Factors shaping the gut microbiome of five species of lizards from different habitats

6   **Diana S. Vasconcelos**[1,2,3]**, D. James Harris**[1,3]**, Isabel Damas-Moreira**[4]**, Ana**
7   **Pereira**[1,2,3]**, Raquel Xavier**[1,3]

9   **1** CIBIO - Centro de Investigação em Biodiversidade e Recursos
10 Genéticos, *InBIO* Laboratório Associado, Campus de Vairão da Universidade do Porto,
11 Vairão, Portugal
12 **2** Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Porto,
13 Portugal
14 **3** BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO - Campus de
15 Vairão, Vairão, Portugal

16 **4** Department of Behavioural Ecology, Bielefeld University, Germany

18 **Corresponding authors:**

19 Raquel Xavier
20 Rua Padre Armando Quintas nº 7, Vairão, 4485-661, Portugal
21 Email address: raq.xavier@cibio.up.pt

22 Diana S. Vasconcelos
23 Rua Padre Armando Quintas nº 7, Vairão, 4485-661, Portugal
24 Email address: dianasv@cibio.up.pt

Abstract

Background. Host-gut microbiota interactions are complex and can have a profound impact on the ecology and evolution of both counterparts. Several host traits such as systematics, diet and social behavior, and external factors such as prey availability and local environment are known to influence the composition and diversity of the gut microbiota.

Methods. In this study, we investigate the influence of systematics, sex, host size, and locality/habitat on gut microbiota diversity in five lizard species from two different sites in Portugal: *Podarcis bocagei* and *Podarcis lusitanicus*, living in syntopy in a rural area in northern Portugal (Moledo); the invasive *Podarcis siculus* and the native *Podarcis virescens*, living in sympatry in an urbanized environment (Lisbon); and the invasive *Teira dugesii* also living in an urban area (Lisbon). We also infer the potential microbial transmission occurring between species living in sympatry and syntopy. To achieve these goals, we use a metabarcoding approach to characterize the bacterial communities from the cloaca of lizards, sequencing the V4 region of the 16S rRNA.

Results. Habitat/locality was an important factor explaining differences in gut bacterial composition and structure, with species from urbanized environments having higher bacterial diversity. Host systematics (i.e., species) influenced gut bacterial community structure only in lizards from the urbanized environment. We also detected a significant positive correlation between lizard size and gut bacterial alpha-diversity in the invasive species *P. siculus*, which could be due to its higher exploratory behavior. Moreover, estimates of bacterial transmission indicate that *P. siculus* may have acquired a high proportion of local microbiota after its introduction. These findings confirm that a diverse array of host and environmental factors can influence lizards' gut microbiota.

63
64
65
66 **Introduction**
67

68 A myriad of microorganisms can be found living in the gastrointestinal tract of all animals.
69 Studies on gut microbiome dynamics have shown that gut microbial assemblages are
70 composed by host-adapted 'core' microbial taxa as well as a mixture of transient microbes
71 (Shapira, 2016). These microbial commensals are known to have a significant impact on
72 host biology, influencing a variety of processes that affect host fitness (Shapira, 2016;
73 Cryan & Dinan, 2012; Thaiss et al., 2016), with the hologenome theory of evolution being
74 recently proposed, where selection acts upon host genome and its associated
75 microbiome as a single evolutionary unit (Zilber-Rosenberg & Rosenberg, 2008). Gut
76 microbiome is known to be involved in disease resistance, besides being important for
77 xenobiotics metabolism, nutrient uptake and energy acquisition (e.g., Vavre & Kremer,
78 2014; Rowland et al., 2018). Moreover, gut microbiota may also contribute towards host
79 adaptation to environment changes by enabling a response to new challenges, such as
80 exploitation of novel food sources (Delsuc et al., 2014; Hammer & Bowers, 2015).
81 Ultimately, gut microbiome may have a major impact on host development, behaviour and
82 fitness, with cascading effects to the dynamics of ecosystems (Thaiss et al., 2016, Cusick
83 et al., 2021, Jia et al., 2021, Chiang et al., 2022). In turn, gut microbial communities may
84 also be modulated by various host traits, such as host evolutionary history, sex and size
85 (Youngblut et al., 2019; Song et al., 202; Bunker, Arnold & Weiss, 2022). External
86 environment, such as habitat or prey availability also affect the gut microbiome of
87 vertebrate species (e.g., Muegge et al., 2011; Xavier et al., 2019; Montoya-Ciriaco et al.,
88 2020; Jin et al., 2021). In addition, social interactions between hosts can also influence
89 the gut microbiome in many animal species, although these mechanisms remain less
90 studied (see review by Archie & Tung, 2015).
91   Gut microbiome studies have been performed in many mammals (e.g., Thaiss et
92 al., 2016), birds (e.g., Hird et al., 2015), fishes (e.g., Xavier et al., 2020) and amphibians
93 (e.g., Bletz et al., 2016). Comparatively, only a few have been performed in reptiles, and
94 only a handful of these addressed lizards. Nevertheless, studies showed that maternal

95   transmission of gut microbiota to offspring can occur in squamate reptiles (Kohl et al.,
96   2017). Additionally, microbiota can be acquired by reptiles through horizontal
97   transmission from the environment or through interaction with other organisms (e.g.
98   predatory encounters, Kohl et al., 2017). Host systematics, and ecology were also seen
99   to be important drivers of gut microbiota diversity in reptiles (e.g. Hong et al., 2011; Smith,
100 Colston & Siler, 2021). For example, feeding habits influence the gut microbiota of the
101 Chinese crocodile lizard, *Shinisaurus crocodilurus* Ahl 1930, with potential effects on host
102 health due to the influence of diet on the abundances of pathogenic or opportunistic gut
103 bacteria (Jiang at al., 2017). Trophic niche was also reported to be an important
104 determinant of the gut bacterial community structure, with differences found between
105 herbivorous and omnivorous lizard species and specific bacterial taxa being linked to
106 adaptation to herbivory (Kohl et al., 2017). Diet and habitat of the Australian water dragon,
107 *Intellagama lesueurii* (Gray, 1831), also had an effect on its gut microbiome, with lizards
108 living in urban areas presenting higher bacterial diversity than populations living in natural
109 habitats (Littleford-Colquhoun et al., 2019). Other habitat characteristics were also seen
110 to influence lizard microbiome. For example, altitude was an important factor explaining
111 community composition and structure of the gut microbiota of the lizard *Phrynocephalus*
112 *vlangalii* (Zhang et al., 2018) and temperature changes also altered the composition of
113 the common lizard (*Zootoca vivipara*) gut microbiome, with warming temperatures
114 resulting in a decrease in diversity and an increase in pathogenic bacteria (Bestion et al.,
115 2017).

116   Here, we analyze and compare the diversity, composition and structure of gut
117 bacterial communities of five related lacertid species captured in Portugal. Individuals of
118 *Podarcis bocagei* (Lopez-Seoane, 1885) and *P. lusitanicus* Geniez, Sá-Sousa,
119 Guillaume, Cluchier and Crochet, 2014, were sampled in Moledo (North of Portugal)
120 where they live in syntopy. Invasive *P. siculus* (Rafinesque-Schmaltz, 1810) and native
121 *P. virescens* Geniez, Sá-Sousa, Guillaume, Cluchier and Crochet, 2014, were sampled
122 from Parque das Nações (Lisbon) where they live in sympatry. Finally, a population of the
123 introduced *Teira dugesii* (Milne-Edwards, 1829) was sampled in the Alcantara Docks in
124 Lisbon. All five species exhibit sexual dimorphism, with males usually being larger than
125 females. They are mostly insectivorous (Geniez et al., 2014; Carretero, Galán & Salvador,

126 2015), although *P. siculus* and *T. dugesii* may also occasionally consume some fruits or

127 flowers (Mačát, Veselý & Jablonski, 2015). *Podarcis* species are considered model

128 organisms to study ecotoxicology, immune/histochemical reactions, among other

129 processes (e.g., Bicho et al., 2013; Luís et al., 2019); however, microbiome studies are

130 still largely lacking, with only three studies available to date. Two studies investigated two

131 species endemic to the Balearic Islands (Spain), *P. lilfordi* (Günther, 1874) and *P.*

132 *pityusensis* (Bosca, 1883), with results indicating that islet, time since separation from

133 mainland, and seasonality are significant factors contributing to their gut microbiome

134 (Baldo et al., 2018, Alemany et al., 2022). Another recent study compared the gut

135 microbiota of two Italian populations of *P. siculus* (mainland vs island) demonstrating that

136 there were considerable differences between the two (Buglione et al., 2022).

137   Our main objective was to determine whether locality, which also corresponded to

138 two different habitats (rural vs urbanized) and host factors such as species, size, and sex,

139 modulate the gut bacterial diversity of these five lizards. To achieve this, we used cloacal

140 swabs to obtain a proxy for gut bacterial communities, which were characterized by

141 sequencing the V4 region of the 16S rRNA gene. Swabs were preferred to fecal samples

142 as these more accurately reflect microbial communities residing in lower gut and cloacal

143 tissues (Bunker, Martin & Weiss, 2022).

144

145

146 **Methods**

147 **Sample collection:**

148   A total of 103 adult lizards from five different species were sampled in September

149 2020: *Podarcis bocagei* (males = 22; females = 9), *P. lusitanicus* (males = 6; females =

150 2), *P. siculus* (males = 13; females = 6), *P. virescens* (males = 16; females = 6), and *Teira*

151 *dugesii* (males = 7; females = 13) (supplementary table 1). These lacertid species are

152 small-sized, with captured individuals measuring on average 6.96 cm snout-vent length

153 (SVL) in *P. siculus*, 5.55 cm in *P. virescens*, 5.03 cm in *P. bocagei*, 4.65 cm in *P.*

154 *lusitanicus*, and 6.06 cm in the introduced species *T. dugesii* (supplementary table 1).

