Honey wasps differ from other wasps in possessing large gut communities dominated by host-restricted bacteria

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ABSTRACT

Honey-feeding social bees, including honey bees, bumbles bees, and stingless bees, possess distinctive gut bacterial communities that provide benefits to hosts, such as defense against pathogens and parasites. Members of these communities are transmitted through social interactions within colonies. The Mexican honey wasp (*Brachygastra mellifica)* represents an independent origin of honey-storing within a group of social Hymenoptera. Honey wasps feed on and store honey, but, unlike bees, they prey on other insects as a protein source, and do not consume pollen. We surveyed the gut bacterial communities of Mexican honey wasps across sites within Texas using 16S rRNA profiling, and we estimated bacterial titer per bee using qPCR. For comparison, we also surveyed non-honey feeding wasps from six families, collected in the same region. We found that honey wasp communities are dominated by characteristic bacterial species. In contrast, other wasps had lower absolute titers and more variable communities, dominated by environmental bacteria. Honey wasps from all sampled nests contained strains of *Bifidobacterium* and *Bombilactobacillus* that were closely related to symbionts of bumble bees and other bees, suggesting acquisition via host-switching. Some individuals also harbored a close relative of *Candidatus* Schmidhempelia bombi (Orbaceae), an uncultured bumble bee symbiont, again suggesting host-switching. The most prevalent species was an uncultured *Lactobacillus* that potentially represents an independent acquisition of environmental *Lactobacillus*. The transition to honey feeding, combined with a highly social life history, appears to have facilitated the establishment of a bacterial community with similarities to those of social bees.

IMPORTANCE

Honey-feeding social insects such as honey bees and bumble bees have conserved gut bacterial communities that are transmitted among nestmates. These bacteria benefit hosts by providing defense against pathogens, and potentially by contributing to pollen digestion. The bacterial communities of

wasps are less studied. Whereas most wasps are carnivorous and consume nectar, honey wasps (*Brachygastra* spp.) store and eat honey. Here, we address the consequences of this dietary shift for the gut community. Using field collections of Mexican honey wasps and other co-occurring wasps, we found that honey wasps have distinctive gut bacterial communities. These include several bacteria most closely related to bacteria in bumble bees, suggesting their acquisition via host-switching. Solitary wasps and social wasps that do not make honey have smaller gut communities dominated by environmental bacteria, suggesting that honey feeding has shaped the gut bacterial communities of honey wasps.

INTRODUCTION

Gut microbial communities often provide numerous benefits to insect hosts (1). They can protect against pathogens or parasites (2–4) enhance or become essential for physiological development (5), metabolize indigestible substrates consumed by the host (6, 7) or neutralize toxic compounds (8, 9). Depending on the host species, microbial community members can be highly host-specific or can consist of bacteria widespread in the environment. Well-studied examples of host-restricted bacterial communities are found in the honey-feeding bees, which belong to a clade consisting of honey bees (genus *Apis*), bumble bees (genus *Bombus*) and stingless bees (tribe Meliponini). These social bees are associated with a bacterial community that is relatively consistent among different species, reflecting a shared evolutionary history (10). Most species harbor representatives of five bacterial lineages that appear to have colonized a common ancestor 80 million years ago and to have subsequently diversified with hosts, though a few bee lineages have lost ancestral associations (11). For example, in the best-studied host, the western honey bee (*Apis mellifera*), adult guts are dominated by these five bacterial groups (sometimes called the “core microbiome”), with several other “accessory” bacterial species present more erratically. These bacteria have been implicated in digestion and detoxification of the nectar, honey, and pollen diet of their hosts (8, 12–16).

67 Conversely, solitary bees (such as non-corbiculate bees) have more variable gut communities containing
68 bacteria commonly associated with diverse bee lineages, flowers (nectar and pollen), and other
69 environmental sources (17–21). Bee gut microbial communities can vary with pathogens (22) and
70 urbanization (23, 24), although some of these bacteria may also protect against pathogens (25).
71 Dietary shifts are frequently associated with a corresponding shift in gut microbial communities
72 (26, 27). A new diet may facilitate or necessitate the acquisition of new symbionts including symbionts
73 that can contribute to nutritional processing. For example, omnivorous, litter-feeding cockroaches have
74 similar gut bacteria regardless of diet composition (28); however, the switch to wood-feeding in
75 *Cryptocercus* cockroaches was accompanied by the acquisition of lignocellulose-degrading protists (29).
76 These symbionts are maintained in the wood-feeding lower termites but were later lost in the soil and
77 litter-feeding higher termites, which possess polysaccharide-degrading fungal and bacterial symbionts
78 that resemble the microbial communities of litter-feeding cockroaches (7, 30–32). On a shorter
79 evolutionary timeline, the vulture bee, *Trigona necrophaga*, switched from pollen and honey to an
80 obligately necrophagous diet. This diet shift is linked to a higher abundance of environmentally acquired
81 acidophiles and more variable bacterial communities compared to related honey-feeding bees (33).
82 Routes of transmission can also affect microbial community composition. Social and gregarious
83 insects can transfer symbionts among group members by direct contact (34), as documented for honey
84 bees (35) and other groups (36). Sociality, however, is not essential for maintaining a conserved, host-
85 restricted microbial community. *Xylocopa* carpenter bees have conserved bacterial communities but
86 limited social interactions within communal nests (37, 38). Brood provisions serve as a microbial
87 transmission route for some solitary bees (21, 39). However, the gut communities of many solitary bees
88 are acquired from environmental sources (pollen and nectar) and are thus heterogeneous (20).
89 Here, we investigate the gut bacterial communities of a honey-feeding wasp, representing an
90 independent evolution of honey-feeding. Our focal species is the Mexican honey wasp, *Brachygastra*

91 *mellifica*. As for honey bees, their colonies contain thousands of individuals, can live for several years,
92 and new nests are established by swarming (40, 41). The honey wasps (*Brachygastra* spp.*,* Vespidae
93 paper wasps) store and feed on honey rather than nectar, as documented in three species (*B. mellifica*,
94 *Brachygastra lecheguana*, and *Brachygastra scutellaris*) (41, 42). This diet shift is rare among wasps; only
95 *Polistes annularis* is known to store honey to overwinter (43), although *Mischocyttarus* spp. store honey
96 short-term (44). To address how this shift may have affected bacterial community composition, we
97 characterized the gut bacteria of Mexican honey wasps across their range in Texas (Fig. 1). We
98 sequenced the V4 region of the 16S rRNA gene to determine the bacterial community composition of 63
99 honey wasps from 13 nests. Honey wasp gut bacterial communities were compared to those of social
100 *Polistes* paper wasps and several solitary wasps. Our findings indicate that honey wasps acquired a
101 unique gut community dominated by bacterial lineages most closely related to bacteria associated with
102 honey-feeding bees, plus an uncultured species of *Lactobacillus*. This composition differs from that
103 observed for other sympatric wasps, including social paper wasps (genus *Polistes,* Vespidae) and solitary
104 wasps, both of which have more variable gut bacterial communities consisting of bacteria found in other
105 environments.
106

107 # RESULTS

108 ## Gut bacterial communities of honey wasps

109 We sampled 63 Mexican honey wasps, including three to six workers sampled from 13 nests,
110 and five workers collected from flowers in Austin (Fig. 1). For comparison, we sampled 47 other wasps,
111 but only 32 of these wasp samples (15 social *Polistes* paper wasps and 17 solitary wasps) had adequate
112 read counts to be included in the analysis (Table S1-S2, explanation of process in Supplementary
113 materials and methods). One honey wasp sample was also removed, due to low read numbers after

114 filtering (Table S1-S2). Three blank samples were included as negative controls to detect contamination
115 during DNA extractions and sequencing. This resulted in 13 - 589 reads from 11 ASVs after filtering
116 (Table S2).

