The scent of symbiosis: gut bacteria may affect social interactions in leaf-cutting ants

Serafino Teseo, Jelle van Zweden, Luigi Pontieri, Pepijn Kooij, Søren Sørensen, Tom Wenseleers, Michael Poulsen, Jacobus Boomsma, Panagiotis Sapountzis

The scent of symbiosis: gut bacteria may affect social interactions in leaf-cutting ants

Serafino Teseo[1,2]*, Jelle S. van Zweden[3]*, Luigi Pontieri[4]*, Pepijn W. Kooij[4,5], Søren J. Sørensen[6], Tom Wenseleers[3], Michael Poulsen[4], Jacobus J. Boomsma[4] & Panagiotis Sapountzis[4]

[1] Unité de Biologie Fonctionnelle et Adaptative (BFA) UMR8251 - CNRS - Université Diderot, Sorbonne Paris Cité, 4 Rue Marie Andrée Lagroua Weill Hallé, 75013, Paris, France.

[2] School of Biological Sciences, Nanyang Technological University, Singapore, 637551, Singapore

[3] Laboratory of Socioecology and Social Evolution, Department of Biology, KU Leuven, Naamsestraat 59, 3000, Leuven, Belgium

[4] Centre for Social Evolution, Section for Ecology and Evolution, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen East, Denmark.

[5] Comparative Fungal Biology, Department of Comparative Plant and Fungal Biology, Royal Botanic Gardens, Kew, Richmond, TW9 3DS, United Kingdom

[6] Section of Microbiology, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen East, Denmark.

* These authors contributed equally

Correspondence: S. Teseo, School of Biological Sciences, Nanyang Technological University, Singapore, 637551, Singapore; P. Sapountzis, Centre for Social Evolution, Section

for Ecology and Evolution, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen East, Denmark.

Email addresses: steseo@ntu.edu.sg (Serafino Teseo); sapountzis@bio.ku.dk (Panagiotis Sapountzis)

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 therefore 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 16S rRNA-based community profiling, 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 bacterial suppression may alter chemical social cues and triggers aggressive behaviour. Chemical analyses of 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. Non-nestmates fed with faecal droplets from different colonies were unusually aggressive compared to pairs fed with faecal droplets from the same colony. We cannot exclude confounding effects resulting from the potentially harmful action of antibiotics on ant hosts. However, our results suggest a correlation between chemical profiles and the presence of certain microbial strains, which may affect nestmate recognition and division of labour. This opens novel questions about the role of symbiotic microorganisms in the evolution of social behaviour.

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

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 (Archie & Theis, 2011; Ezenwa, Gerardo, Inouye, Medina, & Xavier, 2012; Dance, 2014; Cryan & Dinan, 2012; Archie & Tung, 2015). 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 (Clarke et al., 2013; Heijtz et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011) and social interactions (Buffington et al., 2016). Similarly, an increasing corpus of *Drosophila* studies suggests that gut microbes mediate a plethora of physiological/behavioural processes, including specific appetites for proteins (Leitão-Gonçalves et al., 2017) mate choice and mating dynamics (Sharon et al., 2010; Ringo, Sharon, & Segal, 2011; Arbuthnott, Levin, & Promislow, 2016; Morimoto, Simpson, & Ponton, 2017) and the recognition of kin and familiar individuals (Lizé, McKay, & Lewis, 2014).

The increasing awareness of the behavioural role of microbes begs questions about how microorganisms may affect the behavioural ecology of animals with advanced superorganismal colony life. Such insects, the ants, corbiculate bees, vespine wasps and higher termites, live in family groups with permanent division of reproductive labour, unmated worker castes, and sophisticated chemical

communication (Gadau & Fewell, 2009). At the same time, the collective resource acquisition behaviours of the workers are reminiscent of differentiated somatic cells building a metazoan body, but now elevated to the level of organized collective foraging, brood nursing, nest construction and colony defence (Wheeler, 1928; Boomsma & Gawne, 2017). Across taxa, much of the maintenance of collective social integrity and sustainable infrastructure is ensured by chemically mediated nestmate recognition systems, which rely primarily on cues emanating from long-chain hydrocarbons embedded in the waxy layer of the insect cuticle (henceforth cuticular hydrocarbons, or CHC).

Scattered studies have provided evidence for bacterial ecto- or endo- symbionts (i.e., symbionts living outside or inside the body of the host both intra- and extra-cellularly (Bourtzis & Miller, 2003)) potentially affecting CHC-mediated behaviours, but without producing a coherent, general consensus. *Reticulitermes speratus* termites fed with bacteria extracted from other termites of unrelated colonies were attacked by their nestmates, and manipulation of bacterial communities with antibiotics also affected nestmate recognition behaviour (Matsuura, 2001). In contrast, workers of *Pogonomyrmex barbatus* harvester ants with experimentally augmented cuticular microbiomes were more often rejected by nestmates, but workers smeared with antibiotics were not discriminated against. This suggested that cuticle-dwelling microbes might influence nestmate recognition dynamics (Dosmann, Bahet, & Gordon, 2016). However, smearing the cuticles of workers of the leaf-cutting ant *Acromyrmex subterraneus subterraneus* with antibiotics did not affect cuticular hydrocarbon profiles (de Souza et al., 2013). Similarly, a study on *Camponotus fellah* carpenter ants showed that workers with high titres of *Blochmannia* intracellular

endosymbionts had rather low overall quantities of CHCs but with the same relative proportions of the composing compounds (de Souza, Devers, & Lenoir, 2011). Finally, feeding workers of the Argentine ant *Linepithema humile* with antibiotics affected their interspecific social interactions but not the behaviours expressed towards workers of other colonies of their own species, suggesting that bacterial endosymbionts do not significantly affect nestmate recognition in this ant species (Lester, Sébastien, Suarez, Barbieri, & Gruber, 2017).

Despite providing insights in the possible roles of bacterial symbionts in CHC-mediated nestmate recognition, previous studies either investigated microbial effects with behavioural tests, but without measuring changes in CHC profiles (Matsuura, 2001, Lester et al., 2017), or they compared CHC profiles between antibiotic-treated and control workers without monitoring the correlated social behaviours (de Souza et al., 2011; de Souza et al., 2013; Dosmann et al., 2016). To obtain more encompassing insights in the interplay between gut microbiota and chemically mediated social interactions, we performed an integrated analysis of all three variables: CHC profiles, gut bacterial communities, and nestmate recognition behaviour. We experimentally changed the previously characterized gut microbiota of *Acromyrmex echinatior* leaf-cutting ants (Van Borm, Billen, & Boomsma, 2002; Sapountzis et al., 2015) to investigate correlated changes in gut bacterial communities and socially relevant chemical cues. In a first set of experiments (Round 1), we either mildly or strongly suppressed the native gut microbiota of *A. echinatior* workers using, respectively, a sterile sucrose diet or an antibiotics diet. We then measured the changes in the abdominal microbial communities of worker ants and cuticular chemical profiles and used dyadic aggression trials to evaluate associated changes in aggressive

behaviour. In a second set of experiments (Round 2), we partially restored the original abdominal microbial communities of these worker ants and measured the extent to which changes in CHC-profiles and aggression could be reversed. To achieve these effects, we fed experimentally manipulated worker ants with faecal droplets of untreated conspecifics and analysed their abdominal microbial communities, chemical profiles and aggression behaviours.

Material and methods

Experimental design

We used workers from four *A. echinatior* colonies (Ae150, Ae322, Ae153 and Ae331, hereafter named A, B, C and D, respectively) with an already characterized abdominal microbial community (Andersen, Hansen, Sapountzis, Sørensen, & Boomsma, 2013; Sapountzis et al., 2015) 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 liquid food vial. For each colony, we randomly assigned workers to two treatment groups: 1) ants kept on a sterile 10% sucrose solution diet (150 individuals/colony); and 2) ants kept on the same sucrose solution supplemented with 1mg/ml of the antibiotic tetracycline (225 individuals/colony). After two weeks, 522 workers were tested in a first experimental series (Round 1) including aggression assays and analyses of CHCs (GC-MS, see below) and gut microbiota (qPCR and MiSeq, see below). The remaining workers were used in the second experimental series (Round 2), where we attempted to restore their original microbiota by feeding them with 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 faecal fluid from nestmate workers, whereas colony A and C workers received faecal fluid from non-nestmate workers (i.e. 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 (Figure A1).

Finally, we set up an independent smaller experiment, within which we compared the microbial communities of guts, heads and thoraxes of a subset of workers from all treatment groups, before and after the experimental diet treatment. This experiment used workers from the same ant colonies as in our main experiment and in an identical setup. The only difference was that we used heads and thoraxes instead of guts for bacterial characterization. This aimed to make sure we had not overlooked significant microbial presences in other body parts (Figure A2).

Aggression assays

We tested sucrose- and antibiotic-treated ants from round 1, microbiota-remodelled ants from round 2 and untreated ants against nestmates and non-nestmates from the original colonies (3-5 replicates/combination, total=260 tests in Round 1 and 231 tests in Round 2; 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 (Ottoni, 2000) to quantify the frequency and duration of biting, mandible opening, antennation and absence of contact (Figure A3). For statistical analyses, we excluded the mandible opening behaviour (usually considered as aggressive) because pooling it with biting produced very similar results (data not shown). 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_{141}=3.21, *P*=0.001), we considered only interactions between nestmates kept in different Petri dishes (a total of 220 dyadic encounters for experimental Round 1 and 157 for Round 2).

We analysed data in R using the packages *lme4*, *car* and *multcomp* (Fox & Weisberg, 2011 ; Bates, Mächler, Bolker, & Walker, 2015; Hothorn, Bretz, & Westfall, 2008), 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 statistical model included biting *versus* antennation frequencies as the response variable, the factors nestmate relationship, diet treatment and their interaction were included as fixed explanatory variables, and colony affiliation of the two experimental individuals were included as random effects. The colony origin of experimental individuals was included as random effect term. The model used for Round 2 included the same response variable as in the Round 1 model, and 'faecal droplets' was included there 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 for false discovery rate (FDR).

Effects of diet treatments on survival

We analysed the effect of diet on survival with Cox proportional hazards models (with censoring), using the R package *Survival* (version 3.1.1, (Therneau, 2015)) function 'coxph', following assessment of proportional hazards using 'cox.zph'. Survival in the two experimental rounds was evaluated separately, because the

diets, which were used as cofactor affecting the mortality of ants, changed from Round 1 to Round 2. The other cofactors were colony origin and the tetracycline diet for Round 1 and the colony origin and whether the ants had previously been treated with tetracycline in Round 2. Data were plotted using the survival and the GGally packages in R (Schloerke et al., 2014; Therneau, 2015).