155 *Podarcis bocagei* and *P. lusitanicus* were collected on 28-29th of September 2020 from a

156 semi-natural habitat in Moledo, northern Portugal (Fig. 1A) (41°50'19.2"N 8°52'24.5"W),

157 where they live in syntopy (i.e., occurrence of two species in the same habitat at the same
158 time). This location has limited human disturbance, just a few meters from the beach and
159 has extensive vegetation with natural and artificial shelters (e.g., walls of agricultural
160 properties) that can be used by lizards. Ecological adaptation is considered a major factor
161 favoring the isolation between these two species; *P. lusitanicus* lives more on rocks, while
162 *P. bocagei* is ground-dwelling (Carretero, Galán & Salvador, 2015). The diet of these two
163 species is mainly composed by prey belonging to Hemiptera, Coleoptera, Diptera,
164 Hymenoptera and Araneae, with minimal differences between species or sexes
165 (Kaliontzopoulou et al., 2011). *Podarcis siculus* and *P. virescens* were collected on 15-
166 16th of September 2020 in Lisbon, at Parque das Nações (Fig. 1B$_1$, B$_2$) (38°45'43.6"N
167 9°05'40.2"W; 38°46'10.6"N 9°05'31.5"W), where both live in sympatry (sharing habitat
168 type). This is a highly urbanized area near the Tejo River, characterized by large
169 residential and commercial areas, with considerable daily human disturbance. While *P.*
170 *virescens* is native to this location, *P. siculus* is native to the Italian Peninsula, east
171 Adriatic coast and many nearby islands, being invasive elsewhere, including in Lisbon
172 where it was likely introduced about 25 years ago (González de la Veja et al., 2001; Silva-
173 Rocha et al., 2012). Its plasticity in spatial use of habitat, morphology, behaviour, and diet
174 explains its successful colonization of multiple locations outside its native range (Vervust
175 et al., 2010; Carretero & Silva-Rocha, 2015; Damas-Moreira et al., 2019; 2020). This
176 invasive species can present a more versatile diet, as it can also consume fruits and
177 nectar (Mačát, Veselý & Jablonski, 2015; Vervust et al., 2010), while *P. virescens* is
178 known to be insectivorous and to feed mainly on individuals of the class Arachnida and
179 the orders Hymenoptera, Hemiptera, Coleoptera, and Diptera (Juan, 1997). Finally, we
180 collected *T. dugesii* in a nearby area in Lisbon on the 16th of September 2020, in the
181 Alcantara docks, close to the city port area (Fig. 1C) (38°42'12.0"N 9°09'55.9"W). Similar
182 to the other *Podarcis* spp. captured in Lisbon, *T. dugesii* occupies an anthropogenic area,
183 although less busy, close to railway tracks with limited vegetation cover (Fig. 1C). This
184 species was likely introduced by accident via transport ships from Madeira Island three
185 decades ago, in 1992 (Sá-Sousa, 1995). *Teira dugesii* feeds preferentially on insects but
186 also on small fruits (Sadek, 1981). The average monthly temperature and precipitation in
187 September 2020 was 20–18 °C and 50–100 mm, respectively, in Moledo and 20–22 °C

188   and 25–100 mm in Lisbon (Portuguese Institute of Sea and Atmosphere (IPMA).

189   https://www.ipma.pt/resources.www/docs/im.publicacoes/edicoes.online/20201007/HM

190   KBOLtTSGDcATYMcrNg/cli_20200901_20200930_pcl_mm_co_pt.pdf)).

191

192   Experimental protocols and research were approved by the Portuguese Institute

193   for Conservation of Nature and Forests (ICNF) (License 703/2021/CAPT). No animal

194   experimentation was performed in this study, and all samples were collected non-

195   invasively using sterile cotton swabs (Dryswabs™, Medical Wire and Equipment, UK). All

196   individuals were captured using nooses. Lizards were carefully immobilized, avoiding any

197   human contact with the cloaca. We quickly inserted a sterile cotton swab into the entrance

198   of the cloaca to obtain individual microbial samples. The tips of the swabs were cut into

199   individual tubes and stored immediately in ice boxes in the field, and frozen at -20°C within

200   a maximum of 5 hours after sampling. After finishing the field work, swabs were taken to

201   the laboratory for storage at -80°C until processing. After microbial sampling, each lizard

202   was sexed, and the snout-vent length was measured (SVL; from head to cloaca) using a

203   digital caliper (± 0.01 mm error). No animals died or were euthanized during sampling and

204   all animals were released unharmed at the place of collection.

205

206   **DNA extraction and sequencing:**

207   In the laboratory, DNA was extracted from the swabs using the DNeasy®

208   PowerSoil® Kit (QIAGEN, Hilden, Germany) according to the manufacturer's

209   instructions. DNA concentration and quality were measured with the Epoch™ Microplate

210   Spectrophotometer (BioTek Instruments, Inc.; United States of America). DNA was

211   shipped in dry ice to the Centre for Microbial Systems at the University of Michigan

212   Medical School (USA) where the V4 region of the 16S rRNA gene (~ 250 bp) of the

213   bacterial communities was amplified for each sample, along with the extraction blanks

214   and PCR controls using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and

215   806R (5′-GGACTACHVGGGTWTCTAAT−3′) and following the protocol of Kozich et al.

216   2013. The V4 region of this gene is widely used to characterize bacterial communities in

217   various taxa, including reptiles (e.g., Colston and Jackson, 2016; Chiarello et al., 2018).

218   Amplicons were sequenced in a single Illumina MiSeq run using a MiSeq Reagent Kit V2

219   500 cycles. Raw sequence reads were deposited into NCBI's Short Read Archive

220   (https://dataview.ncbi.nlm.nih.gov/object/PRJNA895230?reviewer=f6k2t8jfl1b9ig8i4n73tf

221   tp3u) under project PRJNA895230.

222

223   **Sequence and statistical analysis:**

224   All analyses were performed using the R Software v.4.1.1 (R Core Team, 2020).

225   Raw FASTQ files were denoised using the DADA2 pipeline (Callahan et al., 2016). After

226   an assessment of read quality plots, the parameters for trimming and filtering were set

227   as: trimLeft = 20, truncLen = c(220, 200), maxN = 0, maxEE = c(2, 2), truncQ = 2. The

228   SILVA 138 database (Pruesse et al., 2007; Quast et al., 2013) was chosen for taxonomic

229   assignment. After quality control and taxonomic assignment, sequences identified as

230   Archaea, Eukaryota, Mitochondria, Chloroplast, as well as sequences unassigned to

231   Bacteria, were removed from the dataset. ASVs present in control samples were also

232   removed from downstream analysis. An Amplicon Sequence Variant (ASV) frequency

233   table was constructed using the R package *phyloseq* (McMurdie & Holmes, 2013).

234   Normalized read counts were obtained using the negative binomial distribution

235   implemented in DESeq2 (Love, Huber & Anders, 2014; McMurdie and Holmes, 2014).

236   We removed ASVs that concomitantly met the two following criteria: had a count of less

237   than 0.001% of the total number of reads (3,586,752 [total number of reads] x 0.001% =

238   36) and that were also present in a single sample (supplementary table 2). The

239   composition and abundance of taxa in the mock community were similar to those

240   described by the manufacturer.

241   Bacterial diversity (alpha-diversity, calculated intra-sample) and structure (beta-

242   diversity, calculated as the dissimilarity or distance between pairs of samples) were

243   estimated using the *phyloseq* and the *picante* packages (McMurdie & Holmes, 2013;

244   Kembel et al., 2010) (see R script provided as supplementary materials). Alpha-diversity

245   was estimated using the number of observed ASVs, the Shannon index, and Faith's

246   Phylogenetic Diversity (PD). Beta-diversity was measured using the Bray-Curtis index

247   and the Unifrac phylogenetic weighted and unweighted distances. Principal Coordinate

248   Analysis (PCoA) were used to visually assess dissimilarity among groups.

249   Statistical differences in alpha-diversity between localities were assessed using
250   species as a random factor using a linear mixed effects model (lmer(alpha-diversity ~
251   locality + (1|species)) with the package *lme4* (Bates et al., 2014). Given the significant
252   effect of locality on alpha-diversity (see results section), differences in alpha-diversity
253   among species and between sexes were further assessed using another linear mixed
254   effects model with locality as a random factor (lmer(alpha-diversity ~ species +
255   species/sex + (1|locality)). The effects of locality and species on microbial beta-diversity
256   were assessed using a permutational analysis of variance (PERMANOVA) with 9,999
257   permutations, with the *adonis2* function of the R *vegan* package (Oksanen et al., 2013),
258   using the formula (adonis2(beta-diversity ~ locality + species)). Since both locality and
259   species significantly affected beta-diversity, the pairwise effects of species and sex were
260   tested for each locality separately using the *pairwise.adonis2* function (Arbizu, 2020)
261   using the model (pairwise.adonis2(beta-diversity ~ species + species/sex)). P-values for
262   multiple comparisons were adjusted with the Bonferroni correction. Differences in the
263   proportions of the most abundant taxa at the phyla and genera levels (represented by
264   ≥ 3% on average of all sequences) were assessed between species and sex for each
265   locality separately using a linear model (lm(bacterial taxa ~ species + species/sex)).
266   Correlations between individual size and bacterial alpha-diversity were also tested using
267   the Pearson correlation test for each species, using the *ggpubr* package (Kassambara &
268   Kassambara, 2020) (see supplementary table 1).

269   To further understand the levels of similarity between sympatric and syntopic
270   species, bi-directional bacterial transmission between each pair of species from Moledo
271   and Parque das Nações was estimated using the FEAST software (Shenhav et al., 2019),
272   by testing the contribution of each species (source) to the microbial diversity to its
273   sympatric congener (sink). To this end, the non-normalized ASV frequency table was
274   used and, due to differences in the number of samples between *P. bocagei* and *P.*
275   *lusitanicus,* only a fraction of the individuals of *P. bocagei* were included (the ones with
276   the most similar sex and SVL ratios to the *P. lusitanicus* samples as possible, with
277   included *P. bocagei* individuals having a mean SVL of 4.6 cm vs *P. lusitanicus* with a
278   mean SVL of 4.7 cm), following the FEAST developers' recommendations to avoid
279   overestimation of transmission.

280

281

282 **Results**

283   After filtering, the final ASV table encompassed 3,923 unique ASVs, included in

284 a total of 39 bacteria phyla. The most abundant phyla among the species studied were

285 Firmicutes, Bacteroidota, Actinobacteroidota, Proteobacteroidota and Campylobacterota.

286   Gut bacterial diversity, measured through alpha-diversity indices, was significantly

287 different between localities considering the number of Observed ASVs and PD indices

288 (Observed ASVs: F-statistics = 39.74, DF = 1, p = 0.02; PD: F-statistics = 51.38, DF = 1,

289 p = 0.02), with species from Lisbon showing consistently higher alpha-diversity indices

290 than the ones from Moledo, (Fig. 2). No differences between localities were found with

291 the Shannon index (F-statistics = 8.33, DF = 1, p = 0.07). Moreover, neither species or

292 sex had a significant effect on microbial alpha-diversity (F > 1.03, DF = 4, p > 0.11 and F

293 > 0.57, DF = 5, p > 0.16, respectively), but *P. siculus* had higher diversity than the native

294 *P. virescens*. Microbial structure, measured through beta-diversity indices, was

295 significantly different between localities (Bray-Curtis: R^2 = 0.07, DF = 1, p = 0.0001;

296 Weighted Unifrac: R^2 = 0.03, DF = 1, p = 0.04; Unweighted Unifrac: R^2 = 0.08, DF = 1, p

297 = 0.0001) and species (Bray-Curtis: R^2 = 0.12, DF = 3, p = 0.0001 and the Weighted

298 Unifrac: R^2 = 0.08, DF = 3, p = 0.03; Unweighted Unifrac: R^2 = 0.11, DF = 3, p = 0.0001)

299 (Fig. 3). In general, pairwise differences in beta-diversity between species were found in

300 species collected from Lisbon (Table 1), while no differences were found between sexes.

