $\pm$  0.7 years old;  $\chi^2_1 = 6.21$ ,  $P = 0.013$ ; see  
456 Results S2 in supplementary material). However, age was not a driver of variation in microbiota  
457 composition in female kittiwakes (see results S2 in supplementary material). Differences in  
458 microbiota composition between breeders and non-breeders may therefore reflect physiological or  
459 behavioural differences that are not strictly related to age. In our study, most non-breeders started  
460 building a nest, but did not complete it (pers. obs.). Nests are made of mud, grass and mosses  
461 collected from humid soil. Shorter time spent in nest-building may thus limit the transfer of soil  
462 bacteria on feathers and gut. In addition, in kittiwakes, non-breeders spend usually less time  
463 attending the nest (Hatch & Hatch, 1988), and consequently might spend more time at sea,  
464 potentially increasing the acquisition of bacteria from the marine environment. Accordingly, several  
465 OTUs belonging to the family *Salinisphaeraceae*, whose members are commonly isolated from  
466 marine and high-salinity environments (Vetriani, Crespo-Medina, & Antunes, 2014), were enriched in  
467 feathers and outer-bill of non-breeders.

468 Non-breeding may also result from physiological constraints (Cam et al., 1998; Reed et al.,  
469 2015) associated with poor foraging ability or poor health. Accordingly, in our study, non-breeding  
470 females had lower body mass than breeding females (Kruskal-Wallis test:  $\chi^2 = 8.17$ ,  $P = 0.004$ ; mean  $\pm$   
471 SE: 390  $\pm$  5 g vs. 412  $\pm$  5 g). Poor condition is commonly associated with dysbiotic microbiota

472 (Clemente et al., 2012; Tizard & Jones, 2018). Some OTUs that best represented non-breeding status  
473 in feathers of kittiwakes belonged to the family *Lachnospiraceae*. *Lachnospiraceae* are usually  
474 isolated from the gastrointestinal tract of mammals and birds, where they can influence short-  
475 chained fatty-acid production and thus be associated with gut health and metabolic syndromes  
476 (Stanley, Hughes, Geier, & Moore, 2016; reviewed in Vacca et al., 2020). Although *Lachnospiraceae*  
477 are often suggested to promote health, different genera and species of this family are increased in  
478 diseases (Vacca et al., 2020). For instance, in kittiwakes, the family *Lachnospiraceae* contains mainly  
479 the genera *Tyzzerella* and *Lachnoclostridium*, which, in other species, are enriched in individuals with  
480 high lifetime risk of cardiovascular diseases (Kelly et al., 2016), Crohn's disease (Olaisen et al., 2021)  
481 or colon-associated cancer (Chun-Sai-Er Wang, Li, Yi-Ming Ma, & Hong Yang, 2018; Liang et al., 2020).

482         The composition of preen feather microbiota did not vary with breeding status. If differences  
483 in microbiota between breeding status is mostly caused by variations in the acquisition of  
484 environmental bacteria, the lack of association between microbiota and breeding status in preen  
485 feathers might arise from preen feathers being protected from the external environment by large  
486 body feathers, and therefore being less prone to environmental contamination than other body sites.  
487 Findings about the acquisition of environmental bacteria by preen feather or preen secretion  
488 microbiota are mixed (Díaz-Lora et al., 2019; Martínez-García et al., 2016; Pearce, Hoover, Jennings,  
489 Nevitt, & Docherty, 2017). In contrast, preen feather microbiota may be highly prone to the quantity  
490 and composition of the lipid-rich and energetically costly preen secretion (Moreno-Rueda, 2017). The  
491 lack of association between preen feather microbiota and breeding status suggests therefore that  
492 non-breeding birds can invest similarly in preen secretion as breeding birds. Further studies are  
493 required to determine whether there is no intrinsic differences between non-breeding and breeding  
494 females in the ability to invest in preen secretion or whether lower investment in reproduction  
495 allows non-breeding females to save energy to invest in preen secretion as much as breeding  
496 females.

498 *Association between microbiota and reproductive performance in breeders*

499 When considering breeders only, the composition and diversity of feather microbiota was associated  
500 with variation in reproductive performance (as represented by a principal component related to  
501 body condition, date of laying, clutch size, egg mass and hatching success) in females. The role of  
502 feather microbiota on host fitness has been scarcely studied. Some feather bacteria can alter feather  
503 structure and thus reduce fitness, via the alteration of thermoregulation, flight and signalling  
504 (Clayton, 1999; Leclaire et al., 2014). To limit the spread of feather-degrading bacteria (Alt, Mägi,  
505 Lodjak, & Mänd, 2020; Moreno-Rueda, 2017), birds spread preen secretions containing antimicrobial  
506 bacteria, lipids or immune components (Bandyopadhyay & Bhattacharyya, 1996; Bodawatta et al.,  
507 2020; Carneiro, Czirják, & Rowe, 2020; Martin-Vivaldi et al., 2010) onto their plumage. However,  
508 investment in preen secretions and preening is energetically costly (reviewed in Moreno-Rueda,  
509 2017). Therefore, better female breeders, being in better physiological state, may be able to invest in  
510 preen secretion, and thus control their feather microbiota, more than poorer female breeders. This  
511 hypothesis is supported by two results. First, we found that the associations between reproductive  
512 performance and microbiota composition or diversity were stronger and more consistent for preen  
513 feathers compared to other feather types. Compared to other feathers, preen feathers are usually  
514 saturated with higher quantity of preen secretions (Leclaire et al., 2019), and their microbiota might  
515 thus be more sensitive to variation in the ability of the host to invest in preen secretions. Second, we  
516 found reduced intra-individual dispersion in microbiota composition across body habitats in females  
517 with higher reproductive performance, suggesting that good breeders are able to "leash" their  
518 microbiota better than poor breeders (K. R. Foster, Schluter, Coyte, & Rakoff-Nahoum, 2017).

