High Gut Microbiota Diversity Provides Lower Resistance against Infection by an Intestinal Parasite in Bumblebees

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ABSTRACT: The microbiome, especially the gut flora, is known to affect the interaction between parasites and their hosts. In this context, a parasitic infection can be viewed as an invasion into the preexisting microbial ecological community. Hence, in addition to the intrinsic defense mechanisms of the host itself, infection success depends on the colonization resistance of the microbiota. In the bumblebee *Bombus terrestris*, the microbiota provides resistance to the intestinal parasite *Crithidia bombi*, yet which properties actually provide protection remains largely unknown. Here, we show that the community structure of the gut microbiota—in terms of bacterial operational taxonomic units (OTUs) of 16S ribosomal RNA gene sequences—before parasite exposure can be informative of the eventual infection outcome. Specifically, higher microbiota OTU diversity is associated with less resistance. However, the microbial community structure does not differ between infected and noninfected individuals or between infected individuals of varying susceptibility. This suggests that parasite infection success depends on the microbiota composition but that subsequent changes occur, although the exact alteration that occurs remains elusive. In fact, the bumblebee microbiota is surprisingly unaffected by parasite exposure and infection. Rather, the microbiota-host interaction before parasite exposure seems to be a key mechanism regulating resistance to infection.

Keywords: host-parasite interactions, parasite-microbiome interactions, colonization resistance, feces versus gut, *Bombus terrestris*, *Crithidia bombi*.

Introduction

There is an increasing awareness of the importance of host-associated microbial communities in the health and disease of an organism (Sekirov et al. 2010). In particular, the intestinal gut microbiota not only interacts closely with the host itself (Hooper et al. 2012) but also interferes directly or indirectly with enteric infections (Stecher and Hardt 2011). If at the time of the parasite encounter the microbial community is fully established, infection by a parasite that enters the host by way of the digestive tract can be viewed as an invasion process into this community—a process that eventually can be considered in the light of ecological theory (Shea and Chesson 2002; Foster et al. 2008; Costello et al. 2012; Stacy et al. 2016). As such, a parasite's infection success depends, on the one hand, on the number of accessible niches within a host that are unoccupied by the microbial community; for example, niches could be the different localities within the gut or the host (Kwong et al. 2014; Stacy et al. 2016). On the other hand, infection success might also depend on the parasite's ability to overcome the colonization resistance of the resident microbiota (Sekirov and Finlay 2009; Stecher and Hardt 2011; Buffie and Pamer 2013). Such colonization resistance can occur in various ways, for example, through direct interactions between the parasite and gut microbes, through competition among microbiota and the parasite for niches or nutrients, or by the microbiota altering the environment for the parasite via interaction with the host immune system (Stecher and Hardt 2011; Thaiss et al. 2014).

Examples of microbiota-associated resistance are indeed known across many different insect species, but the specific underlying microbial composition that mediates this resistance is generally difficult to uncover and has only rarely been described. In *Anopheles* mosquitoes, infection success by the *Plasmodium* parasite is not directly affected by the microbiota; however, the expression of the host immune system (Dong et al. 2009; Boissiere et al. 2011), the parasite life cycle, and thus ultimately *Anopheles* vector competence is influenced by coinfecting bacteria (Pumpuni et al. 1993; Gonzalez-Ceron et al. 2003). Experimental evidence has shown that, for example, a native microbial isolate (*Enterobacter* bacterium) directly inhibits *Plasmodium* parasite oocyst development (Cirimotich et al. 2011). Similarly, in sand flies the presence of gut microorganisms reduces the ability of the *Leishmania* parasite to establish (Sant'Anna

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et al. 2014). These examples illustrate that certain individual microbial species can modulate the establishment success of the parasite. In other cases, such as in locusts, protection is correlated with the gut microbiota community as a whole: an increased taxonomic diversity is more protective and reduces the colonization ability of the bacterial pathogen *Serratia marcescens* (Dillon et al. 2005).