301 In samples collected in Moledo, no differences were found in beta-diversity between

302 species or sexes.

303   Although no differences were found in the proportion of the most abundant phyla

304 between species or sexes, some differences were observed when considering the most

305 abundant genera (Fig. 4). In the case of species in Moledo, sex influenced the proportion

306 of the genus *Corynebacterium* (F-statistics = 4.46, DF = 3, p = 0.02) (Table 2, Fig. 5).

307 Moreover, differences between *P. siculus* and *P. virescens* were also found for genus

308 *Corynebacterium* (F-statistics = 6.66, DF = 2, p = 0.003) and for *Odoribacter* (F-statistics

309 = 10.10, DF = 2, p = 0.0002) (Table 2).

310   Pearson correlation test only showed significantly positive correlations
311 between SVL and bacterial alpha-diversity (for Shannon index, R = 0.58, p = 0.04) for
312 males of the invasive species *P. siculus* (Fig. 6). For all other species, R values varied
313 from -0.082-0.68 with p > 0.05. It is important to note that the significant positive
314 correlation of males of *P. siculus* was reversed when the smallest sized individual (59
315 mm) was removed from the dataset (R = -0.62, p = 0.04).

316   Results from FEAST software indicated that the level of bacterial transmission
317 between sympatric species in both populations (Parque das Nações and Moledo) was
318 high. Nevertheless, while bacterial transmission was balanced in both directions between
319 the syntopic *P. lusitanicus* and *P. bocagei* (estimated transmission from *P. bocagei*
320 towards *P. lusitanicus* was ~ 71% on average, and from *P. lusitanicus* towards *P. bocagei*
321 was ~ 69% on average), between the two sympatric species in Lisbon there was a more
322 biased transmission, with *P. virescens* seemingly having a higher contribution towards *P.*
323 *siculus* gut microbiota (transmission estimates from *P. virescens* towards *P. siculus* was
324 ~ 72% on average, and from *P. siculus* towards *P. virescens* it was ~ 55% on average).

325

326

Discussion

328   In this study, we characterized the gut bacterial microbiota of five lizard species
329 from Portugal (the native *Podarcis virescens*, *P. bocagei* and *P. lusitanicus,* and the
330 introduced *P. siculus* and *Teira dugesii*) using a metabarcoding approach. The most
331 abundant phyla found in the lizard species studied herein were Firmicutes, Bacteroidota,
332 Actinobacteroidota, Proteobacteroidota, and Campylobacterota, with these results being
333 in agreement with what has been found in other studies in lizards using cloaca swabs
334 (Jiang et al., 2017; Bunker, Martin & Weiss, 2022). Likewise, some of the most abundant
335 genera found in the present study (Table 2) have also been shown to be highly abundant
336 in the gut of other lizard species (e.g., *Bacteroides*, *Odoribacter*, and *Parabacteroides*,
337 Zhang et al., 2018; Montoya-Ciriaco et al., 2020). It is noteworthy that the gut microbiota
338 of individuals of *P. siculus* sequenced herein and those from Italy by Buglione et al. (2022)
339 share the most abundant bacterial phyla but differ at the level of the most represented
340 bacterial genera.

341   Our results showed that locality was a major predictor of microbial diversity and
342   structure. Plausibly, differences in habitat may lead to differences in the composition and
343   diversity of the gut microbiome of animals (e.g., Amato et al., 2013; Xavier et al., 2021).
344   The two habitats in which the lizards from this study were captured are very different, with
345   lizards from Lisbon living in a highly urbanized and artificial habitat, with greater
346   environmental disturbance, compared to lizards from Moledo, which live in a semi-natural
347   habitat. Specifically, we detected a consistently higher microbial diversity in the species
348   from Lisbon which could possibly be explained by the higher variety of diet items
349   consumed. *Podarcis siculus* diet is viewed as extremely opportunistic, and can include
350   fruits, other lizards, small carrion and even human food waste (e.g. cheese and pasta)
351   (Mo & Mo, 2021; Mačát, Veselý & Jablonski, 2015; Capula & Aloise, 2011). Additionally,
352   we cannot discard a potential influence of climatic variability between the two localities.

353   Although the species we sampled in urban areas can also reside in rural habitats,
354   and vice-versa, our sampling design did not allow comparisons of gut bacterial
355   communities between conspecifics residing in these two habitats. Nevertheless,
356   urbanization is frequently seen to restructure the gut microbiome of animals (e.g.,
357   Stothart, Palme & Newman, 2019), with increases in microbiome alpha-diversity reported
358   for some reptiles (avian and non-avian) and mammals (Dillard et al., 2022). For example,
359   higher gut bacterial diversities were previously reported in a study from populations of the
360   Australian water dragon residing in urban environments when compared to those
361   inhabiting natural environments, presumably driven by differences in the diet (Littleford-
362   Colquhoun et al., 2019). Additionally, authors hypothesized that environmental
363   microbiota, which may be horizontally transferred to lizards, could also be more diverse
364   in urban habitats than in semi-natural ones (Littleford-Colquhoun et al., 2019). A similar
365   pattern was observed in urban crested anole lizards, white-crowned sparrows, as well as
366   in coyotes (Dillard et al., 2022; Phillips, Berlow & Derryberry, 2018; Sugden et al., 2020).
367   Interestingly, Dillard et al. (2022) found increased similarities between the gut microbiota
368   of these three animals and human populations in urbanized habitats. Different hypotheses
369   have been put forward to explain this trend, including that it could be caused by increased
370   heterogeneity of urban land cover (Phillips, Berlow & Derryberry, 2018), higher
371   consumption of human food waste (Sugden et al., 2020) and acquisition of human

372 microbiota in urban habitats (Dillard et al., 2022). We hypothesize that the higher
373 microbiome diversity in lizards from the urban environment could also be related with the
374 aforementioned factors, but further studies including conspecific lizards from urban and
375 natural habitats are needed to determine the generality of this pattern.

376  Gut microbial diversity (alpha-diversity) and bacterial community structure (beta-
377 diversity) did not differ between the two syntopic species, *P. bocagei* and *P. lusitanicus*,
378 sampled at Moledo. Additionally, our analysis of potential bacterial transmission between
379 these two syntopic lizards indicates a high and balanced bi-directional transmission of
380 bacteria between the two species (ca. 70%), indicating a high similarity between their gut
381 microbiota (Shenhav et al., 2019). This is not surprising as the two species have high
382 dietary overlap and similarity in their habitat occupancy. Moreover, it is likely they
383 consume the same or very similar prey items (Kaliontzopoulou et al., 2011), and also
384 encounter each other frequently. On the contrary, although there were no differences in
385 alpha-diversity between species sampled in Lisbon, species-specific gut bacterial
386 community structure (beta-diversity) were found, indicating ecological differences
387 between these species. Additionally, in comparison with the two species sampled in
388 Moledo, lower and unbalanced estimates for bacterial transmission were uncovered
389 between the two sympatric species, *P. siculus* and *P. virescens*. The invasive *P. siculus*
390 was estimated to receive a higher proportion of bacteria from the native *P. virescens* than
391 vice versa (ca. 72% vs 55% on average). These differences could be related to an
392 increased habitat occupancy and successful adaptation to the environment by the
393 invasive species, which facilitated the acquisition of a higher quantity of local microbiota
394 upon its arrival. These results could also be reflecting an increased ability to exploit a
395 variety of food resources, or most likely a combination of both. Although the populations
396 of *P. siculus* and *P. virescens* are found living in sympatry, occupying roughly the same
397 area, they are rarely in syntopy, although sightings of these two species within a few
398 meters of each other have been reported (Ribeiro & Sá-Sousa, 2018). The proportion of
399 some of the most abundant bacterial genera found in our study also differed between *P.*
400 *virescens* and *P. siculus,* but not between *P. lusitanicus* and *P. bocagei*. The influence of
401 host taxonomy in gut microbiota, which is a proxy not only for host genetics but also its

402   general ecology, has been reported in many animals (Moeller et al., 2013; Moeller et al.,
403   2014), including reptiles (Smith, Colston & Siler, 2021).
404   Overall, host sex had no effect on microbiome diversity and structure, although the
405   proportion of the genus *Corynebacterium* significantly differed between sexes in species
406   collected from Moledo (*P. bocagei* and *P. lusitanicus*). An increased abundance of
407   *Corynebacterium* may have a potential negative impact on the host, as found in a study
408   on birds where this genus was hypothesised to decrease the reproductive performance
409   of females (Leclaire et al., 2022). Here, females of *P. lusitanicus* (n=2) had a higher
410   abundance of *Corynebacterium* compared to males (n=6) (Fig. 5). However, the number
411   of females sampled in the present study was low, so further studies are warranted to
412   confirm this finding and understand whether these bacteria negatively affect reproductive
413   output of this lizard. Another interesting result was that size of males of *P. siculus* was
414   positively related with bacterial diversity. This lizard can reach larger sizes than the other
415   studied species (Carretero & Silva-Rocha, 2015, Damas-Moreira et al., 2019). Links
416   between lizard size and microbiome diversity have been previously established and
417   explained by increased gut space and longer transit time permitting microbes to have
418   more opportunities of colonisation (Reese and Dunn, 2018). Furthermore, *P. siculus* can
419   also be bolder and more aggressive than native *Podarcis* species (Downes & Bauwens,
420   2002), and also more exploratory and better at exploiting food resources when compared
421   to the native *P. virescens* at our study location (Damas-Moreira et al., 2019; 2020). These
422   behaviors can be associated with the displacement of *P. virescens* from gardens now
423   inhabited by *P. siculus* (Ribeiro & Sá-Sousa, 2018) and can also be leading to a wider
424   ecological and trophic niche, and consequently to the correlation found, as well as to the
425   slightly higher average microbiome diversity observed for *P. siculus*. However, it is
426   important to note that the correlation found between the size of *P. siculus* and gut
427   microbiome diversity is influenced by the smallest individual captured, with more studies
428   needed to confirm this tendency.
429
430   **Conclusions**
431   The present study contributes to the existing knowledge on the effects of the
432   environment and host factors on the dynamics of the gut microbiome of lizards. Our

433   results showed that habitat was the major driver of differences in microbiome diversity,

434   with lizards from urban environment displaying higher diversity, which probably results

435   from a combination of factors including increased habitat heterogeneity and a potentially

436   more diverse diet. The effect of host taxonomy was more evident in microbiome structure,

437   with differences found between the two sympatric species, *P. siculus* and *P. virescens*,

438   and also between *T. dugesii* and *Podarcis* spp. Although sex had a minor role in

439   determining gut bacterial assemblages of the lizards studied, *Corynebacterium* was more

440   abundant in females of *P. bocagei*, with potentially deleterious effects for reproduction.

