The scent of symbiosis: gut bacteria affect social interactions in leafcutting ants

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Abstract

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

Keywords: gut microbiota, *Acromyrmex echinatior*, social evolution, cuticular hydrocarbons

Introduction

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

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

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

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

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

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

Material and methods

Experimental design

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

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

Aggression assays

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

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

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

DNA extractions

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

16S rRNA qPCR analyses

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

189 (*wolAcro1,* including two strains: *w*SinvictaA and *w*SinvictaB) 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-1α) 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-1α
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

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

16S rRNA MiSeq analyses

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

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

Cuticular hydrocarbon analyses

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

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

Results

Survival analysis

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

Aggression tests (Round 1)

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

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

317 the highest variance (HOMOVA, p<0.001; Figure S4). While gut bacterial
318 communities of ants reared on fungus gardens or sucrose differed significantly
319 from those of tetracycline-reared ants (p<0.001 and p=0.013, respectively;
320 Figure S4), their communities were different, even though not significantly, from
321 each other (p=0.053). Furthermore, diet had the strongest effect on the
322 differences between bacterial communities of treatment groups (PERMANOVA,
323 $F_{2,53}$=9.162, p<0.001), while colony origin did not (PERMANOVA, $F_{3,53}$=1.758,
324 p=0.931). When examining each of the abundant OTUs individually, *EntAcro1*
325 and *RhiAcro1* decreased in tetracycline- and moderately in sucrose-treated
326 individuals, while *w*SinvictaB was largely unaffected by diet (Figures 3, S5a).
327 *w*SinvictaA (present only in colony C, Data set S2), *EntAcro2* and *EntAcro10*
328 (present respectively in 9 and 20 of 37 tested untreated workers taken from
329 fungus gardens; Data set S2), increased slightly in sucrose- and decreased in
330 tetracycline-treated ants, but these effects were not significant. The head, thorax
331 and gut microbial community comparisons showed that the most marked change
332 were of the gut bacterial communities (detailed results in the Supplementary
333 Information; Figure S5).
334
335 *Gut bacterial communities changes (Round 2)*
336 The NMDS ordination showed that the gut bacterial communities of tetracycline-
337 treated individuals before and after faecal droplet feeding were not significantly
338 different. Contrarily, the gut bacterial communities of sugar-treated individuals
339 were closer to those of untreated workers (from original fungus gardens) after
340 faecal droplet feeding (Round 1), suggesting a shift towards the original
341 communities (Figure S5). Interestingly, the gut bacterial samples of sugar-treated

342 individuals exhibited a clear separation depending on which faecal droplets
343 (nestmates or non-nestmates) they were fed on (PERMANOVA, $F_{1,43}$=23.17,
344 p<0.001 and $F_{1,43}$=12.10, p<0.001, respectively and $F_{1,43}$=5.04, p=0.004 for their
345 interaction), whereas there was no such effect in the tetracycline-treated group
346 (Figure S5). Tetracycline-treated ants undergoing faecal droplet feeding showed
347 an increase of all OTUs but *EntAcro2* and *RhiAcro1*, whose levels were further
348 reduced; sucrose-treated ants showed an increase of all gut bacterial taxa
349 examined (Figure 3), but only changes in *EntAcro1*, *EntAcro2*, *EntAcro10* and
350 *w*SinvictaB (respectively: t=-2.69, p=0.008; t=-2.02, p=0.044; t=-5.32, p<0.001;
351 t=-5.95, p<0.001) were significant.
352
353 *Regression of bacterial changes and aggression*
354 To identify which of the OTUs best explains aggression, and since the high
355 variation both in aggression and gut microbiota composition precluded us from
356 comparing mean effects, we regressed the observed aggression between
357 nestmate test pairs on differences in abundance of their gut bacteria (Figure 4).
358 Foremost of all, this analysis confirmed that more aggression was observed
359 when nestmate pairs differed more in their gut microbial community (1500 MiSeq
360 OTUs, using Bray-Curtis distances between pairs, binomial GLMM with
361 'distance' and 'round' as fixed variables and 'colony' as random variable, z=4.91,
362 p<0.001). For individual taxa we used the more accurate qPCR data focusing on
363 the main five taxa, regressing the observed aggression against their ΔΔCt values
364 for each of the six gut bacterial OTUs (Figure 4). This analysis showed that
365 aggression was not positively affected by either of the two *Wolbachia* strains
366 (Figure 4; *wSinvictaA*: z=-0.66, p=0.509; *wSinvictaB*: 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 ΔΔCt 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.