117 *Bifidobacterium* and *Lactobacillus* (Fig. 2) were present in all honey wasp nests; however, some
118 individuals did not possess these bacteria. Seven *Lactobacillus* ASVs (2, 7, 8, 25, 53, 62, and 72) and
119 three *Bifidobacterium* ASVs (1, 3, and 5) were mostly confined to honey wasps, although the
120 *Lactobacillus* ASV2 was present in very low abundance in four solitary wasps. Honey wasps also
121 possessed *Convivina* (ASV17 and ASV26), *Fructilactobacillus* (ASV13), and *Bombilactobacillus* (ASV21)
122 throughout the Texas range, but these were not present in all individuals (Fig. 2B). Honey wasps from
123 some San Antonio nests had bacteria related to *Ca.* Schmidhempelia bombi (ASV14 and ASV30), an
124 undescribed Neisseriaceae (ASV27), and *Asaia* (ASV36). The honey wasps collected in Austin, from a nest
125 (AMP in Fig. 1) or from flowers, had more variable bacterial communities and were not dominated by
126 *Lactobacillus* and *Bifidobacterium*. Some AMP samples had bacterial communities dominated by
127 *Fructilactobacillus*, while others had high abundance of *Ca*. Stammerula and *Convivina*. Several Austin
128 honey wasps possessed two Enterobacteriaceae (ASV4 and ASV6) also found in solitary wasps.

129 Based on the "core microbiome" analysis, some ASVs of *Lactobacillus*, *Bifidobacterium*,
130 *Bombilactobacilus*, *Fructilactobacillus*, and *Convivina* occur in most honey wasp individuals in high
131 abundance (Fig. 3). An NMDS ordination of the Bray-Curtis dissimilarity index shows that the
132 communities of honey wasps cluster together apart from those of other wasps (Fig. 4A, Table S5,
133 PERMANOVA, $F = 15.968$, $p = 0.001$). Beta dispersions significantly differed among the honey wasps and
134 the combined wasps (Table S6, betadisper, $F = 34.933$, $p = 0.001$).
135
136

137 ## Bacterial communities of *Polistes*, solitary wasps

138 The gut bacterial communities of *Polistes* and solitary wasps sometimes contained
139 *Bifidobacterium* (ASV94) or *Lactobacillus* (ASVs 61, 72, 111). Still, these ASVs were absent from honey
140 wasps (Fig. 2B). *Polistes* and solitary wasps shared Enterobacteraceae ASVs (ASV4 and ASV6) with honey
141 wasps. They more often contained *Aristophania*, *Lactococcus*, *Acinetobacter*, and *Exiguobacterium*,
142 *Pantoea*, and *Spiroplasma* (Fig. 2). "Core microbiome" analysis showed that *Polistes* were typified by the
143 presence of the these bacteria(Fig. 3), but no bacteria were detectable in more than 40% of *Polistes*
144 individuals sampled, indicating that *Polistes* bacterial communities are often absent or tiny. The *Polistes*
145 shared some ASVs with the 18 solitary wasps (Enterobacteriaceae ASV4, *Pantoea* ASV9 and ASV19,
146 *Lactobacillus* ASV61 and ASV72), Enterbacteriaceae (ASV6) had higher relative abundance in the solitary
147 wasp samples compared to *Polistes* samples. Solitary wasp bacterial communities were highly variable
148 and dominated by bacteria such as *Acinetobacter*, *Pantoea*, *Pediococcus*, *Wolbachia*, and *Spiroplasma*
149 (Fig. 2aA). "Core microbiome" analysis indicated the most prevalent bacteria were *Gilliamella* (ASV50),
150 three *Lactobacillus* ASVs (ASVs 61, 72, and 78), and two *Snodgrassella* (ASV81 and ASV106) (Fig. 3).
151 These bacteria were all in low abundance (fewer than 12% of total reads in samples) and are not
152 represented among the ASVs in Fig.2A. Notably, the ten most abundant ASVs in honey wasps were not
153 among the most abundant ASVs in *Polistes* and other wasps.
154

155 ## Absolute titers of honey wasp gut bacterial communities

156 Based on qPCR of a region of the bacterial 16S rRNA gene, honey wasps have substantial gut
157 communities, averaging greater than 10^8 gene copies per wasp individual, with relatively little variability
158 in community size (Fig. 5). In contrast, the absolute titers of gut bacteria in *Polistes* and in solitary wasps
159 were lower and highly variable, ranging from below the limit of detection to 10^{10} copies of the bacterial
160 16S rRNA gene (Fig. 5, Table S8). *Polistes* wasps had a median of 3.1×10^5 copies of 16S rRNA gene,
161 ranging from 10^3 to 10^7 copies, and solitary wasps had a similar wide range of bacterial load (Table S9,
162 Dunn's test, z = -1.662, adjusted p = 0.290).

163 ## Bacterial community diversity among honey wasp nests

164 Honey wasp bacterial composition varied among sample locations and varied significantly
165 among nests (Fig. 4B, Table S5 and S7, PERMANOVA, F = 2.9927, p = 0.001; betadisper, F = 1.2641, p =
166 0.253). No nests had distinctly different gut communities based on pairwise comparisons of honey
167 wasps from all 13 nests (Table S7, adjusted for multiple comparisons). *Bifidobacterium* and *Lactobacillus*
168 were the primary members of the bacterial communities and present in all nests, but there were
169 multiple ASVs for each that were differentially distributed among nests.

170 Honey wasp *Bifidobacterium* was represented by three ASVs. Indicator species analysis revealed
171 that ASV1 was significantly associated with five nests while ASV3 was associated with four different
172 nests (Table S10). *Bifidobacterium* ASV1 and ASV3 rarely co-occurred in the same host, but ASV3
173 typically occurred with *Bifidobacterium* ASV5 (Fig. 2B).

174 *Lactobacillus* ASV2 was present in all nests, but the indicator species analysis associated ASV2
175 with five particular nests (Table S10, indicator value = 0.602, p = 0.022). Other *Lactobacillus* ASVs were
176 associated with a subset of nests. For example, *Lactobacillus* ASV25 was associated with San Antonio

177 nest 6 and ASV43 with the Port Lavaca nest (Table S10; ASV25: indicator value = 0.819, p = 0.03; ASV44:
178 indicator value = 0.869, p = 0.0022).
179 Some bacteria had a limited association with only a few nests. For example, San Antonio nests 1,
180 2, and 3 were characterized by the presence of ASVs clustering with *Ca.* Schmidhempelia (Table S10;
181 ASV14: indicator value = 0.639, p = 0.0418; ASV30: indicator value = 0.636, p = 0.0439). Additionally, the
182 Austin nest showed high abundance of *Fructilactobacillus* (Fig. 2B, Table S3, Table S10:indicator value =
183 0.715, p = 0.0412).

184 Laboratory isolation of honey wasp bacteria

185 To verify that the ASVs from honey wasps represented living bacteria, we isolated pure cultures
186 of some community members. These included a member of the *Ca.* Schmidhempelia cluster (wjB12) and
187 several strains of *Bifidobacterium* (efB7, efB4, wjB1) from two San Antonio nests (SA1 and SA7) grown
188 on heart infusion agar (HIA) with 5% sheep's blood under 5% CO_2 at 35°C. A *Fructilactobacillus vespulae*
189 isolate (sjhB6) and an additional *Bifidobacterium* isolate (sjhB46) were also cultured in MRS broth under
190 anaerobic conditions at 31°C. The identities of these cultures were verified with 16S rRNA gene
191 sequencing.