DNA extractions

Four to eight workers per treatment per colony that had been used in the aggression assays were ice anesthetized and individually dissected in sterile Phosphate Buffered Saline (PBS). For each dissected worker, pooled crop, midgut, hindgut, Malpighian tubules and fat body cells were stored at -20°C until DNA extractions. Malpighian tubules and fat body cells are not part of the intestinal system, but similar to a previous study (Sapountzis et al., 2015), we define the gut microbiomes broadly as the bacterial communities associated with the intestinal system consisting of the digestive tract and associated organs – also referred to as abdominal bacterial communities. For the DNA extractions, we immediately homogenized the frozen samples using sterile pestles 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 were 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 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) (Van Borm et al., 2002; Sapountzis et al., 2015; Zhukova, Sapountzis, Schiøtt, & Boomsma, 2017). These belong to the genus *Wolbachia* (*WolAcro1,* including two strains: *w*SinvictaA and *w*SinvictaB) and the orders Entomoplasmatales (class: Mollicutes, OTUs *EntAcro1*, *EntAcro2* and *EntAcro10*) and Rhizobiales (class: Alpha-Proteobacteria, OTU *RhiAcro1* (Sapountzis et al., 2015)). In order to monitor how the dietary treatment affected the communities, we screened individual worker guts and associated organs with qPCR (detailed methods in Table A1 and Supplementary Information) on the five most abundant OTUs (Data set S2, procedures described in Sapountzis et al., 2015) with Cycle threshold (Ct) means of replicated samples as measures of amplicon abundance. The elongation factor 1 alpha (EF-1α) was used as a reference gene (Andersen, Yek, Nash, & Boomsma, 2015). Each run included two negative controls with no added template for each gene used. Data were ordinated using an unscaled principal coordinate analysis (PCoA) and inter-sample distances were calculated using Canberra, Hellinger and Bray-Curtis methods. For analysis, we initially used a standard curve with PCR products in tenfold dilution series of known concentration (fold change method) to calculate the PCR efficiency using the REST software (Pfaffl, Horgan, & Dempfle, 2002). Data were imported in R and expressed as $\Delta\Delta C_T$ values, i.e. as the fold change relative to the EF-1α control gene (Pfaffl, 2004), always using zero as reference. We used linear mixed models (LMM) with the $\Delta\Delta C_T$ values as response variable, ‘diet treatment’ (i.e., untreated, sugar-treated or tetracycline-treated) and

'experimental round' (i.e. Round 1 or 2) as fixed variables, and 'colony' (A, B, C or D) as random variable. Tukey post hoc tests were performed to evaluate significant differences between groups. We used GLMs to pair aggression data with distances calculated using $\Delta\Delta C_T$ values fold change differences, or with absolute bacterial cell numbers obtained from the qPCR data. All correlation analyses were performed in R using the packages lme4, vegan, effects, Rmisc and ggplot2 (Hope, 2013; Bates et al., 2015; Wickham, 2016; Oksanen et al., 2017; Fox et al., 2017).

16S rRNA MiSeq sequencing and 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 (Caporaso et al., 2012). The PCR mix (20µl) contained: 2.0µl 10x AccuPrime™ PCR Buffer II (15mM MgCl2, Life Technologies), 0.15µl AccuPrime™ Taq DNA Polymerase (2 units/µl, Life Technologies), 1.0 µl of each primer (10µM), 2µl diluted template and water to a total of 20µl. PCR incubation conditions were an initial activation of the hotstart polymerase at 94°C for 2min, followed by 30-35 cycles of 94°C for 20s, 56°C for 20s and 68°C for 30s, and a final extension step at 68°C for 5min. Samples were incubated at 70°C for 3min, and then moved directly to ice to minimize hybridization between specific PCR products and short nonspecific amplicons. To add adapters and Index to DNA fragments, we added 2µl of the diluted PCR products to the PCR mix to amplify ca. 458 bp fragments using different combinations of primers presented in Table A1. PCR was performed for 15 cycles and PCR products were 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 on an Illumina MiSeq.

Data [Genbank: SAMN04261407 - SAMN04261536 and SAMN05362797 - SAMN05362832] were analysed using mothur (Schloss et al., 2009) with two adjustments to the MiSeq standard operating procedure (SOP) due to slight differences to the sequencing protocol. First, after assembling the sequences we used the 'make.contigs' command in the software cutadapt (Martin, 2011) to remove the primer sequences, and continued with the mothur pipeline using the output; for the 'screen.seqs' command the start and the end were at 13862 and 23444 (see MiSeq SOP page). 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 per 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. Correlation analyses were performed in R v3.2.3 using the lme4, vegan, effects, Rmisc and ggplot2 packages (Hope, 2013; Bates et al., 2015; Wickham, 2016; Oksanen et al., 2017; Fox et al., 2017). PERMANOVA tests were done in PAST using 1000 permutations and group differences were evaluated using Bonferroni corrected

multiple comparisons. We estimated the difference in variation among groups using the HOMOVA command (with Bonferroni-adjusted alpha-values) implemented in mothur (Schloss et al., 2009).

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). The injection temperature was 280°C and we used a DB-5ms capillary column (30m × 0.25mm × 0.25µm) with helium as the carrier gas at 1ml min^{-1}. The oven temperature was held at 70°C for 1 min, then increased to 220°C at 25°C min^{-1}, and then to 325°C at 3°C min^{-1}, with a final hold for 15min at 325°C. 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 (Aitchison, 1986). To compare odour profiles among different rounds, we used linear mixed models (LMM) with the Z-transformed abundance of each compound as the response variable, 'diet treatment' and 'experimental round' as fixed variables, and 'colony' as a random variable. We considered linear hypotheses using the *multcomp* R package (Hothorn, Bretz, & Westfall, 2008) function glht to evaluate differences between

diet treatments in the same experimental 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 the package *ade4* to perform coinertia analysis (Dolédec & Chessel, 1994) to check for correlations between CHCs (log-transformed peak areas) and qPCR measures (ΔΔCt values) of the six bacterial OTUs considered (see also details 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 in a Monte-Carlo test with 10000 permutations.

Results

Survival analysis

During the Round 1 experiments, mortality increased in tetracycline-treated workers (Cox proportional hazard model, $P<0.001$), similar to what had been observed in a previous study (Sapountzis et al., 2015). However, this effect disappeared in Round 2, when all ants were fed on faecal droplets (Figure A1), suggesting that the harmful effect of tetracycline lasted only as long as it was administered to the ants.

Round 1, aggression tests

When ants were isolated from their original colonies and fed on sterile sucrose diets with or without antibiotics (Figure 1), the diet treatment had a significant effect on aggression ($\chi^2_4=29.62$, $P<0.001$), whereas being nestmates or non-nestmates did not make a significant difference for dyadic aggression ($\chi^2_1=1.64$, $P=0.18$). We also found a significant statistical interaction between diet treatment and nestmate vs non-nestmate interactions ($\chi^2_2=6.9803$, $P=0.0305$) indicating that the strength of the diet effect differed depending on whether nestmates or non-nestmates interacted. The main effect of diet appeared to be mostly due to the high biting frequency between tetracycline-treated ants and their untreated former nestmates taken from the same fungus gardens, which was significantly higher than biting rates in all other nestmate trials (all $P<0.05$, Figure 2(a), Table A2). However, for tetracycline-treated and sucrose-treated ants, the aggression levels between non-nestmates were not significantly different from those observed in nestmate trials (tetracycline-treated: $z=0.365$, $P=0.715$, Figure 2(a));

sucrose-treated: z=1.284, *P*=0.287). This probably explains the significant overall interaction term between diet and (non)nestmate identities, also because the non-nestmate trials showed higher aggression levels than the nestmate trials in the sucrose- vs. tetracycline-treated groups (z=2.375, *P*=0.045).

Round 1, changes in gut bacterial communities

As expected, the microbiomes of tetracycline-treated individuals were strongly affected, showing the lowest variance among OTUs because several of those were virtually eliminated; on the other hand, untreated individuals collected from fungus gardens showed the highest variance (HOMOVA, *P*<0.001; Figure 3). The gut bacterial communities of ants reared on fungus gardens or sucrose diets differed significantly from those of tetracycline-reared ants (PERMANOVA, multiple corrected Bonferroni comparisons; *P*<0.001 and *P*=0.013, respectively; Figure 3), but their communities were not significantly different in one-to-one comparison (*P*=0.053). Furthermore, while diet had a strong effect on the bacterial communities of treatment groups (PERMANOVA, $F_{2,53}$=9.162, *P*<0.001), colony identity was not important (PERMANOVA, $F_{3,53}$=1.758, *P*=0.931).

The most abundant OTUs of the *A. echinatior* gut microbiota are *WolAcro1* (genus *Wolbachia*, including two strains: *w*SinvictaA and *w*SinvictaB) whose role in attine biology is yet unknown (Andersen, Boye, Nash, & Boomsma, 2012), the Mollicutes *EntAcro1*, *EntAcro2* and *EntAcro10*, which are the most abundant bacteria in the guts and surrounding tissues that may be trophic mutualists (Sapountzis et al., 2015; Zhukova, Sapountzis, Schiøtt, & Boomsma, 2017), and

RhiAcro1 (order Rhizobiales, class: Alpha-Proteobacteria), which has been suggested to complement the ants' nitrogen limited diet (Sapountzis et al., 2015). When examining each of these OTUs individually, *EntAcro1* and *RhiAcro1* decreased substantially in the tetracycline treated workers and moderately so in the sucrose-treated workers, while the *Wolbachia* OTU *w*SinvictaB was largely unaffected by diet (Figure 4). *w*SinvictaA (present only in colony C, Data set S2), *EntAcro2* and *EntAcro10* (present respectively in 9 and 20 of the 37 tested untreated workers taken from fungus gardens; Data set S2), increased slightly in sucrose-treated workers but decreased in the tetracycline-treated ants, but these effects were not significant. Our comparison of microbial communities of heads, thoraces and guts revealed that the most pronounced changes associated with different diets occurred in the guts and related abdominal tissues. In the gut samples a few distinct and abundant OTUs (mainly Mollicutes and Alpha-Proteobacteria), underwent major reductions by the antibiotics diet treatment, while the head and thorax samples showed minor effects on a larger range of other OTUs (including Alpha-Proteobacteria, Actinobacteria, Sphingobacteria, Beta-Proteobacteria; Figure A2).

Round 2, aggression tests

During Round 2, we compared aggression across sucrose-treated pairs, tetracycline-treated pairs and sucrose- vs tetracycline treated pairs. Aggression was low in nestmate encounters of ants fed with nestmate faecal droplets, higher in non-nestmate encounters of ants fed with nestmate faecal droplets, and maximal in encounters of non-nestmate ants each fed with non-nestmate faecal droplets (Figure 2(b)). In particular, the highest aggression levels appeared in

encounters between non-nestmate sucrose-treated ants fed with faecal droplets from different colonies (z_{85}=2.05, *P*=0.041; Figure 2(b), Table A2). In contrast, tetracycline-treated pairs always showed low aggression (Figure 2(b)) regardless of whether they were fed on faecal droplets of workers from the same or a different colony. Also, confrontations between sucrose-treated vs tetracycline-treated ants showed low aggression levels, similar to interactions between tetracycline-treated pairs. We found a significant statistical interaction between faecal droplet supplementation and diet treatment (χ^2_2=7.57, *P*<0.05), but not between diet treatment and nestmate *vs* non-nestmate interactions (χ^2_2=3.36, *P*=0.18; Figure 2(b), Table A2). All three main effects were significant (faecal droplet supplementation or not: χ^2_1=7.99, *P*<0.05; initial diet treatment: χ^2_4=36.97, *P*<0.001; interactions between nestmates *vs* non-nestmates: χ^2_1=5.2437, *P*<0.05).

