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Interactions between fungi and bacteria influence microbial community structure in the *Megachile rotundata* larval gut

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Recent declines in bee populations coupled with advances in DNA-sequencing technology have sparked a renaissance in studies of bee-associated microbes. *Megachile rotundata* is an important field crop pollinator, but is stricken by chalkbrood, a disease caused by the fungus *Ascosphaera aggregata*. To test the hypothesis that some gut microbes directly or indirectly affect the growth of others, we applied four treatments to the pollen provisions of *M. rotundata* eggs and young larvae: antibacterials, antifungals, *A. aggregata* spores and a no-treatment control. We allowed the larvae to develop, and then used 454 pyrosequencing and quantitative PCR (for *A. aggregata*) to investigate fungal and bacterial communities in the larval gut. Antifungals lowered *A. aggregata* abundance but increased the diversity of surviving fungi. This suggests that *A. aggregata* inhibits the growth of other fungi in the gut through chemical or competitive interaction. Bacterial richness decreased under the antifungal treatment, suggesting that changes in the fungal community caused changes in the bacterial community. We found no evidence that bacteria affect fungal communities. *Lactobacillus kunkeei* clade bacteria were common members of the larval gut microbiota and exhibited antibiotic resistance. Further research is needed to determine the effect of gut microbes on *M. rotundata* health.

1. Introduction

Microbes have profound effects on insect health as pathogens, nutritional symbionts and facultative symbionts [1]. For example, desert locusts, *Schistocerca gregaria*, harbour gut bacteria that inhibit colonization by pathogenic fungi during times of starvation, when locusts are otherwise especially susceptible [2]. Bacteria are also known to defend their hosts against fungi outside of the gut in aphids [3], beewolf wasps [4], fungus-growing beetles [5] and fungus-growing ants [6].

While it has long been known that pathogenic microbes can have large effects on the fitness of honey bees [7] and some solitary bees [8,9], whether the gut microbiota of bees mediate resistance to infections has been less explored. Recent studies have shown that the gut microbiota of the bumble bee *Bombus terrestris* can protect its host from the trypanosome parasite *Crithidia bombi* [10,11]. Lactic acid bacteria associated with the honey bee may inhibit pathogens such as *Paenibacillus larvae* [12] and *Melissococcus plutonius* [13], and some core gut microbes may also aid in pollen digestion and pathogen defence [14]. The emerging view is that some microbes have beneficial effects on bee fitness, often by providing defence from pathogens.

Megachile rotundata is a solitary bee whose gut microbiota was extensively studied using culture-based techniques [8,15–18], but no recent non-culture-based studies have been conducted. The second most important field crop pollinator after the honey bee [19], *M. rotundata* is plagued by chalkbrood, a fungal disease of the larvae caused by *Ascosphaera aggregata* [20]. Chalkbrood can be a significant source of mortality in *M. rotundata*, yet no effective control strategy currently exists [21].

In this study, we explore interactions between the gut fungi and bacteria associated with *M. rotundata* larvae. We hypothesize that microbes compete in the gut environment, with some directly or indirectly inhibiting the growth of others. Using non-culture-based methods, we describe here how feeding larvae antifungals, antibacterials and pathogen spores changes the community structure and diversity of the fungi and bacteria of the gut.

2. Material and methods

(a) Study organisms

We collected *M. rotundata* nests from a typical agricultural setting for this bee, managed populations in commercial alfalfa seed fields in Box Elder County, UT, as described in Huntzinger *et al.* [22]. Each *M. rotundata* nest is composed of individual cells that contain a mixture of pollen and nectar collected by the mother bee (and hereafter called a pollen provision), and one immature offspring. After provisioning, the brood receives no further maternal care. We removed individual cells from collected nests, and those containing eggs or first instars were express-mailed to the University of Texas, Austin. We placed individual brood cells into wells of a 96-well tissue culture plate (Becton-Dickson, Franklin Lanes, NJ). The wells are approximately the same size as a brood cell and served to hold cells upright. By this time, 2–3 days had passed, and so we used only those cells that contained eggs or first or second instars. All other cells were discarded. *Megachile rotundata* larvae do not fully emerge from the egg chorion and begin to feed until the second instar [23]. We kept the plates in an incubator at 28.5°C and 50% relative humidity.

