Gut microbes contribute to variation in foraging intensity in the honey bee, *Apis mellifera*.

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Competing Interests

The authors declare no competing interests.

Abstract

Gut microbiomes are increasingly recognized for mediating diverse biological aspects of their hosts, including complex behavioral phenotypes. Many studies have reported that experimental disruptions to the gut microbiome result in atypical host behavior. However, studies that address the role that natural variation in gut microbial communities plays in naturally occurring host behavioral variation are sparse. Here, we show that variation in the gut microbiome influences variation in foraging intensity in honey bees. Gut microbial community structure consistently differed between hive-based nurse bees and bees that leave the hive to forage for floral resources. These differences were associated with variation in the abundance of individual microbes, including *Lactobacillus mellis, Lactobacillus melliventris,* and *Bifidobacterium asteroides*. Manipulations of colony demography and individual foraging experience suggested that differences in microbiome composition were associated with task experience. Moreover, single microbe inoculations with *L. mellis, L. melliventris*, or *B. asteroides* caused changes in foraging intensity. These results demonstrate that gut microbiomes contribute to naturally occurring host behavioral variation, and support a model in which positive feedback interactions cause adaptive changes in both gut microbiome composition and host behavior.

Introduction

Gut microbiomes are emerging as important drivers and modulators of host phenotype [1], with evidence supporting the role of microbiomes in host digestion and nutrition, immune health, development, and, more recently, behavior [2–7]. Over the past decade, the “microbiota-gut-brain axis” [7], which describes bidirectional interactions between the gut microbiome and host brain and behavior, has developed as an increasingly important field of study with implications in understanding the ecology and evolution of host-microbe interactions and animal behaviors. However, current studies of the microbiota-gut-brain axis across animal taxa are largely

correlational, and causal studies exploring the relationship between gut microbes and host behavior are sparse [7].

The functional relationship between hosts and microbiomes varies across host traits. In many cases, this relationship is obligatory, where the microbiome is necessary for normal functioning of the host [7–10]. In other cases, there appears to be a facultative relationship between host and microbiome, such that differences in microbiome cause differences in phenotype between individuals [11]. However, many of these host phenotypes result from dysbiosis or fecal transplants from other species [11, 12], and are either not behavioral [11], or represent behaviors associated with aging and senescence [13–18], severity of neurodevelopmental disorders [19–21], or performance in artificial laboratory-based behavioral tasks [22, 23]. Therefore, studies indicating that natural variation in the gut microbiome can induce variation in adaptive, naturally occurring host behavior are lacking.

We use the European honey bees, *Apis mellifera,* to investigate the causal relationship between microbiome and naturally occurring behavior. Honey bees represent a powerful model to learn more about this relationship due to their complex, yet tractable social behaviors and well-defined gut microbiome. In typical honey bee colonies, worker bees exhibit age-related division of labor, which is based on a pattern of individual behavioral maturation. Typically, adult worker bees perform brood care (“nursing”) and other activities in the hive during the first 1-3 weeks of adult life and then transition to foraging behaviors outside of the hive for the final 1-2 weeks of their life [24, 25]. However, honey bee division of labor is flexible and responsive to changing colony needs, and thus does not exclusively rely on the typical age patterns of behavioral maturation [26–30]. In addition, honey bee foragers exhibit considerable individual variation in preference for nectar or pollen [31, 32], exploratory behavior [33], and general foraging intensity [34]. Therefore, honey bee division of labor involves individual variation in both behavioral maturation and task performance [35].

The honey bee gut microbial community is well-characterized and relatively simple, composed of 9 taxonomic clusters, including ~14 different species of facultatively anaerobic and microaerophilic host-adapted bacteria that are consistently present, but may differ in abundance, across different individuals and populations of honey bees [36]. Of particular interest, the composition of honey bee gut microbial communities can be experimentally manipulated. All species can be cultured [37] and used to inoculate young bees [38–40], who must acquire their microbiome from older bees or hive materials [41]. Furthermore, previous studies indicate an association between the gut microbiome and various aspects of division of labor in the honey bee [42–45]. We took advantage of these behavioral and microbiome features to determine whether gut microbes play a causal role in honey bee division of labor, with a specific focus on behavioral maturation and variation in foraging intensity.

Materials and Methods

Animal Husbandry, treatments, and collections

Honey bee colonies were managed using standard beekeeping techniques at the University of Illinois Bee Research Facility in Urbana, IL. Honey bees in this area are a mixture of subspecies, primarily *Apis mellifera ligustica* and *carnica* subspecies. For collection of nurses and foragers, nurses were identified as those actively feeding brood on a brood frame, and foragers were identified as those returning to the hive with pollen loads on their hind legs or having a distended abdomen due to nectar loads [28, 46]. To reduce genetic variation between workers in the colonies used for the single-cohort colony, big-back colony and single microbe inoculation studies (described below), we used bees derived from queens that were each instrumentally inseminated with sperm from a single drone (SDI). Sample size for 16S sequencing studies was 10 bees per group as in [47]. All bees used in 16S sequencing analyses were washed once with 12.5% bleach

in water and twice with double deionized water and flash frozen. All samples were stored at -80°C until further analysis.

Single- and double-cohort colonies

Single-cohort colonies (SCC) were established as previously described [27, 28, 46] using ~1000 newly eclosed bees (<24 hours old). SCCs were kept outdoors, and bees were collected as typical-age nurses and precocious foragers at approximately one week of age (7-9 days of age), and as over-age nurses and typical-age foragers at three weeks of age. To obtain over-age nurses, frames containing brood were removed and replaced with empty comb two weeks after queen introduction to ensure that a new cohort of bees did not eclose before collections. For the SCC depicted in Fig. 2 and Supplementary Fig. 1, bees from four SDI source colonies were used to construct a single SCC. For each collection (Day 9 and Day 21), 10 bees of each source colony were collected for each behavioral task group. On Day 9, nurses were only able to be collected for two source colonies. For the SCC depicted in Supplementary Fig. 2, bees from a single SDI source colony were used, and groups of 10 bees were collected at each collection (Day 7 and Day 21) for each behavioral task group.

Double-cohort colonies (DCC) were established similarly to SCCs with a new, mated queen, 1 plastic honeycomb frame provisioned with honey and pollen paste, 1 empty plastic honeycomb frame, ~200 7-day old barcoded treatment bees (100 per treatment) and ~800 newly emerged “background” bees (mixed colony backgrounds), whose addition contributed to accelerated behavioral maturation of the older cohort [46, 48].

Big-back colonies

Big-back colonies were established as previously described [27, 46, 48] and kept outdoors. For each colony, ~800-900 newly eclosed bees from a single SDI source colony were used; half with

119 a paint mark on the thorax, and half with a plastic tag attached to the thorax (~3 mm diameter, ~1
120 mm thick; “big-back” bees). Four days later, ~400 newly eclosed bees from ~10 mixed source
121 colonies (headed by naturally mated queens) were introduced in order to increase the proportion
122 of precocious foragers in the first cohort [27, 46, 48]. The entrance to the hive was blocked by a
123 piece of Plexiglas with holes in it to prevent the big-back bees from leaving the hive, but to allow
124 paint-marked bees to come and go freely.

125 Bees were collected at 10 days of age: returning active foragers and nurses collected as
126 described above, and big-back/inactive forager bees collected as they were attempting to leave
127 the hive via the holes in the plastic, indicating they too were in the behavioral state of foraging. 10
128 bees per group were collected from all three colonies, with the exception that only seven nurses
129 from the focal group in colony 2 were found and collected.

130

131 **Single microbe inoculations**

132 To identify effects of individual microbes on behavior, we treated groups of bees with either
133 an inoculum of a microbe of interest or sterile food in order to produce bees whose microbiomes
134 were composed of a single honey bee-associated microbe (single microbe-inoculated) or no
135 honey bee-associated microbes (microbiome-depleted control), respectively.

136 To obtain bees that lack the dominant honey bee bacterial species in the gut, modified
137 methods from [39, 49] were used. Specifically, tan-colored pupae with dark eyes were gently
138 removed from brood frames from three or four source colonies per trial using ethanol sterilized
139 forceps, placed dorsal side down in sterilized 3D-printed dental grade resin pieces fitted into
140 sterilized Plexiglas cages, and kept in a 34°C incubator with 60-65% relative humidity for 2 days
141 [50]. Eclosed bees were moved in groups of 30-40 to sterilized Plexiglas boxes (10 x 10 x 7 cm)
142 [51] in a 32°C incubator at 60-65% relative humidity. Each box was provisioned with a ~1 g
143 sterilized pollen patty and 1.7 mL sterilized 25% sucrose with or without inoculum. An equal
144 number of bees from each source colony were added to each box, such that each treatment group

145 (microbiome-depleted or single microbe inoculated) had an equal number of bees of each colony
146 background.

