1 **Title:** *Anopheles albimanus* natural microbiota is altered within one generation of laboratory

2 colonization

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18   **Abstract**

19   Research on mosquito-microbe interactions may lead to new tools for mosquito and mosquito-

20   borne disease control. To date, such research has largely utilized laboratory-reared mosquitoes

21   that typically lack the microbial diversity of wild populations. A logical progression in this area

22   involves working under controlled settings using field-derived mosquitoes or, in most cases, their

23   progeny. Thus, an understanding of how laboratory colonization affects the assemblage of

24   mosquito microbiota would aid in advancing mosquito microbiome studies and their applications

25   beyond laboratory settings. Using high throughput 16S rRNA amplicon sequencing, we

26   characterized the internal and cuticle surface microbiota of F_1 progeny of wild-caught

27   adult *Anopheles albimanus* from four locations in Guatemala. A total of 132 late instar larvae

28   and 135 2-5day old, non-blood-fed virgin adult females that were reared under identical

29   laboratory conditions, were pooled (3 individuals/pool) and analyzed. F_1 larvae from different

30   maternal sites showed different microbial compositions (p=0.001; $F = 9.5$), but these differences

31   were absent at the adult stage (p=0.12; F =1.6). These results suggest that field-acquired

32   mosquito microbiota may be lost in as early as the first generation of colonization under normal

33   laboratory conditions, thus, requiring adjustments to maintain field-derived microbiota. This is

34   the first time that the microbiota of F_1 progeny of wild-caught mosquitoes has been characterized

35   in relation to maternal collection site. Our findings provide a comprehensive background for

36   studying how parentage and environmental conditions differentially or concomitantly affect

37   mosquito microbiome composition, and how this can be exploited in advancing mosquito

38   microbiome studies and their applications beyond laboratory settings.

39   **Keywords:** Mosquito microbiota, *Anopheles albimanus*, laboratory colonization, mosquito

40   microbiome, next generation sequencing, 16S rRNA gene amplicon sequencing

Background

41

42   Mosquitoes contain microbes that inhabit various tissues, such as the alimentary canal,

43   reproductive organs, and cuticle surface [1]. These microbes are thought to be principally

44   obtained from the mosquito habitat during larval development, and from food sources at the

45   adult stage [1]. In addition to acquisition from larval habitats and/or adult food sources,

46   transovarial bacterial transmission from adult females to their eggs, and transstadial

47   transmission across different immature stages, and into the adult stage, have been

48   demonstrated [2, 3]. As a key component of the mosquito microbiota, environmentally-acquired

49   microbes have been shown to affect mosquito life history traits such as the rate of pupation and

50   adult body size [4]. The mosquito microbiota has also been shown to affect mosquito immunity

51   to human pathogens [5], reproduction [6], insecticide resistance [7, 8], and ultimately vector

52   competence—the mosquito's ability to acquire, maintain and transmit pathogens [5]. These

53   effects on multiple physiological characteristics are being leveraged to develop novel

54   approaches to fight mosquito-borne diseases [9].

55   The use of next generation molecular biology tools has resulted in extensive characterization of

56   mosquito microbiota, with the initial focus on bacterial and archaeal components now expanding

57   to eukaryotic microbes [10, 11] and viruses [12, 13]. These advances in mosquito microbiota

58   research have led to field applications of mosquito symbionts for mosquito control. For *Aedes*

59   *aegypti*, the principal vector of dengue, Zika, chikungunya and yellow fever viruses, mosquito-

60   derived symbionts are now being used to suppress mosquito populations [14] and also being

61   considered to control the spread of pathogens [15]. However, studies exploring mosquito

62   symbionts for malaria control have largely remained at the laboratory stage [16, 17]. Similarly,

63   the microbiota of mosquito vectors in some geographical regions are well characterized and

64   studied compared to those from other regions. In malaria vectors for example, studies on the

3

65   microbiota have largely focused on Sub-Saharan African species—in particular, *Anopheles*

66   *gambiae*—and to a lesser extent on those from Southeast Asia [18]. In contrast, the microbiota

67   of Latin American malaria vectors have only recently been comprehensively characterized [7, 8,

68   19-21], with these studies describing associations between *An. albimanus* microbiota and

69   insecticide resistance [7, 8], and the factors that shape the composition of *An. darlingi*, *An.*

70   *albimanus*, *An. nunetzovari*, *An. rangeli,* and *An. triannulatus* microbiota [19-21].

71   To exploit the mosquito microbiota for malaria and malaria vector control, research must

72   successfully advance from laboratory to field settings, a transition which can be fraught with

73   challenges. For example, some malaria vectors such as *An. darlingi, An. vestitipennis,* and *An.*

74   *gambiae,* breed in sites that are small, temporary and often difficult to find and/or access [22-26],

75   making it hard to obtain sufficient immature field mosquitoes for experiments. Where larval

76   habitats are plentiful and easy to find and/or access, the subsequent rearing of field-collected

77   mosquitoes to obtain uniform characteristics can pose additional challenges [27, 28].

78   Additionally, some malaria vectors belong to species complexes whose members are

79   morphologically indistinguishable [29-32], constituting another layer of complexity that needs to

80   be considered in elucidating mosquito-microbe interactions in malaria vectors.

81   These challenges, which are common to research on mosquito ecology and control, are often

82   not reported or discussed in mosquito microbiome studies. Several failed attempts at collecting

83   and rearing sufficient immature mosquitoes from the field for our previous study on the role of

84   mosquito microbiota in insecticide resistance resulted in ultimately using either wild-caught

85   adults [7] or F_1 progeny derived from field-collected adult mosquitoes [8, 33]. While field-caught

86   adult mosquitoes or their F_1 progeny may offer insights into mosquito-microbe interactions in

87   field scenarios, obtaining adult field-collected mosquitoes with uniform and/or controlled

88   physiological characteristics is usually not feasible. Although geographically associated

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89 heterogeneity in microbiota of field-collected mosquitoes has been previously described [34, 35],

90 there is limited information on the fate of field-acquired microbiota after laboratory colonization of

91 field-collected mosquitoes. However, it has previously been observed that upon eclosion, newly-

92 emerged laboratory-reared adult mosquitoes show a reduction in bacterial diversity in contrast to

93 earlier developmental stages [36]. In addition, a recent study on the fate of field-acquired

94 microbiota in laboratory-colonized *An. gambiae s.l.* showed a reduction in bacterial diversity of

95 the F_5 progeny (the first point of measurement) that were reared in dechlorinated tap water in

96 contrast to F_0 [37]. Another study of *Ae. aegypti* from different geographical locations showed

97 no geographically associated heterogeneity microbiota composition after several generations of

98 laboratory colonization [38]. At what point the microbiota of laboratory-colonized mosquitoes

99 become homogenous and whether the microbiota of F_1 laboratory progeny represent their

100 parental origin—and thus could be used for symbiont-based translational studies—remains

101 largely undescribed.

102 Here, we present a comprehensive characterization of the microbiota of laboratory-reared F_1

103 progeny from field-caught adult *An. albimanus*. These represent unanticipated findings from a

104 larger study that was aimed at characterizing the effects of insecticide exposure and resistance

105 on *An. albimanus* microbiota [8]. We expected little or no immediate loss of geographical

106 heterogeneity in microbial composition upon initial laboratory colonization based on evidence of

107 this type of heterogeneity in field-derived populations [34, 35]. However, our data showed

108 geographical heterogeneity in F_1 larval internal and cuticle surface microbiota vs only in F_1 adult

109 cuticle surface microbiota. These findings lay the foundations for studying how parentage and

110 environmental conditions differentially or concomitantly affect mosquito microbiome composition,

111 and how this can be exploited in advancing mosquito microbiome studies and their applications

112 beyond laboratory settings.

5

Results

114   Using high throughput deep sequencing of the universal bacterial and archaeal 16S rRNA gene,

115   we characterized the microbiota of the internal and cuticle surface microbial niches of laboratory

116   reared *An. albimanus* F_1 larvae (n=132) and adult (n=135) progeny originating from mothers that

117   were collected from four locations in south central Guatemala (Fig 1). Mosquitoes were

118   processed as pools (3 individuals per pool), resulting in a total of 44 larval and 45 adult pools. F_1

119   larvae were from mothers collected in El Tererro, Las Cruces 3 and Las Cruces 4, while F_1

120   adults were from mothers collected in Las Cruces 1, Las Cruces 3 and Las Cruces 4 (Table 1).

121   Following quality control of the resulting sequencing reads, amplicon sequence variants (ASVs)

122   were identified and used in downstream analysis. Since adult mosquito microbiota is distinct

123   from that of immature stages [39, 40], ASVs from larvae and adults were analyzed separately,

124   as were ASVs from each microbial niche.

125

126   **Internal and cuticle surface microbiota of laboratory colonized F_1 *An. albimanus* larvae**

127   **differed by maternal collection site**

128   Non-pairwise Bray-Curtis distance comparison showed significant differences in internal

129   (p=0.001) and cuticle surface (p=0.001) microbiota between F_1 larvae from different maternal

130   collection sites. Thus, irrespective of microbial niche, the microbial community structure

131   (composition and relative abundance of ASVs) of F_1 laboratory-colonized larvae differed by

132   maternal collection location. Pairwise PERMANOVA comparison of Bray-Curtis distances further

133   showed significant differences in microbial community structure in larval internal (q<0.01) and

134   cuticle surface microbiota (q<0.01) between every pair of maternal collection site (Table 2a).

135   This location-driven heterogeneity in microbial community structure was further demonstrated by

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136   principal coordinate analysis (PCoA), where F_1 larval internal and cuticle surface microbiota

137   clustered distinctly by maternal collection site (Fig. 2).

