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1 # Fecal transplant allows transmission of the gut microbiota in honey bees.

2

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10

11 **Keywords**: host-microbe interactions, microbiome, 16S rRNA gene, social insects, *Apis*
12 *mellifera*

13

14 ## Abstract

15 The gut of honey bees is colonized by symbiotic bacteria during the first days of adult life,
16 once bees have emerged from their wax cells. Within five days, the gut microbiota becomes
17 remarkably stable and consistent across individual bees. Yet, the modes of acquisition and
18 transmission of the gut microbiota are to be confirmed. Few studies suggested bees could
19 be colonized via contact with fecal matter in the hive and via social interactions. However,
20 the composition of the fecal microbiota is still unknown. It is particularly unclear whether all
21 bacterial species can be found viable in the feces and can therefore be transmitted to
22 newborn nestmates. Using 16s rRNA gene amplicon sequencing we revealed that the
23 composition of the honey bee fecal microbiota is strikingly similar to the microbiota of entire
24 guts. We found that fecal transplantation resulted in gut microbial communities largely similar
25 to those obtained from feeding gut homogenates. Our study shows that fecal sampling and
26 transplantation are viable tools for the longitudinal analysis of bacterial community

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27 composition and host-microbe interactions. Our results also imply that contact of young bees
28 with fecal matter in the hive is a plausible route for the acquisition of the core gut microbiota.
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31 **Introduction**

32 Over the past decade, honey bees (*Apis mellifera*) have become pivotal insect models for
33 the study of gut microbiota evolution and function[1–3]. This is due to the relatively simple
34 composition and consistency of their gut microbiota, the possibility to study *in-vitro* and *in-*
35 *vivo* defined communities of gut bacteria, as well as the recent opportunity to genetically
36 engineer some of the gut symbionts[4–6]. The honey bee gut microbiota has also attracted a lot
37 of attention due to its important role in shaping the health and behavior of these essential
38 pollinators[7–10].

39 The honey bee gut is subdivided into four distinct sections: the crop and midgut contain
40 few bacteria, while the ileum and rectum, together forming the hindgut, contain most core
41 members of the honey bee gut microbiota in different proportions[4,11]. The core bacteria
42 *Gilliamella* and *Snodgrassella* are predominant in the ileum, where they form a biofilm, while
43 *Bombilactobacillus* Firm-4, *Lactobacillus* Firm-5, and *Bifidobacterium* dominate the rectum
44 community[11–13]. How such stable gut bacterial communities are transmitted between
45 individuals remains unclear in this social insect.

46 Honey bee workers are known to progressively acquire their gut microbiota during the
47 first week of adult life in the hive, after emerging from their wax cells[11,12]. The presence of
48 adult nurse bees[11] or fresh pollen from the hive[14] in the environment of newly emerged bees
49 was shown to promote the acquisition of the core microbiota. Suggested mechanisms in
50 these studies are: (*i*) direct transmission *via* trophallaxis behavior, where bees actively
51 exchange the food content of their crop in a mouth-to-mouth interaction, and (*ii*) indirect
52 transmission *via* contact with the fecal matter of nurse bees deposited in the hive
53 environment. Recent studies found that trophallaxis with nurse bees alone was not sufficient[12]
54 and even unnecessary[14] for newly emerged bees to acquire the core gut microbiota. Instead,
55 exposure to hindgut homogenate successfully led to a gut microbiota community similar to
56 the one of hive bees. Gut homogenates, however, not only contain fecal matter, but also the

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57 communities of bacteria attached to the gut epithelium. The source of gut microbiota
58 transmission thus remains ambiguous. Since honey bees do not systematically defecate in
59 laboratory conditions while kept in cages, the use of hindgut homogenates over isolated fecal
60 matter has so far been predominant in the field; whether it is to investigate the mechanisms
61 underlying microbiota transmission or to inoculate microbiota-free (MF) individuals in the
62 context of *in vivo* experiments. Nonetheless, work carried out by our group and others
63 established protocols for routine feces sampling of honey and bumble bees,
64 respectively[5,15,16]. It remains uncertain whether all gut microbiota phylotypes, especially those
65 preferentially colonizing the ileum and forming biofilms, are viable and present in sufficient
66 quantities in fecal matter to allow microbiota transmission across individuals.

67 Thus, our investigation set out to validate the hypothesis that the honey bee gut
68 microbiota can be naturally transmitted through contact with fecal matter by quantifying the
69 relative transmission of the different bacteria present in feces. Using qPCR quantification and
70 amplicon sequencing targeting the 16S rRNA gene, we compared the bacterial taxonomic
71 composition in the feces and guts collected from the same nurse bees (generation no. 1) to
72 understand whether the feces of honey bees provide a robust proxy for their gut microbiota
73 (**Fig. 1**). We then analyzed the bacterial taxonomic composition in the gut of bees fed with
74 feces or gut homogenate a week post-inoculation to determine whether ingestion of feces
75 allows transmission of the microbiota from adults to newly emerged microbiota-free bees
76 (generation no. 2). Our results demonstrate that the gut microbiota composition can be non-
77 invasively monitored using fecal sampling, and that transplantation of fecal matter into
78 microbiota-free bees is a reliable and ecologically relevant method to study microbiota
79 transmission and host-microbe interaction.
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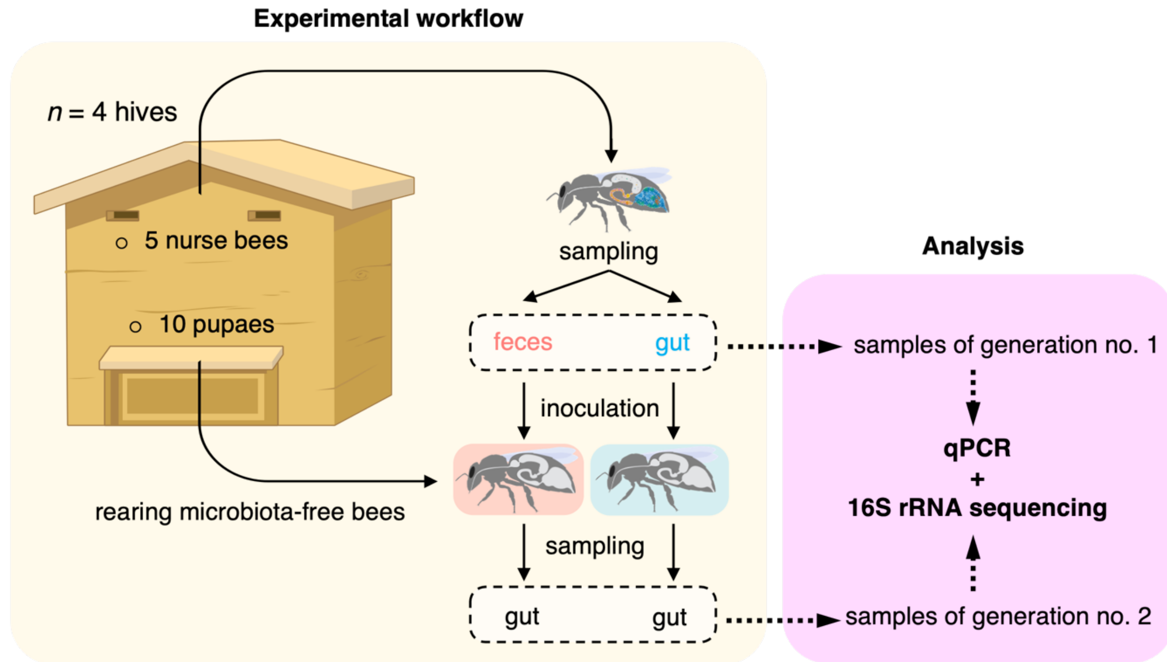

