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Microbiome Structure Influences Infection by the Parasite *Crithidia bombi* in Bumble Bees

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ABSTRACT Recent declines in bumble bee populations are of great concern and have prompted critical evaluations of the role of pathogen introductions and host resistance in bee health. One factor that may influence host resilience when facing infection is the gut microbiota. Previous experiments with *Bombus terrestris*, a European bumble bee, showed that the gut microbiota can protect against *Crithidia bombi*, a widespread trypanosomatid parasite of bumble bees. However, the particular characteristics of the microbiome responsible for this protective effect have thus far eluded identification. Using wild and commercially sourced *Bombus impatiens*, an important North American pollinator, we conducted cross-wise microbiota transplants to naive hosts of both backgrounds and challenged them with a *Crithidia* parasite. As with *B. terrestris*, we find that microbiota-dependent protection against *Crithidia* operates in *B. impatiens*. Lower *Crithidia* infection loads were experimentally associated with high microbiome diversity, large gut bacterial populations, and the presence of *Apibacter*, *Lactobacillus* Firm-5, and *Gilliamella* spp. in the gut community. These results indicate that even subtle differences between gut community structures can have a significant impact on a microbiome's ability to defend against parasite infections.

IMPORTANCE Many wild bumble bee populations are under threat due to human activity, including through the introduction of pathogens via commercially raised bees. Recently, it was found that the bumble bee gut microbiota can help defend against a common parasite, *Crithidia bombi*, but the particular factors contributing to this protection are unknown. Using both wild and commercially raised bees, we conducted microbiota transplants to show that microbiome diversity, total gut bacterial load, and the presence of certain core members of the microbiota may all impact bee susceptibility to *Crithidia* infection. Bee origin (genetic background) was also a factor. Finally, by examining this phenomenon in a previously uninvestigated bee species, our study demonstrates that microbiome-mediated resistance to *Crithidia* is conserved across multiple bumble bee species. These findings highlight how intricate interactions between hosts, microbiomes, and parasites can have wide-ranging consequences for the health of ecologically important species.

KEYWORDS *Bombus impatiens*, trypanosomatid, gut microbiota, host-microbe interaction, symbiosis

As one of the most common insect pollinators of flowering plants, bumble bees fulfill vital roles in both natural ecosystems and agricultural croplands. However, bumble bee populations have experienced recent decline, most likely due to land use change and the spread of pathogens (1, 2). One protozoan parasite, *Crithidia bombi*, infects multiple bumble bee species (3). *Crithidia bombi* increases bumble bee mortality under stressful conditions (4) and reduces colony-founding success of infected queens by 40%, while also leading to significant reductions in mass among infected queens, colony size, male production, and overall fitness (5).

Recently, it has been found that the presence of the bumble bee gut microbiota can

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protect against *C. bombi* infection (6) and that variation in this protective capability is driven at least as much by the microbiota as by host genetics (7). Neither the precise aspects of the microbiome that confer this defensive benefit nor its mechanism of action are known (8). Unlike honey bees, lone bumble bee queens establish new colonies annually; thus, the microbiome of a bumble bee colony reflects that of its founding queen, as well as bacteria acquired during foraging and contact with nestmates (9–11). The single-queen generational bottleneck and the relatively small colony size (typically $<$100 individuals, depending on species) are hypothesized to give rise to microbiome heterogeneity between bumble bee colonies (7, 12).

In this study, we conducted controlled microbiota transplants from different queens into sterile worker bees to determine how *C. bombi* infection loads are affected by microbiome composition via measurements of diversity, bacterial load, and abundances of bacterial phylotypes (closely related clusters corresponding to species or species groups). Furthermore, we compared bees of wild and commercial (captive-bred) origins. Bumble bees have been bred for use as commercial pollinators since 1988 and have been used to supplement or even replace other pollination methods (13). Commercial bumble bees often have higher pathogen infection rates than wild bumble bees and can spread those diseases to wild bumble bee populations (14). Such transmission routes may be a contributing factor in the decline of wild bees (14, 15–19). Both host genetics and the microbiomes of wild versus commercial bees could differ in their ability to confer resistance to *C. bombi*, and we tested these factors using a crosswise experimental design.

Previous studies showing the protective effect of the microbiota against *C. bombi* were conducted with *Bombus terrestris*, a common European bumble bee (6–8). Here, we found that this effect also extends to a species endemic to North America, *Bombus impatiens*. *B. terrestris* and *B. impatiens* are the major commercial species bred in their respective regions (13). We found that bees of commercial origin have reduced susceptibility to the tested *C. bombi* strain and that parasite infection load is negatively correlated with microbiome diversity and bacterial load. Finally, we identified several bacterial phylotypes that are strongly associated with *C. bombi* resistance; these represent potentially beneficial microbes that warrant further investigation.

RESULTS

The following is a brief outline of the experiment: newly eclosed bees (adult bees that emerge from pupation), which lack gut bacteria (6), were inoculated with 5 different microbiota treatments (harvested from the guts of queen bees of different origins). These were then challenged with the *Crithidia* parasite. After 7 days, guts were removed, the parasites were enumerated, the bacterial community compositions were determined by 16S rRNA gene sequencing, and absolute bacterial loads were quantified by quantitative PCR (qPCR). A detailed description of the experiment and treatments can be found in Materials and Methods.

