

1 **Microbial diversity in stingless bee gut is linked to host wing size and influenced by the**
2 **environment**

3

4 Hongwei Liu^{1*}, Mark A. Hall¹, Laura E. Brettell^{1,2}, Juntao Wang¹, Megan Halcroft³, Scott Nacko¹,
5 Robert Spooner-Hart¹, James M Cook¹, Markus Riegler¹, Brajesh Singh^{1,4*}

6

7 ¹Hawkesbury Institute for the Environment, Western Sydney University, Penrith, NSW 2753,
8 Australia.

9 ²Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool, L3 5QA,
10 United Kingdom.

11 ³PO Box 474, Lithgow, 2790.

12 ⁴Global Centre for Land-based Innovation, Western Sydney University, Penrith, NSW, Australia.

13 *Corresponding author: Hongwei Liu h.liu2@westernsydney.edu.au; Brajesh Singh
14 b.singh@westernsydney.edu.au.

15 **Abstract**

16 Stingless bees are important social corbiculate bees, fulfilling critical pollination roles in many
17 ecosystems. However, their gut microbiota, particularly the fungal communities associated with
18 them, remains inadequately characterised. This knowledge gap hinders our understanding of bee
19 gut microbiomes and their impacts on the host fitness. We collected 121 samples from two species,
20 *Tetragonula carbonaria* and *Austroplebeia australis* across 1,200 km of eastern Australia. We
21 characterised their gut microbiomes and investigated potential correlations between bee gut
22 microbiomes and various geographical and morphological factors. We found their core
23 microbiomes consisted of the abundant bacterial taxa *Snodgrassella*, *Lactobacillus* and
24 Acetobacteraceae, and the fungal taxa Didymellaceae, *Monocilium mucidum* and *Aureobasidium*
25 *pullulans*, but variances of their abundances among samples were large. Furthermore, gut bacterial
26 richness of *T. carbonaria* was positively correlated to host forewing length, an established
27 correlate to body size and fitness indicator in insects relating to flight capacity. This result indicates
28 that larger body size/longer foraging distance of bees could associate with greater microbial
29 diversity in gut. Additionally, both host species identity and management approach significantly
30 influenced gut microbial diversity and composition, and similarity between colonies for both
31 species decreased as the geographic distance between them increased. We also quantified the total
32 bacterial and fungal abundance of the samples using qPCR analyses and found that bacterial
33 abundance was higher in *T. carbonaria* compared to *A. australis*, and fungi were either lowly
34 abundant or below the threshold of detection for both species. Overall, our study provides novel
35 understanding of stingless bee gut microbiomes over a large geographic span and reveals that gut
36 fungal communities likely not play an important role in host functions due to their low abundances.

37

38 **Key words**

39 *Austroplebeia australis*; bacterial and fungal communities; core microbiome; forewing size;

40 geographic variation; stingless bee; *Tetragonula carbonaria*

41 **Introduction**

42 Insect guts harbour many microorganisms across the three primary regions; foregut, midgut and
43 hindgut (Chapman and Chapman, 1998). These microorganisms have various host functions that
44 include aiding nutrient extraction from foods (Engel and Moran, 2013), detoxification of harmful
45 compounds (Ceja-Navarro et al., 2015) and protection against parasites and pathogens (Endt et al.,
46 2010). Social corbiculate bees in particular, are known to possess characteristic gut microbiomes.
47 Honey bee (*Apis* spp.) guts, for example, consist of a core bacterial community including
48 *Snodgrassella*, *Gilliamella*, *Lactobacillus* Firm-4 and -5 and *Bifidobacterium* (Koch and Schmid-
49 Hempel, 2011; Kwong et al., 2017), which is acquired mostly through social transmission and
50 from the hive environment (e.g. the hive surface) (McFrederick et al., 2017; Liu et al., 2019).
51 Increasing evidence shows that, like other insects, corbiculate bees may have formed mutualistic
52 relationships with their microbial gut symbionts. The bees benefit from the gut microbiome
53 primarily through defence against enemies and regulation of growth and development (Vásquez
54 and Olofsson, 2009; Koch and Schmid-Hempel, 2011; Zheng et al., 2017). Conversely, changes
55 to the gut microbiome composition of social bees, such as those caused by antibiotic exposure, can
56 lead to dysregulated immune systems and reduced ecological fitness (the overall health and well-
57 being of a bee colony or individual bee) (Liu et al., 2019).

58

59 Among the corbiculate bees, stingless bees (Apidae: Meliponini) comprise >500 species globally,
60 of which 11 recognised species occur in Australia, under two genera: *Austroplebeia* and
61 *Tetragonula* (Dollin and Dollin, 1997; Dollin et al., 2015). They are important pollinators of
62 natural plants and crops (Heard, 1999; Hall et al., 2020), and can be harnessed by beekeepers either
63 through rescuing colonies from felled trees, or propagation in man-made hives (Halcroft et al.,

64 2013). In contrast to the managed stingless bees that are kept and cared for by humans in hives,
65 wild stingless bees are those living and building nests in natural settings, such as forests, jungles,
66 or other types of undisturbed habitats. *Austroplebeia* and *Tetragonula* spp. are similar in body size
67 and colour and occur along the east coast of Australia. However, they belong to different
68 phylogenetic clades, and *Austroplebeia* tends to occur further inland into semi-arid habitats (Heard,
69 1999). Their behaviour also differs; for example, *T. carbonaria* is more active in flight and
70 evidently collects more resin and pollen than *A. australis* (Leonhardt et al., 2014). In contrast, *A.*
71 *australis* colonies are more likely to focus on collecting high-quality nectar (e.g., of high sugar
72 concentrations) (Leonhardt et al., 2014). Such distinct behaviour, along with differences in
73 available floral resources within their habitats can thus shape different gut microbiomes (Vásquez
74 et al., 2012). Previous studies of Australian stingless bees have identified a novel clade of host-
75 specific lactic acid bacteria (*Lactobacillus*) (Leonhardt and Kaltenpoth, 2014) and showed that
76 bacterial communities can change rapidly with site movement (Hall et al., 2021). However, these
77 studies used relatively few samples and to date there is limited comparison of gut microbial
78 communities across species and geographic ranges. Additionally, like other animal gut
79 microbiome studies, fungal communities in the guts of insects, including stingless bees, have
80 received little attention (de Paula et al., 2021). Insect-associated fungi, including moulds and yeasts,
81 can contribute to host nutrient provision (Menezes et al., 2015). For instance, the intracellular
82 symbiotic fungi of beetles, *Symbiotaphrina* spp., can both aid in food digestion and detoxify a
83 variety of plant materials (Dowd and Shen, 1990). Despite their importance, fungal community
84 composition and diversity, interactions with the host and drivers of fungal community assembly
85 remain poorly understood. One of our primary objectives in this study was to characterize the gut

86 fungal community of stingless bees. By doing so, we aimed to gain a more comprehensive
87 understanding of the stingless bee gut microbiome, beyond just the gut bacterial communities.

88

89 Wing size in insects is an essential functional trait for flight performance (flying ability for
90 foraging, mating, and finding new nesting sites) (Wootton, 1992), foraging, dispersal and
91 migration (Johansson et al., 2009). Maximum flight distances of stingless bees were highly
92 correlated with wing size in six stingless bee species, suggesting that flight capacity is a function
93 of their wing size, and thus, bees with larger wings may be able to fly further to forage on more
94 diverse plant resources, resulting a greater number of microbial species in their gut (Casey et al.,
95 1985; Byrne et al., 1988; Araújo et al., 2004). Similarly, it was found that bees with larger body
96 size (as indicated by intertegular span) had larger foraging distances than smaller bees (Greenleaf
97 et al., 2007). However, to date no link has been found between insect morphological traits, such
98 as wing, tibia (where the pollen basket is found) and body sizes, and gut microbial diversity.
99 Despite a correlation between morphological traits and gut microbial diversity does not necessarily
100 imply a causal relationship, understanding the potential relationship in stingless bees could help to
101 shed light on the factors contributing to microbial diversity in different insect species. It could also
102 have implications for agriculture and other areas where insect populations play a critical role.
103 Given that microbiomes are evidently linked to the health and vitality of insect species, we then
104 aimed to determine whether there is a correlation between bee gut microbiomes and morphological
105 traits, such as wing and body size. We hypothesized that a larger gut area or increased foraging
106 distance in stingless bee may support a greater number of gut microbial species.

107

108 In this study, we collected 121 stingless bee samples from the two species, *T. carbonaria* and *A.*
109 *australis* in Australia. We investigated the bee gut microbial abundances and diversity using qPCR
110 analyses and amplicon sequencing (targeting the 16S rRNA and ITS genes). We measured bee
111 morphological traits and tested their correlations with the gut microbial diversity. As research has
112 shown, both bacteria and fungi reside in the gut of insect pollinators, but in honey bees, for example,
113 the abundance of fungal communities is typically lower than that of bacterial communities (Engel
114 and Moran, 2013). Although this pattern may also exist for stingless bees, there is currently a lack
115 of studies investigating the fungal community and its level of abundances. We therefore tested the
116 hypotheses that (i) the bee gut microbiome structure is influenced by host species, geographic
117 location and by whether they are wild or managed (cultivated in hives); (ii) characteristics
118 associated with flight and foraging capacities, such as forewing size, positively correlate to host
119 gut microbiome diversity; and (iii) gut fungal communities are of lower abundances relative to
120 bacterial communities.