Figure 1. Schematic outline of the experimental workflow. The feces and gut from five nurse bees were collected to compare their bacterial composition (generation no. 1) and to inoculate five microbiota-free newly emerged bees (generation no. 2). A week post-inoculation, the guts of inoculated bees were collected, and their bacterial composition assessed. Bacterial total and relative abundances in the feces and gut samples were measured by quantitative PCR and 16s rRNA gene amplicon sequencing, respectively. The experiment was replicated four times using distinct hives.

82

83 ## Results

84 ### Characterization of the honey bee fecal microbiota.

85 To establish whether feces of honey bees are a robust proxy for their gut microbiota,
86 we compared the microbial communities present in feces *versus* gut samples of nurse honey
87 bees from four distinct hives (**Fig. 2**).

88 Honey bee feces were rich in bacteria, with a median bacterial load of $1.58 \cdot 10^6$ cells
89 μl^{-1} of feces (95% CI [$9.20 \cdot 10^5$, $2.59 \cdot 10^6$]) (**Supplementary Fig. 1**). More importantly, the
90 bacterial communities present in feces were remarkably similar to the ones found in the guts
91 of naturally colonized honey bees (**Fig. 2**). The predominant genera of the gut microbiota of

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92 honey bees were detected in both gut and fecal samples, namely *Bombilactobacillus* Firm-4,
93 *Lactobacillus* Firm-5, *Gilliamella*, *Snodgrassella*, *Bifidobacterium*, *Frischella, Bartonella,*
94 *Commensalibacter* and *Apilactobacillus* (formerly *Lactobacillus kunkeei*) **(Fig. 2a)** [1,17,18]. This
95 was the case for all samples across the different hives tested, with the exception however of
96 two bees from hive 15, which appeared to have very low bacterial complexity. We considered
97 these samples as outliers that may have arisen from technical errors considering further
98 analysis discussed below.

99 Diversity of the gut and fecal bacterial communities appeared overall comparable, as
100 measured by alpha- and beta-diversity metrics. Alpha-diversity, which considers species
101 richness and evenness within samples, was significantly higher in the gut samples compared
102 to the fecal samples as measured using the Shannon index (Wilcoxon matched-pairs test, Z
103 = 179, *p*-value = 0.0042) and Simpson metric (paired *t*-test, $t_{(19)} = 3.39$, *p*-value = 0.0031;**Fig.**
104 **2b**). A differential analysis revealed that only chloroplasts differed significantly in relative
105 abundance between the fecal and gut samples likely because gut samples contained more
106 pollen material (**Supplementary Fig. 2;** 13,711,506-fold change, adjusted *p*-value < 0.0001).
107 Yet, the significant difference in alpha-diversity metrics remained after removing chloroplast
108 DNA from the analysis (Shannon index: Wilcoxon matched-pairs test, Z = 177, *p*-value =
109 0.0056; Simpson metric: paired t-test, $t_{(19)} = 2.7551$, *p*-value = 0.0126). This difference was
110 expected as feces constitute a subset of the gut samples. However, there was no significant
111 difference between the microbiota structure of gut and fecal samples (PERMANOVA test
112 based on Bray-Curtis dissimilarities, *p*-value = 0.1; **Fig. 2c**). Interestingly, Bray-Curtis
113 dissimilarity matrices of the fecal and gut samples were positively correlated (Mantel test, r =
114 0.5, *p*-value = 0.0041). Consistently, a Procrustes analysis revealed a significant concordance
115 between the feces and gut datasets (**Fig. 2d**; Procrustes randomization test, $m^2 = 0.45$, p-
116 value = 0.0060) indicating that fecal samples were on average more similar to the gut samples
117 collected from the same individuals than to gut samples belonging to different individuals.

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Finally, we observed a strong positive correlation in the absolute abundance of the most prevalent taxonomic groups between the gut and fecal samples, confirming that the gut colonization level of a given amplicon-sequence variant (ASV) was reflected by its concentration in the feces (Pearson correlation coefficient R = 0.82, *p*-value < 0.0001; **Fig. 2e**). Taken together, our results demonstrate that feces provide a robust proxy for the honey bee gut microbiota composition. Fecal samples allow to infer both the community membership (*i.e.* presence/absence of a bacteria) as well as to estimate the absolute bacterial abundances (*i.e.* levels of gut colonization) in the gut of individual bees.

