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1 Assembly processes of bacterial endophytic and epiphytic communities in the canopy of
2 neotropical trees

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26 Abstract:

27 Bacterial phyllosphere communities play a key role in the fitness of plants. Understanding the
28 factors and mechanisms shaping them is necessary to predict how trees would respond to a
29 variety of stresses or disturbances. In tropical forests, the steep vertical gradients in light,
30 rainfall or temperature within the canopy are likely to drive the assembly of bacterial
31 communities in leaves. We explored the assembly processes driving epiphytic and endophytic
32 bacterial communities within the canopy of three Neotropical tree species in French Guiana.
33 We used 16S high-throughput sequencing to characterize leaf bacterial communities and
34 measured foliar traits of host trees to describe variations within the canopy. We found that
35 selection by foliar traits was more important in endophytic communities with a particular effect
36 of traits related to space and available nutrients. Epiphytic communities were also subjected to
37 selection by foliar traits but were more prone to dispersal limitation on top of the canopy. This
38 study provides evidence that the assembly bacterial communities in leaves of tropical trees is
39 jointly driven by determinism and stochasticity. Moreover, the clear differences found between
40 endophytic and epiphytic communities emphasize the major role of the plant host, and provide
41 new perspectives to further investigating the phyllosphere-bacteria interactions.

42

43 Key-words: assembly rules, bacterial phyllosphere communities, foliar traits, 16S
44 metabarcoding, endophytes, epiphytes, neotropical tree, within-canopy vertical gradient

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51 Plants have a long history of co-evolution with microorganisms and their interactions have
52 proved a key asset to the colonization of land by plants (Strullu-Derrien *et al.*, 2018). Trees, as
53 all plants, interact with microbes. These interactions do not only take place in tree roots, but
54 also in leaves, an environment called the phyllosphere. A huge diversity of microorganisms
55 colonizes the phyllosphere, either on the surface (epiphytes) or within the internal tissues of
56 leaves (endophytes, Rosado *et al.*, 2018). These microbial communities encompass most
57 microbial life forms (including filamentous fungi, yeasts and protists), but are largely
58 dominated by bacteria (Rosado *et al.*, 2018; Stone *et al.*, 2018; Chaudhry *et al.*, 2021).
59 Phyllosphere microbial communities (PMCs) have received growing attention in recent years
60 due to their influence on plant fitness (Vorholt, 2012; Vacher *et al.*, 2016; Rosado *et al.*, 2018).
61 In particular, PMCs can modify photosynthesis by inducing an increase in leaf chlorophyll
62 content, or hydraulics through an increase of the stomatal conductance (Rho & Kim, 2017;
63 Rosado *et al.*, 2018). PMCs produce several beneficial effects on plant/insect interactions by
64 reducing predator insect developmental rate, causing feeding deterrence, or by reducing their
65 survival and oviposition (Omacini *et al.*, 2001; Fernandez-Conradi *et al.*, 2018; Vacher *et al.*,
66 2021). Last, PMCs upregulate host defensive pathways leading to increased seedling survival
67 and a better plant defense (Arnold *et al.*, 2003; Álvarez-Loayza *et al.*, 2011; Christian *et al.*,
68 2017). Disentangling the factors and mechanisms shaping PMCs should thus improve our
69 understanding of how trees would respond to a variety of stresses or disturbances.

70 As for communities of macro-organisms, the assembly of PMCS is driven by four fundamental
71 processes: selection (niche-based mechanisms recruiting a particular set of species, i.e.
72 environmental filtering and biotic interactions, Vellend, 2010), ecological drift (neutral
73 stochastic demographic processes, Chase & Myers, 2011; Hubbell, 2011), dispersal (movement
74 and establishment of species across space preventing local extinction through immigration or
75 mass effect, Chase & Myers, 2011; Hanson *et al.*, 2012) and evolutionary diversification

76 (processes generating new genetic variation, Nemergut *et al.*, 2013). In the case of PMCs, the
77 host may participate in selection and dispersion, e.g. by controlling the quantity and quality of
78 the nutrient pool available, or by producing antimicrobial molecules, thereby selecting
79 particular microbial taxa. The host traits responsible for these selective pressures can be
80 conserved within host phylogenetic clades and leave an imprint on the structure of the microbial
81 community (Kohl, 2020). In the case of a selection by the host, the composition of PMCs should
82 partly result from interactions with the host plant and the host response to its environment,
83 rather than stochastic processes (Brooks *et al.*, 2016; Kohl, 2020). Hence, the effects of host
84 phylogeny and environmental determinants on the beta-diversity of microbial communities
85 have been disentangle in several studies (for a review see Lim & Bordenstein, 2020). However,
86 host genotype or physiology can also exert strong effects on communities independently of host
87 phylogeny (Amato *et al.*, 2019a,b), or environmental conditions may obscure signals (Brooks
88 *et al.*, 2016). Last, priority effects could also play a role in the assembly of plant microbiomes
89 (Zheng & Lin, 2020).

90 In tropical trees, PMCs remain understudied (Harrison & Griffin, 2020). So far, available
91 studies showed variations of PMCs among tree species (Redford *et al.*, 2010; Kembel *et al.*,
92 2014), but few have analyzed the processes driving their assembly (Donald *et al.*, 2020).
93 Because of the huge diversity in tree species (Steege *et al.*, 2013) which develop a wide range
94 of ecological strategies (Fortunel *et al.*, 2012; Allié *et al.*, 2015) , partly translated into the
95 chemistry and the morphology of their leaves (Hättenschwiler *et al.*, 2008; Courtois *et al.*,
96 2012), neotropical trees could thus recruit either generalist or specialist PMCs. Recent studies
97 report, however, contradicting conclusions on the effect of the identity of the host plant species,
98 with some studies reporting a significant effect (Laforest-Lapointe *et al.*, 2016, 2017; Gomes
99 *et al.*, 2018; Yao *et al.*, 2019, 2020), while others reporting no effect (Griffin *et al.*, 2019). Part
100 of this inconsistency may lie in that previous studies did not necessarily consider environmental

