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Running head: Microbiota and fitness in a seabird

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

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Abstract

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

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

Introduction

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

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

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

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

Methods

Study site

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

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

Reproductive outcome

We defined two indices of reproductive outcome. First, we considered breeding status, i.e. breeders *vs*. non-breeders. Individuals were considered non-breeders when they were regularly seen occupying a nesting site, but did not lay eggs. In our study, all non-breeders had mating partners and attempted to breed. In contrast, breeders built a nest and laid 1 or 2 eggs. All birds that were 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 refereed 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

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

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

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

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

Statistics

We performed all statistical analyses using the R statistical software (R Core Team, 2017). To analyze differences in microbiota composition between breeding status (breeder *vs.* non-breeder) or breeder performance ($PC1_{RepPerf}$), we performed PERMANOVAs ("Adonis2" function in the VEGAN package in R, with 5000 permutations and the "margin" option), based on the PhILR transform distance matrix. In the models, breeding status and date of sampling, or $PC1_{RepPerf}$ and the difference between laying date and capture date, were included as predictors. To account for the non-independence of the two members of a pair (see result section below), we carried out PERMANOVAs within each sex. The two members of a breeding pair were never both sampled for tracheal or choanal microbiota, but tracheal and choanal microbiotas of females were sampled later during the breeding season than 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 avoid confounding the sex and date effects, we carried out PERMANOVAs within each sex for tracheal and choanal microbiota also. We did not test for associations between tracheal and choanal microbiota composition and $PC1_{RepPerf}$ as the sample size within each sex was very low (Table 1). Cloacal samples collected in 2016 were analysed in a different Illumina run compared to the cloacal

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

To predict the OTUs that were best associated with breeding status and breeding performance, we used two different procedures. First, we used correlation indices with the point biserial coefficient of association (multipatt procedures in the indicspecies package in R) (De Cáceres & Legendre, 2009) to determine a small set of OTUs that best characterize breeding status or breeding performance. The multipatt procedure cannot associate OTU to a continuous variable. Therefore, $PC1_{RepPerf}$ was split into two categories of equal sample size. In the multipatt analyses, we did not adjust for multiple comparisons, as the purpose of these analyses was exploratory in order to generate, rather than confirm, hypotheses (Bender & Lange, 2001; Ranstam, 2019). We also used the selbal procedure, which compares the average abundance of two groups of microbial species (selbal package in R) (Rivera-Pinto et al., 2018). In the multipatt and selbal procedures, we considered OTUs that were present in at least 10 % of the individuals and whose the mean relative abundance was > 0.025 % across samples. Correlation indices and Selbal cannot be compared as they rely on two different concepts. In correlation indices, microbial signature is defined as a combination of individual taxa, while in selbal, it is defined as a compositional balance between two groups of taxa.

To determine whether α-diversity varied with breeding status or breeder performance, we used linear mixed models with reproductive outcome (breeding status or $PC1_{RepPerf}$), sex, date (sampling date or the difference between laying date and sampling date), year (for the analysis on cloacal microbiota only) and the interaction between reproductive outcome and sex as fixed effects and we included nest identity as a random factor. For tracheal and choanal microbiota, we used linear models. Normality of the residuals was checked using Shapiro-Wilk normality tests. Homogeneity of variance was checked visually (for continuous variables) or using Levene's tests (for dichotomous variables). α-diversity in cloacal microbiota was square-root transformed in the 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,

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

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

Results

Association between microbiota and breeding status

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

The composition of flank feather microbiota and neck feather microbiota varied with 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. 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. 2b and 2c). Correlation indices showed that several of the OTUs best representing the differences between breeders and non-breeders in females belonged to the families *Lachnospiraceae* and *Salinisphaeraceae* and were enriched in non-breeders (Table S1). To determine whether, more