147 For single microbe inoculations, single colonies of bacteria (*B. asteroides* DSM 20089, *L.*
148 *mellis* DSM 26255, *L. melliventris* DSM 26256, DSMZ Leibniz-Institut, Germany) were cultured in
149 de Mann, Rogosa, Sharpe (MRS) (*B. asteroides*) or MRS + 20g/L fructose (*Lactobacillus* species)
150 broth shaking under anaerobic conditions at 35°C for two nights, spun down (3000 rpm for 5 min)
151 and resuspended in 1x PBS to an OD of 1. Every day for 5 days, 50 μl of a fresh culture solution
152 was added to 1.7 mL sterile 25% sucrose water in a new inverted microtube for each treatment
153 box. For microbiome-depleted bees, 50 μl of sterile 1x PBS was added to 1.7 mL 25% sucrose
154 water in a new inverted microtube for each treatment box. Bees were then fed sterile 50% sucrose
155 water for 2 days following the 5-day inoculation period. Bees were kept in treatment boxes until 7
156 days old, when they were randomly chosen for 16S analysis or behavioral assays. Microbe
157 survival in 25% sucrose water was ensured by placing 50 μl of each microbial culture (OD ~1) in
158 1.5 mL 25% sucrose water overnight and then plating this solution using species specific culturing
159 conditions. 16S rRNA sequencing and qPCR validations of microbiome-depleted bees and single
160 microbe inoculated bees after the 7-day treatment period are found in Supplementary Fig. 3A-B.
161

162 **Gut microbiome DNA extraction, 16S rRNA sequencing and analysis**

163 Frozen honey bee guts were dissected under sterile conditions on dry ice. Combined mid- and
164 hind- guts of individual bees were homogenized by maceration with a disposable sterile pestle
165 (VWR, Radnor, PA, USA). The homogenate was added to a PowerSoil Pro Bead Solution tube
166 (Qiagen, Germantown, MD, USA), and DNA was extracted using a DNeasy PowerSoil Pro DNA
167 isolation kit (Qiagen), following manufacturer's instructions. The hypervariable V4 region of the
168 16S rRNA gene was amplified by PCR in triplicates, with a negative control (no DNA), using
169 Platinum Hot Start PCR Master Mix (Invitrogen, Waltham, MA, USA), primers and barcodes

170 designed in [52] with a final concentration 0.25 μM, and the following cycling conditions: 94° 3
171 min, 35x[94° 45s/50° 60s/72° 90s], 72° 10 min. Before sequencing, PCR products were visualized
172 on agarose gels to confirm negative controls did not have amplification, while all samples had
173 expected amplification. All samples met these criteria, indicating no contamination. Samples were
174 pooled based upon concentrations and sequenced on an Illumina MiSeq with 2x250bp paired-
175 end reads. The samples were split among three sequencing runs: #1: SCCs (Dec 2020), #2: Big-
176 back colonies (July 2021), #3: Typical colony nurses and foragers, single microbe inoculated bees
177 (Dec 2021). Reads from these sequencing runs can be found in NCBI's Sequence Read Archive
178 PRJNA958253. Sequences for typical colonies from naturally mated queens were retained from
179 [43].

180 Samples were demultiplexed using QIIME2, and paired end reads were truncated at the
181 first base with a quality score of <Q3 using DADA2 [53]. Paired-end reads were merged and
182 amplicon sequence variants (ASV) were identified using DADA2 [54]. Chimeric ASVs were
183 removed and the remaining ASVs were taxonomically classified using the BEExact database [55].
184 ASVs that were taxonomically identified as a bee specific genus by the BEExact database, but
185 were unclassified at the species level, were subsequently classified to species level if possible,
186 using NCBI megaBLAST. To do this, representative 16S V4 sequences for each ASV obtained
187 from the QIIME2 rep-seqs.qza object were BLASTed against the entire NCBI nucleotide
188 database, and unclassified ASVs were called as the first identified full species with the lowest E-
189 score, if query cover > 80% and percent identity > 92%.

190 For sequencing run #1, 3,220,034 (1,610,017 pairs) total sequence reads were obtained,
191 1,431,503 pairs were filtered, merged and identified as non-chimeric (90%), and 627 ASVs were
192 identified. For sequencing run #2, 945,310 (472,655 pairs) total sequence reads were obtained,
193 424,785 pairs were filtered, denoised, merged and identified as non-chimeric (90%), and 110
194 ASVs were identified. For sequencing run #3, 2,538,722 (1,269,361 pairs) total sequence reads

195 were obtained, 1,141,046 pairs were filtered, denoised, merged and identified as non-chimeric
196 (90%), and 832 ASVs were identified.

197 To estimate the abundance of individual honey bee-associated microbial species in each
198 sample, the read counts for all ASVs that matched the same species were combined. Honey bee-
199 associated species (*Bartonella apis*, *Bifidobacterium asteroides*, *Bifidobacterium coryneforme*
200 [syn. *Bifidobacterium indicum*), *Bombella apis* [previously *Parasaccharibacter apium*), *Frischella*
201 *perrara*, *Gilliamella apicola*, *Lactobacillus apis*, *Lactobacillus helsingborgensis*, *Lactobacillus*
202 *kullabergensis*, *Lactobacillus* [syn. *Bombilactobacillus*) *mellifer*, *Lactobacillus* [syn.
203 *Bombilactobacillus*) *mellis*, *Lactobacillus melliventris*, and *Snodgrassella alvi*) [36, 56, 57] were
204 retained. All other ASV reads, representing non-honey bee-associated microbes and each ASV
205 comprising 0-1% of total reads per sample, were combined and labeled as "other." The variation
206 between samples in the abundance of "other" microbes is similar to previously published datasets
207 [38, 44]. To calculate the proportion of each species in each sample, that species' read count was
208 divided by the total read count for each sample. Raw read counts and/or proportion data were
209 used in analyses as described below.

210 For beta-diversity analyses, we used clr-transformations of ASV raw read counts in
211 statistical tests and Aitchison distance for visualization [58, 59]. To estimate the differential relative
212 abundance of individual microbial species between samples ("relative abundance"), we used
213 Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC) [60] on raw read
214 counts and visualized using proportion data. To estimate absolute bacterial species abundances
215 in individual samples ("absolute abundance"), we quantified the bacterial load in each sample
216 using quantitative PCR (qPCR), as in [38, 61]. We calculated primer efficiencies ($E = 10^{(-1/slope)}$)
217 and normalized each sample's raw 16S gene copy numbers ($n = E^{(intercept-Ct)}$x(DNA extraction
218 elution volume/3)) to the *A. mellifera Actin* gene, as in [38, 61]. We then multiplied the normalized
219 number of 16S rRNA gene copies per sample by the relative proportion of each microbe in that
220 sample (as determined by 16S rRNA sequencing data) to obtain number of genome equivalents

221 of each microbe, as in [38]. Log_{10} values of genome equivalents, after replacing 0s with 1s, were
222 used in statistical analyses.

223 PCRs were performed in triplicate using 10 μl reactions of 0.5 μM primers targeting the
224 16S rRNA gene (F: AGGATTAGATACCCTGGTAGTCC, R: YCGTACTCCCCAGGCGG) or the
225 *A. mellifera Actin* gene (F: TGCCAACACTGTCCTTTCTG, R: AGAATTGACCCACCAATCCA)
226 [39] in 1X *Power*SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) with 3
227 μl of DNA. Each primer set included a no template control. Reactions were performed in 384 well
228 plates using a QuantStudio 6 Flex (Applied Biosystems) with the following cycling conditions: 50°
229 2 min, 95° 10 min, 40x[95° 15 sec/60° 1 min], melt curve: 95° 15 sec, 60° 1 min, 95° 15 sec.
230 Standard curves using serial dilutions of plasmids (TOPO pCR2.1 (Invitrogen)) containing the
231 target sequence (10^8-10^3 copies per 3 μl) were calculated from the molecular weight of the
232 plasmid and the DNA concentration of the plasmid.

233 We found an effect of colony replicate on gut microbial community structure across all
234 experiments, indicating that bees from different colonies have different gut microbial communities
235 and that early environment and/or genetics influences the composition of the gut microbiome.
236 This was the case when comparing bees from different typical colonies (Supplementary Fig. 1A-
237 B), among SCC bees originating from different colony sources (Supplementary Fig. 1C-D), and
238 across bees from different big-back colonies (Supplementary Fig. 1E). Colony differences in
239 honey bee microbiome composition have been previously reported [47].

240

241 **Foraging assays and analysis using barcoded bees**

242 To obtain comprehensive records of foraging activity, we used an automated behavioral tracking
243 system ("bCode") that uses a custom matrix barcode, enabling the unique identification of
244 individual bees [62]. After single microbe inoculations, 7-day old inoculated and microbiome-
245 depleted bees were cold-anaesthetized, a unique barcode was affixed to their thorax using super

246 glue, and a spot of colored paint associated with their treatment group was placed on their
247 abdomen. An equal number (~100) of bees from a single microbe inoculated group and a
248 corresponding microbiome-depleted group (same mix of colony backgrounds, same age) were
249 given barcodes from different sets, and were placed together in a DCC. Four colony replicates
250 per microbe treatment were performed. Two experimental days, corresponding with *L. mellis*
251 colony replicate 3 days 1-2 and *L. melliventris* colony replicate 2 days 1-2, experienced bad
252 weather, and thus no foraging occurred on those days.

253 Colonies were kept in a dark, temperature (32°C) and humidity (~50%) controlled building,
254 with access to the outside environment through a plastic tube. To monitor flight activity, an
255 entrance monitor with a video camera (Supplementary Methods) was attached to the hive
256 entrance, on the outside-facing end of this tube (Supplementary Fig. 4). The camera recorded
257 videos of bees entering and leaving the hive from 05:00 to 21:00 daily for a total of six days.
258 Barcodes in these videos were detected as in [62], and detections were filtered to remove tracking
259 errors and misidentifications. A mean of 97.82%, with a range between 96.24%-99.02%, of
260 detections were retained after these filtering steps. We then applied a flight activity detector [63,
261 64] (Supplementary Methods) to the remaining barcode detections to identify passes through the
262 entrance monitor. Computer code for this detector can be found at:
263 https://github.com/gernat/btools.