Discussion

Experimental treatment affects gut microbial communities

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

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

Although we provide evidence for which bacterial taxa are most affected by our 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

481 behaviour observed in our aggression tests may at least partially depend on
482 volatile semiochemicals from bacterial metabolism. In future experiments,
483 nestmate recognition assays should be implemented in which microbiota-
484 remodelled interacting individuals are prevented from relying on non-volatile
485 cuticular social cues.
486

487 *Microbiota remodelling affects social interactions*

488 Encounters between antibiotic-treated and untreated nestmates produced the
489 highest aggression levels, suggesting that the antibiotic treatment affects
490 chemical cues that are relevant for social interactions. Aggression due to diet
491 treatment was clearly higher than aggression due to nestmate status and the
492 treatment effect to aggression was directly dependant to the gut bacterial titre:
493 the diet effect on aggression was always clear in sucrose-treated ants but tend to
494 diminish (contrasts were less clear) in tetracycline-treated ants (Figure 2A:
495 nestmate (NM) and non-nestmate (nNM) aggression levels of Tetracycline vs
496 Tetracycline treated ants were almost identical). This further suggested that the
497 gut bacterial communities (or certain taxa) are having an active role in nestmate
498 recognition which possibly disappears when these bacterial symbionts get
499 eliminated.
500

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

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

505 relatively longer separation of the experimental ant groups (three instead than
506 two weeks in different petri dishes), the lack of significant differences between
507 rounds is consistent with only partial restoration of microbial gut communities. In
508 non-nestmate aggression assays, pairs of tetracycline-treated and tetracycline-
509 vs. sucrose-treated ants again showed low aggression. In contrast, aggression
510 was high in sucrose-treated pairs when interacting individuals were fed with
511 faecal droplets from different colonies. This implies that, although bacterial
512 communities were only partially restored, colony-specific faecal droplets did
513 affect the cues determining behavioural outcomes of the social interactions. This
514 partial restoration also rules out the possibility of changes in behaviour due a
515 direct effect of tetracycline, which is known to impair mitochondrial function and
516 thus potentially could induce confounding effects (64).
517