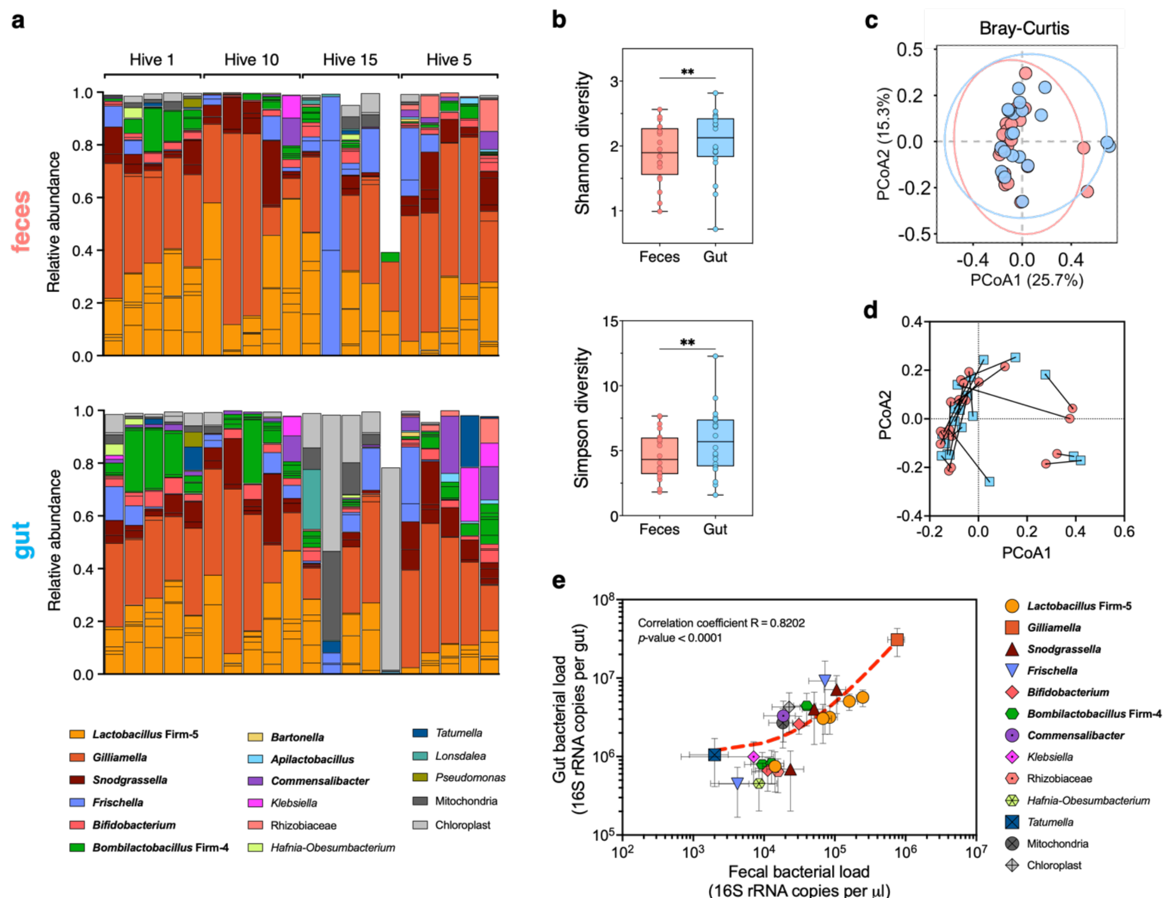

Figure 2. The fecal microbiota of honey bees is a robust proxy for their gut bacterial communities. a Stacked bar plots showing the relative abundance of identified amplicon-sequence variants (ASVs) grouped at the genus level in the feces (top panel) and gut tissues (bottom panel) of hive bees (generation no. 1). Vertically aligned bars represent samples sourced from the same individual. Their hive numbers are indicated. Only ASVs with relative

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abundance above 1% in at least 2 samples are displayed. Prevalent members of the honey bee gut microbiota are in bold. **b** Bacterial α-diversity was significantly higher in the gut than in the feces of hive bees according to both the Shannon (Wilcoxon matched-pairs test (two-tailed)) and Simpson indexes (paired t-test). ** P < 0.005. **c** Principal-coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity index showed no significant difference in β-diversity between the feces (red) and gut (blue) samples (PERMANOVA test, not significant). **d** Procrustes analysis of relative ASV abundances in the feces (red) against gut (blue) samples of hive bees revealed a significant agreement of comparison. Longer lines on Procrustes plots indicate more dissimilarity between samples sourced from the same individual. **e** Scatter plot showing a significant correlation between the absolute abundances of bacteria genera in the gut and the feces samples (Pearson's correlation coefficient and *p*-value are displayed). Only ASVs with absolute abundance above 1% in at least 5 samples are displayed for clarity. The red dotted line represents the linear regression curve (appearing non-linear due to log axes).

127

128 **Transmission of the gut microbiota to microbiota-free honey bees *via* fecal**
129 **transplantation.**

130 We next tested if ingestion of feces would be sufficient and equivalent to gut homogenates
131 for microbiota transmission to newly emerged bees (**Fig. 3**). Five μl of fecal inoculum ($7.89{\cdot}10^5$
132 cells in the inoculum; 95% CI [$4.60{\cdot}10^5$, $1.29{\cdot}10^6$]) was sufficient to successfully seed the gut
133 of MF honey bees, resulting in colonization levels similar to the ones obtained when feeding
134 5 μl of gut homogenate ($3.50{\cdot}10^4$ cells in the inoculum; 95% CI [$1.61{\cdot}10^4$, $4.25{\cdot}10^4$]; Wilcoxon
135 matched-pairs test, Z = 26.00, *p*-value = 0.6226; **Fig. 3a**). The microbial communities in
136 feces- and gut-inoculated bees reached a median of $1.54{\cdot}10^8$ (95% CI [$8.57{\cdot}10^7$, $1.63{\cdot}10^8$])
137 and $1.81{\cdot}10^8$ (95% CI [$1.03{\cdot}10^8$, $2.22{\cdot}10^8$]) cells per gut at day 7 post colonization,
138 respectively. Additionally, the relative abundances of bacterial genera in those communities
139 were again remarkably similar, with all prevalent genera of the bee microbiota found in the
140 gastrointestinal tracts of individuals fed with gut or fecal inoculums (**Fig. 3b**). Even the bees
141 that received fecal and gut inoculums sourced from the individuals of generation no. 1 from
142 hive 15 that appeared to have a remarkably low-diversity microbiota (**Fig. 2a**), harbored a

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143 normal gut bacterial community here (**Fig. 3a**). This suggests that some technical issues may
144 have distorted the gut community profiles of those individuals of generation no. 1 in our
145 previous analysis. Alpha-diversity in the gut, measured with the Shannon and Simpson
146 indexes, did not differ significantly between inoculum types (**Fig. 3c**; paired *t*-test, Shannon:
147 $t_{(18)} = -1.93$, p-value = 0.07; Simpson: $t_{(18)} = -1.45$, p-value = 0.16), although there was a trend
148 towards higher diversity in feces-inoculated bees. There was also no difference in community
149 structure between bees fed the two different inoculum types (PERMANOVA using Bray-Curtis
150 dissimilarities calculated from a matrix of absolute ASV abundance, p-value = 0.057; **Fig. 3d**).
151 Honey bees inoculated with fecal material had on average slightly increased relative
152 abundances of Bifidobacteria and Lactobacilli, which are rectum associated bacteria, than
153 bees inoculated with gut homogenates (**Supplementary Fig. 3**). Yet, we found a robust
154 positive correlation in the absolute abundance of the most prevalent genera composing the
155 gut microbiota between honey bees fed with either gut or fecal inoculums (Pearson
156 correlation coefficient R = 0.89, p-value < 0.0001; **Fig. 3e**). This confirms that feces are a
157 good inoculum source, leading to a gut microbiota composition highly comparable to the one
158 of bees inoculated with a gut homogenate.

