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1 **Title:** Diverse laboratory colonies of *Aedes aegypti* harbor the same adult midgut
2 bacterial microbiome
3

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26
Abstract

27 **Background:** Host-associated microbes, collectively known as the microbiota,
28 play an important role in the biology of multicellular organisms. In mosquito
29 vectors of human pathogens, the gut bacterial microbiota influences vectorial
30 capacity and has become the subject of intense study. In laboratory studies of
31 vector biology, genetic effects are often inferred from differences between
32 geographically and genetically diverse colonies of mosquitoes that are reared in
33 the same insectary. It is unclear, however, to what extent genetic effects can be
34 confounded by uncontrolled differences in the microbiota composition among
35 mosquito colonies. To address this question, we used 16S metagenomics to
36 compare the midgut bacterial microbiome of six recent laboratory colonies of
37 *Aedes aegypti* representing the geographical range and genetic diversity of the
38 species.

39 **Results:** We found that the diversity, abundance, and community structure of the
40 midgut bacterial microbiome was remarkably similar among the six different
41 colonies of *Ae. aegypti*, regardless of their geographic origin. We also confirmed
42 the relatively low complexity of bacterial communities inhabiting the mosquito
43 midgut.

44 **Conclusions:** Our finding that geographically diverse colonies of *Ae. aegypti*
45 reared in the same insectary harbor a similar gut bacterial microbiome supports
46 the conclusion that the gut microbiota of adult mosquitoes is environmentally
47 determined regardless of the host genotype. Thus, uncontrolled differences in
48 microbiota composition are unlikely to represent a significant confounding factor
49 in genetic studies of vector biology.

50 **Keywords:** Mosquito; Microbiota; Vectorial capacity; Metagenomics.

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51 ## Background

52 The mosquito, *Aedes aegypti*, is the main vector of several medically
53 important arboviruses such as Zika, dengue, chikungunya, and yellow fever
54 viruses worldwide. Dengue viruses alone are responsible for 390 million human
55 infections each year [1]. In the absence of vaccines or specific therapeutics for
56 most arboviruses, controlling mosquito vector populations is the primary disease
57 prevention strategy [2]. With the rise of insecticide resistance, the development of
58 novel entomological interventions is underway [3, 4]. Critical to the development
59 of these new vector control methods is an improved understanding of the biology
60 of mosquito vectors such as *Ae. aegypti* [5].

61 Over the last several decades, research efforts have focused on trying to
62 elucidate the genetic [6-8] and environmental [9-12] factors that contribute to
63 variation in the ability of *Ae. aegypti* to transmit human pathogens. Only in recent
64 years, however, has the importance of the microbiota (i.e., host-associated
65 microbes) emerged in vector biology. The gut bacterial microbiota, in particular,
66 influences multiple aspects of the mosquito's biology including vector competence
67 [13, 14] and has become a topic of extensive research. Manipulation of the
68 bacterial species present in the mosquito midgut has been shown to both increase
69 or decrease the amounts of dengue virus, chikungunya virus, or *Plasmodium*
70 *falciparum* [15-19]. The composition of the microbiome (i.e., the collective
71 genomes of the microbiota) found in the midgut of mosquitoes is highly variable
72 and dependent on the environment [20-23] and life stage [24-26].

73 To identify mosquito genetic components of vectorial capacity, researchers
74 often use genetically diverse colonies of mosquitoes reared in the same
75 environment. Observed differences in the vectorial capacity of genetically diverse