In the bumblebee *Bombus terrestris*, a primitively social insect, the microbiota reduces the infection probability and intensity of the common trypanosome gut parasite *Crithidia bombi* (Koch and Schmid-Hempel 2011*b*). Generally, not only does the microbiota protect the bee, but there is also variation in the level of protection against the parasite by different microbiota originating from different colonies (Koch and Schmid-Hempel 2012). Therefore, a certain degree of specificity is mediated by the microbiota-parasite interaction (Koch and Schmid-Hempel 2012). In principle, these results are suggestive of colonization resistance in bumblebees. Closer inspection has shown that several elements seem to play a role, since infection intensity by *C. bombi* in wild-caught bees is lower with an increasing number of taxa (i.e., species richness) in the noncore microbiota but also lower with a higher abundance of a key bacterium (*Gilliamella*; Cariveau et al. 2014). In what way microbiota organization mediates this resistance has not yet been established.

In this study, we aimed at uncovering possible structural properties of the microbiota that can prevent the establishment of an infection by the parasite. Specifically, we focus on the changes in microbiota composition after perturbation by the parasite and how this relates to colonization resistance. For this, we investigated whether there are any properties of the microbiota composition before parasite exposure that are predictive of infection outcome. Complementary to this, we assessed whether the gut microbiota composition after exposure could provide information on the infection outcome. Finally, we assessed the robustness of the microbiota composition to the parasite invasion, which in ecology is sometimes known as resilience (Shade et al. 2012), by analyzing the change from before parasite exposure to after infection establishment. Finally, we study the respective changes at the level of single taxa that make up the microbiota.

Material and Methods

Bee Colonies, Parasite Infections, and Microbiota Sampling

Thirty-three laboratory colonies were raised from healthy *Bombus terrestris* queens that were field caught in the spring of 2014 from two populations in Switzerland (Neunforn and Aesch). Throughout the experiment, the bees were kept under standardized conditions (28° ± 2°C, 60% relative humidity, constant red light illumination) and provided with pollen and sugar water (ApiInvert) ad lib. Mature workers were selected at random from each colony for experimental treatment (fig. 1*A*; table A3; tables A1–A4 are available

