Bacterial density rather than diversity correlates with hatching success across different avian species

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34 Running headline: Peralta-Sánchez, Martín-Platero et al.: Hatching success and bacteria

35 on eggshells

36

37 **SUMMARY:**

38 Bacterial communities within avian nests are considered an important determinant of
39 egg viability, potentially selecting for traits that confer embryos with protection against
40 trans-shell infection. A high bacterial density on the eggshell increases hatching failure,
41 whether this effect could be due to changes in bacterial community or just a general
42 increase in bacterial density. We explored this idea using intra- and interspecific
43 comparisons of the relationship between hatching success and eggshell bacteria
44 characterized by culture and molecular techniques (fingerprinting and high-throughput
45 sequencing). We collected information for 152 nests belonging to 17 bird species.
46 Hatching failures occurred more frequently in nests with higher density of aerobic
47 mesophilic bacteria on their eggshells. Bacterial community was also related to hatching
48 success, but only when minority bacterial OTUs were considered. These findings
49 support the hypothesis that bacterial density is a selective agent of embryo viability, and
50 hence a proxy of hatching failure only within species. Although different avian species
51 hold different bacterial densities or assemblages on their eggs, the association between
52 bacteria and hatching success was similar for different species. This result suggests that
53 interspecific differences in antibacterial defenses are the responsible to keep hatching
54 success at similar levels in different species.

55

56 **Keywords**: ARISA, avian community, bacterial community, bacterial density,
57 comparative analysis, eggshells, hatching success, high-throughput sequencing,
58 Illumina HiSeq, Phylogenetic General Least Square

59

60 **One sentence summary:** Hatching success is predicted by bacterial density in general
61 and by the bacterial community assemblage on eggshells when minority bacterial

62 species are included. Interestingly, hatching success is similar in different species,

63 suggesting interspecific differences in antibacterial defenses..

64

65 **INTRODUCTION**

66

67 Bacteria and other microorganisms are a key component of the environment where
68 animals develop and reproduce and, hence, play a central role in the evolution of life
69 history and antimicrobial defensive traits of animals (McFall-Ngai *et al.*, 2013). For
70 instance, microorganisms have an important role in hatching success, and hence, avian
71 fitness in natural conditions (Cook *et al.*, 2005a, Cook *et al.*, 2005b). Avian eggs are
72 suitable environments for the growth of opportunistic and potential pathogenic bacteria,
73 not only because of their high nutrient content, but also because the temperature
74 necessary for egg incubation is close to the optimal temperature for the exponential
75 growth of these bacteria (Krieg & Holt, 1984, Board & Fuller, 1994, Singleton &
76 Harper, 1998). Bacterial pathogens infect the embryo through the eggshell pores, trans-
77 shell bacterial infection thus requires that bacteria first colonize the eggshell and
78 multiply (Board & Tranter, 1986). Bacteria that can potentially act as pathogens of
79 avian embryos are ubiquitous and have been considered an important evolutionary force
80 selecting for different physical, chemical and behavioral barriers that impede bacterial
81 trans-shell colonization and growth inside the eggs (Board *et al.*, 1994, Deeming, 2002,
82 Wellman-Labadie *et al.*, 2007).

83 Avian incubation reduces bacterial density and provokes changes in bacterial
84 assemblages on eggshells, thereby lowering the risk of embryo and egg content
85 infection (Cook *et al.*, 2005a, Cook *et al.*, 2005b, Shawkey *et al.*, 2009). The role of
86 incubation in bacterial shifts on the eggshells has been corroborated in subsequent
87 studies (Ruiz-de-Castañeda *et al.*, 2011b, Ruiz-De-Castañeda *et al.*, 2012, Potter *et al.*,
88 2013, Brandl *et al.*, 2014, Grizard *et al.*, 2014, Lee *et al.*, 2014, Grizard *et al.*, 2015).
89 These studies suggest that high bacterial densities should be correlated with poor

90 hatching, and will reduce avian fitness overall. However, negative correlation between
91 bacterial densities and hatching success of avian eggs in natural conditions is limited to
92 a few studies, while many others found no association. Hansen *et al.* (2015) found a
93 negative correlation between eggshell bacteria and hatching success within populations
94 of greater white-fronted geese, as did a comparative analysis of hatching success and
95 eggshell bacterial loads from two European bird populations (Soler *et al.*, 2012). Other
96 studies found no association between bacteria on the eggshell and hatching success. For
97 instance, incubation reduced bacterial density on eggshells in the pied flycatcher
98 (*Ficedula hypoleuca*) (Ruiz-De-Castañeda *et al.*, 2012), but this reduction in bacterial
99 density did not covary with hatching success (Ruiz-de-Castañeda *et al.*, 2011a).
100 Similarly, no association was detected in four other Mediterranean species (Peralta-
101 Sánchez *et al.*, 2010, Wang *et al.*, 2011).

102 Variation in the relationship between eggshell bacteria and hatching success may
103 be related to a range of factors that influence the diversity and abundance of eggshell
104 bacteria, including geography, differences in nest construction, and differences in
105 antimicrobial behaviors across bird species. For instance, studies showing that growth
106 of eggshell bacterial communities differ for birds in Mediterranean and tropical areas
107 highlight the importance of considering geographic variation (Cook *et al.*, 2005b, Wang
108 *et al.*, 2011). Moreover, we also know that environmental conditions in avian nests with
109 different structural characteristics (i.e. orientation, open *vs.* hole nests) affect bacterial
110 communities of the eggshells (Goodenough & Stallwood, 2012, Peralta-Sánchez *et al.*,
111 2012), which therefore may alter the association with hatching success. The relationship
112 between characteristics of eggshell bacterial communities and trans-shell bacterial
113 infection or hatching failures would depend on antimicrobial properties of eggshells,
114 which can vary both intra- and interspecifically (Wellman-Labadie *et al.*, 2007, 2008b,

115 Horrocks *et al.*, 2014, Martín-Vivaldi *et al.*, 2014, D'Alba *et al.*, 2016). Thus, detecting
116 associations between the bacterial environment and infection (or hatching failure as its
117 consequence) will probably vary according to environmental conditions and species-
118 specific antimicrobial capabilities.
119 In the present study, we tested the hypothesis that bacteria on eggshells
120 negatively impact hatching success. We explore correlative prediction of this hypothesis
121 by studying bacterial communities of eggshells in an avian community, and exploring
122 the association with hatching success both within and between species while controlling
123 for potentially temporal and geographical confounding effects (i.e. using a single year
124 and location). Bacterial communities were characterized both by traditional culture
125 methods and molecular methodologies. We hypothesize that both bacterial densities and
126 assemblages would be related with hatching success at the intra and the interspecific
127 levels, predicting that nests and species that experience higher bacterial density or a
128 more harmful bacterial assemblage will experience higher hatching failures. However,
129 we cannot dismiss other alternative predictions as a lack of interspecific covariation
130 between hatching success and eggshell bacterial when antimicrobial defenses are on
131 average pretty adjusted to the strength of selection pressure at the species levels.
132 Finally, the use of two different culture independent techniques allows to study their
133 ability to detect the association between hatching success and bacterial community. We
134 hope that this study will provide new evidences for the relationships between
135 microorganisms and birds and help to better understand the selective pressures acting at
136 the nest environment.
137
138
139 **MATERIAL AND METHODS**

140

141 Study system

142 The study area was located in the Hoya de Guadix (37º18'N, 3º11'W), a high elevation
143 plateau, 1000 m a. s. l., with a semi-arid climate. During the breeding season of 2007
144 (March-July), 600 nest-boxes were checked once per week, and an intense search of
145 other nests of wild birds (including hole- and open-nesters) was performed. Nest-boxes
146 were cork-made (height * width * depth: 370 * 200 * 230 mm, bottom-to-hole height:
147 250 mm, hole diameter: 60 mm, height from ground around 2 meters). Once a new nest
148 was found, it was visited every 2-3 days to determine laying date and clutch size. In
149 order to assess hatching success accurately, we visited each nest every day from two-
150 three days before the estimated hatching date of the first egg until two-three days after
151 the end of hatching. This parameter was estimated as the proportion of eggs detected in
152 the nests 2-3 days before hatching (i.e. after possible partial predation events) that
153 successfully hatched in non-predated clutches. Only data from clutches where at least
154 one egg hatched were included in the analyses, in order to avoid possible bias as it is to
155 consider unfertile clutches or nests that experience partial abandonment undetected by
156 us.

157 We got data from 152 clutches from nests belonging to 17 species (Table 1).

158

159 Bacterial sampling and culturing

160 Bacteria from the eggshells were sampled at the beginning of the incubation, a couple of
161 days after clutch was completed. In order to avoid contamination between nests, all
162 samples were collected wearing latex gloves washed with 96% ethanol. Eggs were
163 sampled by gently cleaning their shells with a sterile swab slightly wet with sterile 0.2
164 M sodium phosphate buffer (pH = 7.2). All eggshells from the same clutch were

165 sampled with the same sterile swab. Swabs were preserved in the same phosphate
166 solution in a microcentrifuge tube (1.2 mL) that was stored in a cooler at 4-6ºC. Once in
167 the laboratory, samples were stored at 4ºC until processing (range: 0-45 days). After
168 bacterial sampling, length and width of all eggs were measured with a caliper (accuracy
169 0.02 mm) to estimate sampled surface of eggshell.

