Microbiome structure of a wild *Drosophila* community along tropical elevational gradients and comparison to laboratory lines

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Abstract

Variation along environmental gradients in host-associated microbial communities is not well understood, compared to free-living microbial communities. Because elevational gradients may serve as natural proxies for climate change, understanding patterns along these gradients can inform our understanding of the threats hosts and their symbiotic microbes face in a warming world. In this study, we analysed bacterial microbiomes from pupae and adults of four *Drosophila* species native to Australian tropical rainforests. We sampled wild individuals at high and low elevation along two mountain gradients, to determine natural diversity patterns. Further, we sampled laboratory-reared individuals from isofemale lines established from the same localities, to see if any natural patterns are retained in the lab. In both environments, we controlled for diet to help elucidate other deterministic patterns of microbiome composition. We found small but significant differences in *Drosophila* bacterial community composition across elevation, with some notable taxonomic differences between different *Drosophila* species and sites. Further, we found that field-collected fly pupae had significantly richer microbiomes than laboratory-reared pupae. We also found similar microbiome composition in both types of provided diet, suggesting that the significant differences found among *Drosophila* microbiomes are the product of surrounding environments with different bacterial species pools, possibly bound to elevational differences in temperature. Our results suggest that comparative studies between lab and field specimens help reveal the true variability in microbiome communities that can exist within a single species.

Importance

Bacteria form microbial communities inside most higher-level organisms, but we know little about how the microbiome varies along environmental gradients and between natural host populations and laboratory colonies. To explore such effects on insect-associated microbiomes, we studied gut microbiome in four *Drosophila* species over two mountain gradients in tropical Australia. We also compared these data to individuals kept in the laboratory to understand how different settings changed microbiome communities. We found that field sampled individuals had significantly higher microbiome diversity than those from the lab. In wild *Drosophila* populations, elevation explains a small but significant amount of the variation in their microbial communities. Our study highlights the importance of environmental bacterial sources for *Drosophila* microbiome composition across elevational gradients, and shows how comparative studies help reveal the true flexibility in microbiome communities that can exist within a species.

Introduction

Patterns of diversity over environmental gradients like latitude, elevation, or environmental degradation have long been of interest in community ecology and are of renewed interest for studying the potential consequences of climate change[1–5]. Most studies have focused on animals and plants to investigate these patterns, but bacterial communities are receiving increased attention. Some studies suggest free-living bacteria do not follow the same broad biogeographic patterns as plants and animals[6–8]. Fierer et al.[1] showed that soil bacteria did not change significantly in diversity when sampled across an elevational gradient, in contrast to trends documented in most other taxa. Subsequent studies have found

68 inconsistent patterns in bacterial communities sampled from streams and soils across
69 elevational gradients, with differences usually being attributed to changes in pH and C:N
70 ratio [2,8–10].
71
72 Many insects maintain intimate communities of symbiotic microbes (their 'microbiome').
73 Insect microbiomes can play important roles in host health, digestion, thermal regulation,
74 and protection against natural enemies (reviewed in[11–13]). In turn, many factors can
75 influence insect microbiome composition, some host-dependent (e.g. diet, insect species
76 identity, ontogeny, and parent-to-offspring transmission), others host-independent (e.g.
77 abiotic factors like local environment and temperature)[14–22]. Symbioses between insects and
78 bacteria have been particularly well investigated[23], notably because insect microbiome
79 communities tend to be less complex than those of vertebrates[24]. However, in contrast to
80 environmental microbial communities, the effect of elevational change on insect-associated
81 microbiomes has yet to be investigated in-depth. The most conspicuous aspect of a change
82 in elevation is a difference in mean temperature, creating different environments that can
83 be used as a proxy for climate change scenarios[25,26]. Elevational differences in temperature
84 mean we would expect to see differences in microbiome composition related to
85 temperature-dependent development in both insects[27–31] and bacteria[32–34]. Thus, at
86 different elevations, and in climate change scenarios, insect-associated microbiomes could
87 have different composition[20] with potentially important consequences on microbiome and
88 host functionality.
89

90 *Drosophila* spp. are established models for studying insect-associated microbiomes[35–40]
91 because they are cosmopolitan, occurring in a wide variety of habitats, and easy to maintain
92 in laboratory cultures. *Drosophila*-associated microbiomes have important functional
93 impacts on many aspects of their ecology including thermal tolerance[41], development[42],
94 ability to recognise kin[43], and immunity[38,44]. The microbiomes are of moderate-to-low
95 diversity, making them relatively simple to characterise. Additionally, some *Drosophila*
96 species/populations possess intracellular bacterial symbionts (*Wolbachia* and *Spiroplasma*)
97 that can influence host immunity and protect against natural enemies, including pathogenic
98 fungi, nematodes, and parasitoids[45–49]. This, combined with the well-studied nature of
99 *Drosophila,* makes them ideal candidates for investigating insect-associated microbiomes
100 over elevational gradients.
101
102 Here we examine the effects of elevation change on insect microbiome composition by
103 focusing on the underlying biotic and abiotic factors, including elevation, host species
104 identity, and site location. We sampled wild populations of four focal species of frugivorous
105 *Drosophila* from two mountain gradients in Queensland, Australia: *Drosophila rubida, D.*
106 *pseudoananassae, D. pallidifrons,* and *D. sulfurigaster.* These species occur throughout
107 north Queensland along multiple altitudinal gradients in the Wet Tropics. We chose these
108 four species because they are abundant and occur in sympatry across the full elevational
109 gradient at our focal sites[50]. We hypothesised that variation in microbiome composition
110 between high and low elevation populations will reflect temperature differences at these
111 sites. To control for diet in the field we exclusively sampled pupae from banana-baited
112 bottle traps (see Jeffs et al.[50]). The sampling approach guarantees that each analysed
113 individual originated from an egg laid in our bottle traps and therefore fed solely on yeasted

114 banana. To reinforce our investigation, we analysed lab-reared flies of the same species
115 collected from the same field sites to test if their microbiomes retained any natural
116 differences when reared in the laboratory on a standard, yeast-based diet. We expected *a*
117 *priori* to find high among-individual variation and hypothesised that species identity,
118 elevation, and environment of origin (i.e. lab vs field) would be the primary causes of
119 difference in host microbiome composition[35,39,40,51].

120

121 **2. Material & Methods**

122 *2.1 Study Sites*

123 The Australian Wet Tropics World Heritage Area is a 450 km long, narrow section of
124 rainforest along Queensland's northeast coast between Cooktown and Townsville (15-19'S,
125 145-146.30'E). Samples were collected from two altitudinal gradients: Paluma Range Road
126 (within Paluma Range National Park 19°00'S, 146°14'E) and Kirrama Range Road (within
127 Girramay National Park 18°12'S, 145°50'E). The Paluma gradient ranges from 59 m to 916 m
128 above sea level (a.s.l.) and the Kirrama gradient ranges from 92 m to 770 m a.s.l[50]. We chose
129 sites at high and low elevations (Paluma: 880m, 70m; Kirrama: 730m, 70m) to capture a
130 ~5°C temperature range (mean temperatures 21°C at high elevation, 26°C at low
131 elevation[50]. Temperature was recorded by multiple dataloggers suspended next to bottle
132 traps at each site, which took a reading every hour. Our previous study on these gradients
133 determined ~5°C temperature range and found substantial differences in *Drosophila*
134 community composition at high and low elevation, with some species not present at the low
135 and high ends of the gradient and others changing in abundance[50].