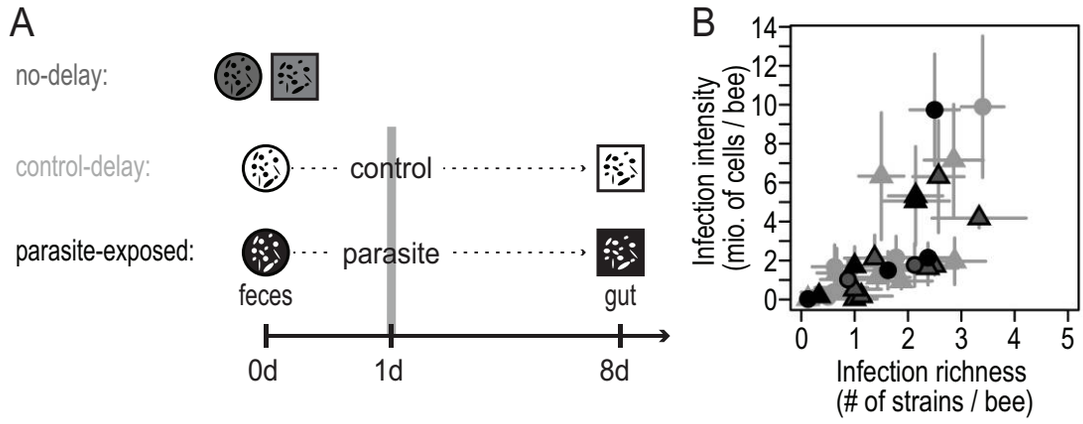

Figure 1: Experimental treatment overview and variation in infection outcome summarized by colony. *A*, A fecal and gut sample was collected from each worker in three experimental treatment lines. For the no-delay treatment, this occurred simultaneously on day 0. Control-delay and parasite-exposed bees received a sham inoculum or a parasite inoculum, respectively, 1 day after the fecal sample was collected. The gut was sampled on day 8, 7 days after parasite exposure. The workers were kept in individual sterile housing boxes for the duration of the experimental period (0–8 days). *B*, Mean infection intensity per colony plotted against mean infection richness 7 days after parasite exposure (error bars show SEM). The parasite inoculum contained five genetically distinct strains. Dark filled symbols highlight colonies that were randomly selected for the analysis of the microbial community of fecal and gut samples. Workers in the control-delay treatment line and the parasite-exposed treatment line originated from the colonies highlighted in black. Workers in the no-delay control treatment line originated from colonies highlighted with black outline. Microbiotas from colonies represented by light gray symbols were not assessed further. The population origin of the colonies is depicted by symbols (circles: Aesch; triangles: Neunforn). mio. = millions.

online), thus randomizing age and excluding any bee with an undeveloped microbiota (Martinson et al. 2012). From this time point onward, all handling materials were either autoclaved or sterilized by washing with 80% ethanol before use. In addition, only filter-sterilized sugar water (pore size, 0.2 μm) and X-ray-radiated pollen (dose, 26.7 kGy; WHO 1997) were given to the experimental bees.

We sampled the gut microbiota for each experimental individual twice (fig. 1*A*). First, a fecal sample was collected nondestructively before the bees were placed individually into a sterile housing container. The following day, after a 2-h starvation period, workers either received an infective dose of 10,000 parasite cells in 10 μL of sugar water (50% sugar w/w) or a control treatment (i.e., an inoculum prepared without parasite cells). The parasite inoculum consisted of 2,000 cells each of five genetically distinct *Crithidia bombi* strains (strain IDs 08.068, 08.075, 08.091, 08.161, and 08.192; Ulrich et al. 2011), as susceptibility to parasite infection in bumblebees is most reliably assessed by infecting individual bees with a cocktail of different *C. bombi* strains (Schmid-Hempel et al. 1999; Ulrich et al. 2011; Näpflin and Schmid-Hempel 2016), which mimics the situation in the field, where multigenotype infections are common (Tognazzo et al. 2012). The second microbiota sample was obtained 7 days after infection, a period long enough for infections of *C. bombi* to establish (Schmid-Hempel and Schmid-Hempel 1993). At that time, the bees were killed and kept frozen at −20°C before the gut was aseptically dissected. We included two types of controls to account for potential differences between fecal and gut microbiota (fig. 1*A*): one to control for differences between the two sample types (no-delay treatment) and another to control for the time delay between parasite exposure and sampling of the microbiota (control-delay treatment).

DNA was extracted from both fecal samples and aseptically dissected guts with the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol, which included a bead-beating step (animal tissue DNeasy 96 protocol). However, DNA was eluted in 100 μL of AE buffer. Parasite infection outcome was evaluated for each bee 7 days after infection. For this, we determined the total number of parasite cells (infection intensity) by quantitative real-time polymerase chain reaction, and we analyzed five *C. bombi* microsatellites (Schmid-Hempel and Reber Funk 2004) to determine the number of different strains (infection richness; appendix, available online; for sample metadata, see table D1 in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.4nm5sh4 [Näpflin and Schmid-Hempel 2018]).

16S Amplicon Sequencing and Processing

We amplified the variable region V3-V4 of the 16S ribosomal RNA gene with universal primers (table A4; Liu et al. 2011; Klindworth et al. 2013) and generated three multiplexed amplicon paired-end libraries from the fecal and gut samples of workers from randomly chosen colonies representing the whole range of infection outcomes (fig. 1*B*; appendix). A total of 58,490,734 16S amplicon sequences were generated on the MiSeq Illumina platform (2 × 300 bp). Raw reads were quality controlled (FastQC ver. 0.11.2), merged (FLASH ver. 1.2.11), primer site trimmed (Cutadapt ver. 1.5), and quality filtered (PRINSEQ-lite ver. 0.20.4). Sequences were clustered on the basis of 97% sequence identity into operational taxonomic units (OTUs) using the UPARSE-OTU algorithm (USEARCH ver. 9.0.2132, 64 bit; Edgar 2010, 2013; table A1; tables D2 and D3 in Dryad: http://dx.doi.org/10.5061/dryad.4nm5sh4 [Näpflin and Schmid-Hempel 2018]).