519 The "Anna Karenina principle" suggests that stressors affect bacterial community  
520 stochastically, resulting in greater variation among dysbiotic bacterial communities compared to  
521 eubiotic communities (Zaneveld et al., 2017). However, in contrast to this hypothesis, we did not find

522 strong evidence for greater inter-individual dispersion in microbiota composition in poorer breeders.  
523 Although several studies in humans or wild animals supported this principle (Altabtbaei et al., 2021;  
524 Stothart et al., 2021), others have found the opposite pattern (Lavrinenko et al., 2020; Ma, 2020). In  
525 kittiwakes, the lack of association between inter-individual dispersion in feather microbiota and  
526 breeding performance may stem from the fact that lower quantity of preen secretions, by increasing  
527 the spread of feather-degrading bacteria, may not affect feather microbiota composition  
528 stochastically.

529         We found that the diversity of preen and flank feather microbiota decreased with increasing  
530 reproductive performance, especially in females. Although numerous studies have shown that high  
531 diversity of gut microbiota is pivotal for host fitness (Larsen & Claassen, 2018; Mosca, Leclerc, &  
532 Hugot, 2016), it has recently been suggested that more diversity is not always better (Reese & Dunn,  
533 2018). Interestingly, in blue petrels (*Halobaena caerulea*), lower microbiota diversity in preen  
534 feathers is associated with higher major histocompatibility complex (MHC) diversity (Leclaire et al.,  
535 2019), which correlates with higher fitness in several vertebrates, including in kittiwake chicks  
536 (Agudo et al., 2012; Pineaux et al., 2020; Smith et al., 2010; Thoss, Ilmonen, Musolf, & Penn, 2011).  
537 Lower bacterial diversity in feathers is also associated with increased body condition in pied  
538 flycatchers (*Ficedula hypoleuca*) (Saag et al., 2011) and brighter coloration in great tits (*Parus major*)  
539 (Kilgas et al., 2012). Higher investment in preen secretions and preening in birds with high  
540 reproductive performance may decrease bacterial niche availability on feathers and therefore reduce  
541 bacterial diversity. Further studies are needed to evaluate whether, in contrast to gut microbiota,  
542 lower diversity in feather microbiota is generally associated with enhanced fitness in birds.

543         In preen feathers, the relative abundance of a *Porphyromonas* OTU was associated with  
544 higher reproductive performance in females. *Porphyromonas* are well-known residents of the oral  
545 microbiota of animals and humans, where they can cause diseases (Acuña-Amador & Barloy-Hubler,  
546 2020; Fournier et al., 2001; Mysak et al., 2014). Recently, some *Porphyromonas* were also detected in

547 other body sites, including lipid-rich glandular secretions of mammals and birds (Leclaire, Jacob,  
548 Greene, Dubay, & Drea, 2017; Rodríguez-Ruano et al., 2015; Theis, Schmidt, & Holekamp, 2012), but  
549 their physiologic functionalities are unknown.

550 Variations in reproductive performance in female breeders were also associated with  
551 variations in the composition of cloacal microbiota. In particular, female breeders with lower  
552 reproductive performance harboured a higher abundance of several strains of the genus  
553 *Corynebacterium*. *Corynebacteria* are widespread in animal microbiota (Callewaert et al., 2013; Sarah  
554 M Hird, Sánchez, Carstens, & Brumfield, 2015; Leclaire et al., 2017), and include commensals, as well  
555 as pathogens (Bernard, 2012; Kanmani et al., 2017). Several *Corynebacteria* are associated with  
556 infertility in humans and other mammals (Alonso et al., 1992; Othman et al., 2016; Riegel et al.,  
557 1995). In birds, the cloaca is the opening of the reproductive tract. Some *Corynebacteria* detected in  
558 female cloacas may thus infect their reproductive system and impair egg formation and  
559 development. Consistently with our finding, a previous study in kittiwakes found that a sexually-  
560 transmitted *Corynebacterium* (named C34) was associated with decreased hatching success in  
561 females, but not in males (W. F. van Dongen et al., 2019). In our study, C34 (OTU\_31) was not one of  
562 the OTUs best representing breeding performance. Still, its relative abundance was negatively  
563 correlated with breeding performance in females (Spearman's correlation test:  $r = -0.51$ ,  $P = 0.007$ ).  
564 Higher reproductive performance may be associated with better immunity that acts as an ecological  
565 filter to limit the spread of the *Corynebacteriaceae* that are potentially pathogenic. As found for C34  
566 (W. F. van Dongen et al., 2019), we did not detect an association between cloacal microbiota and  
567 reproductive performance in males, suggesting that *Corynebacteria* does not impact male fertility.

568

#### 569 *Sex-specific association between microbiota and reproductive outcome*

570 The variations in microbiota with breeding status or reproductive performance were observed in  
571 females, but not in males. Interestingly, in kittiwakes, sex-specific differences between breeders and



572 non-breeders are observed at the physiological level also (Goutte et al., 2010). Non-breeding females  
573 have higher corticosterone levels and lower luteinizing hormone (LH) levels than breeding females,  
574 while there are no differences in hormonal levels between breeding and non-breeding males (Goutte  
575 et al., 2010). It has been suggested that females with high corticosterone levels may have lower  
576 foraging skills and thus unable to cope with the energetic requirement for egg formation, and that  
577 low LH levels may be due to weak social interactions with their mate (Goutte et al., 2010). In  
578 addition, in our study, in contrast to females, there was no difference in body mass between non-  
579 breeding and breeding males (Kruskal-Wallis test:  $\chi^2 = 0.18$ ,  $P = 0.67$ ; mean  $\pm$  SE:  $419 \pm 4$  g vs.  $424 \pm 5$   
580 g, respectively). Altogether, these results suggest that, in kittiwake pairs, reproductive outcome from  
581 the decision to lay eggs to hatching is mainly due to females. Accordingly, in several monogamous  
582 birds with biparental care including kittiwakes (Jacobsen, Erikstad, & Saether, 1995; Leclaire et al.,  
583 2010), females have been suggested to be more determinant of reproductive performance than  
584 males (Peralta-Sánchez, Colmenero, Redondo-Sánchez, Ontanilla, & Soler, 2020; Slagsvold & Lifjeld,  
585 1990).