(b) Treatments

To manipulate gut microbial communities, we randomly applied four treatments to these young larvae by saturating the surface of the pollen provisions with either (*a*) 3 µl of an antibacterial cocktail consisting of 0.003 g ml^{-1} each of rifampicin (Fisher Scientific, Fort Lawn, NJ), tetracycline (Sigma-Aldrich, St Louis, MO), ampicillin (Invitrogen, Grand Island, NY), chloramphenicol (Sigma-Aldrich), and erythromycin (Acros Organics, NJ); (*b*) 3 µl of an antifungal cocktail consisting of Rovral 4F (iprodione, Bayer Cropscience, Kansas City, MO) and fluconazole (Tokyo Chemical Industry, Tokyo, Japan); (*c*) *A. aggregata* spores (obtained from chalkbrood-infected *M. rotundata* cadavers collected from the same field the previous year and stored at room temperature) suspended in sterilized, deionized water (4 µl of 0.04 g spores ml^{-1}) and (*d*) a no-treatment control.

Antimicrobial treatments directly affected bee health and increased mortality, so we tried several treatment schedules and selected the one with the greatest larval survival. This schedule was as follows: antibacterials every other day for 6 days, two *A. aggregata* spore applications (days 1 and 3) and one antifungal treatment on the first day. Larvae were sampled for analysis 7 days after the first day of treatment.

We selected the 10 healthiest appearing, post-defecating larvae (*M. rotundata* larvae have a blind gut and do not defecate until the 5th instar [23]) from each treatment for microbial community sequencing. To avoid contamination, we dissected the intestinal tract of each larva in a new dissecting tray that we sterilized with UV light for 10 min in a laminar flow hood. We dissected the larvae under sterilized water with flame-sterilized dissecting tools. To preserve microbial nucleic acids, we collected each intestinal tract into 250 µl of RNAlater (Ambion, Grand Island, NY) and homogenized the intestines with sterilized pestles. We collected an additional no-template control sample that we exposed to the air during sample collection. We immediately stored the samples at −80°C until ready for DNA extraction and 454 ribotyping.

(c) 454 Pyrosequencing of fungal and bacterial communities

Molecular Research LP (Shallowater, TX) performed the DNA extractions (with 0.1 mm glass beads) and sequencing on a FLX genome sequencer with titanium reagents according to previously published protocols [24–26]. Bacterial 16S rRNA was PCR amplified using the 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′) primer pair, which targets the V1–V3 regions of the 16S gene. The fungal internal transcribed spacer (ITS) was PCR amplified using the ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′) primer pair. The data are publicly available on NCBI's sequence read archive (SRA) under accession number SRP021036.

We analysed the pyrosequencing data using the software program MOTHUR [27,28]. To utilize the UNITE ITS database [29] and to verify our 16S rDNA analyses, we conducted parallel analyses in the program QIIME [30]. For all analyses we followed the standard methods recommended for honey bee gut symbionts [31]. We analysed the 16S rDNA and ITS data separately, but used similar methods (see the electronic supplementary material for detailed descriptions of our analyses). To assign sequences to operational taxonomic units (OTUs), we clustered sequences that shared 97% or greater sequence identity. We removed sequences that did not share 97% or greater sequence identity with any other sequence (i.e. singleton OTUs), as these singleton OTUs may represent sequencing errors. To be certain that we fully explored our data, we also ran all analyses with singleton OTUs included (not reported). For taxonomic assignment, we used the naïve Bayesian classifier (minimum confidence score of 0.8) and the UNITE and greengenes databases for the ITS and 16S rDNA sequences, respectively. To verify these assignments, we conducted additional BLASTn searches against the NCBI nucleotide sequence database.

(d) Microbial community data analysis

We evaluated the effect of treatments on both alpha diversity (microbial diversity within individual guts, i.e. rarefaction curves, the probability of an interspecific encounter (PIE, a diversity index) and Chao1-estimated OTU richness) and beta diversity (microbial diversity across individual guts, i.e. non-metric multi-dimensional scaling (NMDS) of Bray–Curtis dissimilarity matrices), again using the standard methods for honey bee gut symbionts [31] (see online electronic supplementary material for a detailed description). We additionally compared the effects of treatments on bacterial and fungal OTU richness, diversity (PIE), and Chao1-estimated richness (separately) using analysis of variance (ANOVA). We verified that the variances of the error terms were homogeneous. To compare each treatment to the control, we used planned contrasts.