Taxonomic Identification of OTUs

Taxonomic information was assigned to OTUs on the basis of the RDP database (ver. 16) with SINTAX (USEARCH ver. 9.2.64, 32 bit; Cole et al. 2014; Edgar 2016) and cross-referenced against the SILVA database (ver. 128; Pruesse et al. 2012; see table D4 in Dryad: http://dx.doi.org/10.5061/dryad.4nm5sh4 [Näpflin and Schmid-Hempel 2018]). Furthermore, to link OTUs to the previously identified "core" bee microbiome, we downloaded 910 published bee 16S sequences from GenBank (Jeyaprakash et al. 2003; Babendreier et al. 2007; Olofsson and Vásquez 2008; Koch and Schmid-Hempel 2011*a*; Martinson et al. 2011; Moran et al. 2012; Engel et al. 2013; Kwong and Moran 2013; Martinson et al. 2014). We then generated a multiple alignment of these 910 sequences together with our OTUs (for reference sequences, see table D3 in Dryad: http://dx.doi.org/10.5061/dryad.4nm5sh4 [Näpflin and Schmid-Hempel 2018]) using MUSCLE (Edgar 2004) and reconstructed a phylogeny using FastTree (ver. 2.1.9; Price et al. 2010). For each OTU, we identified the identity and distance to the phylogenetically closest published 16S sequence (see table D4 in Dryad: http://dx.doi.org/10.5061/dryad.4nm5sh4 [Näpflin and Schmid-Hempel 2018]). All sequences that were taxonomically classified as mitochondria or chloroplasts were excluded from further analysis.

Excluded Samples

We observed variation in sequencing depth using rarefaction curves (fig. A1). Samples with a sequencing depth of less than 1.5 times the interquartile range below the lower quartile were excluded from the analysis. Furthermore, because we required paired feces and gut samples, nine additional samples from the no-delay treatment group without a matching pair were also excluded. Finally, we dropped one individual from the control-delay treatment group in which we detected an infection of unidentified origin.

Final Data Set

The final data set contained 260 samples, with a total of 36,973,000 reads mapping to 210 OTUs. We rarefied each sample to the smallest library size ($n = 13{,}820$ reads), and this process was replicated 100 times to mitigate effects of the rarefaction process. As such, a rarefied data set contained 166.83 (SD, 4.28) different OTUs on average.

Diversity Measures

We calculated the following alpha diversity measures: species richness (S), the Shannon-Wiener index (H'), and evenness ($E = H'/\ln S$). We measured beta diversity using all pairwise Bray-Curtis dissimilarities between samples. Diversity measures were calculated for each of the 100 rarefied replicates in every sample, and the mean over all replicates in a sample was used in the statistical analysis. Similarly, we determined mean OTU abundance over replicates.

Comparing Feces and Gut Microbial Samples

Fecal microbiota is often considered a proxy for the gut microbiota, yet the validity of this proxy is rarely directly assessed. Because we quantified microbiota composition from a fecal sample before parasite exposure and from gut samples after, the investigation of robustness of the microbial community to parasite exposure required a general assessment of the two sample types. To this end, we compared the mean Bray-Curtis dissimilarity between feces and gut samples of the same bees to the empirical distribution of the mean dissimilarity between feces and gut samples from randomly chosen bee pairs over 10,000 replications. We identified a core set of OTUs (i.e., shared OTUs) that included all OTUs that were detected at least once in both the fecal and the gut sample of a individual in all treatment groups. This within-individual (feces-gut) definition of "core" should not be confused with the definition of "bee core" OTUs based on their taxonomic identity employed in other studies (Moran 2015).