138   Non-pairwise Shannon diversity comparisons showed significant differences in internal

139   (p=0.009) but not cuticle surface (p=0.09) microbiota of F_1 laboratory-colonized larvae from

140   different maternal collection sites, indicating that there was inter-sample variation in the diversity

141   of internal but not cuticle surface microbiota of larvae when all maternal collection sites were

142   taken into consideration. A pairwise Kruskal-Wallis comparison of Shannon diversity indices

143   showed that the inter-sample variation in diversity of larval internal microbiota held true when

144   every pair of maternal collection sites was considered except between Las Cruces 3 and 4

145   (Table 2b and Suppl. 1). Larvae originating from Las Cruces 3 had the highest internal

146   microbiota diversity, followed by Las Cruces 4 and El Terrero (Suppl. 1)

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148   **Cuticle surface, but not internal, microbiota of laboratory colonized F_1 adult *An.***

149   ***albimanus* differed by maternal collection site**

150   Non-pairwise Bray-Curtis diversity comparisons showed significant differences in cuticle surface

151   (p=0.001), but not internal microbiota (p=0.12) between adult F_1 mosquitoes from different

152   maternal collection sites, suggesting a loss of location-driven heterogeneity in microbial

153   community structure in internal but not cuticle surface microbial niche of laboratory-colonized F_1

154   adults. Pairwise PERMANOVA comparisons of Bray-Curtis distances also showed significant

155   differences in microbial community structure of F_1 adult cuticle surface microbiota (q<0.01)

156   between every pair of maternal collection sites (Table 2a). These results were corroborated by

157   PCoA which showed that F_1 adult cuticle surface microbiota, but not internal microbiota,

158   clustered distinctly by maternal collection site (Fig. 2).

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159   Non-pairwise Shannon diversity comparisons showed no differences in the internal (p=0.42) or

160   cuticle surface (p=0.4) microbiota of F_1 adults from different maternal collection sites, indicating

161   that there was little or no inter-sample variation in diversity of F_1 adult microbiota when all

162   maternal collection sites were taken into consideration. Pairwise Kruskal-Wallis comparisons of

163   Shannon diversity indices also detected no inter-sample variation in diversity of F_1 adult cuticle

164   surface or internal microbiota when every pair of maternal collection site was considered (Table

165   2b and Suppl. 1).

166   **Laboratory-colonized F_1 *An. albimanus* larvae comprised a rich and diverse microbiota**

167   **that differed by maternal collection site**

168   Overall, ASVs from larval internal microbiota were assigned to 180 bacterial taxa, and cuticle

169   surface microbiota to 194 bacterial taxa (suppl 2.). A majority of these taxa across all locations

170   (ranging from 118-139 taxa) were shared between the internal and cuticle surface microbiota

171   (Fig 3a), as well as across maternal collection sites (n=110 for cuticle surface and n=117 for

172   internal microbiota) (Fig 3b). While a majority of the identified microbial taxa were shared

173   between both microbial niches, their abundance was generally higher in internal (Fig 4a)

174   compared to cuticle surface (Fig 4b) microbiota. Although a majority of identified microbial taxa

175   in both internal and cuticle surface microbiota were shared across all locations, their abundance

176   differed by maternal location (Fig 4a, 4b and 5).

177   In general, larval internal microbiota was dominated by ASVs identifed as an uncharacterized

178   *Enterobacteriaceae, Leucobacter, Thorsellia,* and *Chryseobacterium* (Fig 4a), together making

179   up over 50% of ASVs (Suppl. 2). In contrast, *Acidovorax,* unchracterized *Comamonadaceae,*

180   and *Paucibacter* (Fig 4b) made up over 50% of ASVs detected on the larval cuticle surface

181   (Suppl. 2).

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182   A few predominant bacterial taxa were present in the larval internal microbial niche across all

183   three maternal collection sites: unclassified *Enterobacteriaceae, Thorsellia, Rhizobium,*

184   *Xantobacter, Acidovorax* and *Pirellula* (Fig 4a.), with remaining taxa showing different patterns of

185   abundance between collection sites (Fig 5 and Suppl. 2). For example, ASVs assigned to the

186   genus *Azoracus* were predominant in larvae from El Terrero, while *Singulisphaera, Paucibacter,*

187   *Ancylobacter, Gemmobacter,* and *Rayranella* were predominant in those from Las Cruces 3.

188   Predominant in larvae from Las Cruces 3 and Las Cruces 4 were *Azospirillum, Bosea* and

189   *Microbacterium*; and in those from El Terrero and Las Cruces 3 were *Terrimicrobium* and

190   *Legionella* (Fig 5). Although outside of the cut off limit set for differential abundance, ASVs

191   assigned to the bacterial genera *Leucobacter* were predominant in the internal microbiota of

192   larvae from Las Cruces 4 and El Terrero, but predominant in only three of the 14 sample

193   replicates from Las Cruces 3 (Fig 4a). Similarly, *Chryseobacterium* was predominant in Las

194   Cruces 4 and 3, but only predominant in six of the 16 pools of larvae from El Terrero. Bacterial

195   taxa that were unique to each location comprised <8% of all taxa in larval internal microbiota

196   (Suppl. 3b), and were below the threshold for inclusion in the heatmap and differential

197   abundance testing (Suppl. 2).

198   Unlike the internal microbial niche, no microbial taxa was predominant in larval cuticle surface

199   microbiota across all three collection sites. However, some taxa showed notable patterns of

200   abundance between locations (Fig 5). These included the genus *Azoarcus,* which was detected

201   at low to moderate frequencies in 13 of 15 pools of larvae from El Terrero, at low frequency in a

202   single pool of larvae from Las Cruces 3, and was not detected at all in Las Cruces 4 (Fig 4b and

203   6). Similarly, ASVs assigned to the genus *Spirosoma* were detected at moderate frequencies in

204   all pools of larvae from Las Cruces 4, but only in a few pools from the other two locations. ASVs

205   assigned to the genus *Paucibacter* were present at relatively higher abundance in larvae from

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206 both Las Cruces 3 and El Terrero compared to those from Las Cruces 4. Those assigned to the

207 genus *Acidovorax* were predominant in larvae from Las Cruces 3 and Las Cruces 4 in contrast

208 to El Terrero. ASVs assigned to *Microbacterium, Bdellovibrio* and *Pelomonas* were present at

209 moderate frequencies in larvae from both Las Cruces 3 and Las Cruces 4 but were not detected

210 in El Terrero(Fig. 5). Bacterial taxa that were unique to each maternal collection site comprised

211 <8% of larval cuticle surface microbiota (Fig 3b), and were below the threshold for inclusion in

212 the heatmap and differential abundance testing (Suppl. 2).

213

214 **Laboratory-colonized adult F$_1$ *An. albimanus* were comprised of sparse internal and**

215 **cuticle surface microbiota that were dominated by ASVs assigned to the genus *Asaia***

216 ASVs from adult internal microbiota were assigned to 62 microbial taxa and cuticle surface

217 microbiota were assigned to 106 microbial taxa. Two of these ASVs which were only present in

218 the cuticle surface microbiota were classified as archaea, while all other remaining ASVs were

219 classified as bacteria (Suppl. 2). Unlike larval microbiota, less than half of the assigned taxa

220 across all locations (ranging from 19-37 taxa) were shared between internal and cuticle surface

221 microbiota (Fig 3a), and only 18 taxa on the cuticle surface and 19 internal taxa were shared

222 across all maternal collection sites (Fig 3b).

223 Overall, ASVs assigned to the bacterial genus *Asaia* dominated both adult internal and cuticle

224 surface microbiota (Fig 6), constituting at least 70% of taxa in each microbial niche (Suppl. 2). A

225 majority of identified taxa in adult internal, but not cuticle surface, microbiota was detected

226 across all three maternal collection sites, with a few of these taxa present in high abundance

227 across all collection sites (Fig. 6). Across all three maternal collection sites, ASVs assigned to

228 the genera *Acinetobacter, Gluconobacter, Pantoea* and *Pseudomonas* were present in

229 moderate to high abundance in adult internal microbiota in addition to *Asaia* (Fig 6).

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230   After excluding low abundance taxa (Suppl. 2), the number of remaining taxa did not meet the

231   requirements for identifying collection site-specific microbial taxa in adult cuticle surface

232   microbiota. This was compounded by dominance (>70%) of ASVs that were assigned to the

233   bacterial genus *Asaia* (Suppl. 2). In addition, the cuticle surface microbiota of adults originating

234   from Las Cruces 4 comprised 43% of all adult cuticle surface microbial taxa (Fig 3b), although a

235   majority were of low abundance.

Discussion

The scientific community is increasingly investigating the role of mosquito microbiota in fighting mosquito-borne diseases [9]. The successful transition of mosquito microbiome research from laboratory to field requires a comprehensive understanding of the dynamics underlying the composition of the microbiota of field-collected mosquitoes and their progeny. We provide a comprehensive characterization of the internal and cuticle surface microbiota of laboratory-reared F_1 progeny from field-caught adult *An. albimanus* that were collected from different locations. Our results show that while location-driven heterogeneity in the microbial community structure of both internal and cuticle surface microbiota was present in the F_1 larvae, this heterogeneity was only evident in the cuticle surface microbiota of adult progeny from the same generation. This work provides comprehensive fundamental data for studying how parentage and environmental conditions differentially or concomitantly affect mosquito microbiome composition.