101 variability. Indeed, PMCs are exposed to strong vertical gradients in light and water availability,
102 which have been previously suggested to influence their composition and diversity (Harrison
103 *et al.*, 2016; Izuno *et al.*, 2016) and could also modify community assembly processes
104 themselves. Such gradients are particularly steep in tropical forests, where the biotic and abiotic
105 conditions in the canopy are very different from those of the understory. The forest top canopy
106 is exposed to strong winds (Bittar *et al.*, 2018), sunlight and high temperatures (Shaw, 2004),
107 while the understory receives only 3% of the light and 30% of the rainfalls (Calder, 2001).
108 These environmental heterogeneities are likely to drive PMCs assembly. In addition, PMCs live
109 in two distinct microenvironments: the internal part (endophytic PMCs) and the surface
110 (epiphytic PMCs) of the leaf more exposed to external environmental conditions (Vacher *et al.*,
111 2016). Consequently, colonization success of the leaf surface may depend on particular
112 microbial functions such as the ability to extract nutrients from the internal tissues while
113 developing on the leaf surface, or live on the few nutrients that leak to the leaf surface, and the
114 ability to withstand dry environments and UVs, through motility or biofilm formation (Vacher
115 *et al.*, 2016; Chaudhry *et al.*, 2021). In contrast, internal tissues of the leaf are richer in nutrients,
116 but contain more plant defense metabolites suggesting that endophytic and epiphytic
117 communities could be driven by different factors. Accordingly, in mangrove tree species,
118 epiphytic PMCs are more strongly shaped by the host than endophytic ones (Yao *et al.*, 2020).
119 Additionally, in Olive trees, variations of nutrients and environmental conditions between
120 internal and external plant tissues determined strong differences in the structure of bacterial
121 communities with notably a dominance of phyla resistant to desiccation and radiation at the
122 surface of leaves (Mina *et al.*, 2020).

123 In this study, we quantified the assembly processes shaping epiphytic and endophytic bacterial
124 communities from the bottom to the top of the canopy of three Neotropical tree species in
125 French Guiana. We used DNA metabarcoding to characterize leaf bacterial communities and

126 measured morphological and chemical foliar traits of host tree to describe variations in the
127 microhabitat of bacteria along the vertical gradient. We specifically tested the hypothesis: (H1)
128 vertical stratification is stronger in epiphytic than in endophytic communities, because
129 epiphytes respond to the within-canopy microclimate gradient ; (H2) the assembly of epiphytic
130 bacterial communities is more deterministic at the top than at the bottom of the canopy, because
131 of harsher abiotic conditions (higher UV radiations and lower humidity) at the top of the canopy
132 ; (H3) foliar traits contribute more to the assembly of endophytic than epiphytic communities,
133 because endophytes are intimately linked to their host tree.

134

135 *Material and Methods:*

136 *Study site and sampling design*

137 Leaf sampling was conducted in October 2017 at the Nouragues Experimental Station (4°02 N,
138 52°414 W) with the help of COPAS (Canopy Operating Permanent Access System) and tree
139 climbers (Figure 1a). Three tree species abundant in the studied area were selected: *Eperua*
140 *falcata* Aubl. (Caesalpiniaceae), *Macrolobium bifolium* Aubl. (Caesalpiniaceae) and
141 *Tetragastris* Gaertn. sp (Burseraceae). *E. falcata* is a shade hemi-tolerant dominant species
142 growing in relatively dense clusters and on a large spectrum of edaphic conditions (Roggy *et*
143 *al.*, 1999; Baraloto *et al.*, 2005). *Macrolobium* is a widespread genus throughout the Amazonian
144 basin (Murphy *et al.*, 2018). *Tetragastris sp.* belongs to the family Burseraceae, an important
145 Neotropical tree lineage known for their chemical defenses including terpenes and phenolics
146 (Fine & Kembel, 2011). The sampling area was approximately 2 ha of homogeneous forest
147 habitat. Five individuals of each tree species were selected, tall enough to have leaves on top
148 of the canopy in full light, and geolocalized (Figure 1b). On each individual, we sampled leaves
149 at 3 heights (Figure 1c): on top of the crown (Top Canopy, TC), in the middle of the crown
150 (Middle Canopy, MC) and on the lower branch of the crown (Bottom Canopy, BC). Three

151 leaves were collected at each height for DNA analysis. Each leaf was collected in a separate
152 plastic bag to avoid cross contaminations, resulting in 135 leaf samples (3 tree species x 5
153 individuals x 3 heights x 3 leaves). In addition, we collected 2 leaves for each height and each
154 individual, that were further used for leaf traits measurements. Leaves were sealed immediately
155 after sampling in pre-weighted zip-lock plastic bags and placed in a cooler until return to the
156 lab.

157

158 *Microclimate measurements*

159 Irradiance at each height (TC, MC, BC) was recorded with a photosynthetically active radiation
160 linear sensor (Acceptor model LP-80 PAR/LAI Ceptometer, Decagon Devices). Instantaneous
161 measurements were realized at the level of sampled leaves for 4 trees and values were then
162 compared to a reference sensor located at the top of a nearby flux tower (NOURAFLUX,
163 nouragues.cnrs.fr), which provide an estimate of the incident irradiance above the canopy (i.e.
164 the full irradiance). For each height, relative irradiance (%) was computed as the ratio of the
165 local and above the canopy irradiance.

166 Air temperature and relative humidity were measured with environmental HOBO sensors
167 (model U23-001, HOBO Pro V2 Temp/RH Data logger, Amanvillers, France) placed at each
168 canopy height (TC, MC, BC) on two trees over 24 hours.