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76 laboratory colonies is generally attributed to host genetics, and not to potential
77 differences in the gut microbiota, but it remains poorly understood what role
78 mosquito genetics plays in shaping the gut microbiome and whether subtle
79 differences in the microbiome could confound genetic studies. It was recently
80 shown that the gut microbiota can be disrupted by genetic modification of
81 mosquitoes [27]. In more natural insect systems such as the relationship between
82 aphids, intracellular bacteria, and parasitic wasps, bacterial symbionts and not the
83 aphid genotype drive the specificity of the interactions between the aphid and the
84 parasitic wasp [28-30]. In other insects, it has been demonstrated that the gut
85 microbiota contributes to host genotype by parasite genotype interactions [31],
86 suggesting that differences in the microbiota should be considered as an
87 additional factor when elucidating the host genetic contribution to a specific trait.
88 In *Ae. aegypti*, previous observations of bacterial taxa specific to certain
89 mosquito lines reared in the same insectary [32, 33] raise the question whether
90 differences in gut microbiota could confound interpretation of phenotypic
91 differences among mosquito colonies. To address this question, we used a
92 targeted metagenomics approach to compare the gut microbiome between six
93 recent colonies of *Ae. aegypti* representing the geographical range and genetic
94 diversity of the species. We performed a comprehensive metagenomics analysis
95 including comparison of bacterial diversity within and between samples as well
96 as identifying bacterial genera that are differentially abundant between colonies.
97 Our results provide empirical evidence that adult *Ae. aegypti* mosquitoes reared
98 in the same insectary harbor a similar gut bacterial microbiome, regardless of
99 their geographic origin.
100

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101 **Results**

102 To test if laboratory colonies of natural populations of *Ae. aegypti* differ in
103 the diversity and composition of their gut microbiome, the V5-V6 variable region
104 of the 16S ribosomal RNA gene was sequenced in 16-18 individual adult female
105 midguts from each of six recent colonies of *Ae. aegypti*. The six colonies chosen
106 represent the geographical range and genetic diversity of the species (Figure 1)
107 and have spent from three to ten generations in the laboratory (Table 1). The
108 experimental design included three replicate adult cages per colony and the
109 individual libraries were randomized across two separate sequencing runs.
110 Individual midguts were aseptically dissected from nulliparous, 4- to 6-day-old
111 females that had been allowed to mate and feed on sugar following emergence.
112 Out of the 96 individual gut microbiomes sequenced, 2,679 operational taxonomic
113 units (OTUs) representing 587 different bacterial genera were identified.
114 Rarefaction curves showed that a sufficient number of sequencing reads was
115 achieved to comprehensively characterize the bacterial communities in the
116 midgut (Supplemental Figure 1).

117 To determine if the gut microbiome of each *Ae. aegypti* colony varies in the
118 diversity of bacterial species present, the within-colony diversity was evaluated
119 by determining the genus richness and the Shannon diversity index. No
120 differences in the levels of richness (Figure 2A) or in Shannon diversity index
121 (Figure 2B) were observed between the colonies (ANOVA: $F = 1.125$, p value =
122 0.353 and $F = 0.522$, p value = 0.759, respectively). In addition, the taxonomical
123 abundance of bacteria was highly similar between the colonies, indicating the
124 dominant bacterial genera in the midgut are not dependent on the colony (Figure
125 3).

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126 To identify dissimilarities in the bacterial community structure between
127 the gut microbiome of laboratory colonies of *Ae. aegypti*, principal coordinates
128 analysis (PCoA) was performed based on a Bray-Curtis dissimilarity matrix. The
129 PCoA showed that the bacterial community structures of all six colonies were
130 highly similar to each other (p value = 0.752) (Figure 4A). In addition, no
131 differences in the bacterial community structure were observed between the
132 replicate cages of each colony, however the bacterial community structure
133 differed between sequencing runs (Supplemental Figure 2). The reason for the run
134 effect is unclear but it could reflect preferential clustering of specific sequences on
135 the flow cell. Although the community structure of gut microbiome of the colonies
136 was similar overall, we tested whether some specific bacterial taxa were
137 differentially abundant. Out of the 587 bacterial genera identified, only zero to six
138 genera were differentially abundant in pairwise comparisons of the six colonies
139 (Supplemental Table 1; Figure 4B) resulting in 98-100% similarity in the
140 abundance of genera present between colonies.