192 Phylogenetic relationships of key honey wasp bacteria

193 We constructed phylogenies for three bacterial groups prevalent in honey wasp bacterial
194 communities belonging to Orbaceae, *Bifidobacteria*, and Lactobacillaceae. Within Orbaceae, the honey
195 wasps possessed two ASVs and a bacterial isolate (strain wjB12) that form a well-supported clade
196 together with *Candidatus* Schmidhempelia bombi, an uncultured Orbaceae known from bumble bees
197 and carpenter bees (Fig. 6A) (37, 45, 46).
198 Three *Bifidobacterium* ASVs and numerous *Bifidobacterium* isolates from honey wasps form a
199 clade with multiple species of *Bifidobacterium* isolated from several species of European bumble bees

200 (*B. commune*, *B. bohemicum*, *B. bombi*) (Fig. 6B). This clade is not closely related to the *Bifidobacterium*
201 *'asteroides'* clade associated with honey bees, bumble bees, carpenter bees, and *Osmia* mason bees (10,
202 37, 55).

203 Honey wasps also possess a variety of bacteria in the family Lactobacillaceae. Eight near-
204 identical ASVs represent an undescribed species of *Lactobacillus* that forms a sister group to the clade of
205 *Lactobacillus* associated with honey bees, bumble bees, stingless bees, and *Osmia* bees (containing
206 *Lactobacillus melliventris* and formerly referred to as "Firm-5") (Fig. 6C). Seven of these *Lactobacillus*
207 ASVs were found primarily in honey wasps, while ASV72 was found in *Polistes* paper wasps.
208 Representatives of this ASV cluster remain uncultured. The 16S rRNA gene amplicon variation suggests
209 there is strain diversity within the honey wasps with ASV2 found in 92% of honey wasps sampled.

210 Another Lactobacillaceae group, *Bombilactobacillus* (containing *Bombilactobacillus mellifer* and
211 formerly called "Firm-4"), was sequenced from multiple honey wasps but remains uncultured (Fig. 6C).
212 Previously the genus was known only from honey bees, bumble bees, stingless bees, and carpenter bees
213 (10, 11, 37, 47). The sequences from honey wasps are close to those from *Bombilactobacillus apium*
214 isolated from the Asian honey bee, *Apis cerana*. Additionally, honey wasps possess the vespid wasp-
215 associated *Fructilactobacillus vespulae*, a *Lactiplantilactobacillus*, and another Lactobacillaceae (Fig. 6C).

216 DISCUSSION

217 Major findings

218 Honey wasps represent an independent evolution of honey storage and feeding in social
219 Hymenoptera, and we explored whether this dramatic shift in diet was linked to changes in the gut
220 bacterial community. We found that honey wasps have bacterial communities that differ from those of
221 other wasps, both social and solitary, but that exhibit some similarities with communities of social bees
222 (Figs 2A, 2B). First, as in the social bees, honey wasps have large bacterial communities with relatively

223 consistent absolute gut titers, as compared to other wasps, which have bacterial titers that are over two
224 orders of magnitude smaller on average and that are highly variable in size (Fig. 5). As estimated by
225 qPCR, sizes of honey wasp bacterial communities are comparable to those of honey bee species, at
226 about 10^8 copies of rRNA genes per bee (10). Second, honey wasp bacterial communities have relatively
227 consistent composition dominated by lineages that are host-restricted, as in social bees. Third, certain
228 members of the honey wasp bacterial communities are most closely related to taxa in microbiomes of
229 social bees (Fig. 6). However, other bacteria characteristic of honey wasp gut communites are not linked
230 to bacteria from social bees.

231 ## Gut bacterial communities of honey wasps

232 Honey wasps have a diverse consortium of Lactobacillaceae that is distinct from that of honey
233 bees or the other wasps included in this study. All honey wasps host an uncultured *Lactobacillus* which
234 belongs to a clade of honey bee-associated *Lactobacillus* near *L. melliventris* (formally Firm-5); multiple
235 ASVs were detected indicating strain diversity in this taxon (Fig. 6C). Despite many efforts, this
236 *Lactobacillus* remains uncultured.

237 *Bombilactobacillus* ASVs in honey wasps are closely related to *B. apium* isolated from *Apis*
238 *cerana* (Fig 6C) (47). Honey wasps also consistently possess three related *Bifidobacterium* ASVs that
239 belong to the same clade as *Bifidobacterium* species (*commune*, *bombi*, *bohemicum*) isolated from
240 European bumble bees (Fig. 6B) (48, 49). We successfully cultured two strains (corresponding to ASV3
241 and ASV5) from honey wasp guts, demonstrating that these ASVs represent living bacteria. Individual
242 wasps had either ASV1 or ASV3 in high abundance, not both, suggesting competition or another
243 incompatibility.

244 Though honey wasp bacterial communities had relatively consistent composition, some bacteria
245 were erratically present. Several nests of honey wasps in San Antonio had ASVs closely related to *Ca.*
246 Schmidhempelia bombi, known from metagenomic samples of *Bombus* impatiens (Fig. 6A)(45). The ASVs

247 from honey wasps nested, with strong support, within a clade previously known only from bumble bees
248 (Fig. 6A). Honey bees, stingless bees, and bumble bees share gut bacteria, including *Lactobacillus*,
249 *Bifidobacterium*, and *Bombilactolacillus* that were acquired by a common ancestor approximately 80
250 million years ago (10), and the bumble bee associate *Ca*. Schmidhempelia was acquired by bumble bees
251 later (50). Associations between honey-feeding bees and their bacterial symbionts predate the evolution
252 of the genus *Brachygastra*, which arose approximately 13.6 million years ago (51). Mexican honey wasps
253 and stingless bees have co-occurred in South, Central and southern parts of North America during that
254 time, and *Bombus* arrived in the Neotropics several million years ago (52), providing ample time for the
255 lineages to interact. The shared food source of nectar may have enabled transfer between honey wasps
256 and honey-feeding bees.

257 Also present in the honey wasps were bacteria commonly associated with other, non-honey-
258 feeding bees. These include *Lactococcus, Convivina*, and some *Lactobacillaceae* such as
259 *Lactiplantibacillus*. Three honey wasps collected individually from Austin had high abundances of ASVs
260 related to *Candidatus* Stammerula and with 99% sequence identity to an uncultured symbiont of
261 tephritid flies (53, 54).

262 While honey wasps appear to have acquired some bacterial lineages found in honey-feeding
263 bees, they lacked members of the *Bifidobacterium "asteroides"*, *Snodgrassella,* or *Gilliamella* clades,
264 which are found in bumble bees, honey bees and many stingless bees (10, 55). Honey wasps also lack
265 the "Firm-5" *Lactobacillus* species (*L. melliventris* group). associated with social bees but possess an
266 undescribed relative. *Snodgrassella* and *Gilliamella* form a biofilm along the ileum of honey bees and
267 bumble bees (12, 50). Also missing are the "accessory" bacteria that are occasionally sampled in honey
268 bee bacterial communities, such as species in the genera *Apilactobacillus*, *Bombella*, *Arsenophonus*,
269 *Bartonella*, *Parasaccharabacter*, *Apibacter*, and *Frischella perrara* (56, 57).