159 Finally, we performed two additional comparisons to assess the level of similarity in
160 the microbiota that established in the gut bees of generation no. 2 and their respective donors
161 in generation no. 1. First, we tested whether pairs of bees of generation no. 2 inoculated with
162 feces and guts collected from the same donor were more similar between them than to other
163 pairs of generation no. 2. Second, we tested whether the composition of the microbiota
164 established in bees of generation no. 2 was more similar to that of the matched donor bees
165 than to that of other bees of generation no. 1, for both feces and gut-inoculated bees
166 independently. Pairs of bees of generation no. 2 inoculated with feces or gut homogenates
167 originating from the same donor bees, were not more similar in gut microbiota composition
168 than other generation no. 2 pairs (**Fig. 3f**; Procrustes randomization test, $m^2 = 0.66$, p-value

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169 = 0.49; Mantel test, r = 0.0032, p-value = 0.47). The lack of similarity between matched pairs
170 was further confirmed when comparing samples across generations (**Supplementary Fig. 4**).
171 There was no significant concordance in the microbiota of donor and receiver bees across
172 the two generations for both the feces (Procrustes randomization test, m^2 = 0.62, p-value =
173 0.15; Mantel test, r = -0.17, p-value = 0.91) and the gut homogenate-inoculated bees
174 (Procrustes randomization test, m^2 = 0.65, p-value = 0.43; Mantel test, r = 0.06, p-value =
175 0.33). The absence of concordance between the community structures observed across
176 generations for matched pairs suggests that community assembly is influenced by other
177 factors distinct from the inoculum source.
178

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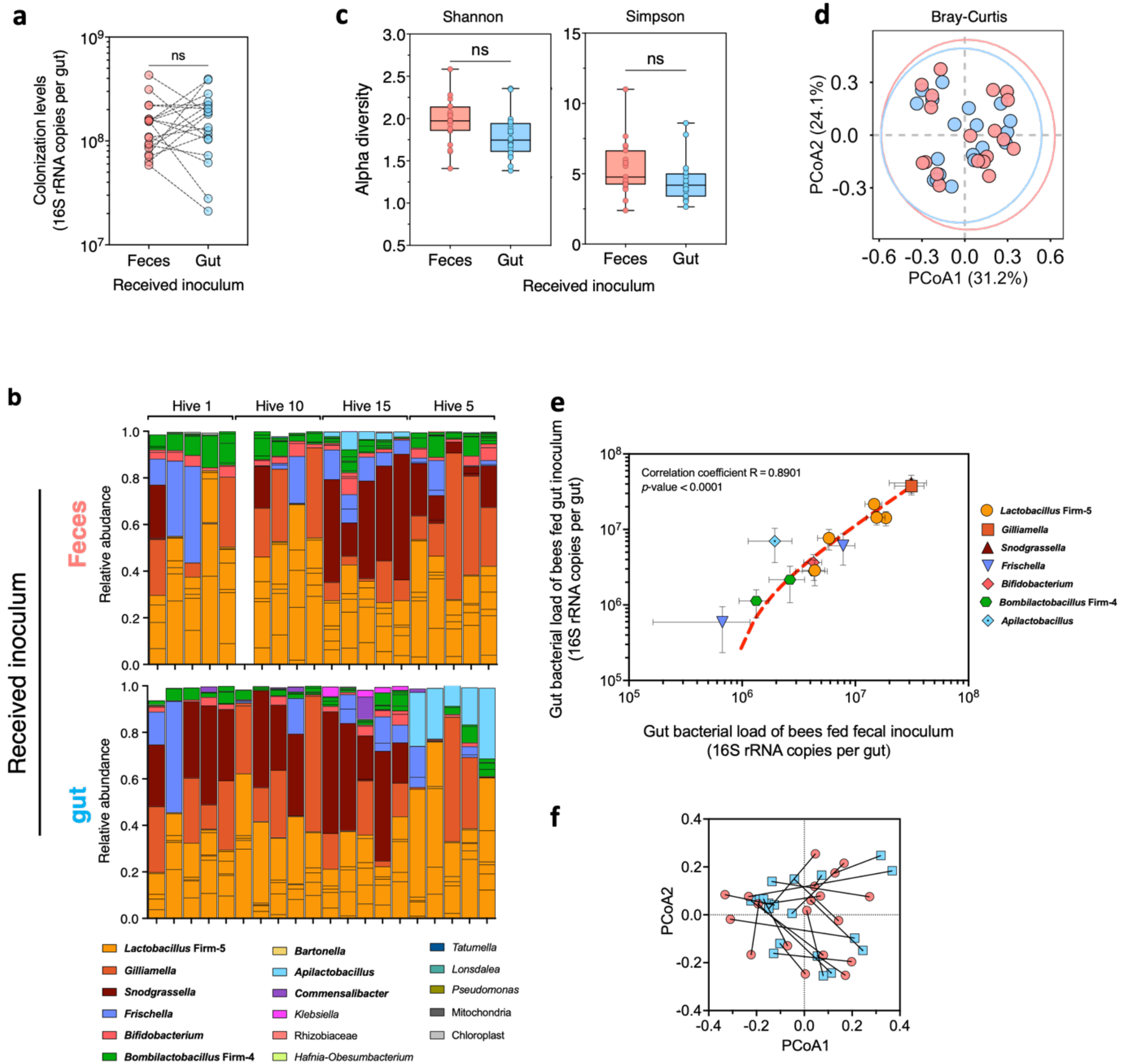

Figure 3. Fecal transplant allows transmission of the honey bee gut microbiota. a Colonization levels of bacteria in the guts of bees inoculated with either a gut homogenate or an aliquot of feces (generation no. 2) did not differ significantly (Wilcoxon matched-pairs rank test, not significant (ns)). Matching samples (*i.e.*, inoculums sourced from the same individuals) are connected by dotted lines. **b** Stacked bar plots showing the relative abundance of amplicon-sequence variants (ASVs), colored by their genus level classification, identified in the gut of bees inoculated with either feces (top panel) or gut homogenates (bottom panel). Vertically aligned bars represent matching samples. Their hive of origin is indicated above. For ease of visualization, only ASVs with a relative abundance above 1% in at least 2 samples are displayed. Prevalent members of the honey bee gut microbiota are shown in bold. One sample from hive ten was lost during the DNA extraction process. **c** Bacterial α-diversity in the gut did not differ significantly between gut-inoculated and feces-inoculated bees based on both the Shannon and Simpson indexes (Unpaired *t*-tests (two-tailed), not significant, ns). **d** Principal-coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity index showed no significant difference in β-diversity