264 Passes through the entrance monitor determined by the flight activity detector were filtered
265 to remove unused barcodes, whose detections either occurred through detection error or due to
266 bee drift between experimental colonies. A mean of 99.19%, with a range of 95.4%-100%, of
267 events were retained after this filtering. Passes were then classified as “incoming,” “outgoing,” or
268 “other.” Since incoming passes had a lower error rate than outgoing passes (Supplementary
269 Table 8), incoming passes alone were used to denote a foraging trip. Individual foraging trips that
270 occurred within 5 min of each other were condensed into a single event. A bee’s first day of
271 foraging was defined as the first day on which it performed at least 4 foraging trips, with at least

272 50% of these trips occurring during peak foraging hours (11:00-15:00 CST), as per previous
273 studies [34, 65]. These criteria yield similar automated thresholds corresponding to human
274 observations of foraging behaviors [34]. All incoming passes from this first day of foraging were
275 counted as foraging trips, as were all subsequent incoming passes [34]. Likewise, an individual
276 bee was considered a "forager" from this day forward.

277 To quantify the degree of skew in foraging intensity among all workers, we calculated the
278 Gini coefficient [34, 66] for each experimental colony across all days and each experimental
279 colony on each day, since colony dynamics change with changes in weather and resource
280 availability, and thus likely changed throughout our studies. The values we obtained (Table 2) are
281 similar to those in previous studies [34, 66], indicating differences in foraging intensity between
282 individuals in each colony. To determine which bees performed the majority of the foraging for
283 each experimental colony on each day, we ranked individuals based on the proportion of total
284 foraging trips they performed for the colony on each day. A group of "elite foragers" was defined
285 as the subset of bees (variation, 5-40% of the foragers) performing $\geq$50% of the foraging trips for
286 each colony on each day [34, 66].

287 Although we monitored our experimental colonies for six days, we reasoned that any
288 behavioral effects seen after the first several days of monitoring may be confounded by changes
289 in colony dynamics due to forager mortality [67], "homogenization" of the microbiome among
290 colony members due to trophallaxis (sharing of liquids containing nutrients and signaling
291 molecules), or acquisition of honey bee-specific microbes from the environment. While we did not
292 test how long it takes for possible changes in microbiome due to these factors to occur, we chose
293 to focus on results for the first half (three days) of the experimental period below. Summary data
294 and statistics for the entire six-day period can be found in Supplementary Table 6.

295

296 **Statistical Analysis**

297 All statistical analyses were performed in R (v 4.2.0) [68]. For all analyses, assumptions (e.g.,
298 normality, homogeneity of variances) were checked before statistical analysis. The following
299 functions were used in the base statistics package: “shapiro.test” (Shapiro-Wilk Normality Test),
300 “fligner.test” (Fligner-Killeen Test of Homogeneity of Variances), “t.test” (Student’s t-test),
301 “wilcox.test” (Mann-Whitney Rank Sum Test), “aov” (ANOVA), “TukeyHSD” (Tukey’s HSD post
302 hoc test), “Kruskal.test” (Kruskal-Wallis test). Kruskal-Wallis post hoc was performed using
303 “kwAllPairsDunnTest”, PMCMRplus package [69]. Microbiome beta diversity was analyzed using
304 Permutation MANOVAs with 999 permutations (“adonis2”, vegan package [70]) on clr-
305 transformed (“transform”, microbiome package [71]) raw read counts, followed by Pairwise
306 Permutation MANOVAs with 999 permutations and FDR p-value adjustment (“pairwise.adonis”,
307 pairwiseAdonis package [72]), and visualized using Principal Components Analysis (“ordinate”,
308 phyloseq package [73]) with Aitchison distance (“distance”, phyloseq package [73]). The relative
309 abundance of individual microbes was analyzed using raw read counts and ANCOM-BC with FDR
310 adjustment (“ancombc2”, ANCOMBC package [60]) with task as a fixed effect and colony as
311 random effect. Relative abundances were visualized using stacked barplots (“ggplot”, ggplot2
312 package [74]). To compare the absolute abundance of individual microbes, Permutation ANOVAs
313 were used with 999 permutations (“perm.anova,” RVAideMemoire package [75]), and p-values
314 were adjusted for multiple comparisons (“p.adjust” with FDR adjustment). Absolute abundance
315 data were visualized using dotplots (“ggplot,” ggplot2 package [74]). Age at onset of foraging data
316 (using each individual bee’s first day of foraging) were analyzed using a Cox Proportional Hazards
317 model (“coxph,” survival package [76]), stratified by replicate [26, 65], and depicted as survival
318 plots using the cumulative proportion of bees from each treatment group that were identified as
319 foragers on each day. Proportions of foraging trips per individual were analyzed as generalized
320 linear mixed-effects models (“glmer,” lme4 package [77]) with log-normal distributions and
321 nAGQ=0, inoculation treatment and day as main factors, and replicate and individual as random
322 factors, followed by “Anova” (car package [78]) to determine main factor significance, and

323 “emmeans” (emmeans package [79]) with Tukey’s p-value adjustment for pairwise comparisons.
324 Two-way mixed ANOVAs with inoculation treatment as a between-subject factor and day as a
325 within-subjects factor, followed by pairwise one-way ANOVAs, were used in the remaining
326 behavioral analyses (“anova_test,” rstatix package [80]). Gini coefficients were calculated using
327 “Gini” (DescTools package [81]). All behavioral data, except age at onset of foraging, were
328 visualized using boxplots (“ggplot,” ggplot2 package [74]).
329

330 # Results

331 ## Nurses and foragers differ in gut microbial community composition

332 To begin to investigate whether gut microbes influence honey bee division of labor, we compared
333 gut microbial communities in nurse and forager bees, which represent two of the canonical
334 behavioral task groups in honey bee division of labor, as well as two discrete time points in
335 behavioral maturation [25]. Nurses and foragers differ markedly in physiology [28, 82–87],
336 neuroanatomy [88], neurochemistry [33, 89, 90], gene expression [29, 91] and gene regulation
337 [65, 92–94]. Therefore, we reasoned that testing for consistent differences in gut microbial
338 community between these two groups would be a powerful way to test for associations between
339 gut microbiome variation and division of labor.

340 We performed 16S rRNA sequencing on gut samples from nurses and foragers from three
341 unrelated honey bee colonies and reanalyzed, using updated methods [53, 54], a similar dataset
342 from a previously published study that used a different 16S sequencing method [43]. We found
343 that nurses and foragers differed significantly in gut microbial community structure in both the
344 previously published dataset (Fig. 1A) and our new dataset (Fig. 1B), indicating robust differences
345 associated with division of labor and independent of sequencing method.

346 We next sought to identify individual microbes that differed in abundance between nurse
347 and forager bees, in order to identify candidate microbes specifically associated with division of

348 labor. Since microbes can vary between individuals in relative and/or absolute abundance, two
349 measures that are not necessarily linked and whose relative effects on host behavior are currently
350 unknown [61], we used both of these abundance measures to get a well-rounded sense of
351 individual microbes that are associated with honey bee division of labor. We found that individual
352 gut microbes significantly differed in both relative and absolute abundance between nurses and
353 foragers (Fig. 1C-D, Supplementary Table 1, Table 1). These results indicate that nurse and
354 forager bees differ significantly in their gut microbial communities. They also identify candidate
355 microbes that may be associated with division of labor.
356

357 **Differences in gut microbial community composition between nurses and foragers**
358 **are dependent upon differences in behavioral state, not age**

359 Under typical colony conditions, nurses and foragers differ in chronological age. However, honey
360 bee division of labor is flexible and responsive to changing colony needs. For example, if a colony
361 experiences a shortage of older bees, some younger individuals will begin to forage at an early
362 age, while a shortage of younger bees will cause bees to continue to act as nurse bees despite
363 advancing chronological age [26–30]. To determine whether the gut microbial differences
364 between nurses and foragers depend on differences in behavioral state and/or age, we exploited
365 this adaptive plasticity to create SCCs, a well-established experimental approach that separates
366 these two factors [27, 28, 46]. In two SCC replicates, we compared gut microbiomes between
367 age-matched nurse and forager bees when they were about one week of age (representing
368 typical-age nurses and precocious foragers) and three weeks of age (representing over-age
369 nurses and typical-age foragers).

370 Age-matched typical-age nurses and precocious foragers did not significantly differ in gut
371 microbial community structure (Fig. 2A, Supplementary Fig. 2A), while age-matched over-age
372 nurses and typical-age foragers did (Fig. 2B, Supplementary Fig. 2B). Similar results were

373 obtained for the abundance of individual microbial species: no microbes consistently differed in
374 abundance between typical-age nurses and precocious foragers across the two SCC replicates
375 (Fig. 2C, Fig. 2E, Supplementary Fig. 2C, Supplementary Fig. 2E, Supplementary Table 2,
376 Supplementary Table 3, Table 1), while two microbes consistently differed in relative abundance
377 and three microbes consistently differed in absolute abundance between over-age nurses and
378 typical-age foragers across the two SCC replicates (Fig. 2D, Fig. 2F, Supplementary Fig. 2D,
379 Supplementary Fig. 2F, Supplementary Table 2, Supplementary Table 3, Table 1). These findings
380 indicate that age-dependent differences in behavioral state are associated with differences in gut
381 microbial communities between nurses and foragers.

382 Together, our typical colony and three-week old SCC data indicate that *Lactobacillus*
383 *mellis* and *Lactobacillus melliventris* were consistently (i.e. identified in three or more analyses
384 across the two abundance measures) more abundant in nurses relative to foragers (Table 1),
385 while *Bartonella apis* and *Snodgrassella alvi* were consistently less abundant in nurses relative to
386 foragers (Table 1). In SCCs alone, *Bifidobacterium asteroides* showed consistent lower relative
387 abundance in over-age nurses compared to typical-age foragers (Table 1) and *Gilliamella apicola*
388 (Table 1) showed consistent higher absolute abundance in over-age nurses compared to typical-
389 age foragers. Such robust associations highlight these six microbes as candidate microbes whose
390 abundances have causal effects on division of labor in honey bees.