136

137 *2.2 Sample Collection and Selection*

138 To ensure a comparable dataset, we selected stratified subsets of samples from the field
139 and laboratory. We primarily collected pupae because they are more easily standardized
140 than larvae and allow us to account for discrepancies in development rate between species.
141 We additionally collected a small number of adult flies to compare microbiome composition
142 between pupae and adults. Based on the results of Jeffs et al.[50], which used pupae samples
143 to identify the natural *Drosophila*-parasitoid food web with COI metabarcoding and
144 Multiplex PCR methods, we selected 214 field samples of the four most abundant
145 *Drosophila* species that occurred at all elevations along both transects: *Drosophila rubida, D.*
146 *pseudoananassae, D. pallidifrons,* and *D. sulfurigaster* (Table 1). Eight *D. rubida* pupae were
147 parasitised by parasitoid wasps, enabling us to test if there are any changes in microbiome
148 richness or unique microbial taxa associated with a developing parasitoid. We subsequently
149 sampled 70 pupae and 70 adults from isofemale (IF) laboratory lines (2-4 lines per species)
150 of these four elevationally ubiquitous species (20 pupae and 20 adults from *D. sulfurigaster,*
151 *D. rubida,* and *D. pseudoananassae*, and 10 pupae and 10 adults from *D. pallidifrons*) to
152 investigate if suspected natural patterns (site- and species-specific influence) were retained
153 in lab-reared flies. Additionally, we took 10 samples of the food source provided to lab-
154 reared *Drosophila* and 20 samples of the banana bait we used in our field sampling, to
155 compare *Drosophila* microbiome samples to a dietary reference and determine how
156 congruent the microbiome communities were between the food source and the insect
157 hosts. Table 1 presents a detailed breakdown of all samples used in this study.

158

159 The *Drosophila* pupae field samples were collected from banana-baited bottle traps placed
160 at low and high elevation sites along both altitudinal gradients. Each bottle trap had a piece

161 of cardboard to assist *Drosophila* larvae in pupation. Bottle traps were left exposed for
162 either 11-12, 14-15, or 24 days, to capture the natural variation in community colonisation
163 and ontogenetic development in different *Drosophila* species[50]. On the day of sampling,
164 these cards were removed and sealed in ziplock bags. The individuals we collected as pupae
165 thus only fed on banana bait. Pupae from each card were sampled by placing the card on a
166 white plastic dinner plate and adding distilled water, using a small paintbrush to remove all
167 pupae. Each pupa was placed into an individual well in 96-well PCR plates and preserved in
168 100% ethanol. Adults were aspirated from bottle traps hung at James Cook University,
169 Townsville (JCU), 2 days after provision of fresh banana bait and placed into individual vials
170 in 100% ethanol. JCU became a supplementary sampling site after Kirrama became
171 inaccessible due to heavy rainfall and landslides. Laboratory-reared pupae were collected
172 with forceps from standard fly food. Adults were collected with an aspirator and sexed, then
173 placed in individual vials with 100% ethanol. Laboratory isofemale lines were established
174 from the same populations sampled in the field (i.e., they were collected at the same sites
175 and shipped live to the lab in Czech Republic in 2017-18, after collection of field samples in
176 2016). Isofemale lines were kept in the lab on a standard *Drosophila* diet medium (corn
177 flour, sugar, agar, yeast, and methyl-4-hydroxybenzoate) for between 18-30 months by the
178 time of sampling.
179
180 Sample DNA was individually extracted using single column GeneAid Blood and Tissue kits
181 according to manufacturer instructions, with one extraction negative control accompanying
182 every 29 samples. For confirmation of *Drosophila* species identification we utilised a
183 multiplex PCR approach[50]. The PCRs were based on the ITS2 and COI regions using custom
184 specific forward and reverse primers. We combined primers into multiplex PCR reactions

185 based on difference in length of product. Gel electrophoresis of PCR products showed
186 product length, allowing us to identify species. When multiplex PCR was inconclusive, we
187 used Sanger sequencing with custom Diptera specific primers to identify the *Drosophila*
188 species. Full details of these processes and primer sequences can be found in the Supporting
189 Information of [50].
190
191 *2.3 Library Preparation & Sequencing*
192 After extraction and identification, all samples were moved to 96-well plates in a
193 randomised order in preparation for bacterial sequencing. DNA templates were stored at -
194 75ºC. These templates were used for amplification of ~400 bp of the V4/V5 hypervariable
195 region of the 16S rRNA gene according to Earth Microbiome Project standards (EMP;
196 http://www.earthmicrobiome.org/protocols-and-standards/16s/). Briefly, sample
197 multiplexing was based on the EMP-proposed double barcoding strategy using the EMP-
198 recommended modifications (12 bp Golay barcodes included on the forward primer 515F
199 (5′-GTGYCAGCMGCCGCGGTAA), and additional 5 bp barcodes on the reverse primer 926R
200 (5′-CCGYCAATTYMTTTRAGTTT)[52,53]. We also added a custom 18S rRNA gene blocking primer
201 (named 926X - 5′ GTGCCCTTCCGTCAATTCCT-C3 3') to counteract the low specificity of EMP
202 primers towards the 16S rRNA gene[22]. PCRs were carried out in triplicate and successful
203 amplification was confirmed with gel electrophoresis. Combined triplicates for each sample
204 were purified with AMPure XP (Beckman Coulter) magnetic beads and equimolarly pooled
205 to a single library (based on DNA concentration measured using a Synergy H1 (BioTek)
206 spectrophotometer). The library was purified using Pippin Prep (Sage Science) from all
207 fragments outside the 300-1100 bp range. To control for contamination, PCR biases and
208 confirm barcoding success, we included four negative controls from the extraction

209 procedure (ENC), eight negative controls from the PCR process (NC), and eight positive
210 controls (PC) of mock microbial communities. PCs were supplied commercially and
211 comprised 4 samples of gDNA templates with equal abundance of 10 bacterial species
212 (ATCC® MSA-1000™) and 4 samples with staggered abundance for the same bacteria (ATCC®
213 MSA-1001™). Altogether the library comprised PCR products from four 96-well plates, each
214 containing one ENC, two NCs, and two different PCs. The library was sequenced in a single
215 run of Illumina MiSeq platform using v3 chemistry with 2 x 300 bp output (Norwegian High
216 Throughput Sequencing Centre, Department of Medical Genetics, Oslo University Hospital).
217

218 *2.4 Data Processing*

219 The sequencing process returned 15,893,914 reads. These raw reads were quality checked
220 (FastQC[54]) and trimmed using USEARCH v9.2.64[55], to keep the quality score above Q20. We
221 trimmed the primers, demultiplexed and merged the reads which resulted in a final
222 amplicon length of 357 bp. We then clustered the reads at 100% identity for a
223 representative set of sequences and used the USEARCH global alignment option at both
224 99% and 97% identity[55] for *de novo* OTU assignment. We subsequently used the BLAST
225 algorithm[56] on the representative sequences, matching them against the SILVA 132
226 database[57] for taxonomic identification, producing a dataset of 1132 OTUs at 97% identity
227 and 1118 at 99% identity. We used the 97% identity OTU table as the primary dataset and
228 the 99% identity table as a supplemental dataset to confirm that the patterns we found
229 were not a product of identity threshold (Fig. S1). Raw sequence data is available on NCBI
230 Raw Sequence Archive (accession no.: PRJNA849960).
231

232 We recovered a mean of 16,898 reads per sample and a median of 14,751 reads (not
233 including negative controls). From our positive controls, we recovered microbiome profiles
234 that matched the expected community composition in each of the 'staggered' and 'even'
235 mock communities. The two low abundant species from the staggered templates (present at
236 0.04%) were successfully recovered from all four staggered mock samples. In the even
237 mocks, there was consistent overrepresentation of *Clostridium beijerinckii* and *Escherichia*
238 *coli* (1.4x - 4.7x expected), leading to reductions in other taxa. Overall, the positive controls
239 in this sequencing run matched our previous sequence outcomes[22,58].
240
241 Any chloroplast, mitochondrial, or eukaryotic OTUs were identified in the OTU table and
242 excluded. Potential bacterial contaminants were systematically evaluated using the R
243 package 'decontam' (V1.5.0[59]). The 'decontam' package uses the prevalence or frequency of
244 OTUs detected in negative controls to remove suspected contaminants. We used the
245 prevalence method, which compares the presence/absence of each sequence in true
246 positive samples compared to the prevalence in negative controls. We used a strict
247 threshold of 0.5, meaning any OTU with a higher proportion of reads in negative controls
248 than test samples was excluded as a contaminant. The prevalence of each OTU is shown in
249 Fig. S2. 43 OTUs were eliminated from the dataset via this process. Singletons were also
250 excluded, as was any OTU that made up less than one percent of the total reads for each
251 sample, which collectively removed 837 OTUs. These procedures resulted in a dataset of
252 110 OTUs and 360 samples.
253
254 *2.5 Statistical Analyses*