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

The composition of outer-bill and choanal microbiota varied also with breeding status in 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 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). Indicator value showed that the some of the OTUs best representing the differences between breeders and non-breeders in the outer-bill microbiota of females belonged to the genus *Salinisphaera* (enriched in non-breeders; Table S1). Indicator value showed that the OTUs best representing the differences between breeders and non-breeders in choanal microbiota belonged to diverse families (Table S1), but interestingly, both indicator value and selbal identified *Pasteurellaceae* OTUs as being enriched in non-breeders compared to breeders (Table S1). However, in females, inter-individual dispersion in outer-bill and choanal microbiota was higher in non-breeders than breeders (see below; Figs. 1d and 1e). These differences in dispersion might thus have contributed to the significant differences obtained in PERMANOVAs (Anderson & Walsh, 2013), especially for outer-bill whose the sample size was not balanced for (6 non-breeding females and 12 breeding females). Although plots (Figs. 1d and 1e) suggest that both composition (group centroid) and dispersion were different between breeding and non-breeding females, further studies including larger sample size are required to ascertain the effect of breeding status on outer-bill and choanal microbiota composition in females.

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

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

In females, inter-individual dispersion in outer-bill and choanal microbiota was higher in non-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-individual dispersion was also found to be higher in breeding males than in non-breeding males in preen feather microbiota ($F_{1,20} = 6.55$, $P = 0.016$). Intra-individual dispersion in microbiota across body sites was not different between breeders and non-breeders either in males or in females ($\chi^2_1 = 2.15$, $P = 0.14$, $n = 20$ and $\chi^2_1 = 2.31$, $P = 0.13$, $n = 16$).

Association between microbiota and breeding performance

The composition of cloacal microbiota varied with $PC1_{RepPerf}$ in females ($F_{1,36} = 2.18$, $R^2 = 0.06$, $P = 0.004$; Fig. 3) but not in males ($F_{1,34} = 0.73$, $R^2 = 0.02$, $P = 0.98$). Correlation indices showed that the majority of the OTUs that best characterized $PC1_{RepPerf}$ belonged to the family *Corynebacteriaceae* and were enriched in the cloacae of females with low $PC1_{RepPerf}$ (Fig. 3 and Table S2). Selbal analysis identified a balance of OTU_73 (*Clostridia*) and OTU_320 (*Corynebacteriaceae*) vs. OTU_12 (*Psychrobacter*) as being associated with $PC1_{RepPerf}$ in female cloaca (Table S2 and Fig. S4a).

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

395 associated with $PC1_{RepPerf}$ 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 $PC1_{RepPerf}$ 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 $PC1_{RepPerf}$ 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 $PC1_{RepPerf}$ (Table S2 and Figs. S4d and S5). The composition of outer bill did not
401 vary with $PC1_{RepPerf}$ (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 $PC1_{RepPerf}$ in
404 females (S = 216, r^2 = -0.80, P = 0.014, n = 9; Fig. 4), while it did not vary with $PC1_{RepPerf}$ in males (S =
405 392, r^2 = -0.37, P = 0.24, n = 12; Fig. 4).

406 α-diversity of preen feather microbiota decreased with $PC1_{RepPerf}$ in both sexes ($F_{1,25}$ = 11.48,
407 P = 0.002; sex * $PC1_{RepPerf}$: $F_{1,23}$ = 1.16, P = 0.29; Fig. 5a). α-diversity of flank feathers decreased with
408 $PC1_{RepPerf}$ in females ($F_{1,1}$ = 7.13, P = 0.020; Fig. 5b), while it decreased marginally with $PC1_{RepPerf}$ in
409 males ($F_{1,1}$ = 3.70, P = 0.073; sex*$PC1_{RepPerf}$: $F_{1,10}$ = 7.42, P = 0.021; Fig. 5b). α-diversity of neck feather,
410 outer-bill and cloacal microbiota did not vary with $PC1_{RepPerf}$ 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 α = 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: *Catellicoccus*) 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

proteobacterium OTU_12 (genus: *Psychrobacter*), the actinobacterium OTU_21 (genus: *Arthrobacter*) and the firmicute OTU_24. Preen feather microbiota was characterized by high levels of the actinobacterium OTU_10 (genus: *Rothia*), while tracheal microbiota was characterized by high levels of the proteobacterium OTU_6 (genus: *Pasteurella*) and the *Bacteroidota* OTU_20 (genus: *Porphyromonas*) and choanal microbiota by high levels of the proteobacterium OTU_7 (genus: *Cardiobacterium*). Preen feather, tracheal and choanal microbiotas were all characterized by high levels of the *Bacteroidota* OTU_2 (genus: *Ornithobacterium*), and the *Proteobacteria* OTU_4 (genus: *Cardiobacterium*). Choanal and preen feather microbiotas were characterized by high levels of the *Bacteroidota* OTU_5 (genus: *Flavobacterium*) and the Bacteroidota OTU_11 (genus: *Riemerella*).