170 Microorganisms were cultured by homogenously spreading 100 μL of the
171 suspensions at serial dilutions on plates with Tryptic Soy Agar (Scharlau Chemie S.A.
172 Barcelona), a broadly used general medium to grow total aerobic and mesophilic
173 bacteria. Agar plates were incubated at 32ºC for 72 hours, and the swab and remains of
174 each sample were frozen at -20ºC for subsequent molecular analyses. Eggshell bacterial
175 density was estimated as the number of Colony Forming Units that grew in Tryptic Soy
176 Agar per egg surface unit (cm^2) for all eggs sampled following Peralta-Sánchez et al.
177 (2012). Detailed information is described in ESM 1.

178

179 <u>DNA extraction</u>

180 DNA extractions were performed by Chelex-based extraction protocol following
181 Martín-Platero et al. (2010). DNA templates were amplified and analyzed by means of
182 ARISA and HiSeq Illumina. Detailed information is described in ESM 1.

183

184 <u>Automatic Ribosomal Intergenic Spacer Analysis (ARISA)</u>

185 PCR amplification of the 16S-23S intergenic spacer region in the rRNA operon was
186 performed with a fluorescence-labeled forward primer (72F: 5'-TGC GGC TGG ATC
187 TCC TT-3', labeled with the phosphoramidite dye 5-FAM; 38R: 5'-CCG GGT TTC
188 CCC ATT CGG-3') (Ranjard *et al.*, 2000). PCR reactions were performed at a final
189 volume of 50 μl and the reaction mix contained 1x PCR buffer (75 mM Tris HCl, pH

190 9.0; 50 mM KCl; 20 mM $(NH4)_2SO_4$), 2.5 mM Cl_2Mg, 200 µM dNTPs, 0.1 µM of each
191 primer, 1 U Taq DNA polymerase (EMBL, Spain) and 10 ng DNA template. PCR
192 started with a denaturing step at 94ºC for 2 min; followed by 30 cycles of amplification
193 of denaturing at 94ºC for 1 min, an annealing step at 55ºC for 30s and an extension step
194 at 72ºC for 1 min; and a final elongation step of 72º C at 5 min. PCRs were performed
195 in an Eppendorf Mastercycler (Eppendorf, Hamburg). The PCR products were checked
196 on 0.9% agarose gels. Determination of amplicon size was performed in the Sequencing
197 Services of the University of Granada using a genetic sequencer ABI Prism 310
198 (Genetic Analyzer, Applied Biosystems).
199 Amplicon profiles were determined with Peak Scanner 1.0 (Applied Biosystems)
200 to set Operational Taxonomic Units (OTUs). *Automaticbinner.r* and *interactivebinner.r*
201 scripts (Ramette, 2009) in R environment (R Core Team, 2015) (range 100-1200 base
202 pairs; min RFI = 0.09; window width = 4; shift = 0.1) were used to obtain a table of 227
203 OTUs (Table 1). Because of inaccuracy of using peak areas in ARISA as indicative of
204 OTUs' abundance, we used the conservative approach of using presence-absence
205 information to build the OTU table. A total of 190 OTUs were used in subsequent
206 analyses. This OTU table comprised information of the bacterial community of each
207 sampled nest (intraspecific approach). Afterwards, this OTU table was used to estimate
208 prevalence of each OTU for each bird species that was used in the interspecific
209 approach.
210
211 TABLE 1 AROUND HERE
212
213 High-Throughput Sequencing
214 16S rRNA variable region 4 (V4) were sequenced in Illumina HiSeq 2000 Platform

215 following the protocols of the Earth Microbiome Project (Gilbert *et al.*, 2010,
216 Thompson *et al.*, 2017). After amplification, primers were trimmed, sequences were
217 demultiplexed and quality filtering was performed, following QIIME software v1.9
218 (Quantitative Insights In Microbial Ecology; Caporaso *et al.*, 2010). Sequences are
219 available in QIITA repository (https://qiita.ucsd.edu/; study ID: 1632). QIIME is a
220 wrapper software that allows to perform both upstream (from the raw sequences files to
221 the OTU table) and downstream analyses (from the OTU table to final publishing
222 results) in a single pipeline (Navas-Molina *et al.*, 2013). Open reference OTU picking
223 procedure (Rideout *et al.*, 2014) was applied to generate the OTU table, clustering
224 sequences against Greengenes database v10_13 (DeSantis *et al.*, 2006, McDonald *et al.*,
225 2012). Subsequently, Archaea, chloroplast, mitochondria and non-phylum assigned
226 OTUs were filtered as well as singletons and OTUs with frequency lower than 0.005%
227 of the total sequence account (Bokulich *et al.*, 2013). In order to control for the
228 sequencing effort, the OTU table was rarefied at 10,569 sequences, and a total of 548
229 OTUs were retained for subsequent analyses (Table 1). More detailed information on
230 this procedure can be found in the Electronic Supplementary Material 1. This OTU table
231 contained information about the bacterial community of each sampled nest (intraspecific
232 approach). Afterwards, this OTU table was used to estimate prevalence of each OTU for
233 each bird species that was used in the interspecific approach.
234
235 Sample size and statistical analyses
236
237 *Intraspecific approach*
238 This approach studied the association between hatching success and bacteria community
239 on eggshells (density and assemblage) at different bird nests after controlling for the

240 species identity in the statistical models.

241 Bacterial density and OTU richness in ARISA were normally distributed after
242 log_{10}-transformation (Kolmogorov-Smirnov tests for continuous variables, $P > 0.05$).
243 OTU richness in HiSeq was normally distributed without transformation. Moreover, the
244 distribution of residuals of arcsine-transformed hatching success in all tested models did
245 not differ from normality (Kolmogorov-Smirnov tests for continuous variables, $P >$
246 0.05), so we were confident in the use of parametric statistical tests with the
247 transformed variables. The predicted influence of bacterial density or OTU richness on
248 hatching success was tested by means of General Lineal Mixed Models (GLMM) with
249 hatching success as the dependent variable, species identities as an independent random
250 factor (random intercept models), and bacterial density or OTU richness as a covariate.
251 We also tested whether the predicted effect of bacterial density differed for different
252 species by estimating the effect of the interaction between species identity (random
253 factor) and bacterial density. Significant interactions indicate that the slopes associated
254 to the relationship between hatching success and bacterial density differ for different
255 species, while non-significant effects suggest similar effects independently of the
256 species identity. All analyses used two-tailed P-values throughout, and were conducted
257 using STATISTICA 10 software.

258 Procrustes ANOVA was used to explore the relationship between hatching
259 success and bacterial assemblage. This permutational statistical test is equivalent to the
260 classical ANOVA test, but in a multivariate setting. First, the Gower distance matrix
261 was calculated from the ARISA OTU table as recommended for binary data
262 (presence/absence) by Kuczynski *et al.* (2010), and Weighted and Unweighted UniFrac
263 distance matrix from Hiseq OTU table (Lozupone & Knight, 2005). UniFrac distance is
264 a measure of the distance between communities based on their phylogenetic structure

265 and is recommended when phylogenetic information of the bacterial community is
266 available (i.e. abundance OTU tables from high-throughput sequencing) (Lozupone &
267 Knight, 2005, Lozupone *et al.*, 2011). Second, Principal Coordinates Analyses (PCoA)
268 axes were calculated from distance matrixes. Third, we used the complete set of PCoA
269 axes as explanatory matrix for the Procrustes ANOVA, that were performed in the R
270 environment (R Core Team, 2015) using the packages 'vegan' (Dixon, 2003) and
271 'geomorph' (Adams & Otarola-Castillo, 2013).
272 Finally, Spearman correlations were performed to explore the relationship
273 between hatching success and OTU abundance only with the HiSeq approach at
274 different taxonomic levels (Phylum, Class, Order and Family) (similar analysis using
275 OTU richness from ARISA was not possible due to we use presence-absence OTU
276 table). False Recovery Rate (FDR) corrections were applied to correct for multiple
277 comparisons.
278
279 *Interspecific approach*
280 For each bird species, we calculated the average values of bacterial density and the
281 prevalence of each OTU from the ARISA and HiSeq OTU tables. Specific information
282 cannot be considered statistically independent due to common ancestry, so we
283 considered phylogenetic relationships between species to perform comparative analyses
284 (Harvey & Pagel, 1991). We used Phylogenetic Generalized Least Square regression
285 (PGLS) analyses (Pagel, 1997, 1999). Implementation and use of the PGLS analyses is
286 thoroughly explained in Møller *et al.* (2011) and Soler *et al.* (2011a). We weighted all
287 species by sample size to avoid potential differences in sampling effort (Garamszegi &
288 Møller, 2010, 2011, Vincze *et al.*, 2013).
289 Geometric mean values of each bacterial density and average hatching success