Analysis of Community Composition and Infection Outcome

To account for differences in the infection severity of individual bees, we use the first principal component (PC1) of the measured infection intensity and infection richness as a compound measure of infection outcome (fig. 1*B*). We assessed the effects of alpha diversity as characterized by evenness (see fig. A2) and inherent variation in colony susceptibility using a linear mixed effects model (LMM) with evenness as a fixed effect and colony identity as a random effect. The significance of the predictor variable in the model was determined by a likelihood ratio test between the full model and a model with intercept only with the same random error structure. The total variance explained by the model is reported by the conditional R^2_c, whereas the marginal R^2_m describes the explained variance attributed only to the predictor variable (Nakagawa and Schielzeth 2013; Bartoń 2016). We adjusted P values for multiple testing if analyses were performed on both full OTU and core OTU data sets (Benjamini and Hochberg 1995). We performed principal component analyses on log-transformed OTU abundances of identified core OTUs including a pseudocount of 1. We then regressed the infection outcome on the principal components to determine the top two components that most significantly contributed to infection outcome. We identified candidate OTUs that underlie the variation in infection outcome as those with the largest eigenvector components along these two principal components.

Microbiota Compositional Change in Response to the Parasite

We calculated the change in alpha diversity from before and after parasite exposure (Δrichness, ΔShannon index, Δevenness), as well as the beta diversity (Bray-Curtis dissimilarities) between the microbial community before and after infection of an individual. We assessed the effect of the change in the microbial community composition due to parasite exposure using an LMM accounting for colony identity as a random effect and PC1 score as a fixed effect.

Statistical Software

All analyses were carried out in R (ver. 3.3.3; R Core Team 2017) using the packages phyloseq (ver. 1.19.1; McMurdie and Holmes 2014), vegan (ver. 2.4.2; Oksanen et al. 2017), lme4 (ver. 1.1.12; Bates et al. 2015), and MuMIn (ver. 1.15.6; Bartoń 2016) as well as respective dependencies.

Data Availability

Raw 16S sequence reads are available from the European Nucleotide Archive (project PRJEB25500). Sample information (table D1), OTU counts (table D2), OTU reference sequences (table D3), and taxonomic classification (table D4) are deposited in the Dryad Digital Repository: https://dx.doi.org/10.5061/dryad.4nm5sh4 (Näpflin and Schmid-Hempel 2018).

Results

Variation in Infection Outcome

On average, within a colony successful infection was observed in 57.50% ± 28.06% (SD) of the workers exposed

to *Crithidia bombi* after a 7-day infection period. Both infection richness (the number of genetically distinct parasite strains that successfully established from the initial five-strain inoculum) and infection intensity (the total number of parasite cells per bee) varied greatly between individual colonies (fig. 1*B*). Figure 1*B* shows a strong correlation between mean infection richness and infection intensity across colonies (Spearman's $\rho = 0.82$, $P < .001$, $n = 33$). The combined effects of individual parasite richness and intensity captured by the first principal component (PC1 score) explained 74.30% and 80.27% of the total variation in *C. bombi* infection success for all colonies ($n = 250$ individuals) and for those colonies ($n = 62$ individuals) that were selected for microbiota analysis before and after infection, respectively. Given this natural variation in infection outcome, we randomly selected 18 colonies covering the whole range (fig. 1*B*) of susceptibilities for the subsequent microbiota composition analysis.