255 Sample analysis was carried out using the packages 'vegan'[60] and 'phyloseq'[61] in R[62]. To
256 measure alpha diversity we calculated the Shannon diversity index, using paired Wilcoxon
257 and paired ANOVA tests to compare values between sets of samples. We calculated Bray-
258 Curtis dissimilarity as a quantitative measure of beta diversity and used these values to
259 create non-metric multidimensional scaling ordinations (NMDS), to simultaneously evaluate
260 the roles of elevation, site, *Drosophila* species, life stage, environment of origin, parasitism,
261 trap identity, duration of trap exposure (and isofemale line and generation number for
262 laboratory samples). To support these ordinations statistically, we calculated PERMANOVA
263 tests on Bray-Curtis dissimilarity values. We applied the Benjamini-Hochberg (B-H)
264 correction on PERMANOVA p-values to control for multiple comparisons and false discovery
265 rate. We also evaluated statistical significance of differences in dispersion among groups
266 using multivariate homogeneity analysis (function "betadisper" in 'vegan'[60]) and ran each of
267 these tests with 999 permutations. In addition, we used DeSEQ differential abundance
268 analysis from the R package 'microbiomeSeq'[63] to compare the relative abundances of
269 bacterial taxa across different sample sets, e.g., differential abundance between *Drosophila*
270 species or between sites.
271
272 To systematically order our analyses, we first tested every variable (environment of origin,
273 elevation, gradient identity, species identity, developmental stage, trap identity, trap
274 duration of exposure, generation number, isofemale line) with all samples to determine the
275 relative importance of all studied factors (results shown below). We subsequently separated
276 the data by environment of origin, i.e., we tested lab *Drosophila* samples separately and
277 field *Drosophila* samples separately. Splitting the dataset in this way meant some variables
278 were not relevant to field samples, and vice versa. For example, when analysing field-only

279 samples we did not include isofemale line or generation number because these only
280 pertained to lab samples. Similarly, when focusing on lab-only samples, we did not include
281 trap identity, trap exposure time, or parasitoid status, because none were parasitised, and
282 traps were only a component of the field study. Furthermore, trap identity and site were
283 correlated variables because each trap was only used at one site. We tested the food/bait
284 samples separately because they were not focal samples and were used to support
285 conclusions about *Drosophila* microbiomes. The only samples positive for parasitoid
286 detection were from *Drosophila rubida* pupae, thus we only compared these samples to
287 other *D. rubida* pupae, and not as part of the core analyses.

288

289 **3. Results**

290 We first tested which of the studied factors influenced microbiome composition in all core
291 field and lab samples combined, using PERMANOVA analysis on Bray-Curtis values. The
292 dominant explanatory variable was environment of origin (i.e., whether a sample came from
293 the lab or field; NMDS mean stress ≈ 0.15, PERMANOVA, $R^2 = 0.150$, Benjamini-Hochberg
294 corrected $p \leq 0.001$; Beta-dispersion $F = 126.8$, $p \leq 0.001$; Table S1, Fig. 1). The R^2 values (see
295 Table S1) indicated that environment of origin was clearly an overarching explanatory
296 variable, thus we decided to further analyse the field and lab samples separately to
297 establish the important deterministic factors within each environment. The main trend in
298 our results was a significant reduction in microbiome richness in lab-reared flies of all
299 species based on a paired Wilcoxon rank sum test between Shannon index values for lab
300 and field samples (B-H corrected $p \leq 0.001$; Fig. 2). This significant trend held when pupae or
301 adult *Drosophila* were analysed separately (Fig. S3, S4, S5).

302

303 *3.1 Microbiome patterns from field samples*

304 When focusing on core field samples, the main factors explaining variation in microbiome
305 structure were trap identity (PERMANOVA R^2 = 0.13, B-H corrected $p \leq 0.001$; Beta-
306 dispersion $p \leq 0.001$) and the interaction between site & trap (PERMANOVA R^2 = 0.13, B-H
307 corrected $p \leq 0.001$; Beta-dispersion $p \leq 0.001$; Table S2), suggesting that local
308 environmental differences in site location, as well as trap identity, have a significant effect
309 on *Drosophila* microbiome composition (Fig. 3). Elevation alone explained a small, but still
310 significant, proportion of the variation observed (R^2 = 0.03). The banana-baited bottle traps
311 were left exposed in the field for different durations (between 11 and 24 days) to ensure we
312 characterized the *Drosophila* community fully. PERMANOVA results suggest that the length
313 of exposure had significant influence on microbiome composition but of lower importance
314 compared to the other factors we identified based on the amount of variation explained (R^2
315 = 0.03). Based on the PERMANOVA analysis, field site was a significant variable but only
316 explained 7% of the variation in diversity (reflected in the minimal differences in average
317 Shannon index value in Fig. S6). We also found evidence of *Drosophila* species-specific
318 differences in microbiome diversity, based on paired Wilcoxon tests between Shannon index
319 values for different species ($p \leq 0.001$; Fig. 4). DeSEQ analysis of differential abundance
320 indicated some bacterial genera were significantly more abundant in some *Drosophila*
321 species but not others, most prominently *Kozakia* and *Corynebacterium* (Table S3). The
322 most abundant bacterial genera (Fig. 5) were evenly distributed throughout all four
323 *Drosophila* species sampled here, including *Acinetobacter,* which was the most dominant
324 genus overall.

325

326 *3.2 Microbiome patterns from laboratory samples*

327 The main factors explaining variation in microbiome structure from the lab-reared samples
328 were *Drosophila* species identity (PERMANOVA R^2 = 0.11, B-H corrected $p \leq 0.001$, Beta-
329 dispersion $p \leq 0.001$), isofemale line (PERMANOVA R^2 = 0.13, B-H corrected $p \leq 0.001$, Beta-
330 dispersion $p \leq 0.001$), and the number of generations an isofemale line had been in the lab
331 (PERMANOVA R^2 = 0.12, B-H corrected $p \leq 0.001$, Beta-dispersion $p = 0.007$; Table S4),
332 suggesting that the duration a lineage of flies had been in the lab environment was
333 important for explaining microbiome composition. The amount of variation explained by
334 species-specific differences can largely be attributed to the presence of *Wolbachia* in *D.*
335 *pseudoananassae* (Fig. 4 & Fig. S7), because the alpha diversity differences between species
336 were not significant (Shannon index values, Fig. 6). All four *Drosophila* species contained
337 high proportions of *Acetobacter*, *Lactobacillus* and *Gluconobacter*, but only *D.*
338 *pseudoananassae* contained *Wolbachia*. The interaction between isofemale line and
339 generation was also highly significant and explained a high proportion of variation relative
340 to other factors, which was expected because IF lines were established at different times,
341 and therefore had been in the lab for varying numbers of generations at the time of
342 sampling. Life stage (i.e., the difference between pupae and adults) was not one of the most
343 important explanatory factors based on the amount of variation explained (PERMANOVA R^2
344 = 0.06). There was no detectable effect of elevation (PERMANOVA R^2 = 0.01), i.e., the
345 elevation of the field site where an isofemale line was collected did not have a significant
346 effect on the microbiome structure in the lab.