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between the gut samples of feces-inoculated (red) and gut-inoculated (blue) bees (PERMANOVA test, not significant). **e** Scatter plot showing a significant correlation in absolute abundances of identified ASVs in the gut between feces-inoculated and gut-inoculated bees (Pearson's correlation coefficient and *p*-value are shown within the plot). Only ASVs with an absolute abundance above 1% in at least 5 samples are displayed for clarity. The red dotted line represents the linear regression curve. **f** Procrustes analysis of relative ASVs abundances in the gut of feces-inoculated bees (red) against gut-inoculated bees (blue) revealed no significant agreement between sample pairs obtained from bees of generation no. 2 inoculated with feces and guts collected from the same donor. Longer lines on Procrustes plots indicate more dissimilarity between matching samples.

179

180 Discussion

181 Here, we characterized the bacterial composition of honey bee feces and found that 182 the fecal microbiota resembles the gut microbiota. Moreover, inoculation of a small volume 183 of feces to MF bees allowed all core microbiota members to establish in the gut, in similar 184 relative and absolute amounts as the ones found in bees inoculated with a gut homogenate.

185 The analysis of the fecal microbiota is commonly used in humans, laboratory rodents, 186 and wild vertebrates to establish correlations between environmental factors, the gut 187 microbiota, and host physiology[19,20]. However, the use of fecal matter as a proxy for gut 188 microbiota composition in humans has been questioned, as the fecal microbiota was found 189 to differ from the mucosa-associated microbiota[21–23]. By contrast, we found that honey bees 190 collected from different hives at the nursing age harbored all core and most prevalent 191 members of the gut microbiota in their feces, in proportions similar to those found in entire 192 guts. Strikingly, bacteria known to colonize the fore part of the gut, namely the ileum, were 193 also detected in the feces. This was unlikely due to the shedding and elimination of dead 194 bacteria as these bacteria were viable and successfully established in the gut of feces-195 inoculated bees. As the honey bee gut microbiota composition changes with age, behavioral 196 task, and nutrition in the field[24,25], it would be interesting to validate that variation in the 197 composition of the fecal microbiota mirrors that of the whole gut under such internal and 198 external constraints. Repeated sampling of feces did not affect bees' survival in a previous 199 study where feces were sampled once per week across three weeks[5]. The possibility of non-

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200 invasively monitoring gut microbiota composition via fecal matter collection will help identify
201 the sources of variation in individual gut microbial communities and link this variation to
202 concomitant changes in host phenotypes[20]. This will facilitate longitudinal field studies on
203 natural populations of honey bees and wild bee species to further characterize ecological
204 and evolutionary processes shaping host-microbe interaction[20,26]. Feces sampling might also
205 be used as a tool to assess pathogen loads in the gut. Copley and colleagues found that the
206 gut parasites *Nosema apis* and *Nosema ceranae* could be detected in the feces of
207 contaminated honey bees[27]. However, the correlation between pathogen abundance in the
208 feces and the gut still needs to be uncovered.

209 The presence of all core bacterial phylotypes in the gut of bees transplanted with a
210 fecal inoculum confirms that the core gut microbiota can be acquired via ingestion of fecal
211 matter, as suggested by previous studies[11,12]. While newly emerged honey bees probably
212 encounter feces on the contaminated hive material[12], coprophagy, a behavior consisting of
213 feces consumption, has not been described in this insect. It is, however, common in
214 gregarious and social insects and allows transmission of the gut microbiota between
215 overlapping generations[28]. Insects may also benefit from the anti-microbial properties of
216 feces via this behaviour[29]. For instance, fecal transplantation in newly emerged bumblebees
217 led to the development of a gut microbiota similar to that of bumblebees from the donor and
218 protected them against the gut parasite *Crithidia bombi*[29,30]. Our results push for the use of
219 fecal transplantation to study the effect of gut microbiota transmission on microbial
220 communities and host phenotypes with a more ecological approach compared to the
221 currently used inoculation of gut homogenate. Given the volume of feces that can be
222 collected from a single bee without altering its physiology (4.8 ± 2.0 µl on average[5]), one can
223 reasonably expect to inoculate at least four MF bees with feces from a single donor in future
224 experiments. Further dilution of fecal material would likely still allow successful seeding of
225 the gut microbiota of MF bees and would enable inoculation of more individuals.

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226 Finally, we also found that while the bacterial communities in the feces and gut were
227 more similar when originating from the same donor bee, such pairing did not transfer to
228 generation no. 2 when analyzing the gut microbiota of bees inoculated with paired samples.
229 Furthermore, paired samples across generations (*i.e.* gut of a bee from generation no. 2 and
230 its inoculum) did not show greater similarity in microbiota composition than unpaired
231 samples. Such decoupling of community structure across generations, likely suggests that
232 community assembly mechanisms and the rearing environment play a greater role than the
233 inoculum source in determining the final composition of the gut communities. Rearing bees
234 of generation no. 2 in cages where social interaction, in particular trophallaxis events, and
235 coprophagy are possible might have influenced the establishment of the bacterial community
236 in the gut of inoculated bees, additionally to other known mechanisms affecting community
237 assembly (*e.g.* interactions between different bacterial community members and between
238 bacteria and the host)[31].

239 In conclusion, our study not only confirms the hypothesis that the honey bee gut
240 microbiota can be transmitted through contact with fecal matter but also opens doors toward
241 longitudinal analyses of individual variation in gut microbiota composition. Feces sampling is
242 a non-invasive method that will reduce the number of animals killed for experimental
243 purposes. This is particularly critical for the study of endangered bee species, or species that
244 are rare or difficult to maintain in laboratory settings. Future studies should yet confirm that
245 feces are a good proxy for gut microbiota composition in other bee species. Fecal
246 transplantation will offer unprecedented opportunities for studying host-microbe interactions
247 in a non-destructive and ecologically relevant manner, as already done in humans and
248 laboratory rodents[19,32].

249

250 Methods

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251 **Honey bee rearing and gut colonization.** Microbiota-free (MF) honey bees *Apis mellifera*
252 *carnica* were obtained from four hives located at the University of Lausanne (VD, Switzerland),
253 as previously described[33]. Briefly, mature pupae were transferred from capped brood frames
254 to a sterile plastic box for each visited hive and they were kept in a dark incubator for 3 days
255 (35 ℃ with 75 % humidity). Adult bees emerging in such laboratory conditions are MF as their
256 gut is free of any symbionts. Bees had unlimited access to a source of sterile 1:1 (w/v) sucrose
257 solution for the duration of the experiment.