169

170 *Morphological and chemical leaf traits measurements*

171 We measured 10 leaf traits that may influence the diversity, composition and function of leaf
172 bacterial communities, because they are indicative of (1) water availability, (2) space available
173 for bacterial colonization, (3) sugars availability, or (4) defense metabolism.

174 To approximate (1) water availability for leaf bacteria, we measured leaf water content (LWC)
175 and stomatal density (SD). The aperture and density of stomata control the exchange of gases

176 (water and carbon dioxide) between the interior of the leaf and the atmosphere, participating in
177 the control of water by the whole plant (Hetherington & Woodward, 2003). Stomata are also a
178 major route of bacterial colonization inside the leaf (Chaudhry *et al.*, 2021). To approximate
179 (2) space available for bacterial colonization, we measured 4 morphological traits: leaf dry
180 matter content (LDMC), leaf mass per area (LMA), leaf thickness (LT) and leaf area (LA). All
181 four are informative of the production of biomass by the plant and the density of leaf (Garnier
182 *et al.*, 2001). To approximate (3) sugars availability for leaf bacteria, we measured Chlorophyll
183 and Nitrogen contents, which reflect the photosynthesis and the metabolism activities of the
184 plant (Field & Mooney, 1986; Evans & Seemann, 1989). Finally, leaf carbon content and leaf
185 construction cost (CCM) were used as proxies of (4) plant defense, given that the defense
186 response involves a wide array of mechanisms which demand a massive redistribution of energy
187 (Chaudhry *et al.*, 2021). One sampled leaf was used to measure SD while the other one was
188 used to measure LWC and all other traits. To measure SD, a band of lamina was fixed with a
189 FAA buffer (5% formalin, 5% acetic acid, 50% ethanol). Stomatal density of the adaxial
190 surfaces of the lamina (SD, number of stomata per cm²) was then determined in the lab with
191 imprints made using transparent nail varnish. The number of stomata was recorded using an
192 optical microscope for each lamina as the average of five randomly selected areas of 1cm² each
193 (Leroy *et al.*, 2009). To measure the other traits, fresh weight (fw, g) was determined by
194 weighing the sealed pre-weighted bags containing the fresh leaves. Leaf area (LA, cm²) of fresh
195 leaves was measured with a scanner and the ImageJ software (Schneider *et al.*, 2012). Leaf
196 thickness (LT, μm) was estimated as the mean of three to eight measurements (four per simple
197 leaf and one per leaflet for compound leaf) with a digital micrometer (Digimatic micrometer,
198 Mitutoyo, Japan). We took care to avoid the main veins for these measurements. Chlorophyll
199 contents (Chl, $\mu\text{g cm}^{-2}$) were estimated with a portable chlorophyll meter (SPAD-502 Konica-
200 Minolta, Osaka, Japan). Four to eight SPAD measurements per leaf (one per leaflet for

201 compound leaf) were averaged to obtain the SPAD estimate of the leaf. We then used the
202 calibration equation proposed by (Coste *et al.*, 2010). Leaves were then dried at 60°C for 72h
203 and weighted (dry weight, g). Leaf water content ($LWC = \frac{fw-dw}{fw}$, %), leaf mass per area
204 ($LMA = \frac{dw}{LA}$, g m⁻²) and leaf dry matter content ($LDMC = \frac{dw}{fw}$, mg g⁻¹) were calculated.
205 Leaves were then ground to determine C, N and ash contents (%) at the USRAVE platform of
206 Bordeaux (France). Leaf specific construction cost (CCM, g glucose g⁻¹ dw) was estimated
207 from C, N and ash content, according to (Poorter, 1994). As in Coste *et al.* (2011), this
208 computation assumes all nitrogen is absorbed as nitrate hence an additional cost for nitrate
209 reduction in non-photosynthetic tissues was taken into account.

210

211 *DNA sampling*

212 To collect epiphytic DNA, the whole upper and lower surfaces of each leaf were carefully wiped
213 with a piece (2x2 cm) of Whatman paper sterilized by autoclaving (120°C, 20 min) and soaked
214 in sterile CTAB buffer (2% cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone, 100
215 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA), within 4 hours after leaves harvesting. The
216 Whatman paper was stored in a 2ml Eppendorf tube filled with CTAB for downstream DNA
217 extraction. Surface-sterilization protocol was performed as recommended by Compant *et al.*,
218 (2021) to collect endophytic communities. Each leaf was surface-sterilized in 0.525% sodium
219 hypochlorite (2 min) and 70% ethanol (2 min) and two segments of 2 cm² area from the lamina
220 were cut and stored in a 2ml Eppendorf tube filled with CTAB for downstream DNA extractions
221 close to a field Bunsen burner to create a sterilized environment. This resulted in 270 DNA
222 samples (3 tree species x 5 individuals 3 heights x 3 leaves x 2 DNA types (epiphytic or
223 endophytic)).