The five different microbiota treatments administered prior to *C. bombi* infection were given to bees from both wild and commercial origins. Four of the treatments involved inoculation with gut microbiota derived from a single wild queen, a pooled combination of four wild queens, a single commercial queen, or a pooled combination of four commercial queens. The fifth inoculum consisted of pooled material from the other four inocula, filtered to remove most cells, while retaining acellular factors that may influence host immunity or parasite resistance. Our rationale behind using several separate microbiome treatment inocula was that different source bees can have different microbiome compositions. Different compositions may give rise to different interactions with the parasite (7). Having this variation is necessary for separation of the factors contributing to parasite resistance. We tested four different inocula (from single bees as well as combined inocula from several bees) with the expectation that there would be differences between them. We used combination inocula in case inocula from single individuals were insufficient to capture the diversity of the bee gut microbiota.

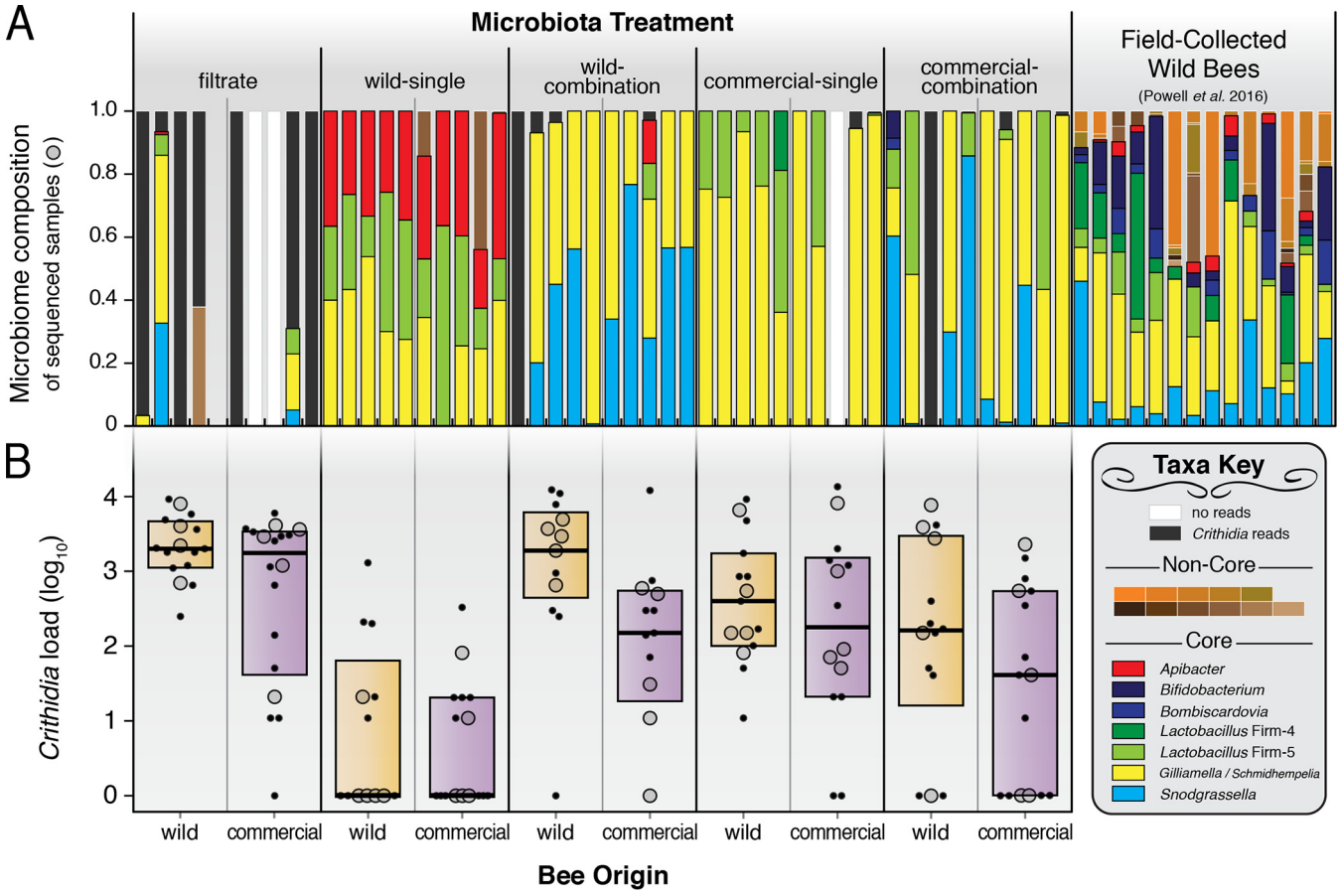

FIG 1 Microbiome composition and *C. bombi* infection load in the bumble bee *B. impatiens*. (A) Gut communities, based on bacterial 16S rRNA gene profiling, of randomly selected bees from five experimental treatments and from wild field-caught bees from a previous study (69) for comparison. *Crithidia*-specific reads were removed prior to downstream analyses. The *y* axis indicates relative abundances (sums to 1 for each sample). (B) *C. bombi* infection loads of individual bees across experimental treatments and host backgrounds (wild or commercial bees). Large markers, microbiota-profiled samples; small markers, unprofiled samples. Boxes show quartiles and medians. *C. bombi* load expressed as number of cells per 10 μl of gut homogenate. Noncore taxa corresponding to colored labels from top left to bottom right in key: *Asaia*, *Enterobacteriaceae*, *Fructobacillus*, *Gluconobacter*, *Lactobacillus kunkeei*, *Leuconostoc*, *Microbacterium*, *Parasaccharibacter*, *Saccharibacter*, *Staphylococcus*, and *Zymobacter*.