270 Our study on honey wasps adds to others suggesting that some vespid wasps have reliable
271 associations with gut bacteria. The subfamily Vespinae contains four genera, of which two have been
272 the focus of research on bacterial associates. *Vespula pensylvanica*, *Vespula vulgaris* and *Vespa velutina*
273 are all associated with several bacteria that we found in honey wasps. *Vespa velutina* nests consistently
274 possess *Lactobacillus* and *Bifidobacterium* in all life stages (58), as we found for honey wasps.
275 *Fructilactobacillus vespulae*, which we found in most honey wasp nests, has been isolated or sequenced
276 from *Vespula vulgaris* in China, *Vespula pensylvanica* in the US (59), and *Vespa velutina* in Belgium. We
277 detected *Convivina* in some individuals from all sampled honey wasp nests, but not in other wasps.
278 *Convivina intestini* has been isolated from *Bombus terrestris* (60) and *Convivina praedatoris* was found in
279 a single *V. velutina* nest collected in Belgium (outside of the Korean native range) (61). Similarly, we
280 detected *Lactiplantibacillus*, another possible member of the *V. velutina* community, in 43% of sampled
281 honey wasps. In a study of bacterial communities of five *Vespa* species in their South Korean native
282 range, *Convivina* and *Lactiplantibacillus* were not among the top 10 bacterial genera (62), so these
283 associations may be site-specific.

284 Gut bacterial communities of other wasps

285 We found that social *Polistes* paper wasps and solitary wasps have relatively small gut
286 communities that vary in composition and absolute bacterial titers. Their bacterial titers, estimated as
287 copies of 16S rRNA operons, were 10^4-10^6, far lower than for honey wasps or honey bees (Fig. 5). These
288 values likely overestimate average community sizes, as almost half of these wasp samples failed to
289 produce sufficient 16S rRNA amplicons and were excluded. In contrast, all samples for honey wasps
290 produced sufficient amplicons. Also, we note that community profiles obtained from PCR amplicons
291 include reads from bacteria present in the food and from extracellular DNA from dead bacteria, in
292 addition to bacteria colonizing the gut. The possibility that these other sources dominate profiles is
293 especially great for low bacterial titer communities consisting of environmental bacteria, such as those

294 in the *Polistes* and solitary wasps. Since many species of bees and wasps can visit the same flowers,
295 where they may contaminate nectar, live or dead cells from other host species may be ingested in
296 food.

297 The *Polistes* paper wasps and solitary wasps had highly variable gut community compositions,
298 often dominated by environmental bacteria, including environmental Lactobacillaceae. They lacked all
299 major members of the honey wasp bacterial community. Some ASVs erratically present in honey wasps
300 were shared with *Polistes* and solitary wasps, including some from Enterobacteriacae, *Pseudomonas*,
301 *Lactococcus*, and some others found in environmental sources such as nectar and other Hymenoptera
302 (20, 37, 56, 59). However, these had low prevalence and low abundance in honey wasps (Fig. 4, Fig. 6).

303 In the four species of social *Polistes,* sequences corresponding to *Aristophania*, *Gilliamella*,
304 *Acinetobacter*, and *Lactobacillus* were found erratically (Fig. 2A). These have been previously detected in
305 flower nectar, solitary bees (17, 63, 64), *Vespa* spp. (62), and *Vespula pensylvanica* (59). We found
306 *Gilliamella* sp. (ASV 50) in a variety of wasps including species from Mutillidae, Crabronidae, Sphecidae,
307 and *Polistes* in low abundance (Table S3). *Gilliamella* species are among the bacteria consistently
308 present in honey bees and bumble bees (58), and sequences from some strains have been detected in
309 native bee species and in nectar (65). These are likely to represent DNA present in ingested nectar, as
310 honey bees are the most common visitors to flowers at all sites.

311 *Polistes* workers also feed on nectar and share it with larvae, as do many solitary wasps,
312 explaining how they acquire some bacteria. However, many adult bees have bacterial communities
313 similar to those in their pollen provisions (20, 21, 23, 24, 63, 65), whereas *Polistes* derive protein from
314 insect prey and do not store pollen. *Polistes* are social and thus have a means of bacterial transmission
315 among nestmates and generations, yet their gut communities are composed of environmental bacteria
316 found in nectar and in solitary bees. It appears that both *Polistes* and solitary wasps acquire bacteria
317 from environmental sources based on the wasps sequenced in this study. Our findings suggest that

318 many wasps, both social and solitary, do not rely on a specific bacterial community, as is the case for
319 some other insects (1, 66).

320 Geographic variation in bacterial communities

321 Mexican honey wasps occur from central Texas south to Panama, so our collection range
322 represents a small part of the *B. mellifica* range. Furthermore, the Mexican honey wasp northern range
323 limit was near the USA/Mexico border until around 1995 (41) and has expanded approximately 500
324 kilometers north over 3 years. We collected 13 nests in Texas and found significant variation in
325 community composition among nests. Bacterial communities are more similar among nestmates.
326 Differences between nests are due to the presence and abundance of particular ASVs, reflecting strain
327 variation among the major microbiome members. Examples include the uncultured *Lactobacillus*,
328 represented by eight ASVs that form a clade (Fig. 6C), and the *Bifidobacterium* with 4 ASVs that varied
329 among hosts.

330 All honey wasp individuals had bacterial communities dominated by *Lactobacillus* and
331 *Bifidobacterium*. The exceptions were wasps from the Austin nest, where three of the five individuals
332 were dominated by *Fructilactobacillus vespulae*. Among the five honey wasps that were collected from
333 flowers in Austin, one had the characteristic bacterial profile, and the four others had a higher
334 abundance of Enterobacteriaceae, *Ca*. Stammerula, and *Conviva*. This variation suggests that honey
335 wasps sometimes lose the conserved, host-restricted set of bacteria, which is replaced by a more
336 variable community containing environmental strains, a pattern observed repeatedly in bumble bees
337 (57). These two Austin collection sites sit close to the northernmost extent of the Mexican honey wasp
338 range, suggesting a change in microbial diversity associated with edge populations (though our sampling
339 is not sufficient for conclusions). Two species of English butterflies have microbiomes with decreased
340 diversity and a shift in composition in the northern part of their range (67). Similarly, the microbial
341 communities of range-expanding plants were more similar to each other than to those of conspecifics in

342 the ancestral range (70). Further research into honey wasps throughout Mexico and Central America
343 would reveal whether the bacterial community varies across its range.

344 Honey wasp diet and bacterial community

345 The transition to long-term honey-feeding and storage may have expedited the acquisition of
346 bee-associated bacteria as honey wasps feed on a sugar-rich diet like that of honey-feeding bees.
347 *Bifidobacterium* spp. have extensive abilities to degrade plant-derived polysaccharides (74), and bee-
348 associated *Bifidobacterium* can degrade and use hemicelluloses (13). Honey wasps likely do not ingest
349 significant amounts of polysaccharides, since they do not eat pollen, so metabolic capabilities of their
350 bacterial communities may be very different from those of honey bees and bumble bees, which
351 consume pollen. Investigation of the functional capabilities of the *Bifidobacterium* strains isolated from
352 honey wasps will offer insights into their metabolic capabilities and potential benefits to hosts.

353 Conclusions

354 The Mexican honey wasp gut bacterial community is an amalgamation of bacteria that are
355 related to those from bumble bees or honey bees, from vespine wasps, or from other environmental
356 sources. These contrast with communities from sympatric polistine wasps and solitary wasps, which
357 have erratic bacterial communities composed of environmentally acquired bacteria. The switch from
358 feeding on nectar and insect prey to a stable, year-long supply of stored honey may have facilitated, or
359 been facilitated by, the acquisition of bacteria associated with honey-feeding bees.