Previous studies on other mosquito species have also shown a loss of field-acquired internal mosquito microbiota following several generations of laboratory colonization [37, 38]. Until now, however, the laboratory generation at which this loss occurs was largely undescribed. Our study represents an initial step in filling this knowledge gap and provides further details regarding the dynamics of changes in both the internal and cuticle surface microbial niches. The homogeneity in internal microbiota of F_1 adults is suggestive of a loss in field-acquired microbiota, which could pose an additional set of challenges for field or semi-field mosquito microbiome studies— particularly studies that rely on laboratory progeny in lieu of field populations to circumvent the challenges involved [25, 27, 28] in obtaining sufficient numbers of wild mosquitoes of uniform physiological characteristics. Previous findings showed that rearing mosquitoes in water from the field could preserve the field-derived internal microbiota in laboratory-colonized adult progeny of

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260   *An. gambiae s. l.* for several generations [37]. In addition, other studies, have shown that the

261   microbiota in larval habitat water significantly influences the internal microbiota of emerging adult

262   mosquitoes [40, 41]. Thus, to preserve wild-type microbiota, care should be taken to ensure that

263   the first laboratory generation are reared in water collected from larval habitats in the field. This

264   first generation would be most suitable for studies that require progeny from field populations.

265   Maternal egg-smearing has been proposed as a mechanism through which adult female

266   mosquitoes and other insects transfer microbes to their progeny [42-45]. This egg-smearing

267   could explain the heterogeneity observed in internal and cuticle surface microbiota between F_1

268   larvae with different maternal origins. On the other hand, a lack of this heterogeneity in the

269   internal microbiota of adult progeny could be attributed to the physiological changes that occur

270   during metamorphosis and adult eclosion, whereby elimination of the larval meconial peritrophic

271   membrane and meconium (midgut and midgut content), along with ingestion of exuvial fluid—

272   which is said to be bactericidal—results in sterile or nearly sterile midguts in newly emerged

273   adults [39, 46]. This, in addition to mechanisms that regulate the composition of adult mosquito

274   internal microbiota [47-49], could explain the observed homogeneity in F_1 adult internal

275   microbiota. Conversely, the heterogeneity in cuticle surface microbiota between adult progeny

276   originating from different maternal sites, suggests that maternally derived microbes in the rearing

277   trays may have colonized adult cuticle surfaces during emergence. The mechanisms underlying

278   the assemblage of the mosquito cuticle surface microbiome are largely undescribed, and thus

279   require further investigation.

280   The low inter-sample variation in microbial diversity observed in this study has largely been

281   described in laboratory mosquito colonies [38, 50]. The microbial composition of laboratory-

282   reared larvae is typically less diverse [50, 51] compared to those of field-derived larvae, but our

283   laboratory-reared larvae exhibited a rich microbial composition that was comparable to those of

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284   field populations [39, 40]. In contrast, our adult progeny had a less diverse microbial composition

285   that was reflective of typical laboratory-reared adult mosquitoes [36, 52]. These findings further

286   suggest that field-acquired microbiota, although transferred to laboratory progeny, may be lost

287   within one generation of laboratory colonization—particularly at the adult stage.

288   In this study, we detected microbial taxa that have previously been identified in *Anopheles* and

289   other mosquito genera [7, 41, 53, 54]. While a majority of the taxa in F_1 larvae were shared

290   between both the internal and cuticle surface microbial niches, a greater abundance of microbial

291   taxa was detected in the internal microbial niche compared to the cuticle surface. The cuticle

292   surface microbiota of mosquitoes and other hematophagous insects are largely uncharacterized

293   and the mechanisms underlying their assemblage remain unknown. As such, we hypothesize

294   that although both internal and cuticle surface niches are exposed to the same water from which

295   the microbiota is derived, a more conducive and protected internal environment could allow for

296   greater proliferation of colonizing bacteria. In F_1 adults however, less than half of the detected

297   microbial taxa were shared between the internal and cuticle surface microbial niches, suggesting

298   differences in physiological conditions that favor microbial colonization, and corroborating

299   findings that point toward a microbial regulatory mechanisms within the mosquito midgut [47-49].

300   This minimal overlap of taxa between internal and cuticle surface microbial niches also suggests

301   a difference in the fate of maternally derived microbes between both niches. Although a few

302   microbial taxa overlapped between adult internal and cuticle surface microbial niches and the

303   most abundant taxa were shared, many of the unshared taxa have been previously detected in

304   adult mosquitoes including *Anopheles* [1, 54, 55], indicating that the cuticle surface microbiota

305   characterized in this study are inherently associated with mosquitoes. Like the larval microbiota,

306   there was a higher abundance of microbial taxa in the adult internal microbial niche compared to

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307   the cuticle surface, further supporting the hypothesis of a more conducive and protected internal

308   environment permitting greater proliferation of colonizing bacteria.

309   With the exception of the adult cuticle surface microbial niche, a majority of all detected microbial

310   taxa overlapped between maternal collection sites in both F_1 larvae and adults, albeit with

311   differing abundances. This reflects restrictions imposed by controlled laboratory environments in

312   the development of mosquito microbiota. In both microbial niches of both larvae and adults,

313   microbial taxa that were specific to maternal collection sites were low in abundance, compared

314   to the moderate to high abundance of those that were shared across all locations. This was

315   particularly true for *Asaia*—notorius for rapidly colonizing laboratory mosquitoes [56]—which

316   constituted at least 70% of both adult internal and cuticle surface microbiota from progeny

317   across all maternal collection sites. These results suggest that field-acquired mosquito

318   microbiota may be lost in as early as the first generation of laboratory colonization.

319   We recognize that not having the microbial community profiles of the mothers from which the F_1

320   progeny were derived is a limitation of this study. However, these are unanticipated findings that

321   arose from a separate study whose focus was on field-derived F_1 progeny reared under

322   controlled settings [8]. The findings herein provide empirical data for further exploring the role of

323   parentage and environmental conditions on the assemblage of the mosquito microbiome, and

324   the fate of field-derived microbes upon laboratory colonization. This is critical for advancing

325   mosquito microbiome studies and their applications beyond laboratory settings.

326 **Methods**

327 The findings presented here extend those of a larger study [8]. Thus, the mosquito collection,

328 processing and sequencing procedures have previously been described in detail [8].

329 **Mosquito collections and laboratory generation of F_1 progeny**

330 Gravid and/or blood-fed adult female *An. albimanus* were sampled across four field sites in the

331 villages of Las Cruces and El Terrero, in La Gomera, department of Escuintla, Guatemala

332 (Figure 1). Field-collected mosquitoes from each location were held in separate paper cups,

333 sustained on 10% sucrose solution, and transported to the insectary at Universidad del Valle de

334 Guatemala in Guatemala City for species identification, oviposition and subsequent rearing of F_1

335 progeny. Mosquitoes that were morphologically identified as *An. albimanus* following

336 identification keys [57] (approximately 300 in total) were subjected to oviposition, and a

337 subsample of the resulting F_1 progeny were used for molecular verification of species identity as

338 described below. A modified oviposition procedure [58] was employed, wherein no more than 70

339 gravid females from the same location were placed in quart size paper ice cream containers with

340 distilled water to a depth of at least 2 cm. The containers were covered with fine mesh fabric that

341 were secured with rubber bands prior to the introduction of gravid field-caught adult *An.*

342 *albimanus*. The mesh was topped with cotton balls soaked in 10% sucrose to sustain the

343 mosquitoes, and subsequently covered with a thick piece of black plastic bag to keep the

344 containers dark and trap in moisture. The oviposition chambers were held under the following

345 insectary conditions; $27\pm2^\circ$C, $80\pm10\%$ relative humidity, and 12-h light-dark cycle. After at least

346 48 h, the adult females were removed and discarded as they were not needed for the original

347 study. The F_1 eggs were collected, pooled by maternal location and reared separately under

348 identical ambient conditions (as described above) and the following larval feeding regimen. Eggs

349   were washed into 18 x 14 x 3 inch plastic larval trays (approximately 200 eggs per tray)

350   containing distilled water to a depth of at least 2 cm, and 3-4 drops of 10% yeast solution.

351   Hatched larvae were sustained on finely ground Koi fish food (Foster & Smith, Inc. Rhinelander,

352   WI) until pupation. At the third to fourth larval instar stage (L3-L4), half of the larvae were

353   separated and used for bioassays in the original study and subsequently processed for

354   microbiota characterization (n=132). Using a stereo microscope, female pupae from the

355   remaining mosquitoes were separated into 8 oz. paper ice cream cups and placed into

356   cardboard cages for adult eclosion. The resulting F_1 adult virgin female mosquitoes were

357   sustained on 10% sucrose solution until they were 2-5 days old and also used for bioassays in

358   the parent study. These were subsequently processed for microbiota characterization (n=135).

359   Post bioassays, larval and adult samples were preserved in RNALater® solution (Applied

360   Biosystems, Foster City, CA), shipped on dry ice to the US Centers for Disease Control and

361   Prevention (CDC) in Atlanta, USA, and stored at -80 °C until further processing. Samples

362   originating from different geographic locations were handled, stored and processed separately.