Round 2, changes in gut bacterial communities

The NMDS ordination suggested that the gut bacterial communities of tetracycline-treated workers before and after faecal droplet feeding were not significantly different. However, after faecal droplet feeding, the gut bacterial communities of sugar-treated workers were closer to those of untreated ones sampled from original fungus gardens in Round 1, suggesting a partial shift backwards towards the original communities (Figure 3). Interestingly, the gut bacterial samples of sugar-treated workers exhibited a clear separation depending on which type of faecal droplets (nestmates or non-nestmates) they were fed with (PERMANOVA, diet treatment: $F_{1,43}$=23.17, *P*<0.001; faecal droplets: $F_{1,43}$=12.10, *P*<0.001; interaction and $F_{1,43}$=5.04, *P*=0.004), whereas

there was no such effect in the tetracycline-treated group (Figure 3). The qPCR showed that tetracycline-treated ants undergoing faecal droplet feeding underwent an increase of all the six most abundant OTUs (except for *EntAcro2* and *RhiAcro1*, whose levels were further reduced); sucrose-treated ants showed an increase of all six gut bacterial OTUs examined (Figure 4), but only changes in *EntAcro1*, *EntAcro2*, *EntAcro10* and *w*SinvictaB were significant (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).

Regression of changes in endosymbiont abundance and aggression

To identify which of the OTUs were associated with worker aggression when bacterial titres were manipulated, we regressed the observed aggression measurements between nestmate test pairs on the differences in abundance of their gut bacteria (Figure 5). This analysis (1500 MiSeq OTUs; Bray-Curtis distances between pairs) confirmed that workers were generally more aggressive in pairwise tests when experimental manipulation had changed their gut microbial community more (binomial GLMM with 'genetic distance' and 'experimental round' as fixed variables and 'colony' as random variable, z=4.91, *P*<0.001). For the six separate OTUs, we used the more accurate qPCR data, regressing observed pairwise aggression against OTU-specific ΔΔCt values (Figure 5). This analysis showed that aggression was not affected by changes in the titres of one of two *Wolbachia* strains (Figure 5; *wSinvictaA*: z=-0.66, *P*=0.509;) and the *EntAcro2* symbiont (z=-0.872, *P*=0.383), but that there were significantly positive correlations for *wSinvictaB* (z=-3.75, *P*<0.001), *EntAcro1* (z=2.10, *P*=0.035), *EntAcro10* (z=6.09, *P*<0.001) and *RhiAcro1* (z=5.85, *P*<0.001). When splitting the data for each of the two rounds, *EntAcro1* showed a

significant positive correlation with aggression in Round 1, while this was so for EntAcro10 in Round 2 (Data set S4). However, only differences in abundance of *RhiAcro1* had a significant effect on aggression in both rounds (Figure 5; Data set S4; Round 1: z=3.25, *P*<0.001; Round 2: z=4.17, *P*<0.001), suggesting that manipulation of Rhizobiales titres has consistent consequences for aggression towards nestmates (see Data set S4). The presented results are based on biting only, but very similar results were obtained when both biting and mandible opening were treated as aggressive behaviours (data not shown).

CHC profiles are associated with diet

Compared to untreated individuals from original fungus gardens, sucrose- and tetracycline-treated workers (data from Round 1 and Round 2 combined) had strong reductions of 4-oxo-octanoic and 4-oxo-decanoic acids in their CHC profiles (Linear Mixed Models (LMMs) with 'diet' and 'round' as fixed variables and 'colony' as random variable. FDR-corrected *P*-values for multiple comparisons were 4-oxo-octanoic acid: untreated *vs.* sucrose-treated, t=15.20, *P*<0.001, untreated *vs*. tetracycline-treated, t=15.01, *P*<0.001; 4-oxo-decanoic acid: untreated *vs*. sucrose-treated, t=-13.10, *P*<0.001, untreated *vs*. tetracycline-treated, t=-13.66, *P*<0.001). Changes to these two compounds were not only significant in overall comparisons, but also in all pairwise comparisons among treatment groups (Data set S4), while changes in two other compounds, the long-chain linear hydrocarbons n-C_{36} and n-C_{40}, were only significant in comparisons between workers treated with antibiotics and the fungus-garden control workers from their home colonies.

Considering the overall association between cuticular chemical profiles and the abundances of symbiont OTUs as measured with qPCR, we found a significant association between these (Coinertia Analysis, RV=0.143, *P*<0.001; Figure A4). When looking at each OTU separately, the strongest pattern was an association between *EntAcro1* and *RhiAcro1* and the two before mentioned 4-oxo-octanoic and 4-oxo-decanoic acids. However, even after we analysed the data without these two acids we recovered a significant overall correspondence between chemical profiles and qPCR gut bacterial abundances (RV=0.141, *P*<0.001). On the other hand, we could not find such a significant association between smell and gut microbiota when we used chemical distance and the difference in qPCR counts between individuals in aggression pairs (RV=0.122, *P*=0.707). Altogether, this suggests that bacterial abundances can explain some of the colony-level changes in cuticular odours that mediate the aggression induced by our manipulative diets, but that aggression in specific dyadic encounters between worker ants cannot be predicted by smell and gut bacteria.

Discussion

Diet and antibiotics treatment affect gut microbial communities

The gut microbial communities of tetracycline-treated ants underwent substantial changes relative to untreated nestmate ants subjected to control experiments (two OTUs decreased strongly, three slightly and one was not affected; Figure 4), whereas the microbiomes of sucrose-treated ants exhibited relatively milder shifts (three OTUs decreased slightly, two increased slightly and one was not affected). These results show that while the antibiotic treatment suppressed most of the endosymbionts in the guts and associated organs of ant workers, the sterile sucrose diet had only a mild effect on relative abundances of OTUs.

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, previously latent OTUs arose (i.e., *EntAcro10*), which may have prevented the original OTUs from re-colonizing the tissues/organs from which they were eliminated (cf., Salem, Florez, Gerardo, & Kaltenpoth, 2015; Anderson, Rodrigues, Mott, Maes, & Corby-Harris, 2015). 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 that some bacterial taxa were more affected by our experimental food treatments than others, amplicon sequencing often does not allow distinguishing bacterial strains because closely related strains have identical 16S sequences (Andersen, Boye, Nash, & Boomsma, 2012; Engel, Stepanauskas, & Moran, 2014; Kuo, 2015). Thus, we cannot exclude that any effect of gut bacteria on behaviour may be driven by the interaction of multiple bacterial strains with different metabolic potential that were captured under the same 97% identity OTUs. Further research should thus implement methods allowing higher resolution.

Diet treatments affect the ant cuticular chemical profiles

Our experiments showed that diet treatments significantly affected ant chemical profiles, and further analyses revealed a correlation between chemical profiles and bacterial abundances. However, we do acknowledge that the experimental removal of gut bacteria likely introduced confounding effects on the wellbeing and survival of the ants because 1) antibiotics may have a direct detrimental effect on mitochondrial functionality (Moullan et al., 2015) and 2) at least some of the gut bacteria will likely have metabolic roles in the production of metabolites that are absent in host diets or in covering direct energy needs (Feldhaar et al., 2007; de Souza, Bezier, Depoix, Drezen, & Lenoir, 2009; Russell et al., 2009; Hu et al., 2017). More work will therefore be needed to unravel direct causation and collateral effects.

With these caveats in mind, we found a consistent and significant decreases of two acids (4-oxo-octanoic and 4-oxo-decanoic) that are known to be specific

products of metapleural gland secretion (Ortius-Lechner, Maile, Morgan, & Boomsma, 2000; Larsen, Fouks, Bos, d'Ettorre, & Nehring, 2014), and in two long-chain linear alkanes (n-C_{36} and n-C_{40}) in the tetracycline-treated individuals. While the latter likely originate as cuticular hydrocarbons of unknown function and production costs, the former have disease-defence functions and are known to be metabolically costly to produce (Poulsen & Boomsma, 2003). The observed changes in chemical profiles may thus have been either directly affected by symbiotic gut bacteria or, more likely, indirectly because of compromised host metabolism and deficiency in resources to produce these compounds. This inference is supported by the fact that CHCs are synthesized in the oenocytes (Ringo, Sharon, & Segal, 2011), which are heavily colonized by *EntAcro1* bacteria (Sapountzis et al., 2015), and are the overall centre of intermediate metabolism in ants (Arrese & Soulages, 2010).

Tetracycline treatment may not only have affected the gut microbes, but also the microbiota on the ants' cuticle, particularly the *Pseudonocardia* actinobacterial symbionts of *Acromyrmex* (Andersen et al., 2013; Andersen et al., 2015), again most likely as an indirect consequence of compromised metabolism affecting nutrient provisioning of cuticular biofilms. In *Drosophila*, cuticular microbes have been hypothesized to affect chemical CHC profiles because compounds embedded in the cuticular wax layer may function as carbon sources and be enzymatically degraded (Ringo, Sharon, & Segal, 2011). Previous research on *Pogonomyrmex* showed that topical antibiotic administration can indeed alter CHC profiles of ant workers, consistent with a possible role of surface microbes in determining CHC profiles (Dosmann et al., 2016). However, the same kind of antibiotic treatment that we used here did not affect CHC profiles when applied

on *Acromyrmex subterraneus subterraneus* (de Souza et al., 2013). Other research suggests that innate immune responses may also affect CHC profiles, both in *Drosophila* (Ryu, Ha, & Lee, 2010) and in the honey bee (Richard, Aubert, & Grozinger, 2008). Multiple factors are therefore conceivably involved and interact with each other, so disentangling the implications for CHC profiles will require extensive further work.

Our correlational evidence for dietary treatments to affect gut bacteria, reflecting a mostly unknown network of causation factors, should also prompt further evaluation of other covariates that could play a role. Across insect taxa, symbiotic bacteria produce a plethora of volatile semiochemicals (Davis, Crippen, Hofstetter, & Tomberlin, 2013; Ezenwa & Williams, 2014), and studies on *Drosophila* suggest that compounds derived from the metabolism of gut microbes can also mediate interactions between host individuals (Lizé et al., 2014; Venu, Durisko, Xu, & Dukas, 2014). Superorganismal ant colonies may have integrated the bacterial metabolism in their chemical-based social dynamics, and ants may thus rely to an unknown extent on a social communication system based on chemicals produced by the colony level community of endosymbiotic bacteria. If these would be sufficiently distinct, this might imply that artificial removal of endosymbionts will handicap social functionality either by triggering inappropriate aggression or by failing to react aggressively to certain challenges, consistent with the effects that emanated from our study. Accordingly, we cannot exclude that the altered behaviour observed in our aggression tests may at least partially depend on volatile semiochemicals related to bacterial metabolism and that social handicaps may not be completely reducible to CHCs on the cuticle. This complicates

recommendations for future experiments. On one hand, it would be important if ways could be found to do nestmate recognition assays in which microbiota-free and -remodelled interacting individuals are prevented from exposure to non-volatile cuticular social cues. On the other hand, taking into account that ants and endosymbionts both have their own genetic and environmentally mediated consequences affecting distinct volatile and cuticular cues, will tend to make experimental designs highly complex when all factors and their interactions are to be unravelled.

