1 **The scent of symbiosis: gut bacteria** 2 **affect social interactions in** 3 **leafcutting ants**

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21

22 Abstract

23 Animal gut microbiota affect host physiology and behaviour. In eusocial
 24 Hymenoptera, where colony-level integrity is preserved via a nestmate
 25 discrimination system based on cuticular hydrocarbon mixtures, microorganismal
 26 effects may influence social dynamics. Although nestmate recognition has
 27 undergone a thorough exploration during the last four decades, few studies have
 28 investigated the putative role of gut microbes. Here we integrate metagenomic,
 29 chemical and behavioural approaches to test whether gut microbes affect
 30 nestmate recognition in *Acromyrmex echinator* leaf-cutting ants. Treating
 31 workers with a sterile diet or with antibiotics resulted in a substantial alteration of
 32 their gut microbial communities. In pairwise social interactions, untreated vs.
 33 antibiotic-treated nestmates behaved more aggressively than other nestmate
 34 and non-nestmate pairs, suggesting that the suppression of microbes indirectly
 35 alters chemical social cues and triggers aggressive behaviour. Chemical
 36 analyses on treated individuals revealed a decrease in the abundance of two
 37 metapleural gland antifungal compounds, and we confirmed the correspondence
 38 between aggression levels and chemical profile differences. Feeding microbiota-
 39 remodelled ants with conspecific faecal droplets partially restored the original
 40 bacterial communities. Furthermore, non-nestmates fed with faecal droplets from
 41 different colonies were unusually aggressive compared to pairs fed with faecal
 42 droplets from the same colony. This suggests that chemicals derived from
 43 microbial strains may shape nestmate recognition, opening novel questions
 44 about the role of microorganisms in the evolution of social behaviour.

45

46 **Keywords:** gut microbiota, *Acromyrmex echinator*, social evolution, cuticular
47 hydrocarbons

48 **Introduction**

49 In the evolution of mutualistic relationships between metazoans and prokaryotes,
50 animals have co-opted the metabolic versatility of microbes to upgrade their
51 physiology, while microorganisms have found favourable environments in animal
52 bodies. As part of the physiology of their animal hosts, symbiotic microbes are
53 also involved in their behavioural processes (1–5). Many insightful discoveries
54 about the physiological and behavioural effects of symbiotic microorganisms
55 stem from the study of germ-free or germ remodelled animals. Research
56 comparing germ-free mice to their untreated counterparts has revealed microbial
57 gut symbionts to affect anxiety-like behaviour (6–8) and social interactions (9).
58 Similarly, a flourishing corpus of *Drosophila* studies suggests that gut microbes
59 mediate a plethora of physiological/behavioural processes, including specific
60 appetites for proteins (10), mate choice and mating dynamics (11–14) and the
61 recognition of kin and familiar individuals (15).

62
63 This increasing awareness about the behavioural role of microbes opens
64 questions about how microorganisms may affect the behavioural ecology of
65 animals with radical social adaptations, such as the eusocial insects. These live
66 in family groups with a permanent division of reproductive labour, worker castes
67 and sophisticated chemical communication (16). Eusocial insect colonies exhibit
68 complex behaviours resulting from the cooperative interactions of individuals,
69 such as foraging, nursing and nest construction, as well as policing and colony
70 defence against unrelated intruders. Across taxa, group-level integrity is ensured

71 by a chemical-based nestmate recognition system, which relies on signals
72 encoded in long-chain hydrocarbons forming a waxy layer on the insect cuticle
73 (cuticular hydrocarbons, or CHC).

74 Our current knowledge about the microbial effects on eusocial insect CHC-
75 mediated social behaviour is limited and inconsistent. *Reticulitermes speratus*
76 termites fed with bacteria extracted from individuals of unrelated colonies are
77 attacked by nestmates, and antibiotic-mediated manipulation of bacterial
78 communities affects nestmate recognition behaviour (17). In *Pogonomyrmex*
79 *barbatus* harvester ants, individuals with experimentally-augmented cuticular
80 microbiomes are rejected by nestmates more than controls, whereas antibiotic-
81 treated individuals are not. This suggests that cuticle-dwelling microbes influence
82 nestmate recognition dynamics (18). Contrarily, however, topical antibiotic
83 administration on *Acromyrmex subterraneus* leafcutter ants does not affect
84 cuticular hydrocarbon profiles (19). Similarly, a study on *Camponotus* carpenter
85 ants revealed a negative correlation between the levels of the bacterial gut
86 symbiont *Blochmannia* and CHC quantities, whereas relative CHC proportions
87 were not affected (20). Finally, antibiotic administration affects interspecific but
88 not intraspecific social interactions in the Argentine ant *Linepithema humile*,
89 suggesting gut microbiota not to be involved in nestmate recognition in this ant
90 species (21).