141

142 **Discussion**

143 We performed a 16S metagenomics analysis to compare the midgut
144 microbiome of six recent colonies of *Ae. aegypti* reared in the same insectary
145 environment. The six colonies were chosen to represent the natural global
146 distribution of the species. Although these colonies represent different genetic
147 backgrounds and different generation times in the laboratory, the gut microbiome
148 was highly similar among all six colonies. We did not observe any differences in
149 the diversity of the bacterial communities or in the bacterial community structure

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150 within the gut. The taxonomical abundance was also similar between the colonies
151 with 98-100% identity in the abundance of bacterial genera present between
152 colonies. The data also confirmed the relatively low complexity of bacterial
153 communities typically found in the gut of insects [34, 35].

154 Other studies that have compared the midgut microbiome of various
155 laboratory colonies of *Ae. aegypti* observed differences in the taxonomical
156 identification of specific bacterial species [32, 33]. Although these studies
157 reported differences in the abundance of specific taxa between colonies of *Ae.*
158 *aegypti*, no difference in the bacterial community structure was in fact observed.
159 Furthermore, the colonies tested in previous studies have been maintained in the
160 laboratory for five to 80 years before their microbiome was examined. It is
161 possible that large differences in the number of generations spent in the
162 laboratory between these studies and ours, resulted in our different observations.
163 Possibly, preferential associations between mosquito genotypes and specific
164 laboratory bacteria may evolve over a long colonization history. This hypothesis
165 remains to be tested.

166 Researchers often use genetically diverse colonies of mosquitoes reared in
167 the same environment to identify mosquito genetic components of vectorial
168 capacity. In such studies, differences in microbiota could confound interpretation
169 of phenotypic differences among mosquito colonies. The present study does not
170 support this hypothesis in the case of *Ae. aegypti*. While this may be the case in
171 some insect systems [28-31], our study provides evidence that the midgut
172 microbiome of colonized *Ae. aegypti* is highly similar and most likely will not
173 confound genetic studies of vector biology.

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174 Although we did not genotype the *Ae. aegypti* colonies used in this study, it
175 is well accepted that *Ae. aegypti* from sub-Saharan Africa belong to a different
176 phylogenetic cluster than pan-tropical *Ae. aegypti* from elsewhere in the world
177 [36-40]. At a more local scale, populations of *Ae. aegypti* sampled from distinct
178 locations are usually genetically distinct [41-43]. Accordingly, we assume that the
179 colonies that we tested in fact represent various genotypes of *Ae. aegypti*. We
180 conclude that mosquito genotype does not influence the microbiome of
181 laboratory-bred *Ae. aegypti*, further demonstrating the importance that the
182 environment plays in shaping the gut microbiome of *Ae. aegypti*. However, this
183 may not be the case in a more natural system. One can imagine that within a given
184 environment, the mosquito genotype may influence the composition of the midgut
185 microbiome and this should be explored further.

186 A limitation of our study was that we only dissected midguts at one time
187 point. Recent results from Short et al. [33] suggest that differences between
188 colonies may exist at different times following adult emergence. It is possible that
189 differences in the gut microbiome between our colonies would have been
190 observed if we had sampled the midguts sooner or later after adult emergence.
191 Since our primary goal was to determine how the gut microbiome of our colonies
192 impacted studies of vector competence, we chose a time point after adult
193 emergence that related to the time when an infectious blood meal is usually
194 offered in vector competence assays.