441   Additionally, the diversity of gut microbiome of males of *P. siculus* seems to increase with

442   host size. Finally, our results set the stage for future research exploring the influence of

443   diet and urbanization on the microbiome of *Podarcis* and the use of sympatric lizards as

444   models to test the effects of behavior on lizard microbial composition.

445

446   **Acknowledgements**

447   The authors would like to thank A. Isabel Ferreira for her help during fieldwork.

448

449

450

451

452

453   **References**

454
455

456   Alemany I, Pérez-Cembranos A, Pérez-Mellado V, Castro JA, Picornell A, Ramon C,

457   Jurado-Rivera JA. 2022. Faecal microbiota divergence in allopatric populations of

458   *Podarcis lilfordi* and *P. pityusensis*, two lizard species endemic to the Balearic

459   Islands. *Microbial Ecology*, 1-14 DOI: 10.1007/s00248-022-02019-3

460   Amato KR, Yeoman CJ, Kent A, Righini N, Carbonero F, Estrada A, Gaskins H R, Stumpf

461   RM, Yildirim S, Torralba M, Gillis M, Wilson BA, Nelson KE, White BA, Leigh SR.

462   2013. Habitat degradation impacts black howler monkey (*Alouatta pigra*)

463   gastrointestinal microbiomes. *The ISME Journal*, 7(7): 1344-1353 DOI:
464   10.1038/ismej.2013.16

465 Arbizu PM. 2020. pairwiseAdonis: pairwise multilevel comparison using adonis. R
466   package version 0.4. Available at: https://github.
467   com/pmartinezarbizu/pairwiseAdonis.

468 Archie EA, Tung J. 2015. Social behavior and the microbiome. *Current Opinion in*
469   *Behavioral Sciences* 6: 28-34 DOI: 10.1016/j.cobeha.2015.07.008

470 Baldo L, Riera JL, Mitsi K, Pretus JL. 2018. Processes shaping gut microbiota diversity in
471   allopatric populations of the endemic lizard *Podarcis lilfordi* from Menorcan islets
472   (Balearic Islands). *FEMS Microbiology Ecology* 94(2): fix186 DOI:
473   10.1093/femsec/fix186

474 Bates D, Mächler M, Bolker B, Walker S. 2014. Fitting linear mixed-effects models using
475   lme4. *Journal of Statistical Software*, 67(1): 1–48 DOI: 10.48550/arXiv.1406.5823

476 Bestion E, Jacob S, Zinger L, Di Gesu L, Richard M, White J, Cote J. 2017. Climate
477   warming reduces gut microbiota diversity in a vertebrate ectotherm. *Nature*
478   *ecology & evolution*, 1(6): 1-3 DOI: 10.1038/s41559-017-0161

479 Bicho RC, Amaral MJ, Faustino AM, Power DM, Rêma A, Carretero MA, Soares AM,
480   Mann RM. 2013. Thyroid disruption in the lizard *Podarcis bocagei* exposed to a
481   mixture of herbicides: a field study. *Ecotoxicology* 22(1): 156-165 DOI:
482   10.1007/s10646-012-1012-2

483 Bletz MC, Goedbloed DJ, Sanchez E, Reinhardt T, Tebbe CC, Bhuju S, Geffers R, Jarek
484   M, Vences M, Steinfartz S. 2016. Amphibian gut microbiota shifts differentially in
485   community structure but converges on habitat-specific predicted functions. *Nature*
486   *communications*, *7*(1): 1-12 DOI: 10.1038/ncomms13699

487 Buglione M, Ricca E, Petrelli S, Baccigalupi L, Troiano C, Saggese A, Rivieccio E,
488   Fulgione D. 2022. Gut microbiota plasticity in insular lizards under reversed island
489   syndrome. *Scientific Reports*, *12*(1): 1-13 DOI: 10.1038/s41598-022-16955-0

490 Bunker ME, Arnold AE, Weiss SL. 2022. Wild microbiomes of striped plateau lizards vary
491   with reproductive season, sex, and body size. *Scientific Reports*, 12(1): 1-12 DOI:
492   10.1038/s41598-022-24518-6

493 Bunker ME, Martin MO, Weiss SL. 2022. Recovered microbiome of an oviparous lizard
494   differs across gut and reproductive tissues, cloacal swabs, and faeces. *Molecular*
495   *ecology resources.* *22*(5): 1693-1705 DOI: 10.1111/1755-0998.13573

496 Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. 2016. DADA2:
497   High-resolution sample inference from Illumina amplicon data. *Nature Methods* 13:
498   581-583 DOI: 10.1038/nmeth.3869

499 Capula M, Aloise G. 2011. Extreme feeding behaviours in the Italian wall lizard, *Podarcis*
500   *siculus*. *Acta Herpetologica* 6(1): 11–14 DOI: 10.1400/179164

501 Carretero MA, Galán P, Salvador A. 2015. Lagartija lusitana–*Podarcis guadarramae*
502   (Boscá, 1916). *Enciclopedia Virtual de los Vertebrados Españoles* (Eds.). Museo
503   Nacional de Ciencias Naturales, Madrid.

504 Carretero MA, Silva-Rocha I. 2015. La lagartija italiana (*Podarcis sicula*) en la península
505   ibérica e islas Baleares. *Ecology* 22: 4829-4841

506 Chiang MH, Ho SM, Wu HY, Lin YC, Tsai WH, Wu T, Lai C, Wu CL. 2022. *Drosophila*
507   model for studying gut microbiota in behaviors and neurodegenerative
508   diseases. *Biomedicines*, *10*(3): 596 DOI: 10.3390/biomedicines10030596

509 Chiarello M, Auguet JC, Bettarel Y, Bouvier C, Claverie T, Graham NAJ, Rieuvilleneuve
510   F, Sucré E, Bouvier T, Villéger S. 2018. Skin microbiome of coral reef fish is highly
511   variable and driven by host phylogeny and diet. *Microbiome* 6(1): 147 DOI:
512   10.1186/s40168-018-0530-4

513 Colston TJ, Jackson CR. 2016. Microbiome evolution along divergent branches of the
514   vertebrate tree of life: what is known and unknown. *Molecular ecology*, 25(16):
515   3776-3800 DOI: 10.1111/mec.13730

516 Cryan JF, Dinan TG. 2012. Mind-altering microorganisms: the impact of the gut
517   microbiota on brain and behaviour. *Nature Reviews Neuroscience* 13: 701-712
518   DOI: 10.1038/nrn3346

519 Cusick JA, Wellman CL, Demas GE. 2021. The call of the wild: using non-model systems
520   to investigate microbiome–behaviour relationships. *Journal of Experimental*
521   *Biology*, *224*(10): jeb224485 DOI: 10.1242/jeb.224485

522 Damas-Moreira I, Riley JL, Carretero MA, Harris DJ, Whiting MJ. 2020. Getting ahead:
523   exploitative competition by an invasive lizard. *Behavioral Ecology and*
524   *Sociobiology* 74: 117 DOI: 10.1007/s00265-020-02893-2

525 Damas-Moreira I, Riley JL, Harris DJ, Whiting MJ. 2019. Can behaviour explain invasion
526   success? A comparison between sympatric invasive and native lizards. *Animal*
527   *Behaviour* 151: 195-202 DOI: 10.1016/j.anbehav.2019.03.008

528 Delsuc F, Metcalf JL, Wegener PL, Song SJ, Gonzalez A, Knight R. 2014. Convergence
529   of gut microbiomes in myrmecophagous mammals. Molecular Ecology 23(6):
530   1301-1317 DOI: 10.1111/mec.12501

531 Dillard BA, Chung AK, Gunderson AR, Campbell-Staton SC, Moeller AH. 2022.
532   Humanization of wildlife gut microbiota in urban environments. *eLife*, *11*: e76381
533   DOI: 10.7554/eLife.76381

534 Downes S, Bauwens D. 2002. An experimental demonstration of direct behavioural
535   interference in two Mediterranean lacertid lizard species. *Animal Behaviour* 63(6):
536   1037-1046 DOI: 10.1006/anbe.2002.3022

537 Geniez P, Sa-Sousa P, Guillaume CP, Cluchier A, Crochet PA. 2014. Systematics of the
538   *Podarcis hispanicus* complex (Sauria, Lacertidae) III: valid nomina of the western
539   and central Iberian forms. *Zootaxa*, 3794(1): 1-51 DOI: 0.11646/zootaxa.3794.1.1

540 González de la Vega JP, González-García JP, García-Pulido T, González-García G.
541   2001. *Podarcis sicula* (Lagartija italiana), primera cita para Portugal. Boletín de la
542   Asociación Herpetológica Española 12 (1): 9

543 Hammer TJ, Bowers MD. 2015. Gut microbes may facilitate insect herbivory of chemically
544   defended plants. *Oecologia* 179(1): 1-14 DOI: 10.1007/s00442-015-3327-1

545 Hird SM, Sanchez C, Carstens BC, Brumfield RT. 2015. Comparative gut microbiota of
546   59 neotropical bird species. *Frontiers in Microbiology* 6: 1403 DOI:
547   10.3389/fmicb.2015.01403

548 Hong PY, Wheeler E, Cann IK, Mackie RI. 2011. Phylogenetic analysis of the fecal
549   microbial community in herbivorous land and marine iguanas of the Galápagos
550   Islands using 16S rRNA-based pyrosequencing. *The ISME journal*, 5(9): 1461-
551   1470 DOI: 10.1038/ismej.2011.33

552 Jiang HY, Ma JE, Li J, Zhang XJ, Li LM, He N, Liu HY, Luo SY, Wu ZJ, Han RC, Chen
553   JP. 2017. Diets Alter the Gut Microbiome of Crocodile Lizards. *Frontiers in*
554   *Microbiology* 8: 2073 DOI: 10.3389/fmicb.2017.02073