(e) Quantifying *Ascosphaera aggregata* spores

To determine the absolute abundance of *A. aggregata* spores in the larval gut samples, we used quantitative real-time PCR (qPCR) of the same DNA extractions as above. Each PCR reaction consisted of 5.0 µl iQ SYBR Green Supermix (Bio-Rad Laboratories, Los Angeles, CA), 3.6 µl molecular grade water, 0.2 µl forward primer, 0.2 µl reverse primer and 1.0 µl sample or standard DNA. We developed *A. aggregata* species-specific primers for amplification of the ITS1 spacer region based on previously published primers [32], by using the complement of the species-specific forward primer (1a-F2) as our reverse primer (5′-CTCGTCGAGGG TCTTTTCC-3′), and using a conserved region (AscoAll1) for our

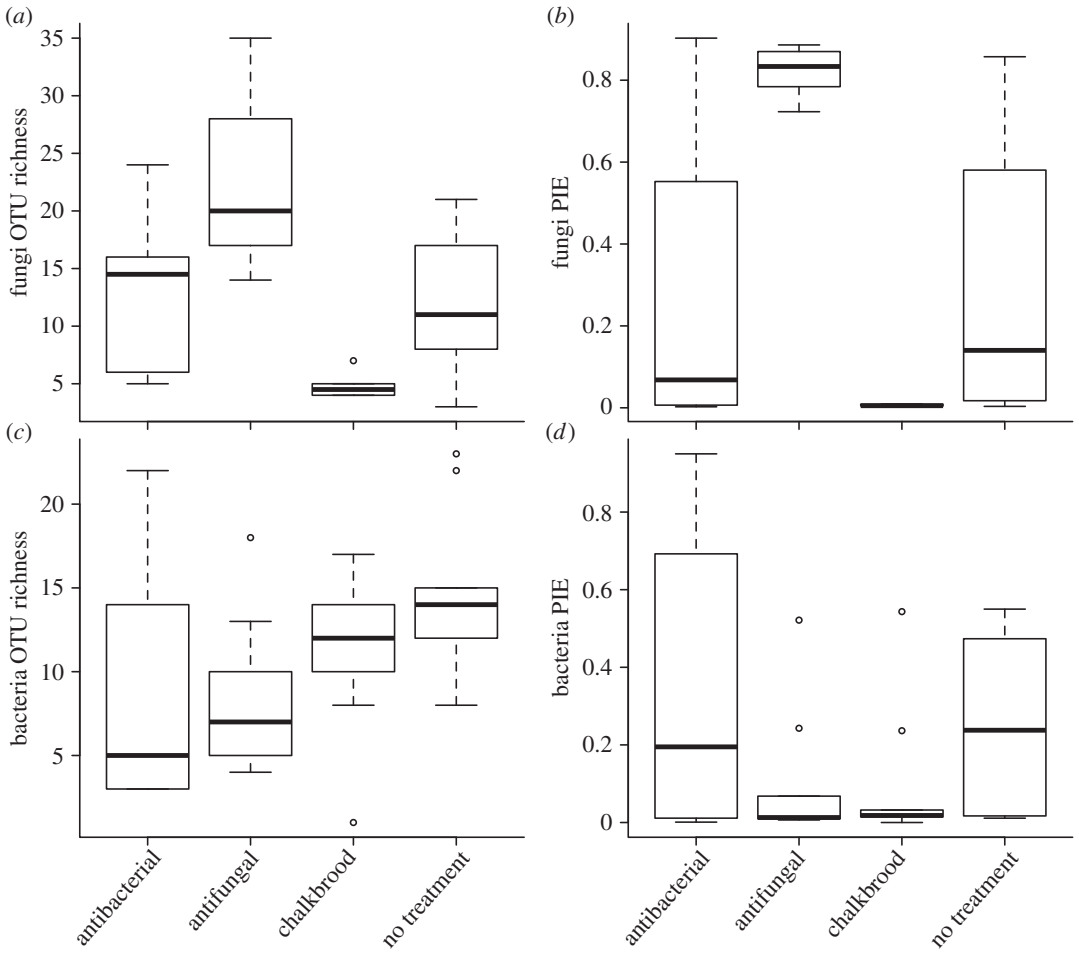

Figure 1. Boxplots of medians, quartiles and outliers of (*a*) fungal operational taxonomic unit (OTU) richness, (*b*) fungal evenness (PIE—the probability of an interspecific encounter, a diversity index), (*c*) bacterial OTU richness, and (*d*) bacterial evenness (PIE) across treatments.

Table 1. Sequence and OTU statistics from the MOTHUR and QIIME analyses.

	fungi		bacteria	
analysis	MOTHUR	QIIME	MOTHUR	QIIME
total reads	118 917	125 299	117 384	200 775
average reads per sample	2972	3030	2935	5019
total OTUs in all samples	171	198	135	169
average OTUs per sample	13	47	11	45

forward primer (5′-GCACTCCCACCCTTGTCTA-3′). This modification created a short amplicon (114 bp) appropriate for qPCR, yet retained the use of the species-specific sequence. The reaction conditions were an initial heating at 95°C for 1 min, followed by 31 cycles of 94°C for 15 s, 56°C for 15 s and 72°C for 15 s.