258 On the third day, five adult nurse honey bees were collected from each of the four
259 original hives (**Figure 1**). They were stunned using CO_2 and immobilized on ice at 4 ℃, and
260 their feces and guts were sampled as described previously[5,16]. Two volumes of 2 µl were
261 collected from each fecal sample and diluted 1:10 (v/v) in either sterile PBS or with 1:1 (v/v)
262 PBS:sucrose solution. Gut samples were homogenized in 1 ml of sterile PBS in bead-beating
263 tubes containing zirconia beads using a FastPrep-25 5G apparatus (MP Biomedicals) set at
264 6 m s^{-1} for 30 sec. Homogenized gut samples were then diluted 1:10 (v/v) to a final volume of
265 100 µl with 1:1 (v/v) PBS:sucrose solution. The PBS-diluted gut and fecal samples were
266 stored at -80 ℃ for further DNA extraction. They constitute the samples of generation no. 1
267 (**Fig. 1**). Feces and gut samples resuspended in PBS-sucrose solution were immediately used
268 for the colonization of MF honey bees.

269 Gut colonization was carried out by individually pipette-feeding MF bees 5 µl of either
270 diluted feces or gut homogenate, which were sourced from bees originating from the same
271 hive. Additionally, each pair of bees colonized with feces or gut sampled from the same nurse
272 bee were marked by a unique color mark painted on their thorax. It enabled the matching of
273 individuals between generations. Colonized bees were kept in groups of 5 individuals in
274 separate sterile cup cages according to their inoculum and hive of origin at 32 ℃ with 75 %
275 humidity. Bees had access to a sterile sucrose solution and pollen sterilized by gamma-
276 irradiation *ad libitum*.

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277 After 7 days, honey bees were immobilized on ice at 4 ℃, sacrificed and their guts
278 were dissected. Gut samples were homogenized as described above and stored at -80 ℃ for
279 further DNA extraction. They were considered samples of generation no. 2 (**Fig. 1**).
280
281 **DNA extraction.** DNA was extracted from the feces of bees from generation no. 1 (**Fig. 1**)
282 and from the gut of bees from generation no. 1 and 2 (**Fig. 1**). Homogenized gut tissues were
283 thawed on ice, and 478 µl of those were used for the DNA extraction procedure. The fecal
284 samples were thawed on ice and diluted by mixing 15 µl of feces with additional sterile PBS,
285 to a final volume of 478 µl. For the following steps, both diluted feces and homogenized guts
286 were treated in the same way.
287 Each sample received 20 µl of 20 mg ml^{-1} proteinase K and 2 µl of s-mercaptoethanol,
288 resulting in 500 µl of source material. Samples were then diluted 2:1 (v/v) with 2X
289 hexadecyltrimethylammonium bromide (CTAB), mixed by bead-beating with glass and
290 zirconia beads using the FastPrep-25 5G set at 6 m s^{-1} for 30 sec and incubated at 56 ℃ for
291 1 h. Samples were mixed with 750 µl of phenol-chloroform-isoamyl alcohol (PCI; ratio
292 25:24:1; pH = 8), and centrifuged at room temperature for 10 min at 16,000 *g.* The upper
293 aqueous layer was transferred to a new tube with 500 µl of chloroform and mixed by
294 vortexing. Samples were centrifuged again at room temperature for 10 min at 16,000 *g.* The
295 upper aqueous layer was mixed with 900 µl of cold 100% ethanol and incubated overnight
296 at -20 ℃ to allow for DNA precipitation. Samples were centrifuged at 4 ℃ for 30 min at 16,000
297 *g* and the supernatant was discarded. DNA pellets were gently washed with 70% ice-cold
298 ethanol, before being centrifuged again at 4 ℃ for 15 min at 16,000 *g.* The supernatant was
299 discarded, and the remaining ethanol was evaporated at room temperature for approximately
300 10 min. Dried DNA pellets were dissolved in 50 µl of nuclease-free water by incubation at 64
301 ℃ for 10 min. Purification of the extracted DNA using CleanNGS magnetic beads (CleanNA)
302 was automated with an Opentrons OT-2 pipetting robot. Briefly, DNA extracts were incubated

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303 with 25 µL of NGS beads at room temperature for 10 min. A magnet was involved to attract
304 the beads and attached DNA at the bottom and clear the supernatant. Beads were rinsed
305 twice with 110 µL of ethanol (80%) and left to dry at room temperature for 10min. The
306 obtained purified DNA extracts were resuspended in 45 µL of Tris-HCl buffer (5 µM; pH 8)
307 and stored at -20 ℃. One sample from hive ten was lost during the DNA extraction process.
308

309 **16S rRNA amplicon-sequencing**. The extracted DNA was used as a template for 16S rRNA
310 amplicon sequencing following the Illumina metagenomic sequencing official guidelines.
311 Briefly, the 16S rRNA gene V4 region was amplified with the primers 515F (5'-
312 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3') and
313 806R (5'-
314 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3'),
315 using a high-fidelity polymerase (Phanta Max, Vazyme). PCR products were purified using
316 CleanNGS magnetic beads (CleanNA) in a ratio of 0.8:1 beads to PCR product. Index PCR
317 was performed using Illumina Nextera Index Kit v2 adapters and resulting amplicons were
318 purified again using CleanNGS magnetic beads. PCR products were purified once more
319 using CleanNGS beads in a ratio of 1.12:1 beads to PCR product. Sample concentrations
320 were normalized based on PicoGreen (Invitrogen) quantification and pooled together. Short-
321 read amplicon sequencing was carried out with an Illumina MiSeq sequencer at the Genomic
322 Technology Facility of the University of Lausanne (Switzerland), producing 2 x 250-bp paired-
323 end reads *via* 150 cycles. Negative controls of DNA extraction and PCR amplification were
324 also sequenced for reference.
325

326 **Microbial community structure analyses.** The bacterial communities present in fecal and
327 gut samples were determined based on analysis of Illumina sequencing, as previously
328 described[8]. Briefly, raw sequencing data were pre-processed by clipping the primer
329 sequences from all reads using Cutadapt[34] (version 4.2 with Python version 3.11.2).

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330 Sequencing data were then processed following the Divisive Amplicon Denoising Algorithm
331 2 pipeline[35] (DADA2; version 3.16) run with R (version 4.2.2). The end of sequences with low
332 quality were further trimmed after 232 and 231 bp, for forward and reverse reads,
333 respectively.