363   **Genomic DNA extraction from mosquito legs, whole mosquito samples, and mosquito**

364   **cuticle surfaces**

365   Stored mosquito samples were thawed overnight at 4°C, and the thawed RNALater® solution

366   was discarded. This was followed by a rinse with nuclease-free water to remove any residual

367   RNALater®. Legs from a subsample of individual F_1 adults were removed using sterile forceps

368   and placed in individual sterile1.5 mL Eppendorf tubes containing 75 μL Extracta DNA Prep

369   solution (Quantabio, Beverly, MA) for DNA extraction following manufacturer's instructions. Both

370   larvae and adult (with and without legs removed) F_1 samples were pooled for whole body and

371   cuticle surface DNA extractions. Each pool comprised 3 individuals, resulting in 44 pools of

372   larvae and 45 pools of adults. Table 1 shows the number of pools (replicates) processed per life

373   stage and maternal collection site. To obtain cuticle surface samples, each pool was submerged

374   in 500 μL of nuclease free water and washed vigorously by agitating with a vortex mixer for at

375   least 15 seconds. The resulting wash water was transferred to new sterile tubes for DNA

376   extraction. The washed sample pools were further surface sterilized using two vigorous washes;

377   first in 70% ethanol, then nuclease free water, each with at least 15 seconds agitation on a

378   vortex mixer. This was followed by one gentle rinse with nuclease free water. Genomic DNA

379   from the surface-sterilized samples and wash water (referred to as internal and cuticle surface

380   respectively) was isolated using the TissueLyser II and DNeasy Blood and Tissue Kit (QIAGEN,

381   Hilden, Germany) as follows; in individual 2 mL sterile tubes, 180 μL of buffer ATL (QIAGEN)

382   and 5 mm diameter stainless steel beads (QIAGEN) were added to each sample pool. With the

383   following settings; 30 hz/s for 8 and 15 minutes for internal and cuticle surface samples

384   respectively, samples were homogenized using the TissueLyser (QIAGEN) fitted with two sets of

385   96-well adapter plates that held the tubes. The plates were rotated every minute during

386   homogenization, and afterwards, samples were transferred into new sterile 1.5 mL Eppendorf

387   tubes for the remaining part of the DNA extraction process. 20 μL of Proteinase K (QIAGEN)

388   was added to each homogenized sample and incubated overnight at 56 °C, after which 200 μL

389   of buffer AL (QIAGEN) was added and incubated for a further 2 h at the same temperature. The

390   remaining steps were performed according to QIAGEN's spin-column protocol for purification of

391   DNA from animal tissues, and the purified DNA was eluted in 70 μL of buffer AE (QIAGEN). Two

392   sets of two blank controls (without samples) were processed alongside the internal and cuticle

393   surface samples, and all steps, along with those described below, were performed under sterile

394   conditions. The purified genomic DNA from samples and blank controls were stored at -80 °C

395   until further processing.

396   **Molecular species confirmation of *An. albimanus* and 16S rRNA amplicon sequencing**

18

397   DNA samples from mosquito legs were used as templates to amplify the second internal

398   transcribed spacer region (ITS2) of the mosquito ribosomal DNA in order to verify morphological

399   identification of *An. albimanus*. This was achieved by conventional PCR using the universal ITS2

400   primers (ITS2 A: TGTGAACTGCAGGACACAT and ITS2 B: TATGCTTAAATTCAGGGGGT) for

401   distinguishing members of *Anopheles* complexes [59]. With a final reaction volume of 25 μL,

402   each PCR comprised $\geq$100 ng/μL DNA template, 15 μM of each primer, 12.5 μL of 2X AccuStart

403   II PCR SuperMix (Quantabio, Beverly, MA), and PCR grade water to final volume. With the

404   following conditions, initial denaturation at 94 °C for 4 min, then 35 cycles of 94 °C for 30 s, 53 °C

405   for 40 s, and 72 °C for 30 s, followed by a final extension for 10 min at 72 °C, the PCRs were

406   performed using a T100™ Thermal Cycler (Bio-Rad, USA). Using EtBr-stained agarose gel

407   electrophoresis, the amplified products sized ~500 bp, confirmed all samples as *An. albimanus*

408   [60].

409   DNA from internal and cuticle surface samples, along with those from blank controls, were used

410   as templates to amplify the universal bacterial and archaeal 16S rRNA gene. This was also

411   achieved by conventional PCR using universal primers targeting the v3-v4 region of the 16S

412   rRNA gene (341F:

413   **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG, and 805R:

414   **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC), each

415   with Illumina® (San Diego, CA USA) adapters (bold typeface). With a final reaction volume of 25

416   μL, each PCR comprised; ≥20 ng/μL DNA template, 5 μM of each primer, 10 μL of 2× KAPA HiFi

417   HotStart PCR mix (Roche, Switzerland), and PCR grade water to final volume. Three negative

418   controls with PCR grade water substituted for DNA template, were processed along with the

419   samples. The PCRs were performed using the T100™ Thermal Cycler (Bio-Rad, USA) under

420   the following conditions; initial denaturation at 95 °C for 3 min, then 25 cycles of 95 °C, 55 °C,

19

421   and 72 °C for 30 s each, followed by a final extension for 5 min at 72 °C. The amplification

422   products, sized ~460 bp, were verified by electrophoreses as described above and quantified

423   using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Amplicons,

424   including PCR products from blanks and negative controls, which yielded no bands following

425   electrophoresis, were purified using Agencourt AMPure XP beads (Beckman Coulter Inc.,

426   Indianapolis, IN, USA) at 0.7X (internal) or 0.875X (cuticle surface) sample volume, and eluted in

427   40 µL of 10 mM Tris buffer (pH 8.5).

428   All purified products, including those of blanks and negative controls, were used as templates for

429   sequencing library preparation. This was accomplished via index PCR using the Nextera XT

430   Index kit v2 sets, A, B and D (Illumina, San Diego, CA). With a final reaction volume of 50 µL,

431   each index PCR comprised; 25 µL NEBNext High-Fidelity 2X PCR master mix (New England

432   Biolabs Inc., Ipswich, MA), 5 µL of each index primer, 10 µL of purified PCR products (0–

433   20 ng/µL) as template, and PCR grade water to final volume. The PCRs were performed under

434   the following reaction conditions; 98 °C for 30 s, then 8 cycles of 98 °C for 10 s, 55 °C and 65 °C

435   for 30 s each, and a final extension at 65 °C for 5 min, and resulting libraries were also cleaned

436   using Agencourt AMPure XP beads (Beckman Coulter Inc., Indianapolis, IN, USA) at 1.2X

437   sample volume and eluted in 25 µL of 10 mM Tris buffer (pH 8.5). The libraries were

438   subsequently analyzed for size and concentration, normalized to 2 nM, pooled and denatured

439   following Illumina guidelines for loading onto flowcells. Sequencing was performed on an

440   Illumina HiSeq 2500 machine, using 2x250 cycle paired-end sequencing kits.

441   **Preprocessing of sequencing reads**

442   Sequencing outputs were demultiplexed and converted to the fastq format for downstream

443   analysis using the bcl2fastq (v2.19) conversion software (Illumina®). A total of 115,250,077

444 demultiplexed paired-end sequencing reads, with a maximum length of 250 bp were initially

445 imported into the 'quantitative insights into microbial ecology' pipeline, QIIME2 v.2017.7.0 [61],

446 and further sequencing read processing and analysis were performed in v.2018.2.0 of the

447 pipeline. Using the DADA2 plugin in QIIME 2 [62], the `denoise-paired` command with the

448 following options; `trunc_len_f: 244, trunc_len_r: 244, and n_reads_learn:`

449 `500000`, was used to correct errors, remove chimeras and merge paired-end reads. The

450 resulting amplicon sequence variants (ASVs; n=30,956,883) were further filtered to remove

451 ASVs with <100 counts and ASVs that were associated with blanks and negative controls. This

452 last filtering step resulted in 17,225,776 ASVs, ranging from 3,277 to 223,222 per sample (mean

453 96,774), that were used for downstream comparison of bacterial composition and taxonomic

454 analysis. Suppl. 3. shows sequencing reads and ASV summary statistics.

455 **Diversity indices**

456 Analysis of microbial diversity within (alpha diversity) and between (beta diversity) samples were

457 performed in QIIME2 using the Shannon diversity index and Bray-Curtis dissimilarity index,

458 respectively. The Shannon diversity indices were calculated using rarefied ASVs counts per

459 sample, in which ASVs per sample were selected randomly without replacement at an even

460 depth (Suppl. 4) for ten iterations. The resulting average Shannon indices are presented and

461 were compared between samples using pairwise Kruskal-Wallis tests with Benjamini-Hochberg

462 false discovery rate (FDR) corrections for multiple comparisons.

463 The Bray-Curtis dissimilarity indices were computed with or without rarefaction, and resulting

464 indices were compared between samples using pairwise PERMANOVA tests (999 permutations)

465 with FDR corrections. There were no discernable differences between results of rarefied and

466 non-rarefied data. Thus, results of Bray-Curtis dissimilarity indices using non-rarefied data were

467   visualized by Principal Co-ordinates Analysis (PCoA) plots in R [63] using the phyloseq R

468   package [64].

469   Significance for both pair-wise analyses was set to $q < 0.05$ (i.e. post FDR p-value corrections).

470   **Taxonomic analysis and differentially abundant microbial taxa**

471   Taxonomic analysis of ASVs was performed using QIIME2's pre-trained Naïve Bayes classifier

472   [65] and q2-feature-classifier plugin [66]. Prior to analysis, the classifier was trained on the

473   QIIME-compatible 16S SILVA reference (99% identity) database v.128 [67], and using the

474   extract-reads command of the `q2-feature-classifier` plugin, the reference sequences

475   were trimmed to the v3-v4 region (425 bp) of the 16S rRNA gene. The relative abundance of

476   annotated ASVs across samples were subsequently visualized using the `qiime feature-`

477   `table heatmap` plugin based on Bray-Curtis distances with the plugin's default clustering

478   method.