195 One potentially important implication of our results is that the same
196 mosquito strain reared in different laboratories might display different
197 phenotypes due to a different gut microbiome. We found that the gut microbiome
198 of mosquito colonies was entirely determined by the insectary environment

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199 regardless of the mosquito genotype. It follows that the same mosquito strain
200 exposed to a different environment could host a different gut microbiota. This
201 could undermine the relevance of reference strains that are shared by different
202 laboratories. It will be interesting in future studies to compare the gut bacterial
203 microbiome of the same mosquito strain reared in different insectaries.
204

205 Conclusions

206 Our finding that geographically diverse colonies of *Ae. aegypti* reared in the
207 same insectary harbor a similar gut bacterial microbiome supports the conclusion
208 that the gut microbiota of adult mosquitoes is environmentally determined,
209 regardless of the host genotype. Thus, uncontrolled differences in microbiota
210 composition are unlikely to represent a significant confounding factor in genetic
211 studies of vector biology.
212

213 Methods

214 Mosquito colonies and sample preparation

215 Six *Ae. aegypti* colonies were chosen to represent the worldwide distribution of
216 the species (Figure 1; Table 1). Eggs from each of these colonies were
217 simultaneously hatched in dechlorinated tap water under reduced air pressure for
218 one hour and 200 first-instar larvae from each colony were sorted into 24 x 34 x
219 9 cm plastic trays. The larvae were fed on a standard diet of Tetramin fish food
220 (Tetra) every other day until pupation. Immediately following emergence, adults
221 (males and females) were randomly separated into three replicate cages per
222 mosquito colony. They were maintained under standard insectary conditions

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223 (28°C, 70% relative humidity and 12h light: 12h dark cycle) for 4-6 days and
224 allowed to mate and feed on sugar.

225 Midguts were dissected from adult females under sterile conditions in a
226 biosafety cabinet. Each mosquito was surface sterilized in 70% ethanol for 3-5
227 minutes and washed three times in sterile 1x phosphate-buffered saline (PBS).
228 Midguts were dissected in a drop of sterile 1x PBS and DNA from individual
229 midguts was extracted as previously reported [9]. Briefly, individual midguts were
230 ground in 300 μl of 20 mg/ml lysozyme dissolved in Qiagen ATL buffer in a sterile
231 tube containing grinding beads. The samples were homogenized for two rounds
232 of 30 seconds at 6,700 RPM (Precellys 24, Bertin Technologies) and DNA was
233 extracted following the Qiagen DNeasy recommended pre-treatment protocol for
234 Gram-positive bacterial samples. To control for contamination of bacteria
235 introduced during the midgut dissections, DNA extractions, and PCR steps,
236 negative controls were made by extracting DNA from blank 1x PBS that was used
237 during the washing steps and by performing negative PCR reactions.

238 **16S sequencing**

239 Custom-made PCR primers were designed to amplify the hypervariable V5-V6
240 region of the bacterial 16S ribosomal RNA gene from midguts as previously
241 described [9]. Purified DNA from each midgut sample was amplified in triplicate
242 by 40 cycles of PCR using Expand High-Fidelity polymerase (Sigma-Aldrich)
243 following manufacturer instructions. To improve PCR sensitivity, 0.15 μl
244 T4gene32 and 0.5 μl 20mg/ml bovine serum albumin (BSA) were added per
245 reaction with 6 μl of template DNA. The three PCR reactions were pooled and the
246 PCR products purified using Agencourt AMPure XP magnetic beads (Beckman
247 Coulter). The purified PCR products were quantified by Quant-iT PicoGreen

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248 dsDNA fluorometric quantification (ThermoFisher Scientific) and pooled for
249 sequencing in paired-end on the Illumina MiSeq platform using the 500-cycle v2
250 chemistry (Illumina). On average, 16-18 individual midguts (6 individuals per
251 adult replicate cage) were sequenced per *Ae. aegypti* colony. In order to achieve
252 enough reads per sample, the sequencing was done in two separate runs. Libraries
253 from each colony and each replicate were dispersed evenly between the two
254 sequencing runs. Five libraries were removed from further analysis due to a low
255 number of reads. Raw sequences were deposited to the European Nucleotide
256 Archive under accession number PRJEB22905.