334 The resulting reads were denoised using the core sample inference algorithm of
335 DADA2, based on error rate learning determined by analyzing 3^8 minimum numbers of total
336 bases from samples picked at random ('nbases' and 'randomize' arguments), and paired-
337 end sequences were merged. Unique sequences outside the 250:255-bp range were
338 removed alongside chimeras. The obtained amplicon-sequence variants (ASVs) were
339 classified using the SILVA reference database (version 138.1)[36]. The taxonomic classification
340 was complemented *via* Blast searches to further discriminate ASVs identified as the genus
341 *Lactobacillus* as either the core phylotypes Firm-5 and Firm-4 of the bee gut microbiota or
342 other non-core *Lactobacillus* species. The dataset was cleaned using Phyloseq[37] (version
343 1.42.0) by removing any unclassified and eukaryotes ASVs. Lastly, the R package
344 Decontam[38] (version 1.18.0) was used to remove contaminants based on prevalence and
345 frequency methods.

346

347 **Bacterial load quantification by qPCR.** Bacterial loads in the gut and feces samples were
348 determined from quantitative PCRs (qPCRs), as previously described[33]. Briefly, universal
349 primers of the 16S rRNA gene were used to determine bacterial load (forward: 5'-
350 AGGATTAGATACCCTGGTAGTCC-3'; reverse: 5'-YCGTACTCCCCAGGCGG-3') and
351 primers specific to the *Actin* gene of *A. mellifera* were employed as control of sample quality
352 (forward: 5'-TGCCAACACTGTCCTTTCTG-3'; reverse: 5'-AGAATTGACCCACCAATCCA-3').
353 Corresponding standard curves were generated using serial dilutions of plasmids bearing the
354 target sequences for the 16S rRNA and *Actin* genes.

355 Purified DNA was used as a template for qPCR reactions by mixing 1 µl of DNA to 5
356 µl of 2X SYBR Select Master Mix (ThermoFisher), 3.6 µl of nuclease-free water, and 0.2 µl of

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357 each appropriate 5 μM primers. Amplification reactions were performed with a QuantStudio
358 5 real-time PCR machine (ThermoFisher), with the following thermal cycling conditions: 50 ℃
359 for 2 min and 95℃ for 2 min for denaturation of DNA, followed by 40 amplification cycles
360 consisting of 95 ℃ for 15 sec and 60 ℃ for 1 min. Each reaction was performed in triplicate.

361 The quantification of gene copy numbers was performed following a published
362 detailed protocol[33]. The slope of the standard curves for each target (*i.e.* universal 16S rRNA
363 gene and *actin*) was used to calculate the primer efficiencies (E) according to the equation: E
364 $= 10^{(-1/slope)}$. The copy number n in 1 μL of DNA was obtained using the formula $n = E^{(intercept-}$
365 $^{Cq)}$. This number was multiplied by the elution volume of the DNA extract to obtain the copy
366 number per gut. Finally, the bacterial 16S rRNA gene copy number was normalized for each
367 sample by dividing it by the corresponding *actin* copy number and multiplying by the median
368 of *actin* copy numbers across all samples.
369

370 **Statistical analyses.** All statistical analyses were performed in R (version 4.2.2). Absolute
371 abundances of each ASV in each sample were calculated by multiplying their proportion by
372 the normalized 16S rRNA gene copy number measured by qPCR. Measures of α diversity
373 (Shannon and Simpson metrics) were obtained with the Phyloseq package[37] (version 1.42.0).
374 Their normal distribution and homoscedasticity were assessed using a Shapiro-Wilk test and
375 a Bartlett test respectively. For normally distributed and homoscedastic data, differences in
376 α diversity metrics between sample types were tested using paired t-tests, otherwise they
377 were analyzed with two-sided Wilcoxon matched-pairs tests. Difference in community
378 structure was assessed based on Bray-Curtis dissimilarities (Phyloseq) using a Adonis and
379 Permutation test (vegan[39]; version 2.6-4). Estimation of correlation between sample pairs was
380 done using Procuste and Mantel tests based on the Pearson correlation method (ade4[40];
381 version 1.7.22 and vegan). ASVs with significant differences in their relative abundances

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382 between sample types in generation no. 1 were determined using the DESeq2 package[41]
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384

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486 ## Acknowledgements

487 We thank Florian Zoppi for his continuous support in the laboratory. We also thank the
488 Genomic Technologies Facility of the University of Lausanne, Switzerland, for performing
489 Illumina sequencing. This work was supported by the Marie Skłodowska-Curie fellowships
490 HarmHoney (grant no. 892574, awarded to A.Ca.) and BRAIN (grant no. 797113, awarded to
491 J.L.), the NCCR Microbiomes (National Centre of Competence in Research), funded by the
492 Swiss National Science Foundation (SNSF, grant no. 180575), and an ERC Starting Grant
493 (MicroBeeOme, grant no. 714804) and SNSF Consolidator grant (grant no. TMCG-3_213860)
494 both awarded to P.E..

495

496 ## Author contributions

497 The original idea for this manuscript emerged from discussions between A.Ca., A.Ch. and
498 J.L.. A.Ca. and A.Ch. conceived the study, designed experiments, carried out bee
499 experiments, and DNA extractions. N.N. performed the purification of DNA samples. L.K.
500 performed qPCR experiments. A.Ch. and J.L. performed AmpliSeq libraries preparations.
501 J.L. and A.Ch. analyzed the amplicon sequencing data and quantitative PCR data with
502 assistance from A.Ca.. A.Ch. plotted the graphs. Y.S. and P.E. supervised the project. A.Ca.
503 and A.Ch. drafted the manuscript. All authors edited subsequent drafts.

504

505 ## Competing interests

506 The authors declare no competing interests.

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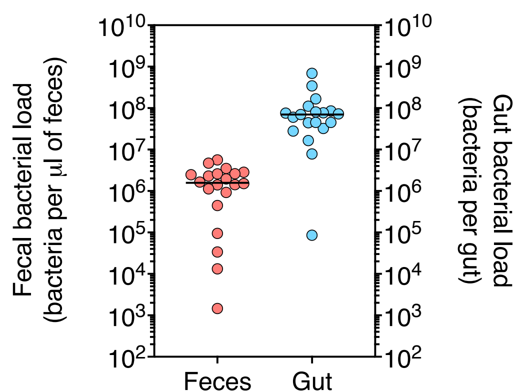
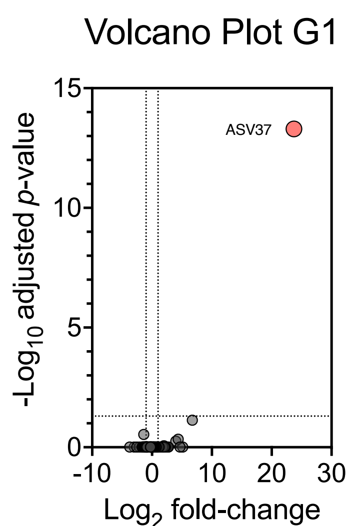
512 # Supplementary material



Supplementary Figure 1. Bacterial loads measured as copies of the 16S rRNA gene in the fecal and gut samples of bees from generation no. 1.