121

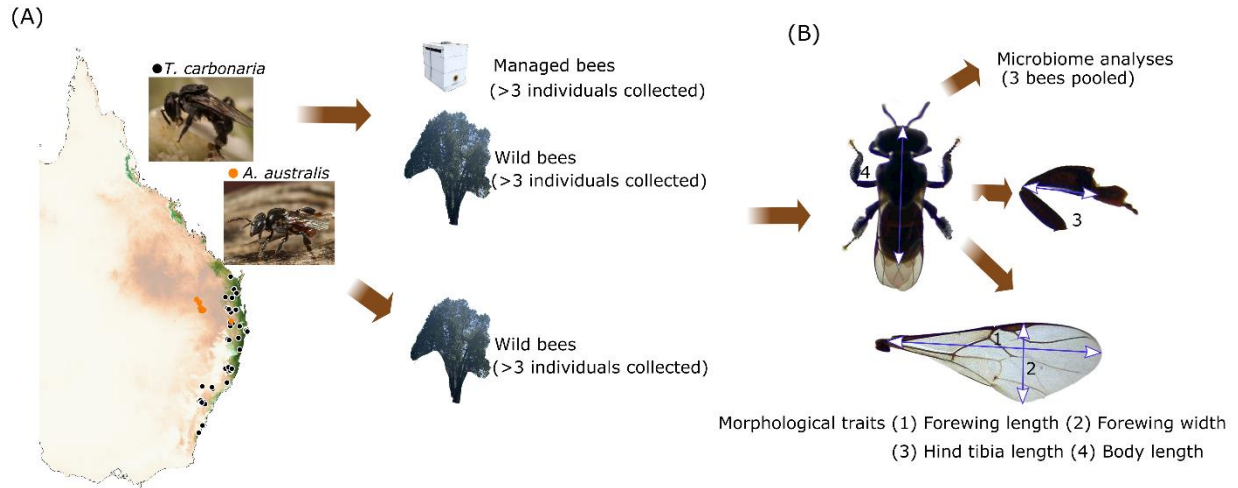
122 **Material and methods**

123 *Specimen collection, measurement and gut dissection*

124 We collected 121 samples (one sample per nest/hive) from the two most common and widespread
125 Australian stingless bee species, *T. carbonaria* and *A. australis*, within their distributional ranges
126 in QLD and NSW, Australia (Fig.1A, Table S1). We collected 3~12 bees per sample so that we
127 had ample bee materials for investigation. Stingless bee foragers were collected from individual
128 managed hives or wild locations (separated by at least 1 km). The *T. carbonaria* samples (n= 80)
129 were collected between September 2018 and January 2020 across a range of 1,200 km in eastern
130 Australia. They comprised 43 samples collected from managed hives and 37 samples collected

131 from the wild (e.g., national parks). The wild and managed bee populations were geographically
132 separated by more than 20 km, making it unlikely that they directly influenced each other through
133 contact or pathogen spillover. The *A. australis* samples (n= 41) had been collected from wild tree-
134 living colonies across 250 km within their natural distributional range (Fig.1A). The geographic
135 range and management types (wild/managed) of species were considered in analyses when
136 comparing between samples. Consequently, *T. carbonaria* was used for analysing management
137 effects on gut microbiomes, and wild bees of both species were compared to investigate species
138 effects. During sampling, geographic coordinates (longitude and latitude) were recorded, and all
139 collected specimens were immediately preserved in 70% ethanol and stored at -20 °C prior to gut
140 dissection and morphological measurement. Three individuals from each sample were used for
141 morphometric measurements that may infer bee flight performance/fitness including forewing
142 length, forewing area (forewing length \times width), hind tibia length and total body length (Wootton,
143 1992) (Fig.1B). These morphological traits were measured using a digital microscope (Leica
144 EZ4W, Leica Microsystems, Buffalo Grove, IL), by the same observer. Digital images of the
145 whole bees were taken for measurement of body length, and forewings and hind tibia were
146 removed, mounted under a cover slip, photographed and measured (Fig.1B). The whole gut of
147 each individual was then dissected on a sterile Petri dish under a microscope using sterilized
148 forceps, and the gut materials of the three bees were pooled and transferred to a 1.5 mL sterile
149 centrifuge tube and preserved at -20 °C prior to DNA extraction for the analyses of gut
150 microbiomes. We decided to use a pooling approach to obtain sufficient DNA for molecular
151 analyses as we found using individuals could be insufficient to achieve this (Hall et al., 2021).

152



153

154 **Fig.1** Sampling locations of stingless bees across eastern Australia and the morphological traits
 155 measured. (A) Sampling locations; and (B) morphological traits measured for the two stingless
 156 bee species.

157

158 *DNA extraction from gut material and library preparation for high throughput amplicon*
 159 *sequencing*

160 DNA was extracted from pooled gut materials using the DNeasy Blood and Tissue Kit (Qiagen)
 161 as per the manufacturer's recommendations. DNA samples were quality checked and quantified
 162 using a Nanodrop 2000 (Thermofisher Scientific, USA) and Qubit (Thermofisher Scientific, USA)
 163 respectively, before being stored at -20°C. Library preparation and bacterial and fungal amplicon
 164 sequencing were then carried out at the Next Generation Sequencing Facility (Western Sydney
 165 University, Australia). The 16S rRNA gene (V3-V4 region) primers 341F (CCT ACG GGN GGC
 166 WGC AG) and 805R (GAC TAC HVG GGT ATC TAA TCC) (Herlemann et al., 2011) and the
 167 fungal ITS2 primers fITS7 (GTG ART CAT CGA ATC TTT G) and ITS4 (TCC GCT TAT TGA
 168 TAT GC) (White et al., 1990; Ihrmark et al., 2012) were used for the amplification and subsequent
 169 sequencing. For library preparation of the Miseq sequencing, the PCR was carried out using the
 170 Eppendorf Master Cycler Pro S system. The thermal conditions comprised an initial denaturation

171 step at 95°C for 3 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing
172 at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension step was at 72°C
173 for 5 minutes. After amplification, the amplicons were purified using Agencourt AMPure XP
174 beads from Beckman Coulter, Inc. Dual indexing was performed on the purified amplicons using
175 the Nextera XT v2 Index Kit from Illumina, following the manufacturer's instructions. The indexed
176 amplicons were quantified using the PicoGreen dsDNA Quantification method. The samples were
177 then pooled at equimolar ratios and sequenced on an Illumina MiSeq instrument at the Western
178 Sydney University's Next-Generation Sequencing facility in Richmond, NSW. The sequencing
179 run included 15% PhiX Control v3 from Illumina and used a MiSeq Reagent Kit v3 (600 cycle
180 kit), as per the manufacturer's instructions. For both the bacterial and fungal sequencing, negative
181 (no template) and positive (a Zymo mock community) controls were sequenced, which worked as
182 expected and were removed from further analyses. All the samples were run on the MiSeq
183 (Illumina) platform, generating 300 bp paired end reads.

184

185 *qPCR analyses for the identification of total bacteria in stingless bee gut*

186 To compare total bacteria between stingless bee gut samples, quantification of 16S rRNA genes
187 was performed relative to the host bee DNA abundance. All DNA samples were normalised to 5.0
188 ng μL^{-1} . 16S rRNA gene was amplified using universal 16S rRNA gene primers 16S-f (AGG ATT
189 AGA TAC CCT GGT AGT CC) and 16S-r (YCG TAC TCC CCA GGC GG) (Kešnerová et al.,
190 2017) while stingless bee actin gene was amplified using primers act-f (CCT GGA ATC GCT
191 GAC AGA ATG C) and act-r (AAG AAT AGA TCC ACC GAT CCA TAC) (Hall et al., 2021).
192 Reactions were carried out in a 10 μL system containing 5.0 μL 1 \times LightCycler 480 SYBR Green
193 I Master mix, 1.5 μL (15 pmol) primer mix, 2.5 μL nucleotide free water, and 1 μL DNA template,

194 and reactions without adding DNA templates were used as negative controls. Thermal cycling
195 conditions included an initial denaturation of 95°C for 5 min, 35 cycles of 95°C for 15 s, 53 °C for
196 15s and 72°C for 30s, followed by a melting curve analysis (Hall et al., 2021). The bacterial
197 abundance for each sample was calculated using the formula below,

$$198 \quad \text{Total 16S rRNA copy numbers} = \frac{(\text{Eff. Actin})^{Ct(\text{Actin})}}{(\text{Eff. 16S rRNA})^{Ct(16S rRNA)}}$$

199 where *Eff.* is the PCR amplification efficiency calculated using LinRegPCR (version 2021.2)
200 (Ruijter et al., 2013). We attempted to determine the total fungal abundance for the samples but
201 found very low fungal abundance (Ct values mostly >35), which made accurate evaluation of the
202 total fungi infeasible.

203

204 *Bioinformatics and statistics*

205 Sequencing files (FASTQ format) were processed using QIIME2 software and its plugins (version
206 2019.7; <http://qiime2.org/>) (Bolyen et al., 2019). Sequencing quality was first assessed using
207 FastQC (Andrews, 2010), then QIIME2 implementation of cutadapt v2019.7.0 was used for
208 removal of primer sequences, and DADA2 v2019.7.0 (Callahan et al., 2016) was used for error-
209 correction, quality filtering, chimera removal and constructing feature tables and final sequence
210 files. DADA2 shows several advantages over other methods including (i) improved accuracy in
211 amplicon sequence variant (ASV) calling and better resolution of closely related ASVs, (ii) higher
212 accuracy compared to methods relying on reference databases, and (iii) DADA2 resolves closely
213 related ASVs with accuracy, which is particularly important for gut microbiome analysis where a
214 high degree of microbial diversity exists (Callahan et al., 2016). Sequencing reads were truncated
215 at 260 bp and 240 bp for forward and reverse reads, respectively, resulting in sequence quality
216 Q>20. The ASVs obtained were summarised and then assigned with taxonomic information using

217 the q2-feature-classifier, a QIIME2 plugin. For the bacterial data, a Naïve Bayes classifier pre-
218 trained on full length Silva sequences (99%) was used to assign taxonomy to each representative
219 sequence. Bee- and plant-associated mitochondria and/or chloroplast sequences were removed
220 from the feature table to retain microbial features only. For the ITS fungal dataset, the classifier
221 was trained to UNITE v8.0 database (99%) (UNITE Community, 2019) (DeSantis et al., 2006).
222 The number of reads for the bacterial and fungal sequencing data was rarefied to 7,125 and 944
223 sequences, respectively, per sample by re-sampling the feature table. The mean number of
224 observed ASVs, Chao1, Simpson's, Shannon and Evenness diversity index values were calculated
225 using QIIME2.