Diet suppresses microbes, potentially affecting social interactions

Encounters between antibiotic-treated and untreated nestmates in Round 1 produced the highest aggression levels, suggesting that the antibiotic treatment affected chemical cues that are relevant for social interactions. Aggression due to changed diet clearly gave a steeper response than aggression due to nestmate status. Sucrose-treated ants gave relatively straightforward behavioural responses after the faecal droplet treatment, but tetracycline-treated ants tended to produce less clear contrasts (Figure 2(a)). Nevertheless, from the aggression results, it can be inferred that tetracycline-treated workers were less aggressive and were aggressed more often. In fact, aggression in tetracycline-treated ants was constantly lower than in all other groups. However, tetracycline vs. untreated workers showed similar aggression levels across the two experimental rounds, suggesting that untreated ants aggressed treated ants and not the other way around. If tetracycline treated workers were more aggressive, aggression in tetracycline treated pairs should have been higher. Tetracycline-treated ants showed low level of aggression in the second round, which was not

unexpected because their gut microbiota were not completely re-established, consistent with behaving similarly to ants tested in the first round. Finally, tetracycline-treated vs. sucrose-treated pairs in the second round showed an intermediate level of aggression between sucrose vs. sucrose (highest) and tetracycline vs. tetracycline pairs (lowest), suggesting that sucrose-treated workers attack tetracycline-treated ones, and not the other way round. Although these results are only correlational, they suggest that tetracycline-treated workers received more aggression than they gave.

As mentioned above, tetracycline negatively affects mitochondria and tissues (Moullan et al., 2015), which makes the effect of the antibiotic itself on behaviour difficult to disentangle from the effects of induced changes in microbial communities, consistent with the higher mortality rates in antibiotic-treated ants in Round 1 (Figure A1; (Sapountzis et al., 2015)). Nevertheless, even if correlative, the connection between observed aggression and gut bacterial abundances was highly significant. A possible interpretation of the increased aggression levels towards nestmates with suppressed gut microbes and abnormal odours may be that those workers are perceived as abnormal or infected. If so, it might be understandable that fungus garden workers would adaptively use aggression to push such deviating workers into foraging and defensive tasks outside or at the periphery of the colony to minimize the spread of perceived putative infections within the colony. On the other hand, lack of aggression between antibiotic-treated non-nestmates may also suggest that that the absence of symbiotic microbes makes such microbially compromised workers unsuitable to patrol territorial boundaries.

The impact of microbiota restoration on social interactions

After faecal droplets administration, dyadic encounters between nestmates revealed only moderate and non-significant aggression levels, barely higher than those observed in Round 1. While these slightly higher responses may have been an effect of the relatively longer separation of the experimental ant groups from their source colonies (three weeks instead than two weeks in petri dishes), the lack of significant differences between experimental rounds is also consistent with the microbial gut communities only having become partly restored. In non-nestmate aggression assays, pairs of tetracycline-treated and tetracycline-treated vs. sucrose-treated ants also showed low aggression levels overall. Sucrose-treated non-nestmate dyads were more aggressive than nestmate dyads, and we could attribute this to a colony-level difference in cuticular hydrocarbon profiles. In contrast, aggression was significantly higher in sucrose-treated pairs when interacting individuals were fed with faecal droplets from different colonies, strongly suggesting an effect of microbes on nestmate discrimination. This seems to imply that, while bacterial communities were indeed partly restored, it was important that this restoration involved the colony's standard mix of microbes and fungal enzymes known to be transferred via faecal fluid (Poulsen & Boomsma, 2005; Schiøtt, Rogowska-Wrzesinska, Roepstorff, & Boomsma, 2010 ; Kooij et al., 2014). However, the partial restoration of gut microbiota suggests that the effect of tetracycline cannot solely explain the observed aggression in Round 1. Even if there were confounding tetracycline effects, the bacterial suppression and partial restoration accounted to a significant degree for the observed aggression.

Conclusions

In this study, we developed an integrative approach to explore the role of gut microbial symbionts in the social dynamics of highly integrated ant colonies with complex division of labour and obligate symbiotic dependence on a co-evolved fungal cultivar. Dietary changes, which resulted in experimental suppression and remodelling of the ant gut microbiota, produced effects on both the cuticular chemicals that ants displayed as socially relevant recognition cues and the social behaviours expressed. Our findings suggest that the observed effects on mutual aggression between individual workers may be related to changes in the abundances of two symbiotic bacterial OTUs, the previously identified major gut bacterial taxa in leaf-cutting ants *EntAcro1* and *RhiAcro1* (Andersen et al., 2013; Sapountzis et al., 2015). The observed differences in the resilience or re-establishment of these two symbionts may reflect differences in their tissue specificity: *EntAcro1* is abundant in the lumen and thus easily removed by antibiotics, while *RhiAcro1* is forming a biofilm in the hindgut and should thus be more resistant to antibiotics (Sapountzis et al., 2015). Further research will be needed to address the mechanisms underlying the link between the changes in relative abundance of these symbionts and the expression of social behaviours, also beyond measuring aggression. Our results suggest that altered microbial communities may result in chemical profiles that are not recognized as nestmates, or that microbiota-free or microbiota-remodelled ants may be recognized as sick and unsuitable for central colony functions such as brood nursing or garden hygiene. Social withdrawal of unhealthy ant workers has been demonstrated for at least two ant species (Bos, Lefèvre, Jensen, & d'Ettorre, 2012; Heinze & Walter, 2015), and it is conceivable that the ostracism of sick

ants is driven by changes in CHCs or other volatile compounds that are important in communication. Regardless, our findings provide evidence that gut bacterial symbionts may be involved in multiple forms of social discrimination in ants so that the concept of a healthy colony-level gut microbiome may have similar far-reaching implications as has been repeatedly suggested for humans and farm animals (Bourtzis & Miller, 2003; Dubilier, Bergin, & Lott, 2008; Hara et al., 2013; Kamada, Chen, Inohara, & Núñez, 2013; Jha & Berrocoso, 2016; Kanji et al., 2018).

Acknowledgements

Funding was provided by the Danish National Research Foundation (DNRF57), an ERC Advanced grant (323085) to J.J.B., and an Intra-European Marie Curie Fellowship to P.S. (300584). As no ethical restrictions apply to insect research under European legislation, no institutional or governmental regulatory bodies reviewed this study.

References

Aitchison, J. (1986). The statistical analysis of compositional data. London, UK: Chapman and Hall.

Andersen, S.B., Boye, M., Nash, D.R., Boomsma, J.J. (2012). Dynamic *Wolbachia* prevalence in *Acromyrmex* leaf-cutting ants: potential for a nutritional symbiosis. *Journal of Evolutionary Biology*, 25(7):1340–1350.

Andersen, S.B., Hansen, L.H., Sapountzis, P., Sørensen, S.J., Boomsma, J.J. (2013). Specificity and stability of the *Acromyrmex–Pseudonocardia* symbiosis. *Molecular Ecology*, 22(16):4307–4321.

Andersen, S.B., Yek, S.H., Nash, D.R., Boomsma, J.J. (2015). Interaction specificity between leaf-cutting ants and vertically transmitted *Pseudonocardia* bacteria. *BMC Evolutionary Biology*, 15:27.

Anderson, K.E., Rodrigues, P.A.P., Mott, B.M., Maes, P., Corby-Harris, V. (2015). Ecological Succession in the Honey Bee Gut: Shift in *Lactobacillus* Strain Dominance During Early Adult Development. *Microbial Ecology*, 71(4):1008–1019.

Arbuthnott, D., Levin, T.C., Promislow, D.E.L. (2016). The impacts of *Wolbachia* and the microbiome on mate choice in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, 29(2):461–468.

Archie, E.A., Tung, J. (2015). Social behavior and the microbiome. *Current Opinion in Behavioral Sciences,* 6 (Supplement C):28–34.

Archie, E.A., Theis, K.R. (2011). Animal behaviour meets microbial ecology. *Animal Behaviour,* 82(3):425–436.

Arrese, E.L., Soulages, J.L. (2010). Insect fat body: energy, metabolism, and regulation. *Annual Reviews of Entomology,* 55:207–225.

Bates, D., Mächler, M., Bolker, B., Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software,* 67(1).

Boomsma, J.J., Gawne, R. (2017). Superorganismality and caste differentiation as points of no return: how the major evolutionary transitions were lost in translation. *Biological Reviews,* 93(1):28–54.

Bos, N., Lefèvre, T., Jensen, A.B., d'Ettorre, P. (2012). Sick ants become unsociable. *Journal of Evolutionary Biology,* 25(2):342–351.

Bourtzis, K., Miller, T.A. (2003). Insect Symbiosis, Boca Raton, FL: CRC Press.

Buffington, S.A., Di Prisco, G.V., Auchtung, T.A., Ajami, N.J., Petrosino, J.F., Costa-Mattioli, M. (2016). Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell,* 165(7):1762–1775.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME Journal,* 6(8):1621–1624.

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R.D., Shanahan, F., et al. (2013). The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Molecular Psychiatry*, 18(6):666–673.

Cryan, J.F., Dinan, T.G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience*, 13(10):701–712.

Dance, A. (2014). Microbes take charge. *Proceeding of the National Academy of Sciences USA*, 111(6):2051–2053.

Davis, T.S., Crippen, T.L., Hofstetter, R.W., Tomberlin, J.K. (2013). Microbial volatile emissions as insect semiochemicals. *Journal of Chemical Ecology*, 39(7):840–859.

de Souza, D.J., Bezier, A., Depoix, D., Drezen, J.M., Lenoir, A. (2009). *Blochmannia* endosymbionts improve colony growth and immune defence in the ant *Camponotus fellah*. *BMC Microbiology*, 9:29.

de Souza, D.J., Devers, S., Lenoir, A. (2011). Blochmannia endosymbionts and their host, the ant *Camponotus fellah*: Cuticular hydrocarbons and melanization. *Comptes Rendus Biologies*, 334(10):737–741.

de Souza, D.J., Lenoir, A., Kasuya, M.C.M., Ribeiro, M.M.R., Devers, S., Couceiro, J.da C., et al. (2013). Ectosymbionts and immunity in the leaf-cutting ant *Acromyrmex subterraneus subterraneus*. *Brain, Behavior, and Immunity*, 28 (Supplement C):182–187.

Dolédec, S., Chessel, D. (1994). Co-inertia analysis: an alternative method for studying species–environment relationships. *Freshwater Biology*, 31(3):277–294.

Dosmann, A., Bahet, N., Gordon, D.M. (2016). Experimental modulation of external microbiome affects nestmate recognition in harvester ants (*Pogonomyrmex barbatus*). *PeerJ*, 4:e1566.

Dubilier, N., Bergin, C., Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology,* 6(10):725-740.

Engel, P., Stepanauskas, R., Moran, N.A. (2014). Hidden diversity in honey bee gut symbionts detected by single-cell genomics. *PLoS Genetics,* 10(9):e1004596.

Ezenwa, V.O., Gerardo, N.M., Inouye, D.W., Medina, M., Xavier, J.B. (2012). Microbiology. Animal behavior and the microbiome. *Science,* 338(6104):198–199.

Ezenwa, V.O., Williams, A.E. (2014). Microbes and animal olfactory communication: Where do we go from here? *BioEssays: news and reviews in molecular, cellular and developmental biology,* 36(9):847–854.

Feldhaar, H., Straka, J., Krischke, M., Berthold, K., Stoll, S., Mueller, M.J., et al. (2007) Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*. *BMC Biology,* 5:48.

Fox, J, Weisberg, S, Friendly, M, Hong, J, Andersen, R, Firth, D, et al. (2017). effects: Effect Displays for Linear, Generalized Linear, and Other Models. https://cran.r-project.org/web/packages/effects/index.html.

Fox, J., Weisberg, S. (2011). An R Companion to Applied Regression. http://socserv.socsci.mcmaster.ca/jfox/Books/Companion.

Gadau, J., Fewell, J. (2009). Organization of Insect Societies: From Genome to Sociocomplexity. Cambridge, MA: Harvard University Press.