We quantified *A. aggregata* spores by comparing the quantification cycle (C_q) of the sample with that of a standard DNA series run at the same time. For the standards, we used a series of *A. aggregata* spore concentrations (1×10^8, 1×10^7, 1×10^6, 1×10^5 spores ml^{-1} water; determined using a haemocytometer), and extracted DNA from 100 µl of each concentration using the UltraClean Plant DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). We conducted our qPCR reactions in 96-well plates, and we included two replicate standard series on each plate. Also, we ran two replicate qPCR reactions for every gut sample, and the spore quantity data reported are the averages of the two. The mean reaction efficiency across all the standards was 2.01 (s.d. = 0.12).

3. Results

(a) 454 Pyrosequencing and community sampling statistics

The fungal and bacterial communities in the guts of larval *M. rotundata* were composed of relatively few OTUs (average of 11–47 OTUs per sample; table 1, figures 1 and 2). Our sampling of most communities was at or near saturation, based on the rarefaction curves (figure 2, electronic supplementary material, S1). The MOTHUR and QIIME analysis pipelines

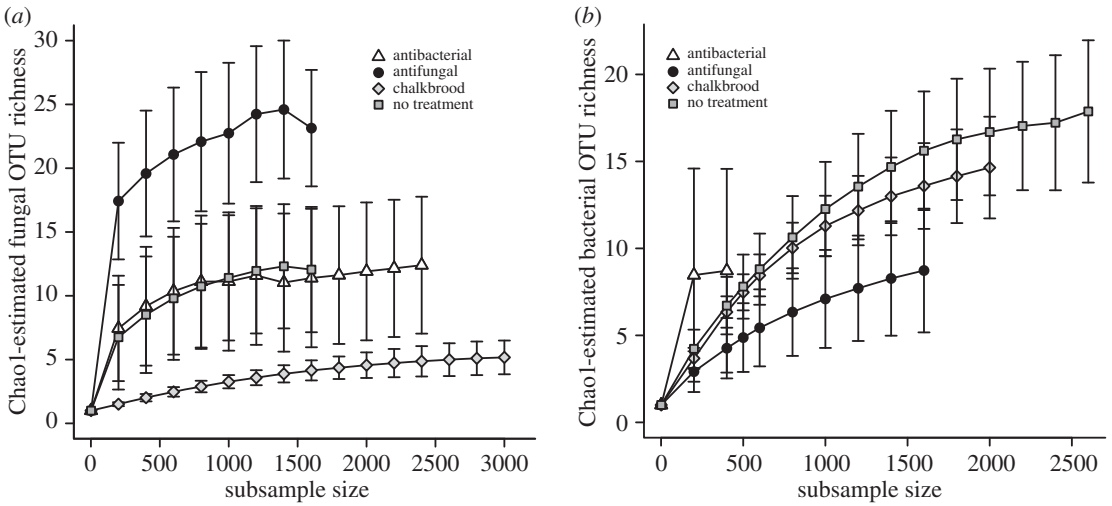

Figure 2. Rarefaction analysis of Chao1-estimated OTU richness of (*a*) fungal and (*b*) bacterial communities, averaged across treatments (means indicated with 95% CIs). The *X*-axis indicates the number of sequences in each subsample (without replacement) in the rarefaction analysis.