479   Differentially abundant microbial taxa across locations were identified using QIIME2's analysis of

480   composition of microbiomes (ANCOM) [68] plugin. The cut-off for differential abundance was set

481   to an effect size of log F≥20 and W≥20, i.e. a taxon was differentially abundant across maternal

482   collection sites if the ratio of its abundance to those of at least 20 other taxa (25% of all included

483   taxa) differed significantly across sites.

484   Only annotated ASVs with counts ≥ 2000 (larvae) or ≥ 1000 (adults) were included in the

485   heatmaps and ANCOM analysis. Prior to each analysis, ASV frequency data was normalized by

486    $\log_{10}$ transformation following the addition of pseudocounts of 1.

487   The outputs of data analyses were aesthetically formatted using Inkscape [69]**.**

22

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: The raw sequencing reads generated from this project, including those from negative controls (blanks), have been deposited in the National Center for Biotechnology Information (NCBI), Sequence Read Archive under the BioProject PRJNA512122.

Competing interests: The authors declare that they have no competing interests

Author contributions: ND† & AL conceptualized and designed the study; NP facilitated and provided facilities for field work; ND†, ACB, FL & JCL performed mosquito collections, and mass rearing; ND† & MS performed molecular analysis and sequencing; ND† analyzed the data; ND† and ND performed the data visualizations; ND† drafted the manuscript; all authors reviewed and approved the final version of the manuscript.

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747   **Supporting Information**

748   **Suppl. 1.** Shannon alpha diversity plots

749   **Suppl. 2.** Frequency and relative abundance of microbial taxa detected in internal and cuticle
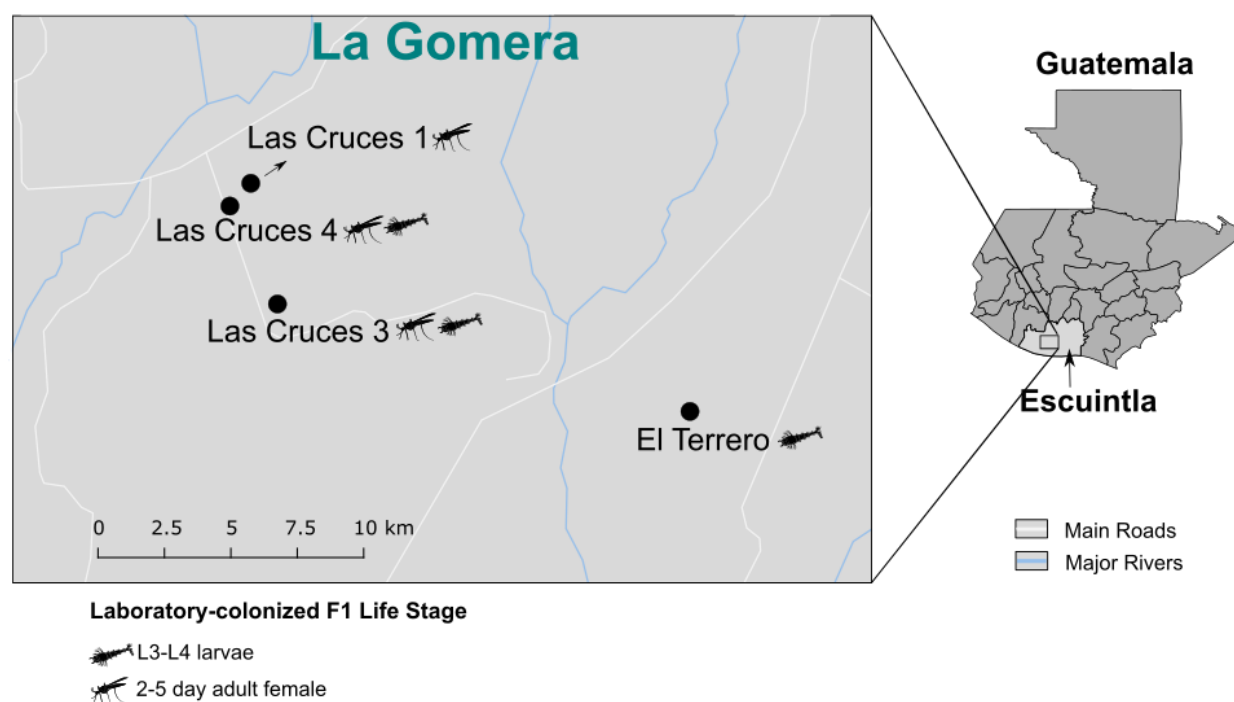
750   surface microbiota of F_1 laboratory colonized *An. albimanus* larvae and adults

751   **Suppl. 3.** Summary statistics of sequencing reads and amplicon sequence variants (ASVs) used

752   for downstream analysis

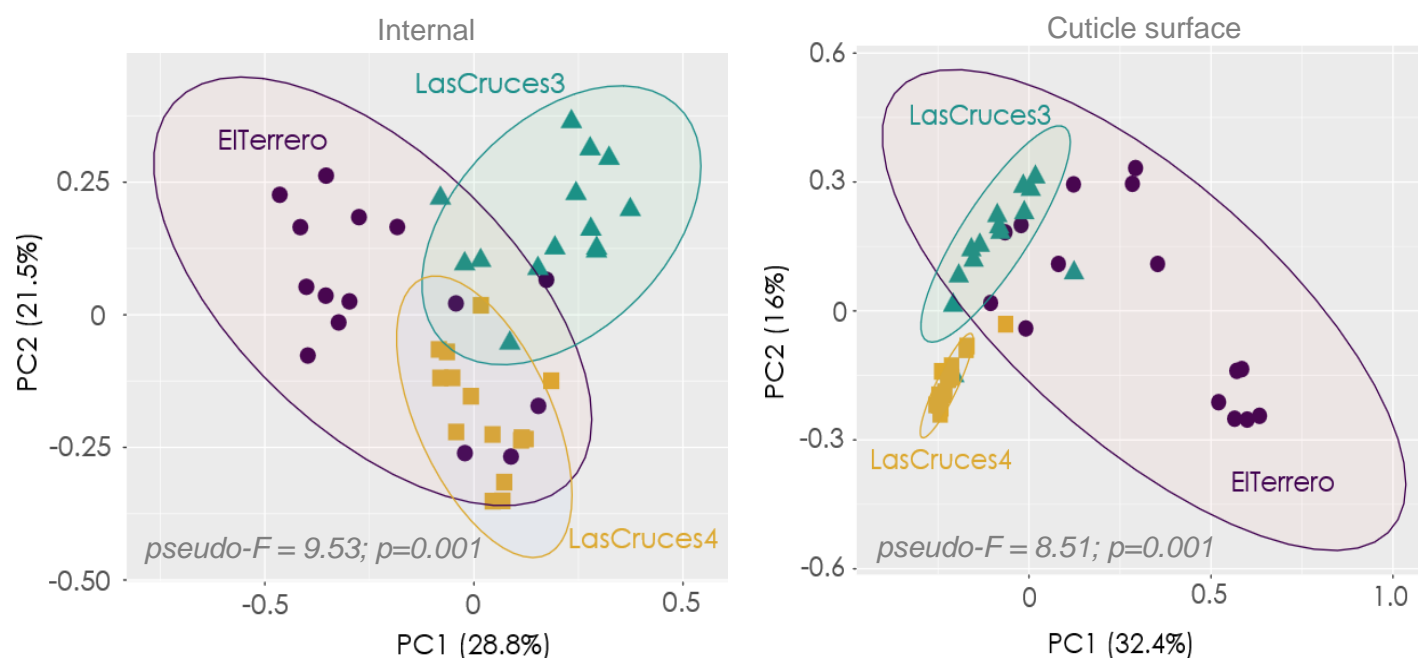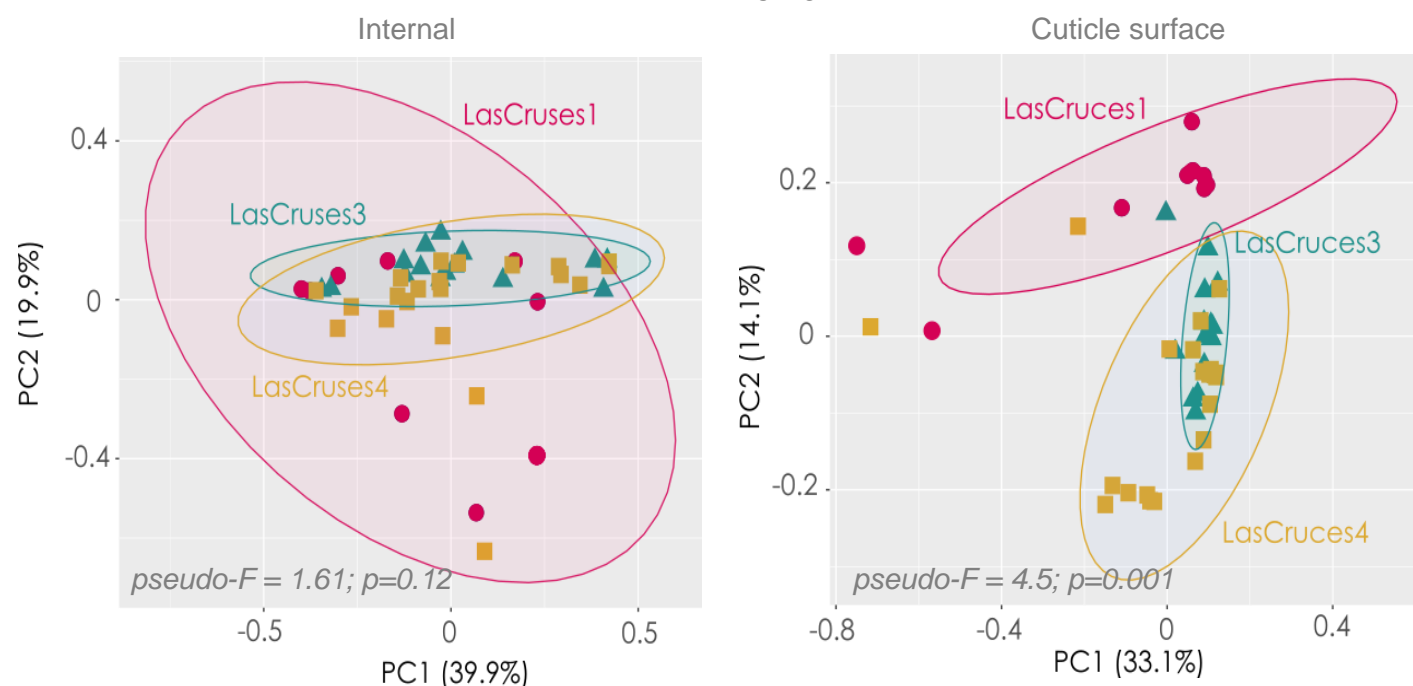753   **Suppl. 4.** Rarefaction depth and plots of sequencing reads

754

Figure 1: Map showing the geographical origins of F_1 *Anopheles albimanus*. F_1 laboratory-colonized mosquitoes were derived from gravid and/or blood fed females collected from each location. Mosquito icons show the geographical origin of the F_1 life stages studied.