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

Microbiota composition varied with sex in cloaca ($F_{1,149}$ = 1.12, R^2 = 0.01, P = 0.048), but not in other body sites (all P > 0.10, except in preen feathers where sex was marginally significant: $F_{1,41}$ = 1.14, R^2 = 0.03, P = 0.058). Indicator values showed that OTU_31 (genus *Coynebacterium*), OTU_94 (family *Hungateiclostridiaceae*), OTU_71 (genus *Parvimonas*) and OTU_129 (genus *Atopobium*) were enriched in female cloaca compared to male cloaca.

Discussion

Using high-throughput sequencing, we investigated the association between fitness-related traits 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 ± SE: 5.5 ± 0.5 years old *vs.* 7.4 ± 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 ±
471 SE: 390 ± 5 g vs. 412 ± 5 g). Poor condition is commonly associated with dysbiotic microbiota

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

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

Association between microbiota and reproductive performance in breeders

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

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

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

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

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

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

Variations in reproductive performance in female breeders were also associated with variations in the composition of cloacal microbiota. In particular, female breeders with lower reproductive performance harboured a higher abundance of several strains of the genus *Corynebacterium*. Corynebacteria are widespread in animal microbiota (Callewaert et al., 2013; Sarah M Hird, Sánchez, Carstens, & Brumfield, 2015; Leclaire et al., 2017), and include commensals, as well as pathogens (Bernard, 2012; Kanmani et al., 2017). Several *Corynebacteria* are associated with infertility in humans and other mammals (Alonso et al., 1992; Othman et al., 2016; Riegel et al., 1995). In birds, the cloaca is the opening of the reproductive tract. Some Corynebacteria detected in female cloacas may thus infect their reproductive system and impair egg formation and development. Consistently with our finding, a previous study in kittiwakes found that a sexually-transmitted *Corynebacterium* (named C34) was associated with decreased hatching success in females, but not in males (W. F. van Dongen et al., 2019). In our study, C34 (OTU_31) was not one of the OTUs best representing breeding performance. Still, its relative abundance was negatively correlated with breeding performance in females (Spearman's correlation test: $r = -0.51$, $P = 0.007$). Higher reproductive performance may be associated with better immunity that acts as an ecological filter to limit the spread of the *Corynebacteriaceae* that are potentially pathogenic. As found for C34 (W. F. van Dongen et al., 2019), we did not detect an association between cloacal microbiota and reproductive performance in males, suggesting that *Corynebacteria* does not impact male fertility.

Sex-specific association between microbiota and reproductive outcome

The variations in microbiota with breeding status or reproductive performance were observed in 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 ± SE: 419 ± 4 g vs. 424 ± 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

been shown to be due to the transfer of cloacal bacteria during copulation (White et al., 2010). However in contrast to feather microbiota, we found a weak sex-difference in cloacal microbiota. Interestingly, female cloacas were enriched with OTUs associated with lower reproductive performance in female kittiwakes (e.g., the C34 bacterium; W. F. van Dongen et al., 2019 and OTU_74 (this study)) or belonging to genera (*i.e.*, *Parvimonas* and *Atopobium*) associated with vaginal infection in women (Brotman et al., 2012; Raimondi et al., 2021), further suggesting that female cloaca host female-specific bacteria that might impact fertility.