360 METHODS

361 Sample Collection

362 Honey wasps from 13 nests, individual honey wasps, *Polistes* wasps, and solitary wasps were collected
363 across sites in central and south Texas (Fig. 1, Table S1). Guts were dissected from specimens using

364 sterile technique and stored in 100% ethanol at -80°C. Additional honey wasp guts were preserved in
365 20% glycerol at -80°C for bacterial isolation.

366 DNA Extractions, Sequencing, and qPCR

367 DNA was extracted from whole guts using the CTAB/phenol-chloroform extraction method (35). The full
368 length or V4 region of the 16S rRNA gene was PCR-amplified and sequenced on an Illumina MiSeq
369 platform at the Genome Sequencing and Analysis Facility (University of Texas at Austin). To estimate
370 total 16S rRNA gene copies per insect as an estimate of bacterial abundance, aliquots of diluted DNA
371 extracts were used as template for qPCR. The observed threshold (CT) cycle values were plotted on a
372 standard curve to determine the absolute copy number.

373 Bacterial Isolation and Identification

374 The preserved honey wasp gut homogenates from two nests (SA1 and SA7) and an individual collected
375 in Austin were grown on HIA + 5% sheep's blood or in MRS broth at 35°C in 5% CO_2 or 31°C in anaerobic
376 conditions for 1-3 days. To identify bacterial isolates, the 16S rRNA gene was PCR amplified, and
377 amplicons were sequenced at the DNA Sequencing Facility (University of Texas at Austin).

378 Data Analysis and Phylogenetic Reconstruction

379 Illumina sequencing files were processed in R using the dada2 pipeline to remove poor quality reads and
380 other sequencing artifacts, and to determine unique amplicon sequence variants (ASVs). Taxonomy was
381 assigned using the SILVA 138.2 database (75) and manually confirmed using NCBI BLASTn. Chloroplasts,
382 mitochondria, and unassigned reads were removed, along with contaminants detected in the three
383 blank samples. Read counts were checked after each filtering step; 16 samples were excluded because
384 they 1) lost more than 50% of reads during a post-dada2 filtering or 2) had fewer than 1500 reads after
385 filtering was complete. These issues suggest contamination due to low bacterial biomass (see

386 Supplementary methods, Table S2)(68, 69). Following filtering, the remaining samples had an average of
387 25,103 reads (range from 2172, to 128,080) (Table S3). To account for uneven sequencing, samples were
388 normalized by rarefying to 95% of the minimum sampling depth, resulting in 2063 reads for each sample
389 (Fig. S2, Table S4). All subsequent analyses were conducted on both the rarefied data set and the full
390 data, and there were no differences in statistical outcomes. The "core microbiomes" of honey wasps,
391 *Polistes*, and solitary wasps were identified using the microbiome package. Indicator species analysis
392 was also performed using the indicspecies package to determine if any ASVs were significantly
393 associated with each honey wasp nest. Statistical differences between gut bacterial communities across
394 honey wasp nests and insect types were analyzed using PERMANOVA (*adonis2*), and assumptions were
395 assessed using beta dispersion analysis. Post-hoc pairwise comparisons with Bonferroni adjustment
396 were performed to directly compare bacterial communities across nests and insect types.
397 The 16S rRNA gene phylogenies were reconstructed for Lactobacillaceae, Orbaceae, and
398 *Bifidobacterium*, the groups containing most ASVs or isolates from honey wasps. Full length 16S rRNA
399 gene sequences were obtained from GenBank. These were aligned using MAFFT with ASV sequences
400 and full length 16S rRNA from the isolated bacteria. Maximum likelihood phylogenies were
401 reconstructed using IQtree with auto-generated substitution models and 100 standard bootstrap
402 alignments.

403 DATA AVAILABILITY

404 Raw 16S rRNA gene amplicon reads are deposited in the National Center for Biotechnology
405 Information Sequence Read Archive under accession PRJNA932820. ASV sequences, taxonomic
406 assignments, read numbers, metadata can be found in the Supplement. Scripts for the analysis can be
407 found on GitHub (https://github.com/annatpham/honey-wasp-microbiome), and insect specimens are
408 deposited at the University of Texas Insect Collection (UTIC).

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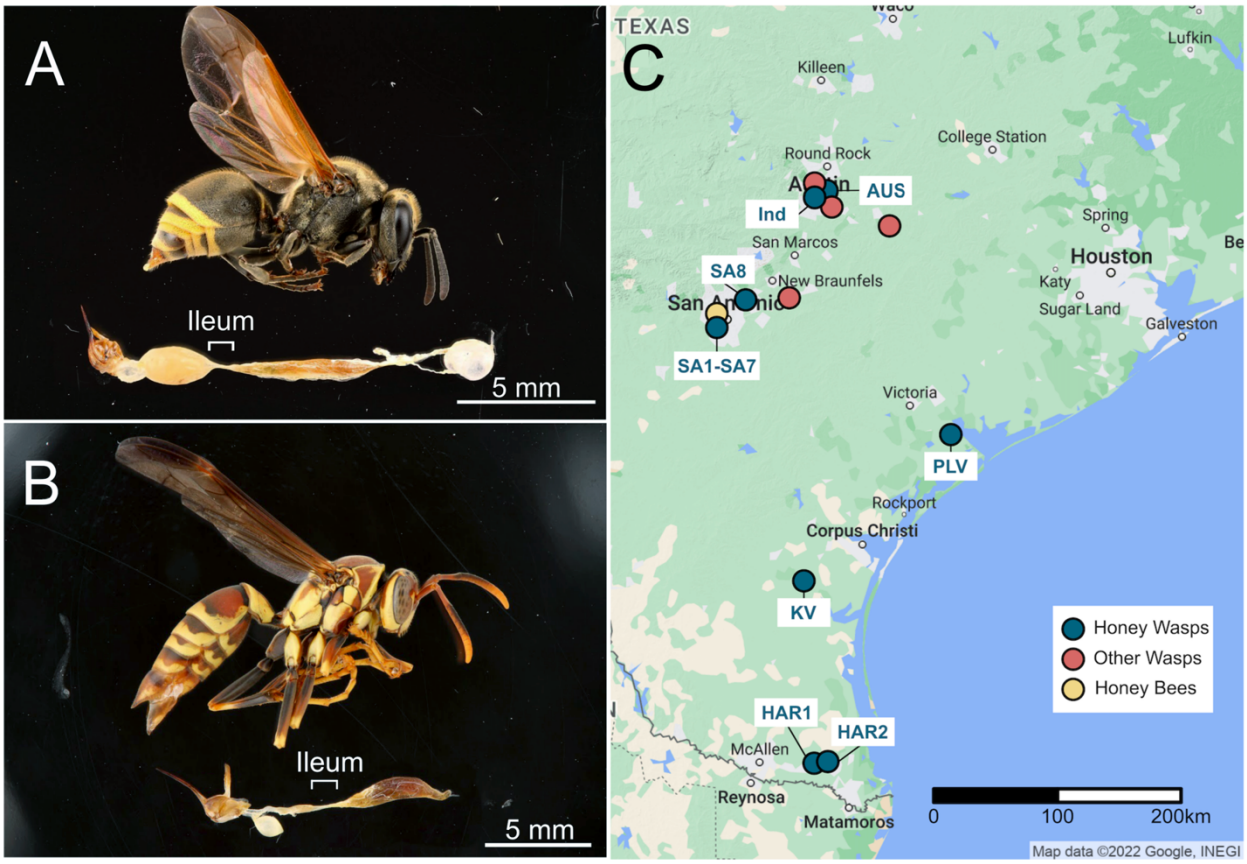
591 # FIGURE LEGENDS