555 Jia Y, Jin S, Hu K, Geng L, Han C, Kang R, Pang Y, Ling E, Tan EK, Pan Y, Liu, W. 2021.
556   Gut microbiome modulates *Drosophila* aggression through octopamine
557   signaling. *Nature communications*, *12*(1): 1-12 DOI: 10.1038/s41467-021-23041-
558   y

559 Jin L, Huang Y, Yang S, Wu D, Li C, Deng W, Zhao K, He Y, Li B, Zhang G, Xiong Y, Wei
560   R, Li G, Wu H, Zhang H, Zou, L. 2021. Diet, habitat environment and lifestyle
561   conversion affect the gut microbiomes of giant pandas. *Science of the Total*
562   *Environment*, 770: 145316 DOI: 10.1016/j.scitotenv.2021.145316

563 Juan F. 1997. La lagartija ibérica (*Podarcis hispanica*) en la Sierra de Segura, Albacete:
564   biometría, etología y folidosis. Al-Basit: Revista de Estudios Albacetenses 40: 111-
565   134

566 Kaliontzopoulou A, Adams DC, van der Meijden A, Perera A, Carretero MA. 2011.
567   Relationships between head morphology, bite performance and ecology in two
568   species of *Podarcis* wall lizards. *Evolutionary Ecology* 26: 825-845 DOI:
569   10.1007/s10682-011-9538-y

570 Kassambara A, Kassambara MA. 2020. Package 'ggpubr'. Available at:
571   https://cran.Rproject.org/web/packages/ggpubr/ggpubr.pdf

572 Kembel S, Cowan P, Helmus M, Cornwell W, Morlon H, Ackerly D, Blomberg S, Webb C.
573   2010. "Picante: R tools for integrating phylogenies and
574   ecology." *Bioinformatics*, 26: 1463–1464 DOI: 10.1093/bioinformatics/btq166

575 Kohl KD, Brun A, Magallanes M, Brinkerhoff J, Laspiur A, Acosta JC, Caviedes-Vidal E,
576   Bordenstein SR. 2017. Gut microbial ecology of lizards: insights into diversity in
577   the wild, effects of captivity, variation across gut regions and
578   transmission. *Molecular ecology*, *26*(4): 1175-1189 DOI: 10.1111/mec.13921

579 Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a
580   dual-index sequencing strategy and curation pipeline for analyzing amplicon
581   sequence data on the MiSeq Illumina sequencing platform. *Applied and*
582   *environmental microbiology* 79(17): 5112-5120 DOI: 10.1128/AEM.01043-13

583 Leclaire S, Pineaux M, Blanchard P, White J, Hatch SA. 2022. Microbiota composition
584   and diversity of multiple body sites vary according to reproductive performance in
585   a seabird. *Molecular Ecology*. DOI: 10.1111/mec.16398

586 Littleford-Colquhoun BL, Weyrich LS, Kent N, Frere CH. 2019. City life alters the gut
587   microbiome and stable isotope profiling of the eastern water dragon (*Intellagama*
588   *lesueurii*). *Molecular ecology*, *28*(20): 4592-4607 DOI: 10.1111/mec.15240

589 Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion
590   for RNA-seq data with DESeq2. *Genome Biology* 15(12): 1-21 DOI:
591   10.1186/s13059-014-0550-8

592 Luís C, Rodrigues I, Guerreiro SG, Fernandes R, Soares, R. 2019. Regeneration in the
593   *Podarcis bocagei* model organism: a comprehensive immune-/histochemical
594   analysis of the tail. Zoomorphology 138: 399-407 DOI: 10.1007/s00435-019-
595   00452-6

596 Mačát Z, Veselý M, Jablonski D. 2015. New case of fruit eating observation in *Podarcis*
597   *siculus* (Rafinesque-Schmaltz, 1810) (Lacertidae) from Croatia. *Biharean Biologist*
598   9(2): 158-159

599 McMurdie PJ, Holmes S. 2013. *phyloseq*: an R package for reproducible interactive
600   analysis and graphics of microbiome census data. *PLoS One* 8: e61217 DOI:
601   10.1371/journal.pone.0061217

602 McMurdie PJ, Holmes S. 2014. Waste not, want not: why rarefying microbiome data is
603   inadmissible. *PLoS computational biology*, *10*(4): e1003531 DOI:
604   10.1371/journal.pcbi.1003531

605 Mo M, Mo E. 2021. Observations of Southern Italian Wall Lizards (*Podarcis siculus*
606   *siculus*) scavenging artificial foods in the Province of Messina, Sicily, Italy. *Reptiles*
607   *& Amphibians*, *28*(2): 292-294

608 Moeller AH, Li Y, Mpoudi Ngole E, Ahuka-Mundeke S, Lonsdorf EV, Pusey AE, Peeters
609   M, Hahn BH, Ochman H. 2014. Rapid changes in the gut microbiome during
610   human evolution. *Proceedings of the National Academy of Sciences*, *107*(51):
611   21947-21948 DOI: 10.1073/pnas.1419136111

612   Moeller AH, Peeters M, Ndjango JB, Li Y, Hahn BH, Ochman H. 2013. Sympatric
613   chimpanzees and gorillas harbor convergent gut microbial communities. *Genome*
614   *Research* 23(10): 1715-1720 DOI: 10.1101/gr.154773.113

615   Montoya-Ciriaco N, Gómez-Acata S, Muñoz-Arenas LC, Dendooven L, Estrada-Torres
616   A, Díaz de la Vega-Pérez AH, Navarro-Noya YE. 2020. Dietary effects on gut
617   microbiota of the mesquite lizard *Sceloporus grammicus* (Wiegmann, 1828) across
618   different altitudes. *Microbiome*, 8(1): 1-19 DOI: 10.1186/s40168-020-0783-6

619   Muegge BD, Kuczynski J, Knights D, Clemente JC, Gonzalez A, Fontana L, Henrissat B,
620   Knight R, Gordon, JI. 2011. Diet drives convergence in gut microbiome functions
621   across mammalian phylogeny and within humans. *Science* 332: 970-974 DOI:
622   10.1126/science.1198719

623   Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara RB, Simpson GL,
624   Solymos P, Stevens MHH, Wagner H, Oksanen MJ. 2013. Package 'vegan'.
625   Community ecology package 2(9): 1-295. Available at: https://cran.r-
626   project.org/web/packages/vegan/index.html

627   Phillips JN, Berlow M, Derryberry EP. 2018. The effects of landscape urbanization on the
628   gut microbiome: an exploration into the gut of urban and rural white-crowned
629   sparrows. *Frontiers in Ecology and Evolution*, 6: 148 DOI:
630   10.3389/fevo.2018.00148

631   Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glockner FO. 2007.
632   SILVA: a comprehensive online resource for quality checked and aligned
633   ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research*
634   35(21): 7188-7196 DOI: 10.1093/nar/gkm864

635   Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO.
636   2013. The SILVA ribosomal RNA gene database project: improved data
637   processing and web-based tools. *Nucleic Acids Research* 41(D1): D590-596 DOI:
638   10.1093/nar/gks1219

639   Reese AT, Dunn RR. 2018. Drivers of microbiome biodiversity: a review of general rules,
640   feces, and ignorance. *MBio*, 9(4): e01294-18 DOI: 10.1128/mBio.01294-18

641   Ribeiro R, Sá-Sousa P. 2018. Where to live in Lisbon: urban habitat used by the
642   introduced Italian wall lizard (*Podarcis siculus*). *Basic and Applied Herpetology* 32:
643   57-70 DOI: 10.11160/bah.101

644   Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, Tuohy K. 2018. Gut
645   microbiota functions: metabolism of nutrients and other food components.
646   *European journal of nutrition*, *57*(1): 1-24 DOI: 10.1007/s00394-017-1445-8

647   Sadek RA. 1981. The diet of the Madeiran lizard *Lacerta dugesii*. *Zoological Journal of*
648   *the Linnean Society* 73(4): 313-341 DOI: 10.1111/j.1096-3642.1981.tb01599.x

649   Sá-Sousa P. 1995. The introduced Madeiran lizard, *Lacerta* (*Teira*) *dugesii* in Lisbon.
650   *Amphibia-Reptilia* 16(2): 211-214

651   Silva-Rocha I, Salvi D, Carretero MA. 2012. Genetic data reveal a multiple origin for the
652   populations of the Italian wall lizard *Podarcis sicula* (Squamata: Lacertidae)
653   introduced in the Iberian Peninsula and Balearic islands. *Italian Journal of*
654   *Zoology*, *79*(4): 502-510 DOI: 10.1080/11250003.2012.680983

655   Shapira M. 2016. Gut microbiotas and host evolution: scaling up symbiosis. *Trends in*
656   *ecology & evolution*, 31(7): 539-549 DOI: 10.1016/j.tree.2016.03.006

657   Shenhav L, Thompson M, Joseph TA, Briscoe L, Furman O, Bogumil D, Mizrahi I, Pe'er
658   I, Halperin E. 2019. FEAST: fast expectation-maximization for microbial source
659   tracking. *Nature Methods* 16(7): 627-632 DOI: 10.1038/s41592-019-0431-x

660   Smith SN, Colston TJ, Siler CD. 2021. Venomous snakes reveal ecological and
661   phylogenetic factors influencing variation in gut and oral microbiomes. *Frontiers in*
662   *Microbiology* 12: 657754 DOI: 10.3389/fmicb.2021.657754.

663   Song SJ, Sanders JG, Delsuc F, Metcalf J, Amato K, Taylor MW, Mazel F, Lutz HL,
664   Winker K, Graves GR, Humphrey G, Gilbert JA, Hackett SJ, White KP, Skeen HR,
665   Kurtis SM, Withrow J, Braile T, Miller M, McCracken KG, Maley JM, Ezenwa VO,
666   Williams A, Blanton JM, McKenzie VJ, Knight R. 2020. Comparative analyses of
667   vertebrate gut microbiomes reveal convergence between birds and
668   bats. *MBio*, *11*(1): e02901-19 DOI: 10.1128/mBio.02901-19

669   Stothart MR, Palme R, Newman AE. 2019. It's what's on the inside that counts: stress
670   physiology and the bacterial microbiome of a wild urban mammal. *Proceedings of*
671   *the Royal Society B*, *286*(1913): 20192111 DOI: 0.1098/rspb.2019.2111

672 Sugden S, Sanderson D, Ford K, Stein LY, St Clair CC. 2020. An altered microbiome in
673   urban coyotes mediates relationships between anthropogenic diet and poor
674   health. *Scientific reports*, *10*(1): 1-14 DOI: 10.1038/s41598-020-78891-1

675 Team RC. (2020) A language and environment for statistical computing. R Foundation
676   for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org/.