224

225 *DNA extraction, amplification and sequencing*

226 DNA was extracted using a CTAB extraction method (Carrell & Frank, 2014). We added 800
227 μ l of CTAB solution to 0.6 g of tissue, incubated for 2 h at 60°C, and homogenized with glass
228 beads for 3 min. Proteins were removed with the addition of an equal volume of chloroform,
229 centrifuged for 10 min at 16 000 g, and placed the top aqueous phase in a sterile tube. Nucleic
230 acids were precipitated with the addition of 1/10 volume of cold 3 M sodium acetate and 1/2
231 volume cold isopropanol, froze them at -20°C for 12 h, and centrifuged for 30 min at 16 000 g.
232 The supernatant was discarded, 700 μ l of 70% ethanol was added to the solution, and
233 centrifuged for 10 min. The air-dried pellet was resuspended with 30 μ l of DNA resuspension
234 fluid (1.0 M Tris-HCL and 0.1 M EDTA) and stored it at -20°C. The V5-V6 region of the
235 bacterial 16S rRNA gene was amplified using the chloroplast-excluding forward primer 799f
236 (Chelius & Triplett, 2001) and the reverse primer 1115R (Reysenbach & Pace, 1995). Forward
237 and reverse primers were tagged in 5' with a combination of two different 8-nucleotide labels.
238 The PCR amplification was done in 25 μ l with 1x buffer, 0.22 mM dNTP each, 0.45 μ M each
239 tagged primer, 2.84 mM MgCl₂, 0.11 mg/ml bovine serum albumin and 0.04 U/ μ l Taq
240 polymerase (Solis Biodyne). The thermocycling conditions were as follows: 5 min at 94°C, 30
241 cycles of 30 sec at 94°C, 30 sec at 58°C and 40 sec at 78°C. The PCR reactions were done for
242 each sample separately and amplicons were quantified with a fluorescence-based method
243 (Qubit 3.0, Invitrogen Life Tech) and pooled in equimolar conditions. The library was built
244 using the FASTERIS MetaFast protocol (FASTERIS SA, Plan-les-Ouates, Switzerland) and
245 sequenced on one run of a MiSeq Illumina platform (FASTERIS SA,) using the paired-end
246 sequencing technology. To control for potential contaminants (Salter *et al.*, 2014) and false
247 positive sequences caused by tag-switching events (Esling *et al.*, 2015) the sequenced
248 multiplexes comprised extractions/PCR negative controls and unused tag combinations.

249

250 *Bioinformatics*

251 A total of 3.181.610 sequencing reads were obtained and curated using the OBITools3 package
252 (Boyer *et al.*, 2016) and the R software version 3.5.3 (R Core Team 2019) following the
253 procedure described in (Zinger *et al.*, 2019). Paired-end reads were assembled and assigned to
254 their respective samples. After dereplication, low quality sequences (i.e. containing Ns, being
255 shorter than 80 pb or singletons) were excluded. PCR/sequencing errors were removed from
256 the dataset and remaining reads were clustered into operational taxonomic units (OTUs) at 97%
257 similarity using the Sumacust algorithm (Kopylova *et al.* 2016). The most abundant read was
258 considered representative of the OTU and taxonomically assigned using the SILVAngs r138.1
259 (Quast *et al.*, 2013). Finally, we checked the taxonomic assignment and kept only sequences
260 assigned to the Bacteria kingdom. Curation procedure yielded to 649,212 reads corresponding
261 to 10152 bacterial OTUs in 203 samples. Finally, because the sequencing depths were uneven
262 across samples (Figure S1), the sequencing depth of each sample was standardized by randomly
263 resampling a number of reads equal to the first quartile of read number across samples (n=1864)
264 which removed 51 samples and kept 152 samples for subsequent analyses.

265

266 *Statistical analyses*

267 All analyzes were conducted using the R software (R Development Core Team) and with
268 packages vegan 2.5-7 (Oksanen *et al.*, 2020), adespatial 1.7-17 (Dray *et al.*, 2021), ape 5.5
269 (Paradis & Schliep, 2019), phyloseq 1.36.0 (McMurdie & Holmes, 2013), picante 1.8.2
270 (Kembel *et al.*, 2010), lme4 1.1-27.1 (Bates *et al.*, 2015), FactoMineR 2.4 (Lê *et al.*, 2008) and
271 ggplot2 3.3.5 (Wickham, 2016, p. 2).

272 The importance of deterministic processes in the assembly of PMCs was assessed by estimating
273 their variation in composition (i.e. beta-diversity) and diversity (i.e. species richness) among
274 host species, within the canopy (TC, MC, BC), within the leaf (epiphytes or endophytes), and
275 across spatial location of the host trees. The diversity and composition of bacterial communities

276 were assessed using exponential of the Shannon index (see Supplementary) and Bray-Curtis
277 distance (Lou Jost & Chazdon, 2010). Because spatial autocorrelation may arise from both
278 spatially structured environmental factors and dispersal, spatial variation was account for by
279 including Principal Components of Neighbours Matrices (PCNM) spatial eigenvectors based
280 on the geographical coordinates of tree individuals in the model. PCNMs significance were
281 tested by computed Moran's I with 1000 permutations and retained only three PCNMs that
282 showed a significant and positive value of Moran's I. Variations in PMCs composition were
283 estimated by performing a Permutational Multivariate Analyses of Variance (PERMANOVA)
284 with 999 permutations (Anderson, 2001) and dissimilarities among samples were visualized
285 using a Principal Coordinate Analyses (PCA). The model included geographical position of
286 trees (3 significant PCNMS), host species (*E. falcata*, *M. bifolium*, *Tetragastris sp.*), vertical
287 position in the canopy (low, middle and top of the canopy), position on the leaf (epiphytes and
288 endophytes) and their interactions as fixed effects. The homogeneity of variance among groups
289 (host species, position in the canopy and on the leaf) groups were tested as PERMANOVA
290 analysis are sensitive to heterogeneity in within-group variance (Oksanen *et al.*, 2020).

291 The relationships between foliar traits and the alpha- (Shannon index) and beta-diversity (Bray-
292 Curtis distance) of endophytic and epiphytic communities were assessed using a linear mixed
293 model and a permutational multivariate analysis of variance, respectively. Both models
294 included leaf traits related to water resources (LWC, SD), available space (LDMC, LMA, LT,
295 LA), available sugars (Chlorophyll and N contents) and defense metabolism (CCM and C
296 content) implemented as fixed effects, and host species implemented as random effect.
297 Homogeneity of variance among host species was also tested after performing the
298 PERMANOVA model.