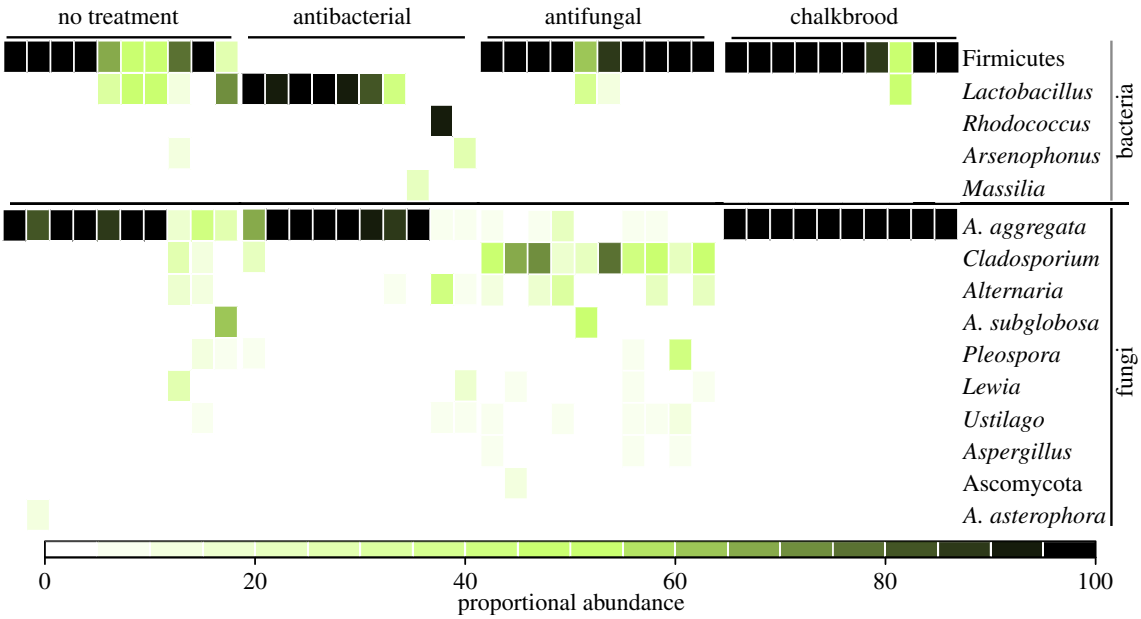

Figure 3. Heatmap of the top five bacterial phylotypes and the top 10 fungal phylotypes found in the *M. rotundata* larval gut. Each column represents an individual gut sample, arranged by treatment. Proportional abundance of each phylotype (rows) within each larval gut microbial community (columns) is represented by colour as indicated in the scale at the bottom of the graph. (Online version in colour.)

were similar in the number of sequences retained after quality control and in the number of OTUs per sample (table 1). Alpha diversity (see electronic supplementary material, figure S1), OTU taxonomy (figure 3, see electronic supplementary material, table S1 for OTU taxonomic assignments from the MOTHUR and QIIME pipelines) and beta diversity (figure 4, electronic supplementary material, S2) were also similar between the two analysis pipelines. Our negative control sample (sampled air, no template) failed to produce PCR amplicons, and was therefore not sequenced.

(b) Fungal community diversity

The experimental treatments had a significant effect on fungal OTU richness (figure 1*a*, $F_{3,36} = 16.56$, $p < 0.001$) and evenness (figure 1*b*, $F_{3,36} = 19.28$, $p < 0.001$). The application of *A. aggregata* spores to the pollen provisions decreased fungal OTU richness (figure 1*a*, $F_{1,36} = 2.92$, $p = 0.006$), while the antifungal treatment increased fungal OTU richness (figure 1*a*, $F_{1,36} = 4.09$, $p < 0.001$). The treatments showed the same pattern of effects on fungal community evenness: the *A. aggregata* treatment decreased fungal OTU evenness (figure 1*b*, $F_{1,36} = 2.54$, $p = 0.02$), while the antifungal treatment increased fungal OTU evenness (figure 1*b*, $F_{1,36} = 4.85$, $p < 0.001$). Increased fungal OTU richness and evenness in the antifungal treatments corresponded with a decrease in the proportional and absolute abundance of *A. aggregata* (figures 3 and 5). The antibacterial treatment, however, did not affect fungal OTU richness (figure 1*a*, $F_{1,36} = 0.195$, $p = 0.85$) or community evenness (figure 1*b*, $F_{1,36} = 0.16$, $p = 0.87$).

To determine whether the differences in fungal OTU richness in the *A. aggregata* and antifungal treatments were due to an inhibitory effect (either direct or indirect) between *A. aggregata* and other fungi in the gut or just a swamping effect of *A. aggregata*, we additionally compared Chao1 estimates of OTU richness between our treatments. To equalize sampling depth, we estimated OTU richness based on a random subsample of 1020 sequences per sample, which

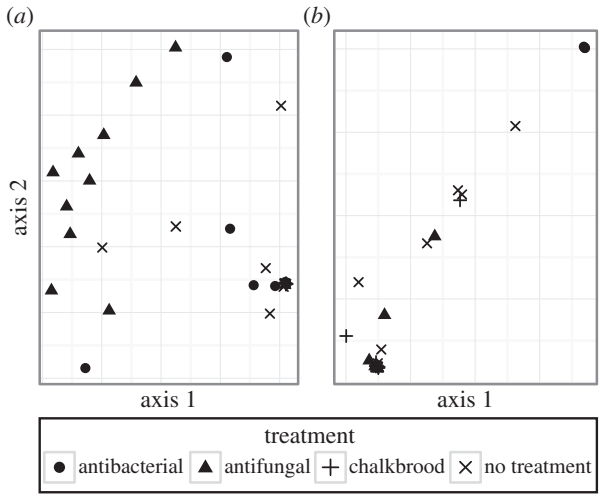

Figure 4. Non-metric multidimensional scaling (NMDS) ordination of (*a*) fungal communities and (*b*) bacterial communities from four treatments. The main fungal cluster is composed of communities dominated by *A. aggregata*, while the main bacterial cluster is composed of communities dominated by the unclassified Firmicute OTU. Three out of 10 bacterial communities from larvae that had been treated with antibacterials generated enough sequences to include in the analysis, these three communities cluster near the top of (*b*). We conducted NMDS analyses with two-dimensional solutions, 500 iterations each, stress = 0.13 for fungal communities and 0.01 for bacterial communities.