Hara, N., Alkanani, A.K., Ir D., Robertson, C.E., Wagner, B.D., Frank, D.N., et al. (2013). The role of the intestinal microbiota in type 1 diabetes. *Clinical Immunology*, 146(2):112–119.

Heijtz, R.D., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., et al. (2011). Normal gut microbiota modulates brain development and behavior. *Proceeding of the National Academy of Sciences USA*, 108:3047–3052.

Heinze, J., Walter, B. (2010). Moribund Ants Leave Their Nests to Die in Social Isolation. *Current Biology*, 20(3):249–252.

Hope, R.M. (2013). Rmisc: Ryan Miscellaneous. https://cran.r-project.org/web/packages/Rmisc/index.html.

Hothorn, T., Bretz, F. and Westfall, P. (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal*, 50(3):346-363.

Hu, Y., Sanders, J.G., Łukasik, P., D'Amelio, C.L., Millar, J.S., Vann, D.R., et al. (2018). Herbivorous turtle ants obtain essential nutrients from a conserved nitrogen-recycling gut microbiome *Nature Communications*, 9, Article number: 964.

Jha, R., Berrocoso, J.F.D. (2016). Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review. *Anim Feed Science and Technology*, 212:18–26.

Kamada, N., Chen, G.Y., Inohara, N., Núñez, G. (2013). Control of Pathogens and Pathobionts by the Gut Microbiota. *Nature Immunology*, 14(7):685–690.

Kanji, S., Fonseka, T.M., Marshe, V.S., Sriretnakumar, V., Hahn, M.K., Müller, D.J. (2018). The microbiome-gut-brain axis: implications for schizophrenia and antipsychotic

induced weight gain. *European Archives of Psychiatry and Clinical Neuroscience,* 268(1):3–15.

Kooij, P.W., Rogowska-Wrzesinska, A., Hoffmann, D., Roepstorff, P., Boomsma, J.J., Schiøtt M. (2014). *Leucoagaricus gongylophorus* uses leaf-cutting ants to vector proteolytic enzymes towards new plant substrate. *ISME Journal,* 8(5):1032–1040.

Kuo, C.H. (2015). Scrambled and not-so-tiny genomes of fungal endosymbionts. *Proceeding of the National Academy of Sciences USA,* 112(25):7622–7623.

Larsen, J., Fouks, B., Bos, N., d'Ettorre, P., Nehring, V. (2014). Variation in nestmate recognition ability among polymorphic leaf-cutting ant workers. *Journal of Insect Physiology*, 70:59–66.

Leitão-Gonçalves, R., Carvalho-Santos, Z., Francisco, A.P., Fioreze, G.T., Anjos, M., Baltazar, C., et al. (2017). Commensal bacteria and essential amino acids control food choice behavior and reproduction. *PLOS Biology,* 15(4):e2000862.

Lester, P.J., Sébastien, A., Suarez, A.V., Barbieri, R.F., Gruber, M.A.M. (2017). Symbiotic bacterial communities in ants are modified by invasion pathway bottlenecks and alter host behavior. *Ecology,* 98(3):861–874.

Lizé, A., McKay, R., Lewis, Z. (2014). Kin recognition in *Drosophila*: the importance of ecology and gut microbiota. *ISME Journal,* 8(2):469–477.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal,* 17(1):10–12.

Matsuura, K. (2001). Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. *Oikos,* 1;92(1):20–26.

Morimoto, J., Simpson, S.J., Ponton, F. (2017). Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biology Letters*, 13(7): 20160966.

Moullan, N., Mouchiroud, L., Wang, X., Ryu, D., Williams, E.G., Mottis, A., et al. (2015). Tetracyclines disturb mitochondrial function across eukaryotic models: a call for caution in biomedical research. *Cell Reports*, pii: S2211-1247(15)00180-1.

Neufeld, K.M., Kang, N., Bienenstock, J., Foster, J.A. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility*, 23(3):255–264, e119.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2017). vegan: Community Ecology Package. R package version 2.3-4. http://CRAN.R-project.org/package=vegan.

Ortius-Lechner, D., Maile, R., Morgan, E.D., Boomsma, J.J. (2000). Metapleural Gland Secretion of the Leaf-cutter Ant *Acromyrmex octospinosus*: New Compounds and Their Functional Significance. *Journal of Chemical Ecology*, 26(7):1667–1683.

Ottoni, E.B. (2000). EthoLog 2.2: a tool for the transcription and timing of behavior observation sessions. *Behavior research methods, instruments, & computers*, 32(3):446–449.

Pfaffl, M.W., Horgan, G.W., Dempfle, L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*, 30(9):e36.

Pfaffl, M.W. Quantification strategies in real-time PCR. A–Z Quant PCR. 2004 89-113.

Poulsen, M., Boomsma, J.J. (2005). Mutualistic fungi control crop diversity in fungus-growing ants. *Science,* 307(5710):741–744.

Poulsen, M., Bot, A.N.M., Boomsma, J.J. (2003). The effect of metapleural gland secretion on the growth of a mutualistic bacterium on the cuticle of leaf-cutting ants. *Naturwissenschaften,* 90(9):406–409.

Richard, F.J., Aubert, A., Grozinger, C.M. (2008). Modulation of social interactions by immune stimulation in honey bee, *Apis mellifera*, workers. *BMC Biology,* 17;6:50.

Ringo, J., Sharon, G., Segal, D. (2011). Bacteria-induced sexual isolation in *Drosophila*. *Fly,* 5(4):310–315.

Russell J.A., Moreau C.S., Goldman-Huertas B., Fujiwara M., Lohman D.J., Pierce N.E. (2009). Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proceeding of the National Academy of Sciences USA*, 106(50):21236–212341.

Ryu, J.H., Ha, E.M., Lee, W.J. (2010). Innate immunity and gut–microbe mutualism in *Drosophila*. *Developmental & Comparative Immunology,* 34(4):369–376.

Salem, H., Florez, L., Gerardo, N., Kaltenpoth, M. (2015). An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society of London B: Biological Sciences,* 282(1804):20142957.

Sapountzis, P., Zhukova, M., Hansen, L.H., Sørensen, S.J., Schiøtt, M., Boomsma, J.J. (2015). *Acromyrmex* leaf-cutting ants have simple gut microbiota with nitrogen-fixing potential. *Applied and Environmental Microbiology,* 81(16):5527-5537.

Schiøtt, M., Rogowska-Wrzesinska, A., Roepstorff, P., Boomsma, J.J. (2010). Leaf-cutting ant fungi produce cell wall degrading pectinase complexes reminiscent of phytopathogenic fungi. *BMC Biology*, 8:156.

Schloerke, B., Crowley, J., Cook, D., Hofmann, H., Wickham, H., Briatte, F., Marbach, M. and Thoen, E. (2014). GGally: Extension to ggplot2. R package version 0.5.0. https://cran.r-project.org/package=GGally.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al. (2009). Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology,* 75(23):7537–7541.

Sharon, G., Segal, D., Ringo, J.M., Hefetz, A., Zilber-Rosenberg, I., Rosenberg, E. (2010). Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceeding of the National Academy of Sciences USA,* 107(46):20051–20056.

Therneau, T. (2015). A Package for Survival Analysis in S. version 2.38. https://CRAN.R-project.org/package=survival.

Van Borm, S., Billen, J., Boomsma, J.J. (2002). The diversity of microorganisms associated with *Acromyrmex* leafcutter ants. *BMC Evolutionary Biology,* 3;2.

Venu, I., Durisko, Z., Xu, J., Dukas, R. (2014). Social attraction mediated by fruit flies' microbiome. *Journal of Experimental Biology,* 217(8):1346–1352.

Wheeler, W.M. (1928). Emergent evolution and the development of societies. New York: W.W. Norton & Co.

Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis. New York: Springer-Verlag.

Zhukova, M., Sapountzis, P., Schiøtt, M., Boomsma, J.J. (2017). Diversity and Transmission of Gut Bacteria in *Atta* and *Acromyrmex* Leaf-Cutting Ants during Development. *Frontiers in Microbiology*, 8:1942.

Tables

Table A1. Primers used for library preparation of the MiSeq sequencing for the main diet experiment.

Forward	
MS_515f_F1	AATGATACGGCGACCACCGAGATCTACACAACCAACGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGAGTGCCAGCMGCCGCGGTAA
MS_515f_F2	AATGATACGGCGACCACCGAGATCTACACAAGAGCTCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCGGGAGTGCCAGCMGCCGCGGTAA
MS_515f_F3	AATGATACGGCGACCACCGAGATCTACACAGATAAGTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGGCCGGGAGTGCCAGCMGCCGCGGTAA
MS_515f_F4	AATGATACGGCGACCACCGAGATCTACACCAACCATCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAATGGCCGGGAGTGCCAGCMGCCGCGGTAA
MS_515f_F5	AATGATACGGCGACCACCGAGATCTACACCCTAGCGATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTATGAATGGCCGGGAGTGCCAGCMGCCGCGGTAA
MS_515f_F6	AATGATACGGCGACCACCGAGATCTACACGCTCTGCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGAGTGCCAGCMGCCGCGGTAA
MS_515f_F7	AATGATACGGCGACCACCGAGATCTACACGGCGTTGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCGGGAGTGCCAGCMGCCGCGGTAA
MS_515f_F8	AATGATACGGCGACCACCGAGATCTACACGTCGCTAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGGCCGGGAGTGCCAGCMGCCGCGGTAA
MS_515f_F9	AATGATACGGCGACCACCGAGATCTACACTGGTTGACTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAATGGCCGGGAGTGCCAGCMGCCGCGGTAA
MS_515f_F10	AATGATACGGCGACCACCGAGATCTACACTTAGATCGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTATGAATGGCCGGGAGTGCCAGCMGCCGCGGTAA
Reverse	
MS_806r_R1	CAAGCAGAAGACGGCATACGAGATAACCAACGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACGGACTACHVGGGTWTCTAAT
MS_806r_R2	CAAGCAGAAGACGGCATACGAGATAACCGAACGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGGGAGGACTACHVGGGTWTCTAAT
MS_806r_R3	CAAGCAGAAGACGGCATACGAGATACTGCTCTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R4	CAAGCAGAAGACGGCATACGAGATATACCAGGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAAACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R5	CAAGCAGAAGACGGCATACGAGATATTGATTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGAAACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R6	CAAGCAGAAGACGGCATACGAGATCAACCTCTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACGGACTACHVGGGTWTCTAAT
MS_806r_R7	CAAGCAGAAGACGGCATACGAGATCAGAATATGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGGGAGGACTACHVGGGTWTCTAAT
MS_806r_R8	CAAGCAGAAGACGGCATACGAGATCCATGCGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R9	CAAGCAGAAGACGGCATACGAGATCCGCTAGGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAAACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R10	CAAGCAGAAGACGGCATACGAGATCGCATCATGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGAAACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R11	CAAGCAGAAGACGGCATACGAGATGAGGAGTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACGGACTACHVGGGTWTCTAAT
MS_806r_R12	CAAGCAGAAGACGGCATACGAGATGCTGGTCACGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGGGAGGACTACHVGGGTWTCTAAT
MS_806r_R13	CAAGCAGAAGACGGCATACGAGATGGAGGCTCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R14	CAAGCAGAAGACGGCATACGAGATGTAAGAAGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAAACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R15	CAAGCAGAAGACGGCATACGAGATGTTAAGTTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGAAACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R16	CAAGCAGAAGACGGCATACGAGATTCGTTCGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACGGACTACHVGGGTWTCTAAT
MS_806r_R17	CAAGCAGAAGACGGCATACGAGATTGAATGGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGGGAGGACTACHVGGGTWTCTAAT
MS_806r_R18	CAAGCAGAAGACGGCATACGAGATTGATCTCAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R19	CAAGCAGAAGACGGCATACGAGATTGGTCCTGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAAACGTCTAACGGACTACHVGGGTWTCTAAT
MS_806r_R20	CAAGCAGAAGACGGCATACGAGATTGGTTGACGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGAAACGTCTAACGGACTACHVGGGTWTCTAAT