592 **Figure 1:** Specimen images and collecting locations. (A) Mexican honey wasp, *Brachygastra mellifica*, (B)
593 common paper wasp, *Polistes exclamans*. Specimens have their guts removed to show the internal
594 structures of the midgut, ileum, and hindgut. The bottom section of the images were cut and rotated to
595 orientate the midguts to the right and sting to the left. (C) Map of Texas showing the collecting sites of
596 honey wasps, *Polistes* paper wasps and solitary wasps, and honey bees.
597
598 **Figure 2:** Relative abundance of (A) the top 16 bacteria genera and (B) the top 50 amplicon sequence
599 variants (ASVs) from honey wasps, other wasps, and honey bees. Honey wasps are grouped by nest,
600 except five individuals collected in Austin (Ind) based on the rarefied data. Nests are named based on
601 their location and organized on the plot from north (top) to south Texas (bottom). Other wasps are
602 grouped by family (Vesp = Vespidae, Mut = Mutillidae, Thynn = Thynnidae, Crab = Crabronidae, Sphec =
603 Sphecidae, Ich = Ichneumonidae, Pomp = Pompillidae). Honey wasps typically possess a characteristic
604 bacterial community that distinguishes them from other wasp species.
605
606 **Fig. 3.** "Core microbiome" analysis of the rarefied data set showing three insect groups (A) honey wasps,
607 (B) *Polistes* paper wasps, and (C) solitary wasps studied. Prevalence is the proportion of insect samples
608 in each group containing ASVs at detection thresholds ranging from 1% to 20% of the total read
609 abundance. Many solitary wasps are excluded due to lack of amplification, and some included samples
610 had low titers, raising the possibility that contaminants or dead bacteria in food dominate.
611
612 **Figure 4:** Nonmetric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarity index of the
613 rarefied data set. (A) Honey wasp gut bacterial communities compared to those of *Polistes* paper wasp
614 and solitary wasps (stress = 0.19). (B) Honey wasp gut bacterial communities categorized by nest of
615 origin (stress = 0.24).
616
617 **Figure 5:** Bacterial 16S rRNA gene copies per individual host/host gut for honey wasps, social *Polistes*
618 paper wasps, and solitary wasps. Dashed line is the median. The gray shaded area is the region below
619 the detection threshold, and the yellow shaded area represents the range of 16S rRNA gene copies for
620 *Apis mellifera* from Kwong et al. (10). Honey wasps (n = 10) were from nests in San Antonio (SA1 and
621 SA3), *Polistes* samples represent five species (n = 19), and solitary wasps represent 7 families (n = 30,
622 including four solitary eumenine vespid wasps). Honey wasp gut bacterial communities significantly
623 differed from those of *Polistes* paper wasps and solitary wasps in all pairwise comparisons (honey
624 wasps:paper wasps, z = -3.417, adjusted p = 0.002; honey wasps:solitary wasps, z = -4.990, adjusted p =
625 1.8×10^{-6}).
626
627 **Figure 6:** Phylogenies of the 16S rRNA gene of the major bacterial taxa from the honey wasps. (A)
628 Orbaceae, (B) Bifidobacteriaceae, and (C) Lactobacillaceae. Phylogenies were inferred by maximum
629 likelihood, with bootstrap support indicated by black circles. Colors represent bacterial genera or clades
630 associated with bees, host source listed in parentheses. ASVs from this study are highlighted, and the
631 asterisks denote bacteria isolated from honey wasps.
632



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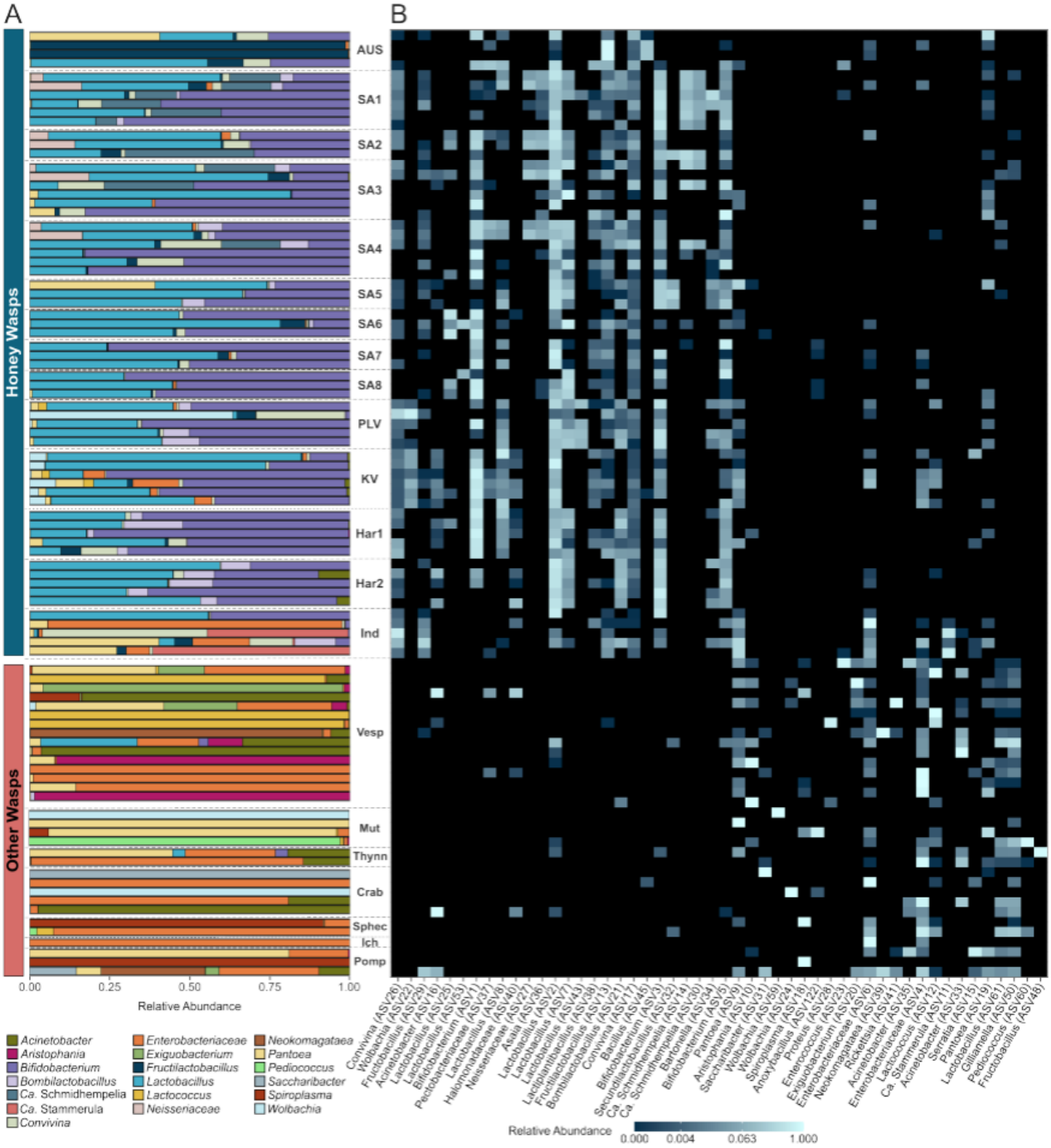