91

92 Despite providing insights into the role of symbiotic microbes in CHC-mediated
93 nestmate recognition, these studies either investigate microbial effects through
94 behavioural tests, without correlating CHC measures (17,21), or compare CHC
95 profiles between antibiotic-treated and control individuals without behavioural
96 tests (18–20). To gain more insights into the interplay between gut microbiota

97 and chemical-based social interactions, we here seek to implement an
 98 integrative analysis of CHC, gut bacterial communities and nestmate recognition
 99 behaviour. We remodelled the previously-characterized gut microbiota of
 100 *Acromyrmex echinator* leafcutter ants (22,23) to investigate the link between gut
 101 bacterial communities and socially relevant chemical signals. Our hypothesis
 102 was that remodelling of the ant gut microbiota would result in a chemical profile
 103 shift with measurable effects on social interactions. Therefore, in a first set of
 104 experiments, we either mildly suppressed or completely eliminated the native gut
 105 microbiota of *A. echinator* using respectively a sterile sucrose diet or antibiotics
 106 (Round 1). We then characterized the effects on the ant gut microbial
 107 communities and chemical profiles, and used dyadic aggression trials to evaluate
 108 treatment-dependent changes of gut microbial communities with chemical
 109 profiles and behaviour. In a second set of experiments, we partially restored the
 110 original ant gut microbial communities with the aim to rescue the chemical and
 111 behavioural effects obtained with the experimental diet and the antibiotics
 112 (Round 2). To achieve this, we fed experimentally-treated individuals with faecal
 113 droplets of untreated conspecifics and again analysed their microbial
 114 communities, chemical profiles and social interactions.

115 **Material and methods**

116 *Experimental design*

117 We used workers from four *A. echinator* colonies (Ae150, Ae322, Ae153 and
 118 Ae331, hereafter named respectively A, B, C and D) with an already
 119 characterized gut microbiota (24,25) for the experiments (Figure 1). Colonies
 120 were collected in Gamboa, Republic of Panama in 2003-2010, and kept at 25°C,
 121 70% RH and 12:12 L:D photoperiod. Individuals were taken from fungus gardens
 122 (375 individuals/colony, N=1500) and placed in groups of 15 in sterile Petri
 123 dishes (Ø 90×15mm; 25 dishes/colony, N=100) including a food container. For
 124 each colony, we randomly assigned workers to two treatment groups: 1) ants
 125 kept on a sterile 10% sucrose solution diet (150 individuals/colony); 2) ants kept
 126 on the same sterile 10% sucrose solution supplemented with 1mg/ml of the
 127 antibiotic tetracycline (225 individuals/colony). After two weeks, 522 individuals
 128 were tested in a first series of experiments (Round 1) including aggression
 129 assays and analyses of CHC (GC-MS, see below) and gut microbiota (qPCR
 130 and MiSeq, see below). The remaining individuals underwent the experiments in
 131 Round 2, where we attempted to restore their original microbiota by feeding ants
 132 on a sterile 10% sucrose solution supplemented with faecal droplets (0.033
 133 droplets/μl) obtained by squeezing abdomens of untreated workers from the
 134 source colonies. Colony B and D workers received nestmate droplets, whereas A
 135 and C workers received non-nestmate droplets (from colonies B and D,
 136 respectively, Figure 1). After one week, individuals were tested in aggression
 137 assays and used for CHC and microbiota analyses. Throughout the experiments,
 138 we monitored survival to determine the effects of our treatments on ant mortality
 139 (details in the Supplementary information, Figure S1).

140 Finally, we set up an independent smaller-scale experiment in which we
141 compared the microbial communities of ant guts, heads and thoraxes before and
142 after the experimental diet treatment (details in the Supplementary Information,
143 Figure S2).

144

145 *Aggression assays*

146 We tested microbiota-remodelled and untreated ants against nestmates and
147 non-nestmates from the original colonies, in both experimental rounds (3-5
148 replicates/combination, total=260 tests; Figure 1, Data set S1). Assays consisted
149 of dyadic encounters in Petri dishes with clean filter paper on the bottom. For two
150 minutes after first contact, an observer (blind with respect to ant treatment and
151 original colony) used the software Etholog 2.25 (26) to quantify the frequency
152 and duration of biting, mandible opening, antennation and absence of contact
153 (Figure S3). For statistical analyses, we excluded the mandible opening
154 behaviour (usually considered as aggressive) because pooling it with biting
155 produced similar results (Supplementary Information). In addition, because
156 keeping ants in the same Petri dishes for three weeks resulted in a significant
157 effect on aggression (Binomial GLMM with ‘Petri dish’ as fixed and ‘colony’ as
158 random variables, $z=3.21$, $Df=141$, $p=0.001$), we considered only interactions
159 between nestmates kept in different Petri dishes.