257 **Data analysis**

258 To account for possible contamination at various steps in the sample-processing
259 pipeline, the sequencing reads were corrected with the reads from the negative
260 controls. The sequencing reads from each sample were mapped to the reads found
261 in the negative controls using Bowtie2 [44]. Reads that mapped to reads in the
262 negative controls were removed from the analysis. Read filtering, OTU clustering
263 and annotation were performed with the MASQUE pipeline
264 (https://github.com/aghozlane/masque) as previously described [45]. A total of
265 2,679 OTUs were obtained at 97% sequence identity threshold. Genus richness
266 and Shannon diversity index were compared by analysis of variance (ANOVA). All
267 other statistical analyses were performed with SHAMAN (shaman.c3bi.pasteur.fr)
268 as previously described [9]. Briefly, the normalization of OTU counts was
269 performed at the OTU level using the DESeq2 normalization method. After
270 normalization, an additional six individuals were removed due to low size factors.
271 In SHAMAN, a generalized linear model (GLM) was fitted and vectors of contrasts
272 were defined to determine the significance in abundance variation between

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273 sample types. The GLM included the main effect of the *Ae. aegypti* colony, the main
274 effect of replicate cage, the main effect of sequencing run and the interaction
275 between colony and replicate. The resulting *p* values were adjusted for multiple
276 testing according to the Benjamini and Hochberg procedure [46]. Principal
277 coordinates analysis (PCoA) was performed with the ade4 R package (v1.7.6)
278 using a Bray-Curtis dissimilarity matrix. Permutational multivariate analysis of
279 variance (PERMANOVA) was performed in the vegan R package (v2.4.3) as a
280 distance-based method to test the statistical significance of the association
281 between bacterial community structure and mosquito colony.
282
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297 **Authors' contributions**

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298 LBD and LL designed the study. AVR, ID, DJ, CP, MNM, AK, JJL, and VD provided
299 mosquito specimens to initiate colonies. LBD carried out the experiments. LBD,
300 CB, and LM performed the sequencing. LBD, AG, SV, and LL analyzed the data. LBD
301 and LL wrote the manuscript. All authors read and approved the final manuscript.

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438 **Figure Legends**

439 **Figure 1**: World map showing the origin of the *Ae. aegypti* colonies used in the
440 study overlaid with the approximate global distribution of *Ae. aegypti* adapted
441 from Kraemer et al. [47, 48]. The colonies were initiated on different years and
442 represent different generation times in the laboratory (Table 1).

443

444 **Figure 2**: Genetic diversity of the gut bacterial communities is similar between
445 diverse colonies of *Ae. aegypti*. The genus richness (a) and Shannon diversity index
446 (b) were calculated for each colony representing 16-18 individual midguts from 3
447 replicate cages dissected 4-6 days after adult emergence. Genus richness is the
448 number of bacterial genera identified in each colony. The Shannon diversity index
449 accounts for the relative abundance of each bacterial genus. Error bars represent
450 95% confidence intervals. No difference in richness (ANOVA: F = 1.125, *p* value =
451 0.353) or in Shannon index (ANOVA: F = 0.522, *p* value = 0.759) was detected
452 between colonies.

453

454 **Figure 3**: The dominant bacterial genera found in the midgut are similar among
455 diverse colonies of *Ae. aegypti*. The abundance of the 12 most abundant genera is
456 shown for each colony representing 16-18 individual midguts from 3 replicate
457 cages dissected 4-6 days after adult emergence. Bacterial genera were assigned to
458 OTUs clustered with a 97% cutoff using the SILVA database (https://www.arb-
459 silva.de).