The inocula were sequenced along with samples from treatment bees at the end of the experiment to determine their community compositions.

All bees were infected with a common strain of *C. bombi* administered 7 days after microbiota treatment. The gut microbiome compositions of five randomly chosen samples from each origin-treatment combination were assessed 7 days after *C. bombi* exposure by PCR amplification and high-throughput sequencing of the bacterial 16S rRNA gene (Fig. 1A). Following quality filtering, a total of 2,190,342 sequences were retrieved across 49 samples. These formed 30 operational taxonomic units of 97% or greater sequence identity ($OTUs_{97}$). For 13 of these clusters, the top BLASTN hits corresponded to sequences of phylotypes previously sampled from bees. Seven typical bee-associated phylotypes were represented by single OTU_{97} (*Apibacter*, *Bifidobacterium*, *Bombiscardovia*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, *Saccharibacter*, or *Snodgrassella*), while another typical bee-associated phylotype was represented by six $OTUs_{97}$ (*Gilliamella*). *Gilliamella* OTUs may also comprise "*Candidatus* Schmidhempelia" (20), a closely related bacterium which was not distinguishable by our short-read data set.

Each treatment resulted in bees with distinct microbiome profiles (Fig. 1A), as assessed by community dissimilarity metrics (Bray-Curtis analysis of similarity [ANOSIM] $R = 0.55$, $P < 0.001$, PERMDISP $F_4 = 2.5$, $P = 0.068$; Sørensen-Dice ANOSIM $R = 0.57$, $P < 0.001$, PERMDISP $F_4 = 0.57$, $P = 0.69$). Community compositions were not

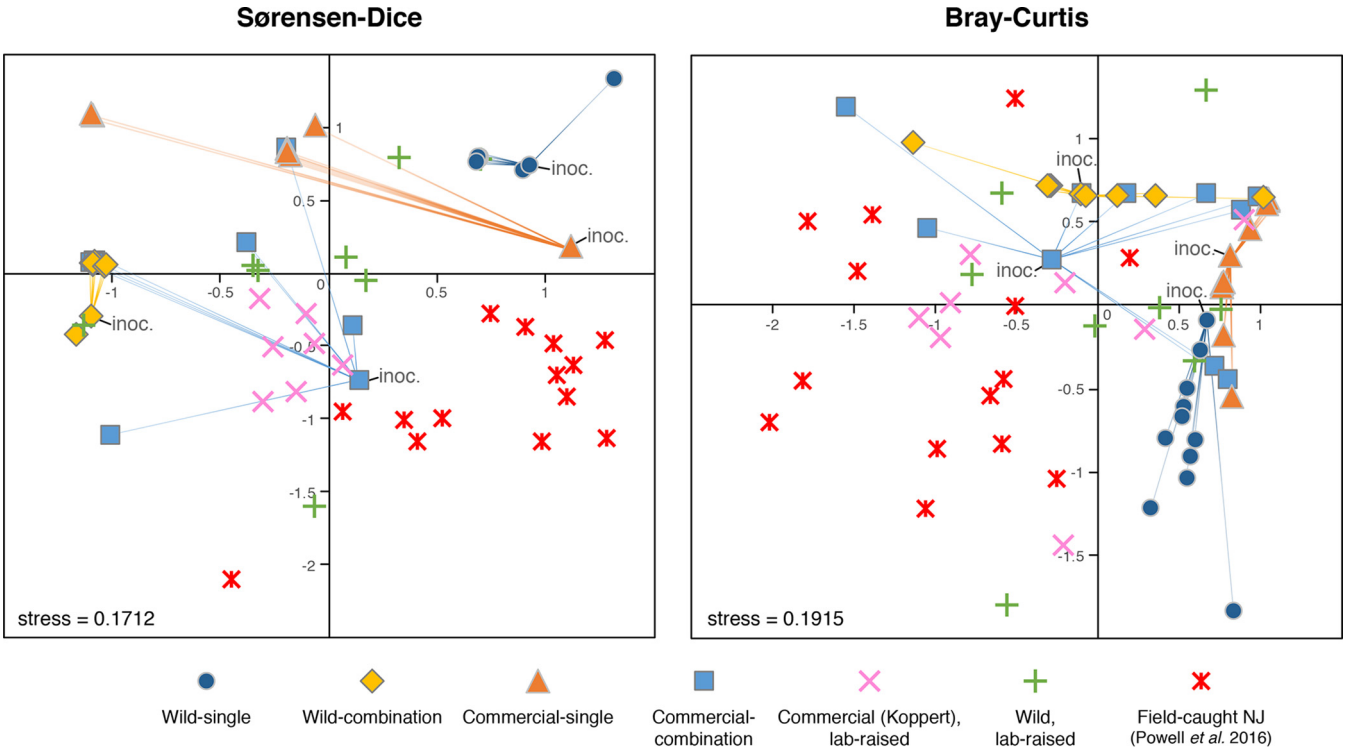

FIG 2 Nonmetric multidimensional scaling (NMDS) plots of community dissimilarities based on Sørensen-Dice and Bray-Curtis distances. Input samples used for inoculation of experimental microbiota treatments are denoted by "inoc." and by lines connecting to the resulting output microbiomes. Communities were compared at a depth of 5,000 reads per sample; samples with <600 reads and samples from the filtrate treatment were excluded.