677 Thaiss CA, Zmora N, Levy M, Elinav E. 2016. The microbiome and innate immunity.
678   *Nature* 535: 65-74 DOI: 10.1038/nature18847

679 Vavre F, Kremer N. 2014. Microbial impacts on insect evolutionary diversification: from
680   patterns to mechanisms. *Current opinion in insect science*, *4*: 29-34 DOI:
681   10.1016/j.cois.2014.08.003

682 Vervust B, Pafilis P, Valakos ED, Van Damme R. 2010. Anatomical and physiological
683   changes associated with a recent dietary shift in the lizard *Podarcis sicula*.
684   *Physiological and Biochemical Zoology*, *83*(4): 632-642 DOI: 10.10.86/651704

685 Xavier R, Mazzei R, Perez-Losada M, Rosado D, Santos JL, Verissimo A, Soares MC.
686   2019. A risky business? Habitat and social behavior impact skin and gut
687   microbiomes in caribbean cleaning gobies. *Frontiers in Microbiology* 10: 716 DOI:
688   10.3389/fmicb.2019.00716

689 Xavier R, Pereira A, Pagan A, Hendrick GC, Nicholson MD, Rosado D, Soares MC,
690   Pérez-Losada M, Sikkel PC. 2020. The effects of environment and ontogeny on
691   the skin microbiome of two *Stegastes* damselfishes (Pomacentridae) from the
692   eastern Caribbean Sea. *Marine Biology* 167(7): 1-12 DOI: 10.1007/s00227-020-
693   03717-7

694 Xavier R, Soares MC, Silva SM, Banha F, Gama M, Ribeiro L, Anastácio P, Cardoso SC.
695   2021. Environment and host-related factors modulate gut and carapace bacterial
696   diversity of the invasive red swamp crayfish (*Procambarus
697   clarkii*). *Hydrobiologia*, *848*(17): 4045-4057 DOI: 10.1007/s10750-021-04623-9

698 Youngblut ND, Reischer GH., Walters W, Schuster N, Walzer C, Stalder G, Ley RE &
699   Farnleitner AH. 2019. Host diet and evolutionary history explain different aspects
700   of gut microbiome diversity among vertebrate clades. *Nature communications*,
701   10(1): 1-15 DOI: 10.1038/s41467-019-10191-3

702 Zhang W, Li N, Tang X, Liu N, Zhao W. 2018. Changes in intestinal microbiota across an
703   altitudinal gradient in the lizard *Phrynocephalus vlangalii*. *Ecology and evolution*,
704   8(9): 4695-4703 DOI: 10.1002/ece3.4029
705 Zilber-Rosenberg I, Rosenberg E. 2008. Role of microorganisms in the evolution of
706   animals and plants: the hologenome theory of evolution. *FEMS microbiology*
707   *reviews*, 32(5): 723-735 DOI: 10.1111/j.1574-6976.2008.00123.x

708

709
710
711
712
713
714
715
716

717 **Legends**

718
719 **Figure 1**: **Sampling sites of five lizard species from Portugal.** Map of Portugal with
720 aerial photographs of sampling sites for A) *P. bocagei* and *P. lusitanicus,* B_1) *P. viriscens,*
721 B_2) *P. siculus and* C) *T. dugesii.* Specific collection areas are delimited by yellow lines.
722 Map data ©2021 Google.

723 **Figure 2: Boxplots of alpha-diversity of the studied lizards' gut microbiome.**
724 Boxplots of the alpha-diversity indices (Faith's phylogenetic diversity, Shannon diversity
725 and the number of observed ASVs) for the gut microbiome of the studied lizards. Lines
726 within each boxplot indicate the median and whiskers display the range. Open dots
727 represent data points and black dots represent outliers.

728 **Figure 3: Principal coordinate analysis (PCoA) plots of the gut bacterial beta-**
729 **diversity of the studied lizards.**
730 PCoA plots representing Bray–Curtis and Unweighted Unifrac distances, grouped by
731 lizard species with a 95% confidence interval ellipse.

732

733   **Figure 4: Most abundant bacterial genera from the gut of the five studied lizard**

734   **species**. Relative abundance of the most abundant bacterial genera ($\geq$ 3% on average of

735   all sequences) in the gut microbiome of the studied lizard species from Lisbon (A) and

736   from Moledo (B). Lines within each boxplot indicate the median and whiskers display the

737   range. Open dots represent data points and black dots represent outliers.

738

739   **Figure 5: Relative abundance of *Corynebacterium* in the gut microbiota of species**

740   **from Moledo between sexes.** Differences in the proportion of the most abundant genus

741   *Corynebacterium* between males and females of *P. bocagei* and *P. lusitanicus*.

742

743   **Figure 6: Linear regression for effects of size on bacterial diversity.** Linear

744   regression plot between size (SVL) and gut bacterial alpha-diversity (Shannon index) for

745   *P. siculus.* The colored area represents a 95% confidence limit.

Figure 1

Sampling sites of five lizard species from Portugal.

Map of Portugal with aerial photographs of sampling sites for A) *P. bocagei* and *P. lusitanicus,* B$_1$) *P. viriscens,* B$_2$) *P. siculus and* C) *T. dugesii.* Specific collection areas are delimited by yellow lines. Map data ©2021 Google.

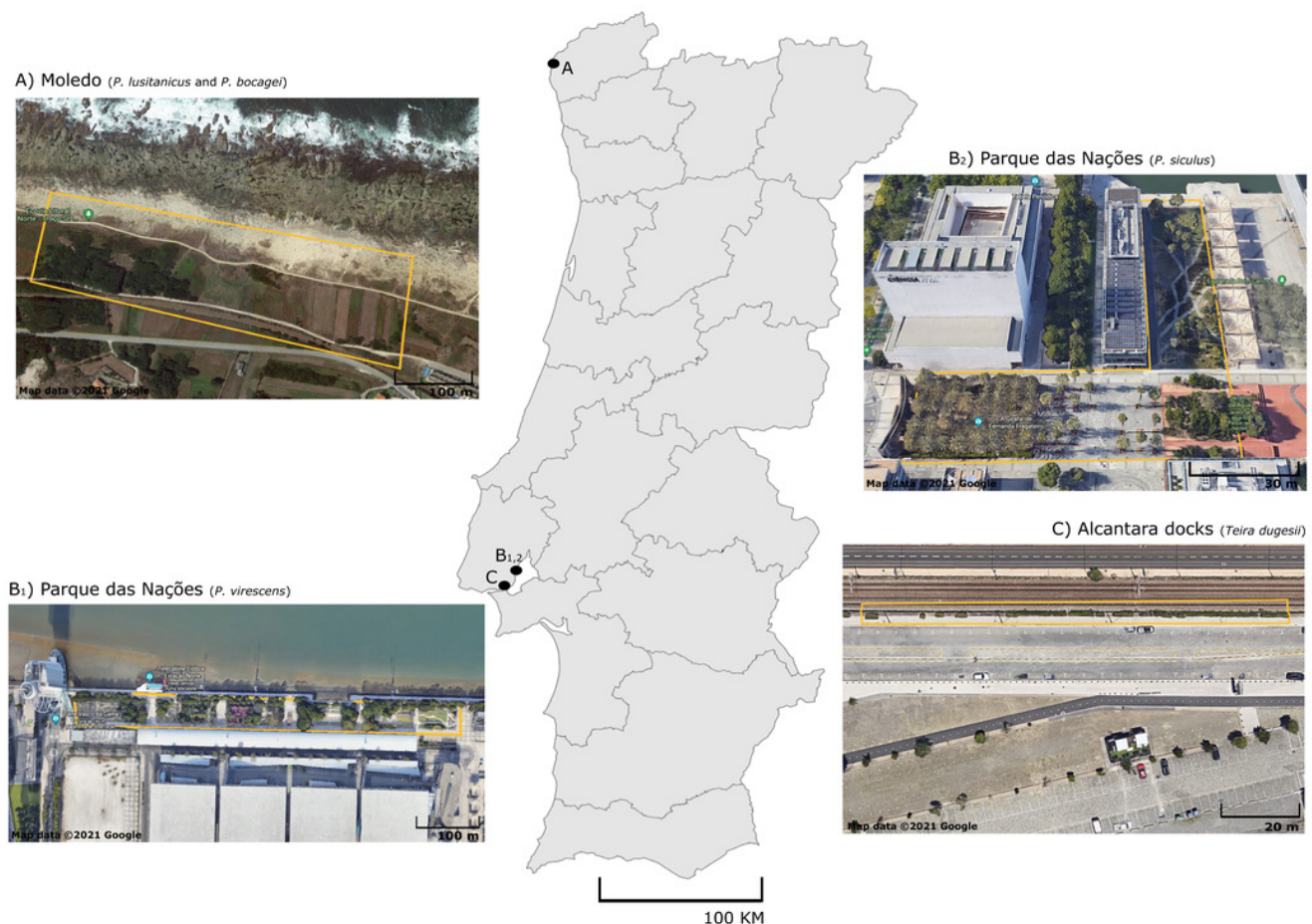



Figure 2

Boxplots of alpha-diversity of the studied lizards' gut microbiome.

Boxplots of the alpha-diversity indices (Faith's phylogenetic diversity, Shannon diversity and the number of observed ASVs) for the gut microbiome of the studied lizards. Lines within each boxplot indicate the median and whiskers display the range. Open dots represent data points and black dots represent outliers.