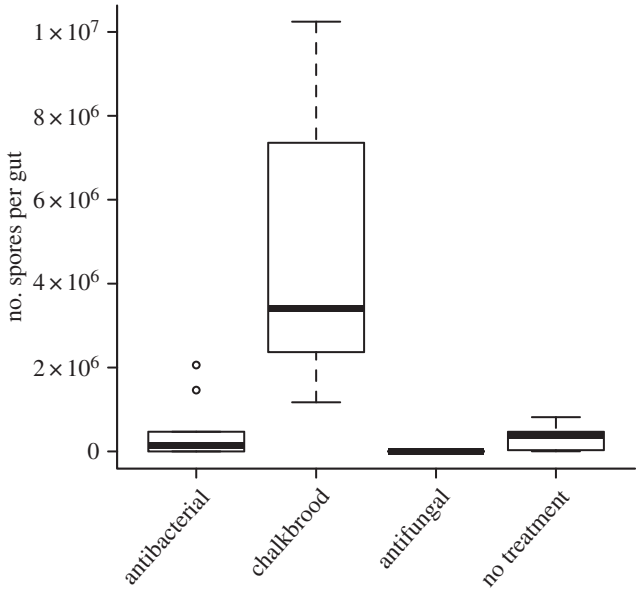

Figure 5. Boxplot of median, quartiles and outliers for the number of *A. aggregata* spores from larval guts by treatment as determined by quantitative PCR using absolute quantification.

allowed us to include most samples and base our Chao1 estimates on a large number of sequences (figure 2). The estimated OTU richness differed significantly across treatments (figure 2*a*, $F_{3,36} = 15.85$, $p < 0.001$). The *A. aggregata* treatment decreased fungal OTU richness in comparison to the no-treatment control ($F_{1,36} = 2.86$, $p = 0.007$) whereas the antifungal treatment increased fungal OTU richness ($F_{1,36} = 3.98$, $p < 0.001$). The antibacterial treatment did not affect fungal OTU richness ($F_{1,36} = 0.11$, $p = 0.91$).

Based on the proportional abundance of the ITS sequences, *A. aggregata* was the most abundant fungal taxon present (figure 3). *Ascosphaera aggregata* abundance, however, was lower in all of the communities treated with antifungals, two communities treated with antibacterials and three control communities. Besides *A. aggregata*, several saprophytic fungi, plant pathogenic fungi, an unclassified ascomycete fungus and two other members of the *Ascosphaera* genus (*A. subglobosa* and *A. asterophora*) were also represented in one or more communities in proportional abundances between 8 and 77% (figure 3). One larval gut contained an OTU that shared 99% sequence identity to *Beauveria bassiana*, the asexual form of *Cordyceps bassiana* and an entomopathogen with a broad host range (49).

NMDS ordination of the Bray–Curtis dissimilarity matrix also showed that fungal communities differed by treatment (figure 4). All *A. aggregata*-treated communities grouped into one tight cluster, along with several of the antibacterial and no-treatment control samples, all of which were dominated by *A. aggregata*. Three no-treatment control and two antibacterial-treated communities were placed well outside of this cluster, as were all the communities in the fungicide treatment. All of the communities outside of the *A. aggregata* cluster had lower proportional abundance of *A. aggregata*.

The mean number of *A. aggregata* spores per larval gut as determined by qPCR differed between our treatments (figure 5, $F_{3,36} = 17.69$, $p < 0.001$). Unsurprisingly, the *A. aggregata* spore treatment showed the highest number of *A. aggregata* spores (figure 5, $F_{1,36} = 5.86$, $p < 0.001$). No *A. aggregata* spores were detected by qPCR in the antifungal treatments, while the number of spores in the antibacterial treatments did not differ from the no-treatment control (figure 5, $F_{1,36} = 0.24$, $p = 0.81$).