Figure 2. Principal coordinate analysis (PCoA) ordinations of internal and cuticle surface microbiota from F₁ laboratory-colonized *An. albimanus*. The PCoA plots are based on Bray-Curtis distances between the microbiota of mosquitoes with differing maternal collection sites. Each point on the plot represents the microbial composition of a pool of three individuals, and mosquito pools are color-coded by their origin/maternal location. For larvae, the first two principal component (PC) axes captured 50% (internal) and 48% (cuticle surface) of the

variance in the data, with both internal and cuticle surface microbiota clustering distinctly by maternal collection site. For adults, the first two PC axes captured 59% (internal) and 47% (cuticle surface) of the variance in the data, with cuticle surface but not internal microbiota clustering distinctly by maternal collection site. PERMANOVA statistics are presented at the bottom of each plot.
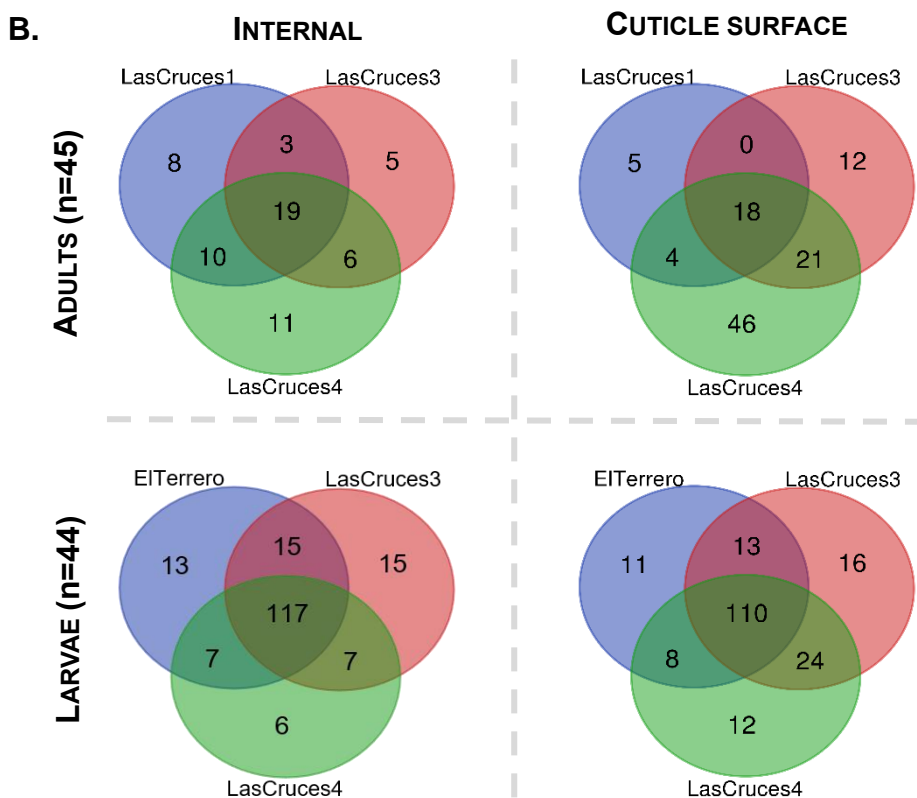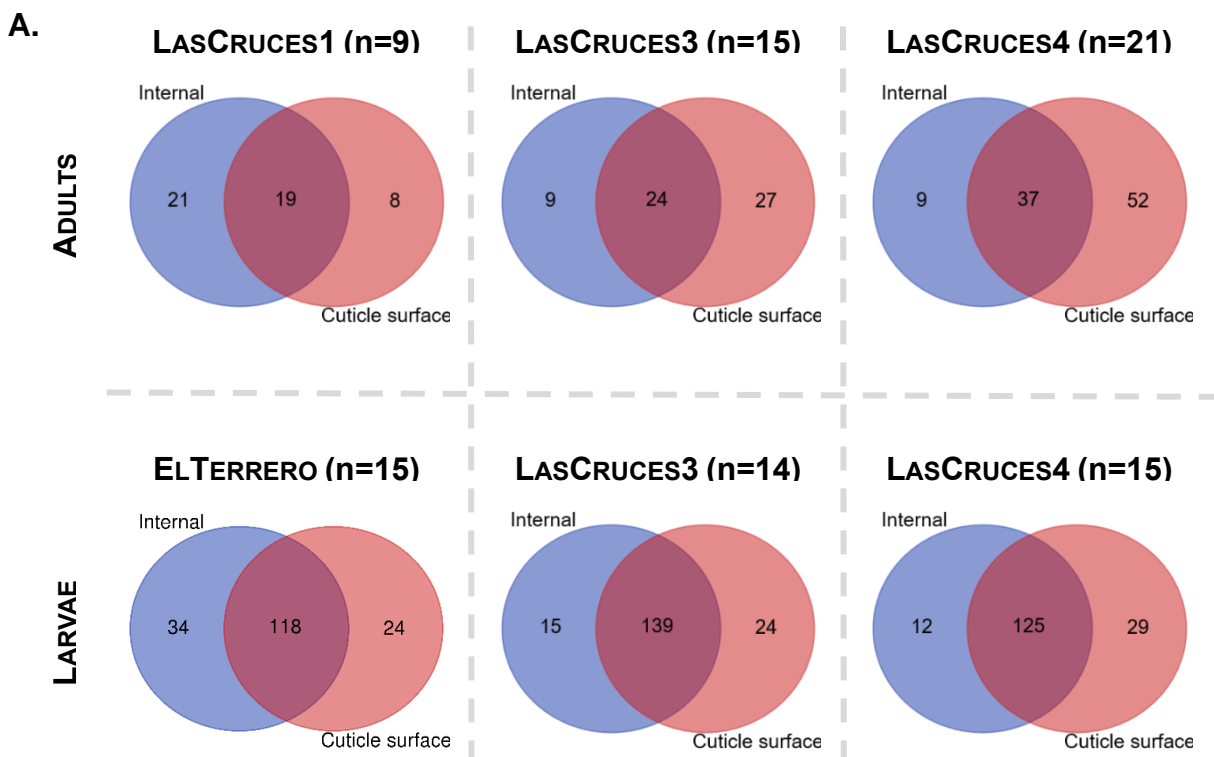
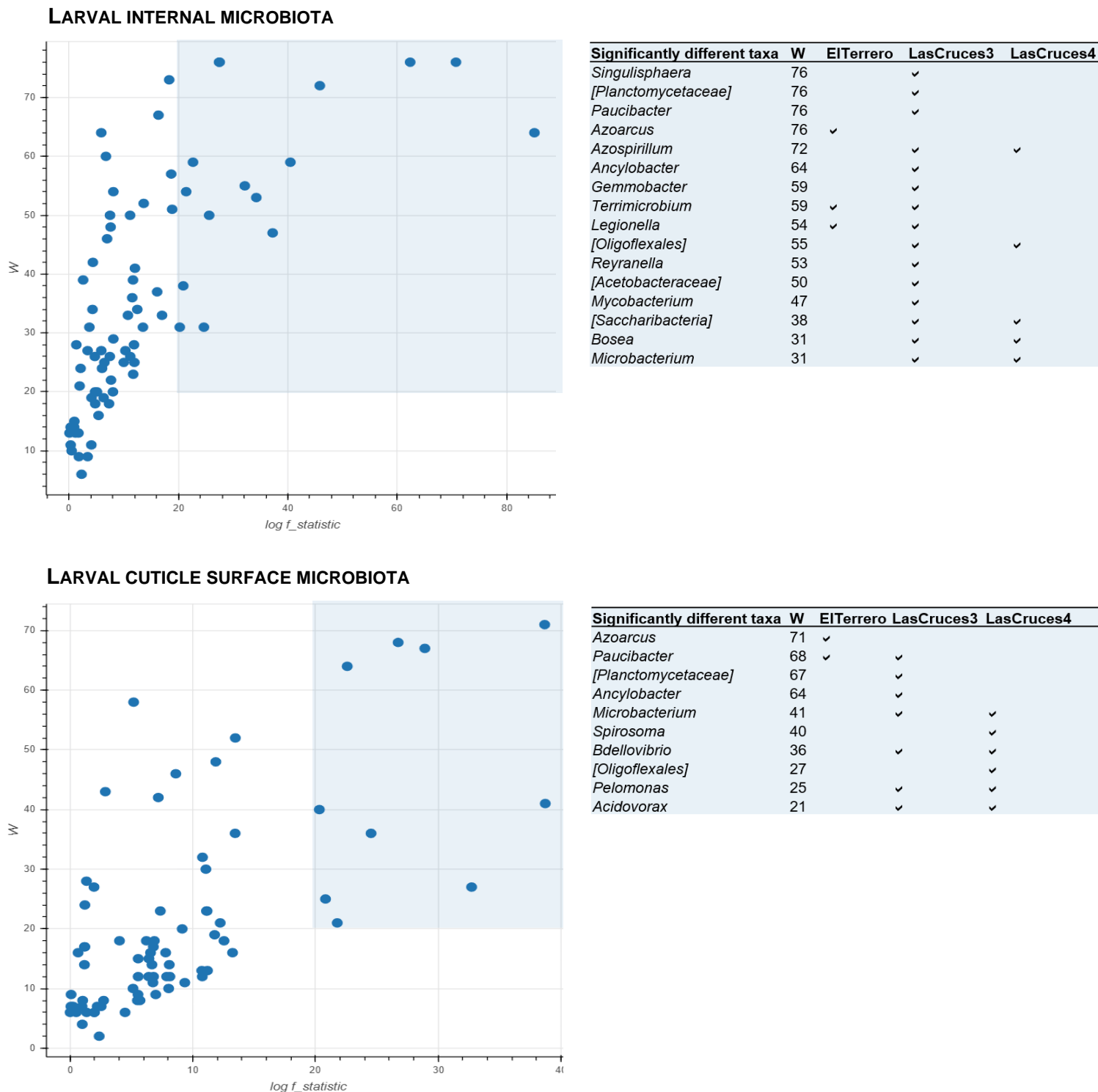**Figure 3. Number of unique and shared microbial taxa between microbial niches (A); number of unique and shared microbial taxa between maternal collection sites (B).** The number of taxa shown in the Venn diagram represent bacterial taxa except in the cuticle surface microbiota of adults from Las Cruces 4, where two archaeal taxa were present. n = pools of mosquito samples analyzed per location or microbial.