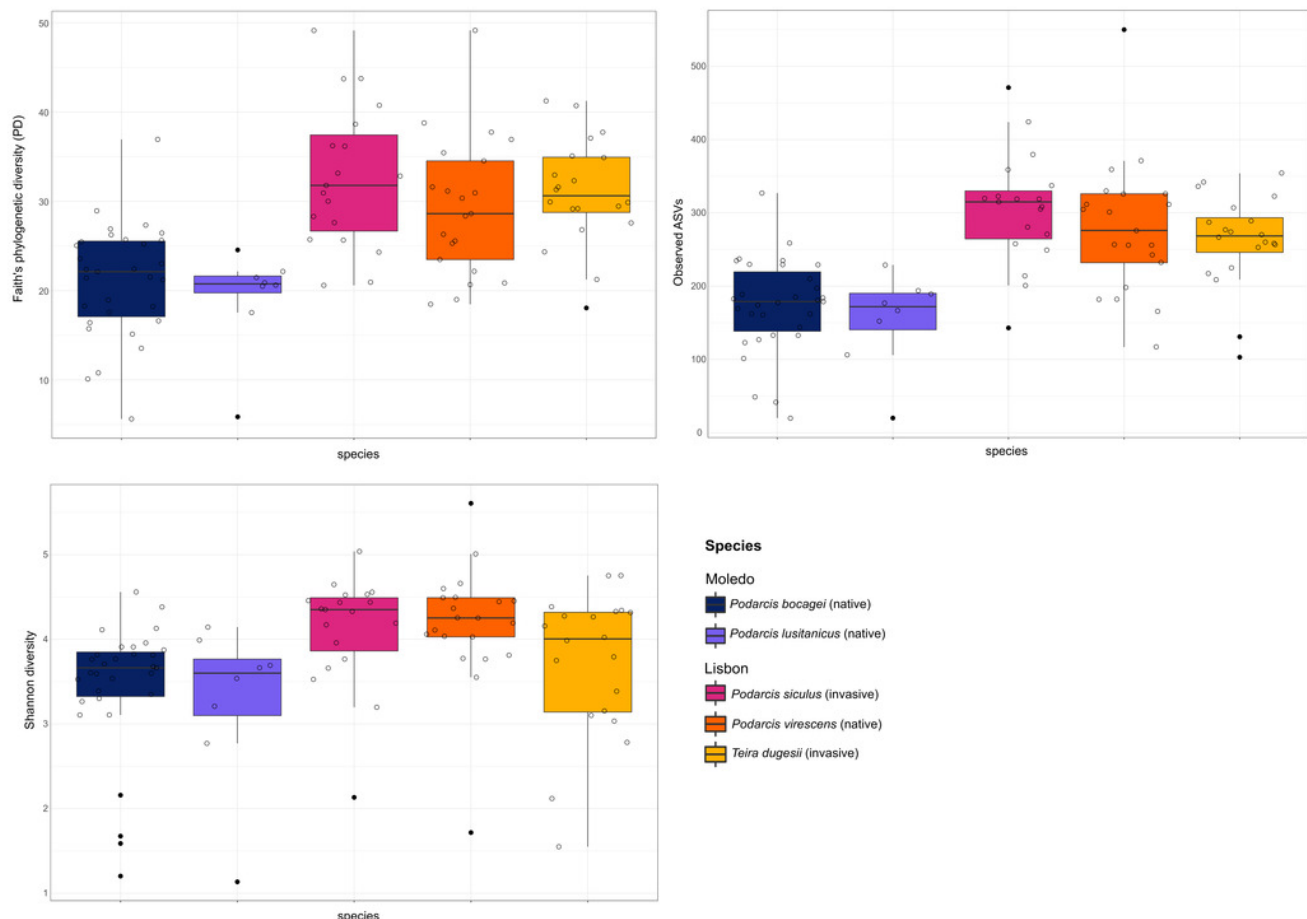



Figure 3

Principal coordinate analysis (PCoA) plots of the gut bacterial beta-diversity of the studied lizards.

PCoA plots representing Bray–Curtis and Unweighted Unifrac distances, grouped by lizard species with a 95% confidence interval ellipse.

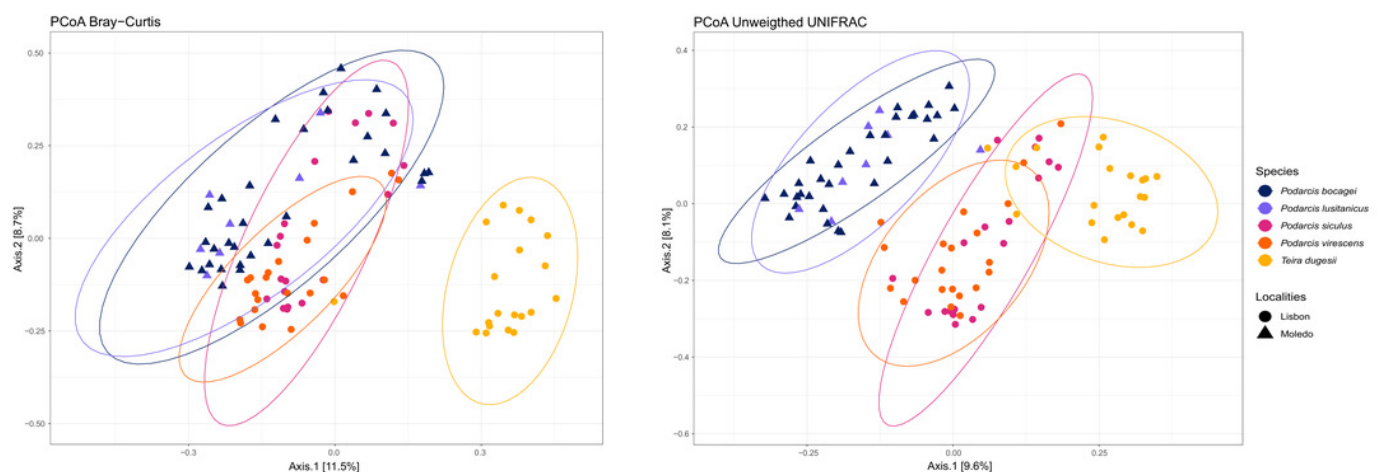



Figure 4

Most abundant bacterial genera from the gut of the five studied lizard species.

Relative abundance of the most abundant bacterial genera (≥ 3% on average of all sequences) in the gut microbiome of the studied lizard species from Lisbon (A) and from Moledo (B). Lines within each boxplot indicate the median and whiskers display the range. Open dots represent data points and black dots represent outliers.

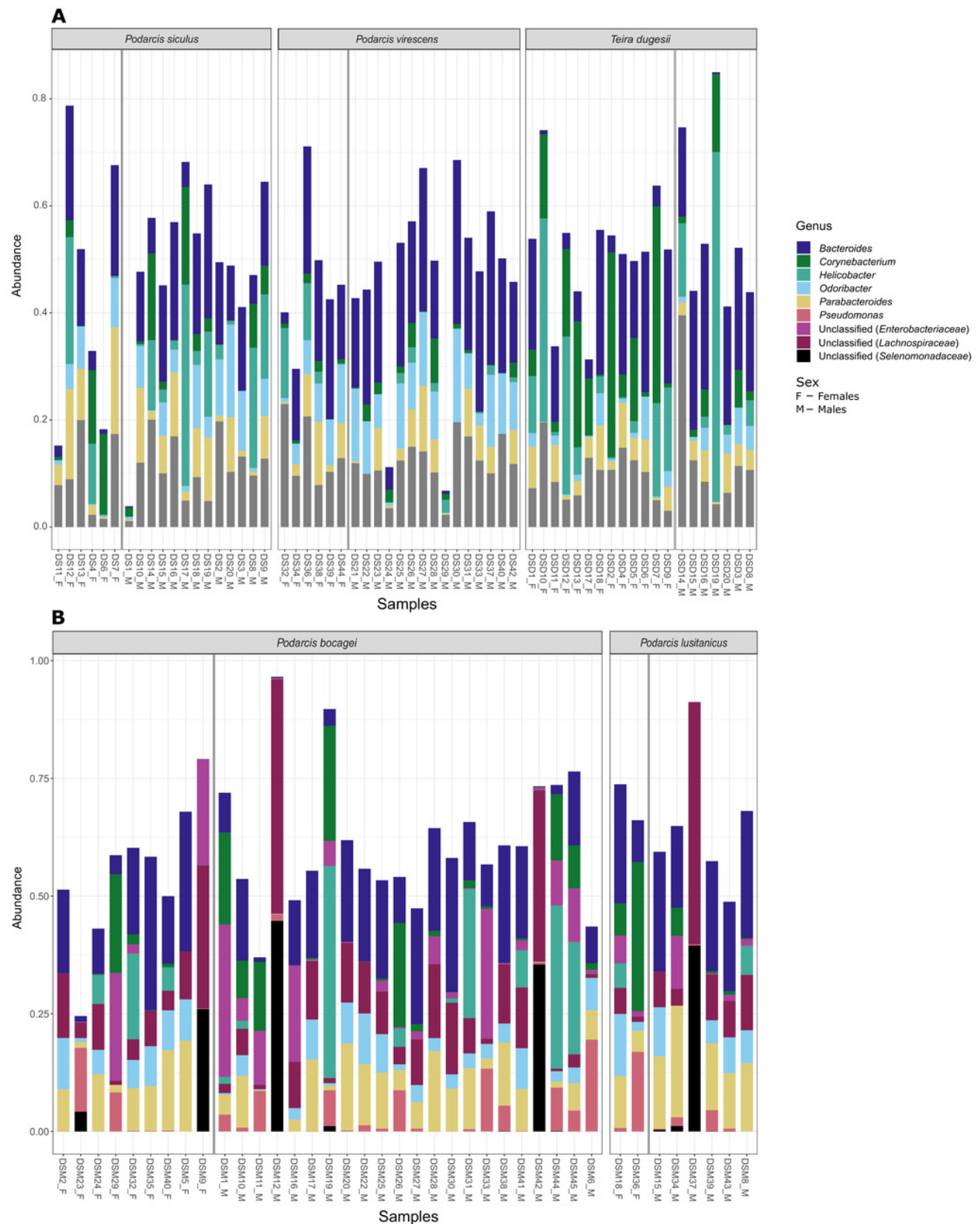



Figure 5

Relative abundance of *Corynebacterium* in the gut microbiota of species from Moledo between sexes.

Differences in the proportion of the most abundant genus *Corynebacterium* between males and females of *P. bocagei* and *P. lusitanicus*.