460

461 **Figure 4**: The midgut bacterial community structure is similar between diverse
462 colonies of *Ae. aegypti*. Bacterial community structures between colonies are

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463 compared by (a) principal coordinates analysis (PCoA) and (b) pairwise
464 differential abundance analysis. PCoA is based on a Bray-Curtis dissimilarity
465 matrix and indicates a lack of overall differences (PERMANOVA: p value = 0.752).
466 Results of differential abundance analysis are shown for each pair of colonies as
467 the proportion of all bacterial genera identified (n=587) that were non-
468 significantly differentially abundant after correction for multiple testing.
469
470 **Supplemental Table 1:** Identification of bacterial genera that are differentially
471 abundant in pairwise comparisons of colonies. The lack of a comparison between
472 two colonies indicates that no bacterial genera were significantly different
473 between them.
474
475 **Supplemental Figure 1**: Rarefaction curves for the individual samples used in the
476 analysis at the genera level. The curves show the number of detected bacterial
477 genera as a function of the number of reads analyzed per sequencing library. Each
478 curve represents a single midgut sample.
479
480 **Supplemental Figure 2**: The midgut bacterial communities are highly structured
481 by sequencing run. The cluster dendrogram of individual midgut samples based
482 on a Bray-Curtis dissimilarity matrix shows that sequencing run, and not the
483 identity of the mosquito colony, determines bacterial community relatedness.
484 Midgut samples are represented by numbers color coded by sequencing run. Dark
485 blue samples were sequenced in the first run, whereas light blue samples were
486 sequenced in the second run.
487

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488 **Table**

489

490 Table 1. *Aedes aegypti* colonies included in this study. The country and region of
491 origin, year of collection, and number of generations spent in the laboratory prior
492 to the study are shown.

Country	**Region**	**Year**	**Generation**
Australia	Cairns	2013	10
Cambodia	Phnom Penh	2015	7
French Guiana	Cayenne	2015	4
Gabon	Bakoumba	2014	10
Guadeloupe	Saint François	2015	5
Uganda	Zika	2016	3