513

514
515



Supplementary Figure 2. Volcano plot presenting significance vs. fold-change based on relative abundances of all amplicon sequence variants (ASVs) in the gut compared to the fecal samples of nurse bees from generation no. 1. The colored ASV was significantly different in DESeq2 analyses (FDR-corrected $P<0.05$).

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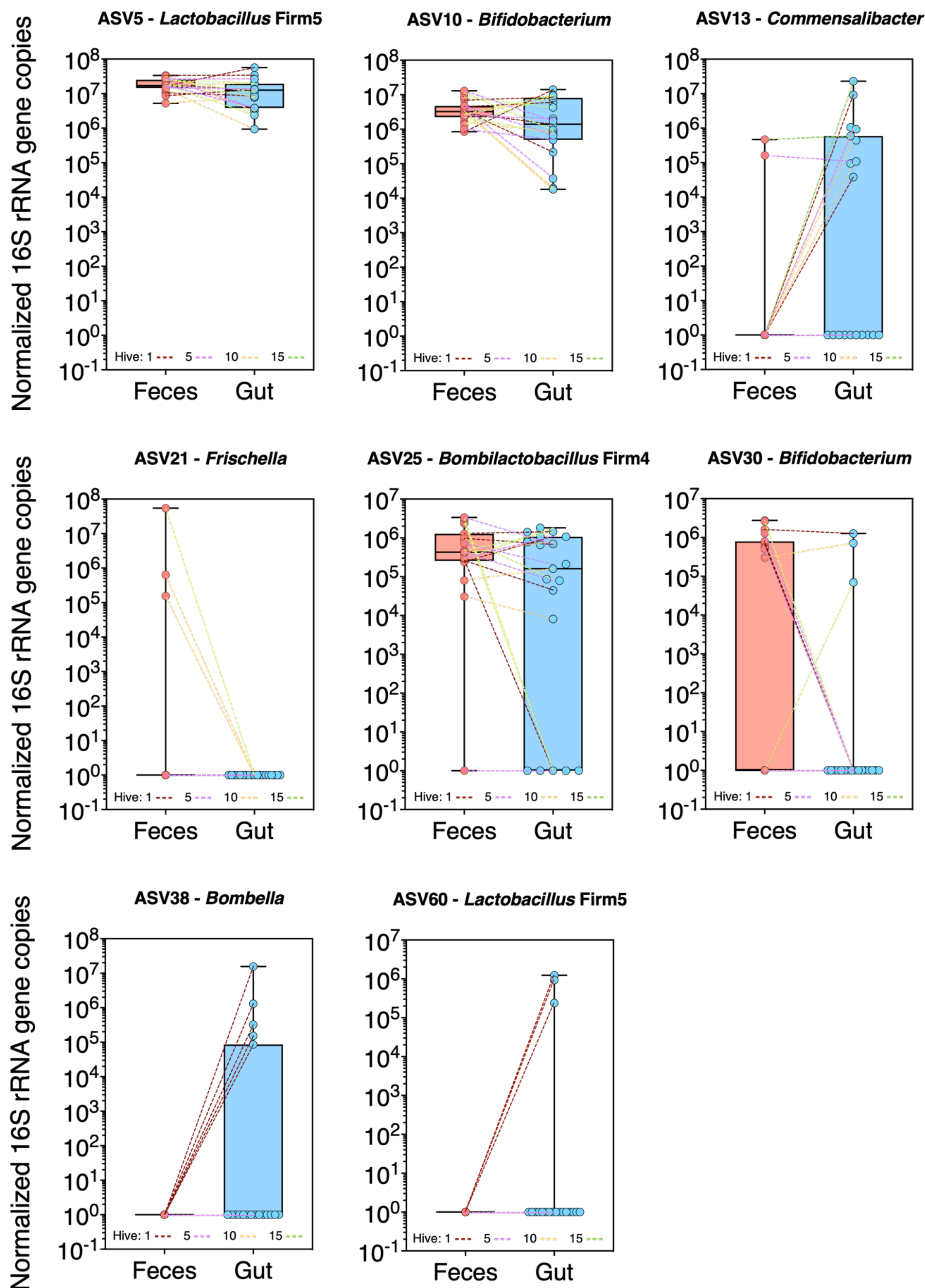

Supplementary Figure 3. Significantly different 16S rRNA gene copies of various ASVs in the gut of bees inoculated with either feces or gut homogenate.

517

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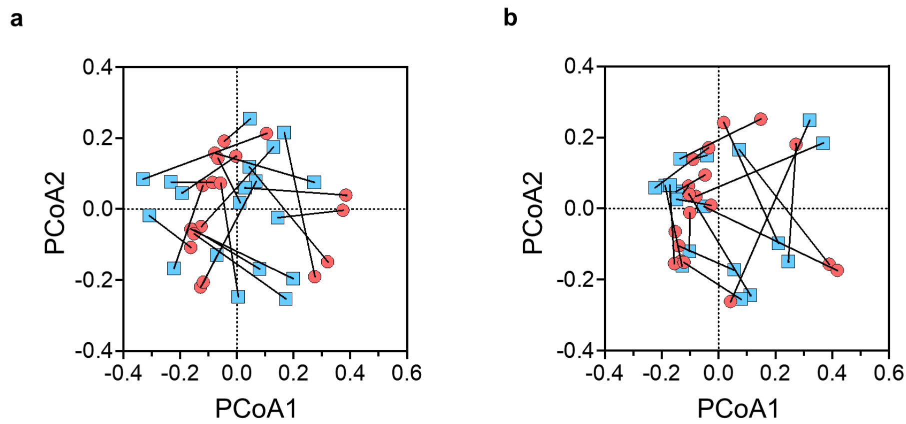
518



519

520 **Supplementary Figure 4**. Procrustes analysis of relative ASVs abundances in the fecal **(a)**
521 or gut **(b)** samples of bees from generation no. 1 (red) against the gut of bees from generation
522 no. 2 (blue) was obtained from PCoA and revealed no significant agreement of comparison.