586         Although the variation in feather microbiota according to reproductive outcome are sex-  
587 specific, we did not detect sex-differences in feather microbiota composition. In birds, evidence for  
588 sex-differences in microbiota is mixed (Ambrosini et al., 2019; Goodenough et al., 2017; Grieves,  
589 Gloor, Kelly, Bernards, & MacDougall-Shackleton; Pearce et al., 2017; Saag et al., 2011). Sex-  
590 differences in avian microbiota have been attributed to sex-differences in behaviour or hormonal  
591 levels (Corl et al., 2020; Leclaire et al., 2019). In kittiwakes, during the pre-laying period, males and  
592 females differ in numerous behavioural and physiological traits, such as nest attendance  
593 (Helfenstein, 2002) and the chemical composition of preen secretions (Leclaire et al., 2011).  
594 However, we found great similarity between mates in the microbiota of most body sites. High rates  
595 of bacterial transfer between pair-mates due to allo-preening and nest sharing (Kulkarni & Heeb,  
596 2007; Danielle J Whittaker et al., 2016) might therefore mask any potential pre-existing sex-  
597 differences in feather microbiota. In kittiwakes, similarity in cloacal microbiota between mates has

598 been shown to be due to the transfer of cloacal bacteria during copulation (White et al., 2010).  
599 However in contrast to feather microbiota, we found a weak sex-difference in cloacal microbiota.  
600 Interestingly, female cloacas were enriched with OTUs associated with lower reproductive  
601 performance in female kittiwakes (e.g., the C34 bacterium; W. F. van Dongen et al., 2019 and  
602 OTU\_74 (this study)) or belonging to genera (*i.e.*, *Parvimonas* and *Atopobium*) associated with  
603 vaginal infection in women (Brotman et al., 2012; Raimondi et al., 2021), further suggesting that  
604 female cloaca host female-specific bacteria that might impact fertility.

605

#### 606 *Differences in microbiota across body sites and sexes*

607 We found that the microbiota, although specific to individuals, varies across body habitats in black-  
608 legged kittiwakes, which is consistent with other studies on animal microbiomes (Alfano et al., 2015;  
609 Costello et al., 2009; Ferretti et al., 2018). In adult kittiwakes, cloacal microbiota was dominated by  
610 bacteria belonging to the phyla *Firmicutes* and *Actinobacteria*. The bacterial phyla composition of  
611 bird cloaca or gut varies greatly across species (Sarah M. Hird, Ganz, Eisen, & Boyce, 2018; Sarah M  
612 Hird et al., 2015; Laviad-Shitrit, Izhaki, Lazar, & Halpern, 2019), but our result is in line with a  
613 previous study on the same population of kittiwakes (W. F. D. van Dongen et al., 2013). In addition,  
614 the most abundant OTU detected in cloaca of kittiwakes was *Catellibacterium marimammalium* (OTU\_1),  
615 which is also the most common bacteria in the cloaca of other gull species from North America,  
616 South Africa and Europe (Koskey, Fisher, Traudt, Newton, & McLellan, 2014; Laviad-Shitrit et al.,  
617 2019; Lu, Santo Domingo, Lamendella, Edge, & Hill, 2008; Merkevicene et al., 2017). A *Lactobacillus*  
618 (OTU\_3) was found to be the second most abundant OTU in the cloaca of kittiwakes, which is  
619 common for the gastrointestinal tract of birds and other vertebrates (Grond, Sandercock,  
620 Jumpponen, & Zeglin, 2018; Maurice et al., 2015). Finally, a *Cetobacterium* (OTU\_16) was also  
621 enriched in the cloaca of kittiwakes, and members of this genus had already been isolated from the  
622 gut of other marine animals (G. Foster et al., 1995; Godoy-Vitorino et al., 2017).

623 Flank feather, neck feather and outer-bill microbiota of kittiwakes were characterized by high  
624 levels of an OTU belonging to the genus *Psychrobacter* (OTU\_12). *Psychrobacter* are commonly  
625 isolated from cold and saline habitats (Bowman, Cavanagh, Austin, & Sanderson, 1996; Rodrigues et  
626 al., 2009), and high abundance has also been detected in the feathers of other arctic or subantarctic  
627 seabirds (Leclaire et al., 2019; Shawkey et al., 2006). Interestingly, the microbiota of outer-bill was  
628 more similar to that of neck and flank feathers, than that of other body regions. Similarity in  
629 microbiota might be due to the outer-bill being a keratinized structure like feathers, and being  
630 frequently in contact with feathers during preening and sleeping, when birds tuck their bill into their  
631 feathers.

632 Some of most abundant OTUs in kittiwake trachea and choanae belong to the genera  
633 *Ornitobacterium* (OTU\_2), *Riemerella* (OTU\_11) and *Pasteurella* (OTU\_6), and to the family  
634 *Cardiobacteriaceae* (OTU\_7 and OTU\_4). Some members of these genera and this family are  
635 commonly present in the upper part of the respiratory tract of birds or mammals (Barbosa et al.,  
636 2020; Holman et al., 2017), and can cause diseases. For instance, *Ornithobacterium rhinotracheale*,  
637 *Riemerella anatipestifer*, *Pasteurella multocida* and *Sutonella ornithocola* are well-known respiratory  
638 avian pathogens, that can cause economic loss when infecting poultry and be major threat for  
639 endangered wild birds (Jaeger et al., 2018; Lawson et al., 2011; Sandhu, 2008; Van Empel & Hafez,  
640 1999). Many infectious bacteria are low-abundance residents and cause diseases only when dysbiosis  
641 occurs. Further studies are needed to determine the infection potential of these bacteria in  
642 kittiwakes.