347

348 *3.3 Comparisons between field and laboratory samples and microbiome of the food source*

349 There was no significant difference in microbiome composition of laboratory food samples
350 from Czech Republic and the banana bait that we used in the field in Australia (Paired
351 Wilcoxon test on Shannon index values, $p = 0.09$; Fig. S8). Banana bait and lab fly food both
352 contained high relative proportions of *Acetobacter* and *Lactobacillus,* with some lab food
353 samples containing *Gluconobacter* (Fig. S9). Despite the physical differences in food sources,
354 the most abundant bacterial genera were the same, and the food source microbiome
355 diversity was low. In lab-reared flies, these three bacterial genera dominated the
356 microbiomes of pupae and adults (Fig. S7). In the field, however, *Acetobacter* and
357 *Lactobacillus* were not the most abundant genera of either pupae or adult gut microbiomes.
358 *Acetobacter* and *Lactobacillus* had the greatest difference in relative abundance between
359 field and lab samples (summary statistics in Table 2, Fig. S10). Whilst still present in field
360 samples, the relative abundance of *Acetobacter* and *Lactobacillus* was proportionally lower
361 in their taxa-rich microbiomes (Fig. 1).
362
363 *3.4 Robustness of results*
364 There was no detectable difference in microbiome diversity of parasitised pupae of *D.*
365 *rubida,* compared to unparasitized pupae (Paired Wilcoxon test on Shannon index values, p
366 = 0.25; Fig. S11), nor were there bacterial genera unique to parasitised samples. We also
367 found that the 99% identity OTU table produced qualitatively the same results as the 97%
368 identity OTU table. For example, in an NMDS on all samples the dominant explanatory
369 variable was still environment of origin (NMDS mean stress ≈ 0.17, PERMANOVA, $R^2 = 0.17$,
370 B-H corrected $p \leq 0.001$; Beta-dispersion $p \leq 0.001$; Fig. S1). Removing *Wolbachia* from the
371 dataset also did not qualitatively change the outcome of statistical tests, e.g., the main
372 factors explaining microbiome structure in lab-reared samples were *Drosophila* species

373 identity (PERMANOVA R^2 = 0.11, B-H corrected $p \leq 0.001$, Beta-dispersion $p \leq 0.001$),
374 isofemale line (PERMANOVA R^2 = 0.17, B-H corrected $p \leq 0.001$, Beta-dispersion $p \leq 0.001$),
375 and the number of generations an isofemale line had been in the lab (PERMANOVA R^2 =
376 0.12, Benjamini-Hochberg corrected $p \leq 0.001$, Beta-dispersion $p \leq 0.001$).
377

378 **4. Discussion**

379 Our results revealed small but significant variation in microbiome structure between
380 *Drosophila* populations from high and low elevation across both gradients. We expected to
381 find greater differences across elevation, because there is well-documented evidence of
382 both insects and bacteria developing differently according to differences in temperature of
383 >5°C[29–31,33,34,64]. This finding could be a result of the *Drosophila* species sampled here being
384 ubiquitous across elevation without forming sufficiently distinct populations at high and low
385 elevation sites, or because the ~5°C temperature shift between our sites was not strong
386 enough to drastically alter microbiome composition. Standardising diet may have
387 homogenised *Drosophila* microbiomes to some extent, but the overall lack of similarity in
388 microbiome composition between food microbiome and field-caught pupae suggests that
389 diet was not a dominant variable in structuring *Drosophila* microbiomes.
390

391 The most pronounced differences in microbiome composition were between individuals
392 raised in the laboratory and those raised in the field. Interestingly, the two food sources
393 (banana bait in the field and yeast-based *Drosophila* medium in the lab) had very similar
394 microbiome profiles, suggesting that dietary factors were not primarily responsible for the
395 observed differences between environments. The bacterial community from lab food

396 matches well with the microbiomes found within lab-reared pupae and adult flies. This was
397 expected, because it reflects a well-established pathway of insect microbiome colonisation -
398 *Drosophila* ingest food and acquire bacteria associated with that food source[36]. Yet in the
399 field, *Drosophila* microbiomes do not correspond well with the bacterial communities found
400 on banana bait samples. The observed pattern can be explained by significant differences in
401 microbiome colonisation from environmental bacterial species pools[65,66]. The flies sampled
402 from the lab come from a highly regulated environment, with a specific and consistent food
403 source provided into heat-sterilised glass vials, so the only 'available' bacteria for colonising
404 their microbiomes come from the diet, surrounding lab environment, and vertically
405 inherited endosymbionts (e.g., *Wolbachia* in *D. pseudoananassae*). In contrast, the bacterial
406 species pool in Australian tropical rainforest comprises much greater diversity and
407 abundance of different taxa, creating a greater variety of possible microbiome composition
408 within *Drosophila* hosts. This diversity of taxa creates more room for ecological drift,
409 dispersal, and selection to act on microbiome communities, in turn creating greater among-
410 individual and between-species variation in wild flies. Consistently higher diversity in wild
411 *Drosophila* microbiomes suggests that microbiomes are predominantly colonised from the
412 wider environment and dependent on local species pool diversity. For instance, bottle traps
413 were visited by other organisms - e.g., staphylinid beetles, neriid flies, Lepidoptera - which
414 could also have been a source of bacteria colonising the microbiome of the *Drosophila*
415 sampled in this study.

416

417 There was consistently low microbiome diversity found in both lab-reared pupae and lab-
418 reared adults, suggesting that low diversity within pupae is an accurate representation of
419 lab-reared microbiomes. This result was surprising because we anticipated some stage-

420 specific microbiome community patterns, given that *Drosophila* are holometabolous and
421 thus undergo substantial gut remodelling during complete metamorphosis[67]. The
422 consistency across life stages from lab-reared individuals provides further evidence for the
423 depauperate nature of the lab microbial environment. In contrast to the lab, the field-
424 caught adults of *D. rubida* lacked congruence with the field-caught pupae, and both stages
425 lack similarity with the banana bait (Fig. S12, S13). The lack of geographic variation in
426 microbiome composition suggests that different sites are unlikely to fully explain the
427 discrepancy. With adult flies we cannot rule out that they might have fed on a substance
428 other than our yeasted banana bait prior arriving at our bottle traps, making dietary
429 variation a parsimonious explanatory factor in life-stage differences[36,68]. It is also possible
430 that the high diversity we found in pupal samples reflects their metabolic activity (despite
431 their lack of feeding), given that complete metamorphosis is an intense period of organismal
432 change[69]. The substantial differences in microbiomes between lab and field specimens
433 suggests that future studies should be cautious and specific with the types of microbiome-
434 related questions investigating laboratory flies. Interpreting microbiome community
435 composition from lab-kept specimens, particularly those from cultures maintained across
436 multiple generations, is unlikely to yield data entirely representative of natural
437 microbiomes[70,71], except perhaps in scenarios where microbiome communities from lab-
438 reared specimens are a subset of the more diverse microbiomes found in the field (as our
439 results show in Figure 5).
440
441 Previous studies on *Drosophila* have demonstrated high intra- and interspecific variation in
442 microbiome community composition from both wild-caught and lab-reared flies[39,40,70,72,73],
443 which our results corroborate. Controlling for diet in both scenarios allowed us to recognise

444 this species-specificity more accurately. We found a significant effect of species identity
445 (11% variation explained) but it did not explain as much variation as the results from Adair
446 et al.[40] who found species identity explaining 42% and 70% variation in two different sets of
447 *Drosophila spp*. This discrepancy could be a product of the species themselves (i.e., we used
448 a different set of *Drosophila* species) or the number of species studied (we studied four
449 species here; Adair et al.[40] eighteen), but the evidence from both studies suggests that
450 species-specificity is maintained in the lab, albeit not in a consistent manner. Statistically
451 significant differences in microbiome alpha diversity measures between different *Drosophila*
452 species in the field were not reflected in the lab. From the field, the only significant
453 difference in microbiome richness was between *D. pseudoananassae* and *D. rubida*, and this
454 was non-significant in the lab. Moreover, there was a highly significant difference in richness
455 between *D. rubida* and *D. sulfurigaster* in the lab, but not in the field. Some of our
456 significant PERMANOVA results were accompanied by significant Beta-dispersion, which
457 suggests that there was some heterogeneous dispersion in the variables we measured. This
458 was expected based on the high variation typically exhibited by microbiome community
459 data and typically found when sampling wild individuals. Even with greater and more even
460 sample size, we would still expect to find a large, significant difference in community
461 richness and composition between field and lab *Drosophila* microbiomes.