391 The observed age-dependent microbiome differences between nurses and foragers
392 suggest that associations between gut microbiome and behavioral state depend on worker
393 experience. Since SCCs are composed of bees of a single age cohort, three-week old SCCs are
394 composed of bees that have been performing their respective tasks for prolonged periods (1-2
395 weeks longer) compared to one-week old SCCs. Furthermore, the lack of gut microbial community
396 differences between one-week-old SCC nurses and foragers suggests that differences in
397 behavioral maturation rate are not associated with differences in gut microbial community
398 composition. Therefore, we hypothesized that task experience plays an important role in gut

399 microbial community composition. In the next section, we tested this by focusing on foraging
400 experience since it is known to have strong effects on behavior, brain chemistry, brain structure,
401 and brain gene expression [66, 89, 95, 96].
402

403 **Foraging experience influences gut microbial community structure, while**
404 **behavioral state influences the abundance of individual microbes**

405 In order to determine the causal effects of behavioral state and foraging experience on honey bee
406 gut microbiome composition, we controlled for behavioral state while manipulating foraging
407 experience. To do this, we created "big-back colonies" in which some foragers were freely able
408 to leave the colony ("active foragers"), while other bees, who appeared at the entrance and
409 showed an inclination to forage, were prevented from leaving due to the presence of a thick plastic
410 tag on their backs ("inactive foragers") [46, 48]. We were thus able to compare bees of the same
411 age and behavioral state that differed in foraging experience.

412 We found that nurses and inactive foragers did not differ in gut microbial community
413 structure, while active foragers and inactive foragers did (Fig. 3A). This finding indicates that
414 foraging experience plays an important role in defining differences in gut microbial community
415 structure. In contrast to these community structure results, which take below-species-level
416 diversity into account [54], we did not find any microbial species that differed in relative or absolute
417 abundance between age-matched active and inactive foragers (Fig. 3B-C, Supplementary Table
418 4). This suggests that the abundance of individual microbes is defined by behavioral state, and
419 not experience foraging outside of the hive. Although these results are seemingly contradictory to
420 our SCC data, which indicate that differences in the abundance of individual gut microbes may
421 be dependent upon worker experience (Fig. 2D,F, S2 Fig. D,F), it is possible that honey bee gut
422 microbes and foraging behaviors interact through a feedback model. For example, gut microbes
423 may change in abundance due to foraging experience, possibly incrementally (and thus

424 indiscernibly) at first and building with more experience, and this change in microbiome may then
425 feed back to influence host foraging behaviors. In the next section, we test one part of this model
426 by assessing the effects of inoculation with individual microbes on host foraging behavior.
427

428 Inoculation with individual microbes affects group- and individual-level foraging 429 intensity

430 To determine whether variations in gut microbiome composition play a causal role in
431 honey bee division of labor, we performed single microbe inoculations with three out of the six
432 individual microbes robustly associated with division of labor as described above: *B. asteroides*,
433 *L. mellis* and *L. melliventris*. Although it is a simplification of the natural microbiome, we chose to
434 test the effects of each microbe in isolation in order to increase experimental power. In addition
435 to the strong patterns of association reported above, we chose these three microbes due to *B.*
436 *asteroides*' known effects on host physiology with links to behavior and the *Lactobacillus* species'
437 high metabolic output [39], since gut microbial metabolites likely contribute to the microbiota-gut-
438 brain axis [7]. Using an automated tracking system [62], we determined the effect of inoculation
439 with these three microbes on two measures of foraging behavior important in division of labor in
440 honey bees: rate of behavioral maturation (age at onset of foraging) and foraging intensity
441 (amount of foraging trips performed by a group or individual) [35].

442 *B. asteroides* inoculated bees did not differ from microbiome-depleted bees in behavioral
443 maturation rate (Fig. 4A) across experimental replicates, but did differ in foraging intensity.
444 Overall, *B. asteroides* inoculated bees performed the majority of the foraging trips for the colony
445 on the first day after colony formation (Fig. 4B, Supplementary Table 5). These group-level effects
446 were not due to a difference in number of foragers between inoculated and microbiome-depleted
447 bees (Fig. 4C, Supplementary Table 5). Rather they were due to a difference in individual-level
448 foraging intensity, as individual *B. asteroides* inoculated foragers performed a majority of the

449 foraging trips for the colony on the first day after colony formation (Fig. 4D, Supplementary Table
450 5). Likewise, *B. asteroides* experimental colonies displayed a degree of skew in foraging intensity
451 among individuals (Table 2) and *B. asteroides* inoculated bees represented a higher proportion
452 of "elite forager" bees, i.e., a small subset of foragers that performed ≥50% of the colony's foraging
453 trips [34, 66], relative to microbiome-depleted bees on the first two days after colony formation
454 (Fig. 4E, Supplementary Table 5).

455 Similar to *B. asteroides* inoculated bees, *L. mellis* inoculated bees did not differ from
456 microbiome-depleted bees in behavioral maturation rate (Fig. 5A), but did differ in foraging
457 intensity. However, *L. mellis* inoculation caused an opposite effect on foraging intensity compared
458 to *B. asteroides* inoculation. Overall, *L. mellis* inoculated bees performed the minority of foraging
459 trips for the colony on the third day after colony formation (Fig. 5B, Supplementary Table 5).
460 Again, these group-level effects were not due to a difference in number of foragers between
461 inoculated and microbiome-depleted bees (Fig. 5C, Supplementary Table 5). Rather they were
462 due to a difference in individual-level foraging intensity, as *L. mellis* inoculated bees performed a
463 minority of foraging trips for the colony on the first day after colony formation (Fig. 5D,
464 Supplementary Table 5). Additionally, they could also be due to skew in foraging intensity
465 between individual foragers (Table 2), as *L. mellis* inoculated bees displayed a trend (with
466 marginal significance on the second day, Supplementary Table 5) of representing a lower
467 proportion of elite foragers than microbiome-depleted bees on all three days after colony
468 formation (Fig. 5E, Supplementary Table 5).

469 *L. melliventris* inoculated bees showed similar results to *L. mellis* inoculated bees. *L.*
470 *melliventris* inoculated bees did not differ from microbiome-depleted bees in behavioral
471 maturation rate (Fig. 6A), but did differ in foraging intensity. Overall, *L. melliventris* inoculated
472 bees performed the minority of foraging trips for the colony on the second day after colony
473 formation (Fig. 6B, Supplementary Table 5). Yet again, this group-level effect was not due to a
474 difference in number of foragers between inoculated and microbiome-depleted bees (Fig. 6C,

475 Supplementary Table 5). Rather, it was due to a difference in individual-level foraging intensity,
476 as individual *L. melliventris* inoculated foragers performed a minority of the foraging trips for the
477 colony on the second day after colony formation (Fig. 6D, Supplementary Table 5). Likewise, *L.*
478 *melliventris* colonies displayed a degree of skew in foraging intensity between individual foragers
479 (Table 2), and *L. melliventris* bees represented a lower proportion of elite foragers than
480 microbiome-depleted bees on the second day after colony formation (Fig. 6E, Supplementary
481 Table 5).

482 Overall, these results indicate that inoculation with individual gut microbes is sufficient to
483 cause increases or decreases in foraging intensity between worker bees at both the group- and
484 individual- level.

485

486 **Discussion**

487 Here, we show that natural variation in the gut microbiome can induce variation in a
488 naturally occurring host behavior. Specifically, our results indicate that differences in honey bee
489 gut microbial communities influence honey bee division of labor by affecting foraging intensity,
490 but not rate of behavioral maturation. This conclusion is supported by both microbe abundance
491 data and behavioral data: *B. asteroides* is found in higher abundance in foragers compared to
492 nurses, and inoculation with this species caused bees to have increased foraging intensity.
493 Similarly, *L. mellis* and *L. melliventris* are found in lower in abundance in foragers compared to
494 nurses, and inoculation with either of these species caused bees to have decreased foraging
495 intensity.

496 From a technical perspective, the observation that single microbe inoculations can cause
497 an increase or a decrease in foraging intensity dependent upon the microbe indicates that it is
498 unlikely that the results reported here are due to an artifact of the microbiological or behavioral
499 methods we used. In addition, the congruence of our microbe abundance and behavioral data
500 indicate that single microbe inoculations, which do not represent natural microbiome variations,

501 provide a useful method for functional analysis. Together, our results suggest that naturally
502 occurring increases in *B. asteroides*, which occur simultaneously with naturally occurring
503 decreases in *L. mellis* and *L. melliventris,* act synergistically to increase the intensity of foraging
504 behavior in individual bees. Honey bees, with a complex eusocial life history, live in large perennial
505 colonies and therefore collect large amounts of nectar and pollen to ensure colony survival during
506 periods when floral resources are not available. Thus, the influence of these three microbes on
507 honey bee foraging intensity indicates that host-microbe interactions likely help sustain the
508 perennial lifestyle of this eusocial insect.

509 While previous studies report correlational differences in gut microbiome associated with
510 honey bee behavioral task [42, 44, 61] and behavioral maturation [45], here, we provide evidence
511 that gut microbiomes play a causal role in defining behavioral differences between honey bees.
512 Given the age-related nature of honey bee division of labor, this research also shows that gut
513 microbiomes can drive adaptive variations in host behavioral phenotypes over the lifetime of
514 individual animals.