(c) Bacterial community diversity

Bacterial OTU richness was significantly affected by our treatments (figure 1*c*, $F_{3,36} = 3.24$, $p = 0.03$). Antibacterial treatments (figure 1*c*, $F_{1,36} = 2.55$, $p = 0.02$) and antifungal treatments (figure 1*c*, $F_{1,36} = 2.77$, $p = 0.008$) both lowered bacterial OTU richness in comparison to the no-treatment control, but *A. aggregata* treatments did not (figure 1*c*, $F_{1,36} = 1.39$, $p = 0.17$). Although bacterial community evenness appeared lower in the antibacterial and *A. aggregata* treatments (figure 1*d*), evenness and contrasts of treatments to the control were not significantly affected by our experimental treatments ($F_{3,36} = 2.37$, $p = 0.09$). Chao1 estimates of bacterial OTU richness were significantly different between treatments (figure 2*b*, $F_{2,26} = 6.21$, $p = 0.006$), with lower estimated richness in the antifungal samples compared to the controls (figure 2*b*, $F_{1,26} = 2.00$, $p = 0.002$). Seven of the 10 samples treated with antibacterials generated fewer than 1000 sequences (electronic supplementary material, figure S1). We included all antibacterial-treated samples in the alpha diversity analyses except for the Chao1 ANOVA, from which we excluded all antibacterial samples in order to maintain balance across treatments. We excluded the seven samples with fewer than 1000 sequences from our ordination analysis.

The most abundant bacterial taxon across all samples was an unclassified Firmicute bacterium as assigned by the naïve Bayesian classifier (figure 3, electronic supplementary material, table S1). This bacterium had 93% sequence similarity to an unclassified bacterium isolated from the gut of *Apis mellifera* [33] and 84% sequence similarity to an unclassified *Clostridium* species isolated from the gut of the moth *Spodoptera littoralis* [34]. This unclassified Firmicute was proportionally abundant in nearly all antifungal and *A. aggregata*-treated samples and in seven of the no-treatment control samples,

but was missing from all of the antibacterial treatments. The second-most abundant taxon shared 100% sequence identity to a *Lactobacillus* strain belonging to the *L. kunkeei* clade and found originally in association with the sweat bee *Augochlora pura* [35]. This *L. kunkeei* relative was proportionally abundant in the antibacterial-treated samples and in a few of the no-treatment control samples (figure 3, electronic supplementary material, table S1). Although not as prevalent or abundant, an OTU with 99% sequence similarity to several sequences from the secondary endosymbiont *Arsenophonus* was associated with three larvae (top BLAST hit NCBI accession number DQ115536.1, figure 3, electronic supplementary material, table S1). An even more rare OTU exhibited 99% sequence identity to a *Sodalis*-like symbiont of a stinkbug (NCBI accession number AB571330.1, electronic supplementary material, table S1) and 98% sequence identity to several *Sodalis glossinidius* sequences (e.g. NCBI accession number AP008232.1).

NMDS ordination of the Bray–Curtis dissimilarity matrix showed that bacterial communities from larvae treated with antibacterials differed from the other treatments. Only three of the gut samples from larvae treated with antibiotics returned more than 1000 bacterial sequences, and these three samples clustered separately from the other samples (figure 4). Gut bacterial communities from the control, antifungal and *A. aggregata* treatments were mostly dominated by the unclassified Firmicute and formed a tight cluster in the NMDS plot (figure 4). Those gut bacterial communities from the control, antifungal and *A. aggregata* treatments that were not dominated by the unclassified Firmicute did not closely cluster with each other or the three antibacterial treatments (figure 4).

4. Discussion

Ascosphaera aggregata appears to affect the growth of other fungi in the larval gut of the alfalfa leafcutting bee *M. rotundata*. We do not currently know whether this effect is caused by competition dynamics between *A. aggregata* and other fungi, by antibiotic compounds secreted by *A. aggregata*, or is simply a byproduct of *A. aggregata* metabolism. Many of the fungi we detected are known saprophytes, and may compete with *A. aggregata* for resources in the larval gut. We hypothesize that the ability of *A. aggregata* to affect the growth of these other fungi may be the result of selection on *A. aggregata* to be an effective competitor in the larval gut.

Alternatively, fungi present as only a few cells may not have been detected in the *A. aggregata* spore treatments and no-treatment controls, but these rare fungi may have been detected in the antifungal treatments when *A. aggregata* relative abundance was diminished. Two lines of evidence, however, suggest that was not the case. First, the Chao1 estimate of richness, which is based on the number of rare taxa sampled from a community, displayed the same significant patterns of richness as our observed OTU counts. Second, the 454-pyrosequencing assay detected very low levels of *A. aggregata* in the antifungal treatments; the pyrosequencing assay was therefore more sensitive than the qPCR assay. Thus, the pyrosequencing assay should be sensitive enough to detect very low levels of other fungi, ruling out the above explanation that the patterns we detected were due to sampling error instead of interactions between *A. aggregata* and other fungi.