a.

b.
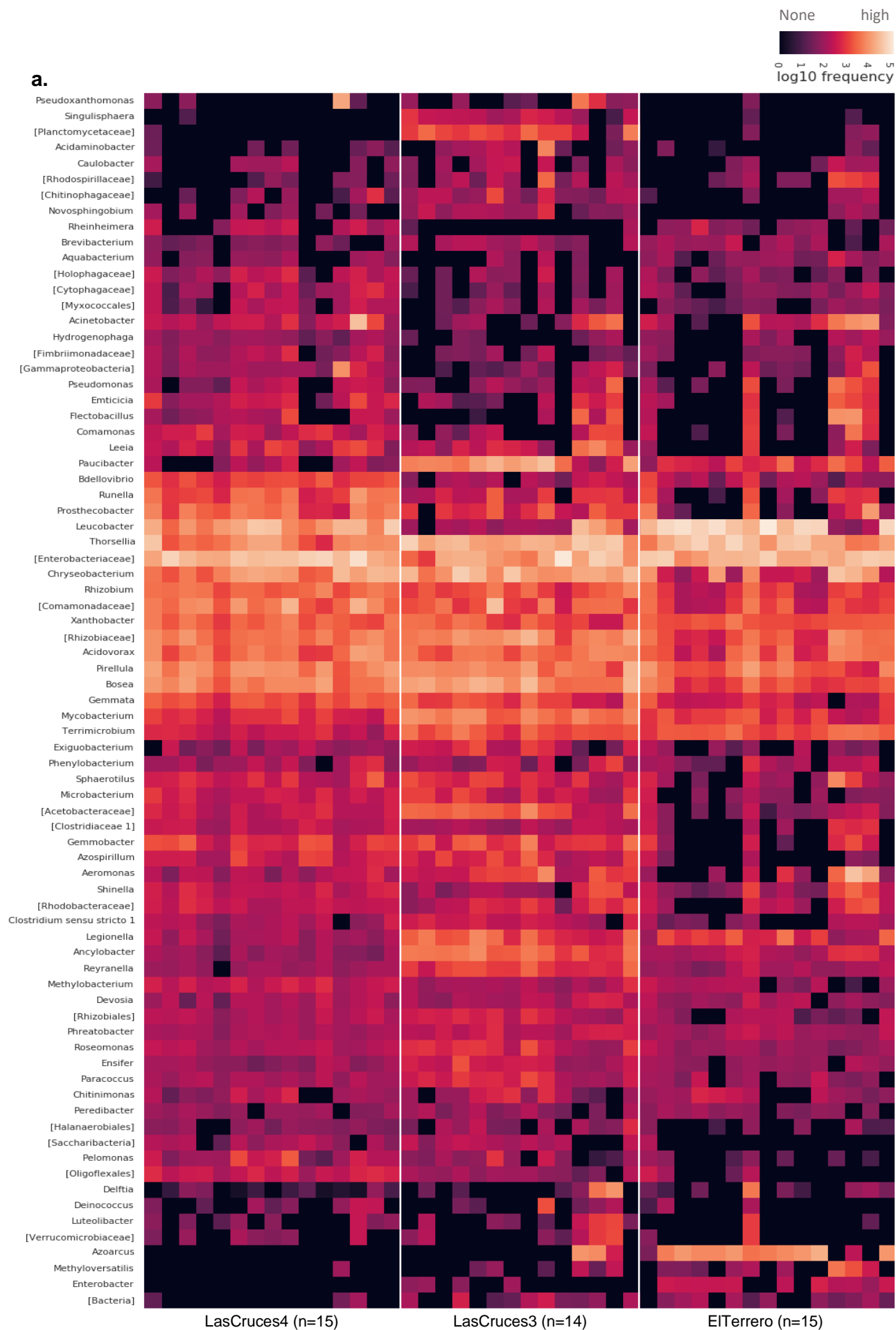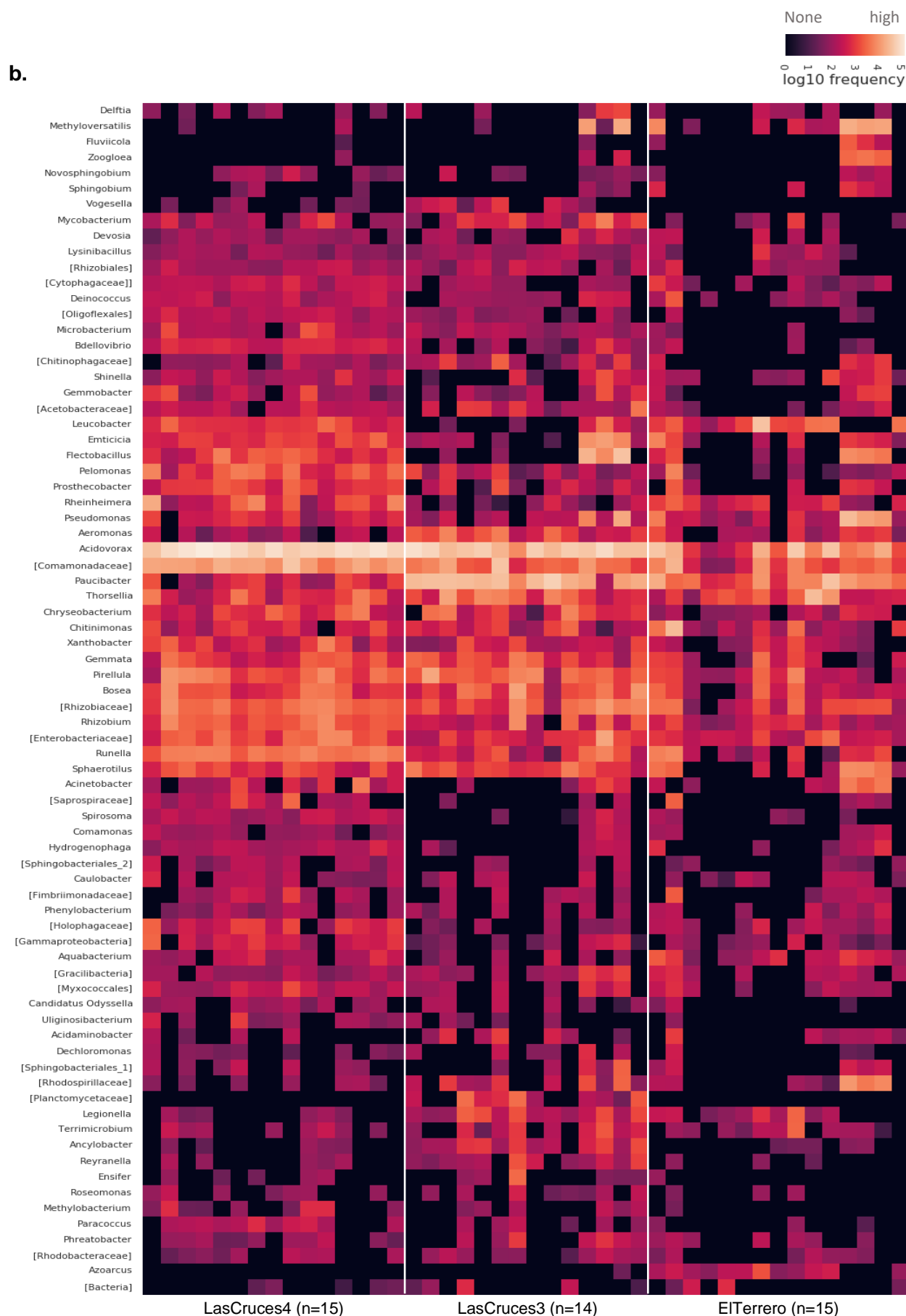
Figure 4. Frequency of ASVs from the internal (a) and cuticle surface (b) microbiota of laboratory-colonized *An. albimanus* F$_1$ larvae originating from different maternal collection sites. ASVs were annotated to the genus level or the lowest possible taxonomic level (in square brackets) and are clustered by the average nearest-neighbors chain algorithm. Only taxonomically annotated ASVs with frequencies ≥2000 are presented.