299 Significant variations of the leaf traits mentioned above were beforehand tested individually by
300 performing an analysis of variance (ANOVA, $p < 0.01$). The model included the vertical

301 positions in the crown, the host species and their interactions. Normality of each variable was
302 tested with a Shapiro test and variables were transformed with a box-cox when necessary.
303 To disentangle the relative contribution of niche-based and neutral processes in the assembly
304 of epiphytic and endophytic communities in leaves separately, we implemented a null modeling
305 approach as proposed by Stegen *et al.* (2013) and Vass *et al.* (2020). Pairwise phylogenetic
306 turnover between communities were calculated with the mean-nearest-taxon-distance
307 (β MNTD) metric (Fine & Kembel, 2011) to infer the strength of selection. MUSCLE (Edgar
308 2004) with default parameters was used to align sequences found in communities of each
309 vertical position (TC, MC, BC). Pairwise distances were computed with a Juke-Cantor model
310 (JC69) and built using a phylogenetic tree with the UPGMA method for each community. The
311 pairwise phylogenetic turnover between communities was calculated as the mean nearest taxon
312 distance metric (β MNTD) while accounting for taxa relative abundance. According to Stegen
313 *et al.* (2013), the degree to which β MNTD deviates from a null model expectation should reflect
314 the degree to which community composition is driven by selection, which is expected to recruit
315 phylogenetically related OTUs due to niche conservatism. To quantify the deviation, we built
316 a null model by shuffling randomly taxa names and abundances across tips of the phylogeny,
317 and then calculated β MNTD. This allowed us to obtain the null distribution of β MNTD, after
318 999 randomizations. Then, β -nearest taxon index (β NTI) was computed as the difference
319 between observed β MNTD and the mean of the null distribution in units of s.d. of the null
320 distribution. For this procedure we used the R codes provided by (Tripathi *et al.*, 2018).
321 According to Stegen (2013), when $|\beta$ NTI|>2, the observed difference in phylogenetic turnover
322 between communities is the result of selection. When $|\beta$ NTI|<2, the observed difference in
323 phylogenetic community composition should result from dispersal limitation, ecological drift
324 or homogenizing dispersal. We quantified these processes by computing Bray-Curtis based
325 Raup-Crick metric (RC_{bray}) on pairwise comparisons when $|\beta$ NTI|<2. Following the method

326 proposed by Dini-Andreote *et al.* (2015), the relative contribution of each process to variations
327 in community composition were estimated as follow: dispersal limitation was estimated as the
328 percentage of pairwise comparisons with $|\beta\text{NTI}| < 2$ and $\text{RC}_{\text{bray}} > +0.95$; homogenizing dispersal
329 was estimated as the percentage of pairwise comparisons with $|\beta\text{NTI}| < 2$ and $\text{RC}_{\text{bray}} < -0.95$;
330 and ecological drift was estimated as the percentage of pairwise comparisons with $|\beta\text{NTI}| < 2$
331 and $|\text{RC}_{\text{bray}}| < 0.95$. We then computed the contribution of each process (selection, dispersal
332 limitation, homogenizing dispersal or ecological drift) for each position in the canopy in
333 epiphytic and endophytic communities.

334

335 *Results*

336 *Variations in leaf microclimate, physiology and phenology within the canopy and across* 337 *tropical tree species*

338 Temperature and relative humidity showed no significant differences between the top and the
339 bottom of the canopy (86% +/- 12 % of relative humidity and 26 +/- 3°C), but the relative
340 irradiance varied from 77 % +/- 34% of the PAR at the top of the canopy to 1.55% +/-1.5% on
341 the soil (Figure 1c). All leaf traits exhibited strong and significant variations among host species
342 and positions within the canopy (Figure 2). Interactions between both factors were only
343 significant for C, N contents and CCM (Figure 2). Most leaf trait variations followed the same
344 trends across the different tree species. Leaf SD, LDMC, LMA, LT and chlorophyll content
345 decreased from the top to the bottom of the canopy while LWC and LA increased.

346

347 *Leaf bacterial community composition associated to tropical tree species*

348 Leaf bacterial communities were dominated by 6 classes including Alphaproteobacteria
349 (49.06%), Actinobacteria (23.64%), Sphingobacteria (5.79%), Gammaproteobacteria (4.50%),
350 Chloroflexia (5.43%) and Cytophagia (2.44%) (Figure S2). 5 orders represented 76% of the

351 OTUs: Rhizobiales, Actinomycetales, Sphingomonadales, Sphingobacteriales and
352 Rhodospirillales (Figure S3). We were able to assign 200 families and 630 genera with mainly
353 *Methylobacterium* (12.60%), and *Sphingomonas* (10.58%).

354

355 *Factors driving leaf bacterial communities at the leaf, the canopy and tree scales*

356 Bacterial community composition varied significantly across host species, geographical
357 position and within the leaf (Table 1). Host species was the main driver of the bacterial
358 assemblage in leaves, explaining 10.7% (Table 1) of bacterial composition and influencing
359 significantly its diversity (Table S1). However, tree species showed no deep differences
360 regarding their bacterial communities at the Class or Family taxonomic rank (Figure S2)
361 indicating that variations of bacterial communities among their host species occur at lower
362 taxonomic ranks. Surprisingly, the variation in community composition within the canopy was
363 less important than differences between leaf inside tissues and its surface suggesting that
364 community assemblage of Bacteria in tropical tree species occur at the leaf spatial scale.

365

366 *Assembly processes in endophytic and epiphytic communities*

367 In endophytic communities, deterministic assembly processes ($\beta\text{NTI} < |2|$) represented 50.3%,
368 52.1% and 43.7% at the top, middle and bottom of the canopy respectively (Figure 3a). Overall,
369 no significant difference of pairwise comparisons of βNTI was observed suggesting that
370 deterministic and stochastic factors are equally important in endophytic communities within
371 the canopy. However, when estimating the effect of each process within the canopy we found
372 that endophytic communities were mainly driven by selection (52.10% at TC, 43.7% at MC
373 and 50.3% at BC) and ecological drift (41% at TC, 39.5% at MC and 47 % at BC) whereas
374 dispersal limitation had limited effects at the top (6.8%) and bottom of the canopy (2.6%)
375 (Figure 4a). Interestingly, the latest had more effect in the middle of the canopy (16.8%).