Table A2. Aggression tests: post hoc planned contrasts among tests between treatment groups

Round	Comparisons		Estimate	Std. Error	z value	*P*	Significance
1	NM S vs. S	nNM S vs. S	-0.6976	0.5431	-1.284	0.28744	
1	NM S vs. T	nNM S vs. T	-24.867	10.472	-2.375	0.04566	*
1	NM T vs. T	nNM T vs. T	0.1853	0.5078	0.365	0.71521	
1	NM S vs. S	NM S vs. T	17.638	10.708	1.647	0.18479	
1	NM S vs. S	NM T vs. T	-0.4822	0.5310	-0.908	0.43002	
1	NM S vs. S	NM T vs. FG	-15.691	0.4628	-3.391	0.00429	**
1	NM S vs. S	NM S vs. FG	0.5509	0.7061	0.780	0.47154	
1	NM T vs. T	NM S vs. T	22.460	10.454	2.149	0.06862	
1	NM T vs. T	NM T vs. FG	-10.869	0.3990	-2.724	0.02098	*
1	NM T vs. T	NM S vs. FG	10.331	0.6674	1.548	0.19761	
1	NM S vs. T	NM S vs. FG	-12.129	11.444	-1.060	0.37594	
1	NM S vs. T	NM T vs. FG	-33.329	10.120	-3.293	0.00429	**
1	NM S vs. FG	NM T vs. FG	-21.200	0.6146	-3.449	0.00429	**
2	NM S vs. S - Same FD	nNM S vs. S - Same FD	-11.145	0.4867	-2.290	0.035242	*
2	NM S vs. S - Same FD	nNM S vs. S - Different FD	-27.164	0.5421	-5.011	8.65e-06	***
2	nNM S vs. S - Same FD	nNM S vs. S - Different FD	-16.019	0.5664	-2.828	0.009639	**
2	NM T vs. T - Same FD	nNM T vs. T - Same FD	-0.8886	0.4715	-1.885	0.079312	.
2	NM T vs. T - Same FD	nNM T vs. T - Different FD	-11.513	0.5451	-2.112	0.050444	.
2	nNM T vs. T - Same FD	nNM T vs. T - Different FD	-0.2627	0.5477	-0.480	0.631526	
2	NM S vs. T - Same FD	nNM S vs. T - Same FD	-24.492	0.7589	-3.227	0.003331	**
2	NM S vs. T - Same FD	nNM S vs. T - Different FD	-29.090	0.8198	-3.548	0.001691	**
2	nNM S vs. T - Same FD	nNM S vs. T - Different FD	-0.4598	0.5008	-0.918	0.441340	
2	NM S vs. FG - Same FD	NM S vs. S - Same FD	0.2959	0.4502	0.657	0.583937	
2	NM S vs. FG - Same FD	NM T vs. T - Same FD	0.2696	0.4506	0.598	0.586242	
2	NM S vs. FG - Same FD	NM S vs. T - Same FD	18.124	0.7737	2.342	0.034063	*
2	NM S vs. FG - Same FD	NM T vs. FG - Same FD	-10.381	0.3683	-2.819	0.009639	**

Abbreviations. FG: Fungus Garden ants; S: sucrose-treated ants; T: Tetracycline-treated ants; FD: faecal droplets; NM: Nest Mate; nNM: non-Nest Mate.

Figure legends

Figure 1. Experimental design. Large workers of *A. echinatior* were collected from fungus gardens and kept for two weeks on artificial sucrose or sucrose + tetracycline diets before aggression trials were performed after two weeks to compare workers having received artificial diets with control workers on their normal fungus-garden diet (Round 1). The guts of all experimental workers were then dissected to estimate absolute abundances of symbionts by qPCR and MiSeq on 16s rRNA, whereas remaining body parts (thoraxes and heads) were used for extractions of cuticular hydrocarbons and subsequent GC-MS analyses. In Round 2, a comparable set of workers from Round 1 that were still alive were transferred to a new experimental diet consisting of sucrose and faecal fluid from control workers of their own colony or one of the other (unrelated) colonies. In both rounds, the worker ants were also used in aggression trials to assess whether their social behaviours changed after the two diet treatments. Abbreviations. FG: Fungus Garden ants; S: sucrose-treated ants; T: Tetracycline-treated ants; fd: faecal droplets; NM: Nest Mate; nNM: non-Nest Mate.

Figure 2. Effects of sucrose and tetracycline treatments on aggression in dyadic 1:1 encounters. (a). Round 1. (b). Round 2. Aggression was quantified as the mean rate of biting divided by the mean rate of antennation (base-line friendly exploration) with 95% upper and lower confidence limits as error bars. The same lower case letter marks pairwise comparisons that were not significant on average, while different letters mark significant differences. Red letters refer to comparisons within treatment categories and black letters to comparisons across treatment categories. = and ≠ symbols refer to diets with faecal fluid from the

same or a different colony, respectively. Abbreviations. FG: Fungus Garden ants; S: sucrose-treated ants; T: Tetracycline-treated ants; fd: faecal droplets; NM: Nest Mate; nNM: non-Nest Mate.

Figure 3. (a). Non-metric dimensional scaling (NMDS) of Bray-Curtis distances using the rarefied MiSeq reads of the five most abundant gut bacterial OTUs. Differentially coloured ellipses represent the three treatments (black: ants picked from fungus-gardens, yellow: ants fed on sucrose, green: ants fed on tetracycline). Continuous lines: Round 1; dashed lines: Round 2. Tetracycline-treated individuals separate the most from untreated conspecifics, whereas the OTU profile of sucrose-treated individuals largely overlaps with that of untreated individuals. Round 2 samples are similar to those of Round 1, confirming that faecal droplet feeding restored only partially the original gut microbial communities. (b). Round 1: non-metric dimensional scaling (NMDS) of Bray-Curtis distances using the rarefied MiSeq reads of the five most abundant OTUs (same as (a)) using S and T samples. (c). Non-metric dimensional scaling (NMDS) of Bray-Curtis distances using the rarefied MiSeq reads of the five most abundant OTUs for Round 2 samples. Grey shadows highlight the sample clustering based on the combination of treatments and the faecal droplet feeding group to which they belonged (sucrose-treated and tetracycline-treated ants fed with faecal droplets of workers from colony B or D).

Figure 4. Changes in qPCR-estimated bacterial titres expressed as fold changes for the six most abundant bacterial OTUs after experimental Rounds 1 and 2 after a sucrose diet (yellow bars) and a Tetracycline diet (green bars). Counts

were normalized relative to EF-1α copies to produce unbiased fold-changes (ΔΔCt) using the OTU counts obtained from untreated conspecifics reared on a normal fungal diet as reference level (grey y=0 values), all with 95% CI. Asterisks indicate significant differences (*P*<0.05) between treatment groups.

Figure 5. Association between aggression levels and ΔΔCt abundance differences due to diet treatment for all six abundant bacterial taxa in the gut microbiomes and six of the specific symbionts estimated with GLMM regressions. (a). All six abundant taxa. (b). wSinvictaA. (c). wSinvictaB. (d). EntAcro10. (e). EntAcro1. (f). EntAcro2. (g). RhiAcro1. Data are plotted for the pooled data obtained in experimental rounds 1 and 2. Asterisks indicate significance levels: * *P*<0.05, ** *P*<0.01, and *** *P*<0.001. Statistically significant correlations between observed aggression and bacterial taxa (besides the one accounting for all six abundant taxa; top left) were produced when using the Mollicutes *EntAcro1* or *Entacro10*, the two most common symbionts of leaf-cutting ants and the Rhizobiales hindgut endosymbiont *RhiAcro1* (Sapountzis et al. 2015).

Figure A1. Survival curves of ants kept on artificial diets. (a). Colony A. (b). Colony B. (c). Colony C. (d). Colony D. For each colony, two survival curves are represented: one for ants reared on sucrose diet in Round 1 and then on sucrose + faecal droplets in Round 2 (red) and one for the ants reared initially on tetracycline diet in Round 1 and on sucrose + faecal droplets in Round 2 (blue). Crosses indicate censored data (days 14 and 21) and dashed lines the confidence intervals for each survival curve. A Coxph model showed that during

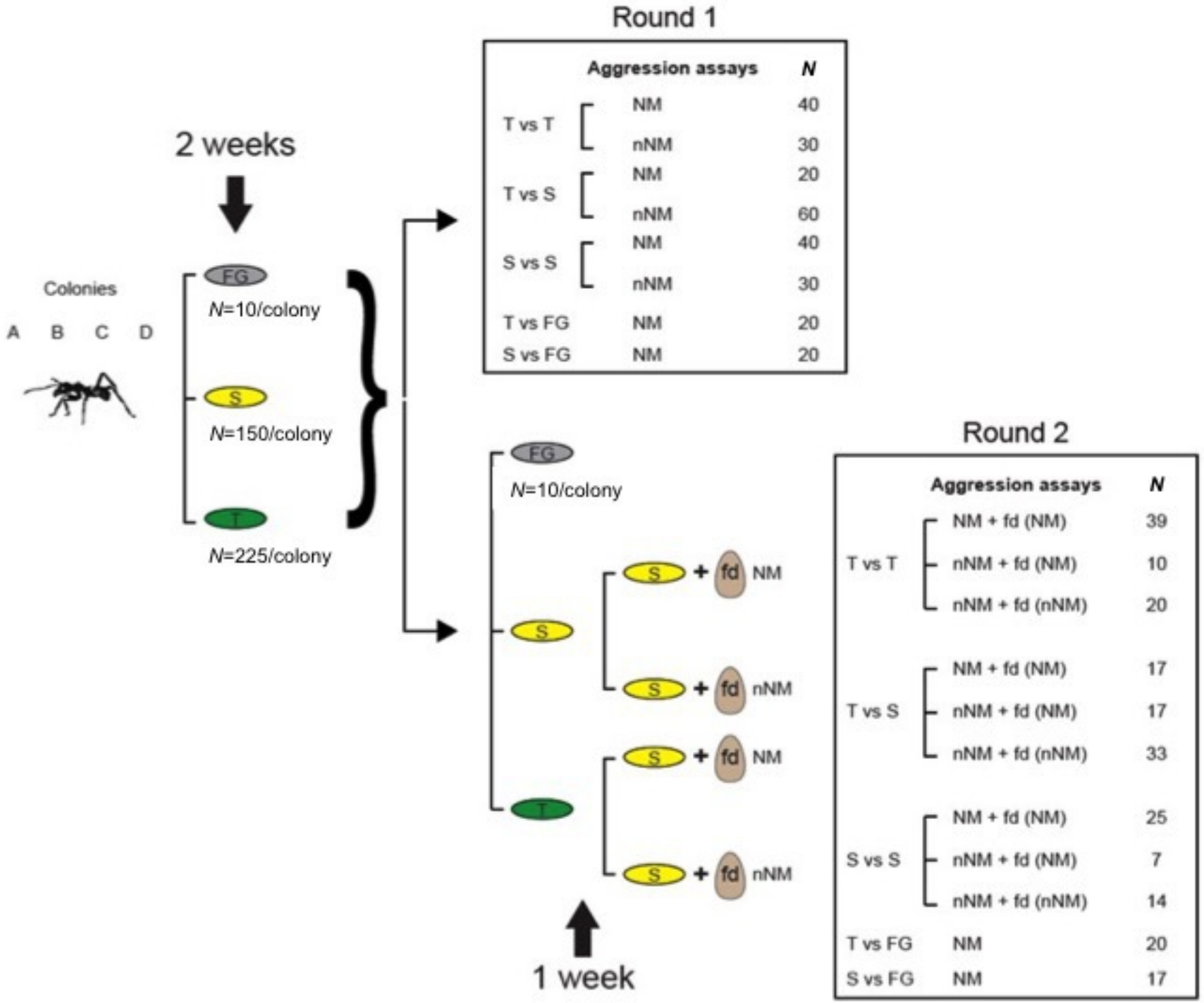
Round 1 (days 0-14) tetracycline significantly affected the survival of the ants (P<0.0001) but during Round 2 (days 15-21) this effect disappeared (P=0.3293). Colony origin also seemed to affect the mortality in both rounds to a lesser degree (Round 1: Colony B: F=0.38, P<0.001; Colony D: F=0.42, P<0.001; Round 2: Colony C: F=0.43, P=0.017; Colony D: F=0.46, P=0.049).