290 for each bird species were calculated. Distribution frequencies of these variables did not
291 differ from normality after log_{10}- and arcsine transformations, respectively
292 (Kolmogorov-Smirnov normality tests, $P > 0.15$). Bacterial density significantly varied
293 between species (GLM, bacterial density as dependent variable, species identity as
294 factor, $F_{16,125} = 1.83$, $P = 0.034$), which justified the use of mean values in our
295 comparative analyses (Peralta-Sánchez *et al.*, 2012).
296 Prevalence of each OTU in each bird species was calculated from ARISA and
297 HiSeq OTU tables. Following similar steps as in the intraspecific approach, Gower
298 distance matrix were calculated from ARISA OTU table, and Weighted and
299 Unweighted UniFrac distance matrixes from HiSeq OTU table. Principal Coordinates
300 Analyses (PCoA) were performed from these distance matrixes and the complete set of
301 axes was used in the Procrustes ANOVA.
302 Phylogenetic relationships were based in Thuiller *et al.* (2011) and modifications
303 in the phylogenetic tree were performed using TreeGraph2 free software 2.11.1-654
304 beta (Figure 1).
305 Phylgenetic General Least Square (pGLS) models were performed in the R
306 environment (R Core Team, 2015). For bacterial density analyses, 'MASS' (Venables
307 & Ripley, 2002), 'ape' (Paradis *et al.*, 2004) and 'mvtnorm' (Genz *et al.*, 2009)
308 packages were used as well as an additional function by R. Freckleton (University of
309 Sheffield) implemented in the package 'caic'. For bacterial assemblages analyses,
310 'vegan' (Dixon, 2003) and 'geomorph' (Adams & Otarola-Castillo, 2013) packages
311 were used.
312
313 *Relationship between bacterial density and bacterial community*
314 At the intraspecific level, GLMM were performed comparing bacterial density and OTU

315 richness and species as random factor and Procrustes ANOVA for comparing bacterial
316 density and bacterial assemblages including species identity as factor (ARISA and
317 HiSeq). Bacterial density correlated positively with bacterial species richness as well as
318 with bacterial assemblages when consider HiSeq bacterial richness, but this was not the
319 case for ARISA bacterial richness (ESM 2). For comparative analyses a similar
320 approach was performed: pGLS tested the association between bacterial density and
321 OTU richness and bacterial assemblages. No significant relationship was found between
322 those variables (ESM 2).
323
324
325 **RESULTS**
326
327 *Microbiology of avian eggshells*
328
329 Gammaproteobacteria (58.0% average abundance, phylum Proteobacteria) was the most
330 dominant class in bacterial community on eggshells followed by Actinobacteria (18.1%,
331 phylum Actinobacteria), Bacilli (11.9%, phylum Firmicutes) and Sphingobacteriia
332 (5.6%, phylum Bacteroidetes). This dominant community is common among and within
333 species (Figure 1). However, Actinobacteria or Bacilli dominated over other classes in
334 some communities as well as Sphingobacteriia did in only one clutch (Figure 1).
335 *Pseudomonadaceae* was one of the most dominant families in the bacterial communities
336 on eggshells (94.1% of samples, average abundance 31.0%). Within
337 *Pseudomonadaceae*, the genera *Pseudomonas* were the most dominant (94.1% of
338 samples, average abundance 30.4%). The other two most dominant families were
339 *Micrococcaceae* (96.3% of samples, average abundance 9.8%) and *Enterobacteriaceae*

340 (92.6% of samples, average abundance 2.50%). *Arthrobacter* was the most dominant
341 genera of the family *Micrococcaceae* (93.2% of samples, average abundance 5.60%)
342 while one unidentified species was the most dominant of the family *Enterobacteriaceae*
343 (94.85% of samples, average abundance 8.30%). Abundance of different taxa are shown
344 in Figure 1 and ESM 4.
345
346 FIGURE 1
347
348 *Intraspecific variation in hatching success and in bacterial density and community*
349 Hatching success was negatively correlated with the density of aerobic mesophilic
350 bacteria after controlling for bird species identity (Table 2; Figure 2). That effect of
351 bacterial density on hatching success did not depend on species identity (GLMM,
352 interaction species identity x bacterial density, $F_{17,118} = 0.67$, $P = 0.824$).
353
354 TABLE 2 AROUND HERE
355
356 FIGURE 2 ABOUT HERE
357
358 After controlling for bird species identity, hatching success was only
359 significantly explained when using Unweighted UniFrac (Table 2, Figure 3). Finally,
360 none of the OTUs were correlated significant with hatching success at different
361 taxonomic levels (ESM 3).
362
363 FIGURE 3 AROUND HERE
364

365 *Interspecific variation in bacterial assemblages and hatching success*

366 Bacterial assemblages on eggshells were very similar among bird species (Table 2,
367 ESM 6). Interspecific variation in eggshells bacterial density was not associated with
368 differences in hatching success (Table 3). Moreover, among species variation in
369 bacterial assemblages was not significantly related with interspecific variation in
370 hatching success with any of the molecular approaches (Table 3).

371

372 TABLE 3 AROUND HERE

373

374 FIGURE 4 AROUND HERE

375

376

377 **DISCUSSION**

378

379 The main goal of this manuscript was to explore the effects of bacterial community of
380 the avian eggshells on the hatching success in a community of birds. Our main findings
381 are that (i) variation in eggshell density of mesophilic bacteria is negatively related to
382 within species variation in hatching success of wild birds and that (ii) this effect was
383 similar for all species considered in our study. Moreover, (iii) Unweighted UniFrac
384 distance between bacterial communities covaried with hatching success. At the
385 interspecific level, neither (iv) eggshell bacterial densities, nor (iv) bacterial
386 assemblages covaried with hatching success. These results suggest that bacteria on the
387 eggshell influence reproductive success of birds, and that this effect is similar in
388 different taxa.

389 Although a very low percentage of the bacterial diversity grow in mesophilic

390 media in aerobic conditions (Amann *et al.*, 1995, Pace, 1997), mesophilic bacterial
391 counts have been used to quantify bacterial abundance (Cook *et al.*, 2003, Cook *et al.*,
392 2005a, Cook *et al.*, 2005b). Moreover, conclusions achieved by culture-dependent
393 estimations of bacterial abundance on eggshells have been validated by using molecular
394 characterization (i.e. diversity) of communities (Spanggaard *et al.*, 2000, Cook *et al.*,
395 2005a, Shawkey *et al.*, 2009). In addition to traditional culture techniques, we also
396 employed two different molecular methods: ARISA and Hiseq. Both techniques differ
397 in the rRNA regions (ARISA amplifies the ITS region; and in our case HiSeq amplified
398 the V2-V4 region of 16s rRNA), their ability to detect OTUs (from 190 using ARISA to
399 548 using HiSeq) and, consequently, the possibility to identify different taxonomic
400 groups influencing hatching success. ARISA provide a fingerprinting of the bacterial
401 community while Hiseq return the phylogenetic information of the OTUs (i.e. bacterial
402 group identity) (Fisher & Triplett, 1999, Corneo *et al.*, 2013). Especially important is
403 the comparison between ARISA and Unweighted Unifrac results as both
404 approximations use OTU presence/absence information. We found a significant effect
405 of the bacterial assemblage in hatching success only when Unweighted Unifrac was
406 tested. Both approaches have been proposed as complementary (Lee *et al.*, 2014),
407 although in our study Hiseq has performed better than ARISA. Although ARISA has
408 been used extensively in microbial ecology for characterizing bacterial community, the
409 advantages of next generation sequencing make this technique more recommendable in
410 studies of microbial ecology.
411
412 *Taxonomical composition of bacterial communities*
413 Few studies have explored the bacterial community on avian eggshells using culture-
414 independent methods (Shawkey *et al.*, 2009, Grizard *et al.*, 2014, Lee *et al.*, 2014,

415 Grizard *et al.*, 2015). The bacterial community found on eggshells in the present study
416 resembles those described in previous papers. The bacterial profile present in our
417 samples, rich in Gammaproteobacteria, has been previously reported in the feral dove,
418 *Columba livia* (Grizard *et al.*, 2014), or the magpie *Pica pica* (Lee *et al.*, 2014).
419 Moreover, Shawkey *et al.* (2009) found a rich community of Betaproteobacteria in the
420 pearl-eyed thrush *Margarops fuscatus*. The similarities in eggshell bacterial
421 composition between ours and previous studies also apply to lower taxonomic levels.
422 *Pseudomonadaceae* is a common family on eggshells and nests of blue tits, *Cyanistes*
423 *caeruleus* and great tits, *Parus major* (Goodenough & Stallwood, 2010), house wrens
424 Troglodytes aedon (Singleton & Harper, 1998) and common magpies (Lee *et al.*, 2014).
425 *Pseudomonas*, a saprophytic and opportunistic bacteria (Bergey *et al.*, 1984) that is
426 commonly found in eggshells of these species and those analyzed in our study has
427 traditionally been related to infection and disease in birds (Benskin *et al.*, 2009). The
428 detected anti-*Pseudomonas* activity of egg contents (Kovacs-Nolan *et al.*, 2005)
429 confirms that this bacteria seems to play an important role in the viability of bird eggs.
430 However, we can only speculate that particular strains could be related with hatching
431 failure, and, only in some particular environmental conditions, they are able to infect
432 egg contents. For instance, *Neisseria sp.* is the main strain producing hatching failures
433 in the arctic goose *Anser nivalis* (Hansen *et al.*, 2015) suggesting the importance of
434 other bacteria in addition to *Pseudomonas*. In other cases, common and commensal
435 stains are related with egg content infection as *Escherichia coli* and *Enterococcus sp.* (in
436 the saker falcon *Falco cherrug*: Janos *et al.*, 2007), or *Escherichia coli and*
437 *Staphylococcus epidermitis* (in the house sparrow *Passer domesticus*: Pinowski *et al.*,
438 1994). Some of these genera were found in low abundance in the present study,
439 although they were not significantly related with hatching failure.