643 We found that the major OTUs of preen feathers were more similar to those of the upper  
644 respiratory tract than to those of other feather types. Respiratory microbiota was analysed using  
645 different DNA extraction protocols and sequencing runs compared to the microbiota of other body  
646 sites. Similarity in respiratory and preen feather microbiota might thus be an artefact due the  
647 methods used for respiratory microbiota analyses specifically enriching the OTUs detected in preen  
648 feathers. Alternatively, similarity in respiratory and preen feather microbiota might come from the

649 transfer of bacteria between the preen feathers and the inner bill (whose microbiota might be similar  
650 to that of the respiratory tract), when birds collect preen secretion with their bill before preening  
651 their feathers. Previous studies characterizing the microbiota of preen feathers or preen secretions  
652 seem to show strong variations across species (e.g., Leclaire et al., 2019; Videvall et al., 2021;  
653 Danielle J. Whittaker & Theis, 2016). For instance, similarly to kittiwakes, in Leach's storm-petrels  
654 (*Oceanodroma leucorhoa*), preen gland microbiota is dominated by *Proteobacteria*, and includes high  
655 abundance of *Moraxellaceae* (Pearce et al., 2017). In contrast, preen microbiota is dominated by the  
656 phylum *Firmicutes* (mainly the class *Clostridia*) in hoopoes (Rodríguez-Ruano et al., 2018), and by  
657 *Actinobacteria* in blue petrels (Leclaire et al., 2019).

658

### 659 *Conclusion*

660 Altogether, our findings are consistent with the idea that natural variation in the microbiota is  
661 associated with differences in host fitness in wild birds. Our study is correlative, and the causal  
662 relationship between fitness and microbiota is unknown. Further studies are therefore required to  
663 determine whether abnormal microbiotas cause dysbioses that impair reproductive performance, or  
664 whether differences in microbiota are due to reproductive-performance associated variations in diet,  
665 physiology or behaviour. A better understanding of the association between fitness and microbiota  
666 in wild animals is important to appreciate the evolution of several behavioural or physiological traits.  
667 For instance, bacteria with deleterious effect on fitness can select for the evolution of antibacterial  
668 defences, including immune and behavioural mechanism such as preening. In addition, an  
669 association between fitness and the abundance of bacteria affecting plumage coloration and odours  
670 of the host (Archie & Theis, 2011) might lead colours or odours to evolve as sexual ornaments.

671

### 672 **Acknowledgements**

673 We thank Anaïs Pessato, Mathieu Bergès, Axelle Denis, Lucie Abolivier and Thomas Pagnon for their  
674 help in the field. We thank Guillaume Croville and the virology group of the Veterinary school of

675 Toulouse (ENVT) for help and advice during the DNA extraction stage. We are grateful to the  
676 genotoul bioinformatics platform Toulouse Occitanie (Bioinfo Genotoul,  
677 <https://doi.org/10.15454/1.5572369328961167E12>) for providing computing and storage resources.  
678 The study was financed by grants from the French Polar Institute Paul-Emile Victor (IPEV 'Programme  
679 1162 SexCoMonArc2' to SL) and from the Fondation Fyssen (to SL). This study is part of the  
680 'Laboratoire d'Excellence' (LABEX) entitled TULIP (ANR-10-LABX-41). This experiment was conducted  
681 under the approval of the USGS Alaska Science Center Animal Care and Use Committee, in  
682 accordance with US law and under permits from the US Fish and Wildlife Service and the State of  
683 Alaska.

684

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#### 1154 **Data accessibility and benefit sharing statement**

1155 The data supporting the findings of this study (OTU count tables, metadata, OTU phylogenetic tree,  
1156 and OTU sequences) are archived in the publicly accessible repository "Open Science Framework".

1157 DOI: 10.17605/OSF.IO/WHCXN

1158

#### 1159 **Author contribution**

1160 SL conceived and designed the study, performed the statistical analyses and wrote the manuscript.

1161 MP and SL collected the data and carried out the molecular analyses. SAH is responsible of the long-

1162 term monitoring on Middleton Island and gave access to the study area. All authors contributed  
1163 comments to the manuscript and gave final approval for publication.

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1165 **Conflict of interest**

1166 The authors declare no conflicts of interest.

1167 **Table 1:** Sample size per sample type. All samples were collected in 2017, except cloacal samples  
 1168 which were collected both in 2016 and 2017.

	Non-breeders			Breeders		
	Females	Males	Number of pairs in which the two members were captured	Females	Males	Number of pairs in which the two members were captured
Flank feathers	7	11	5	15	20	14
Neck feathers	7	10	4	14	20	13
Preen feathers	6	8	5	15	14	12
Outer-bill	6	10	4	12	18	12
Choanae	6	10	0	7	9	0
Trachea	6	10	0	7	10	0
Cloaca 2016	32	18	8	21	17	9
Cloaca 2017	11	13	5	18	21	13

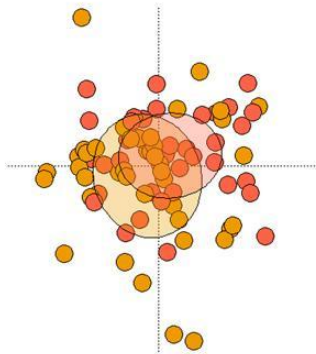
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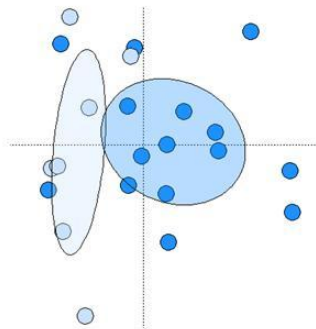


1171 **Figure 1:** Unconstrained distance-based redundancy analysis plots showing differences between non-  
1172 breeders and breeders in the microbiota of (a) cloaca, (b) flank feathers, (c) neck feathers, (d) outer  
1173 bill and (e) choanae in females. In each plot, the lighter colour represents non-breeders, while the  
1174 darker colour represents breeders. For all microbiota shown, breeding and non-breeding females had  
1175 different bacterial composition (all  $P < 0.035$ ).