160 We analysed data in R using the packages *lme4*, *car* and *multcomp* (27–29),
161 fitting generalized linear mixed models (GLMMs). Classifying biting as
162 aggressive behaviour and antennal contact as non-aggressive allowed measures
163 to be analysed as a single binomial response variable. For Round 1, the initial
164 model included biting/antennation frequencies as response variable, whereas

165 nestmate, diet treatment and their interaction were included as fixed factors. The
 166 colony origin of experimental individuals was included as random effect term.
 167 The model used for Round 2 included the same response variable as Round 1
 168 model, and 'faecal droplets' was included as an additional fixed factor (two
 169 levels: from nestmates or non-nestmates). We tested the significance of fixed
 170 effects using the *car* function 'Anova'. Where needed, we conducted post hoc
 171 planned contrasts between groups of interest using the *multcomp* function 'glht',
 172 correcting alpha values with false discovery rate (FDR).

173

174 *DNA extractions*

175 Four to eight workers per treatment per colony used in the aggression assays
 176 were ice anesthetized and individually dissected in sterile Phosphate Buffered
 177 Saline (PBS). Pooled crop, midgut, hindgut, Malpighian tubules and fat body
 178 cells were stored at -20°C until DNA extractions, and immediately homogenized
 179 after thawing in 200µl ATL buffer supplemented with 20µl proteinase K (Qiagen)
 180 using sterile pestles. Subsequently, Ø0.45 mm glass beads were added and
 181 tubes vortexed for 30s, after which the samples were incubated at 56°C
 182 overnight under constant agitation. DNA was extracted using the Qiagen Blood
 183 and Tissue kit, and all samples were eluted in 100µl AE elution buffer.

184

185 *16S rRNA qPCR analyses*

186 The gut microbiota of *A. echinator* normally consists of five predominant OTUs
 187 (Operational Taxonomic Units, representing a cluster of bacterial 16S rRNA
 188 sequences of ≥97% similarity) (22,25,30). These belong to the genus *Wolbachia*

189 (*wolAcro1*, including two strains: *wSinictaA* and *wSinictaB*) and the orders
190 Entomoplasmatales (class: Mollicutes, OTUs *EntAcro1*, *EntAcro2* and
191 *EntAcro10*) and Rhizobiales (class: Alpha-Proteobacteria, OTU *RhiAcro1*
192 (25,31–33)). In order to monitor how the dietary treatment affected the
193 communities, we screened individual worker guts with qPCR (detailed methods
194 in Supplementary Information, text and Table S1) on the five most abundant
195 OTUs (Data set S2, procedures described in (25)), with Cycle threshold (Ct)
196 mean of replicated samples used as a measure of amplicon abundance. The
197 elongation factor 1 alpha (EF-1 α) was used as a reference gene (34). Each run
198 included two negative controls with no added template for each gene used. Data
199 were ordinated using an unscaled principal coordinate analysis (PCoA) and inter-
200 sample distances were again calculated using Canberra, Hellinger and Bray-
201 Curtis methods. We estimated the difference in variation among groups using the
202 HOMOVA command (with Bonferroni-adjusted alpha-values) implemented in
203 mothur (35). For analysis, we initially used a standard curve with PCR products
204 in tenfold dilution series of known concentration (fold change method) to
205 calculate the PCR efficiency using the REST software (36). Data were imported
206 in R and expressed as $\Delta\Delta C_T$ values, i.e. as the fold change relative to the EF-1 α
207 control gene (37), always using zero as reference. We used linear mixed models
208 (LMM) with the $\Delta\Delta C_T$ values as response variable, ‘diet treatment’ (i.e.,
209 untreated, sugar-treated or tetracycline-treated) and ‘experimental round’ (i.e.
210 Round 1 or 2) as fixed variables, and ‘colony’ (A, B, C or D) as random variable.
211 Tukey post hoc tests were performed to evaluate significant differences between
212 groups. We used GLMs to pair aggression data with distances calculated using
213 ΔC_T values fold change differences, or with absolute copy numbers calculated

214 using the qPCR data. All correlations were performed in R using the lme4,
215 vegan, effects, Rmisc and ggplot2 packages (27,38–41).