226

227 **Statistical analyses**

228 R version 4.0.3 (2020-10-10) was used for analyses unless otherwise stated. Correlations between
229 stingless bee traits and gut microbial alpha diversity (the diversity of microbial species within a
230 sample, calculated in QIIME2) were examined using multiple linear regression and visualised in
231 R. The effect of stingless bee species and management types on gut microbial community
232 composition and diversity were investigated using permutational multivariate analysis of variance
233 (PERMANOVA, permutation=9999), and visualised with principal component analysis (PCA)
234 using the Vegan package (v.2.5-6) (Oksanen et al., 2013). Fitting bee traits onto PCA ordination
235 was then performed using function *envfit* in Vegan (v.2.5-6). The ggplot2 package (version 3.3.3)
236 (Ginestet, 2011) was used to produce the stacked graph at phylum level.

237

238 For core microbiome analysis and random forest test, we used an online microbiome analyses tool
239 (MicrobiomeAnalyst, <https://www.microbiomeanalyst.ca/>) following recommended parameters

240 (Chong et al., 2020). Some previous studies identified those members occurring in >20% hosts at
241 abundance of >0.1% within a defined host population as their core microbiomes (Bereded et al.,
242 2020). In this study we calculated core microbiomes in stingless bees using 20% threshold but also
243 analysed with a 40% and 60% threshold to increase the likelihood of biological relevance of the
244 gut microbiome. For analysing gut microbial community variation over spatial gradients (latitude
245 and longitude of each sample), we constructed geographic and environmental distance-decay
246 relationships based on our spatially highly resolved set of samples (Soininen et al., 2007). This
247 analysis reveals how the similarity in host microbiome composition between communities varies
248 with geographic distance. The R package geosphere (1.5-10) (Hijmans, 2019) was used to
249 calculate distance (km) between locations based on geographic coordinates for each sample. The
250 *vegdist* function in the Vegan package (v.2.5-6) was used to calculate Bray-Curtis similarity (1-
251 Bray-Curtis dissimilarity). Distance-based multivariate analysis for a linear model was then
252 performed to investigate correlations between the Bray-Curtis similarity and geographic distance
253 between samples.

254

255 Lastly, structural equation models (SEM) were used to evaluate the effects of morphometric traits
256 and management approaches of bees on the bacterial richness (the number of different types of
257 microorganisms present in a sample) in their gut, which was conducted using AMOS17.0 (AMOS
258 IBM, USA). The measurement of richness can provide insights into the overall health and stability
259 of the gut microbiome, as well as the availability of different types of microbes that may impact
260 the bee's health and fitness. The maximum-likelihood estimation was fitted to the SEM modelling,
261 and Chi-square and approximate root mean square error were calculated to examine model fit.
262 Adequate model fits were determined according to a non-significant chi-square test ($P > 0.05$),

263 high goodness fit index (GFI) (> 0.90), low Akaike value (AIC) and root square mean error of
264 approximation (RMSEA) (< 0.05) as previously described (Delgado-Baquerizo et al., 2016).

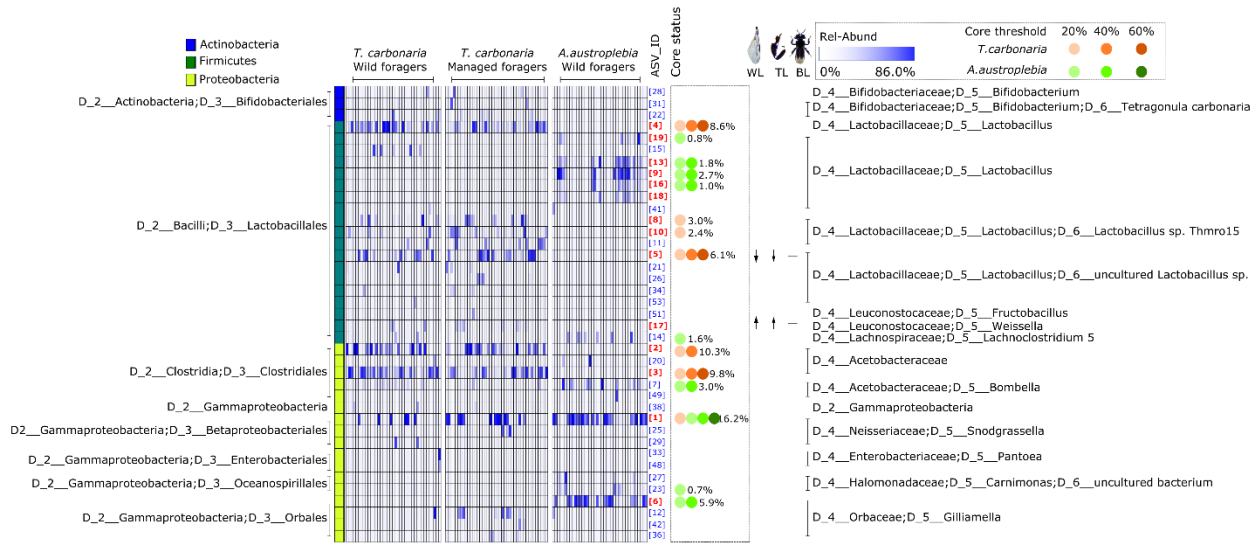
265

266 **Results**

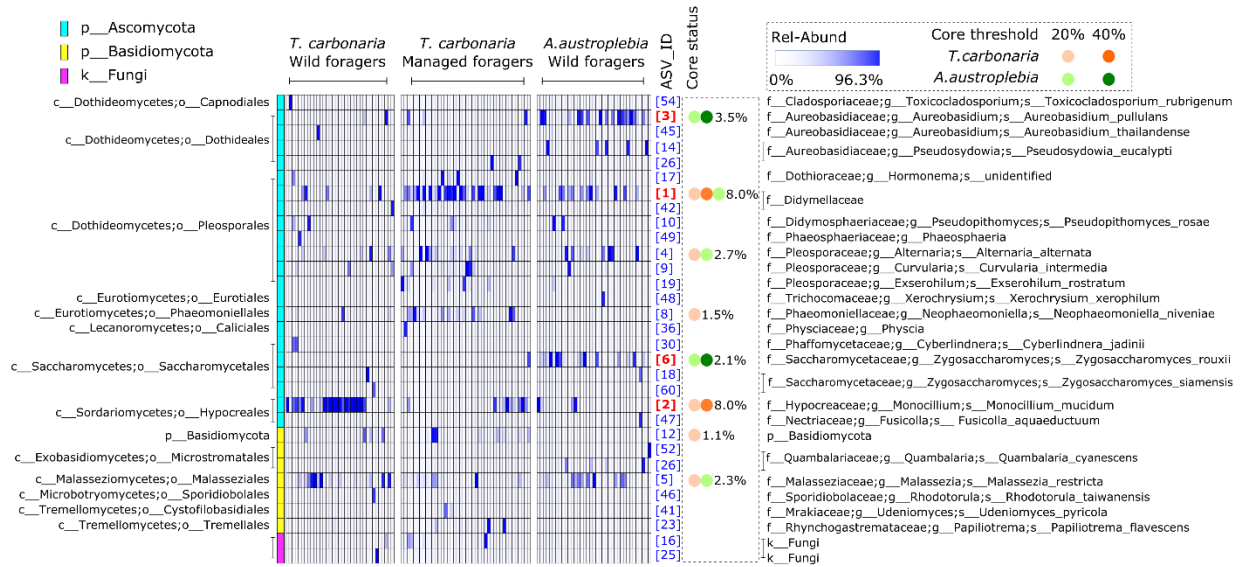
267 *Core microbiome analyses of the stingless bee gut microbial communities*

268 The gut microbial communities of the two bee species were characterised using high throughput
269 amplicon sequencing. At the phylum level, gut bacterial communities of both species were
270 dominated by Proteobacteria, Firmicutes and Actinobacteria, along with less abundant
271 Bacteroidetes, Verrucomicrobia, Tenericutes, Acidobacteria, Gemmatimonadetes and other
272 unidentified taxa (Fig.S1A). The fungal community was dominated by Ascomycota and
273 Basidiomycota, with Chytridiomycota, Mucoromycota and other unidentified taxa also common
274 (Fig.S1B). The stingless bee gut microbiome composition appears to be highly variable within
275 host species and colonies, as shown by the heatmaps (Figs.2 and 3). When the presence threshold
276 was set at 20% for defining the core microbiome, we found seven (four *Lactobacillus* spp., two
277 Acetobacteraceae and a *Snodgrassella* sp.) core bacterial ASVs for *T. carbonaria* and nine (four
278 *Lactobacillus* spp., a *Lachnoclostridium* sp., a *Bombella* sp., a *Snodgrassella* sp., a *Carnimonas* sp.
279 and a *Gilliamella* sp.) for *A. australis* (Fig.2). When further increased to 60%, only three (two
280 *Lactobacillus* spp., and an Acetobacteraceae) and one (*Snodgrassella* sp.) core taxa were observed
281 for *T. carbonaria* and *A. australis* (Fig.2). We also identified the core fungal species; with the 20%
282 threshold, six (a Didymellaceae, an *Alternaria* sp., a *Neophaeomoniella* sp., a *Monocillium* sp., a
283 Basidiomycota and a *Malassezia* sp.) core fungal taxa were identified for *T. carbonaria* and five
284 (an *Aureobasidium* sp., a Didymellaceae, an *Alternaria* sp., a *Zygosaccharomyces*, and a