statistically different between wild or commercial bees (Bray-Curtis ANOSIM $R = 0.00$, $P = 0.94$, PERMDISP $F_1 = 0.060$, $P = 0.81$; Sørensen-Dice ANOSIM $R = 0.00$, $P = 0.90$, PERMDISP $F_1 = 0.19$, $P = 0.68$), or between source colonies (Bray-Curtis ANOSIM $R = 0.06$, $P = 0.16$, PERMDISP $F_9 = 0.51$, $P = 0.85$; Sørensen-Dice ANOSIM $R = 0.02$, $P = 0.37$, PERMDISP $F_9 = 0.42$, $P = 0.91$) in our experiment. The wild-single treatment produced microbiomes with considerably greater alpha diversity than the other treatments, as measured by $OTUs_{97}$ (average, 4.0 OTU_{97}; Bonferroni-adjusted *t* tests, $P \leq 0.01$) and Shannon's H (average, 1.58; Bonferroni-adjusted *t* tests, $P \leq 0.001$). However, all treatments had less diverse microbiomes than those of wild field-caught *B. impatiens* from a previous study (average, 10.3 OTU_{97}, 2.65 Shannon's H; Fig. 1A). The microbiomes of untreated bees in lab-reared *B. impatiens* colonies (both newly established from the wild and from commercial origins) were also found to differ from those of field-caught bees and treatment bees (Fig. 2; see also Fig. S1 in the supplemental material).

Crithidia bombi infection levels were measured for 138 samples (13 to 18 samples from each origin-treatment combination; Fig. 1B). Infection loads varied significantly according to microbiome treatment [two-way analysis of variance (ANOVA), $F(4,138) = 19.0$, $P < 0.0001$] and bee origin [two-way ANOVA, $F(1,138) = 10.8$, $P = 0.0013$], but no interaction between bee origin and microbiome treatment was detected [two-way ANOVA, $F(4,138) = 0.598$, $P = 0.66$]. The filtrate treatment produced, on average, bees with the greatest *C. bombi* infection loads (2,495 cells per 10 μl), while the wild-single treatment resulted in the lowest infection loads (78 cells per 10 μl).

Both bee origin and microbiome diversity were significant correlates of *C. bombi* infection load. Commercial captive-raised bees had lower *C. bombi* loads than did wild bees (Fig. 3A). Bees with more diverse microbiomes also had fewer *C. bombi* parasites (Fig. 3B and C). To investigate the relationship between bacterial abundance and *C. bombi* abundance, we performed quantitative PCR (qPCR) targeting the bacterial 16S rRNA gene as a proxy to quantify absolute loads of the gut microbiota. A strong