462

463 In other insect species, host transmission of extracellular symbionts (like those in the gut)
464 have been hypothesised to result in long-term associations between insect and microbe[74–
465 76]. The long-term laboratory survival of our four *Drosophila* species (minimum 24
466 generations) with radically different microbiome composition (compared to their
467 counterparts from the field) suggests that their symbiotic relationship with bacteria is not

468 highly specialised[77], corroborating previously published findings. For example, Wong et al.[37]
469 found no consistent evidence for a core microbiome in multiple *Drosophila* species and
470 Storelli et al.[78] described axenic *Drosophila* returning to normal growth and development in
471 the presence of a single bacteria species. Furthermore, Coon et al.[79,80] showed that any
472 bacterium was sufficient to facilitate mosquito larval development, whereas axenic
473 mosquito larvae do not develop past the first instar. Thus, our results provide further
474 evidence of satisfactory microbiome function being provided by a limited number of
475 bacterial species, which poses questions about the advantages of diverse microbiomes
476 within wild insects and the links between microbiome community diversity and host fitness.
477
478 Overall, we found significant differences in microbiome diversity of field-caught and lab-
479 reared *Drosophila*, which were consistent across species and life stage. We hypothesise that
480 these differences in diversity are the products of environments with markedly different
481 bacterial species pools. To elucidate functional conclusions from insect-microbiome
482 analyses, more in-depth molecular analysis (e.g., metagenomics, transcriptomics) is
483 required. We suggest that comparative studies between lab and field specimens help reveal
484 the true variability in microbiome communities that can exist within a single species.

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Table 1: Breakdown of the sample set used in this study. PL = Paluma Low, PH = Paluma High, KL = Kirrama Low, KH = Kirrama High, JCU = James Cook University campus in Townsville.

Species	Stage	Environment of origin	Sites	Number of samples	Core or Additional
D. rubida	pupae	field	PL, PH, KL, KH	71	Core
D. rubida (parasitised)	pupae	field	PL, PH, KL, KH	8	Core
D. rubida	adult	field	JCU	14	Core
D. pseudoananassae	pupae	field	PL, PH, KL, KH	48	Core
D. pallidifrons	pupae	field	PL, PH, KL, KH	39	Core
D. sulfurigaster	pupae	field	PL, PH, KL, KH	10	Core
D. rubida	pupae	lab	PL, PH, KL, KH	20	Core
D. rubida	adult	lab	PL, PH, KL, KH	20	Core
D. pseudoananassae	pupae	lab	PL, KL, KH	20	Core

D. pseudoananassae	adult	lab	PL, KL, KH	20	Core
D. pallidifrons	pupae	lab	PH, KH	10	Core
D. pallidifrons	adult	lab	PH, KH	10	Core
D. sulfurigaster	pupae	lab	PL, PH, KL, KH	20	Core
D. sulfurigaster	adult	lab	PL, PH, KL, KH	20	Core
banana bait	na	field	PL, PH, JCU	20	Additional
lab fly food	na	lab	na	10	Additional

Figure 1: NMDS analysis of microbiome communities from all samples in this study, from the lab (blue) and the field (red). Samples of *Drosophila rubida*, *D. pseudoananassae*, *D. pallidifrons*, *D. sulfurigaster*, and food bait are indicated by different shapes.

Figure 2: Comparison of Shannon index values for all samples in this study, split by environment of origin. Lab samples are shown in blue and field samples in red. The triple asterisk (***) shows high significance ($p > 0.001$) of a paired Wilcoxon test.

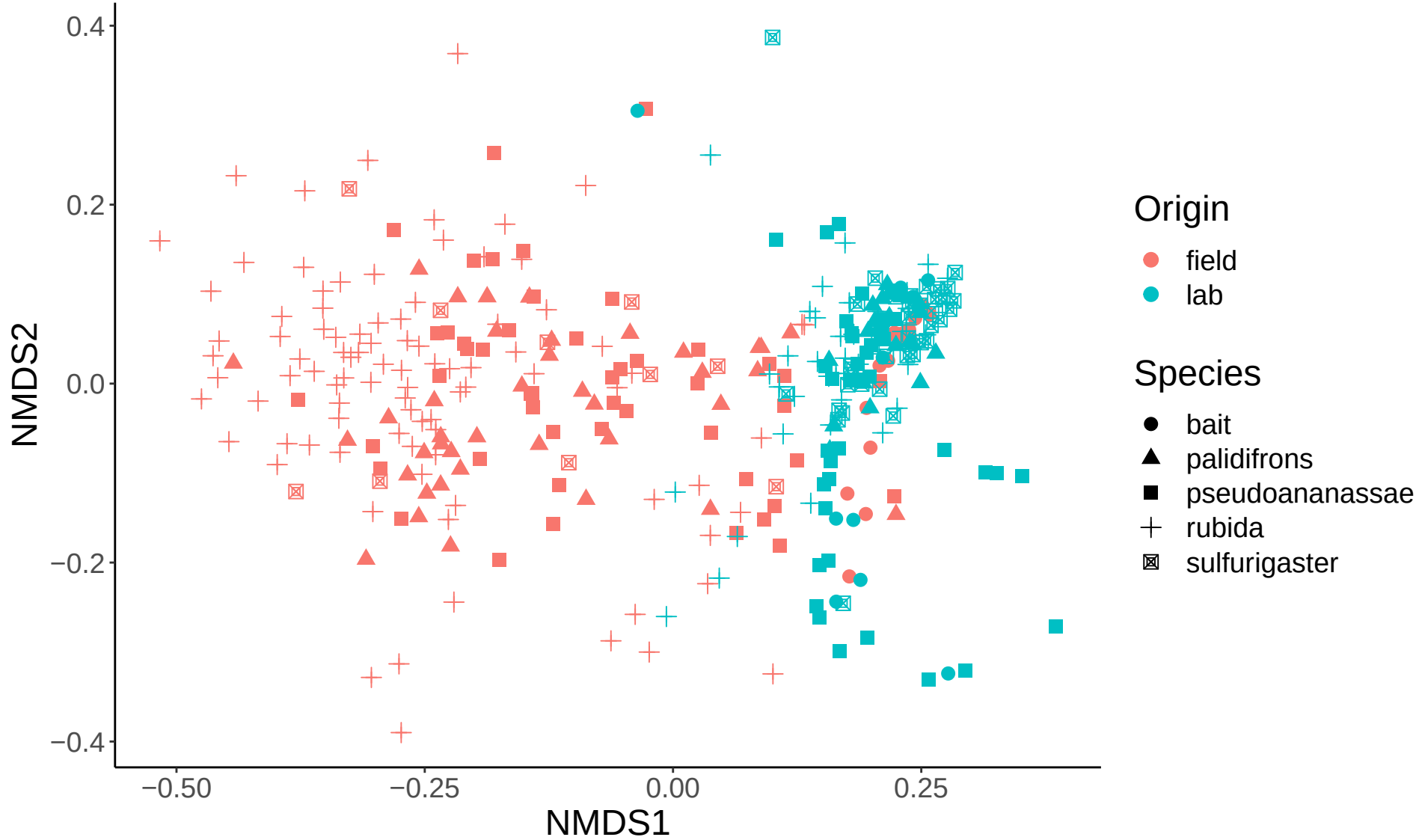
Figure 3: PCoA analysis of microbiome communities from the core field samples. Samples of *Drosophila rubida*, *D. pseudoananassae*, *D. pallidifrons*, and *D. sulfurigaster* are indicated