518 ## Conclusions

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

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

Figures

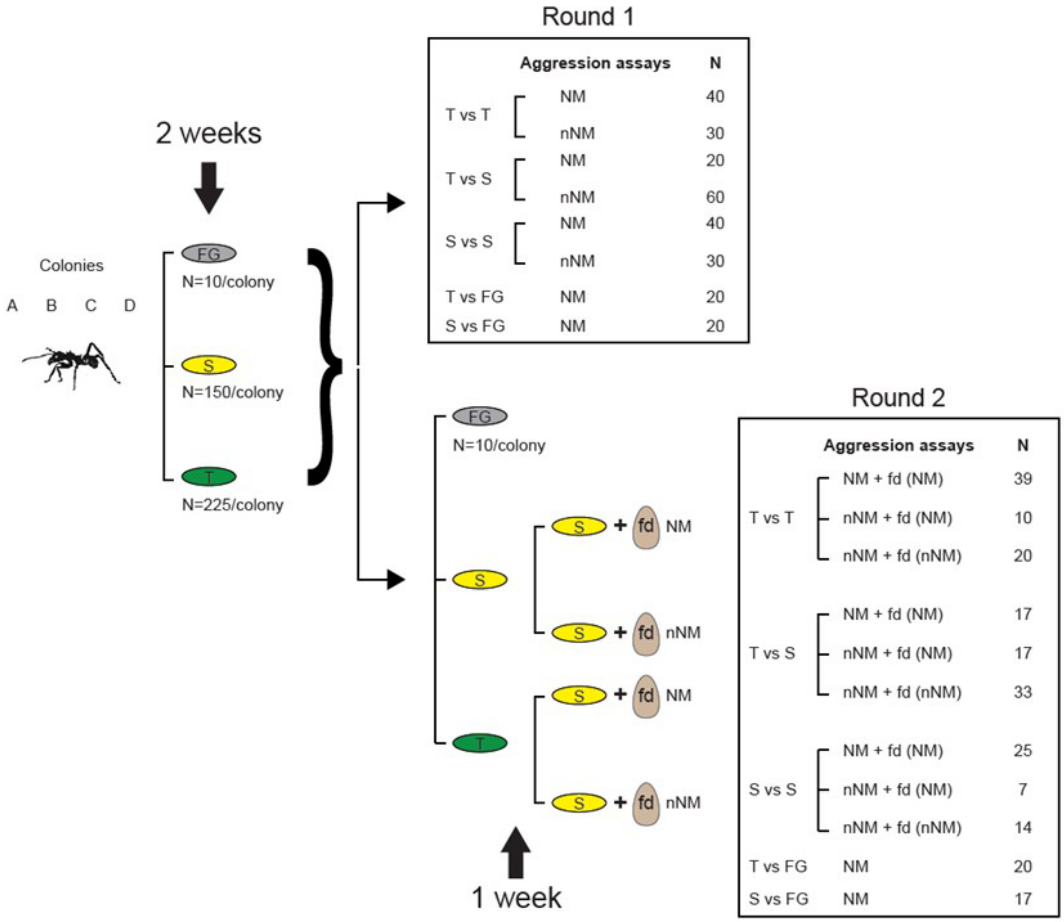


Figure 1. Experimental design. Ants were collected from fungus gardens (FG) and kept for two weeks on a sucrose (S) or Tetracycline (T) diet (Round 1). After two weeks we performed aggression trials among the three treatment groups. Individual guts were then dissected for gut bacterial characterization (16S-qPCR 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

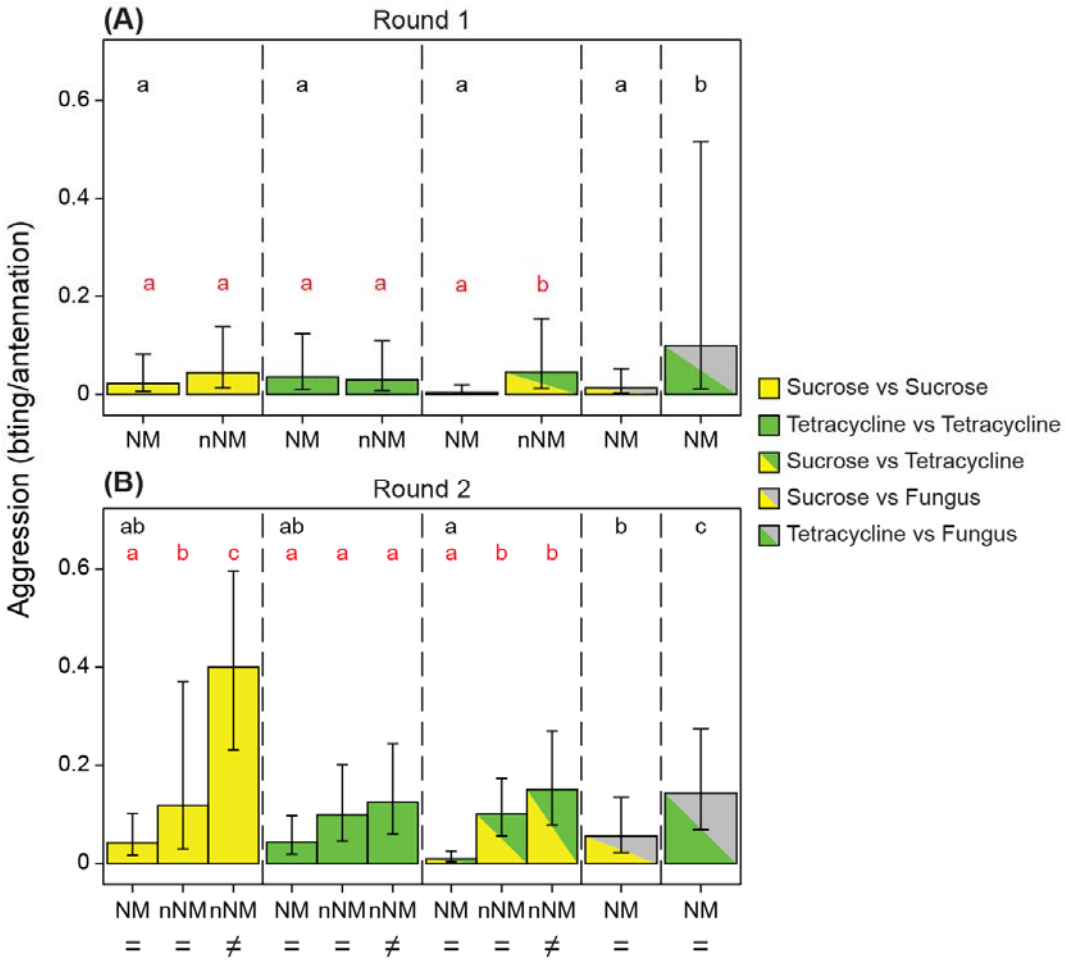
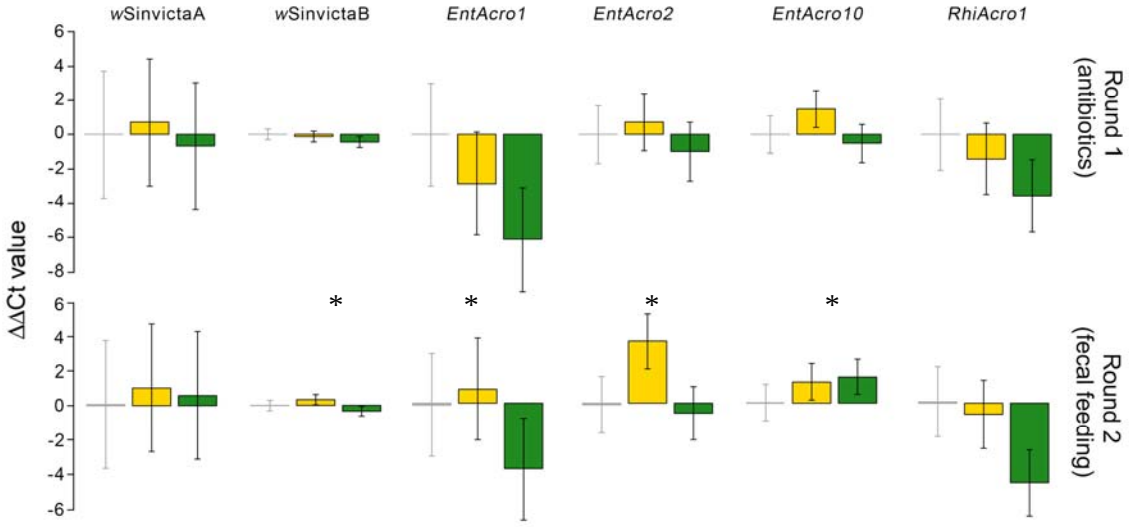