285 *Malassezia* sp.) for *A. australis* (Fig.3). Only two fungal taxa were detected for both the bee species
 286 when the threshold increased to 40% and no fungal taxa were detected at a 60% threshold (Fig.3).
 287



289 **Fig.2** Heatmap summarising variation in the composition of bacterial communities in the stingless
 290 bee gut. Each amplicon sequence variant (ASV) has a unique numeric identifier shown in square
 291 brackets that is consistent with the main text and those shown in other figures. ASVs that were
 292 present at $\geq 10\%$ relative abundance in any sample were included. ASVs highlighted in red
 293 significantly differed in relative abundance between the two bee species; up and down arrows
 294 besides ASVs marked significant correlations between the ASV with the bee morphological trait
 295 ($P < 0.01$). The dots on the right summarise the core conditions of that ASV in the stingless bee
 296 gut bacterial communities. In this case, 20%, 40% and 60% occurrences across samples were tested.
 297 The phylum of each ASV is indicated by the colours on the left of the heatmap, and the percentage
 298 besides dots indicates its contribution to the total number of reads obtained from sequencing. The
 299 text on the left shows bacterial taxonomy at class (D_2) and order (D_3) level, while text on the
 300 right shows bacterial taxonomy at the family (D_4), genus (D_5) and species (D_6) level.
 301



302

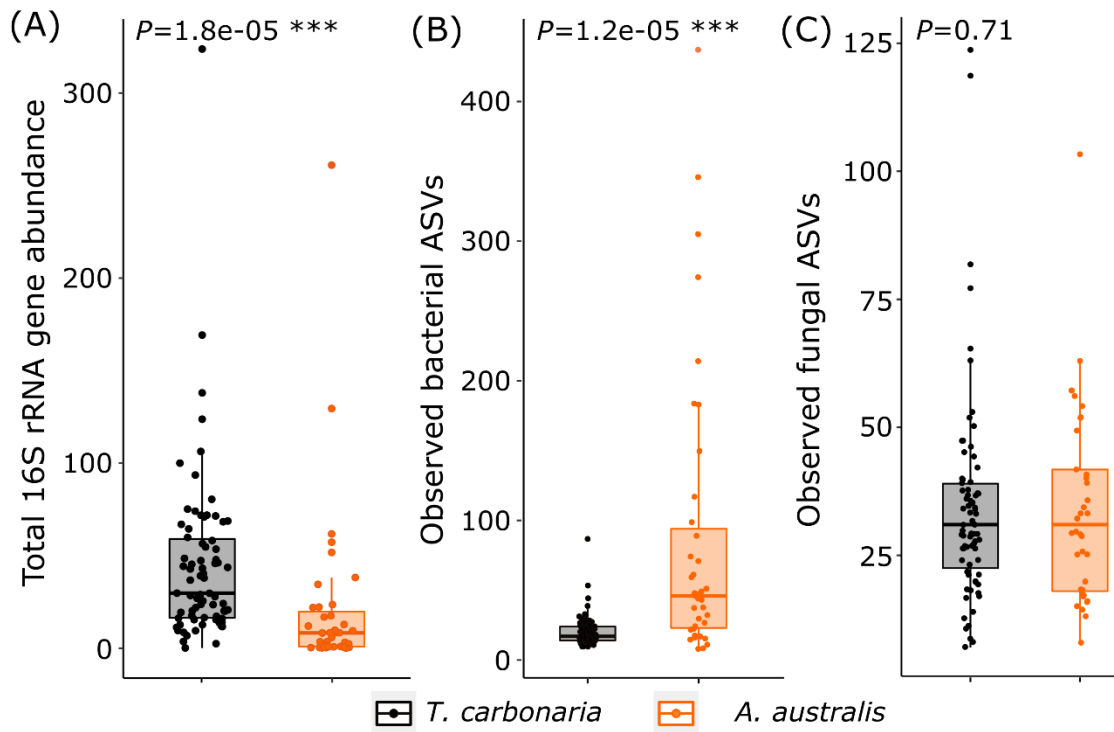
303 **Fig.3** Heatmap summarising variation in the composition of fungal communities in the stingless
 304 bee gut. Each amplicon sequence variant (ASV) has a unique numeric identifier shown in square
 305 brackets that is consistent with the main text and those shown in other figures. ASVs that were
 306 present at $\geq 10\%$ relative abundance in any sample were included. ASVs highlighted in red
 307 significantly differed in relative abundance between the two bee species. The dots on the right
 308 summarise the core conditions of that ASV in the stingless bee gut bacterial communities. In this
 309 case, 20%, 40% and 60% occurrences across samples were tested. The phylum of each ASV is
 310 indicated by the colours on the left of the heatmap, and the percentage besides each ASV indicates
 311 its contribution to the total number of reads obtained from sequencing. The text on the left shows
 312 bacterial taxonomy at class (c_) and order (o_) level, while text on the right shows bacterial
 313 taxonomy at the family (f_), genus (g_) and species (s_) level.

314

315 *Total bacterial and fungal abundance, and alpha diversity of the stingless bee gut microbiomes*

316 We compared wild (*T. carbonaria* & *A. australis*) and managed (*T. carbonaria* only) samples
 317 within their geographic range to detect species and management effects on gut microbiomes. We
 318 found that the total gut bacterial abundance was significantly higher in *T. carbonaria* and was
 319 correlated to their geographic locations, including both the latitude ($R=-0.21$, $P=0.0009$) and
 320 longitude ($R=0.43$, $P<0.0001$) (Fig.4A). However, we found no evidence that gut bacterial
 321 abundance correlated to any of the phenotypic traits of stingless bees (Table 1). In contrast, total
 322 fungi within bee gut samples were very low and we could not detect effective amplifications in $>80\%$

323 samples using qPCR method ($Ct > 35$). Multiple alpha indices were then compared among samples
324 to provide detailed insight into the gut microbial diversity (Fig.4B,C). There was a significantly
325 higher bacterial diversity in the gut of *A. australis* than *T. carbonaria* in terms of the observed
326 ASVs, Chao1 and Shannon (Table 1, Fig.4B). Alpha diversity of the gut fungal community of *A.*
327 *australis* was also significantly higher than that of *T. carbonaria* in terms of Evenness and Simpson
328 but not the observed ASVs (Table 1, Fig.4C). For bacterial communities of *T. carbonaria*, wild
329 bees had higher diversity than managed bees (Observed ASVs, Chao1 and Evenness) while
330 diversity of the fungal community did not differ between management approaches (Table 1).
331



332

333 **Fig.4** Alpha diversity and total bacterial abundance of the stingless bee gut microbiomes. Total
334 bacterial abundance measured by qPCR analyses (A). Observed amplicon sequence variants
335 (ASVs) in the stingless bee gut (B: bacteria, C: fungi). Boxplots indicate the first and third quartiles
336 with the median value indicated by a horizontal line. Asterisks denote statistically significant
337 differences between the two species.

338 **Table 1** Differences of the microbial community diversity and composition among samples and their correlations to stingless bee
 339 phenotypes, latitude and longitude. Significant *P* values are highlighted in bold (*P*<0.05).

	Management (Wild ~Managed)		Species (<i>T. carbonaria</i> ~ <i>A. australis</i>)		Wing length		Tibia length		Body length		Wing size		Latitude		Longitude	
	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>
Total bacterial abundance	0.024	0.88	24.31	1.75e-05	0.68	0.41	0.17	0.68	1.56	0.22	0.14	0.71	28.67	0.0009	11.70	4.75e-07
Bacterial community (alpha-diversity)																
Observed ASVs	7.23	0.014	57.29	2.93e-10	0.019	0.89	0.21	0.64	3.87	0.054	0.78	0.38	0.93	0.34	41.42	3.07e-09
Chao1	6.70	0.004	35.68	1.43e-07	2.49	0.12	1.01	0.12	1.10	0.30	1.48	0.23	3.29	0.072	20.50	1.48e-05
Shannon	0.21	0.78	7.12	0.01	0.0004	0.98	0.16	0.69	0.42	0.52	0.004	0.95	0.70	0.40	6.28	0.014
Evenness	5.31	0.03	1.64	0.21	0.001	0.97	0.19	0.67	0.056	0.94	0.29	0.59	0.18	0.67	4.26	0.041
Simpson	2.10	0.26	0.93	0.34	0.14	0.71	0.001	0.99	0.052	0.82	0.096	0.92	0.44	0.51	0.17	0.68
Fungal community (alpha-diversity)																
Observed ASVs	1.54	0.22	1.29	0.26	0.81	0.37	0.72	0.40	0.087	0.77	0.64	0.43	0.063	0.80	1.68	0.20
Shannon	0.81	0.37	4.71	0.034	0.50	0.48	0.21	0.65	0.24	0.63	0.22	0.634	1.34	0.25	2.30	0.13
Evenness	0.0009	0.98	11.38	0.0014	0.059	0.81	2.33	0.13	0.071	0.79	0.14	0.71	1.40	0.24	7.14	0.63
Simpson	0.37	0.55	5.26	0.025	0.58	0.45	3.02	0.087	0.011	0.97	0.12	0.73	1.10	0.30	2.90	0.091
Bacterial community beta-diversity																
Community composition	2.40	0.0097	22.31	0.0001	-	0.0001	-	0.001	-	0.20	-	0.004	-	0.0001	-	0.0001
Unifrac distance (weighted)	1.55	0.20	26.53	0.0001	-	0.001	-	0.001	-	0.73	-	0.002	-	0.001	-	0.001
Unifrac distance (unweighted)	5.42	0.011	41.26	0.0001	-	0.001	-	0.001	-	0.022	-	0.010	-	0.001	-	0.001
Fungal community beta-diversity																
Community composition	4.22	0.0001	4.13	0.0001	-	0.001	-	0.001	-	0.54	-	0.073	-	0.70	-	0.001