440

441 *Intraspecific approach*

442 At the intraspecific-level, our findings support the predicted negative
443 relationship between the density of aerobic mesophilic bacteria on the eggshell at the
444 beginning of the incubation and hatching success. This result suggests two possible
445 scenarios. The first one is that bacterial density per se affects hatching success
446 independently of the composition of the bacterial community. The second scenario
447 suggests a link between potentially harmful bacteria that were present on the eggshells
448 at the time of sampling (i.e. the start of the incubation) and bacterial density and
449 probability of embryo death. In this sense, we detect a significant association between
450 eggshell bacterial assemblage and hatching success, but only when using the
451 Unweighted UniFrac metric of bacterial community. Both Weighted and Unweighted
452 UniFrac distance matrixes were calculated because we do not know a priori the relative
453 importance of rare bacterial taxa. Weighted UniFrac gives more weight to the most
454 abundant bacterial taxa while Unweighted UniFrac gives similar weight to all bacterial
455 species present (Lozupone *et al.*, 2007). The fact that only Unweighted UniFrac was
456 significantly relate to hatching success point out the importance of minority and rare
457 bacterial taxa on the embryos' viability, although we did not find a significant
458 correlation between prevalence of any individual OTU and hatching success.
459 Supporting these speculations, bacterial density and community assemblage were
460 related and, thus, it is still possible that the negative association between hatching
461 success and bacterial density for mesophilic bacteria is due to the presence of certain
462 bacterial groups on the eggshells, the consortium of some rare bacterial species or
463 maybe facilitating others to pass through the eggshell even though they are not harmful
464 by themselves. It is worth to mention here that for characterizing bacterial communities

465 we used richness values and OTU assemblages as a whole (i.e. distance matrixes). We
466 just speculate that only a few bacterial strains are able to penetrate the eggshells (Cook
467 *et al.*, 2005a, Wang *et al.*, 2011), which may be at lower density on eggshells but their
468 presence could be related to bacterial abundance. This idea is really attractive, but
469 empirical evidence and experimental studies are necessary for disentangling these
470 relationships.

471 Birds by means of early incubation are able to control bacterial growth on the
472 eggshell (Cook *et al.*, 2005a, Shawkey *et al.*, 2009) and induce changes in their bacterial
473 communities (Shawkey *et al.*, 2009, Potter *et al.*, 2013, Brandl *et al.*, 2014, Grizard *et*
474 *al.*, 2014, Lee *et al.*, 2014) that favor proliferation of less harmful bacterial strains
475 (Cook *et al.*, 2005a, Brandl *et al.*, 2014, Lee *et al.*, 2014), thereby reducing probability
476 of hatching failures (Cook *et al.*, 2003, 2005b). These beneficial effects of incubation
477 are likely mediated by the associated drying effect of the incubation (D'Alba *et al.*,
478 2010, Ruiz-de-Castañeda *et al.*, 2011c), particularly at the beginning of the incubation
479 period, when most of the trans-shell bacterial infections occur (Cook *et al.*, 2005a, Cook
480 *et al.*, 2005b, Shawkey *et al.*, 2009, but see Wang *et al.*, 2011). Trying to reduce the
481 effect of incubation on bacterial communities of eggshells, eggs were sampled after the
482 laying stage in our study. However, some of the species investigated start to incubate
483 before the end of egg laying, and these species tend to harbor more bacteria on their
484 eggshells than those starting to incubate with the last eggs (Peralta-Sánchez *et al.*,
485 2012). Thus, because these species could also vary in hatching success, interspecific
486 variation in incubation behavior could explain the detected intraspecific association
487 between bacteria and hatching success. This possibility is, however, unlikely; first
488 because the detected positive association between bacterial density and hatching success
489 was corrected for the random effect of species identity and, second, because the slope of

490 the association between hatching success and bacterial density was similar in different
491 species. Our results are in any case correlative, and experiments would be necessary for
492 establishing further cause-effect relationships.
493
494 *Interspecific approach*
495 Associations between bacterial density and hatching success have been detected
496 in poultry (Board & Fuller 1994), for one tropical bird species, *Margarops fuscatus*
497 (Cook *et al.*, 2005b), and in a comparative study of European birds (Soler *et al.*, 2012).
498 In the present study, we found that species with the highest eggshell bacterial densities
499 were not those with the lowest rates of hatching success. Moreover, richness and
500 phylogenetic structure of bacterial communities of different species was not
501 significantly related to hatching success. These results may suggest that species
502 experiencing a high risk of bacterial infections (i.e. eggshell bacterial densities) are also
503 those laying eggs with more effective defenses against trans-shell bacterial infection
504 (Soler *et al.*, 2011a), breaking up the expected association. Interspecific variation in
505 number and size of eggshell pores and thickness (Becking, 1975, Ar *et al.*, 1979,
506 Mallory & Weatherhead, 1990, Zimmermann & Hipfner, 2007), in diversity and
507 quantity of antibacterial proteins on the eggshells (Panheleux *et al.*, 1999), cuticle
508 (Wellman-Labadie *et al.*, 2007, 2008a), and albumen of the eggs (Wellman-Labadie *et*
509 *al.*, 2007, Shawkey *et al.*, 2008, Wellman-Labadie *et al.*, 2008b, Hirose *et al.*, 2011),
510 and in diversity and concentration of maternal antibodies in the egg yolk (Hasselquist &
511 Nilsson, 2009) could affect probability of embryo infection (Board & Fuller, 1994). We
512 have not considered these antimicrobial defenses that have to be adjusted to the risk of
513 bacterial infections related to environmental conditions (Cook *et al.*, 2003, Cook *et al.*,
514 2005a, Soler *et al.*, 2011b, Wang *et al.*, 2011), and that might explain the absence of an

515 interspecific correlation between bacterial assemblage and hatching success. Previous
516 evidence supporting such a relationship in Soler *et al.* (2012) did not use hatching
517 success of the nests that were sampled for bacterial density on the eggshell, but
518 collected data from the literature instead. Soler *et al.* (2012) also used a larger list of
519 species sampled in Spain and Denmark. However, other scenarios are also possible. For
520 instance, (i) the subset of species included in our study is not completely representative
521 of bacteria-bird relationships (i.e. only includes altricial species and mainly nesting
522 above ground); (ii) this effect is not that strong in relatively dry environments like the
523 south of Spain (in comparison with tropical areas) (Wang *et al.*, 2011); (iii) or this
524 association is driven by other components of bacterial composition diversity (i.e.
525 functional diversity) or interspecific interactions within the community (for the same
526 Unifrac or richness different effects may be found depending on the
527 antagonistic/mutualistic interactions of certain bacteria).
528
529 *Final Remarks*
530 Even though we failed to detect a negative association between bacterial density
531 on eggshells and hatching success across 17 avian species, we found the predicted
532 association at the intraspecific level, which supports the extended idea that bacteria are
533 important selective agents for wild bird eggs (Deeming, 2002, Cook *et al.*, 2005a, Cook
534 *et al.*, 2005b). Similarly, we found some evidences of the effect of bacterial community
535 composition (Unweighted UniFrac) on hatching success within species, indicating the
536 potential importance of rare bacterial groups in this context. Our findings support the
537 importance of the Rare Biosphere (Pedrós-Alió, 2012) as well as open new avenues to
538 understand their role in bird fitness.
539

540

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557

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764

1 FIGURE CAPTIONS

2

3 Figure 1. Summary plots representing prevalence of bacterial phyla and classes on bird
4 eggshells for 17 avian species (A); prevalence of bacterial phyla on eggshells per bird
5 nest (B); prevalence of bacterial Genera for 17 avian species (C); prevalence of bacterial
6 for 152 clutches of 17 avian species (D). Avian phylogenetic tree was modified from
7 Thuiller et al. (2011).