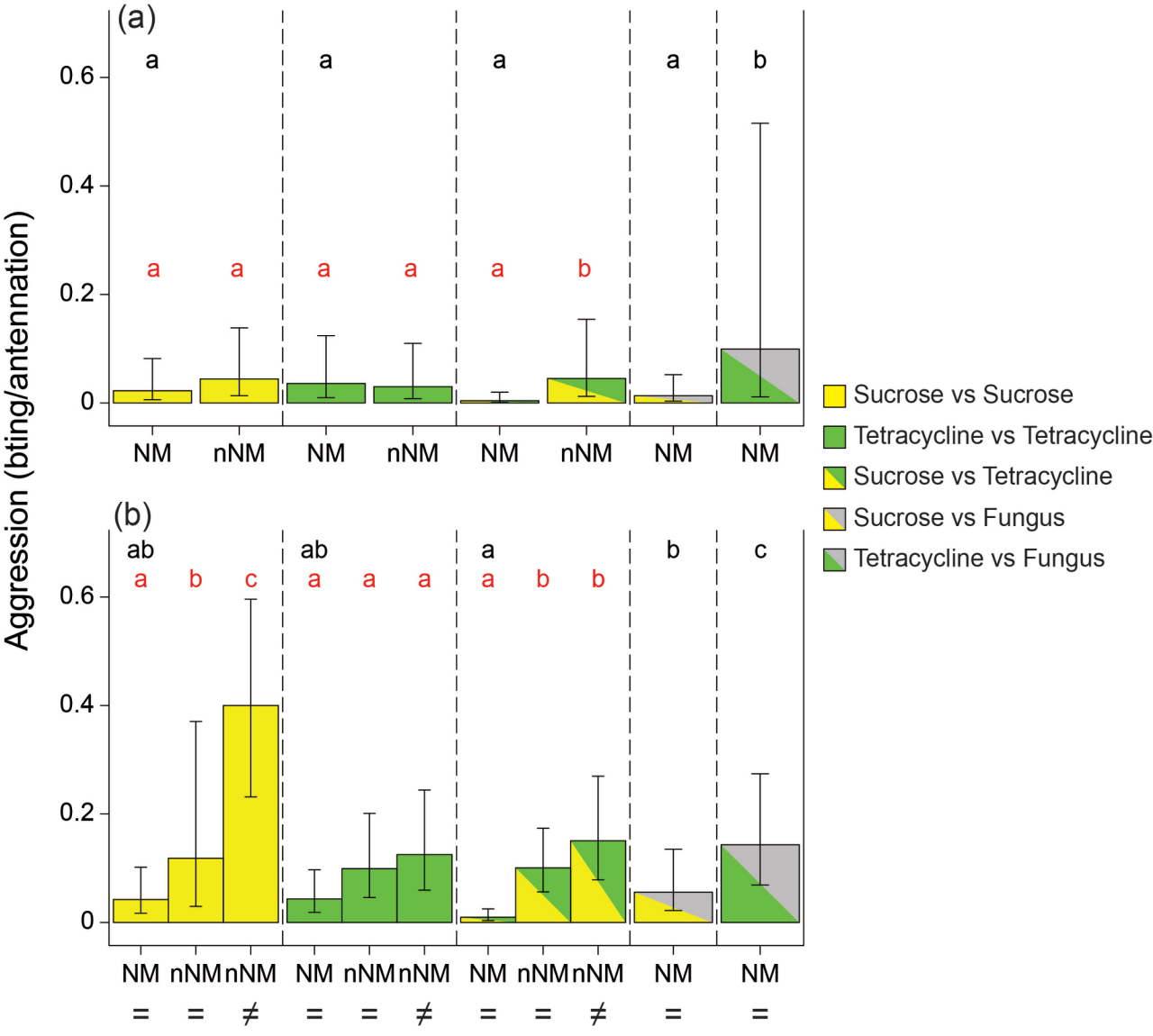
Figure A2. Diet treatments have different effects on bacteria in the guts and the rest of the tissues (heads and thoraces). Using three out of four colonies (A, B and D; Figure 1) that were used for the main experiment, we performed an additional separate experiment with similar setup (Figure 1) but with the only purpose of monitoring the bacterial changes in the gut (abdominal) tissues compared to heads and thoraxes. After the end of the experiment tissues and compartments were collected, DNA was extracted and bacterial community characterization was performed with qPCR and 16S-MiSeq sequencing (SAMN05362797-SAMN05362832) using previously described methods (Sapountzis et al., 2015). (a). Non-metric dimensional scaling (NMDS) of Bray-Curtis distances using the rarefied 16S-MiSeq reads of the twenty most abundant bacterial OTUs jointly in both guts and the rest of the body tissues. The light grey shadow shows the clustering of the gut samples, while the dark grey shows clustering of the head and thorax tissue samples. While gut bacterial communities are dominated by Wolbachia, RhiAcro1 and Mollicutes OTUs (Sapountzis et al., 2015), bacterial composition in heads and thoraces consists mostly of Actinobacteria, Wolbachia and Sphingobacteria OTUs. (b) Heat map showing the relative abundances of the most abundant 56 out of 411 OTUs after rarefaction (none of the remaining OTUs had more than 400 reads in total and

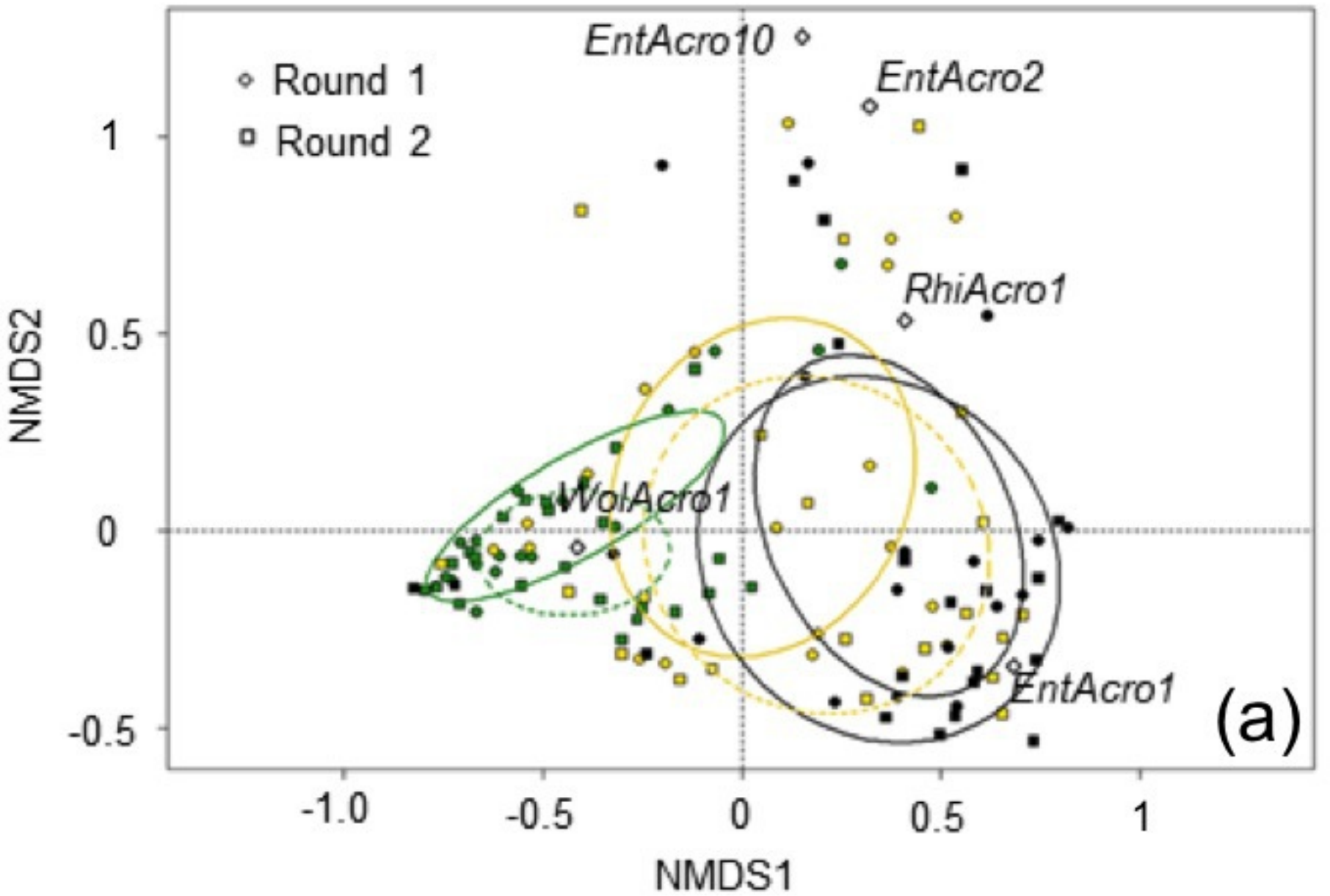
therefore, when plotted on a heat map similar to (b), they appeared as white blocks). OTUs were grouped by class: the first five belong to Alpha-Proteobacteria, the next three to Mollicutes, then Actinobacteria, Gamma-Proteobacteria, Sphingobacteria, Beta-Proteobacteria and others. The top half of the heat map shows relative abundances of OTUs in gut tissues, while the bottom half shows relative abundances of OTUs in the remaining tissues (heads and thoraxes). The diet treatments from which the ant samples originate are shown on the right: FG=fungus garden, T=tetracycline-treated, S=sucrose treated. The visual inferences of (a) and (b) suggested that the tetracycline and sugar diets affected, respectively strongly and moderately, RhiAcro1 and the Mollicutes OTUs (EntAcro1, EntAcro2, and EntAcro10) in the gut, similarly to a previous study (Sapountzis et al., 2015). We did not detect any treatment-related major differences in WolAcro1 abundances in the gut or any of the major taxa present in the head and thorax tissues (b). The above visual inferences suggesting that the major effects in microbiota due to diet treatments occur in the gut were confirmed by qPCR: in gut tissues, differences in the abundant bacterial taxa due to diet treatments were always highly significant and several-fold change (χ^2_4=18.86, P<0.001; χ^2_4=80.59, P<0.001; χ^2_4=41.99, P<0.001; χ^2_4=26.82, P<0.001 for EntAcro1, wSinvictaA, wSinvictaB and RhiAcro1, respectively). However, in the heads and thoraces, out of the three most abundant taxa (the joint average abundance of wSinvictaA, wSinvictaB and ActAcro1 in the guts of ants reared on fungus is 46-57 %), only the less than 1-fold differences in wSinvictaB were significant among treatments (χ^2_4=96.55, P<0.001), while the differences in wSinvictaA and ActAcro1 were either marginally significant or not at all (χ^2_4=10.43, P=0.033 and χ^2_4=0.93, P=0.919 respectively).