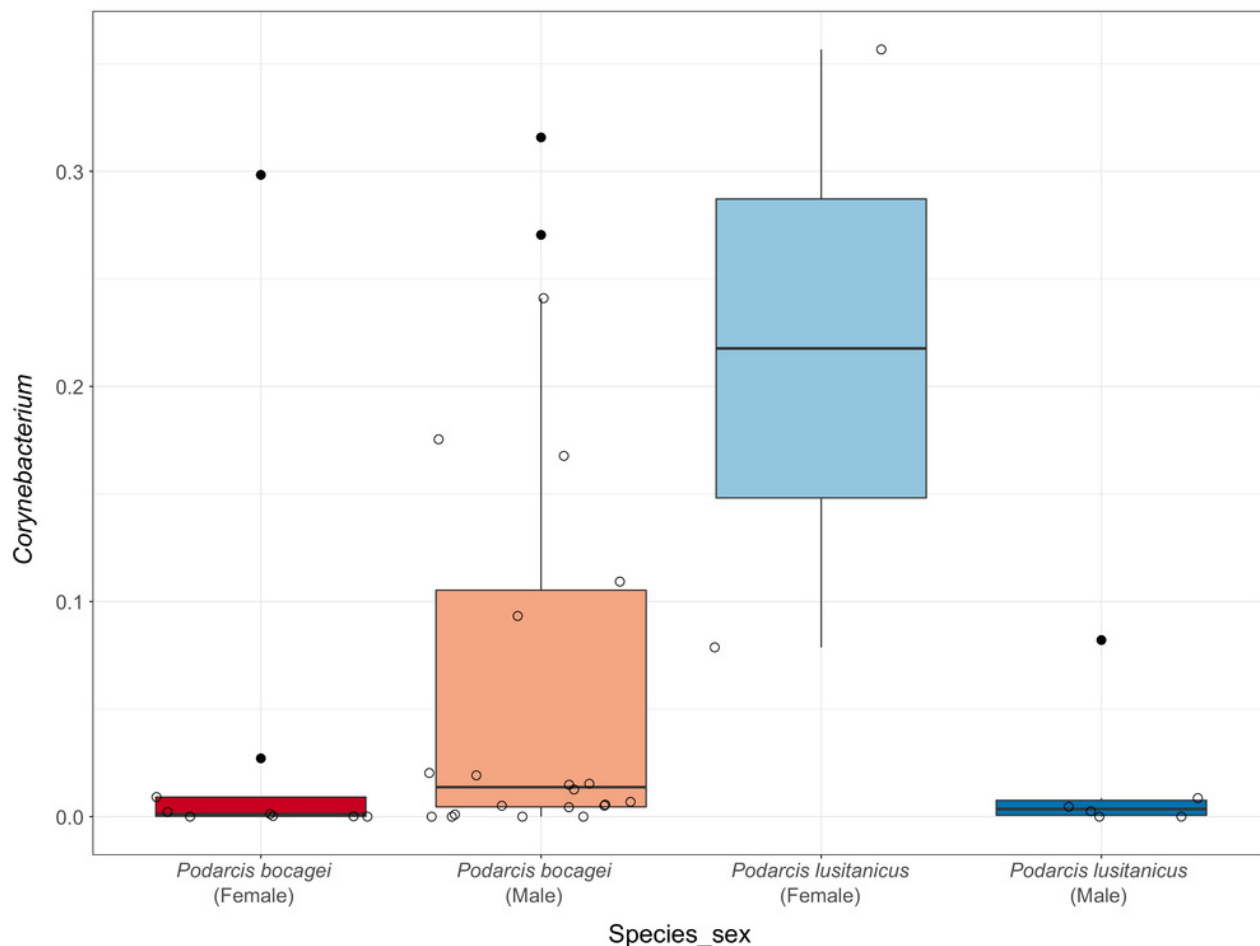
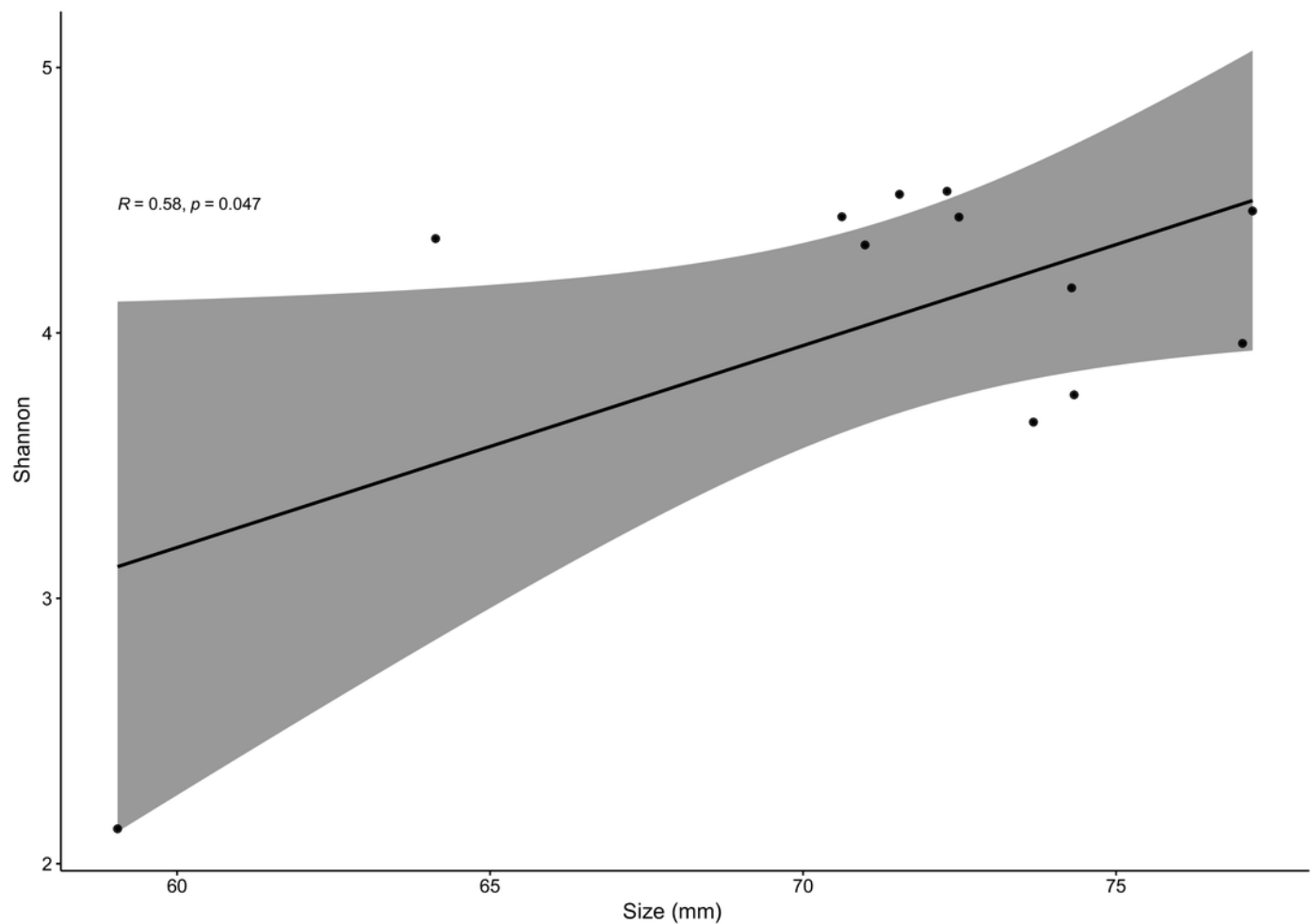



Figure 6

Linear regression for effects of size on bacterial diversity.

Linear regression plot between size (SVL) and gut bacterialalpha-diversity (Shannon index) for P. siculus. The colored area represents a 95% confidence limit.

Results from the pairwise PERMANOVA testing the effect of species and sex in gut microbial beta-diversity.

Results presenting R^2 and respective adjusted p-values. Significant results are depicted in bold.

2 **Table 1** - **Results from the pairwise PERMANOVA testing the effect of species and**
3 **sex in gut microbial beta-diversity.** Results presenting R^2 and respective adjusted p-
4 values. Significant results are depicted in bold.

			Unifrac phylogenetic weighted	Unifrac phylogenetic unweighted	Bray–Curtis
Lisbon	*Podarcis siculus* vs. *Podarcis virescens*	species	**R^2 = 0.10; p = 0.03**	**R^2 = 0.18; p = 0.0003**	**R^2 = 0.12; p = 0.003**
		sex	R^2 = 0.01; p = 0.99	**R^2 = 0.11; p = 0.02**	R^2 = 0.06; p = 0.24
	Podarcis siculus vs. *Teira dugesii*	species	R^2 = 0.03; p = 0.37	**R^2 = 0.44; p = 0.0001**	**R^2 = 0.49; p = 0.0001**
		sex	R^2 = 0.01; p = 0.98	**R^2 = 0.07; p = 0.04**	R^2 = 0.04; p = 0.17
	Podarcis virescens vs. *Teira dugesii*	species	**R^2 = 0.10; p = 0.01**	**R^2 = 0.45; p = 0.0001**	**R^2 = 0.52; p = 0.0001**
		sex	R^2 = 0.02; p = 0.90	R^2 = 0.06; p = 0.06	R^2 = 0.02; p = 0.44
Moledo	*Podarcis bocagei* vs. *Podarcis lusitanicus*	species	R^2 = 0.02; p = 0.41	R^2 = 0.02; p = 0.92	R^2 = 0.02; p = 0.75
		sex	R^2 = 0.05; p = 0.41	R^2 = 0.06; p = 0.26	R^2 = 0.05; p = 0.73

Results from the linear models testing the effect of species and sex in the proportion of the most abundant genera for each locality.

Order/Family of genera that remained unclassified is presented between brackets. Significant results are depicted in bold.

3 **Table 2** - **Results from the linear models testing the effect of species and sex in the**
4 **proportion of the most abundant genera for each locality.** Order/Family of genera
5 that remained unclassified is presented between brackets. Significant results are
6 depicted in bold.

	Lisbon		Moledo	
	Species (DF = 2)	Sex (DF = 3)	Species (DF = 1)	Sex (DF = 2)
Odoribacter	**F = 10.10; p = 0.0002**	F = 1.16; p = 0.33	F = 0.72; p = 0.40	F = 0.81; p = 0.45
Corynebacterium	**F = 6.66; p = 0.003**	F = 2.09; p = 0.11	F = 0.13; p = 0.72	**F = 4.46; p = 0.02**
Helicobacter	F = 2.96; p = 0.06	F = 0.32; p = 0.82	F = 1.01; p = 0.32	F = 0.35; p = 0.71
Parabacteroides	F = 2.11; p = 0.13	F = 0.53; p = 0.67	F = 2.53; p = 0.12	F = 0.68; p = 0.51
Bacteroides	F = 2.43; p = 0.10	F = 1.11; p = 0.35	F = 1.33; p = 0.26	F = 0.03; p = 0.97
Pseudomonas	NA	NA	F = 0.04; p = 0.85	F = 1.88; p = 0.17
Unclassified (*Selenomonadaceae*)	NA	NA	F = 0.11; p = 0.74	F = 0,26; p = 0.76
Unclassified (*Lachnospiraceae*)	F = 1.42; p = 0.25	F = 0.18; p = 0.91	F = 0.18; p = 0.68	F = 0.76; p = 0.48
Unclassified (*Enterobacteriaceae*)	F = 0.27; p = 0.77	F = 0.66; p = 0.58	F = 0.93; p = 0.34	F = 0.06; p = 0.94