LARVAL INTERNAL MICROBIOTA

Significantly different taxa	W	ElTerrero	LasCruces3	LasCruces4
Singulisphaera	76		✔	
[Planctomycetaceae]	76		✔	
Paucibacter	76		✔	
Azoarcus	76	✔		
Azospirillum	72		✔	✔
Ancylobacter	64		✔	
Gemmobacter	59		✔	
Terrimicrobium	59	✔	✔	
Legionella	54	✔	✔	
[Oligoflexales]	55		✔	✔
Reyranella	53		✔	
[Acetobacteraceae]	50		✔	
Mycobacterium	47		✔	
[Saccharibacteria]	38		✔	✔
Bosea	31		✔	✔
Microbacterium	31		✔	✔

LARVAL CUTICLE SURFACE MICROBIOTA

Significantly different taxa	W	ElTerrero	LasCruces3	LasCruces4
Azoarcus	71	✔		
Paucibacter	68	✔	✔	
[Planctomycetaceae]	67		✔	
Ancylobacter	64		✔	
Microbacterium	41		✔	✔
Spirosoma	40		✔	
Bdellovibrio	36		✔	✔
[Oligoflexales]	27			✔
Pelomonas	25		✔	✔
Acidovorax	21		✔	✔

Figure 5. Volcano plots of differentially abundant bacterial taxa in F$_1$ laboratory colonized *An. albimanus* larvae originating from three different maternal collection sites. The plots show results of analysis of composition of microbiomes (ANCOM) tests for differentially abundant microbial taxa between maternal collection site, with an effect size set to log F≥20,

and a cut-off of differential abundance set to W≥20 (i.e. a taxon was differentially abundant across maternal collection sites if the ratio of its abundance to those of at least 20 other taxa (25% of all included taxa) differed significantly across sites). Differentially abundant taxa are highlighted (blue shaded area) and the taxa names and locations in which they were most abundant are presented in the adjoining tables.
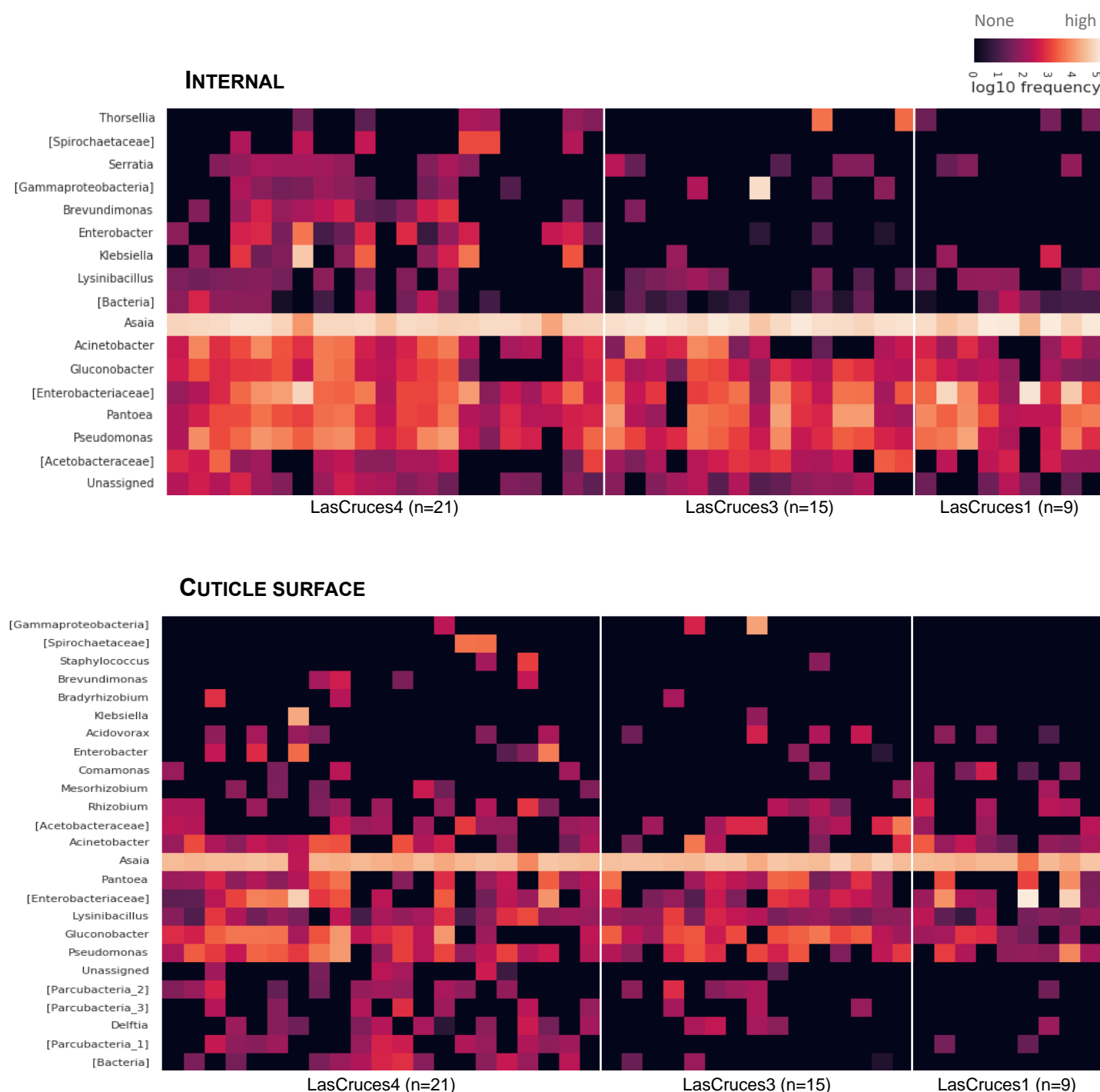
Figure 6. Frequency of ASVs from the internal and cuticle surface microbiota of laboratory-colonized F₁ adult *An. albimanus* originating from different locations. ASVs were annotated to the genus level or the lowest possible taxonomic level (in square brackets) and are clustered by the average nearest-neighbors chain algorithm. Only taxonomically annotated ASVs with frequencies ≥1000 are presented.

Table 1. Descriptive statistics of laboratory colonized F_1 *An. albimanus* processed, per life stage and maternal collection site.

Maternal collection site	Number of mosquitoes (number of pools)	
	L3-L4 larvae	Adult ♀s
El Terrero	45 (15)	-
Las Cruces 1	-	27 (9)
Las Cruces 3	42 (14)	45 (15)
Las Cruces 4	45 (15)	63 (21)

Table 2. Pairwise alpha and beta diversity comparisons of laboratory colonized F$_1$ *An. albimanus* microbiota from different maternal collection sites. a. Pairwise beta (Bray-Curtis) diversity comparison showed significant differences in larval internal and cuticle surface microbiota between maternal collection sites. In contrast, only adult cuticle surface but not internal microbiota were significantly different across maternal collection sites. **b.** Pairwise alpha (Shannon) diversity comparison showed significant differences in larval internal but not cuticle surface microbiota between maternal collection sites (two of the three pairs). In contrast, there was no significant difference in adult internal or cuticle surface microbiota between maternal collection sites. Pairwise alpha and beta diversity comparisons were conducted using Kruskal-Wallis and PERMANOVA (999 permutations) tests respectively, with Benjamini-Hochberg FDR correction (q-value). Significance was determined at q < 0.05.

a.

	Group 1	Group 2	Internal			Cuticle surface		
			pseudo-F	p-value	q-value	pseudo-F	p-value	q-value
Adults	LasCruces1	LasCruces3	2.21	0.088	0.161	**5.80**	**0.001**	**0.003**
	LasCruces1	LasCruces4	1.92	0.107	0.161	**4.97**	**0.004**	**0.005**
	LasCruces3	LasCruces4	0.85	0.443	0.443	**3.07**	**0.005**	**0.005**
Larvae	ElTerrero	LasCruces3	**9.81**	**0.001**	**0.001**	4.48	0.001	0.001
	ElTerrero	LasCruces4	**7.98**	**0.001**	**0.001**	11.43	0.001	0.001
	LasCruces3	LasCruces4	**10.88**	**0.001**	**0.001**	11.37	0.001	0.001

b.

	Group 1	Group 2	Internal			Cuticle surface		
			H	p-value	q-value	H	p-value	q-value
Adults	LasCruces1	LasCruces3	0.00	0.98	0.98	1.09	0.30	0.41
	LasCruces1	LasCruces4	0.49	0.48	0.72	1.23	0.27	0.41
	LasCruces3	LasCruces4	1.77	0.18	0.55	0.67	0.41	0.41
Larvae	ElTerrero	LasCruces3	**6.63**	**0.01**	**0.02**	4.45	0.03	0.10
	ElTerrero	LasCruces4	**6.94**	**0.01**	**0.02**	1.87	0.17	0.26
	LasCruces3	LasCruces4	0.49	0.48	0.48	1.10	0.29	0.29