515 Our findings suggest that positive feedback interactions cause adaptive changes in both
516 gut microbiome composition and host behavior, such that changes in gut microbe abundance and
517 host behavior interact to strengthen one another and increase foraging intensity. Modeling
518 interactions in this way can provide the basis for future studies that probe the neural and metabolic
519 mechanisms by which *B. asteroides*, *L. mellis* and *L. melliventris* affect host behavior. Overall,
520 our findings highlight the importance of feedback mechanisms in microbiota-gut-brain
521 interactions, provide evidence that variation in gut microbiomes can drive variation in naturally
522 occurring behaviors, and support gut microbiomes as important drivers of host phenotypic
523 variation and plasticity.

524 Previous studies have shown that gut microbes may influence host behavior through a
525 variety of mechanisms including production of substances that cause changes in host brain gene
526 expression or host production of neurotransmitters and hormones [7]; these general mechanisms

are also associated with the regulation of honey bee division of labor [33, 35, 65, 87–90, 93, 95, 97, 98]. Likewise, previous studies indicate that some host factors associated with honey bee division of labor, such as social interactions and diet [99, 100], may also modulate gut microbiome composition [7]. Therefore, future research will work to identify specific mechanisms by which honey bees and their gut microbiomes interact. Overall, studies using the honey bee as a model have the potential to further elucidate the microbiota-gut-brain axis, and achieve a more comprehensive understanding of interkingdom interactions.

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Competing Interests

The authors declare no competing interests.

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552 10.17632/f2s47y3nhn). Computer code for the barcode detector is available at
553 https://github.com/gernat/btools.
554

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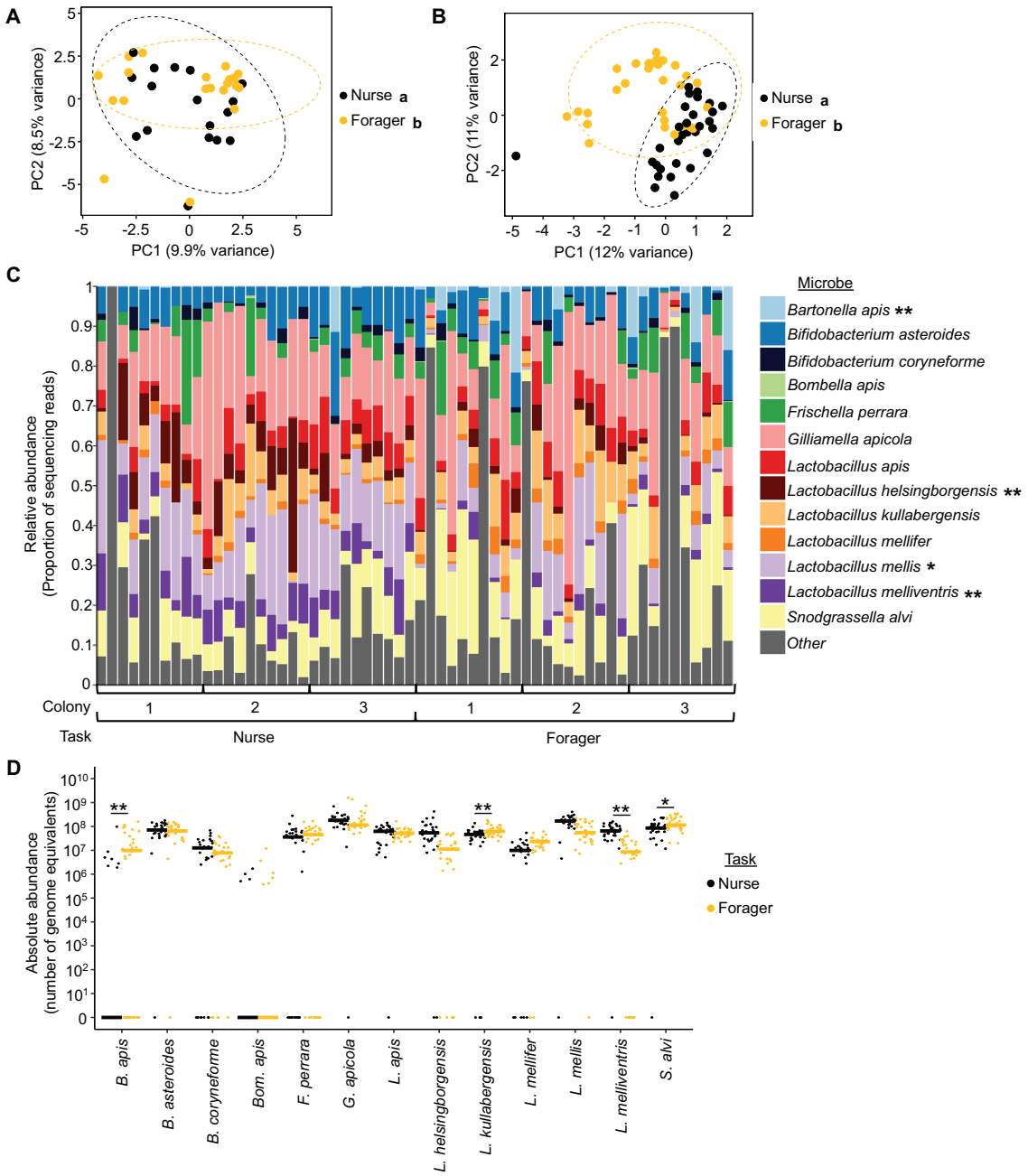
802

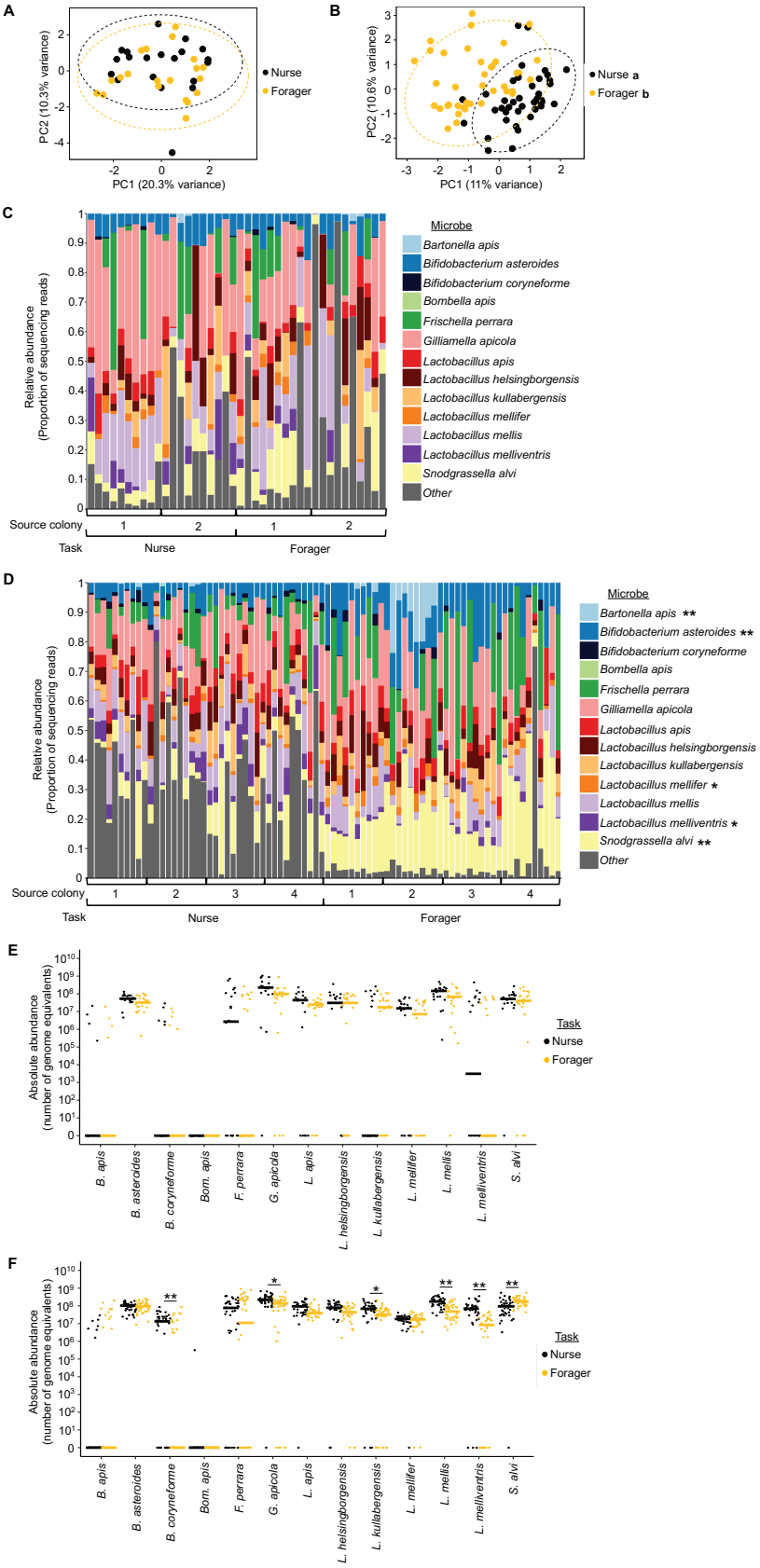
803 # Figures



804

805 **Figure 1. Nurses and foragers from typical honey bee colonies differ in gut microbial**
806 **community. (A-B)** Nurses and foragers differed in overall gut microbial community structure. (A)
807 Reanalyzed data from Kapheim et al 2015. Two-way Permutation MANOVA using Aitchison
808 Distance, Task: $F_{1,38}$ = 1.51, R^2 = 0.036, p = 0.018; Colony: $F_{4,38}$ = 1.72, R^2 = 0.165, p = 0.001,