216

217 *16S rRNA MiSeq analyses*

218 To investigate whether novel OTUs appeared with the dietary treatment, we
219 screened ant worker guts individually with 16S rRNA MiSeq sequencing.
220 Amplicons were generated using the 515F/806R primers targeting the 16S rDNA
221 V4 region (42), purified using the Agencourt AMPure XP (Beckman Coulter) and
222 quantified using Quant-iT dsDNA High-Sensitivity Assay Kit and Qubit
223 fluorometer (Invitrogen) to allow for dilution and mixing in equal concentrations
224 before sequencing. Sequencing (for details see the Supplementary Information)
225 took place in the Section of Microbiology at the University of Copenhagen using
226 an Illumina MiSeq. Data [Genbank: SAMN04261407 - SAMN04261536 and
227 SAMN05362797 - SAMN05362832] were analysed using mothur (35). Details on
228 the mothur procedure are given in the Supplementary Information. After
229 filtering/processing the sequencing data and clustering at 97%, rarefaction tables
230 were constructed using pseudo-replicate OTU datasets containing 1-272000
231 sequences with 1000 iterations/pseudo-replicate, and the resulting curves were
232 visualized in Microsoft Excel 2013. The final OTU Table was rarefied at 5000
233 reads after manual inspection of the rarefaction curves, which reduced the
234 number of OTUs to 1500. We used the MiSeq data to calculate Canberra and
235 Bray-Curtis distances, after which we used Non-metric MultiDimensional Scaling
236 (NMDS) to ordinate and visualize the effects. We used GLMs to pair aggression
237 data with Bray-Curtis or Canberra distances calculated from the 16S rRNA

238 MiSeq data. Correlations were performed in R v3.2.3 using the lme4, vegan,
239 effects, Rmisc and ggplot2 packages (27,38–41).

240

241 *Cuticular hydrocarbon analyses*

242 CHCs were extracted by immersing the dissected heads and thoraces of
243 aggression test individuals first in 150 µl HPLC-grade hexane, and then in 150 µl
244 HPLC-grade chloroform (chemicals from Sigma-Aldrich, Belgium), both for 10
245 min under continuous agitation. The two extracts were mixed, and the solvent
246 evaporated at room temperature in a laminar flow cupboard. The dry extract was
247 then dissolved in 30µl hexane, of which 3µl were injected in a Shimadzu QP2010
248 Ultra GC-MS (splitless injector mode). Details on the GC-MS settings are given
249 in the Supplementary Information. Our initial integration analysis of GC-MS runs
250 detected 137 peaks, of which we selected 73 that had a relative abundance
251 larger than 0.1% (Data set S3). Peak areas of cuticular compounds were
252 integrated using R v3.1.0 (using package *xcms*, script available upon request)
253 and normalized using a Z-transformation (43). To compare odour profiles among
254 different rounds, we used linear mixed models (LMM) with the relative
255 abundance of each compound as the response variable, ‘diet treatment’ and
256 ‘experimental round’ as fixed variables, and ‘colony’ as a random variable. We
257 conducted linear hypotheses using the *multcomp* R package (44) function *glht* to
258 evaluate differences between diet treatments in the same Rounds, and between
259 the same diet treatments across Rounds. All p-values were corrected for false
260 discovery rate (FDR) given that we had conducted 73 separate tests per
261 contrast. We used a coinertia analysis (45) to check for correlations between
262 CHCs (transformed to logarithmic data) and qPCR measures (ΔC_t values) of the

263 six most abundant bacterial taxa (see details about these taxa below). In short,
264 we generated two independent data matrices (either using the individual profiles
265 or the pairwise differences for each trial), performed PCA analyses and paired
266 them using the coinertia analysis using a Monte-Carlo test with 10000
267 permutations.
268

269 **Results**

270 *Survival analysis*

271 During Round 1, mortality increased in tetracycline treated workers (Cox
272 proportional hazard model, $p < 0.001$), similar to what had been observed in a
273 previous study (25). However, this effect disappeared in Round 2, when all ants
274 were fed on faecal droplets (Figure S1), suggesting that the harmful effect of
275 tetracycline lasted only as long as it was administered to the ants.

276

277 *Aggression tests (Round 1)*

278 In Round 1, where ants were isolated from their original colonies and fed on
279 sterile sucrose diets with/without antibiotics (Figure 1), we found a significant
280 nestmate*treatment interaction ($\chi^2=6.9803$, $df=2$, $p=0.0305$). The diet treatment
281 had a significant effect on aggression, whereas being non-nestmates did not
282 (diet treatment: $\chi^2=29.62$, $df=4$, $p<0.001$; nestmates vs. non-nestmates: $\chi^2=1.64$,
283 $df=1$, $p=0.18$; $N=260$ dyadic aggression assays). This result was mostly due to
284 the high biting frequency between tetracycline-treated ants and their untreated
285 former nestmates taken from fungus gardens, which was significantly higher
286 from all other nestmate trials (all $p<0.05$, Figure 2A, Table S2). For tetracycline-
287 treated and sucrose-treated ants, aggression levels between non-nestmates
288 were not significantly different from those observed in nestmate trials
289 (tetracycline-treated: $z=0.365$, $p=0.715$, Figure 2A; sucrose-treated: $z=1.284$,
290 $p=0.287$). Contrarily, in the sucrose- vs. tetracycline-treated groups, non-
291 nestmate trials showed higher aggression than nestmate trials ($z=2.375$,
292 $p=0.045$).