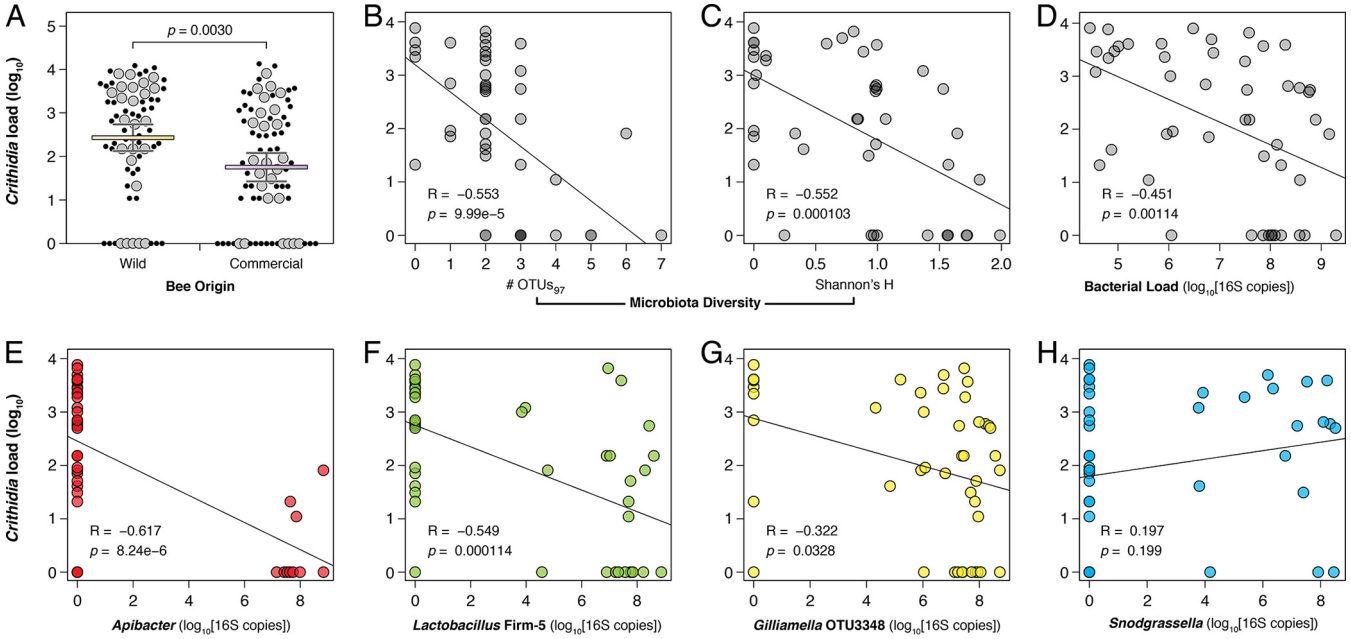

FIG 3 Correlations between *C. bombi* infection load and host and microbiome characteristics. (A) Lower *C. bombi* susceptibility in bees of commercial origin (two-tailed *t* test). Large markers, microbiota-profiled samples; small markers, unprofiled samples. Means and 95% confidence intervals shown. (B and C) Lower *C. bombi* loads in bees with high microbiome diversity. (D) Lower *C. bombi* loads in bees with larger gut bacterial populations. (E to G) Lower *C. bombi* loads associated with greater abundance of *Apibacter*, *Lactobacillus* Firm-5, and *Gilliamella* in the gut. (H) No impact of *Snodgrassella* abundance on *C. bombi* infection load. Pearson correlation statistics shown. *C. bombi* load expressed as number of cells per 10 μl of gut homogenate.

negative correlation was found between *C. bombi* load and bacterial load (Fig. 3D), suggesting that the strength of the bumble bee microbiota's protective effect scales with the number of gut bacteria present.

However, this trend might also be driven by specific bacterial phylotypes. To assess this possibility, we examined the relationship between *C. bombi* load and the absolute abundances of each OTU. Four OTUs were significantly correlated with lower parasite loads: *Apibacter* (Fig. 3E), *Lactobacillus* Firm-5 (Fig. 3F), and two *Gilliamella* OTUs (Fig. 3G and S2). *Apibacter* had the largest effect, and clustering of reads at 100% identity showed that a single *Apibacter* strain was predominant in the wild-single treatment (see Data Set S2 in the supplemental material). *Snodgrassella alvi*, another common bumble bee-associated bacterial phylotype, showed no relationship with *C. bombi* infection load (Fig. 3H).

The observed correlations were not solely driven by the wild-single microbiota treatment (the treatment with the most pronounced outcome in our experiment). Diminished *C. bombi* load with increasing microbiota diversity, bacterial load, and abundances of *Lactobacillus* Firm-5 and *Gilliamella*, as well as the neutral effect of *Snodgrassella* spp., were all trends that persisted following the removal of wild-single samples from our analyses (Table S1). The effect size and statistical significance of the associations were reduced in these cases, however. Since our experimental microbiota inoculations preceded *C. bombi* infection, the results presented here point toward causative relationships, not mere correlations. The microbiomes of posttreatment bees closely resembled those of their respective input inocula (Fig. 2 and S1), suggesting that exposure to *C. bombi* is not responsible for the observed gut community differences but rather that the microbiota induces changes to *C. bombi* susceptibility.

DISCUSSION

Of the five microbiome treatment groups, the wild-single microbiome had the greatest protective effect against *C. bombi*, followed by the commercial-combination treatment (Fig. 1B). The microbiome treatments explained 33.3% of the observed variation in parasite load, whereas bee origin (likely reflecting host genetic differences)

explained only 4.7% of the observed variation (Fig. 3A). This extends previous findings for *B. terrestris* that the gut microbiome is a much more important determinant of *C. bombi* resistance than host background (7). In our experiment, *B. impatiens* bees of commercial origin were less susceptible to *C. bombi* than wild *B. impatiens* bees. This finding is consistent with the hypothesis that commercial captive-bred bees (where *C. bombi* infection prevalence is generally higher) are better able to tolerate parasites, leaving wild bees vulnerable to pathogen spillovers from commercial operations (19). However, our experiments do not allow us to claim a general difference between commercial and wild bees, since we only sampled from limited sources for either category. Also, different parasite-host combinations can result in diverse infection outcomes (7), and we only tested a single *C. bombi* strain; further experiments are needed to validate the resilience of commercial bees.

The more important factor in infection resistance, microbiome composition, displayed substantial variation between our treatments (Fig. 1A). This allowed us to tease apart the components of gut community structure that are most influential in promoting *C. bombi* resistance. A potentially important component in host health is gut microbiome diversity; for instance, low microbiome diversity in humans is associated with inflammatory bowel disorder and *Clostridium difficile* infections (21). We found that greater microbiome diversity was associated with lower infection loads (Fig. 3B and C). Our experimental treatments generally had lower diversity than field-collected bees (Fig. 1A), which is consistent with observations that bees acquire "noncore" environmental bacteria (from foraging on flowers, etc.) when not confined to the indoors (22, 23). In contrast, our experimental bees had microbiomes almost exclusively consisting of the "core" gut bacteria. While it appears that a higher diversity of the core microbiota can protect against *C. bombi*, the same may not be true of noncore bacteria: previous surveys of wild bumble bees showed increased *C. bombi* infection with increasing noncore diversity (24, 25). Surveys of gut microbiota of *Bombus* species in China and North America indicate that all species possess the core phylotypes, including *Gilliamella*, *Snodgrassella*, and *Lactobacillus* Firm-5, but that some individual bees of each species are dominated by noncore environmental bacteria (25, 26). Potentially, retention of the core phylotypes is more important than diversity *per se* in resisting pathogen colonization.