341 *Factors correlated to the beta-diversity of the stingless bee gut microbiomes*

342 Bacterial and fungal community composition (beta-diversity, differences in microbial community

343 composition between samples) were influenced by both host species identity (Bacterial: $R^2=0.23$,

344 $P=0.0001$, fungal: $R^2=0.054$, $P=0.0001$) and management approach (managed/wild, assessed in *T.*

345 *carbonaria* only) (Bacterial: $R^2=0.031$, $P=0.0097$, fungal: $R^2=0.053$, $P=0.0001$) (Table 1,

346 Fig.5A,B). When both stingless bee species were considered together, the gut bacterial community

347 composition was closely associated with the host forewing length, forewing size, tibia length as

348 well as latitude and longitude (Table 1, Fig.5A). However, none of these correlations were

349 significant when the two species were analysed independently, indicating that phenotype

350 differences between the two species may have driven the occurrence of such correlations. Similarly,

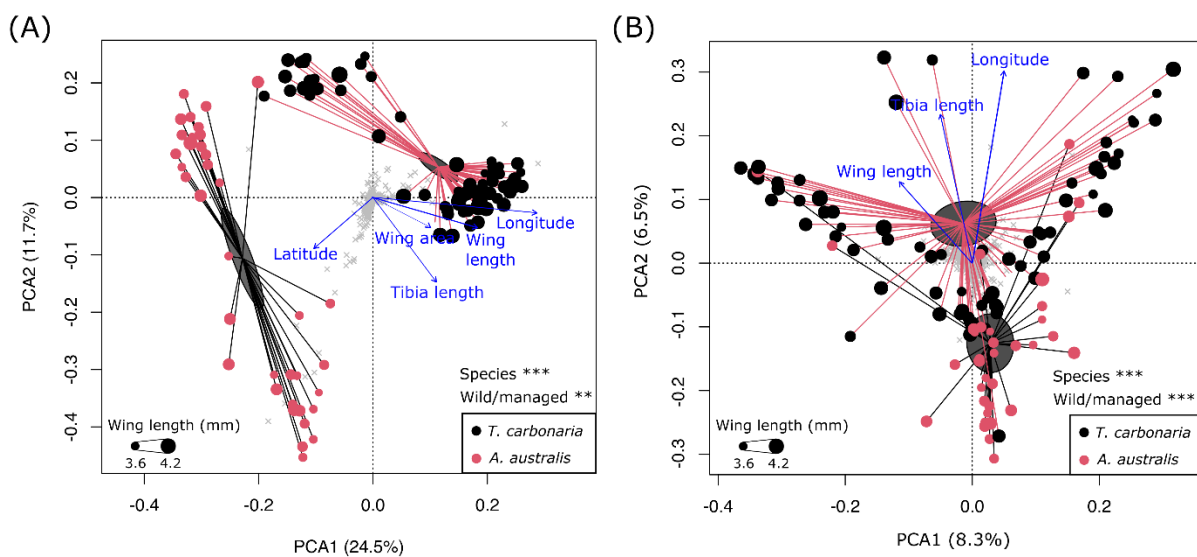
351 the fungal community composition also significantly correlated to the host forewing length, tibia

352 length, latitude and longitude when the two species were analysed together (Table 1, Fig.5B).

353 When examining species separately, only body length was associated with the fungal community

354 composition of *A. australis* ($R^2=0.16$, $P=0.021$).

355



356

357 **Fig.5** Principal component analyses (PCA) of the gut bacterial (A) and fungal (B) microbial
358 community composition. Ellipses show the standard error of the mean. Blue arrows represent the
359 direction of environmental gradients, with length proportional to strength of the correlation.
360

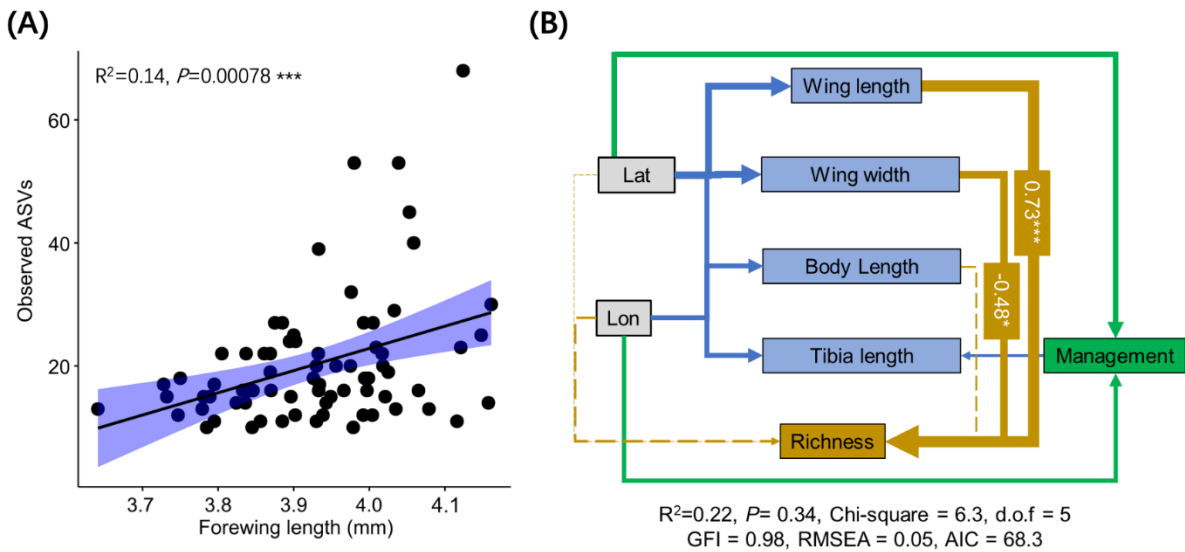
361 We then used Bray-Curtis similarity index to determine the influence of geographic distance (km)
362 between samples on gut bacterial and fungal community composition (Fig.S2). A significant linear
363 decrease in composition similarity with increasing geographic distance was seen between samples
364 for *T. carbonaria* gut bacterial communities ($R^2=0.005$, $P<0.0001$). In addition, there was a
365 similarity decrease with increasing geographic distance for fungal communities for both *T.*
366 *carbonaria* ($R^2=0.006$, $P<0.0001$) and *A. australis* ($R^2=0.01$, $P=0.002$) (Fig.S2A,C,D). These
367 results show that the microbiome composition varies more between samples from geographically
368 distant locations. However, the adjusted R^2 for each correlation was small (0.005~0.01), indicating
369 only a weak link. No significant correlation was seen for gut bacterial communities in *A. australis*
370 (Fig.S2B). The *A. australis* samples covered a relatively smaller area, and so the power to detect
371 small changes may have been reduced compared to *T. carbonaria*.

372

373 *Correlation of stingless bee gut microbial richness with host morphological traits and geography*

374 Interestingly, gut bacterial richness (as indicated by observed ASVs, $R=0.38$, $F=11.89$, $P=0.00078$)
375 and Chao1 diversity index (an estimate of the total number of bacterial species, $R=0.38$, $F=11.91$,
376 $P=0.00094$) in *T. carbonaria* showed a significant positive correlation with host forewing length
377 (Fig.6A); a similar trend was also observed for the forewing area (observed ASVs, $R=0.34$, $F=8.06$,
378 $P=0.0059$; Chao1, $R=0.30$, $F=7.43$, $P=0.008$). Total body length, hind tibia length, latitude and
379 longitude showed no correlation with gut bacterial richness or any other alpha diversity indices.
380 The forewing area/body length ratio, which is believed to determine flight capacity, also correlated
381 with gut bacterial richness in *T. carbonaria* ($R=0.24$, $F=5.66$, $P=0.021$). SEM analysis that

382 considered morphological traits, geographical factors and management effects together showed
 383 consistent results with a dominant effect of forewing length on gut bacterial richness in *T.*
 384 *carbonaria* being revealed (Fig.6B). Lastly, linear models showed that forewing length and area,
 385 tibia length and body length were all positively correlated with one another (e.g. $R_{\text{wing length-area}}=0.85$; $R_{\text{wing length-body length}}=0.38$; $R_{\text{wing length-tibia length}}=0.72$; $P<0.0001$ in all cases), indicating that
 386 larger bees possess longer wings (Fig.S3A,B,C).
 387
 388



389
 390 **Fig.6** A significant linear correlation between gut observed ASVs with host forewing length in *T.*
 391 *carbonaria*. Person correlation (A) and structural equation modelling (SEM) summarising
 392 correlations of the bee morphometric trait, geographical factor and management approach with the
 393 observed ASVs in the gut of *T. carbonaria* (B). In A, the black line shows regression with shading
 394 area representing 95% confidence intervals, and data points were shown with black dots. In B,
 395 solid arrows indicate significant effect sizes ($P<0.05$, dashed lines $P>0.05$) and width of the
 396 arrow represents the strength of the relationship. The colour of the arrows corresponds to each
 397 targeted factor. Standardised path coefficient values are shown besides the significant pathways.
 398 Lon: longitude; Lat: latitude.
 399

400 **Discussion**

401 We characterised the whole gut microbiome of two stingless bee species, *T. carbonaria* and *A.*
402 *australis*, from 121 locations spanning a large geographic range in eastern Australia. We quantified
403 total gut bacterial and fungal abundance using qPCR and found that bacterial abundance was
404 higher in *T. carbonaria* (45.11±46.15) compared to *A. australis* (21.64±46.48), and fungi were
405 either lowly abundant or below the threshold of detection. We also tested whether gut microbiomes
406 are linked to host traits, geographic location (longitude and latitude), host species and management
407 type (wild vs managed) to understand the factors that shape the gut microbiome. This
408 understanding may provide insights into how hosts and their microbiomes have evolved in
409 response to different environmental conditions. For *T. carbonaria*, we observed a positive
410 correlation between both forewing length and area with host gut bacterial richness (the number of
411 bacterial species in bee gut). For both species, microbiomes consistently became more distinct
412 from one another in their composition with increasing geographic distance between samples,
413 which suggests a role of geographic factors in shaping stingless bee gut microbiomes. Additionally,
414 variations in climate (e.g., temperature) across the 1200km range where samples were collected
415 have also likely contributed to the differences in microbiomes observed between samples. Overall,
416 by combining analyses of both the bacterial and fungal communities with host traits and geography,
417 we provide novel understandings of the stingless bee gut microbiomes.