809 Task*Colony: $F_{2,38}$ = 1.17, R^2 = 0.056, p = 0.105. n = 5 colonies. (B) New data. Two-way
810 Permutation MANOVA using Aitchison Distance, Task: $F_{1,59}$ = 4.52, R^2 = 0.069, p = 0.001; Colony:
811 $F_{2,59}$ = 2.35, R^2 = 0.072, p = 0.001, Task*Colony: $F_{2,59}$ = 1.22, R^2 = 0.037, p = 0.114. n = 10
812 bees/colony, 3 colonies. Depicted as Principal Components Analysis (PCA) plots. Lowercase
813 letters in legends denote statistically significant groups. **(C)** Nurses and foragers differed in
814 relative abundance of four individual microbial species (new data only). Depicted as stacked bar
815 plots, with each bar representing a single bee's microbiome. Asterisks in legend: *, p≤0.05, **,
816 p≤0.01, ANCOM-BC between nurses and foragers. See Supplementary Table 1 for all p-values.
817 **(D)** Nurses and foragers differed in absolute abundance of four individual microbial species (new
818 data only). Depicted as dot plots with all data points plotted, line represents median, n = 10
819 bees/colony, 3 colonies. *, p≤0.05, **, p≤0.01, Permutation ANOVA Test between nurses and
820 foragers. See Supplementary Table 1 for all p-values.
821


A
PC2 (10.3% variance)
PC1 (20.3% variance)
Nurse
Forager
B
PC2 (10.6% variance)
PC1 (11% variance)
Nurse a
Forager b
C
Relative abundance
(Proportion of sequencing reads)
Microbe
Bartonella apis
Bifidobacterium asteroides
Bifidobacterium coryneforme
Bombella apis
Frischella perrara
Gilliamella apicola
Lactobacillus apis
Lactobacillus helsingborgensis
Lactobacillus kullabergensis
Lactobacillus mellifer
Lactobacillus mellis
Lactobacillus melliventris
Snodgrassella alvi
Other
Source colony
Task
Nurse
Forager
D
Relative abundance
(Proportion of sequencing reads)
Microbe
Bartonella apis **
Bifidobacterium asteroides **
Bifidobacterium coryneforme
Bombella apis
Frischella perrara
Gilliamella apicola
Lactobacillus apis
Lactobacillus helsingborgensis
Lactobacillus kullabergensis
Lactobacillus mellifer *
Lactobacillus mellis
Lactobacillus melliventris *
Snodgrassella alvi **
Other
Source colony
Task
Nurse
Forager
E
Absolute abundance
(number of genome equivalents)
Task
Nurse
Forager
B. apis
B. asteroides
B. coryneforme
Bom. apis
F. perrara
G. apicola
L. apis
L. helsingborgensis
L. kullabergensis
L. mellifer
L. mellis
L. melliventris
S. alvi
F
Absolute abundance
(number of genome equivalents)
Task
Nurse
Forager
B. apis
B. asteroides
B. coryneforme
Bom. apis
F. perrara
G. apicola
L. apis
L. helsingborgensis
L. kullabergensis
L. mellifer
L. mellis
L. melliventris
S. alvi

822

823 **Figure 2. Age-matched nurses and foragers differ in gut microbial community. (A-B)** Age-
824 matched typical-age nurses and precocious foragers from a single cohort colony did not differ in
825 gut microbial community structure at about one week of age (A), but age-matched over-age
826 nurses and typical-age foragers significantly differed in gut microbial community structure at three
827 weeks of age (B). 1 week: Two-way Permutation MANOVA using Aitchison Distance, Task: $F_{1,39}$
828 = 0.93, R^2 = 0.023, p = 0.523; Source colony: $F_{3,39}$ = 2.66, R^2 = 0.065, p = 0.001, Task*Colony:
829 $F_{1,39}$ = 1.17, R^2 = 0.028, p = 0.259. n = 10 bees/source colony, 2 source colonies. 3 weeks: Two-
830 way Permutation MANOVA using Aitchison Distance, Task: $F_{1,79}$ = 6.48, R^2 = 0.073, p = 0.001;
831 Source colony: $F_{3,79}$ = 1.89, R^2 = 0.065, p = 0.001, Task*Colony: $F_{3,79}$ = 1.22, R^2 = 0.042, p =
832 0.094. n = 10 bees/source colony, 4 source colonies. Depicted as PCA plots. Lowercase letters
833 in legends denote statistically significant groups. **(C-D)** Age-matched nurses and foragers from a
834 single cohort colony did not differ in relative abundance of individual microbial species at one
835 week of age (C), but did differ in relative abundance of five individual microbial species at three
836 weeks of age (D). Depicted as stacked bar plots, with each bar representing a single bee's
837 microbiome. Asterisks in legend: *, $p \leq 0.05$, **, $p \leq 0.01$, ANCOM-BC between nurses and foragers.
838 See Supplementary Table 2 for all p-values. **(E-F)** Age-matched nurses and foragers from a single
839 cohort colony did not differ in absolute abundance of individual microbial species at one week of
840 age (E), but did differ in absolute abundance of six microbial species at three weeks of age (F).
841 Depicted as dot plots with all data points plotted, line represents median, n = 10 bees/source
842 colony, 2 source colonies (1 week) or 4 source colonies (3 weeks). *, $p \leq 0.05$, **, $p \leq 0.01$,
843 Permutation ANOVA Test between nurses and foragers. See Supplementary Table 2 for all p-
844 values.
845

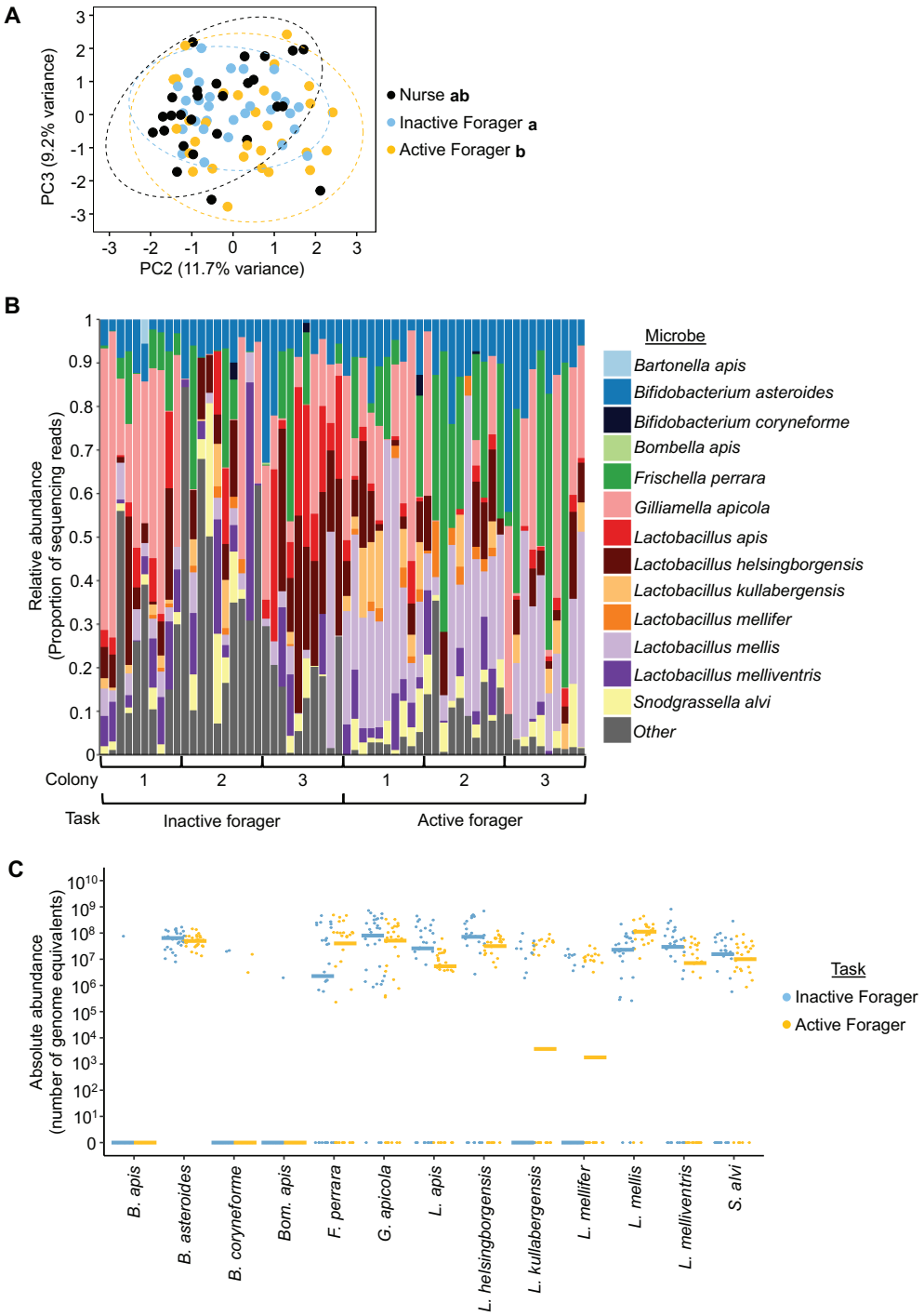


Figure 3. Age-matched inactive and active foragers differ in gut microbial community structure, but not the abundance of individual microbes. (A) Age-matched nurses and inactive foragers were similar in gut microbial community structure, while age-matched active and