Differences in microbiota across body sites and sexes

We found that the microbiota, although specific to individuals, varies across body habitats in black-legged kittiwakes, which is consistent with other studies on animal microbiomes (Alfano et al., 2015; Costello et al., 2009; Ferretti et al., 2018). In adult kittiwakes, cloacal microbiota was dominated by bacteria belonging to the phyla *Firmicutes* and *Actinobacteria.* The bacterial phyla composition of bird cloaca or gut varies greatly across species (Sarah M. Hird, Ganz, Eisen, & Boyce, 2018; Sarah M Hird et al., 2015; Laviad-Shitrit, Izhaki, Lalzar, & Halpern, 2019), but our result is in line with a previous study on the same population of kittiwakes (W. F. D. van Dongen et al., 2013). In addition, the most abundant OTU detected in cloaca of kittiwakes was *Catellicoccus marimammalium* (OTU_1), which is also the most common bacteria in the cloaca of other gull species from North America, South Africa and Europe (Koskey, Fisher, Traudt, Newton, & McLellan, 2014; Laviad-Shitrit et al., 2019; Lu, Santo Domingo, Lamendella, Edge, & Hill, 2008; Merkeviciene et al., 2017). A *Lactobacillus* (OTU_3) was found to be the second most abundant OTU in the cloaca of kittiwakes, which is common for the gastrointestinal tract of birds and other vertebrates (Grond, Sandercock, Jumpponen, & Zeglin, 2018; Maurice et al., 2015). Finally, a *Cetobacterium* (OTU_16) was also enriched in the cloaca of kittiwakes, and members of this genus had already been isolated from the 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

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

Conclusion

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

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Data accessibility and benefit sharing statement

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

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Author contribution

SL conceived and designed the study, performed the statistical analyses and wrote the manuscript. MP and SL collected the data and carried out the molecular analyses. SAH is responsible of the long-

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

Conflict of interest

The authors declare no conflicts of interest.

Table 1: Sample size per sample type. All samples were collected in 2017, except cloacal samples 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