Figure 2. Effects of sucrose and tetracycline treatment on aggression. Mean biting/antennation ratio, with upper and lower confidence limits as error bars. **A.** Round 1. Tetracycline-treated vs. untreated nestmate ant pairs show the highest aggression levels, surpassing non-nestmate trials. **B.** 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-1α 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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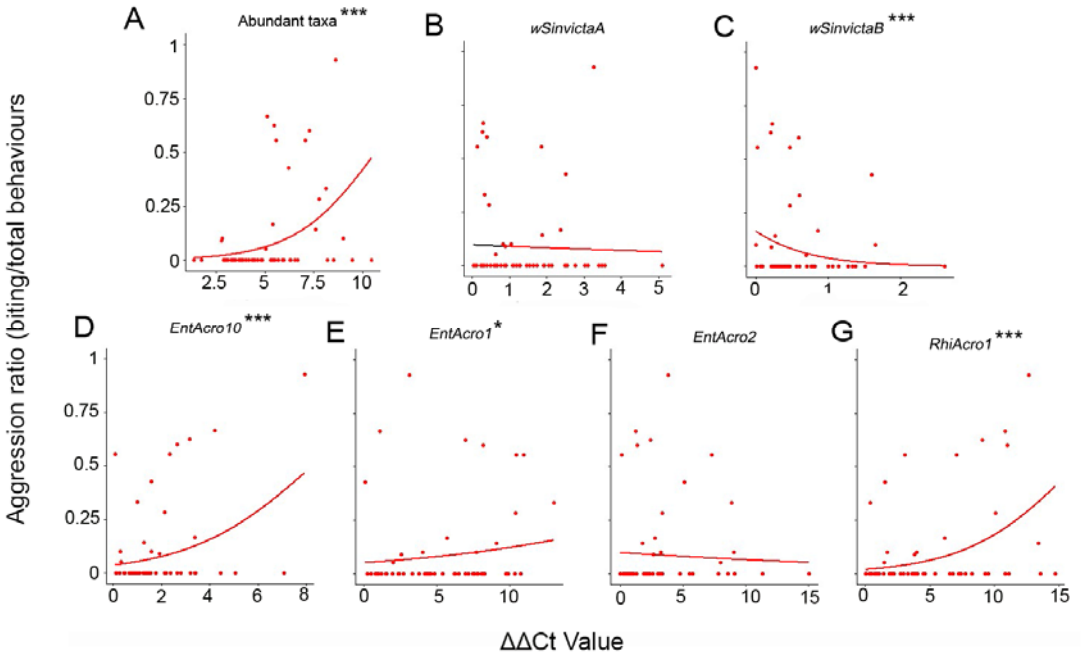

Figure 4. A. Association between aggression level and differences in the abundant gut bacteria using GLMMs. The relationship between biting frequency and bacterial abundances measured using the qPCR fold change (Ct distance) for both rounds for all six or for individual bacterial taxa. Curves represent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

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