850 inactive foragers differed in gut microbial community structure. Two-way Permutation MANOVA
851 using Aitchison Distance, Task: $F_{2,86}$ = 1.82, R^2 = 0.038, p = 0.011; Colony: $F_{2,86}$ = 5.90, R^2 =
852 0.122, p = 0.001, Task*Colony: $F_{4,86}$ = 0.798, R^2 = 0.033, p = 0.896. n = 10 bees/colony, 3
853 colonies. Depicted as PCA plot. Lowercase letters in legends denote statistically significant
854 groups as determined by Pairwise Permutation MANOVA. **(B)** Age-matched inactive and active
855 foragers did not differ in the relative abundance of individual microbial species (B). Depicted as
856 stacked bar plots, with each bar representing a single bee's microbiome. Asterisks in legend: *,
857 p≤0.05, **, p≤0.01, ANCOM-BC between inactive and active foragers. See Supplementary Table
858 4 for all p-values. **(C)** Age-matched inactive and active foragers did not differ in absolute
859 abundance of individual microbial species. Depicted as dot plots with all data points plotted, line
860 represents median, n = 10 bees/colony, 3 colonies. *, p≤0.05, **, p≤0.01, Permutation ANOVA
861 Test between inactive and active foragers. See Supplementary Table 4 for all p-values.
862

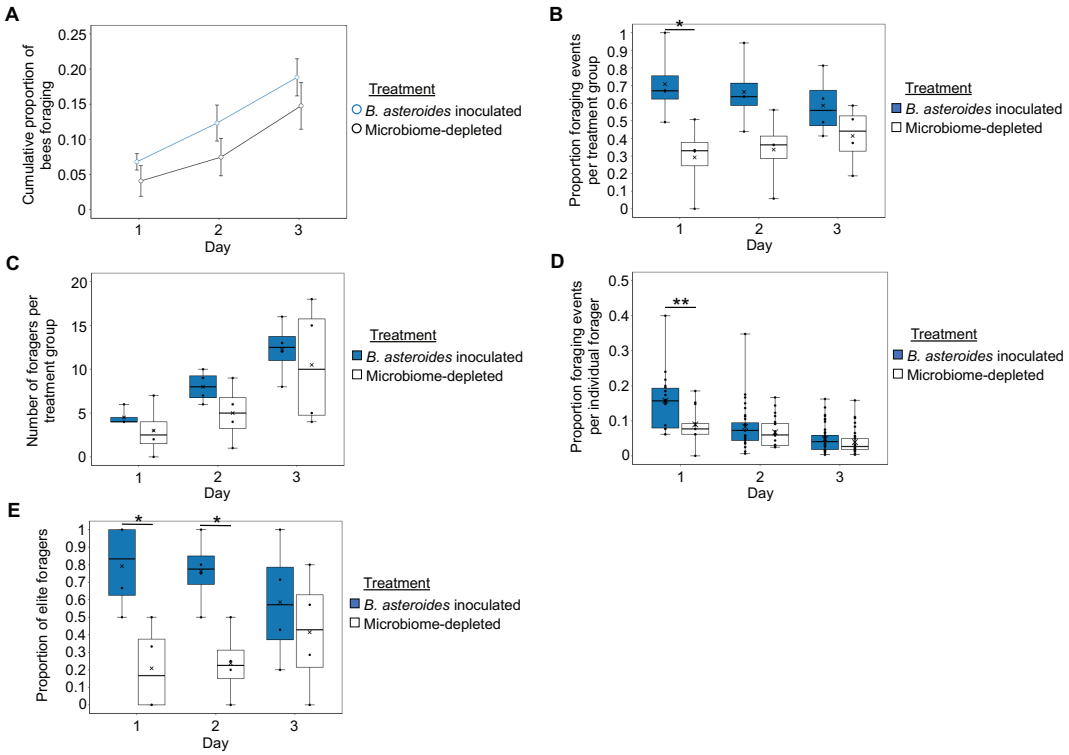


Figure 4. Bees inoculated with *B. asteroides* do not differ from microbiome-depleted bees in behavioral maturation (age at onset of foraging), but do differ in foraging intensity. (A) *B. asteroides* inoculated bees did not differ from microbiome-depleted bees in age at onset of foraging. Cox Proportional Hazards, z = -0.935, p = 0.350. **(B)** *B. asteroides* inoculated foragers, as a group, performed the majority of foraging trips for the colony the first day after experimental colony construction. Two-way Mixed ANOVA, Treatment: $F_{1,6}$ = 5.86, p = 0.050, Day: $F_{2,12}$ = 0, p = 1, Treatment*Day: $F_{2,12}$ = 2.64, p = 0.112. See Supplementary Table 5 for Pairwise comparisons. **(C)** *B. asteroides* inoculated bees represented a similar number of foragers as microbiome-depleted bees on all three days after experimental colony construction. Two-way Mixed ANOVA, Treatment: $F_{1,6}$ = 1.02, p = 0.351, Day: $F_{1.07,6.42}$ = 14.01, p = 0.008, Treatment*Day: $F_{1.07,6.42}$ = 0.15, p = 0.726. See Supplementary Table 5 for Pairwise comparisons. **(D)** Individual *B. asteroides* inoculated foragers performed a majority of foraging trips for the colony the first day after experimental colony construction. Generalized Linear Mixed Effects Model, Treatment: χ^2 =

877 3.44, $p = 0.064$, Day: $\chi^2 = 92.39$, $p < 0.001$, Treatment*Day: $\chi^2 = 8.03$, $p = 0.018$. See
878 Supplementary Table 5 for Pairwise comparisons. **(E)** *B. asteroides* inoculated bees represented
879 a higher proportion of elite foragers than microbiome-depleted bees on the first two days after
880 experimental colony construction. Two-way Mixed ANOVA, Treatment: $F_{1,6} = 9.597$, $p = 0.021$,
881 Day: $F_{2,12} = 0$, $p = 1$, Treatment*Day: $F_{2,12} = 1.78$, $p = 0.210$. See Supplementary Table 5 for
882 Pairwise comparisons. (A) depicted as survival plot. All other data depicted as box plots with data
883 points plotted, line represents median, x represents mean, and whiskers represent the minimum
884 and maximum values, n = 4 colonies. Asterisks used to denote comparisons between treatment
885 groups on each day only: *, $p \leq 0.05$, **, $p \leq 0.001$.
886
887

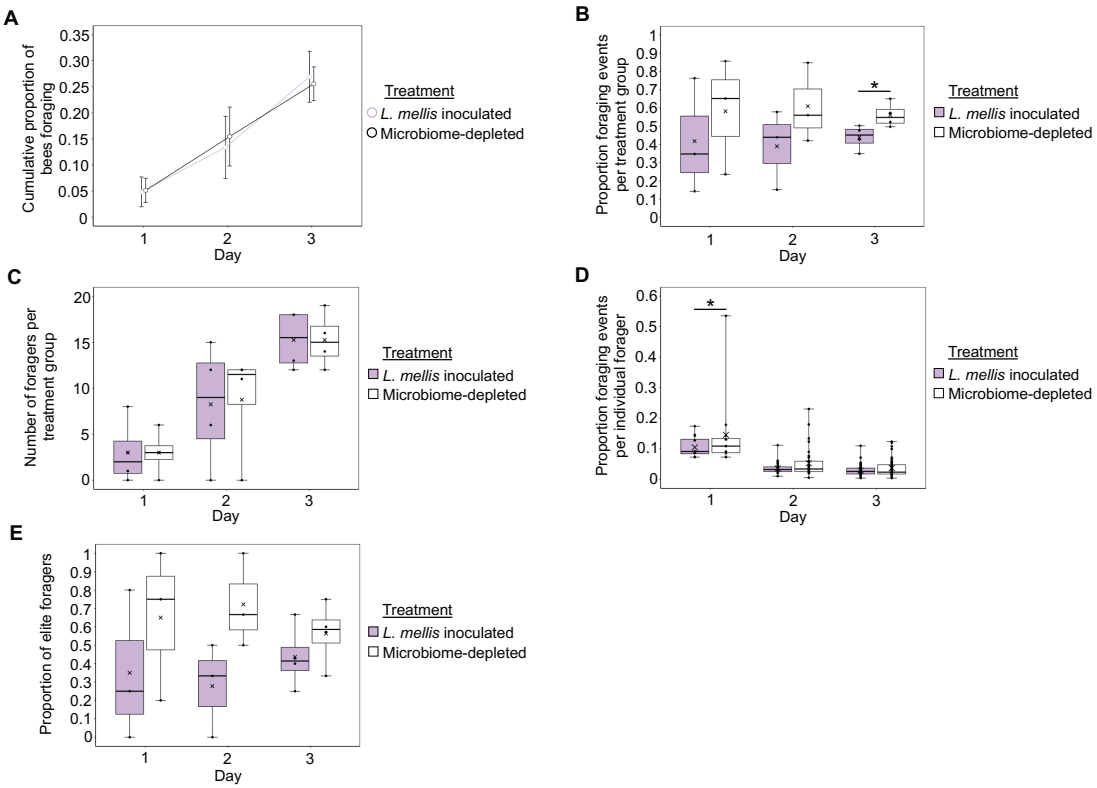


Figure 5. Bees inoculated with *L. mellis* do not differ from microbiome-depleted bees in behavioral maturation (age at onset of foraging), but do differ in foraging intensity. (A) *L. mellis* inoculated bees did not differ from microbiome-depleted bees in age at onset of foraging. Cox Proportional Hazards, z = 0.118, p = 0.906. (**B)** *L. mellis* inoculated foragers, as a group, performed the minority of foraging trips for the colony the third day after experimental colony construction. Two-way Mixed ANOVA, Treatment: $F_{1,4}$ = 1.17, p = 0.340, Day: $F_{2,8}$ = 0, p = 1, Treatment*Day: $F_{2,8}$ = 0.30, p = 0.750. See Supplementary Table 5 for Pairwise comparisons. **(C)** *L. mellis* inoculated bees represented a similar number of foragers as microbiome-depleted bees on all three days after experimental colony construction. Two-way Mixed ANOVA, Treatment: $F_{1,6}$ = 0.01, p = 0.946, Day: $F_{2,12}$ = 24.20, p < 0.001, Treatment*Day: $F_{2,12}$ = 0.01, p = 0.987. See Supplementary Table 5 for Pairwise comparisons. **(D)** Individual *L. mellis* inoculated foragers performed a minority of foraging trips for the colony on the first day after experimental colony construction. Generalized Linear Mixed Effects Model, Treatment: χ^2 = 4.56, p = 0.032, Day: χ^2 =