Correlation of absolute abundances of microbial phylotypes with *C. bombi* load revealed three bacterial taxa as promising candidates for inhibiting *C. bombi*: *Apibacter*, *Lactobacillus* Firm-5, and *Gilliamella* (Fig. 3 and S2). *Lactobacillus* Firm-5 and *Gilliamella* are well-known core members of the honey bee and bumble bee gut microbiota (27–31), and they participate in the digestion of complex polysaccharides in the bee hindgut (32–35). Gut colonization by *Gilliamella* spp. has been associated with decreased *Crithidia* infection levels in field-caught bees, but the effect was small (25) or not significant (6). Another core microbiota member, *Snodgrassella alvi* (36), was found to be negatively associated with *C. bombi* in one survey of field-caught bees (6) but not in another (25). In our experimental transplant study, we found no correlation between *Snodgrassella* and *C. bombi* levels in the gut (Fig. 3H). A recent study in honey bees also showed no benefit of *Snodgrassella* spp. in suppressing infection by a related protozoan parasite, *Lotmaria passim* (37).

Apibacter spp. exhibited the strongest negative correlation with *C. bombi* infection (Fig. 3E). Previously classified as an unidentified *Bacteroidetes/Flavobacteriales* bacterium (7, 25, 30, 38), representatives of the *Apibacter* clade have now been cultivated from both honey bees and bumble bees (39, 40). Their biological role within the gut remains uncharacterized, although our results suggest that *Apibacter* spp. can colonize to high numbers in a given individual ($>$30% relative abundance). In field-caught bees, *Apibacter* spp. constituted a much smaller proportion of the gut community (average, 2.6%; Fig. 1A). Interestingly, *Apibacter* spp. were only present in the wild-single treatment, which resulted in significantly lower levels of *Crithidia* infection in both wild and commercial bees. The wild-combination treatment, which failed to reduce infection loads, lacked *Apibacter* spp. (save for a single sample, which had $<$500 reads and was

removed from the analysis). This suggests substantial heterogeneity in the microbiomes of overwintered queens, which may result in bee colonies with differing abilities to resist various pathogens and parasites (7).

A previous experimental microbiome transplant study by Koch and Schmid-Hempel (7) in the European bumble bee *B. terrestris* offers evidence consistent with our results. Although not noted by the authors at the time, their bees with the highest *Crithidia* infection loads were found in a treatment group lacking *Lactobacillus* Firm-5 (as "*Lactobacillus* sp."), and the lowest infection loads were recorded in a group harboring *Apibacter* spp. (as "*Bacteroidetes*") (see Fig. 4 and S2 in reference 7). That both studies point to these two bacteria as key players in the interaction between microbiome, host, and parasite is compelling evidence of a conserved interaction network present across bumble bee species.

Several possibilities for the mechanism behind microbiota-mediated resistance should be explored going forward. Since both *C. bombi* and the microbiota colonize the bumble bee hindgut (41), it is possible that the microbiota directly inhibits *C. bombi* through the secretion of antimicrobial compounds, competition for resources, or spatial interference. Indirect interaction via the host may also play a role: the bee gut microbiota can stimulate the immune system, with different combinations of microbes provoking different immune responses (8, 42, 43). Host innate immunity is likely a key component in combatting *C. bombi* (44–46), but working out the mechanisms, especially as applied to wild bee populations, requires further study of the complex interplay between environmental influences, host genotype, parasite genotype, and the microbiota (47).

There is considerable evidence of the gut microbiota promoting host health in other insects and affecting the growth of eukaryotic parasites. In mosquitoes, gut bacteria are essential for host development (48) and can affect the infection cycle of *Plasmodium falciparum*, the causative agent of malaria (49). In sand flies, the diversity of the gut microbiome decreases over the course of *Leishmania* infection but is also essential for the parasite's survival, as shown by *Leishmania* growth suppression in antibiotic-treated flies (50). In bumble bees, *Crithidia* can be transmitted between members of a colony by fecal-oral contact and can spread across multiple colonies via contact at shared floral resources (51). Infection prevalence increases (up to ~80%) and then decreases over the course of a year, reflecting the annual growth and decline of bumble bee colonies (52). *Crithidia* survival in overwintering queens seeds the cycle in the following year (47).

Our results indicate a potential for coevolutionary interactions between gut microbiomes and parasites in overwintering queens. While the effect of infection intensity on the spread of *Crithidia* spp. has not been explicitly tested, higher *Crithidia* loads are associated with the higher transmission rates typically seen early in the colony cycle (52). By lowering *Crithidia* abundance at the individual level, particular microbiomes might decrease overall infection rates across a colony. If so, why have "protective" microbiomes not become the norm? Perhaps any given bacterial strain can only inhibit specific *Crithidia* strains (7) and *Crithidia* genotype frequencies constantly shift to escape inhibition. There might also be fitness tradeoffs for possessing certain microbiome compositions, independent of *Crithidia*. Furthermore, it has been hypothesized that *Crithidia* infection alters bee-foraging activities, potentially leading to self-medication (51, 53). How such behavioral changes induced by *Crithidia* spp. affect the microbiome is unknown. Microbiome changes after *Crithidia* infection also may impact fitness; however, in this study, we only tested the effect of the microbiome prior to parasite exposure.