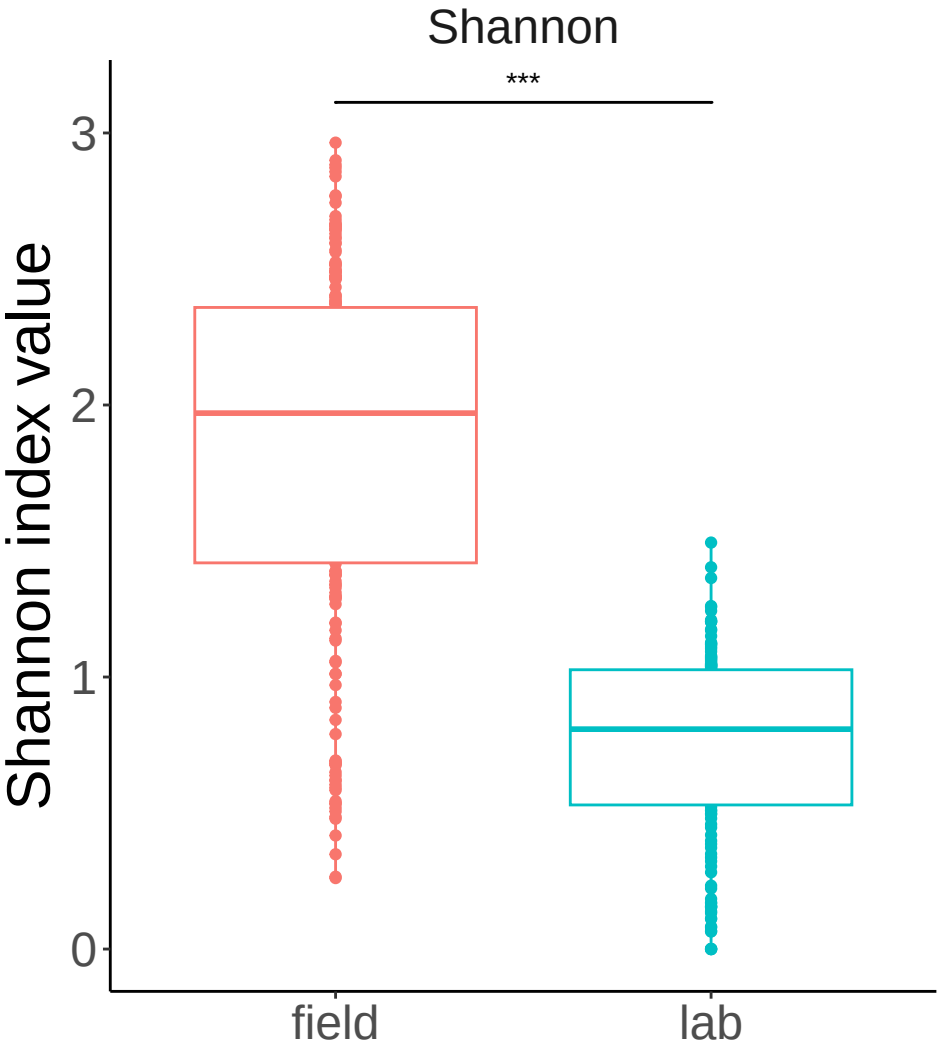
740 by different colours, and the different sites of collection are indicated by shape. K = Kirrama,
741 P = Paluma, JCU = James Cook University, L = Low [elevation], H = High [elevation]. Samples
742 of *D. rubida* collected at JCU were all adults.
743
744 **Figure 4: Comparison of Shannon index values from each species of *Drosophila* from (A)**
745 **field and (B) lab environments.** Each colour represents a different species. Statistical
746 comparisons come from paired Wilcoxon tests. Three asterisks (***) denotes a highly
747 significant result ($p \leq 0.001$). Two asterisks (**) indicates a result of moderate significance
748 (between $p < 0.1$ and $p \leq 0.001$). One asterisk (*) denotes a marginally significant result ($p <$
749 0.1). NS. = not significant.
750
751 **Figure 5: The most abundant bacterial genera from all samples in this study.** Different
752 colours mark different bacterial genera. <2.5% is a conglomerate category of low abundance
753 taxa that made up less than 2.5% of the median number of reads. Each individual column
754 represents an individual sample. Relative abundance is on the y axis.
755
756 **Figure 6: NMDS analysis of microbiome communities from core lab samples.** Samples of
757 *Drosophila rubida*, *D. pseudoananassae*, *D. pallidifrons*, and *D. sulfurigaster* are indicated by
758 different colours and shapes.

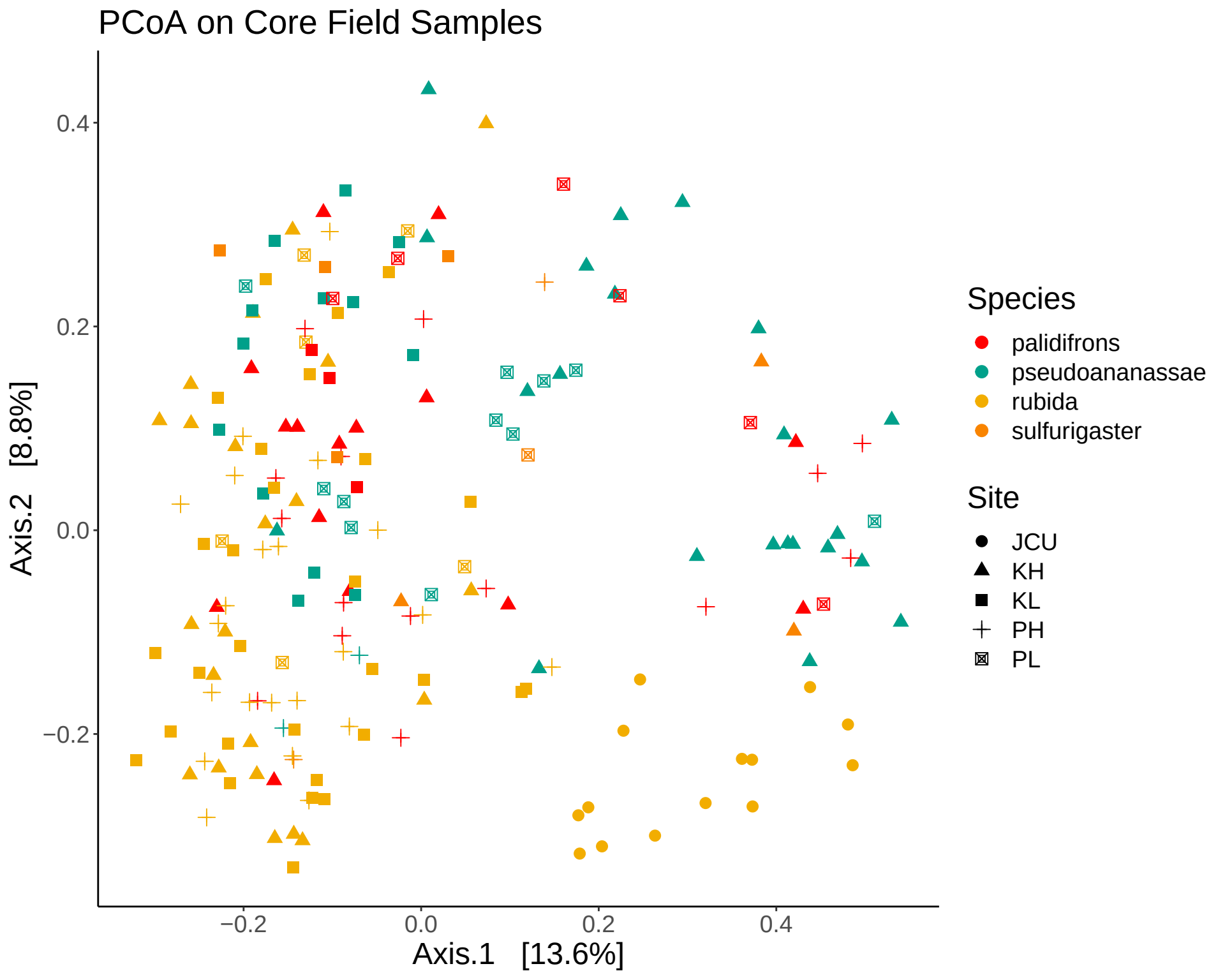
Table 2: Summary output of DeSEQ differential abundance analysis showing the top 5 bacterial genera by origin of sample. Positive test statistic values indicate a genus significantly more abundant in lab samples and negative test statistics indicate a genus significantly more abundant in field samples.