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

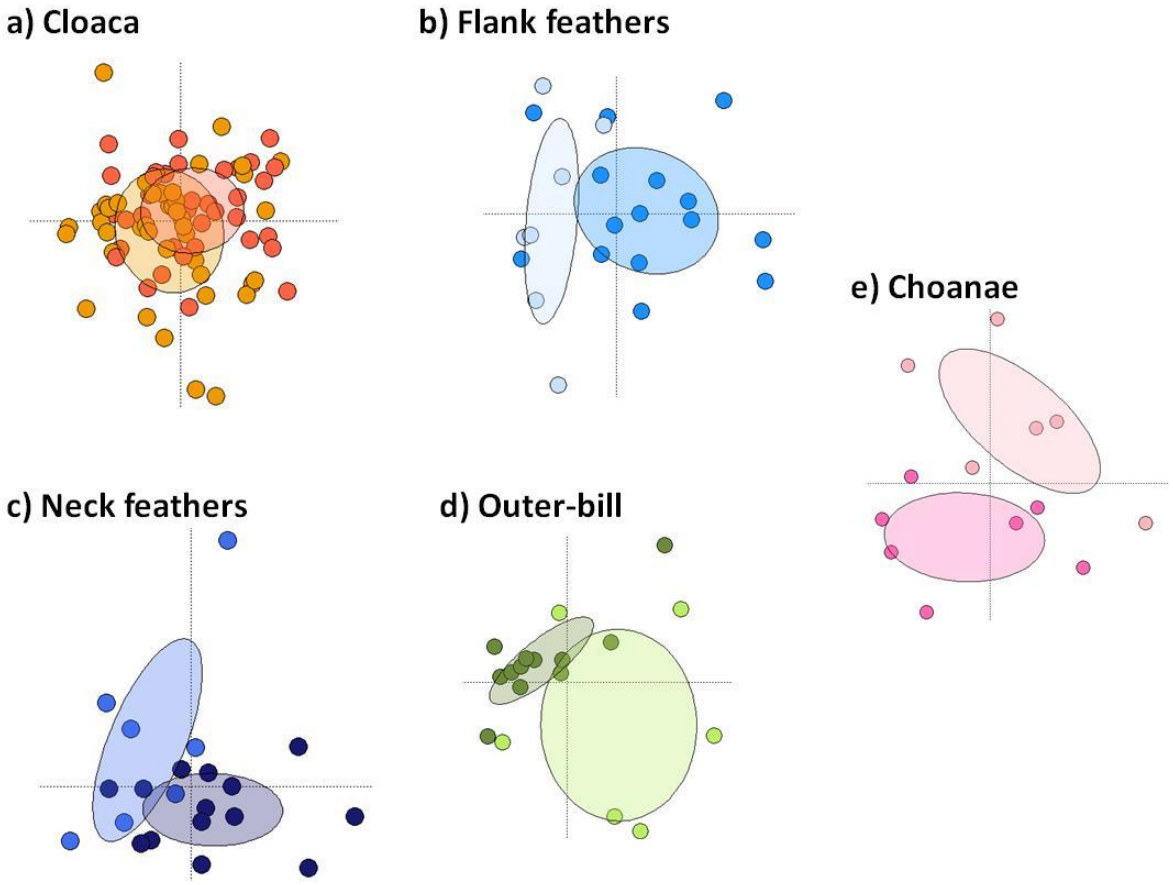

Figure 2: Boxplots showing the relative abundance of the OTUs belonging to the families (a) *Lachnospiraceae* and (b) *Salinisphaeraceae* in the flank and neck feathers of non-breeding and breeding females. Compared to breeding females, non-breeding females had higher abundance of *Lachnospiraceae* in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