8

9 Figure 2. Correlation between hatching success (percentage of eggs that hatch in a
10 clutch) and log-transformed of mesophilic bacterial density on eggshells at the
11 beginning of the incubation after correcting for differences among species. Bacterial
12 density was calculated by number of colony forming units that grew in Tryptic Soy
13 Agar plates divided by egg surface (cfu/cm^2). Line represents the trend line ($R^2 = 0.02$).

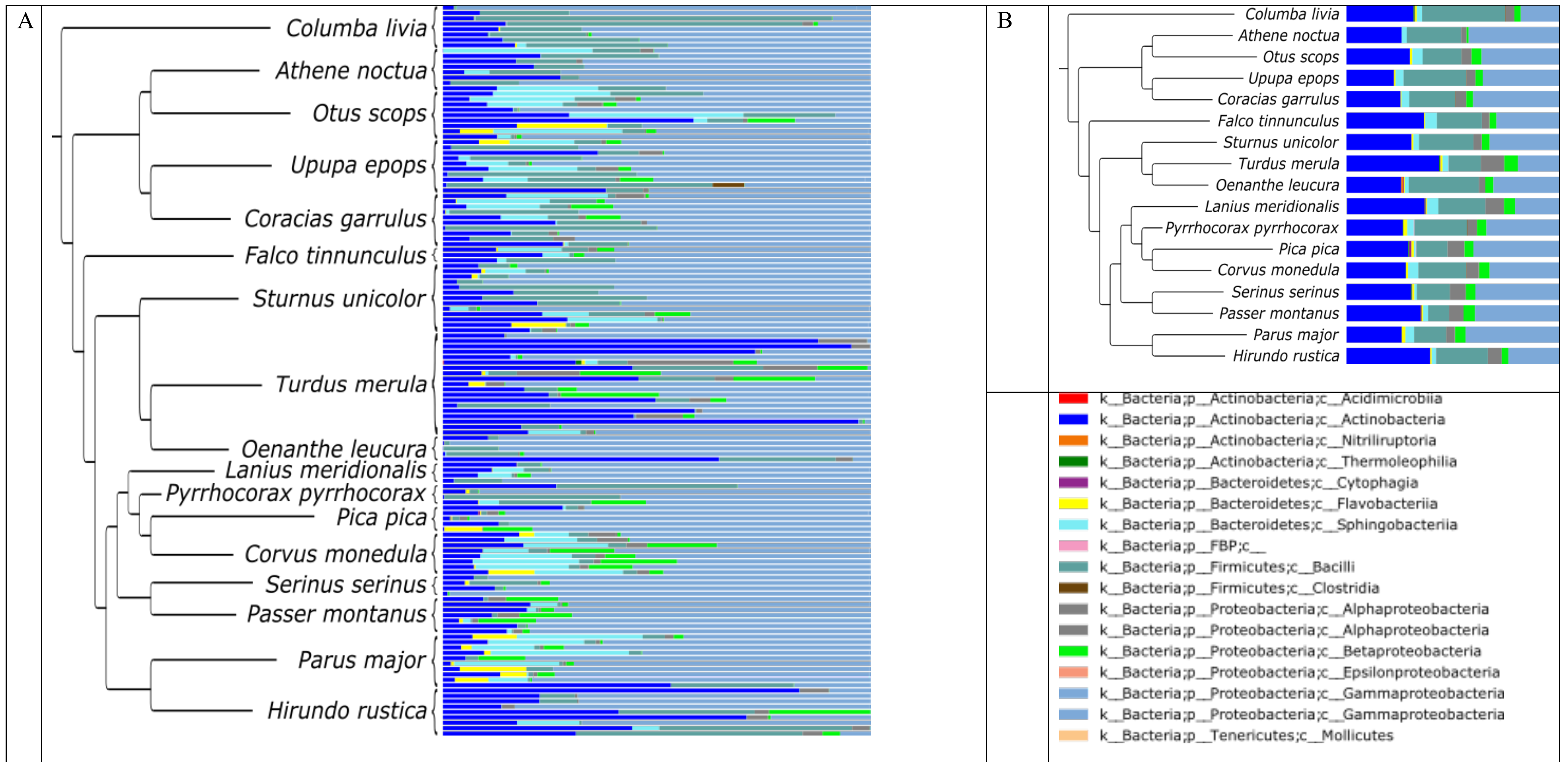
14

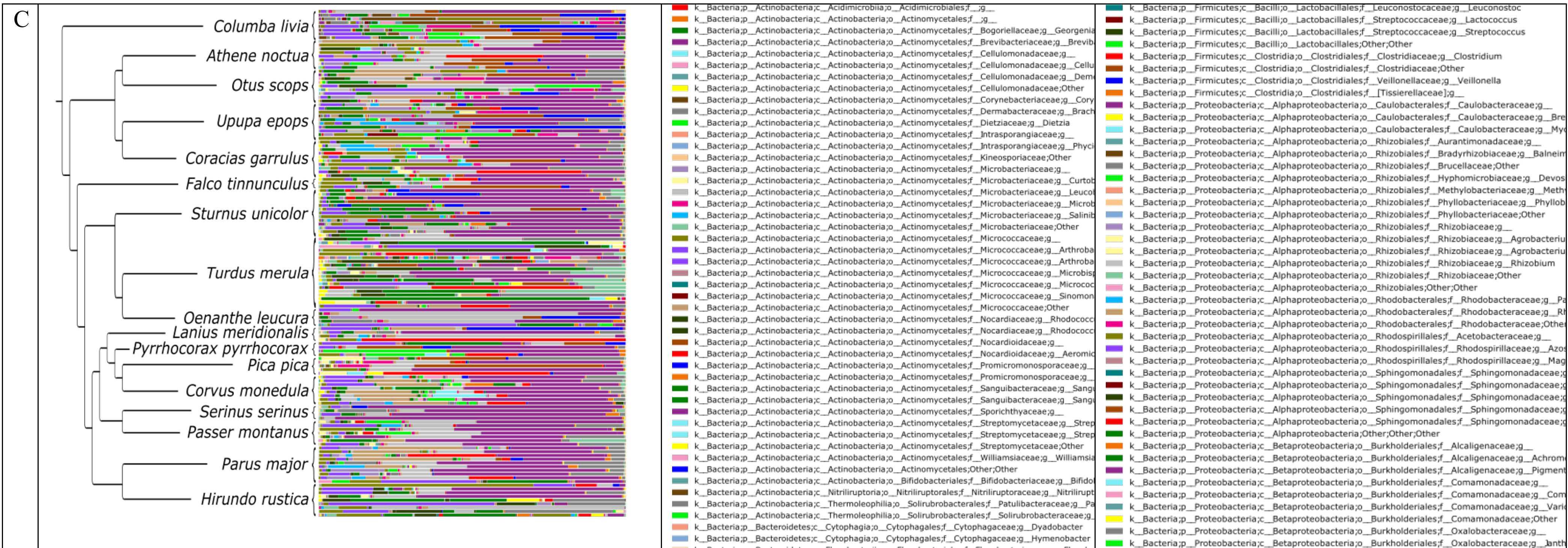
15 Figure 3. 3D plots showing the first three axes of Principal Coordinate Analyses
16 (PCoA) of bacterial community of bird eggshells. Percentages show the proportion of
17 explained variance for each axis. Each dot represents the bacterial community of the
18 complete clutch in a nest. A and D represent bacterial community estimated by Gower
19 distance of ARISA, B and E by means of Unweighted UniFrac distance, and C and F by
20 means of Weighted UniFrac. A, B and C are colored by avian species while D, E and F
21 are colored by hatching success gradient.

22

23 Figure 4. 3D plots showing the first three axes of Principal Coordinate Analyses
24 (PCoA) of bacterial community on eggshells of different species of birds. Percentages
25 show the proportion of explained variance for each axis. Each dot represents the

26 prevalent bacterial community of each avian species. Each OTU prevalence was
27 calculated as the percentage of presence of that OTU in the nest of each avian species.
28 A represents bacterial community estimated by Gower distance of ARISA, B by means
29 of Unweighted UniFrac distance, and C by means of Weighted UniFrac.