Genus	baseMean	log2foldchange	lfcSE	Test statistic	Adjusted p value
Acetobacter	436.99	3.59	0.38	9.46	≤ 0.001
Lactobacillus	183.75	3.16	0.38	8.34	≤ 0.001
Acinetobacter	195.62	-7.91	0.33	-23.70	≤ 0.001
Klebsiella	105.22	-7.01	0.37	-18.99	≤ 0.001
Chishuiella	59.69	-6.19	0.41	-14.97	≤ 0.001

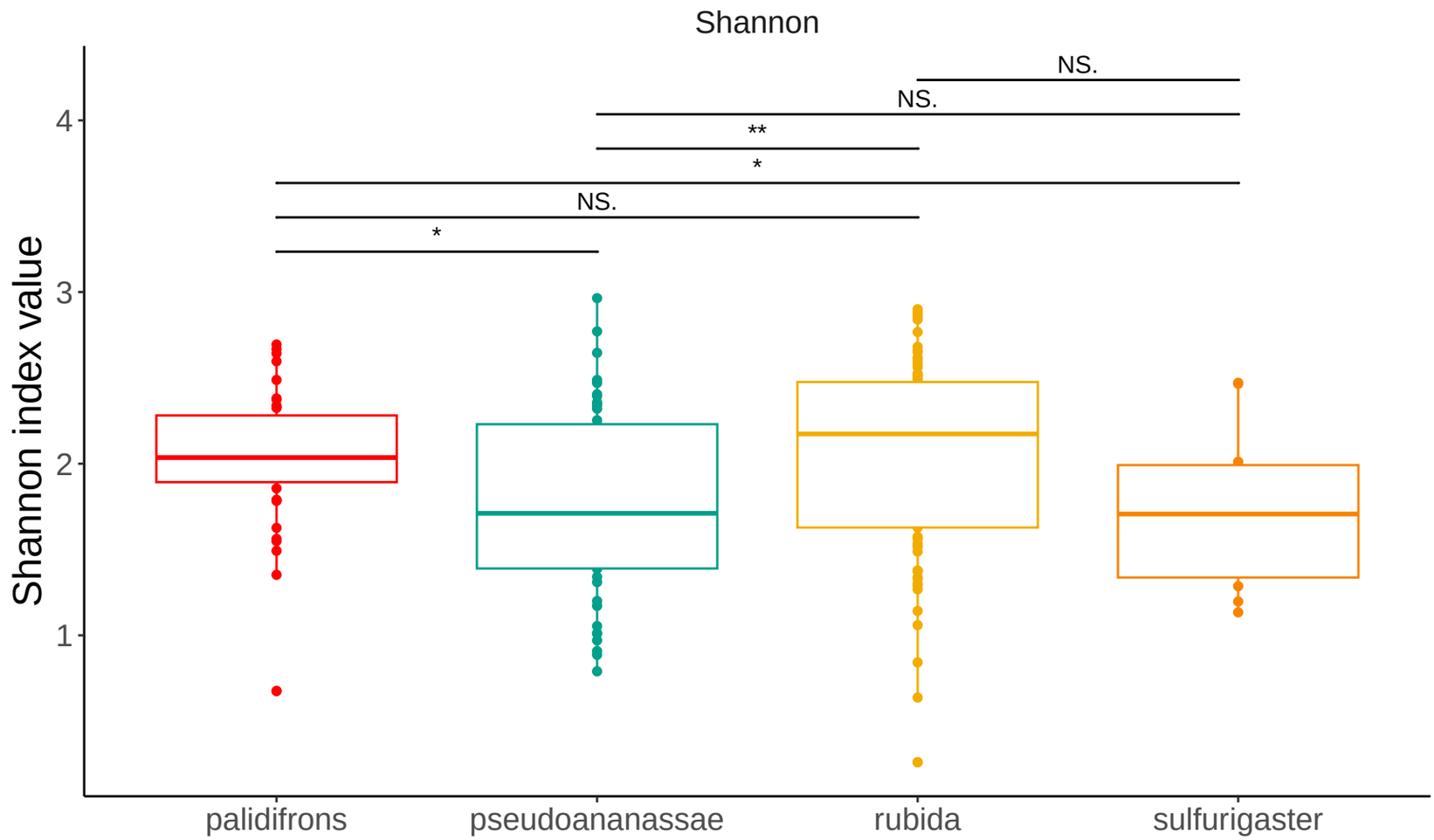
759


0.4
0.2
0.0
−0.2
−0.4
NMDS2
−0.50
−0.25
0.00
0.25
NMDS1
Origin
field
lab
Species
bait
palidifrons
pseudoananassae
rubida
sulfurigaster