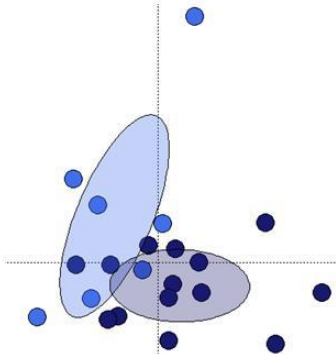
a) Cloaca



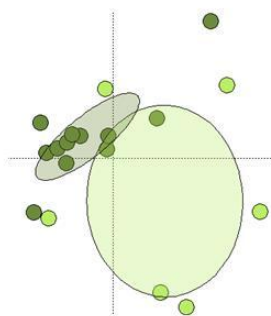
b) Flank feathers



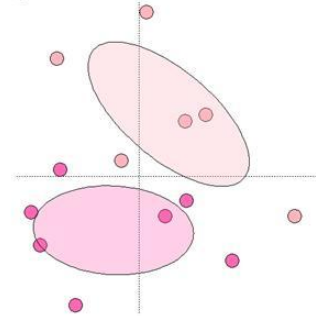
c) Neck feathers



d) Outer-bill



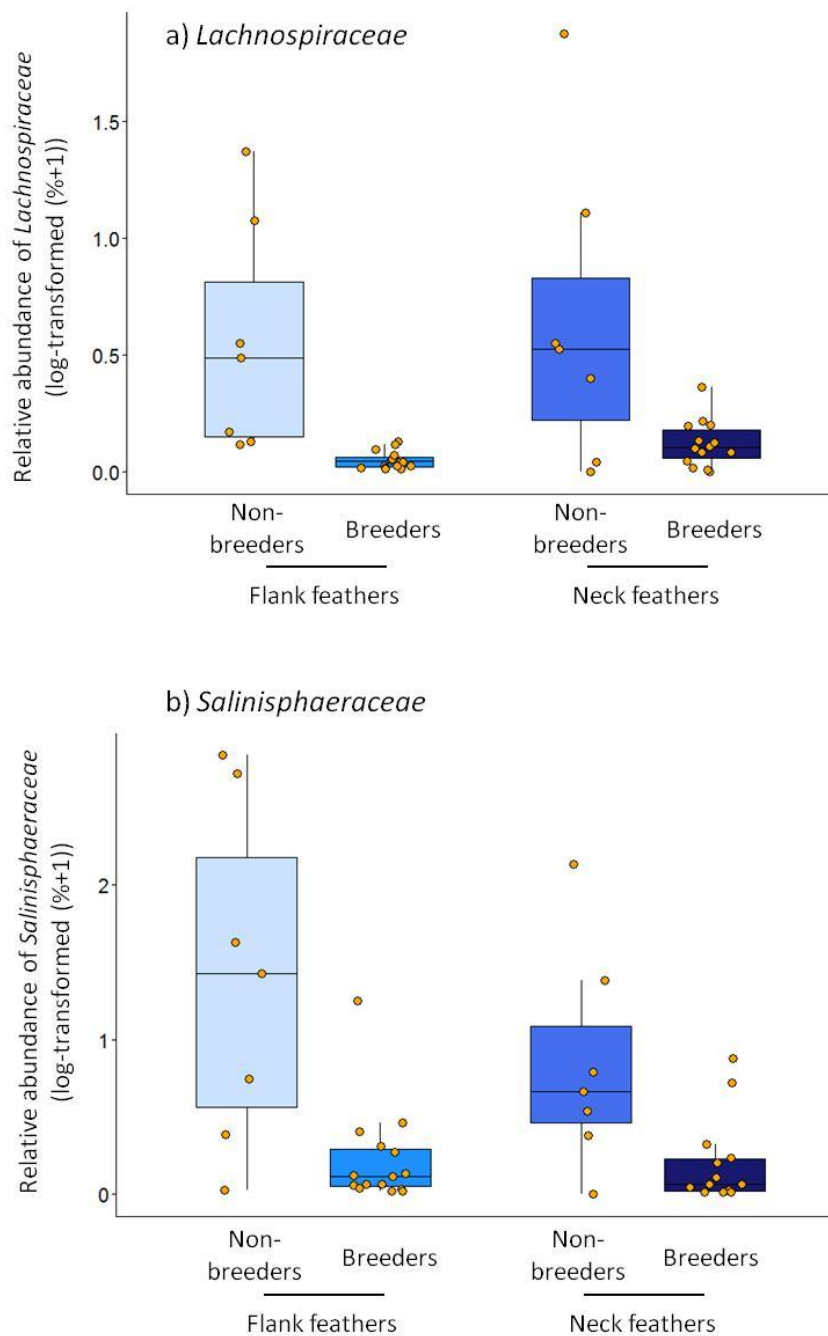
e) Choanae



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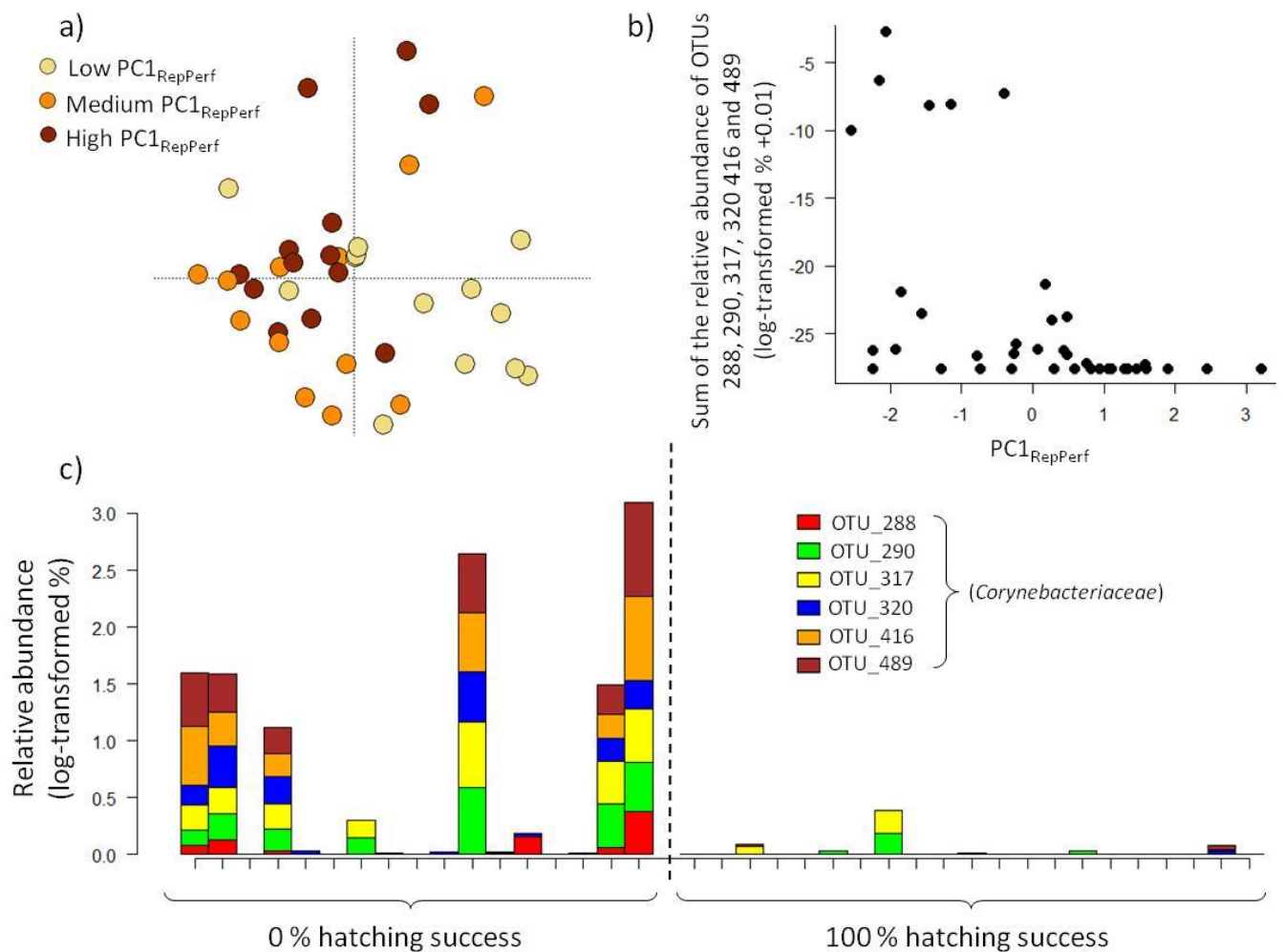
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1178 **Figure 2:** Boxplots showing the relative abundance of the OTUs belonging to the families (a)  
 1179 *Lachnospiraceae* and (b) *Salinisphaeraceae* in the flank and neck feathers of non-breeding and  
 1180 breeding females. Compared to breeding females, non-breeding females had higher abundance of  
 1181 *Lachnospiraceae* in flank feathers ( $\chi^2_1 = 12.67$ ,  $P < 0.001$ ) and *Salinisphaeraceae* in flank and neck  
 1182 feathers ( $\chi^2_1 = 6.26$ ,  $P = 0.012$  and  $\chi^2_1 = 4.36$ ,  $P = 0.037$ ). They tended also to have higher abundance  
 1183 of *Lachnospiraceae* in neck feathers ( $\chi^2_1 = 3.43$ ,  $P = 0.067$ ).