418

419 *Stingless bee gut microbial diversity correlates with host forewing size*

420 Our findings revealed that *Lactobacillus* spp., Acetobacteraceae, and *Snodgrassella* spp. were
421 consistently present in both bee species. The core fungal species identified were less consistent
422 among samples than bacteria, but they likely included a *Neophaeomoniella* sp., a *Monocillium* sp.,

423 a Basidiomycota, and a *Malassezia* sp. Further investigations are needed to determine their origins,
424 such as whether they were acquired from the environment or inherited from parents, or from
425 interactions with other bees. We observed a positive correlation between gut bacterial species
426 richness and host forewing length and size of *T. carbonaria*, as indicated by using a linear model
427 and SEM approach. Insect wing sizes are closely related to their flight capacity (DeVries et al.,
428 2010); longer wings favour wider variation in speed and increase capacity for longer flight duration
429 and energy saving (DeVries et al., 2010). These factors potentially increase the capacity of bees to
430 collect diverse floral resources. Furthermore, insects with larger wings are more successful in host-
431 seeking and their location of oviposition sites (Berwaerts et al., 2002; Davis and Holden, 2015).
432 Therefore, there are two possible drivers of the link between wing length and bacterial richness:
433 (i) bees with larger wings have larger bodies (supported by our data), so may have a larger gut area
434 for bacteria to colonize, and (ii) bee foragers with larger wings are to encounter more bacterial
435 species when accessing more diversified floral resources across an area.

436

437 As found in this study, stingless bee guts contained bacterial genera such as *Pantoea*,
438 *Sphingomonas*, *Stenotrophomonas*, *Gilliamella* spp. (Graystock et al.,
439 2017), *Saccharibacter* spp. (McFrederick et al., 2012), *Massilia* spp. (Graystock et al., 2017)
440 and *Acinetobacter* spp. (Graystock et al., 2017), which are commonly found on all parts of
441 flowering plants, suggesting plant visits might be key to the microbial acquisition by stingless bees
442 and support the second hypothesis. But additional research is needed to test the above hypotheses
443 in detail, and to determine whether the findings here apply to other insects. Gaining this knowledge
444 should aid a better understanding of microbial ecology in insect pollinators. The composition and
445 distribution of gut microbiomes in stingless bees are likely to vary between gut regions, similar to

446 other insect species such as honeybees. The stingless bee foregut or crop may exhibit more
447 variability due to exposure to the environment and diverse food sources, but the exact patterns of
448 gut microbiome distribution in stingless bees are not yet fully characterized.

449

450 *Bacterial communities*

451 In this study, Proteobacteria and Firmicutes were found to be the dominant bacterial phyla in the
452 gut of both stingless bee species, followed by Actinobacteria; a pattern also observed in honey
453 bees and bumble bees (Kakumanu et al., 2016; Wang et al., 2019). However, it is unclear whether
454 the composition and diversity of gut microbiomes are comparable across bee species in different
455 gut regions. Such microbial similarity among species supports a strong host selection of the
456 microbial environment by eusocial bees. The core bacterial phylotype, *Lactobacillus*, has
457 important functions in the host, such as protection against pathogens and food digestion, as has
458 been demonstrated for honey bees in previous studies (Engel and Moran, 2013; Liu et al., 2019;
459 Kwong and Moran, 2016; McFrederick et al., 2012). They are common in the gut system of
460 bumblebees worldwide (Kwong and Moran, 2016), suggesting that mutualisms with *Lactobacillus*
461 exist throughout the eusocial bees across different geographic regions. *Lactobacillus* spp. are the
462 main indicator taxon distinguishing the stingless bee gut microbiomes of the two species, which
463 suggests a great variance in phylogeny and abundance of the *Lactobacillus* genus at species/strain
464 levels between the two species. *Snodgrassella* spp., another core bacterial genus in the stingless
465 bee gut microbiome, also features in the core microbiome of both honey bees and bumble bees
466 (Koch and Schmid-Hempel, 2011). *Snodgrassella* spp. are saccharolytic fermenters and have been
467 implicated in the protection of bumble bees against *Crithidia bombi* (Koch and Schmid-Hempel,
468 2011). Laboratory studies indicated that glyphosate (the primary herbicide used worldwide) can
469 perturb the strain abundance of core gut *Snodgrassella alvi* in honey bees, which led to higher rates

470 of mortality when glyphosate-treated bees were exposed to the opportunistic pathogen *Serratia*
471 *marcescens* (Motta et al., 2018), highlighting the importance of this bacterium in the maintenance
472 of host health. As with *Lactobacillus*, the relevance of the species/strain diversity of *Snodgrassella*
473 spp. in the stingless bee gut is not yet understood but may correspond to differences in host
474 metabolic capabilities. Interestingly, a recent study surveyed gut microbiomes of Brazilian
475 stingless bees by sampling multiple species within the genus *Melipona*, and showed that stingless
476 bees can lose the core symbioses of *Snodgrassella* (Cerqueira et al., 2021). This suggests that
477 strong ecological shifts or functional replacements in the stingless bee gut microbiome can occur.

478

479 The gut bacterial species richness of *A. australis* was significantly higher than that of *T. carbonaria*.
480 Such microbial difference may relate to the distinct foraging behaviour of the two species. *T.*
481 *carbonaria* evidently collects more protein enriched food (e.g. pollen) than *A. australis* which
482 likely focuses on high-quality nectar (carbohydrate enriched) (Leonhardt et al., 2014). This higher
483 level of carbohydrate foraging may be linked with the higher bacterial richness seen in *A. australis*.
484 Hive managed bees seem likely to possess less gut bacterial diversity than wild bees, which may
485 indicate a less diverse food composition. A previous study also found that gut bacterial diversity
486 of fruit fly (*Bactrocera tryoni*) larvae was significantly lower in laboratory populations compared
487 with field populations (Deutscher et al., 2018). We observed that stingless bee gut microbiomes
488 vary greatly among samples. This aligns with a previous study on whole-body bacterial and fungal
489 communities of managed *T. carbonaria* (Hall et al., 2021). Temporal and spatial changes of the
490 bee microbiome composition observed in that study may also, to some extent, explain the high
491 variability of microbiome composition we saw across a geographic gradient. For example, Hall et
492 al. (2021) saw dramatic increases in the relative abundances of *Bombella* and *Zymobacter* and

493 almost complete depletion of *Snodgrassella* when colonies were moved from a florally resource-
494 rich site to a resource-poor site. All the above findings, along with previous studies (5, 6, 8, 19),
495 suggest that stingless bees are prone to compositional shifts, putatively influenced by food
496 resources, both spatially and temporally, physiological status, origin of the colony and climate at
497 different geographic locations.

498

499 *Fungal communities*

500 The dominant fungal phyla observed, Ascomycota and Basidiomycota, usually fulfil a
501 decomposing role in most land-based ecosystems, by breaking down organic materials such as
502 large molecules of cellulose or lignin, and in doing so play important roles in carbon and nitrogen
503 cycling (Dighton, 2016). We identified core fungal taxa in *T. carbonaria* and *A. australis*.
504 Interestingly, two of these, *Malassezia restricta* and *M. globosa*, are also among the most abundant
505 fungal species in the human gut (as indicated by their large presence in faecal samples and
506 intestinal mucosa), and have been identified in association with gut diseases including colorectal
507 cancer (Coker et al., 2019). Another core fungal genus, *Monocillium* spp. has previously been
508 isolated from soil, dead leaves and wood and some species (e.g., *M. curvisetosum*) originate from
509 aphids. There is evidence that *Monocillium* spp. are able to antagonise a plant parasitic nematode
510 by colonising their cysts (Ashrafi et al., 2017). The detected *Alternaria alternata* can be an
511 opportunistic fungal pathogen on plants causing leaf spots, rots and blights (Tsuge et al., 2013);
512 however, its function in the bee gut and whether it may be vectored between plants by bees is
513 currently unknown. Further investigations are needed to determine how gut-colonising fungi
514 interact with co-occurring bacteria, and the implications for host nutrition and fitness. The low

515 amount of the total fungi in the stingless bee gut demands future investigations into whether these
516 fungi have functional roles in the fitness of stingless bees.