493

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Figure 1

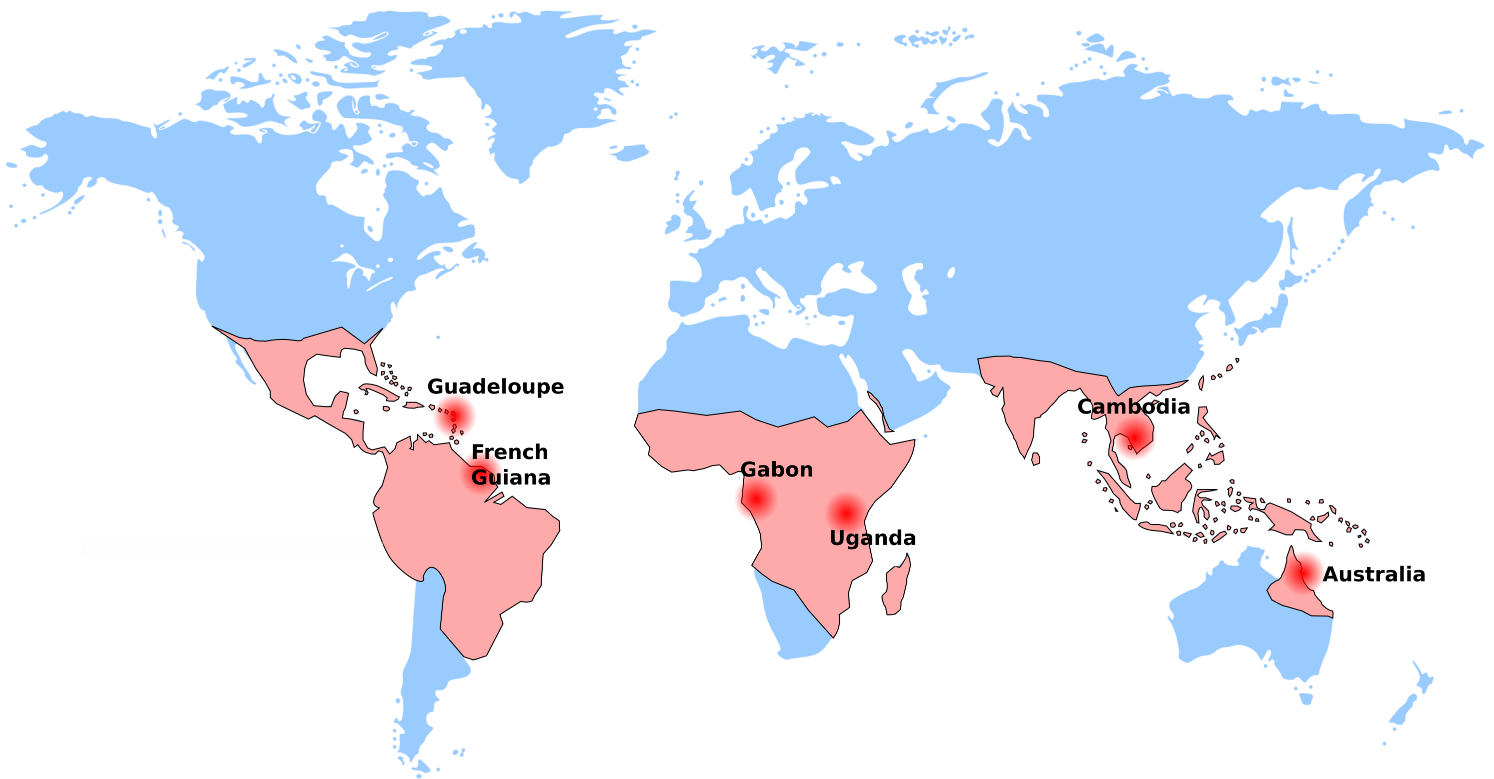

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Figure 2