376 In epiphytic communities, deterministic assembly processes ($\beta\text{NTI} < |2|$) represented 34.1%,
377 49.6% and 43.1% at the top, middle and bottom of the canopy respectively (Figure 3b). We
378 found significant differences of pairwise comparisons of βNTI values overall among the three
379 vertical positions (Figure 3b). As for endophytic communities, epiphytic communities were
380 also mainly driven by selection (34% at TC, 49.6% at MC and 43.1% at BC) and ecological
381 drift (46.5% at TC, 44% at MC and 50.6% % at BC). Dispersal limitation had increasing effect
382 on epiphytic communities from the top to the bottom of the canopy (19.4% at TC, 6.55% at MC
383 and 6.25% at BC, Figure 4b).

384

385 *Effects of leaf traits in bacterial epiphytic and endophytic communities:*

386 Leaf traits accounted for 21.6 % and 18.4% of the variation in endophytic and epiphytic
387 community composition, respectively (Table 2). In endophytic communities, 9.17% of the
388 variation were explained by LDMC (3.6%), Chlorophyll (2.97%) and C contents (2.60%). In
389 epiphytic communities, 9.17% of the variation were significantly explained by LT (2.57%),
390 Chlorophyll (1.88%), N (2.07%) and C (2.48%) contents. Leaf traits had no significant effects
391 on endophytic and epiphytic alpha diversity (Table S2).

392

393 *Discussion*

394 *Host species is a strong driver of bacterial leaf communities*

395 Bacterial communities in the phyllosphere were largely dominated by Alphaproteobacteria,
396 Actinobacteria and Bacteroidetes which is in line with previous observations in tropical trees
397 (Kembel *et al.*, 2014; Griffin & Carson, 2018; Donald *et al.*, 2020). Proteobacteria are known
398 to carry methylotrophy, nitrification or nitrogen fixation, Bacteroidetes phyllosphere bacteria
399 are often aerobic and pigmented suggesting a good adaptation to this environment and
400 Actinobacteria are plant pathogens, nitrogen-fixers, fungal antagonists or decomposers (Stone

401 *et al.*, 2018). *Sphingomonas* (Sphingomonales) and *Methylobacterium* (Rhizobiales), the two
402 most abundant genera, are common in phyllosphere communities (Bulgarelli *et al.*, 2013).
403 *Methylobacterium* uses the methanol produced by the leaf metabolism as source of carbon and
404 energy and *Sphingomonas* produces multiple transport proteins suggesting an ability to
405 scavenge diverse plant metabolites (Delmotte *et al.*, 2009). In line with previous studies on
406 tropical tree species (Kembel *et al.* 2014, Laforest-Lapointe *et al.* 2016, Harrison and Griffin
407 2020), host species were the most important drivers of PMC, together with the geographical
408 factors. Simultaneously, the position on the leaf was also significant to a lesser extent, contrary
409 to what was observed in Laforest-Lapointe *et al.* (2016) and Herrmann *et al.* (2021). Contrary
410 to our first hypothesis, no prominent impact of the vertical stratification on diversity or
411 composition was observed, neither in endophytic or in epiphytic communities.

412

413 *Deterministic and stochastic processes equally govern the assembly of endophytic and*
414 *epiphytic communities across the vertical gradient*

415 According to the β -nearest taxon index (β NTI), both deterministic and stochastic processes are
416 jointly responsible for the assembly of leaf PMCs (Stegen *et al.* 2013, Vass *et al.* 2020, Zhou &
417 Ning 2017, Vacher 2016). Niche-based factors included ecological filters imposed by the
418 environment, here we showed a wide variation in microclimate (mainly irradiance) from the
419 top of the canopy to the base of the crown, but also by the host, namely the physiological and
420 morphological variations of leaves across tree species and induced by the irradiance vertical
421 gradient. Overall, and contrary to our expectations, separate analyses of endophytic and
422 epiphytic bacterial communities showed that endophytic communities were more prone to
423 determinism (46%) than epiphytic ones (37%), but with variations according to the position in
424 the canopy. The assembly of the endophytic and epiphytic was also significantly driven by
425 stochastic processes such as dispersal limitation, ecological drift or diversification. The

426 importance of these effects differed between epiphytes and endophytes communities and across
427 the vertical gradient. Dispersal limitations had more effects on endophytic PMCs assembly in
428 the middle of the crown of the tree whereas it had higher effects at the top of the canopy for
429 epiphytic PMCs. Beside the seed and germination environment, bioaerosols are one of the
430 major sources of the colonization of the leaf by bacteria (Bulgarelli *et al.*, 2013); together with
431 rainfall and subsequent splashing of raindrops (Morris, 2002) or transmission by herbivorous
432 insects or animals (Vacher *et al.*, 2016, 2021). While it is easy to imagine that the top of the
433 canopy is more subject to wind and rainfalls (Bittar *et al.*, 2018), their effects inside the crown
434 are more difficult to understand. Moreover, the contribution of ecological drift (or evolutionary
435 diversification) on the turnovers was similar on endophytic or epiphytic communities and
436 whatever the positions. Here, we cannot distinguish between ecological drift and evolutionary
437 diversification. It is assumed that ecological drift should occur in communities where the
438 strength of deterministic processes is low (Chase & Myers, 2011), which is rather not the case
439 in this study, and interactions between ecological drift and other processes may be important in
440 host-associated microbial communities (Kohl, 2020). On the other hand, host-associated
441 communities should evolve in time in a manner that is congruent with host phylogeny (Brooks
442 *et al.*, 2016; Kohl, 2020; Lim & Bordenstein, 2020).