293

294 *Aggression tests (Round 2)*

295 During Round 2, across sucrose-treated pairs, tetracycline-treated pairs and
 296 sucrose- vs tetracycline treated pairs, aggression was low in nestmate
 297 encounters fed with nestmate faecal droplets, higher in non-nestmate encounters
 298 fed with nestmate faecal droplets and maximal in non-nestmate encounters fed
 299 with non-nestmate faecal droplets (Figure 2b). In particular, the highest
 300 aggression levels appeared in encounters between non-nestmate sucrose-
 301 treated ants fed with faecal droplets from different colonies ($z=2.05$, $df=85$,
 302 $p=0.041$; Figure 2b, Table S2). Tetracycline-treated pairs showed instead low
 303 aggression (Figure 2b), regardless of whether they were fed on the same or
 304 different faecal droplets. Sucrose-treated vs tetracycline-treated ants exhibited
 305 low aggression levels, similar to tetracycline-treated pairs. We found a significant
 306 effect of the interaction faecal droplet*treatment ($\chi^2=7.57$, $df=2$, $p<0.05$), but not
 307 diet treatment*nestmate ($\chi^2=3.36$, $df=2$, $p=0.18$; Figure 2b, Table S2). All three
 308 main effects were significant (faecal droplets: $\chi^2=7.99$, $df=1$, $p<0.05$; diet
 309 treatment: $\chi^2=36.97$ $df=4$, $p<0.001$; Nestmate: $\chi^2=5.2437$ $df=1$, $p<0.05$).

310

311 *Gut bacterial communities changes (Round 1)*

312 We examined the changes of the gut bacterial communities using both 16S
 313 rRNA MiSeq amplicon sequencing and 16S rRNA qPCR to measure the levels of
 314 the five most abundant OTUs (Data set S2; (22,25,30). Microbiomes of
 315 tetracycline-treated individuals were most strongly affected, showing the lowest
 316 variance, whereas untreated individuals collected from fungus gardens showed

the highest variance (HOMOVA, $p < 0.001$; Figure S4). While gut bacterial communities of ants reared on fungus gardens or sucrose differed significantly from those of tetracycline-reared ants ($p < 0.001$ and $p = 0.013$, respectively; Figure S4), their communities were different, even though not significantly, from each other ($p = 0.053$). Furthermore, diet had the strongest effect on the differences between bacterial communities of treatment groups (PERMANOVA, $F_{2,53} = 9.162$, $p < 0.001$), while colony origin did not (PERMANOVA, $F_{3,53} = 1.758$, $p = 0.931$). When examining each of the abundant OTUs individually, *EntAcro1* and *RhiAcro1* decreased in tetracycline- and moderately in sucrose-treated individuals, while *wSinictaB* was largely unaffected by diet (Figures 3, S5a). *wSinictaA* (present only in colony C, Data set S2), *EntAcro2* and *EntAcro10* (present respectively in 9 and 20 of 37 tested untreated workers taken from fungus gardens; Data set S2), increased slightly in sucrose- and decreased in tetracycline-treated ants, but these effects were not significant. The head, thorax and gut microbial community comparisons showed that the most marked change were of the gut bacterial communities (detailed results in the Supplementary Information; Figure S5).

Gut bacterial communities changes (Round 2)

The NMDS ordination showed that the gut bacterial communities of tetracycline-treated individuals before and after faecal droplet feeding were not significantly different. Contrarily, the gut bacterial communities of sugar-treated individuals were closer to those of untreated workers (from original fungus gardens) after faecal droplet feeding (Round 1), suggesting a shift towards the original communities (Figure S5). Interestingly, the gut bacterial samples of sugar-treated

individuals exhibited a clear separation depending on which faecal droplets (nestmates or non-nestmates) they were fed on (PERMANOVA, $F_{1,43}=23.17$, $p<0.001$ and $F_{1,43}=12.10$, $p<0.001$, respectively and $F_{1,43}=5.04$, $p=0.004$ for their interaction), whereas there was no such effect in the tetracycline-treated group (Figure S5). Tetracycline-treated ants undergoing faecal droplet feeding showed an increase of all OTUs but *EntAcro2* and *RhiAcro1*, whose levels were further reduced; sucrose-treated ants showed an increase of all gut bacterial taxa examined (Figure 3), but only changes in *EntAcro1*, *EntAcro2*, *EntAcro10* and *wSinivictaB* (respectively: $t=-2.69$, $p=0.008$; $t=-2.02$, $p=0.044$; $t=-5.32$, $p<0.001$; $t=-5.95$, $p<0.001$) were significant.