Understanding the factors affecting bumble bee health is vital to agriculture and to the sustainability of natural ecosystems. Wild bumble bees are among the most common and important nonmanaged insect pollinators (54), and *B. impatiens* in particular is the most valuable species in its range across eastern North America (55). It is also currently the primary species sold commercially across North America and provides highly efficient pollination services for various greenhouse crops, including

tomatoes (56), muskmelons (57), and sweet peppers (58), as well as for a number of field crops (54, 59, 60). As commercial reliance on captive-bred bees grows (13, 61), the impact of pathogen exposure on wild bee populations, and the corresponding methods to alleviate such stressors, will require increasingly urgent and critical evaluation.

MATERIALS AND METHODS

Bumble bee colonies. Wild *B. impatiens* queens were collected in Polk County, TX (30.7449, −94.6218) in March 2015, coinciding with their emergence from winter hibernation. The wild queens were kept in individual cages in a laboratory incubator at 28°C and 60% humidity, fed a diet of sterile irradiated pollen and sucrose water (1:1 [wt/vol]), and allowed to start a colony. Commercial bumble bees were ordered from Biobest (Westerlo, Belgium; Leamington, Ontario, Canada, for North America) and kept in the lab on a diet of sterile irradiated pollen (Betterbee, Greenwich, NY) and sucrose water.

Preparation of treatment groups. Worker *B. impatiens* from both wild and commercial colonies were inoculated with one of five microbiome treatments derived from wild-caught or commercially produced queens: wild-single, wild-combination, commercial-single, commercial-combination, and filtrate. In order to generate the wild-single microbiome treatment, one whole gut was removed from a wild queen and homogenized in 250 μl of 10 mM phosphate-buffered saline (PBS). Of this, 100 μl was set aside for the filtrate treatment. Glycerol was added to the remaining 150 μl, resulting in a total volume of 300 μl at 15% (vol/vol) glycerol. This mixture was then separated into 5-μl aliquots and frozen at −80°C.

In order to generate the wild-combination treatment, whole guts were removed from four wild queens and homogenized in 1,000 μl PBS. Of this, 400 μl was set aside for the filtrate treatment. The remaining 600 μl was diluted in glycerol to yield 1,200 μl at 15% (vol/vol) glycerol. The mixture was then separated into 5-μl aliquots and frozen at −80°C.

The above-mentioned process was followed to generate the commercial-single and commercial-combination microbiome treatments from commercial queens. The filtrate treatment was generated from 75 μl of homogenized guts set aside from each treatment. The guts were filtered through 4 layers of cheese cloth and then brought to a 15% (vol/vol) glycerol concentration. This method of filtration was done to remove intact bacterial cells while retaining other nonbacterial gut particulates that may affect pathogen infectivity or host immune response. We attempted separation with 0.2-μm-pore-size filters. However, these filters were easily clogged, and we were able to obtain only a few microliters of liquid from ~200 μl of bee guts. This volume was insufficient for administering our treatments, so we used folded cheese cloth, which yielded sufficient material while removing the vast majority of bacterial cells. The final filtrates were combined and then divided into 5-μl aliquots and frozen at −80°C. This treatment was a control for potential unintended effects of feeding bees homogenized gut material (e.g., if the gut material itself induced protection against the parasite or if there was an acellular agent present that made the bees sick). While not all filtrate-fed bees were completely bacteria-free by the end of the experiment (Fig. 1A), a comparison of absolute bacterial loads (see Data Set S3 in the supplemental material) shows that filtrate-fed bees had a much lower load of bacteria, often at or below the detection limit of our method.

In order to generate germfree bumble bees, *B. impatiens* worker cocoons were removed from the wild and commercial colonies. Four wild colonies and six commercial colonies were used for the experiment. The pupae were removed from the cocoon and allowed to mature under sterile conditions at 28°C and 60% humidity. This method has been previously used and validated to generate microbiota-free bees (6, 62). Upon eclosion, they were transferred to individual sterile plastic cages and inoculated with one of the five treatments. Bees from each colony were assigned randomly to treatment groups. The prepared 5-μl treatment aliquots were thawed on ice and then combined with 10 μl of sterile sugar water for a final 15-μl inoculum. To encourage feeding, bees were starved for 3 to 5 h prior to inoculation. The bees were monitored to ensure that they consumed the inoculum. Following the inoculation, workers were fed filter-sterilized sucrose water and gamma-irradiated pollen *ad libitum* and kept at room temperature in individual plastic cages. To produce sterile pollen, pollen purchased from Brushy Mountain Bee Farm (NC, USA) was irradiated at 30 kGy with gamma radiation (Sadex Corporation, IA, USA). Sterilization was validated by mixing ~100 mg irradiated pollen in 1 ml PBS, plating 100 μl on lysogeny broth agar, and observing for growth of microbial colonies.

***Crithidia* infection.** After allowing 7 days for the administered microbiota to establish in the gut, bees were removed from their cages and starved for 3 to 5 h in preparation for *Crithidia* infection. The *C. bombi* strain used for infection was isolated from a *B. impatiens* worker bee collected in West Haven, CT, USA, in September 2012 (*C. bombi* strain 12.6 from reference 63). *Crithidia* was grown in Insectagro DS2 medium (Corning, Inc.) supplemented with 5% fetal bovine serum at 28°C and 3% CO_2. The concentration of the *Crithidia* culture was determined using a hemocytometer (Neubauer improved cell counting chamber), and a 2:1 solution of sucrose water to culture medium was prepared so that the bees were fed a total of 15,000 *Crithidia* cells each. The bees were monitored to ensure that they consumed the 15 μl of infection mixture.