443

444 *Epiphytic communities are selected by similar traits as endophytic communities in leaves*

445 The identity of host species and leaf traits related to the plant economics spectrum strongly
446 shaped the composition (beta-diversity) of both endophytic and epiphytic communities. We
447 expect this to be mainly due to the physiology of the leaf, as suggested by our results, as already
448 shown by (Kembel *et al.*, 2014). The impact of leaf resource uptake strategies on bacterial
449 communities can be explained through their impact on local leaf nutrients and water availability
450 for bacteria (Kembel *et al.*, 2014; Lajoie & Kembel, 2021). As expected, foliar traits explained

451 up to 21.6% and 18.4 % of the variation of beta-diversity between samples in endophytic and
452 epiphytic bacterial communities respectively, which suggests a more important role of leaf
453 attributes on endophytic than on epiphytic communities. Leaf dry matter content for endophytic
454 communities and leaf thickness for epiphytic ones, i.e. traits related to space for colonization,
455 explained similar proportions of variation. The environment at the surface of a leaf and its
456 chemistry (cuticle layer, stomata, hydathodes) is known to create more stressful and nutrient-
457 poor conditions for bacteria. To cope with them, bacteria develop growth strategies such as
458 forming large aggregates, key to successful colonization (Vorholt, 2012; Vacher *et al.*, 2016;
459 Chaudhry *et al.*, 2021). On another hand, stomatal chambers or apoplastic spaces inside leaves
460 are large intercellular spaces known to host microbial communities (Vacher *et al.*, 2016) which
461 can explain the effect of leaf thickness we showed. These host-adapted colonizers are also more
462 tolerant to harmful UV radiation oxidative stress and desiccation, particularly important for top-
463 canopy leaves (Delmotte *et al.*, 2009; Vorholt, 2012). Epiphytic communities were more
464 strongly shaped by traits related to available sugars (3.95% of the variation), namely
465 chlorophyll and nitrogen contents than epiphytic communities (2.97 %), and similarly by traits
466 linked to defense such as carbon content (2.48% and 2.6% of the variation in epiphytic and
467 endophytic communities respectively). These results suggest a selection by nutrients resulting
468 from photosynthesis. Indeed, leaf nitrogen content is linked to the maximum photosynthetic
469 rate (Cornelissen 2003) and correlates with composition of bacterial communities (Kembel *et*
470 *al.*, 2014; Laforest-Lapointe *et al.*, 2016; Lajoie *et al.*, 2020). Leaf nutrients include also volatile
471 organic compounds such as methanol exported to the surface of leaf (Vacher *et al.*, 2016;
472 Lemanceau *et al.*, 2017), which may explain the slightly higher abundance of *Methylobacterium*
473 we found in epiphytic communities. Interestingly, leaf traits related to water content (leaf water
474 content and stomatal density) showed no impact on communities contrary to what is expected
475 in the literature. Leaf surface microtopographic features is known to regulate the assembly of

476 the bacterial communities (Vorholt, 2012); in particular stomatal density of leaf vein density
477 because they improve the supply of available water to the surrounding microorganisms (Yan *et*
478 *al.*, 2022). Last, most of the leaf traits investigated decreased or increased significantly with the
479 vertical light gradient within the canopy, which was expected (Markestijn *et al.*, 2007; Poorter
480 *et al.*, 2019), and suggest an indirect effect of the irradiance on the composition of endophytic
481 and epiphytic communities in leaves.

482

483 To conclude, our study provides evidence that the assembly of bacterial communities in tropical
484 leaves was governed by selection imposed on endophytic communities; and with epiphytic
485 communities more exposed to dispersal limitation on top of the canopy. We found that internal
486 features of leaf were selective factors for epiphytic communities in the same way as for
487 endophytic ones. This emphasizes the global selective pressure (i.e. the phyllosphere effect) of
488 the plant on microbial communities, but with marked differences. However, stochastic process
489 is also important and ecological drift (or evolutionary diversification) was similar in both
490 communities within the canopy, which raises questions about how strongly the phylogeny of
491 the host might also drives the composition of bacterial communities.

492

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497

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500 the manuscript with inputs from all authors. All authors gave approval for publication.

501

502 Data availability: Leaf traits measurements, all metadata on tree individuals, processed
503 sequence data used for this manuscript, R scripts is available from the Zenodo repository, under
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505

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Tables:

Table 1: Bacterial community structure explained by host species, vertical position in the canopy, position on the leaf (epiphyte vs endophyte), their interactions and geographical position. PERMANOVA analysis of Bray-Curtis dissimilarities. Statistical significance was assessed from 999 permutations

| | Df | SumSq | R ² | F | Pr(>F) |
|----------------------------|----|-------|----------------|-------|--------------|
| Host species (H) | 2 | 5.167 | 0.107 | 9.676 | 0.001 |
| Position in the canopy (C) | 2 | 0.484 | 0.010 | 0.907 | 0.605 |

| | | | | | |
|--------------------------|-----|--------|--------------|-------|--------------|
| Position in the leaf (L) | 1 | 0.857 | 0.018 | 3.208 | 0.001 |
| MEM1 | 1 | 1.13 | 0.023 | 4.233 | 0.001 |
| MEM2 | 1 | 0.35 | 0.007 | 1.311 | 0.123 |
| MEM3 | 1 | 0.425 | 0.009 | 1.593 | 0.043 |
| (H)x(C) | 4 | 0.92 | 0.019 | 0.861 | 0.797 |
| (H)x(L) | 2 | 2.503 | 0.052 | 4.688 | 0.001 |
| (C)x(L) | 2 | 0.589 | 0.012 | 1.103 | 0.287 |
| (H)x(C)x(L) | 4 | 0.819 | 0.017 | 0.767 | 0.961 |
| Residual | 131 | 34.979 | 0.725 | | |
| Total | 151 | 48.223 | 1.000 | | |