Regression of bacterial changes and aggression

To identify which of the OTUs best explains aggression, and since the high variation both in aggression and gut microbiota composition precluded us from comparing mean effects, we regressed the observed aggression between nestmate test pairs on differences in abundance of their gut bacteria (Figure 4). Foremost of all, this analysis confirmed that more aggression was observed when nestmate pairs differed more in their gut microbial community (1500 MiSeq OTUs, using Bray-Curtis distances between pairs, binomial GLMM with 'distance' and 'round' as fixed variables and 'colony' as random variable, $z=4.91$, $p<0.001$). For individual taxa we used the more accurate qPCR data focusing on the main five taxa, regressing the observed aggression against their $\Delta\Delta Ct$ values for each of the six gut bacterial OTUs (Figure 4). This analysis showed that aggression was not positively affected by either of the two *Wolbachia* strains (Figure 4; *wSinivictaA*: $z=-0.66$, $p=0.509$; *wSinivictaB*: $z=-3.75$, $p<0.001$), or

367 *EntAcro2* ($z=-0.872$, $p=0.383$), but showed significant positive effects for
 368 *EntAcro1* ($z=2.10$, $p=0.035$), *EntAcro10* ($z=6.09$, $p<0.001$) and *RhiAcro1*
 369 ($z=5.85$, $p<0.001$). However, only differences in abundance of *RhiAcro1* had a
 370 strong effect on aggression in both rounds of the experiment (Figure 4; Data set
 371 S4; Round 1: $z=3.25$, $p<0.001$; Round 2: $z=4.17$, $p<0.001$), suggesting that
 372 Rhizobiales are a driving force in the recognition of nestmates (see Data set S4;
 373 similar results were obtained when both biting and mandible opening were
 374 treated as aggressive behaviors).

375

376 *Effects of diet on CHC profiles*

377 Compared to untreated individuals from original fungus gardens, sucrose- and
 378 tetracycline-treated individuals of Round 1 exhibited a strong reduction of 4-oxo-
 379 octanoic and 4-oxo-decanoic acids (LMMs with 'diet' and 'round' as fixed
 380 variables and 'colony' as random variable, FDR-corrected p-values for multiple
 381 comparisons; 4-oxo-octanoic acid: untreated vs. sucrose-treated, $t=15.20$,
 382 $p<0.001$, untreated vs. tetracycline-treated, $t=15.01$, $p<0.001$; 4-oxo-decanoic
 383 acid: untreated vs. sucrose-treated, $t=-13.10$, $p<0.001$, untreated vs. tetracycline-
 384 treated, $t=-13.66$, $p<0.001$). However, changes to these compounds were
 385 significant in all pairwise comparisons of all treatment groups, suggesting that it
 386 is more related to the isolation than the removal of bacteria (Data set S4). On the
 387 other hand, changes in n-C36 and n-C40 were only significant in pairwise
 388 comparisons of ants treated with antibiotics and ants reared on their original
 389 colonies.

390

391 Considering all experimental individuals of Rounds 1 and 2, we found a
 392 significant correspondence between chemical profiles (CHC) and qPCR data
 393 (RV=0.143, $P<0.001$; Figure S6). The OTUs *EntAcro1* and *RhiAcro1* co-varied
 394 the most with the two metapleural gland acids. There was a significant
 395 correspondence between chemical profiles and qPCR gut bacterial abundances
 396 also when the acids from the metapleural gland were excluded (RV=0.141,
 397 $p<0.001$). Contrarily, we found no significant correspondences when instead of
 398 using the individual CHC and qPCR profiles, we used each aggression pair as a
 399 single sample and for input we used the odour differences and $\Delta\Delta C_t$ values in
 400 aggression pairs (RV=0.122, $p=0.707$). This suggested that bacterial
 401 abundances can explain some of the changes in odour that occurred in the diet
 402 treatments (because there is a highly significant correlation between the two data
 403 sets), but not to such an extent that they accurately reflect odour differences
 404 between individuals that may lead to (a lack of) recognition.

405 Discussion

406 *Experimental treatment affects gut microbial communities*

407 The gut microbial communities of tetracycline-treated ants underwent the most
408 substantial deviation from those of untreated nestmates (two taxa decreased
409 strongly, three slightly and one was not affected; Figure 3), whereas those of
410 sucrose-treated ants exhibited a relatively milder shift (three taxa decreased
411 slightly, two increased slightly and one was not affected). These results show
412 that while the antibiotic suppressed most of the ant microbial community, the
413 sterile sucrose diet affected only mildly the various taxa, altering their relative
414 abundance.