517

518 *Distance-decay relationship between stingless bee gut microbiomes and geographic distance of*
519 *bee sampling*

520 Our data showed that microbial biogeographic patterns (a distance-decay relationship) could be
521 applied to stingless bee gut microbiomes on a geographic scale of 250~1,200 km. While we
522 predicted decreasing community similarity with greater distance due to dispersal limitation of
523 stingless bee microbiomes (Soininen et al., 2007; Nemergut et al., 2013), evidence for such a
524 relationship in bee microbiomes was previously lacking. An analysis of relative abundances of
525 *Snodgrassella* and *Gilliamella* across *Bombus* and *Apis* hosts found poor correlation with
526 geography (Koch et al., 2013). Previous studies also found limited effects of geographic location
527 on microbiota composition probably due to small sample sizes and/or geographic distances
528 (Kwong et al., 2017). However, the bacterial and fungal distance-decay relationship detected in
529 our study, although significant, explained only a small amount ($R^2=0.005\sim 0.01$) of observed
530 variation, perhaps smaller than those typically observed for plants and other animals. The number
531 of samples, geographic area covered and sequencing depth all could affect the differences,
532 highlighting the need to couple high-throughput sequencing methods with wide geographical
533 coverage.

534

535 **Conclusions**

536 We characterised the gut microbiomes of two stingless bee species from different genera across
537 1,200 km, spanning large parts of their geographic ranges in eastern Australia. We found the gut

538 microbial richness of *T. carbonaria* correlated to key host morphological traits, namely forewing
539 length and area, that can affect foraging behaviour. In addition, total fungi in the stingless bee gut
540 appears to be in low abundance and were hardly detected in most of the bee samples. Overall, our
541 findings, esp. the observed correlation between gut microbiomes and bee fitness traits may provide
542 a novel framework to test functional interactions between insect pollinators and their gut
543 microbiome.

544

545 **Data availability**

546 The data that supports the findings of this study are available in the supplementary material of this
547 article. The raw sequencing data have been deposited in the NCBI Sequence Read Archive (SRA)
548 under Bioproject code PRJNA698658.

549

550 **Author contributions**

551 HL, BKS, JC, RS, MR and LB conceived the idea; Mark H, Megan H, SN and HL collected wild
552 and managed bee samples along eastern Australia; HL conducted bee gut dissections, bee
553 measurement and DNA extraction, and analysed the sequencing data; Mark H drew Fig.1a and JW
554 conducted the SEM analyses; HL led writing of the manuscript, and all authors contributed to
555 manuscript edits and approved final version for submission.

556

557 **Acknowledgements**

558 This work was supported by the Hort Frontiers Pollination Fund [PH15001] (Healthy bee
559 populations for sustainable pollination in horticulture) and host microbiome and multitrophic
560 interaction fund (Australian Research Council (DP190103714; DP210102081)). We thank Dr

561 Jasmine Grinyer for contributing an additional stingless bee specimen from the Blue Mountains.
562 Megan also provided records collected by the late Allan Biel, who recovered hundreds of stingless
563 bee colonies, particularly around the Tara region of QLD. We thank him for his passion and
564 commitment to Australian native bees.

565

566 **Conflict of interests**

567 The authors declare that there is no conflict of interests.

568

569 **Reference**

570 Andrews S. FastQC: a quality control tool for high throughput sequence data. Available online at:

571 <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>. Accessed on 09/02/2020.

572 Araújo E, Costa M, Chaud-Netto J, Fowler HG. Body size and flight distance in stingless bees

573 (Hymenoptera: Meliponini): inference of flight range and possible ecological implications.

574 *Braz J Biol* 2004;64(3B):563-568. <https://doi.org/10.1590/S1519-69842004000400003>.

575 Ashrafi S, Stadler M, Dababat AA, Richert-Pöggeler KR, Finckh MR, Maier W. *Monocillium*

576 *gamsii* sp. nov. and *Monocillium bulbillosum*: two nematode-associated fungi parasitising

577 the eggs of *Heterodera filipjevi*. *MycKeys* 2017(27):21-38.

578 <https://doi.org/10.3897/mycokeys.27.21254>

579 Bereded NK, Curto M, Domig KJ, Abebe GB, Fanta SW, Waidbacher H, Meimberg H.

580 Metabarcoding analyses of gut microbiota of Nile tilapia (*Oreochromis niloticus*) from lake

581 awassa and lake chamo, Ethiopia. *Microorganisms* 2020;8(7):1040.

582 <https://doi.org/10.3390/microorganisms8071040>.

583 Berwaerts K, Van Dyck H, Aerts P. Does flight morphology relate to flight performance? An
584 experimental test with the butterfly *Pararge aegeria*. *Funct Ecol* 2002;16(4):484-491.
585 <https://doi.org/10.1046/j.1365-2435.2002.00650.x>.

586 Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm
587 EJ, Arumugam M, Asnicar F. Reproducible, interactive, scalable and extensible microbiome
588 data science using QIIME 2. *Nat Biotechnol* 2019;37(8):852-857.
589 <https://doi.org/10.1038/s41587-019-0209-9>.

590 Byrne DN, Buchmann SL, Spangler HG. Relationship between wing loading, wingbeat frequency
591 and body mass in homopterous insects. *J Exp Biol* 1988;135(1):9-23.
592 <https://doi.org/10.1242/jeb.135.1.9>.

593 Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-
594 resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;13(7):581-583.
595 <https://doi.org/10.1038/nmeth.3869>.

596 Casey TM, May ML, Morgan KR. Flight energetics of euglossine bees in relation to morphology
597 and wing stroke frequency. *J Exp Biol* 1985;116(1):271-289.
598 <https://doi.org/10.1242/jeb.116.1.271>.

599 Ceja-Navarro JA, Vega FE, Karaoz U, Hao Z, Jenkins S, Lim HC, Kosina P, Infante F, Northen
600 TR, Brodie EL. Gut microbiota mediate caffeine detoxification in the primary insect pest of
601 coffee. *Nat Commun* 2015;6(1):7618. DOI: 10.1038/ncomms8618.

602 Cerqueira AES, Hammer TJ, Moran NA, Santana WC, Kasuya MCM, da Silva CC. Extinction of
603 anciently associated gut bacterial symbionts in a clade of stingless bees. *ISME J*
604 2021;15:2813–2816. <https://doi.org/10.1038/s41396-021-01000-1>.

605 Chapman RFChapman RF, 1998. *The insects: structure and function*, Cambridge university press.

606 Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional,
607 and meta-analysis of microbiome data. *Nat Protoc* 2020;15(3):799-821.
608 <https://doi.org/10.1038/s41596-019-0264-1>.

609 Coker OO, Nakatsu G, Dai RZ, Wu WKK, Wong SH, Ng SC, Chan FKL, Sung JJY, Yu J. Enteric
610 fungal microbiota dysbiosis and ecological alterations in colorectal cancer. *Gut*
611 2019;68(4):654-662. <http://dx.doi.org/10.1136/gutjnl-2018-317178>.

612 Davis AK, Holden MT. Measuring intraspecific variation in flight-related morphology of monarch
613 butterflies (*Danaus plexippus*): which sex has the best flying gear? *J Insects* 2015;2015.
614 <https://doi.org/10.1155/2015/591705>.

615 de Paula GT, Menezes C, Pupo MT, Rosa CA. Stingless bees and microbial interactions. *Curr*
616 *Opin Insect Sci* 2021;44:41-47. <https://doi.org/10.1016/j.cois.2020.11.006>.

617 Delgado-Baquerizo M, Maestre FT, Reich PB, Jeffries TC, Gaitan JJ, Encinar D, Berdugo M,
618 Campbell CD, Singh BK. Microbial diversity drives multifunctionality in terrestrial
619 ecosystems. *Nat Commun* 2016;7(1):1-8. DOI: 10.1038/ncomms10541.

620 DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P,
621 Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench
622 compatible with ARB. *J Appl Environ Microbiol* 2006;72(7):5069-5072.
623 <https://doi.org/10.1128/AEM.03006-05>.

624 Deutscher AT, Burke CM, Darling AE, Riegler M, Reynolds OL, Chapman TA. Near full-length
625 16S rRNA gene next-generation sequencing revealed *Asaia* as a common midgut bacterium
626 of wild and domesticated Queensland fruit fly larvae. *Microbiome* 2018;6(1):85.
627 <https://doi.org/10.1186/s40168-018-0463-y>.

628 DeVries PJ, Penz CM, Hill RI. Vertical distribution, flight behaviour and evolution of wing
629 morphology in *Morpho* butterflies. *J Anim Ecol* 2010;79(5):1077-1085.
630 <https://doi.org/10.1111/j.1365-2656.2010.01710.x>.

631 Dighton J, 2016. *Fungi in ecosystem processes*, CRC press.
632 <https://doi.org/10.1201/9781315371528>.

633 Dollin AEDollin LJ. Australian stingless bees of the genus *Trigona* (Hymenoptera: Apidae).
634 *Invertebr Syst* 1997;11(6):861-896. <https://doi.org/10.1071/IT96020>.

635 Dollin AE, Dollin LJ, Rasmussen C. Australian and New Guinean stingless bees of the genus
636 *Austroplebeia* Moure (Hymenoptera: Apidae)—a revision. *Zootaxa* 2015;4047(1):1-73.
637 <https://doi.org/10.11646/zootaxa.4047.1.1>.

638 Dowd PFShen SK. The contribution of symbiotic yeast to toxin resistance of the cigarette beetle
639 (*Lasioderma serricorne*). *Entomologia Experimentalis et Applicata* 1990;56(3):241-248.
640 <https://doi.org/10.1111/j.1570-7458.1990.tb01402.x>.

641 Endt K, Stecher B, Chaffron S, Slack E, Tchitchek N, Benecke A, Van Maele L, Sirard J-C,
642 Mueller AJ, Heikenwalder M, Macpherson AJ, Strugnell R, von Mering C, Hardt W-D. The
643 microbiota mediates pathogen clearance from the gut lumen after non-typhoidal *Salmonella*
644 diarrhea. *PLOS Pathog* 2010;6(9):e1001097. <https://doi.org/10.1371/journal.ppat.1001097>.

645 Engel PMoran NA. The gut microbiota of insects – diversity in structure and function. *FEMS*
646 *Microbiol Rev* 2013;37(5):699-735. <https://doi.org/10.1111/1574-6976.12025>.