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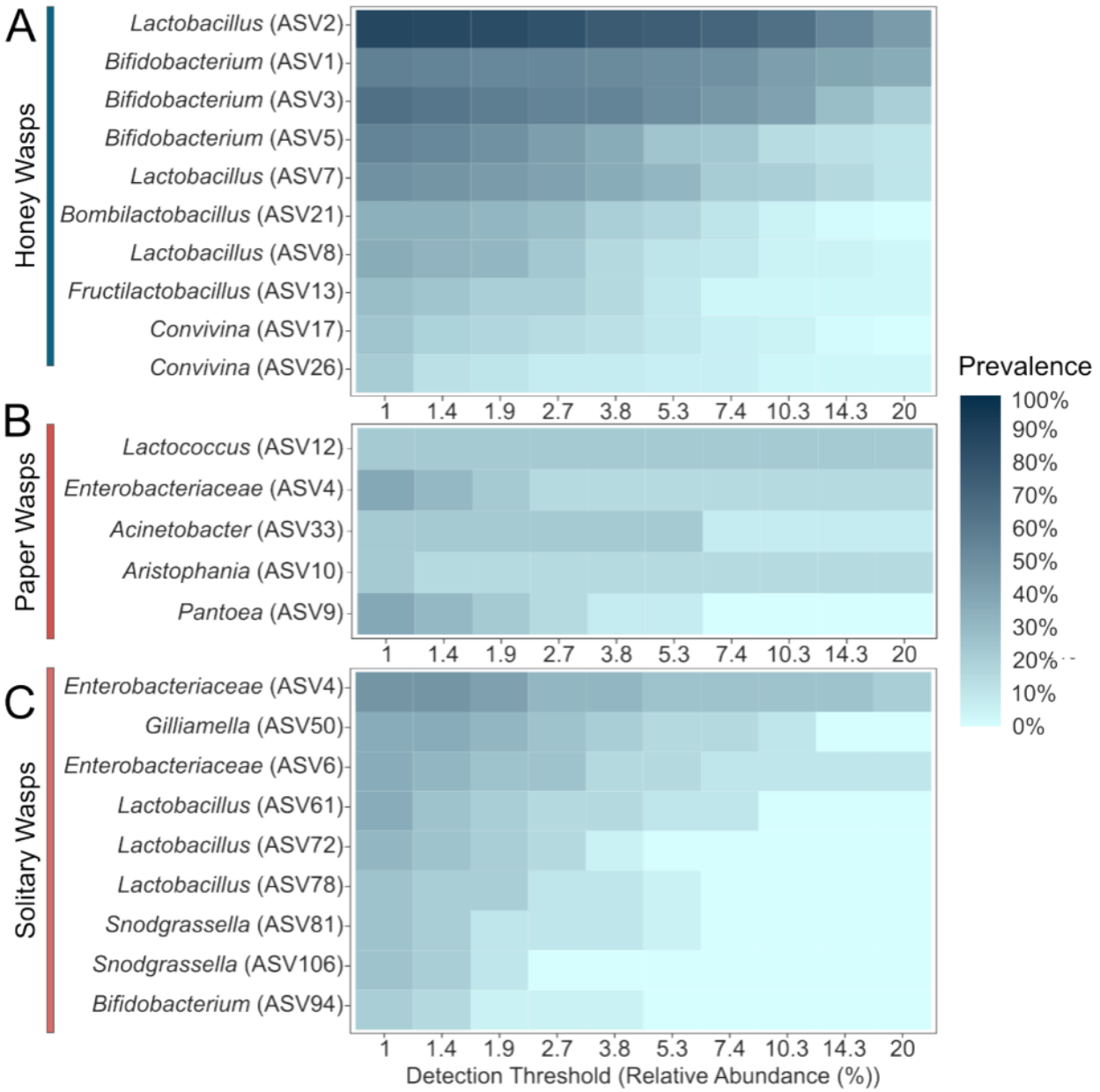

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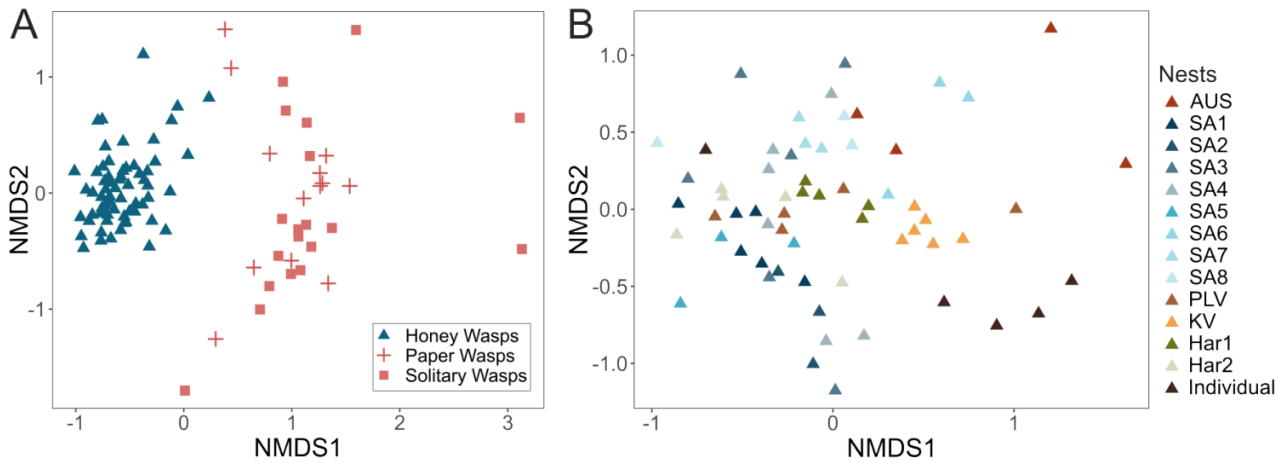