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788 Table 2: Results of a PERMANOVA testing the effects of 10 leaf traits categorized in 4 groups on dissimilarity matrices of endophytic and
 789 epiphytic communities, host species was implemented as random factor and 1000 permutations were done. LWC: Leaf water content, SD: Stomatal
 790 density, LDMC: Leaf Dry Matter Content, LMA: Leaf Mass per Area, LA: Leaf Area, LT: Leaf Thickness, CCM: Leaf construction cost.
 791 Significant p-values (<0.05) and associated Rsquare are indicated in bold. The direction of variation of values of foliar traits from the top (TC) to
 792 the bottom (BC) of the canopy is also indicated (but see also Figure 2)

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| | | Variations | ENDOPHYTES | | | | | EPIPHYTES | | | | |
|------------------------|------|------------|------------|-------|-------|----------------|--------------|-----------|-------|-------|----------------|--------------|
| | | from TC to | Df | SS | F | R ² | Pr(>F) | Df | SS | F | R ² | Pr(>F) |
| | | BC | | | | | | | | | | |
| Leaf water resources | LWC | increase | 1 | 0.316 | 0.943 | 0.0164 | 0.511 | 1 | 0.234 | 0.846 | 0.0081 | 0.606 |
| | SD | decrease | 1 | 0.326 | 0.972 | 0.0169 | 0.526 | 1 | 0.422 | 1.526 | 0.0146 | 0.139 |
| Space for colonization | LDMC | decrease | 1 | 0.693 | 2.069 | 0.036 | 0.059 | 1 | 0.612 | 2.215 | 0.0212 | 0.641 |
| | LMA | decrease | 1 | 0.345 | 1.031 | 0.0179 | 0.817 | 1 | 0.518 | 1.875 | 0.018 | 0.441 |
| | LA | increase | 1 | 0.333 | 0.993 | 0.0173 | 0.527 | 1 | 0.434 | 1.571 | 0.015 | 0.139 |
| | LT | decrease | 1 | 0.403 | 1.203 | 0.0209 | 0.155 | 1 | 0.741 | 2.682 | 0.0257 | 0.006 |

| | | | | | | | | | | | | |
|--------------------|---------------------|----------|----|--------|-------|---------------|--------------|----|--------|-------|---------------|--------------|
| Available sugars | Chlorophyll content | decrease | 1 | 0.571 | 1.704 | 0.0297 | 0.015 | 1 | 0.541 | 1.958 | 0.0188 | 0.028 |
| | N content | stable | 1 | 0.326 | 0.972 | 0.0169 | 0.458 | 1 | 0.598 | 2.164 | 0.0207 | 0.018 |
| Defense metabolism | C content | stable | 1 | 0.5 | 1.493 | 0.026 | 0.037 | 1 | 0.714 | 2.584 | 0.0248 | 0.004 |
| | CCM | decrease | 1 | 0.333 | 0.994 | 0.0173 | 0.326 | 1 | 0.475 | 1.72 | 0.0165 | 0.055 |
| Residual | | | 45 | 15.08 | | 0.784 | | 85 | 23.494 | | 0.816 | |
| Total | | | 55 | 19.226 | | 1 | | 95 | 28.785 | | 1 | |

795

796 Figures captions

797 Figure 1: a) Localization of the sampling site in French Guiana, b) localization of each tree in
798 the sampling area, c) variation of light, temperature and humidity levels along the vertical
799 gradient, d) Correlation circle of variables on the two first axis from a PCA performed on the
800 10 leaf traits. e) PCA of individual leaves on the two first PCA axes with open circles, filled
801 circles and triangles corresponding to leaves from *E.falcata*, *M.bifolium* and *T. sp.* respectively.
802 Axes 1 and 2 represent, respectively, 50 and 17% of the overall leaves' inertia. e and f)
803 Contributions of each variable to the two first dimensions of the PCA.

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805 Figure 2: Variations of each individual foliar leaf trait in the canopy and between host species.
806 Differences between host species, position in the canopy and their interactions were tested with
807 an analysis of variance. Normality of each variable was tested with a Shapiro test and the
808 variable was transformed with a box-cox when necessary. Significant p-values are indicated
809 ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; NS, non-significant. LWC: Leaf water content in percent,
810 SD: Stomatal density in number.cm², LDMC: Leaf Dry Matter Content in mg.g⁻¹, LMA: Leaf
811 Mass per Area in g.m⁻², LA: Leaf Area in cm², LT: Leaf Thickness in μm , Chlorophyll content
812 in $\mu\text{g.cm}^{-2}$, CCM: Leaf construction cost in g of Glucose.g⁻¹, C and N: Carbon and Nitrogen
813 contents in g.g⁻¹. E.f = *Eperua falcata*; M.b = *Macrolobium bifolium*; T.sp = *Tetragastris sp*;
814 white = top canopy, light grey = middle canopy, dark grey = bottom canopy.

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816 Figure 3: Patterns of between-community nearest taxon intext (βNTI) in bacterial endophytic
817 (a) and epiphytic (b) communities within the top (TC), the middle (MC) and the bottom (BC)
818 of the canopy. Box plots of βNTI distributions show the median (thick line), the first quartile
819 (lower box bound), the third quartile (upper box bound), the range of data that deviate from the
820 box no more than 1.5 times the height of the box (vertical lines). p-values resulting from a

821 multiple comparisons Kruskal-Wallis test are shown. Horizontal dashed red lines indicate
822 upper and lower significance threshold at $bNTI = +2$ and -2 respectively.

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824 Figure 4: Relative contribution of deterministic (selection by environment, biotic interactions
825 or nonrandom dispersal) and stochastic processes (dispersal limitation, mass effect, ecological
826 drift or diversification) for each vertical position in the assembly of bacterial endophytic (a)
827 and epiphytic (b) communities. White color corresponds to dispersal limitation or mass effect,
828 grey to ecological drift or diversification and dark grey to selection.