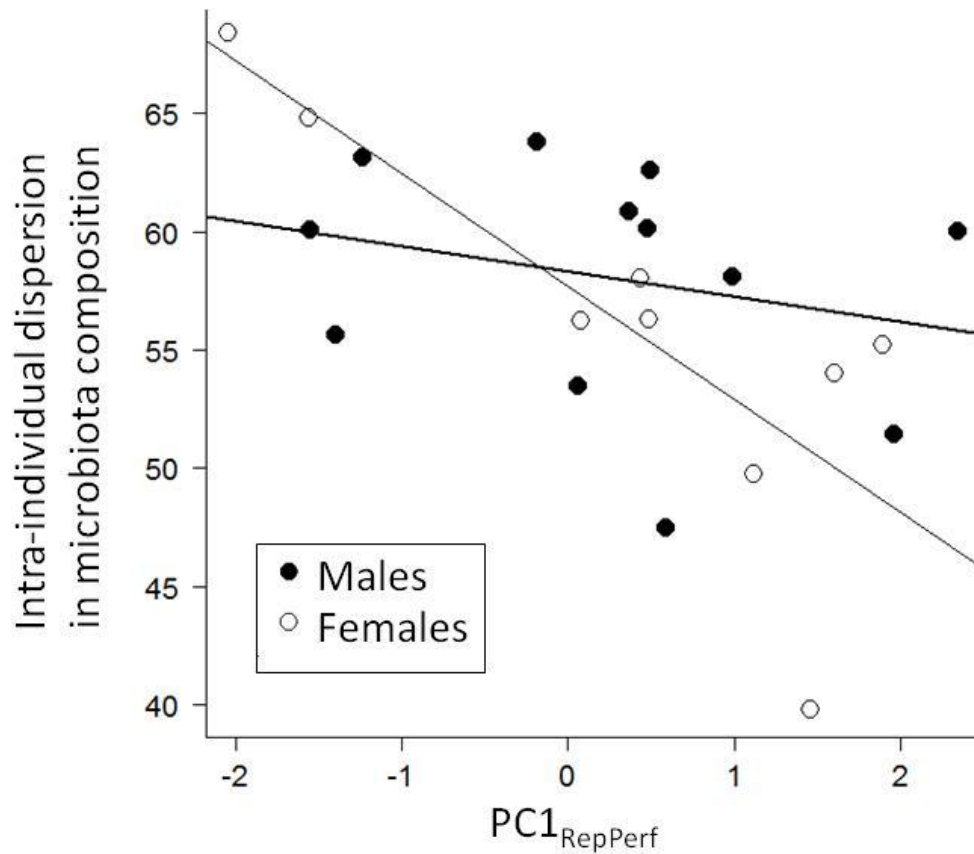
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1185 **Figure 3:** Plots showing variations in cloacal microbiota according to reproductive performance in  
 1186 female breeders. (a) Unconstrained distance-based redundancy analysis plots showing differences in  
 1187 female cloacal microbiota according to PC1<sub>RepPerf</sub>. Female cloacal microbiota composition varied with  
 1188 PC1<sub>RepPerf</sub> in females ( $F_{1,36} = 2.19$ ,  $R^2 = 0.06$ ,  $P = 0.004$ ). Although PC1<sub>RepPerf</sub> was analyzed as a  
 1189 continuous variable (see text), it is displayed here as a categorical variable for illustrative purposes.  
 1190 We categorized PC1<sub>RepPerf</sub> into three groups of equal sample size. Axis 1 and 3 are shown. (b) Sum of  
 1191 the relative abundance of the six *Corynebacteriaceae* OTUs that correlated with PC1<sub>RepPerf</sub> with  $P <$   
 1192 0.02 in the correlation index analysis. (c) Barplot representing the relative abundance of these  
 1193 *Corynebacteriaceae* OTUs in each female according to hatching success.  
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1195  
1196

1197 **Figure 4:** Intra-individual dispersion in microbiota composition according to  $PC1_{RepPerf}$  in males (black  
1198 dots and bold line) and females (white dots and thin line). Because not all body sites were sampled  
1199 on each single individual, we restricted the analyses on individuals for which cloaca, flank feathers,  
1200 neck feathers, preen feathers and outer-bill were sampled. Intra-individual dispersion varied with  
1201  $PC1_{RepPerf}$  in females, but not in males ( $S = 216$ ,  $r^2 = -0.80$ ,  $P = 0.014$  and  $S = 392$ ,  $r^2 = -0.37$ ,  $P = 0.24$ ).