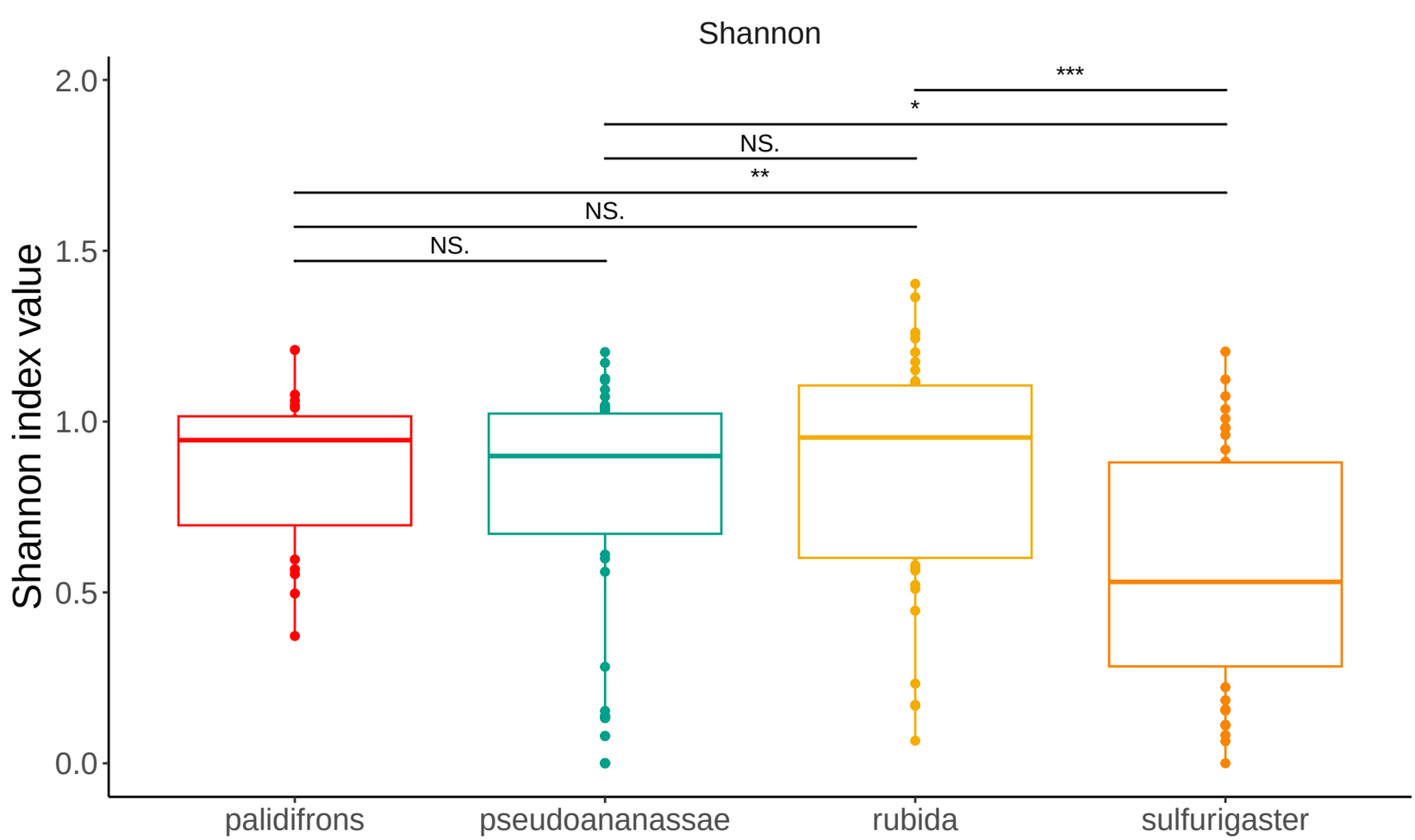

Shannon
***
Shannon index value
3
2
1
0
field
lab

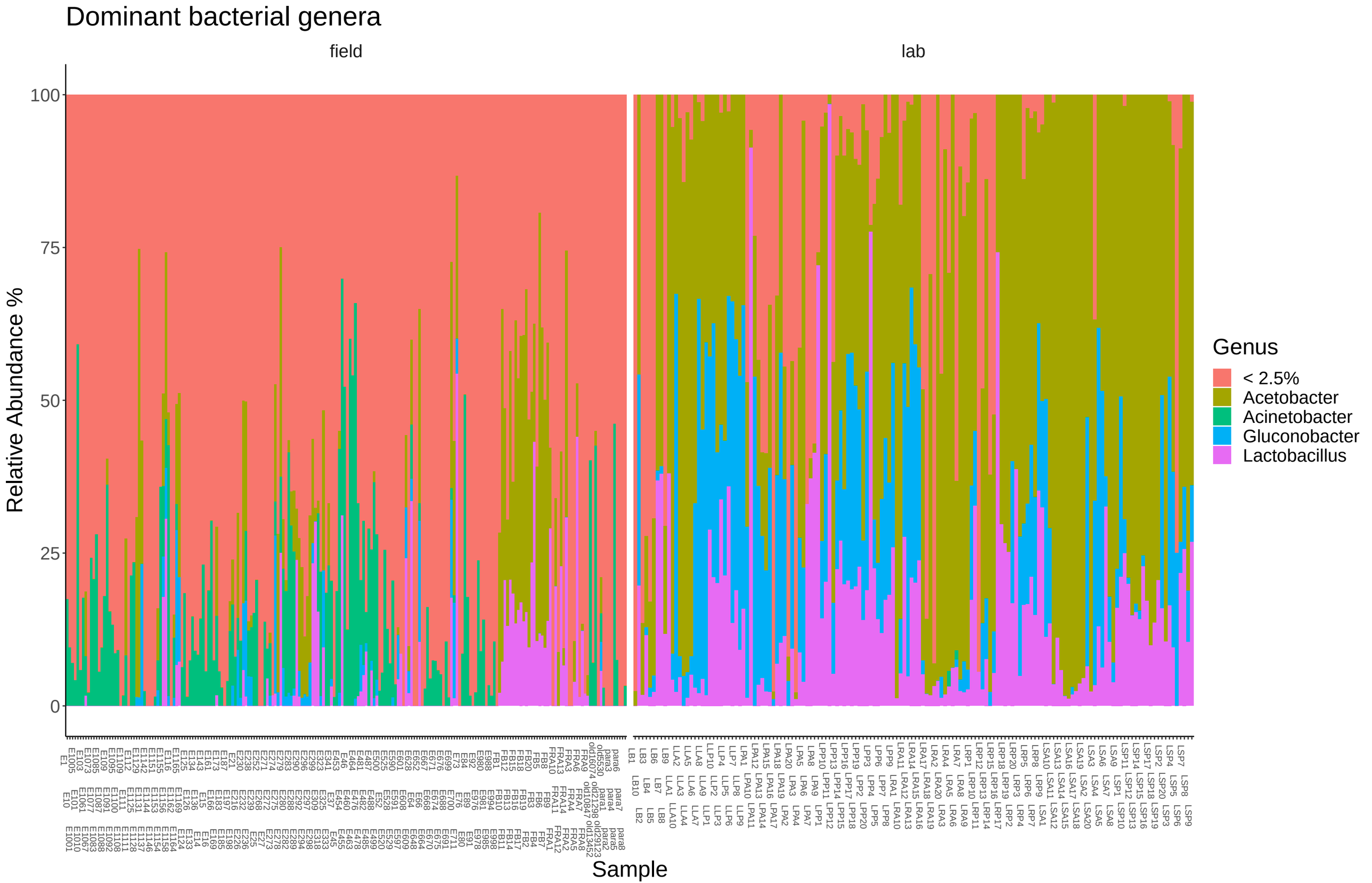

PCoA on Core Field Samples
0.4
0.2
0.0
−0.2
Axis.2 [8.8%]
−0.2
0.0
0.2
0.4
Axis.1 [13.6%]
Species
palidifrons
pseudoananassae
rubida
sulfurigaster
Site
JCU
KH
KL
PH
PL

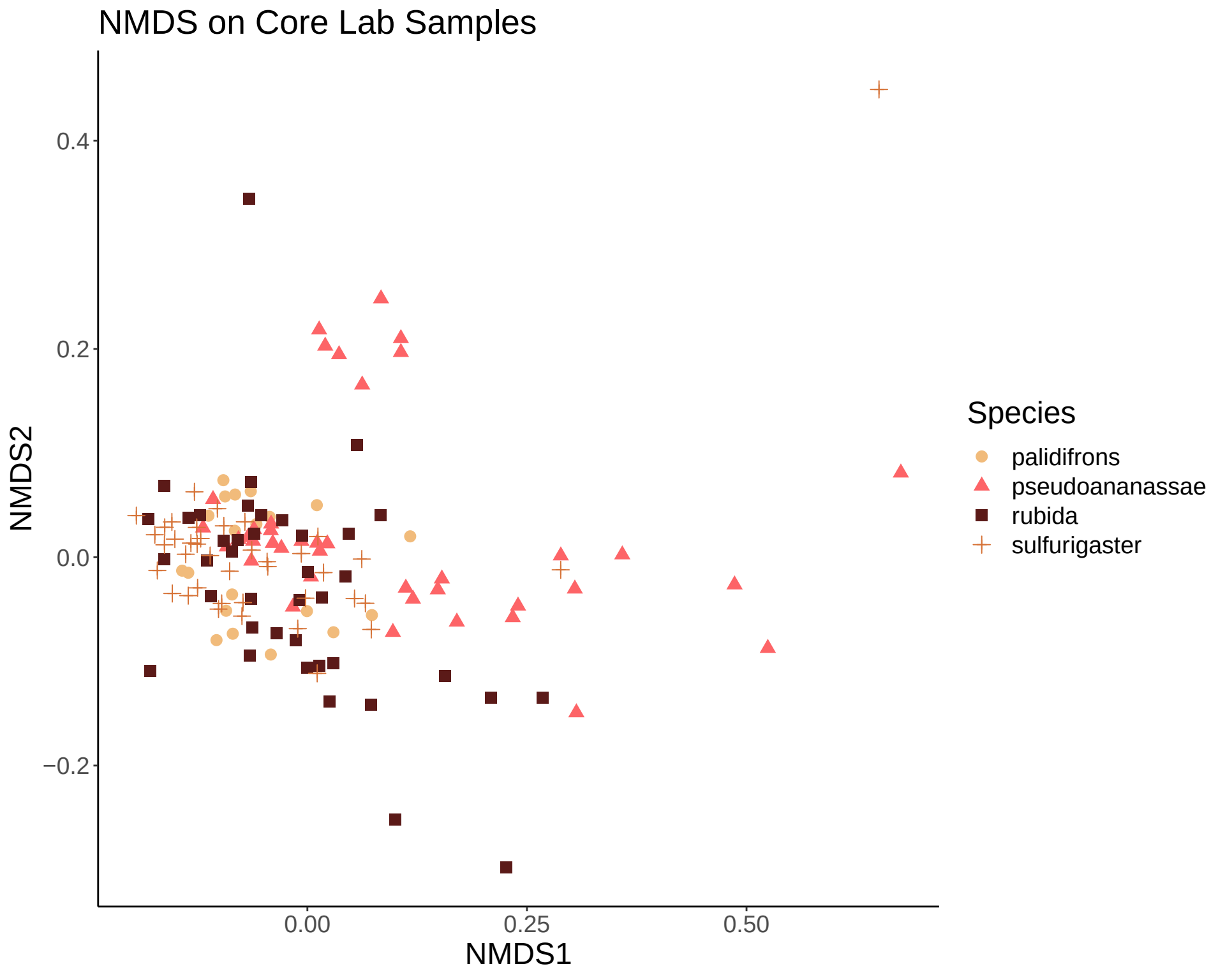
A: Field


Shannon
NS.
NS.
**
*
NS.
*
4
3
2
1
Shannon index value
palidifrons
pseudoananassae
rubida
sulfurigaster

B: Lab


Shannon
***
*
NS.
**
NS.
NS.
2.0
1.5
1.0
0.5
0.0
Shannon index value
palidifrons
pseudoananassae
rubida
sulfurigaster


Dominant bacterial genera
field
lab
Relative Abundance %
100
75
50
25
0
Sample
Genus
< 2.5%
Acetobacter
Acinetobacter
Gluconobacter
Lactobacillus


NMDS on Core Lab Samples
0.4
0.2
0.0
−0.2
NMDS2
0.00
0.25
0.50
NMDS1
Species
palidifrons
pseudoananassae
rubida
sulfurigaster