A
Columba livia
Athene noctua
Otus scops
Upupa epops
Coracias garrulus
Falco tinnunculus
Sturnus unicolor
Turdus merula
Oenanthe leucura
Lanius meridionalis
Pyrrhocorax pyrrhocorax
Pica pica
Corvus monedula
Serinus serinus
Passer montanus
Parus major
Hirundo rustica
B
Columba livia
Athene noctua
Otus scops
Upupa epops
Coracias garrulus
Falco tinnunculus
Sturnus unicolor
Turdus merula
Oenanthe leucura
Lanius meridionalis
Pyrrhocorax pyrrhocorax
Pica pica
Corvus monedula
Serinus serinus
Passer montanus
Parus major
Hirundo rustica
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia
k__Bacteria;p__Actinobacteria;c__Actinobacteria
k__Bacteria;p__Actinobacteria;c__Nitriliruptoria
k__Bacteria;p__Actinobacteria;c__Thermoleophilia
k__Bacteria;p__Bacteroidetes;c__Cytophagia
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia
k__Bacteria;p__FBP;c__
k__Bacteria;p__Firmicutes;c__Bacilli
k__Bacteria;p__Firmicutes;c__Clostridia
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria
k__Bacteria;p__Tenericutes;c__Mollicutes


C
Columba livia
Athene noctua
Otus scops
Upupa epops
Coracias garrulus
Falco tinnunculus
Sturnus unicolor
Turdus merula
Oenanthe leucura
Lanius meridionalis
Pyrrhocorax pyrrhocorax
Pica pica
Corvus monedula
Serinus serinus
Passer montanus
Parus major
Hirundo rustica

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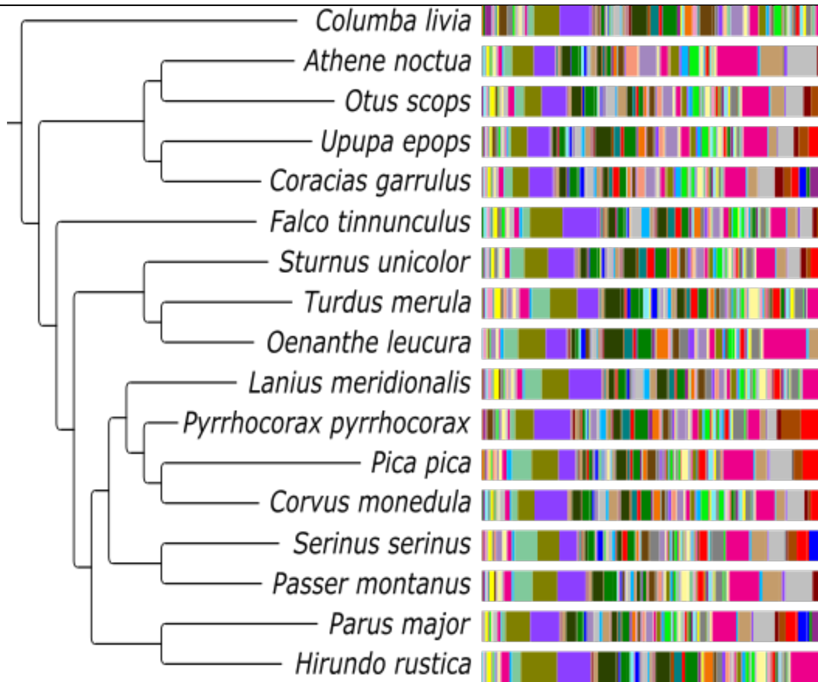
<td>
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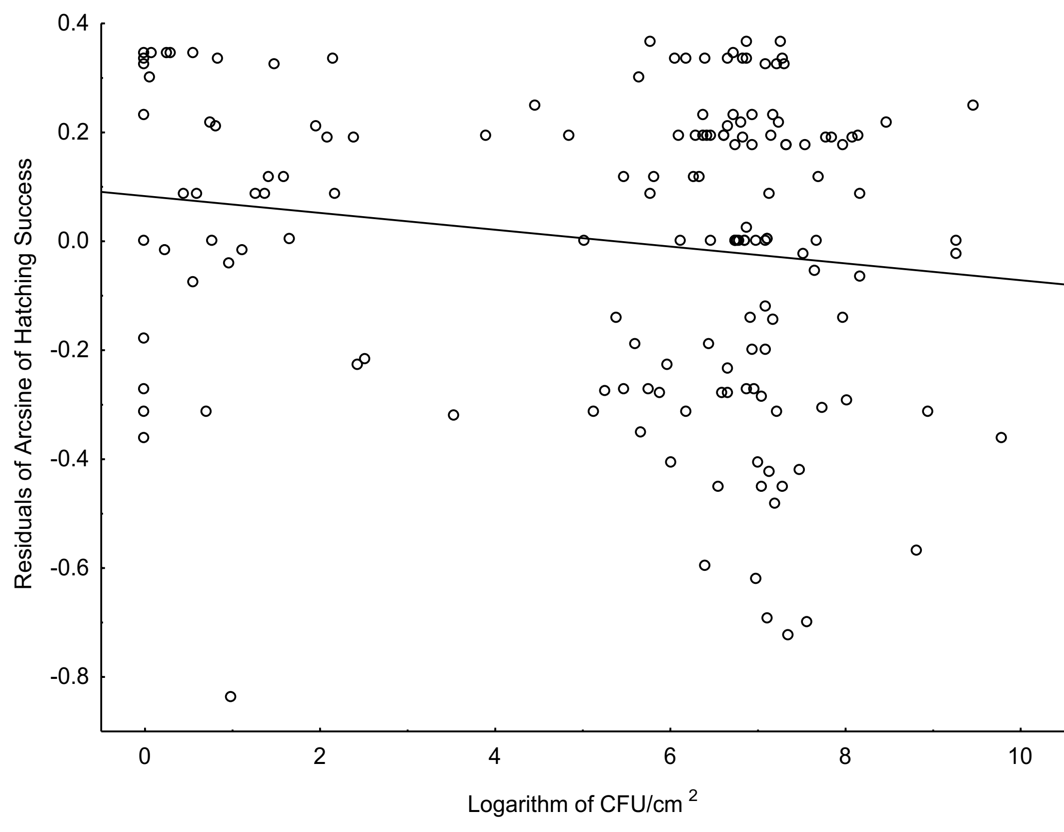

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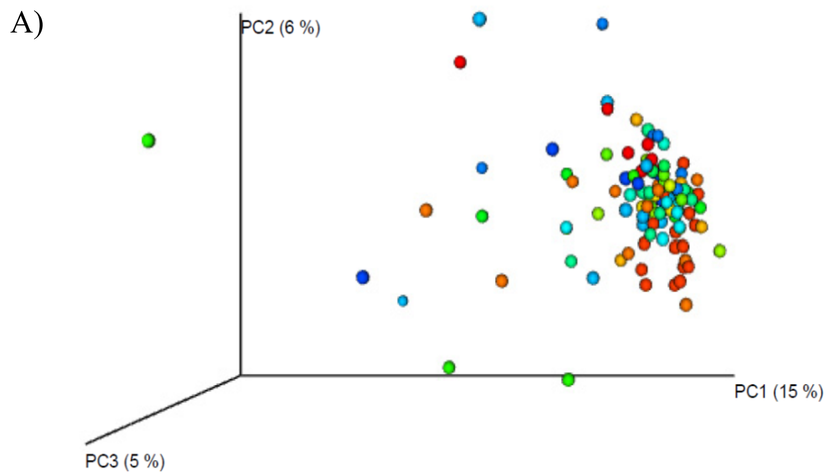
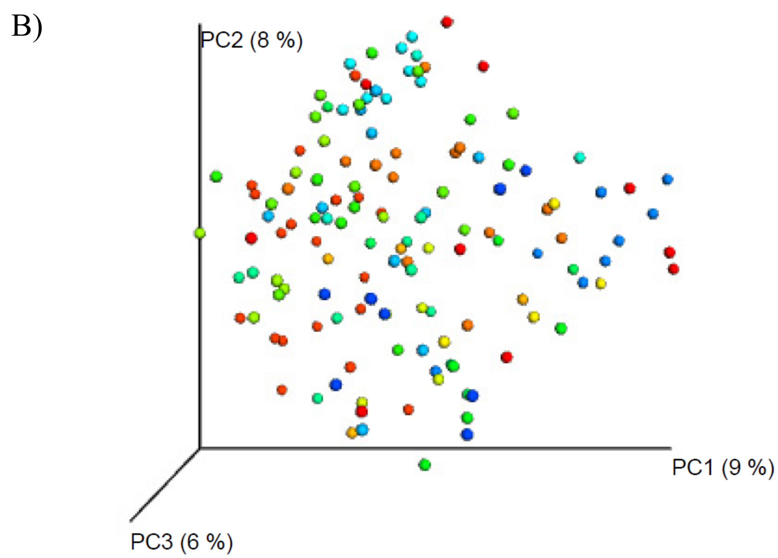
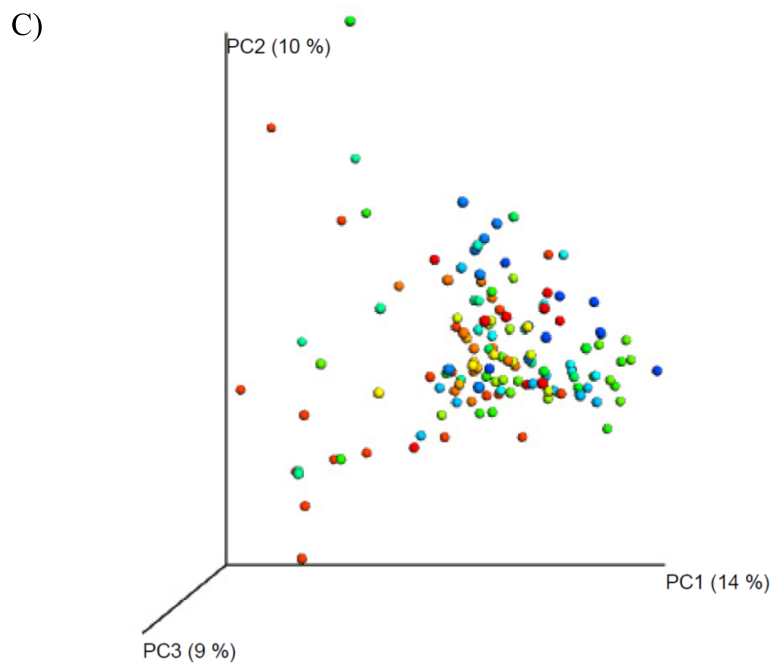
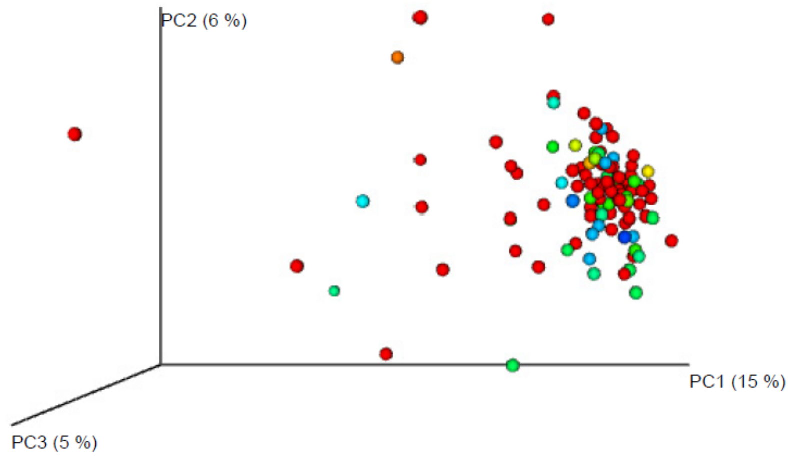