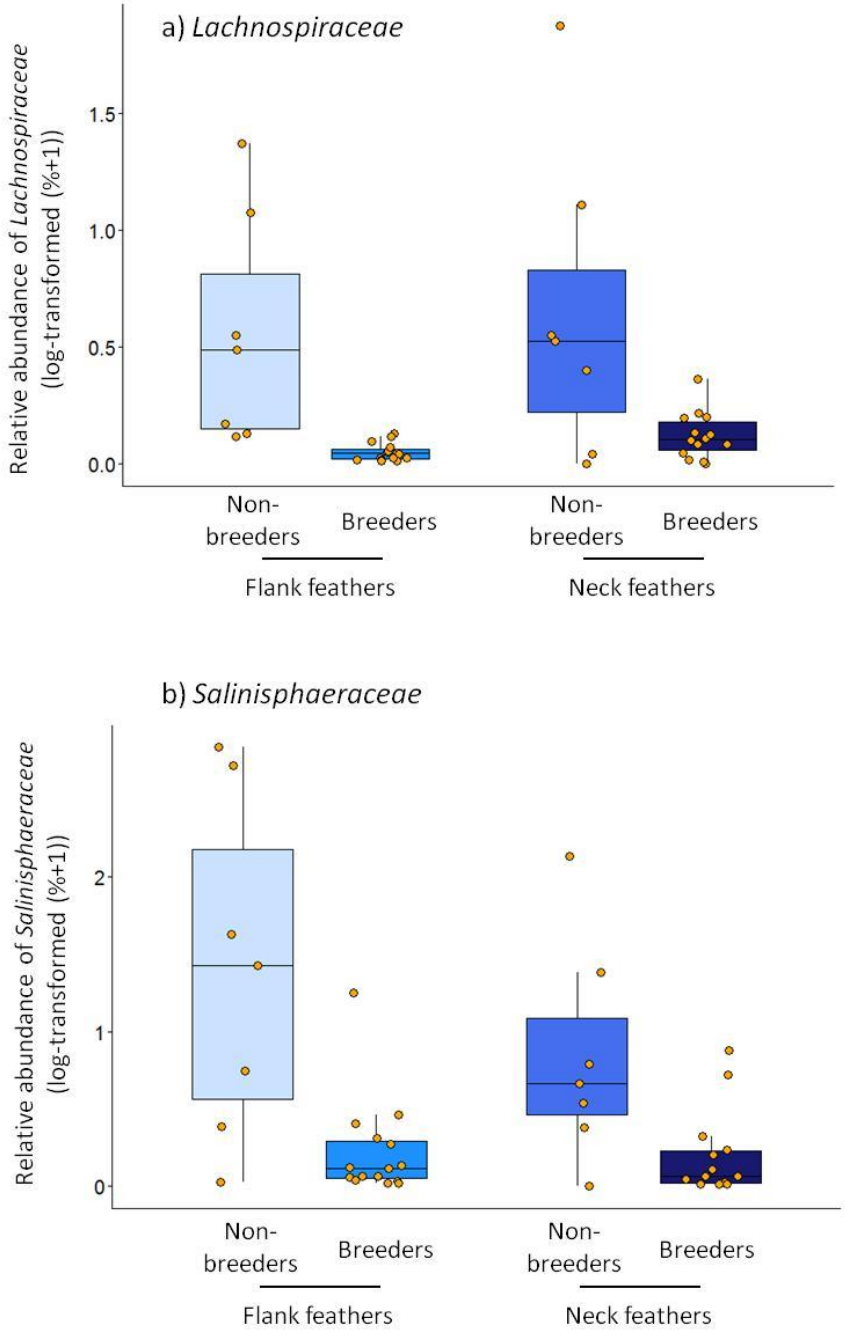

Figure 3: Plots showing variations in cloacal microbiota according to reproductive performance in female breeders. (a) Unconstrained distance-based redundancy analysis plots showing differences in female cloacal microbiota according to $PC1_{RepPerf}$. Female cloacal microbiota composition varied with $PC1_{RepPerf}$ in females ($F_{1,36}$ = 2.19, R^2 = 0.06, P = 0.004). Although $PC1_{RepPerf}$ was analyzed as a continuous variable (see text), it is displayed here as a categorical variable for illustrative purposes. We categorized $PC1_{RepPerf}$ into three groups of equal sample size. Axis 1 and 3 are shown. (b) Sum of the relative abundance of the six *Corynebacteriaceae* OTUs that correlated with $PC1_{RepPerf}$ with P < 0.02 in the correlation index analysis. (c) Barplot representing the relative abundance of these *Corynebacteriaceae* OTUs in each female according to hatching success.