902 126.64, $p < 0.001$, Treatment*Day: $\chi^2 = 1.926$, $p = 0.382$, See Supplementary Table 5 for Pairwise
903 comparisons. **(E)** *L. mellis* inoculated bees represented a similar proportion, though with a trend
904 of lower proportion, of elite foragers as microbiome-depleted bees all three days after
905 experimental colony construction. Two-way Mixed ANOVA, Treatment: $F_{1,4} = 4.18$ $p = 0.111$, Day:
906 $F_{2,8} = 0$, $p = 1$, Treatment*Day: $F_{2,8} = 0.813$, $p = 0.477$. See Supplementary Table 5 for Pairwise
907 comparisons. (A) depicted as survival plot. All other data depicted as box plots with data points
908 plotted, line represents median, x represents mean, and whiskers represent the minimum and
909 maximum values, n = 4 colonies. Asterisks used to denote comparisons between treatment
910 groups on each day only: *, $p \leq 0.05$, **, $p \leq 0.001$.
911

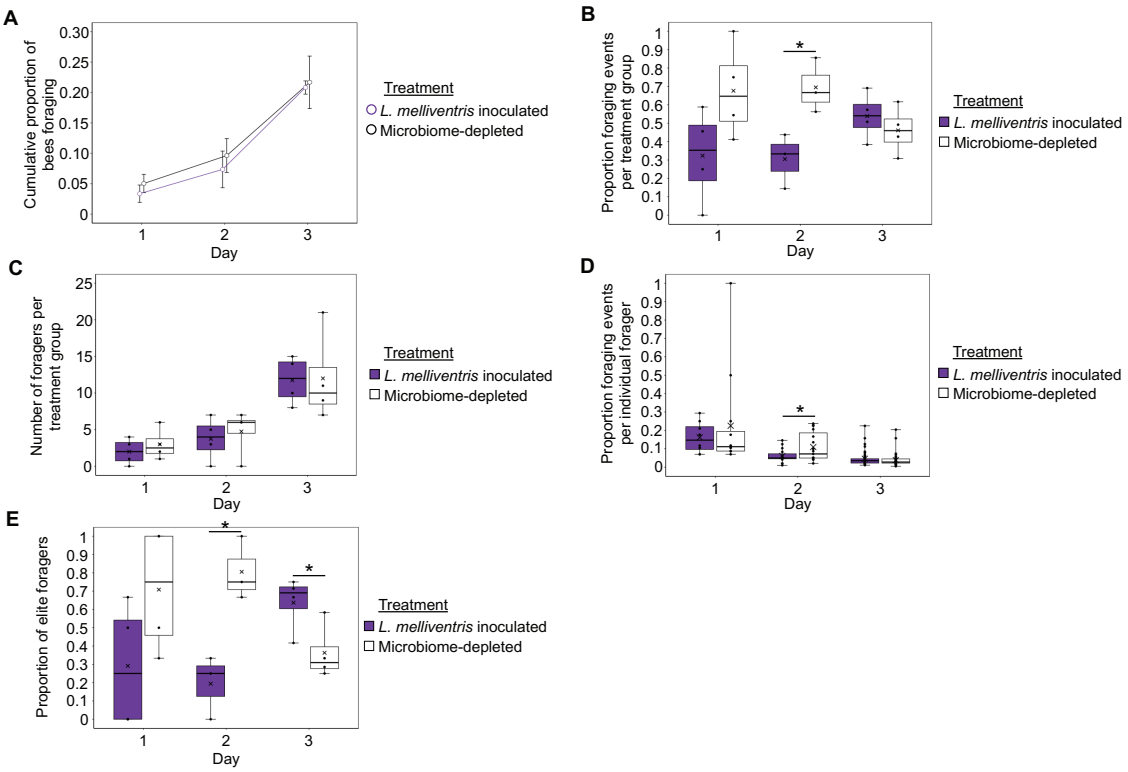


Figure 6. Bees inoculated with *L. melliventris* do not differ from microbiome-depleted bees in behavioral maturation (age at onset of foraging), but do differ in foraging intensity. (A) *L. melliventris* inoculated bees did not differ from microbiome-depleted bees in age at onset of foraging. Cox Proportional Hazards, z = -0.383, p = 0.702. (**B**) *L. melliventris* inoculated foragers, as a group, performed the minority of foraging trips for the colony the second day after experimental colony construction. Two-way Mixed ANOVA, Treatment: $F_{1,4}$ = 5.76, p = 0.074, Day: $F_{2,8}$ = 0, p = 1, Treatment*Day: $F_{2,8}$ = 3.09, p = 0.101. See Supplementary Table 5 for Pairwise comparisons. **(C)** *L. melliventris* inoculated bees represented a similar number of foragers as microbiome-depleted bees on all three days after experimental colony construction. Two-way Mixed ANOVA, Treatment: $F_{1,6}$ = 0.163, p = 0.700, Day: $F_{1.13,6.76}$ = 22.43, p = 0.002, Treatment*Day: $F_{1.13,6.76}$ = 0.04, p = 0.869. See Supplementary Table 5 for Pairwise comparisons. **(D)** Individual *L. melliventris* inoculated foragers performed a minority of foraging trips for the colony the second day after experimental colony construction. Generalized Linear Mixed Effects

926 Model, Treatment: χ^2 = 1.14, p = 0.286, Day: χ^2 = 86.85, p < 0.001, Treatment*Day: χ^2 = 5.79, p
927 = 0.055, See Supplementary Table 5 for Pairwise comparisons. **(E)** *L. melliventris* inoculated bees
928 represented a lower proportion of elite foragers than microbiome-depleted bees on the second
929 day after experimental colony construction, but a higher proportion on the third day after
930 experimental colony construction. Two-way Mixed ANOVA, Treatment: $F_{1,4}$ = 4.65, p = 0.097,
931 Day: $F_{2,8}$ = 0, p = 1, Treatment*Day: $F_{2,8}$ = 3.71, p = 0.072. See Supplementary Table 5 for
932 Pairwise comparisons. (A) depicted as survival plot. All other data depicted as box plots with data
933 points plotted, line represents median, x represents mean, and whiskers represent the minimum
934 and maximum values, n = 4 colonies. Asterisks used to denote comparisons between treatment
935 groups on each day only: *, p≤0.05, **, p≤0.001.
936

	Relative abundance			Absolute abundance		
Microbe	**Typical colonies**	**SCC Replicate 1 (week 3)**	**SCC Replicate 2 (week 3)**	**Typical colonies**	**SCC Replicate 1 (week 3)**	**SCC Replicate 2 (week 3)**
B. apis	lower abundance	lower abundance		lower abundance		
B. asteroides		lower abundance	lower abundance			
B. coryneforme					higher abundance	
Bom. apis						
F. perrara						
G. apicola					higher abundance	higher abundance
L. apis						higher abundance
L. helsingborgensis	higher abundance					
L. kullabergensis				lower abundance	higher abundance	higher abundance
L. mellifer		lower abundance				
L. mellis	higher abundance				higher abundance	higher abundance
L. melliventris	higher abundance	higher abundance		higher abundance	higher abundance	
S. alvi		lower abundance	lower abundance	lower abundance	lower abundance	

Table 1: Summary table of individual microbe abundance data. Microbes that significantly differed in relative (left) and absolute (right) abundance measures in nurses relative to foragers in typical colonies and individual single cohort colony replicates. Higher abundance, higher in abundance in nurses relative to foragers (pink). Lower abundance, lower in abundance in nurses relative to foragers (blue).

Test Microbe	Colony Replicate	Overall Colony Gini Coefficient	Day 1 Gini Coefficient	Day 2 Gini Coefficient	Day 3 Gini Coefficient
B. asteroides	1	0.376	0.056	0.116	0.425
B. asteroides	2	0.362	0.352	0.320	0.278
B. asteroides	3	0.415	0.133	0.445	0.368
B. asteroides	4	0.305	0.199	0.303	0.242
L. mellis	1	0.339	0.130	0.320	0.304
L. mellis	2	0.459	0.304	0.453	0.355
L. mellis	3	0.310	NA	NA	0.310
L. mellis	4	0.302	0.126	0.230	0.363
L. melliventris	1	0.398	0.186	0.271	0.365
L. melliventris	2	0.283	NA	NA	0.281
L. melliventris	3	0.419	0.167	0.386	0.415
L. melliventris	4	0.391	0.174	0.453	0.267

Table 2: Gini coefficients for each experimental colony depicted in Figures 4-6. Gini coefficients, representing the degree of skew in foraging intensity between individual foraging bees, where 0 represents complete equality in foraging intensity between individuals and 1 represents complete inequality in foraging intensity between individuals, measured for each experimental colony across all days ("Overall Colony Gini Coefficient"), and for each experimental colony on each individual day ("Day X Gini Coefficient"). NAs represent days with bad weather in which foraging did not occur.