A)
PC2 (6 %)
PC1 (15 %)
PC3 (5 %)

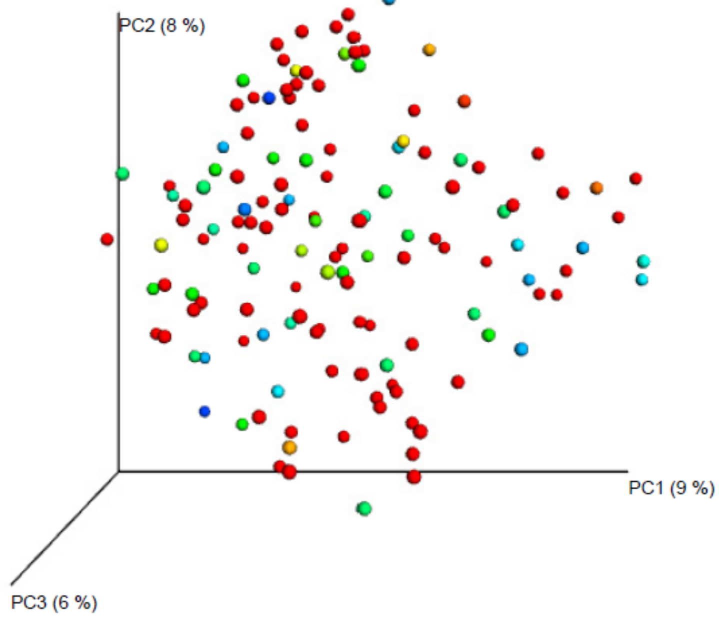

B)
PC2 (8 %)
PC1 (9 %)
PC3 (6 %)
Athene noctua
Columba livia
Coracias garrulus
Corvus monedula
Falco tinnunculus
Hirundo rustica
Lanius meridionalis
Oenanthe leucura
Otus scops
Parus major
Passer montanus
Pica pica
Pyrrhocorax phyrrhocorax
Serinus serinus
Sturnus unicolor
Turdus merula
Upupa epops


C)
PC2 (10 %)
PC1 (14 %)
PC3 (9 %)

D)



E)



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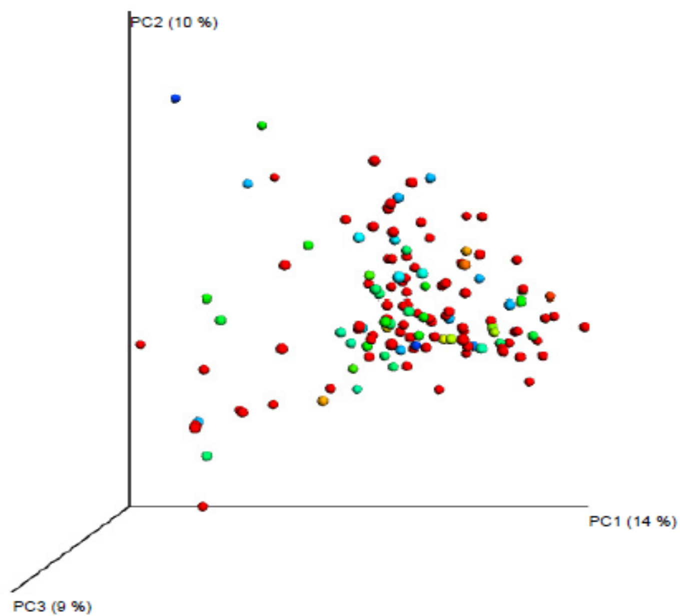
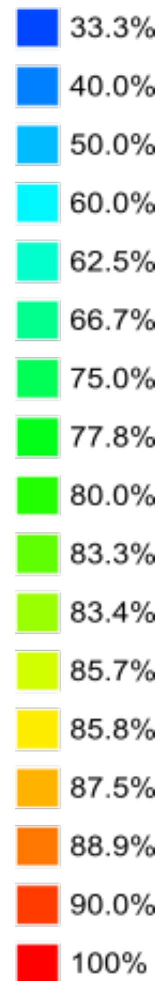
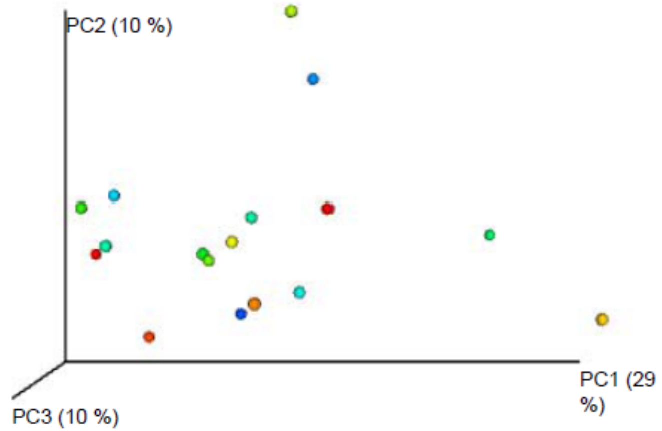
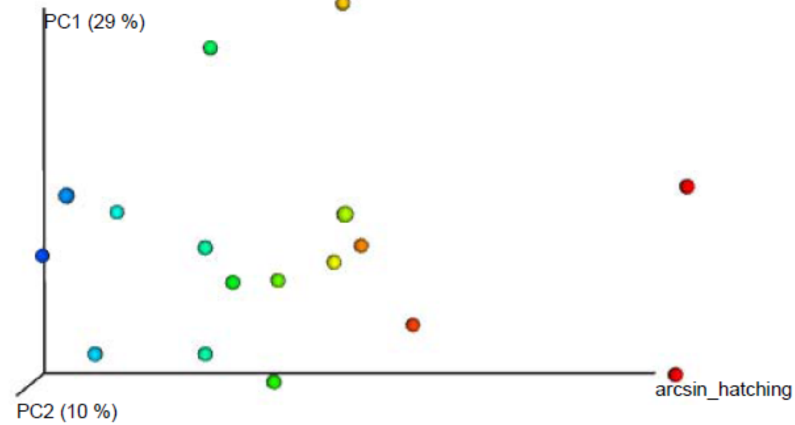