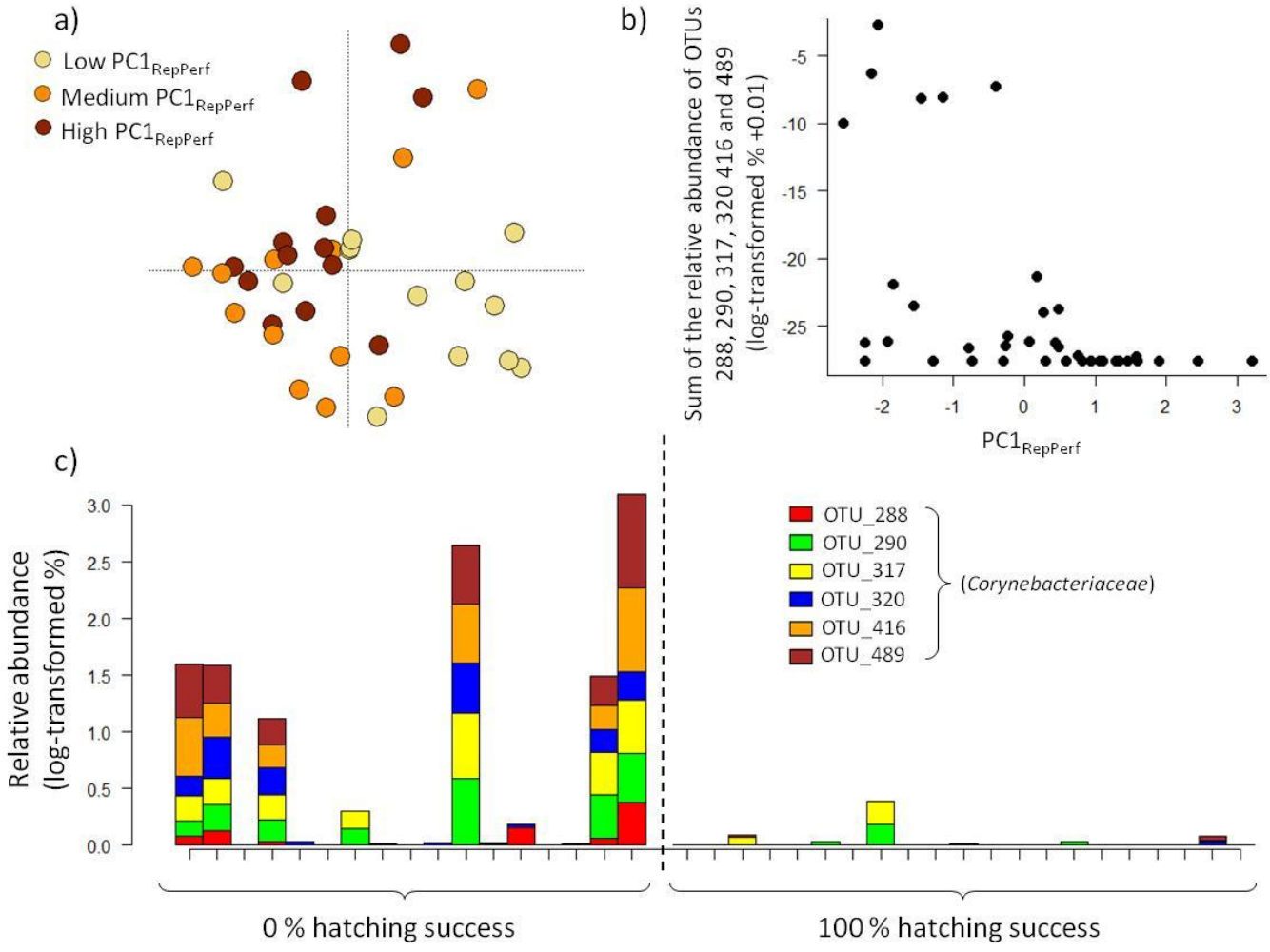

Figure 4: Intra-individual dispersion in microbiota composition according to $PC1_{RepPerf}$ in males (black dots and bold line) and females (white dots and thin line). Because not all body sites were sampled on each single individual, we restricted the analyses on individuals for which cloaca, flank feathers, neck feathers, preen feathers and outer-bill were sampled. Intra-individual dispersion varied with $PC1_{RepPerf}$ 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).