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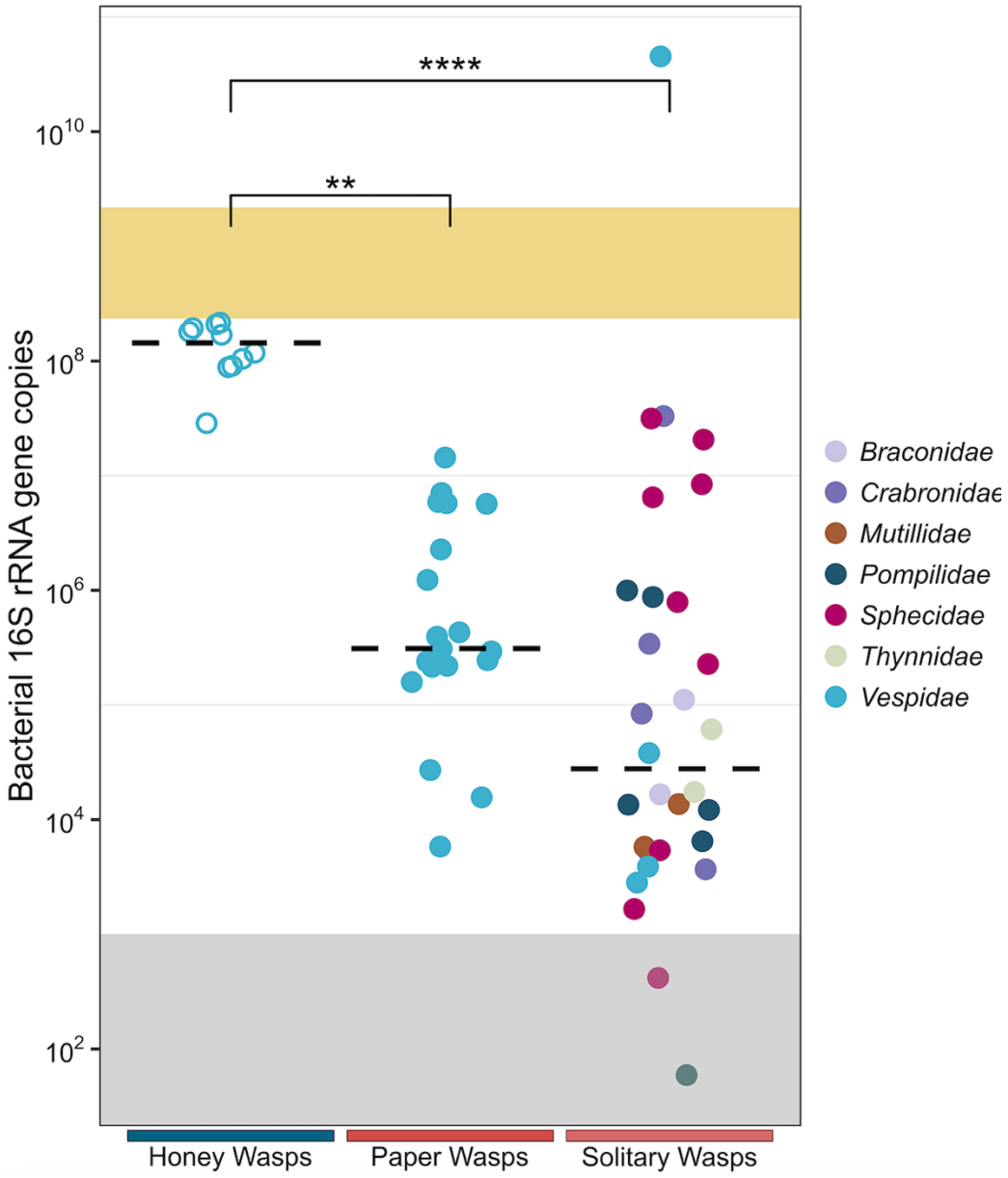

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688 **Figure 5:** Bacterial 16S rRNA gene copies per individual host for honey wasps, social *Polistes* paper
689 wasps, and solitary wasps. Dashed line is the median. The gray shaded area is the region below the
690 detection threshold, and the yellow shaded area represents the range of 16S rRNA gene copies reported
691 for *Apis mellifera* (10). Honey wasps (n = 10) were from nests in San Antonio (SA1 and SA3), *Polistes*
692 samples represent five species (n = 19), and solitary wasps represent 7 families (n = 30, including four
693 solitary eumenine vespid wasps). Honey wasp gut bacterial communities significantly differed from
694 those of *Polistes* paper wasps and solitary wasps in all pairwise comparisons (honey wasps:paper wasps,
695 z = -3.417, adjusted p = 0.002; honey wasps:solitary wasps, z = -4.990, adjusted p = 1.8×10^{-6}).
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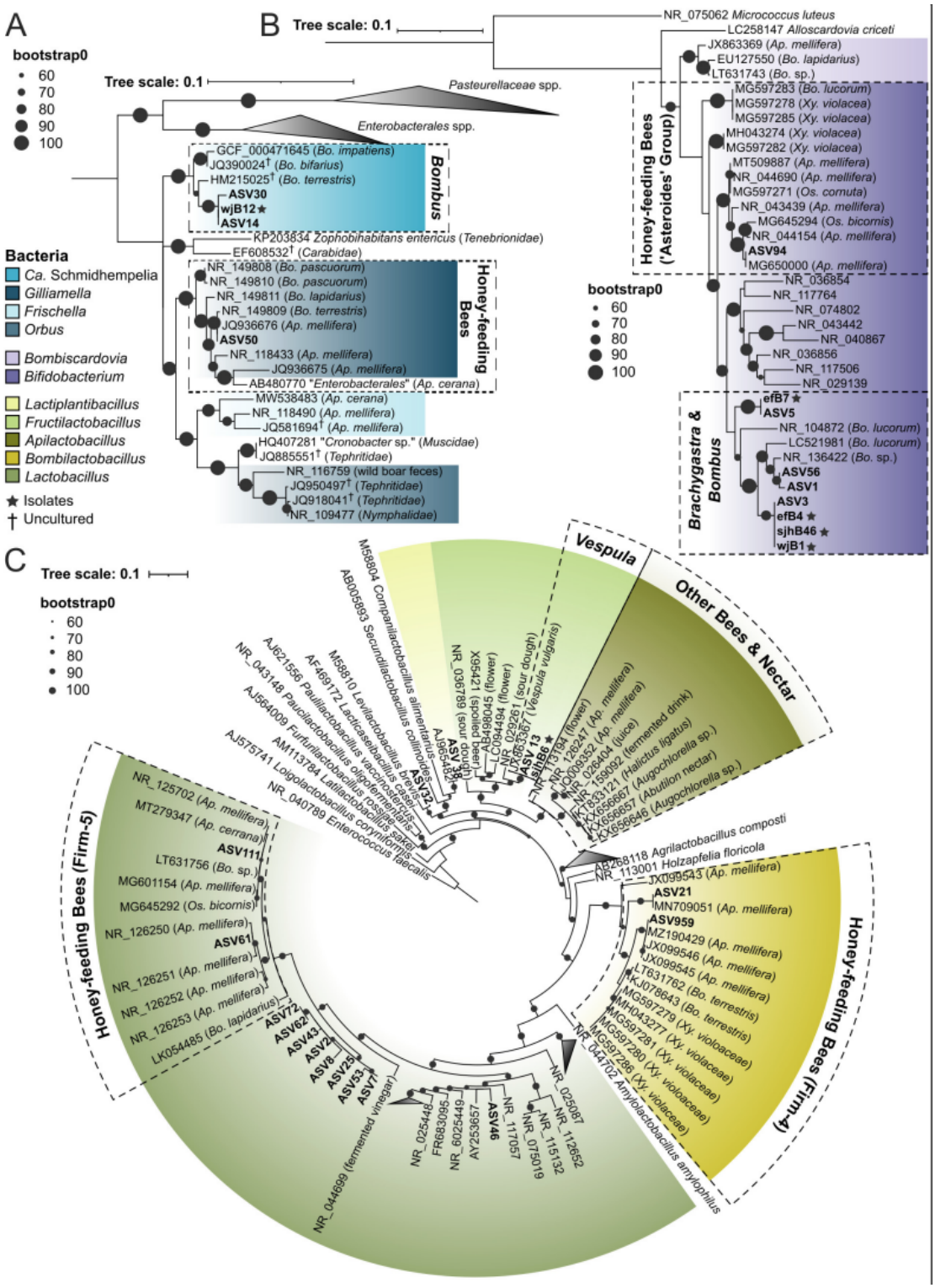

Figure 6: Phylogenies of the 16S rRNA gene of the major bacterial taxa from the honey wasps. (A) Orbaceae, (B) Bifidobacteriaceae, and (C) Lactobacillaceae. Phylogenies were inferred by maximum likelihood, with bootstrap support indicated by black circles. Colors represent bacterial genera or clades associated with bees, host source listed in parentheses. ASVs from this study are highlighted, and the asterisks denote bacteria isolated from honey wasps.