415
416 Feeding workers with conspecific faecal droplets only partially restored the
417 original gut microbial communities of sucrose- and tetracycline-treated ants,
418 suggesting that the elimination of strains such as *RhiAcro1* and *EntAcro2* was
419 irreversible. This may imply that some bacteria need to be established during
420 larval development or early adult life and cannot be reintroduced later. In
421 addition, new OTUs emerged (i.e., *EntAcro10*) which may have prevented the
422 original OTUs from tissue re-colonization (cf., 46,47). We also cannot exclude
423 that the tetracycline effect lasted for some time even after its administration was
424 suspended (one week before the preparation of the corresponding microbial
425 DNA samples), potentially interfering with the faecal droplet-mediated microbial
426 gut re-colonization.

427
428 Although we provide evidence for which bacterial taxa are most affected by our
429 experimental treatments, amplicon sequencing often does not allow

distinguishing bacterial strains with identical 16S sequences (33,48,49). Thus, we cannot exclude the effect of gut bacteria on behaviour to be driven by the interaction of multiple bacterial strains with different metabolic potential, and further research should implement methods that allow considering this.

Microbiota remodelling affects ant cuticular chemical profiles

Gut bacteria play essential metabolic roles in several ant species, such as the production of metabolites that are absent in host diets or help cover energy needs (50–53), and bacterial removal may thus impair metabolism functions and ant wellbeing. Social withdrawal of unhealthy ant workers has been demonstrated for at least two ant species (54,55), and it is conceivable that the ostracism of the sick ants is driven by changes in CHCs (or other volatile compounds important in communication). We found consistent and significant decreases of two metapleural gland acids (4-oxo-octanoic and 4-oxo-decanoic) (56,57) in sucrose-treated ants, and reductions in these compounds and two linear alkanes (n-c36 and n-c40) in tetracycline-treated individuals. These chemical profile differences may have been caused by symbiotic gut bacteria affecting CHC biosynthesis, either directly by contributing to the CHC pool, or indirectly by affecting host metabolism and therefore host CHC production. This inference is further supported by the fact that CHCs are synthesized in the oenocytes (12), which are heavily colonized by *EntAcro1* bacteria (25) and are the centre of the intermediate metabolism in ants (58).

Tetracycline treatment may not only have affected the gut microbes, but also those living on the ant cuticle (such as the Actinobacteria *Pseudonocardia*

(24,34)). Such effects may result from the direct actions of the antibiotic or from indirect effects, such as changes in host metabolism or production of nutrients sustaining cuticular bacterial growth. In *Drosophila*, cuticular microbes have been hypothesized to affect chemical profiles by using CHC as a carbon source or a substrate for degradative enzymes (12), and previous research on *Pogonomyrmex* ants showed that topical antibiotic administration can alter CHC profiles, confirming a possible role of surface microbes in CHC profile determination (18). However, the same kind of antibiotic treatment applied on *Acromyrmex subterraneus* did not affect CHC profiles (19). Work on *Drosophila* suggests that the innate immune response may also mediate CHC profiles (59), and innate immune responses to bacteria affects CHC profiles in honey bees (60). Thus, multiple factors are conceivably involved and likely interact to form worker CHC profiles, and disentangling this complex of host-symbiont interactions and contributions will require extensive further work.

Although we provide correlational evidence for our dietary treatments to act on gut bacteria, which may indirectly affect metapleural gland acids and cuticular hydrocarbons via the metabolism, we cannot exclude other types of effects. Across insect taxa, symbiotic bacteria produce a plethora of volatile semiochemicals (61,62), and studies on *Drosophila* suggest compounds deriving from the metabolism of gut microbes to mediate interactions between individuals (15,63). Eusocial Hymenoptera may have integrated the bacterial metabolism in their chemical-based social dynamics, and ants may thus rely on a social communication system based on chemicals produced by its commensal bacteria. This would allow discriminating against unfamiliar odours from different bacterial communities and would be complementary to the well-studied CHC-mediated nestmate recognition system. Accordingly, we cannot exclude that the altered

behaviour observed in our aggression tests may at least partially depend on volatile semiochemicals from bacterial metabolism. In future experiments, nestmate recognition assays should be implemented in which microbiota-remodelled interacting individuals are prevented from relying on non-volatile cuticular social cues.

Microbiota remodelling affects social interactions

Encounters between antibiotic-treated and untreated nestmates produced the highest aggression levels, suggesting that the antibiotic treatment affects chemical cues that are relevant for social interactions. Aggression due to diet treatment was clearly higher than aggression due to nestmate status and the treatment effect to aggression was directly dependant to the gut bacterial titre: the diet effect on aggression was always clear in sucrose-treated ants but tend to diminish (contrasts were less clear) in tetracycline-treated ants (Figure 2A: nestmate (NM) and non-nestmate (nNM) aggression levels of Tetracycline vs Tetracycline treated ants were almost identical). This further suggested that the gut bacterial communities (or certain taxa) are having an active role in nestmate recognition which possibly disappears when these bacterial symbionts get eliminated.