Two competing explanations may underlie the diminished bacterial OTU richness in our antifungal treatments. The antifungal treatments decreased abundance of *A. aggregata*, which suggests that *A. aggregata* may positively affect some bacteria. *Ascosphaera aggregata* may provide necessary metabolites for certain bacteria or inhibit the growth of other fungi that outcompete or otherwise interfere with certain bacteria in the larval gut. Alternatively, although we chose antifungals that specifically target fungi and not bacteria, we cannot rule out the possibility that the antifungals also negatively affected certain bacteria.

We did not find evidence that bacteria affect fungi. The most proportionally abundant bacteria did not correlate with the abundance of *A. aggregata*. The *Arsenophonus* OTU occurred in only three samples, all of which exhibited low *A. aggregata* abundance, although one of these samples was treated with antifungals. The genus *Arsenophonus* includes secondary endosymbionts (including son-killers), host-specific primary beneficial endosymbionts, non-specific putative mutualists and plant pathogens [36,37]. Future study is needed to determine whether the *Arsenophonus* OTU associated with *M. rotundata* adopts son-killing, mutualism or some other strategy.

In a culture-based study of interactions between *A. aggregata* and other microbes, Inglis *et al.* [17] were unable to detect any inhibitory relationship between the rest of the *M. rotundata* microbiota and *A. aggregata*, but did find that the prevalence of chalkbrood (the disease itself) was greater in larvae with their naturally occurring microbiota in comparison to larvae raised on axenic or near-axenic pollen provisions. Naturally occurring microbes therefore seemed to enhance chalkbrood infection.

We found similar fungal communities, but different bacterial communities, compared to previous culture-based *M. rotundata* gut microbiota surveys [15,16]. Thus, the fungal species present in the larval guts are more readily cultured than the bacterial species. Both Goerzen [16] and Inglis *et al.* [15] found *Alternaria alternata* and *Aspergillus* species in their surveys, which we also detected as common associates of *M. rotundata*. Inglis *et al.* [15] further reported *Cladosporium cladosporiodes* as a dominant fungus in *M. rotundata* pollen and provisions, and we found this fungus to be common in the larval guts. As previously reported [21], we likewise found *A. aggregata* to be more common in our US derived larvae than in the Canadian populations of Goerzen [16], where chalkbrood occurs at low levels.

Besides *A. aggregata*, we also found some interesting putative pathogenic fungi associated with *M. rotundata* larval guts. Although only found in one larva, we detected the presence of the entomopathogenic *B. bassiana*. This fungus can infect *M. rotundata* immatures [38] and adults [39], although infections have only been seen in laboratory bioassays and not in the field. This fungus is also a very common soil microbe. We also detected two other species of *Ascosphaera*: *A. asterophora* [20] and *A. subglobosa* [40], although at much lower abundance than *A. aggregata*.

Host-specificity of gut microbes is a condition conducive to coevolution and mutualism [14]. The unclassified Firmicute OTU we found is not closely related to any known bacteria, and thus may be specific to the *M. rotundata* gut environment. Some members of the core gut microbiota of honey bees and bumble bees likewise appear to be host-specific and mutualistic [10,11,14]. However, such host-specificity contrasts with the *L. kunkeei* clade OTU, which inhabits the guts of a broad range of bee species and has also been isolated from flowers [35,41].

We found several gut bacteria with resistance to a broad range of antibiotics, including the proportionally abundant

L. kunkeei OTU. US honey bees have been treated with tetracycline for decades and their gut microbes have acquired multiple tetracycline resistance genes [42]. *Lactobacillus kunkeei* is a common associate of the honey bee foregut (crop) and hive materials [43–45]. To persist in those environments *L. kunkeei* may have also acquired antibiotic resistance. This suggests that pesticide application to honey bees may have far-reaching effects on the microbiota of native bee populations.

Future research is needed to understand the mechanisms that underlie the interactions that we found here. For example, bioassays could determine if *A. aggregata* directly inhibits other *M. rotundata*-associated fungi via chemical secretions. Overlapping resource requirements of *A. aggregata* and the other gut fungi would suggest that competition for limiting resources is important. Genomic or transcriptomic analyses would determine the antibiotic and competitive potential of *A. aggregata*. Further studies are needed to determine whether the decrease in bacterial richness in the antifungal treatments was due to fungi interacting with bacteria or caused by the antifungals directly affecting bacteria. Research determining the roles of these microbes in *M. rotundata* health and development also promises to be rewarding.

Data accessibility. DNA sequences: NCBI Sequence Read Archive accession number SRP021036.

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