After another 7 days, shown to be within the peak period of *Crithidia* infection (64), the bees were placed on ice and dissected. The gut was removed and homogenized in 200 μl PBS. *Crithidia* infection load was recorded by counting the number of *Crithidia* cells present in 10 μl of homogenized gut using a hemocytometer (Data Set S3). The remaining homogenized gut and bee carcass were stored at −80°C. *Crithidia* cells actively infected and multiplied within *B. impatiens*, as indicated by some bees having

higher counts at the end of the experiment than what was expected from an initial infection inoculum of 15,000 cells.

Microbiome analysis. Five individual guts from each of the 10 treatment groups were randomly selected for DNA extraction according to Engel et al. (65). The microbiome inocula for each treatment group were also similarly processed to assess whether the transplant of the original microbiota to naive bees was successful (Fig. 2 and S1). DNA concentrations were determined using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Inc.). Samples were diluted to 10 ng/μl and sent to the University of Texas at Austin Genomic Sequencing and Analysis Facility, where the V4 region of the bacterial 16S rRNA gene was amplified by PCR using universal bacterial primers 515F and 806R. Amplifications were carried out in triplicate and the products pooled to minimize PCR jackpot effects. Amplicon libraries were sequenced on the Illumina MiSeq platform with a 2 $\times$ 250-bp read design. The sequenced samples are listed in Data Set S3.

Sequences were processed and analyzed using QIIME version 1.9.1 (66). Primer sequences were trimmed from the reads, and forward and reverse reads were joined with SeqPrep. Quality filtering using split_libraries_fastq.py was performed using the settings Phred q $\geq$ 30, maximum $N = 0$, and read length fraction minimum of 0.8. Reads of $<$230 bp or $>$270 bp were removed (expected read length, $\sim$250 bp). Sequences were then clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the pick_de_novo_otus pipeline. Any OTUs accounting for less than 0.5% of the reads for that sample were excluded from the analysis, since they could result from multiplexing barcode assignment errors. Taxonomic assignment of representative OTUs was manually determined using BLASTN against the GenBank nr database. The recovered OTUs were further filtered to remove reads derived from plastid, mitochondrial, and eukaryotic (e.g., *Crithidia* or host) sources. Samples with fewer than 500 total reads were also removed from the analysis. Alpha- and beta-diversity analyses were performed at a read depth of 550 reads per sample; the OTU corresponding to *Crithidia* was retained for the beta-diversity analysis to permit ordination of samples lacking any bacterial reads. Read/rarefaction thresholds were chosen based on sequencing depth and by maximizing the number of samples retained. There were no more than 12 OTUs in any sample. Visual inspection of rarefaction curves showed that gut community diversity was adequately captured. The OTU tables and representative sequences generated in this study are presented in Data Set S4.

Additional analysis of *Apibacter* reads was performed using CD-HIT-EST (67). Reads from the wild-single treatment were clustered at 100% identity. Clusters with fewer than 5 reads were excluded. *Apibacter*-specific clusters were identified by BLAST using the *Apibacter* representative sequence from the 97% OTU clustering as a query. The cluster sizes and sequences are listed in Data Set S2.

Quantitative PCR (qPCR) was performed according to Cariveau et al. (25) to determine the absolute number of 16S rRNA gene copies present per gut sample, as a proxy for the number of bacteria present in each gut. Total copy numbers were adjusted by the relative abundance output from OTU clustering to obtain absolute abundances of bacterial OTUs in each sample. Statistical analyses were conducted in R 3.2.3 (68) and Prism 6 (GraphPad Software, Inc.) and used $\log_{10}(x + 1)$-transformed *C. bombi* and bacterial load values. We acknowledge the potential for error in both the amplicon sequencing and the qPCR (due to PCR primer bias and different 16S rRNA gene copy numbers for each bacterial genome), and thus assessments of OTU abundance, particularly in a comparison between OTUs, should be interpreted cautiously.

Lab-reared *B. impatiens* microbiomes. We additionally surveyed the microbiomes of lab-reared *B. impatiens* colonies on which we did not conduct experimental microbiome manipulations. Two adult workers each from each colony were sampled and processed as described above. Four colonies were established from wild queens collected in Polk County, TX, in March 2015, and another four colonies were purchased from a commercial bumble bee supplier (Koppert Biological Systems, The Netherlands; bumble bee production in Howell, MI, USA). The microbiomes were compared to that of wild field-caught wild *B. impatiens* bees collected in New Jersey, USA, from a previous study (69).

Accession number(s). Parasite counts and qPCR data are placed in Data Sets S1 and S3 in the supplemental material. The 16S rRNA gene sequence reads are deposited under NCBI BioProject no. PRJNA371284.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02335-17.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.5 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.5 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.

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B.K.M. and N.A.M.; resources and supervision, N.A.M.; data curation, formal analysis, visualization, and writing of the original draft, B.K.M. and W.K.K.; and review and editing of the writing, B.K.M., W.K.K., N.A.M., and H.K.

We declare no competing interests.

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