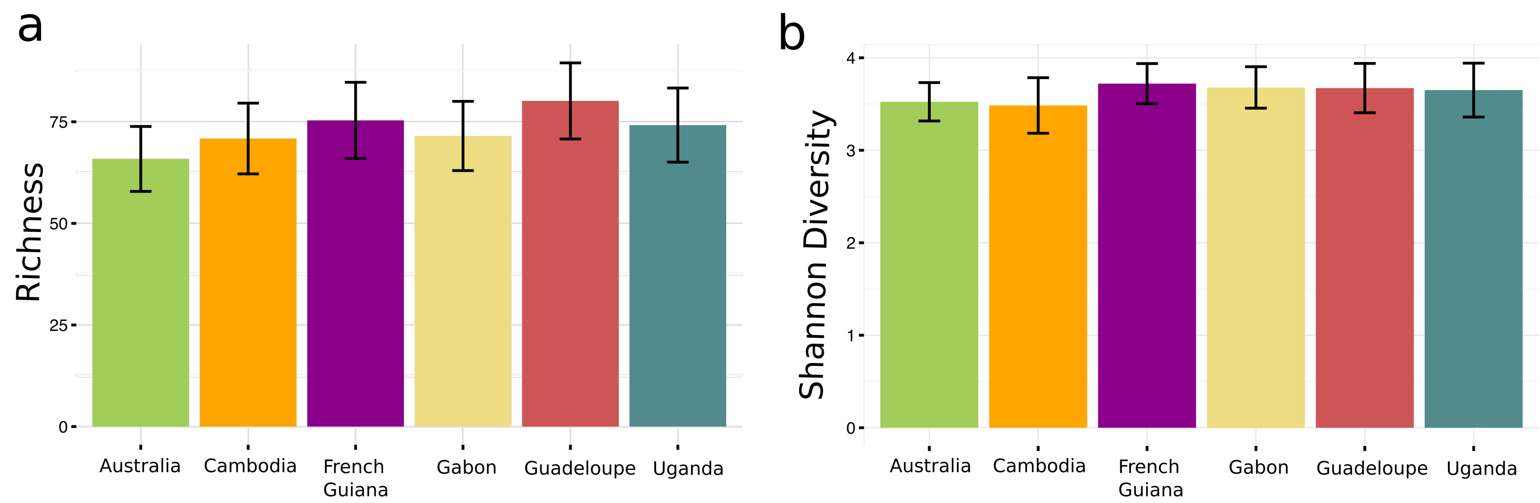

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Figure 3

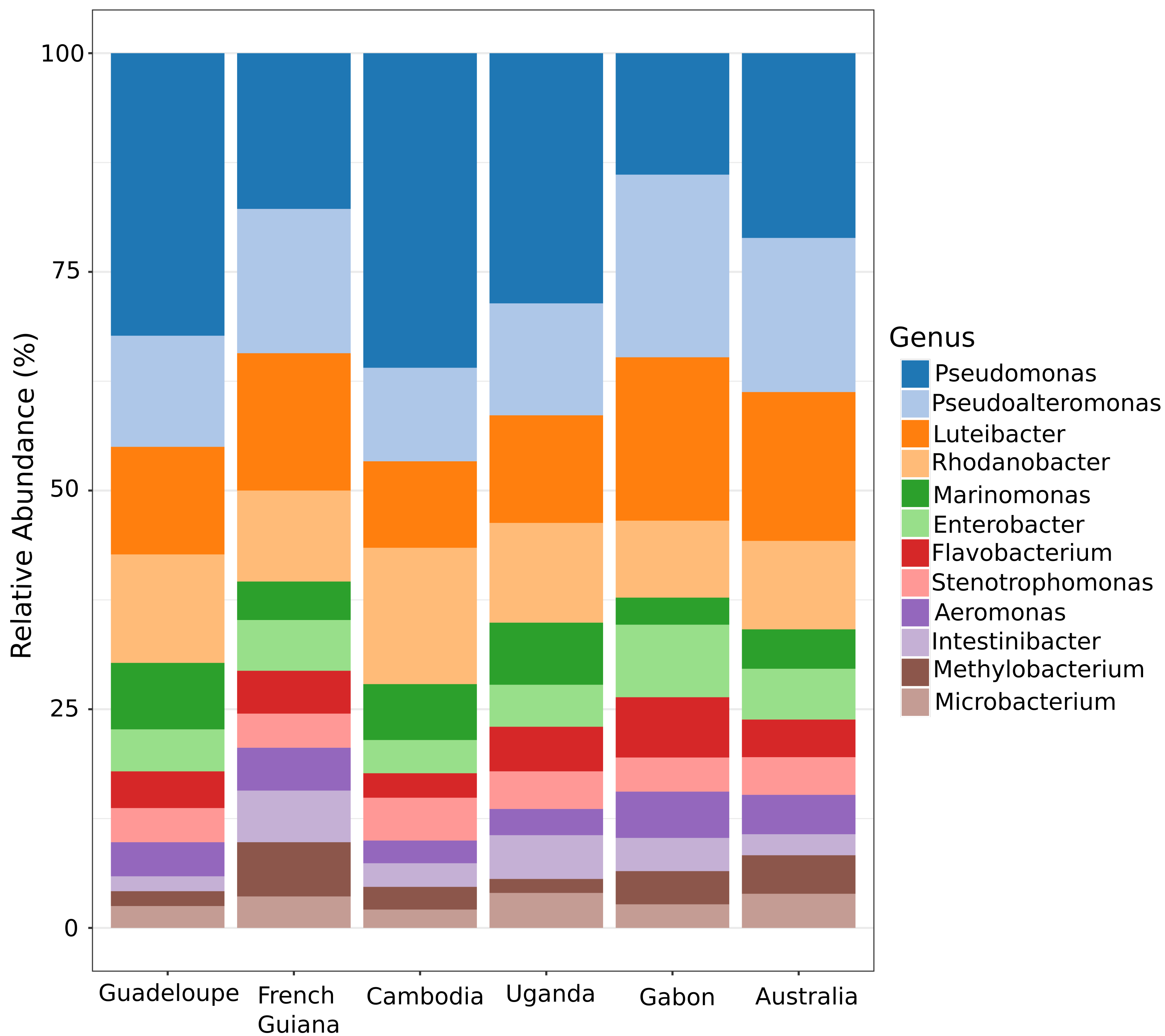

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Figure 4