The impact of partial restoration of the gut microbiota on social interactions

After faecal droplets administration, nestmate dyadic encounters revealed aggression levels only moderately (and non-significantly) higher than those of Round 1. While this moderate increase in aggression may be an effect of the

relatively longer separation of the experimental ant groups (three instead than two weeks in different petri dishes), the lack of significant differences between rounds is consistent with only partial restoration of microbial gut communities. In non-nestmate aggression assays, pairs of tetracycline-treated and tetracycline- vs. sucrose-treated ants again showed low aggression. In contrast, aggression was high in sucrose-treated pairs when interacting individuals were fed with faecal droplets from different colonies. This implies that, although bacterial communities were only partially restored, colony-specific faecal droplets did affect the cues determining behavioural outcomes of the social interactions. This partial restoration also rules out the possibility of changes in behaviour due a direct effect of tetracycline, which is known to impair mitochondrial function and thus potentially could induce confounding effects (64).

517

518 **Conclusions**

In this study, we seek to take an integrative approach to explore the role of gut microbial symbionts in the social dynamics of a eusocial organism. Remodelling of the ant gut microbiota produced effects on both the chemicals ants display as socially-relevant recognition signals and their resulting behaviours. Our findings suggest that the observed effects mostly depend on two bacterial taxa, the previously identified major gut symbionts of leafcutting ants *EntAcro1* and *RhiAcro1*. Further research will be needed to address the mechanisms underlying the link between these symbionts and behavioural modifications. Either the altered microbial communities may result in chemical profiles that cause individuals to look more like non-nestmates, or microbiota-remodelled ants may be recognized as sick, and aggression towards such individuals could serve

530 to prevent the spread of infections to optimize colony health and efficiency.
 531 Regardless, our findings provide evidence that gut bacterial symbionts may be
 532 involved in kin recognition by contributing to shape ant CHC signatures, with
 533 implications for our understanding of social insect-symbiont evolution.

534 Figures

535
 536 **Figure 1. Experimental design.** Ants were collected from fungus gardens (FG)
 537 and kept for two weeks on a sucrose (S) or Tetracycline (T) diet (Round 1). After
 538 two weeks we performed aggression trials among the three treatment groups.
 539 Individual guts were then dissected for gut bacterial characterization (16S-qPCR
 540 and 16S-MiSeq), whereas thoraxes and heads were used for chemical

541 extractions and subsequent GC-MS analyses. In Round 2, the remaining
542 individuals were fed for one week on faecal droplets (fd) either from their original
543 nestmates (NM) or non-nestmates (nNM) from a different colony. These ants
544 were then tested in aggression trials similar to Round 1 and collected for gut
545 microbiota characterization and GC-MS analyses.

546

Figure 2. Effects of sucrose and tetracycline treatment on aggression.

Mean biting/antennation ratio, with upper and lower confidence limits as error bars. **A.** Round 1. Tetracycline-treated vs. untreated nestmate ant pairs show the highest aggression levels, surpassing non-nestmate trials. **B.** Round 2. Encounters between individuals fed on faecal droplets. In nestmate trials, tetracycline-treated vs. untreated individuals still show the highest aggression. Letters indicate significance in pairwise comparisons (different letters = statistically significant differences). Red font indicates comparisons within treatment category (e.g. sucrose vs sucrose, tetracycline vs tetracycline) whereas black font shows comparisons across treatment categories (e.g. trials between sucrose-treated nestmates compared to trials between tetracycline-

559 treated nestmates). In non-nestmate encounters, sucrose-treated individuals fed
 560 with faecal droplets from different colonies ($\neq$) show the highest aggression
 561 levels, whereas non-nestmates fed with faecal droplets from the same colonies
 562 (=) exhibit low aggression. NM = nestmates; nNM = non-nestmates; FD= Faecal
 563 droplets. See Table S2 for details on statistical comparisons among treatment
 564 groups.

565

566 **Figure 3. A. qPCR bacterial titres fold change of the six most abundant**
567 **bacteria after experimental Rounds 1 and 2.** Fold change is normalized to ant
568 EF-1 α copies, and the microbial strain levels of untreated conspecifics (ants
569 reared on FG) are used as reference level ($y=0$). Scale bars represent standard
570 errors. Asterisks indicate significance levels, $p < 0.05$. Yellow: sucrose-treated;
571 green bars: tetracycline-reared ants.

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Figure 4. A. Association between aggression level and differences in the abundant gut bacteria using GLMMs. The relationship between biting frequency and bacterial abundances measured using the qPCR fold change (Ct distance) for both rounds for all six or for individual bacterial taxa. Curves represent predicted values from the models. Asterisks indicate significance levels: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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