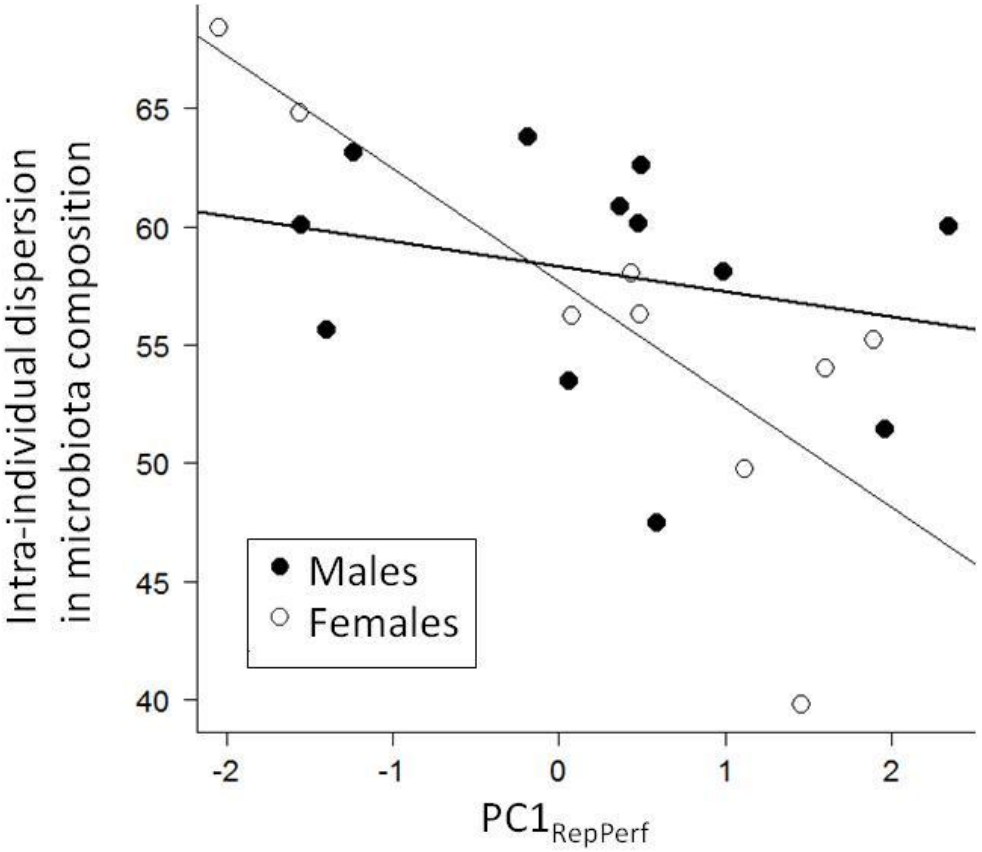

Figure 5: α-diversity of (a) preen feather microbiota and (b) flank feather microbiota in relation to $PC1_{RepPerf}$ in males and females. α-diversity (Faith's PD index) was calculated on rarefied samples. In preen feathers, the relationship between α-diversity and $PC1_{RepPerf}$ did not vary with sex ($PC1_{RepPerf}$ *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 shown, while in flank feathers, the relationship between α-diversity and $PC1_{RepPerf}$ varied with sex ($PC1_{RepPerf}$ *Sex: $F_{1,10}$ = 7.42, P = 0.021), thereby sex-specific regression lines are shown (females: $F_{1,1}$ = 7.13, P = 0.020 and males: $F_{1,1}$ = 3.70, P = 0.073).

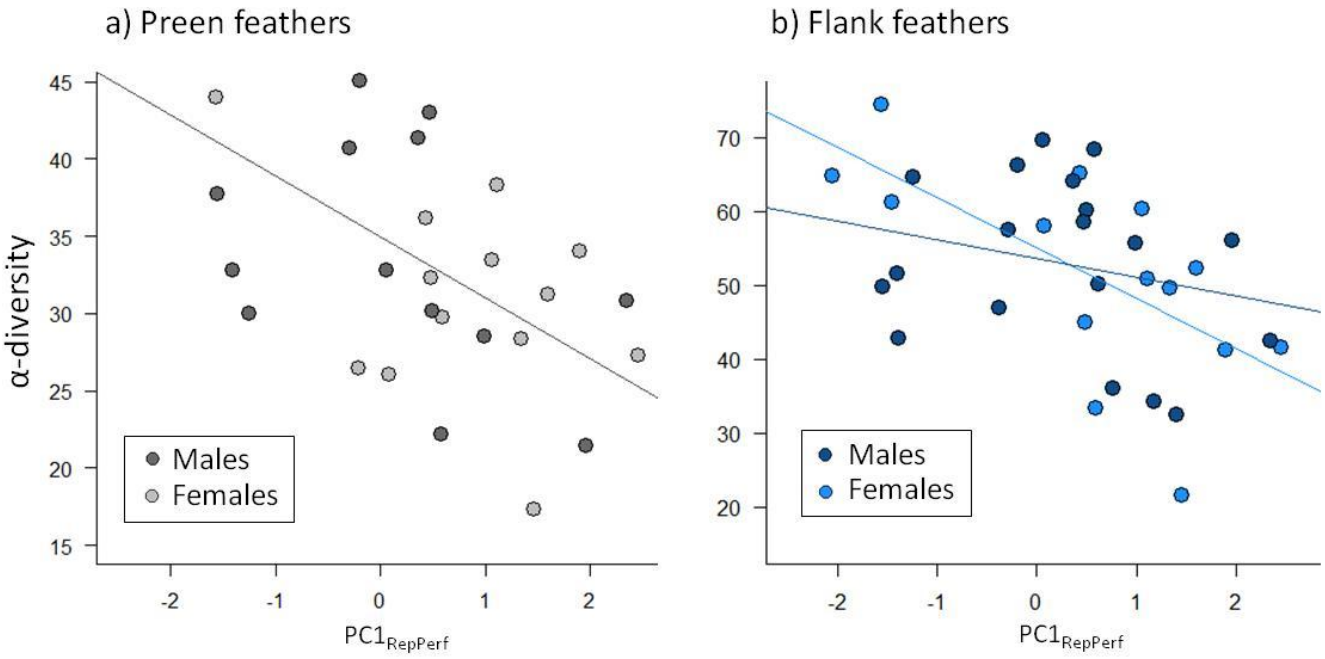

Figure 6: Plots showing differences in microbiota between body sites. a) Unconstrained distance-based redundancy analysis plots showing separation of microbiota by body sites. b) Mean relative abundance of the most abundant phylum in the different body sites. c) Mean relative abundance of the most abundant OTUs in the different body sites and neighbour-joining phylogenetic tree based on the V5-V6 16S rRNA sequences of these most abundant OTUs (NA: not affiliated). Correlation indices showed that all the OTUs shown in Fig. 6c, except OTU_43, differed significantly between body sites (all $P < 0.0002$).