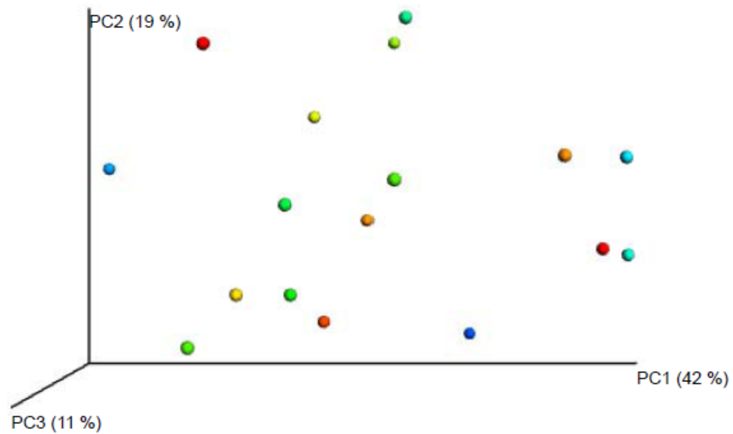


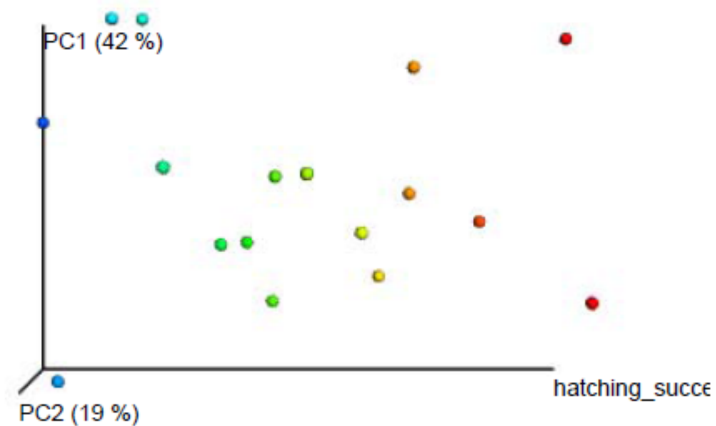
ARISA

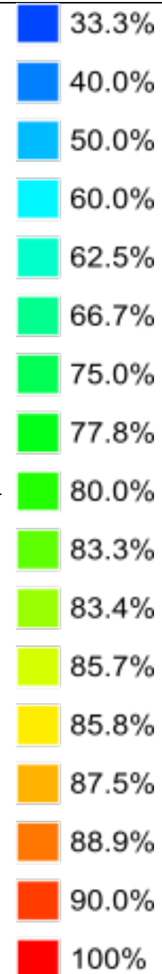




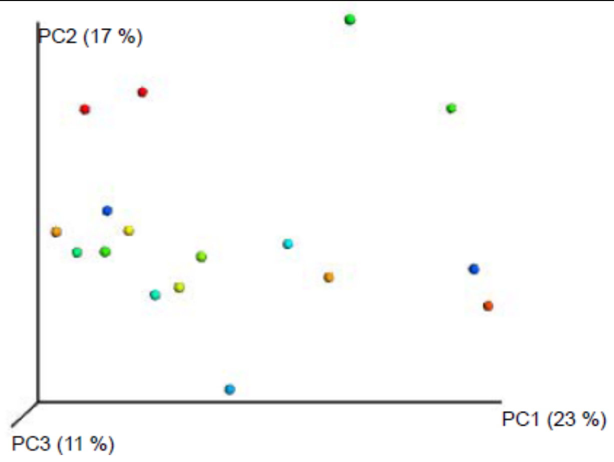
WEIGHTED UNIFRAC







UNWEIGHTED UNIFRAC



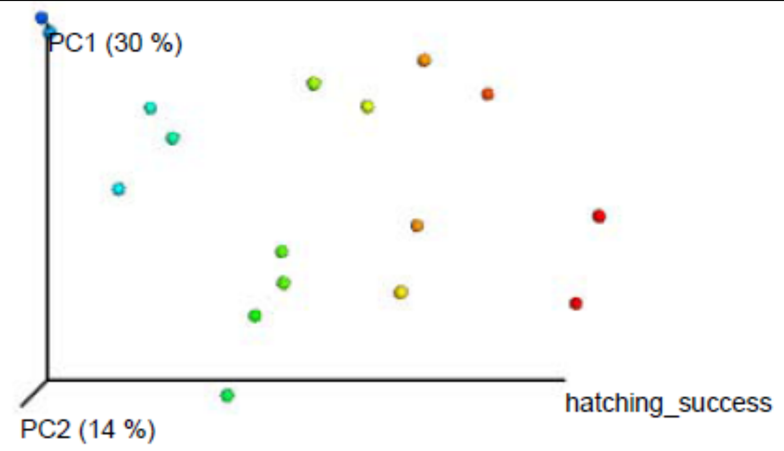


Table 1. Values of arcsin of hatching success (percentage of hatched eggs from the clutch), log-transformed values of the geometric means of bacterial density on the eggshells (log10-tranformed number of colonies corrected for eggshell surface and dilution titer that grew in media for mesophilic bacteria) and OTU richness in ARISA (Automated Ribosomal Intergenic Spacer Analysis) and Hiseq (Highthoughtput sequencing using HiSeq1000 Illumina platform). Sample sizes (N) used in different analyses are also reported.

Bird species	Hatching success	Cultures			ARISA		HiSeq	
		N	Log10 Density	Density (CFUs/cm^2)	N	OTU richness	N	OTU richness
Athene noctua	100.0%	8	6.773	5929253.25	8	87	8	308
Columba livia	99.2%	9	3.836	6854.88	8	90	8	371
Coracias garrulus	97.5%	10	6.287	1936421.96	10	132	10	391
Corvus monedula	94.8%	8	5.961	914113.24	6	61	8	382
Falco tinnunculus	100.0%	3	7.501	31695674.63	3	39	3	303
Hirundo rustica	95.0%	9	5.849	706317.55	8	84	9	349
Lanius meridionalis	91.2%	4	4.996	99083.19	4	16	4	337
Oenanthe leucura	93.7%	6	6.644	4405548.64	5	65	5	264
Otus scops	98.6%	9	5.291	195433.95	9	128	9	384
Parus major	96.4%	10	6.602	3999447.50	9	82	10	386
Passer montanus	95.2%	7	6.085	1216186.00	7	63	7	330
Pica pica	92.0%	15	1.128	13.43	2	11	5	306
Pyrrhocorax pyrrhocorax	90.4%	4	5.428	267916.83	4	39	4	271
Serinus serinus	95.7%	5	4.913	81846.48	5	70	4	292
Sturnus unicolor	91.3%	13	5.799	629506.18	12	105	13	434
Turdus merula	89.2%	19	5.662	459198.01	17	112	19	469
Upupa epops	82.3%	13	6.625	4216965.03	8	80	10	404

Table 2. Statistical analyses performed in order to explore the effects of bird species identity and bacterial density (log-transformed), OTU richness and bacterial assemblages on hatching success (arcsine transformed). Bacterial density on eggshells was estimated as the number of colony forming units per cm^2 of mesophilic bacteria and General Linear Mixed Models (GLMM) was applied. OTU richness was estimated as the number of OTUs in ARISA and HiSeq. OTU richness from ARISA approach was log-transformed in order to reach normality. Bacterial assemblages were estimated by means of Automatic Ribosomal Intergenic Spacer Analysis (ARISA) or by means of highthroughput sequencing (Illumina HiSeq platform). For comparison of bacterial assemblages, Pearson similarity index was applied for ARISA data, and Weighted and Unweighted UniFrac distances for HiSeq data and Procrustes ANOVAs (PA) were performed.

Approach	Statistical Method	Predictors	F	d.f.	P
Cultures	GLMM	Bird species identity	1.10	16,134	0.103
		Bacterial density	5.29	1,134	**0.023**
ARISA	GLMM	Bird species identity	1.27	16,107	0.219
		OTUs richness	2.37	1,107	0.126
	PA	Bird species identity	1.23	16,46	0.298
		Bacterial assemblage (GOWER)	1.09	62,46	0.070
HiSeq	GLMM	Bird species identity	1.20	16,118	0.282
		OTUs richness	0.11	1,118	0.736
	PA	Bird species identity	1.80	16,15	0.273
		Bacterial assemblage (Unweighted UniFrac)	2.07	104,15	**<0.001**
	PA	Bird species identity	1.03	16,50	0.273
		Bacterial assemblage (Weighted UniFrac)	0.76	69,50	0.488

Table 3. Phylogenetic General Least Square (pGLS) analyses evaluating the effects bacterial density or community on bird eggshells in hatching success in a comparative approach. Bacterial density was estimated as the geometric mean of colony forming units per cm^2 per bird species. Bacterial assemblages for each bird species was calculated as the prevalence of every OTU in each bird species (ratio of an OUT is present in an avian species), for both Automatic Ribosomal Intergenic Spacer Analysis (ARISA) and high-throughput sequencing (Illumina HiSeq platform). Pearson similarity index was applied to ARISA for calculating the distance matrix and Weighted and Unweighted UniFrac distance from HiSeq.

Approach	Variable/matrix	R^2 adjusted	F	d.f.	P
Cultures	Bacterial density	-0.02	0.76	1,16	0.461
ARISA	Gower distance matrix	0.07	1.07	1,16	0.397
HiSeq	Unweighted UniFrac distance matrix	0.10	1.64	1,16	0.475
	Weighted UniFrac distance matrix	0.05	0.83	1,16	0.583