Figure A3. Behaviours observed in the aggression trials. (**a)** avoidance, (**b)** biting, (**c)** antennation, (**d)** mandible opening.

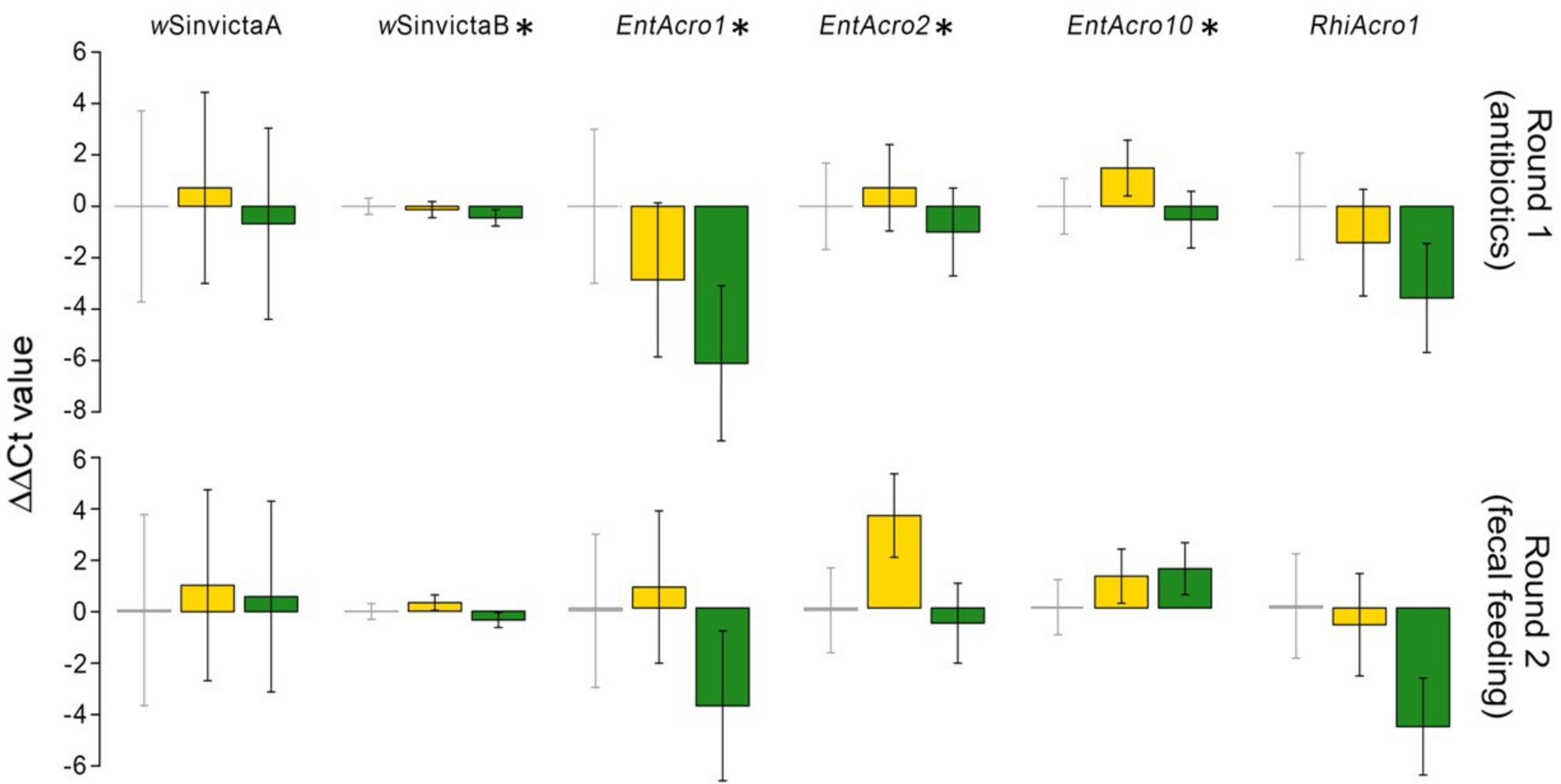
Figure A4. Coinertia analysis of PCA of gut bacteria *vs.* PCA of odour profile. **(a)** The relationship between the bacterial abundances of the six abundant taxa in the gut (qPCR data fold change, ΔCt values and the 73 cuticular GC-MS peaks analysed. **(b)** An expansion of the areas within the dashed square of A. The bacterial taxa are being presented as red vectors.

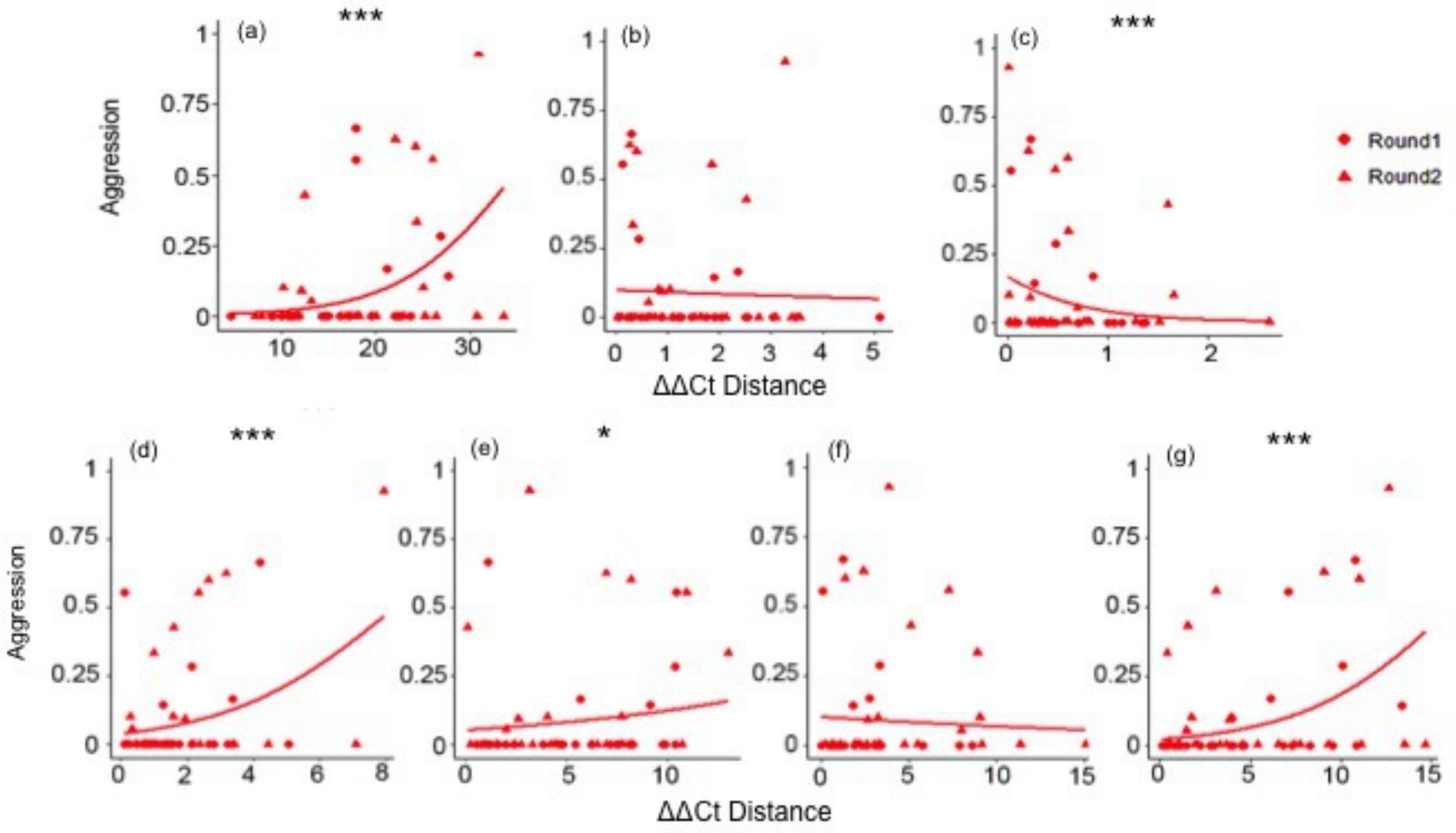

Round 1
Aggression assays
N
T vs T
NM
40
nNM
30
T vs S
NM
20
nNM
60
S vs S
NM
40
nNM
30
T vs FG
NM
20
S vs FG
NM
20
2 weeks
Colonies
A B C D
FG
N=10/colony
S
N=150/colony
T
N=225/colony
FG
N=10/colony
S
S + fd NM
S + fd nNM
S + fd NM
T
S + fd nNM
1 week
Round 2
Aggression assays
N
T vs T
NM + fd (NM)
39
nNM + fd (NM)
10
nNM + fd (nNM)
20
T vs S
NM + fd (NM)
17
nNM + fd (NM)
17
nNM + fd (nNM)
33
S vs S
NM + fd (NM)
25
nNM + fd (NM)
7
nNM + fd (nNM)
14
T vs FG
NM
20
S vs FG
NM
17


(a)
(b)
Aggression (bting/antennation)
0
0.2
0.4
0.6
NM
nNM
=
≠
Sucrose vs Sucrose
Tetracycline vs Tetracycline
Sucrose vs Tetracycline
Sucrose vs Fungus
Tetracycline vs Fungus


EntAcro10
EntAcro2
Round 1
Round 2
RhiAcro1
WolAcro1
EntAcro1
(a)
NMDS2
NMDS1
1
0.5
0
-0.5
-1.0
-0.5
0
0.5
1

EntAcro1
Stress: 0.10
D
WolAcro1
B
RhiAcro1
EntAcro10
Sucrose
Tetracycline
EntAcro2
(b)
NMDS2
0.5
0
-0.5
-1
-1
0
1
2
EntAcro1
Stress: 0.11
D
WolAcro1
B
RhiAcro1
EntAcro10
Sucrose
Tetracycline
EntAcro2
(c)
NMDS2
NMDS1
0.5
0
-0.5
-1
-1.5
-1
-0.5
0
0.5
1
1.5


wSinvictaA
wSinvictaB *
EntAcro1 *
EntAcro2 *
EntAcro10 *
RhiAcro1
ΔΔCt value
6
4
2
0
-2
-4
-6
-8
Round 1
(antibiotics)
Round 2
(fecal feeding)


(a)
***
(b)
(c)
***
Aggression
ΔΔCt Distance
Round1
Round2
(d)
***
(e)
*
(f)
(g)
***
Aggression
ΔΔCt Distance