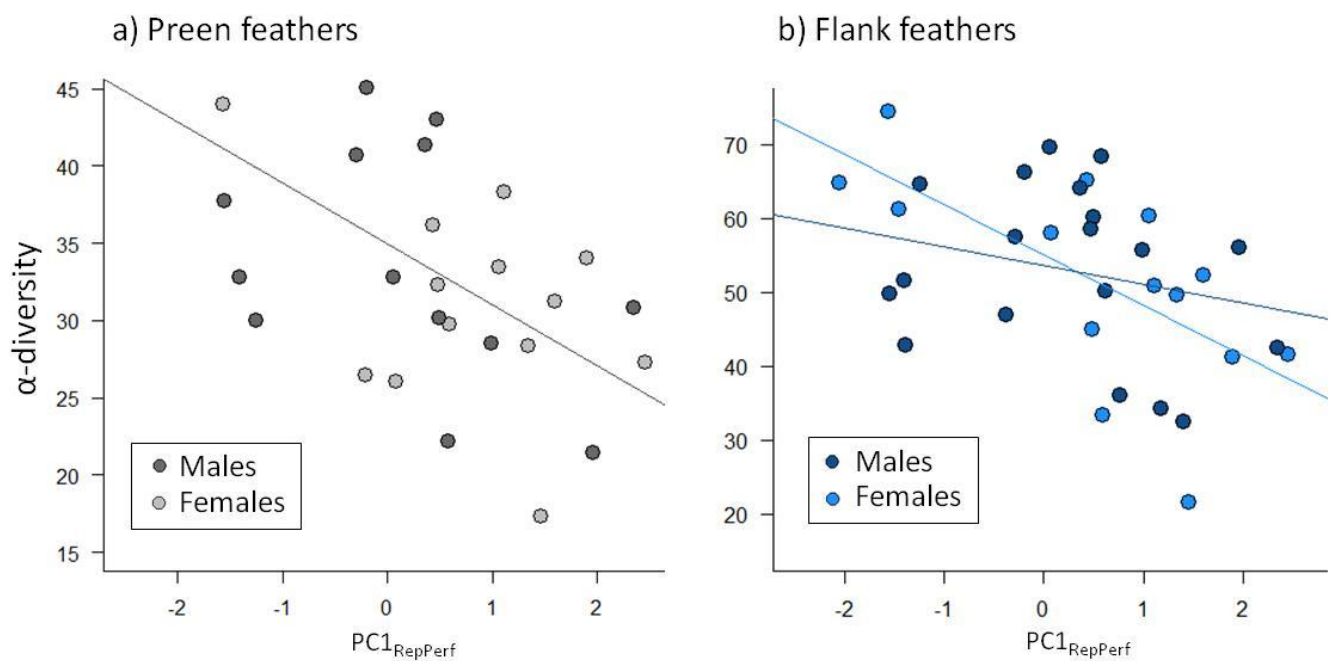


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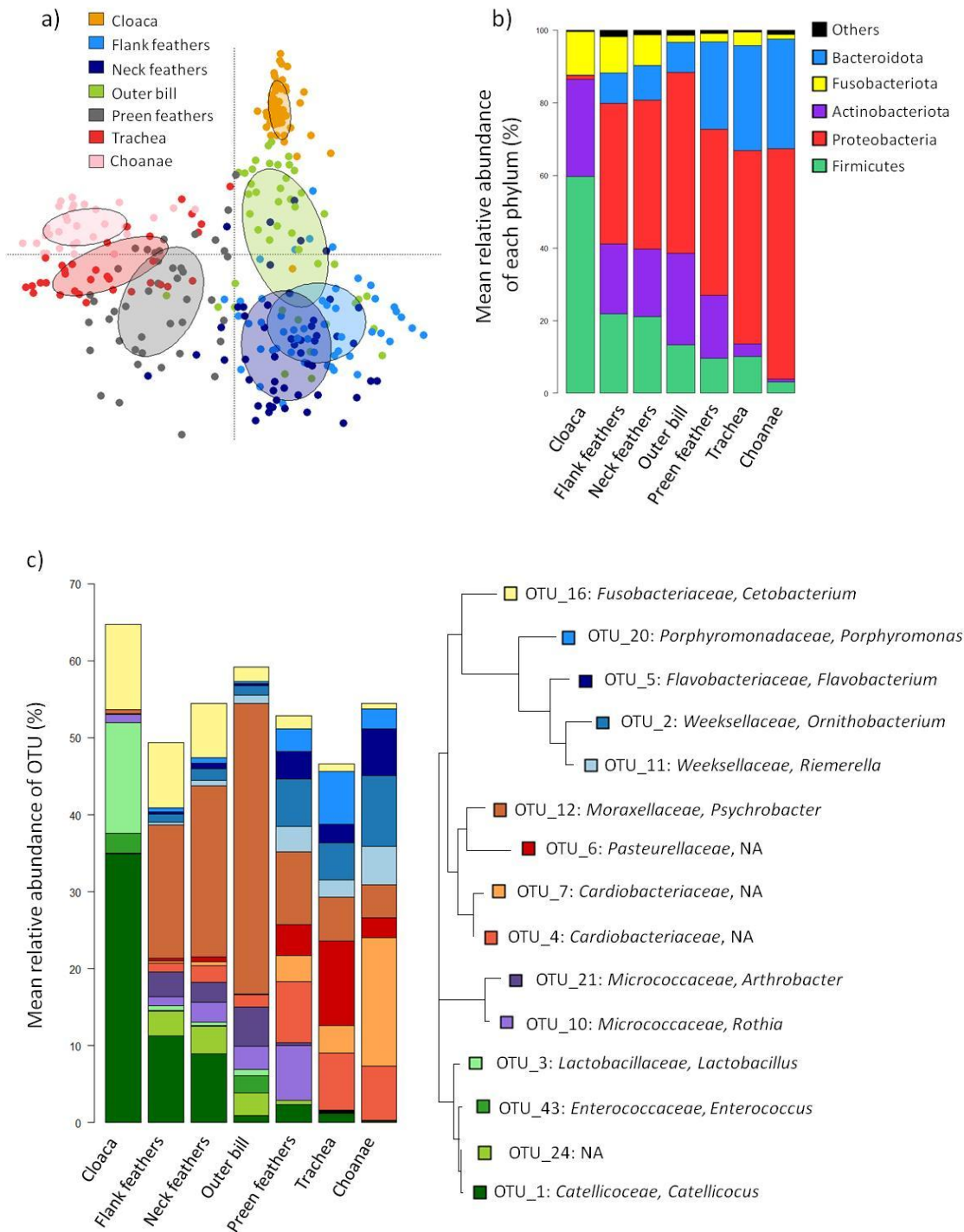
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1205 **Figure 5:**  $\alpha$ -diversity of (a) preen feather microbiota and (b) flank feather microbiota in relation to  
1206  $PC1_{RepPerf}$  in males and females.  $\alpha$ -diversity (Faith's PD index) was calculated on rarefied samples. In  
1207 preen feathers, the relationship between  $\alpha$ -diversity and  $PC1_{RepPerf}$  did not vary with sex ( $PC1_{RepPerf}$   
1208 \*Sex:  $F_{1,23} = 1.16$ ,  $P = 0.29$ ;  $PC1_{RepPerf}$ :  $F_{1,25} = 11.48$ ,  $P = 0.002$ ), thereby a single regression line is  
1209 shown, while in flank feathers, the relationship between  $\alpha$ -diversity and  $PC1_{RepPerf}$  varied with sex  
1210 ( $PC1_{RepPerf}$  \*Sex:  $F_{1,10} = 7.42$ ,  $P = 0.021$ ), thereby sex-specific regression lines are shown (females:  $F_{1,1} =$   
1211  $7.13$ ,  $P = 0.020$  and males:  $F_{1,1} = 3.70$ ,  $P = 0.073$ ).



1212

1213 **Figure 6:** Plots showing differences in microbiota between body sites. a) Unconstrained distance-  
 1214 based redundancy analysis plots showing separation of microbiota by body sites. b) Mean relative  
 1215 abundance of the most abundant phylum in the different body sites. c) Mean relative abundance of  
 1216 the most abundant OTUs in the different body sites and neighbour-joining phylogenetic tree based  
 1217 on the V5-V6 16S rRNA sequences of these most abundant OTUs (NA: not affiliated). Correlation  
 1218 indices showed that all the OTUs shown in Fig. 6c, except OTU\_43, differed significantly between  
 1219 body sites (all  $P < 0.0002$ ).



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