647 Ginestet C. *ggplot2: elegant graphics for data analysis*: Wiley Online Library; 2011.
648 https://doi.org/10.1111/j.1467-985X.2010.00676_9.x.

649 Graystock P, Rehan SM, McFrederick QS. Hunting for healthy microbiomes: determining the core
650 microbiomes of *Ceratina*, *Megalopta*, and *Apis* bees and how they associate with microbes

651 in bee collected pollen. *Conserv Genet* 2017;18(3):701-711. DOI:10.1007/s10592-017-
652 0937-7.

653 Greenleaf SS, Williams NM, Winfree R, Kremen C. Bee foraging ranges and their relationship to
654 body size. *Oecologia* 2007;153(3):589-596. DOI: 10.1007/s00442-007-0752-9

655 Halcroft MT, Spooner-Hart R, Haigh AM, Heard TA, Dollin A. The Australian stingless bee
656 industry: a follow-up survey, one decade on. *J Api Res* 2013;52(2):1-7.
657 <https://doi.org/10.3896/IBRA.1.52.2.01>.

658 Hall MA, Jones J, Rocchetti M, Wright D, Rader R. Bee visitation and fruit quality in berries under
659 protected cropping vary along the length of polytunnels. *J Econ Entomol* 2020;113(3):1337-
660 1346. <https://doi.org/10.1093/jee/toaa037>.

661 Hall MA, Brettell LE, Liu H, Nacko S, Spooner-Hart R, Riegler M, Cook JM. Temporal changes
662 in the microbiome of stingless bee foragers following colony relocation. *FEMS Microbiol*
663 *Ecol* 2021;97(1). <https://doi.org/10.1093/femsec/fiaa236>.

664 Heard TA. The role of stingless bees in crop pollination. *Annu Rev Entomol* 1999;44(1):183-206.
665 <https://doi.org/10.1146/annurev.ento.44.1.183>.

666 Herlemann DP, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF. Transitions in
667 bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J*
668 2011;5(10):1571-1579. <https://doi.org/10.1038/ismej.2011.41>.

669 Hijmans RJ. *geosphere: Spherical trigonometry*. R package version 1.5–7 2017. Available from:
670 URL: <https://CRAN.R-project.org/package=geosphere>. 2019.

671 Ihrmark K, Bödeker I, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, Strid Y, Stenlid J,
672 Brandström-Durling M, Clemmensen KE. New primers to amplify the fungal ITS2 region–

673 evaluation by 454-sequencing of artificial and natural communities. *FEMS Microb Ecol*
674 2012;82(3):666-677. <https://doi.org/10.1111/j.1574-6941.2012.01437.x>.

675 Johansson F, Söderquist M, Bokma F. Insect wing shape evolution: independent effects of
676 migratory and mate guarding flight on dragonfly wings. *Biol J Linn Soc* 2009;97(2):362-
677 372. <https://doi.org/10.1111/j.1095-8312.2009.01211.x>.

678 Kakumanu ML, Reeves AM, Anderson TD, Rodrigues RR, Williams MA. Honey bee gut
679 microbiome is altered by In-hive pesticide exposures. *Front Microbiol* 2016;7(1255).
680 <https://doi.org/10.3389/fmicb.2016.01255>.

681 Kešnerová L, Mars RAT, Ellegaard KM, Troilo M, Sauer U, Engel P. Disentangling metabolic
682 functions of bacteria in the honey bee gut. *PLoS Biol* 2017;15(12):e2003467.
683 <https://doi.org/10.1371/journal.pbio.2003467>.

684 Koch HSchmid-Hempel P. Socially transmitted gut microbiota protect bumble bees against an
685 intestinal parasite. *Proc Natl Acad Sci USA* 2011;108(48):19288-19292.
686 <https://doi.org/10.1073/pnas.1110474108>.

687 Koch H, Abrol DP, Li J, Schmid-Hempel P. Diversity and evolutionary patterns of bacterial gut
688 associates of corbiculate bees. *Mol Ecol* 2013;22(7):2028-2044.
689 <https://doi.org/10.1111/mec.12209>.

690 Kwong WKMoran NA. Gut microbial communities of social bees. *Nat Rev Microbiol*
691 2016;14(6):374-384. <https://doi.org/10.1038/nrmicro.2016.43>.

692 Kwong WK, Medina LA, Koch H, Sing K-W, Soh EJY, Ascher JS, Jaffé R, Moran NA. Dynamic
693 microbiome evolution in social bees. *Sci Adv* 2017;3(3):e1600513. DOI:
694 10.1126/sciadv.1600513.

695 Leonhardt SD, Kaltenpoth M. Microbial communities of three sympatric Australian stingless bee
696 species. *PLoS One* 2014;9(8):e105718. <https://doi.org/10.1371/journal.pone.0105718>.

697 Leonhardt SD, Heard TA, Wallace H. Differences in the resource intake of two sympatric
698 Australian stingless bee species. *Apidologie* 2014;45(4):514-527.
699 <https://doi.org/10.1007/s13592-013-0266-x>.

700 Liu H, Macdonald CA, Cook J, Anderson IC, Singh BK. An ecological loop: host microbiomes
701 across multitrophic interactions. *Trends Ecol Evol* 2019;34(12):1118-1130.
702 <https://doi.org/10.1016/j.tree.2019.07.011>.

703 McFrederick QS, Wcislo WT, Taylor DR, Ishak HD, Dowd SE, Mueller UG. Environment or kin:
704 whence do bees obtain acidophilic bacteria? *Mol Ecol* 2012;21(7):1754-1768. DOI:
705 10.1111/j.1365-294X.2012.05496.x.

706 McFrederick QS, Thomas JM, Neff JL, Vuong HQ, Russell KA, Hale AR, Mueller UG. Flowers
707 and wild megachilid bees share microbes. *Microb Ecol* 2017;73(1):188-200.
708 <https://doi.org/10.1007/s00248-016-0838-1>.

709 Menezes C, Vollet-Neto A, Marsaioli AJ, Zampieri D, Fontoura IC, Luchessi AD, Imperatriz-
710 Fonseca VL. A Brazilian social bee must cultivate fungus to survive. *Curr Biol*
711 2015;25(21):2851-2855. DOI: 10.1016/j.cub.2015.09.062.

712 Motta EV, Raymann K, Moran NA. Glyphosate perturbs the gut microbiota of honey bees. *Proc*
713 *Natl Acad Sci USA* 2018;115(41):10305-10310. <https://doi.org/10.1073/pnas.1803880115>.

714 Nemergut DR, Schmidt SK, Fukami T, O'Neill SP, Bilinski TM, Stanish LF, Knelman JE, Darcy
715 JL, Lynch RC, Wickey P, Ferrenberg S. Patterns and processes of microbial community
716 assembly. *Microbiol Mol Biol Rev* 2013;77(3):342-356. DOI: 10.1128/MMBR.00051-12.

717 Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin P, O'hara R, Simpson G, Solymos P,
718 Stevens M, Wagner H. Community ecology package. R package version 2013;2(0). URL
719 <http://cc.oulu.fi/~jarioksa/>. Accessed on 13/02/2020.

720 Ruijter JM, Pfaffl MW, Zhao S, Spiess AN, Boggy G, Blom J, Rutledge RG, Sisti D, Lievens A,
721 De Preter K. Evaluation of qPCR curve analysis methods for reliable biomarker discovery:
722 bias, resolution, precision, and implications. *Methods* 2013;59(1):32-46.
723 <https://doi.org/10.1016/j.ymeth.2012.08.011>.

724 Soininen J, McDonald R, Hillebrand H. The distance decay of similarity in ecological communities.
725 *Ecography* 2007;30(1):3-12. <https://doi.org/10.1111/j.0906-7590.2007.04817.x>.

726 Tsuge T, Harimoto Y, Akimitsu K, Ohtani K, Kodama M, Akagi Y, Egusa M, Yamamoto M, Otani
727 H. Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*.
728 *FEMS Microbiol Rev* 2013;37(1):44-66. <https://doi.org/10.1111/j.1574-6976.2012.00350.x>.

729 Vásquez AOlofsson TC. The lactic acid bacteria involved in the production of bee pollen and bee
730 bread. *J Apic Res* 2009;48(3):189-195. <https://doi.org/10.3896/IBRA.1.48.3.07>.

731 Vásquez A, Forsgren E, Fries I, Paxton RJ, Flaberg E, Szekely L, Olofsson TC. Symbionts as
732 major modulators of insect health: lactic acid bacteria and honeybees. *PLOS one*
733 2012;7(3):e33188. <https://doi.org/10.1371/journal.pone.0033188>.

734 Wang L, Wu J, Li K, Sadd BM, Guo Y, Zhuang D, Zhang Z, Chen Y, Evans JD, Guo J, Zhang Z,
735 Li J. Dynamic changes of gut microbial communities of bumble bee queens through
736 important life stages. *mSystems* 2019;4(6):e00631-19. DOI:
737 <https://doi.org/10.1128/mSystems.00631-19>.

738 White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA
739 genes for phylogenetics. PCR protocols: a guide to methods and applications
740 1990;18(1):315-322. DOI:10.1016/B978-0-12-372180-8.50042-1.

741 Wootton RJ. Functional morphology of insect wings. *Annu Rev Entomol* 1992;37(1):113-140.
742 <https://doi.org/10.1146/annurev.en.37.010192.000553>.

743 Zheng H, Powell JE, Steele MI, Dietrich C, Moran NA. Honeybee gut microbiota promotes host
744 weight gain via bacterial metabolism and hormonal signaling. *Proc Natl Acad Sci USA*
745 2017;114(18):4775-4780. <https://doi.org/10.1073/pnas.1701819114>.

746