Comparing Fecal and Gut Microbial Communities

We found that feces and gut microbiota samples from the same individual bee were more similar to each other than were feces and gut microbiota samples from randomly chosen bees on the basis of beta diversity, irrespective of the sampling interval: no-delay versus delay-control treatment (permutation test of Bray-Curtis dissimilarity; no-delay treatment: empirical $P = .001$; delay-control treatment: empirical $P < .001$; fig. A3). Furthermore, alpha diversity between feces and gut was significantly correlated (more so with sampling delay) for the Shannon index (no-delay treatment: Pearson's correlation $r = 0.47$, $P = .003$; delay-control treatment: $r = 0.62$, $P < .001$; fig. 2*B*) and evenness (no-delay treatment: $r = 0.40$, $P = .013$; delay-control treatment: $r = 0.58$, $P = .001$; fig. 2*C*) but not for species richness (no-delay treatment: $r = 0.55$, $P < .001$; delay-control treatment: $r = 0.50$, $P = .006$; fig. 2*A*; see also fig. A4). Only a subset of 47 OTUs (51 OTUs if the parasite-exposed treatment is included; fig. 2*D*) from a total of 210 OTUs were observed at least once in both sample types from the same individual. These OTUs represent the most abundant OTUs in the data set (fig. A5) and include the taxonomic core groups of the social bee gut microbiome as defined by Kwong and Moran (2016; table D4 in Dryad: http://dx.doi.org/10.5061/dryad.4nm5sh4 [Näpflin and Schmid-Hempel 2018]). Here, we defined the 16 OTUs contained in the intersection of all treatment groups to be the core microbial subset for this study (fig. 2*D*). Furthermore, these core OTUs are more closely related to known core bee 16S sequences than are the other 31 shared OTUs (see "Material and Methods" and table D4 in Dryad: http://dx.doi.org/10.5061/dryad.4nm5sh4 [Näpflin and Schmid-Hempel 2018]).

Fecal Microbiota before Infection: Does the Microbiota Predict Infection Outcome?

We analyzed the fecal microbiota community of individual workers before parasite exposure (fig. 1*A*; parasite-exposed treatment) in order to identify any properties of the microbiota that are predictive of the infection outcome. We found that infection outcome varied with evenness (LMM; $\chi^2_1 = 4.00$, $P_{\text{adjusted}} = .046$, $\beta = 2.54 \pm 1.24$ [SE], sample size–corrected Akaike information criterion [AICc] = 193.6, $R^2_m = 0.06$, $R^2_c = 0.31$; fig. 3*A*). Most interestingly, individuals with higher evenness scores (i.e., higher diversity) showed higher—rather than lower—susceptibility to the parasitic infection. This relationship is even more pronounced if only core OTUs are considered (LMM; $\chi^2_1 = 6.66$, $P_{\text{adjusted}} = .020$, $\beta = 3.10 \pm 1.15$ [SE], AICc = 190.9, $R^2_m = 0.10$, $R^2_c = 0.32$; fig. A6). We identified candidate core OTUs that contribute most to infection

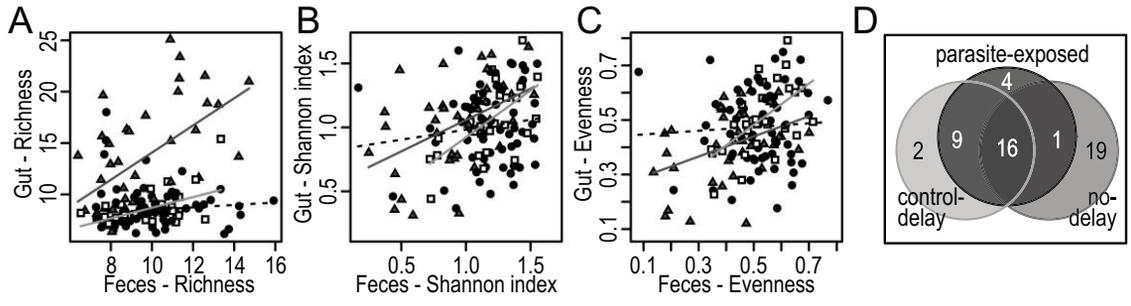

Figure 2: Similarities between fecal and gut microbiota. *A–C* show alpha diversity measures: species richness (*A*), Shannon index (*B*), and evenness (*C*) of the individual gut microbiotas plotted against the respective diversity measure of the fecal microbiota. Each point represents an individual bee (no-delay treatment: $n = 38$, dark gray triangles; control-delay treatment: $n = 29$, open squares; parasite-exposed treatment: $n = 61$, black circles). The correlation is visualized by solid (significant) and dotted (nonsignificant) fitted regression lines. *D*, Venn diagram of the number of operational taxonomic units that were observed at least once in both sample types of the same individual.

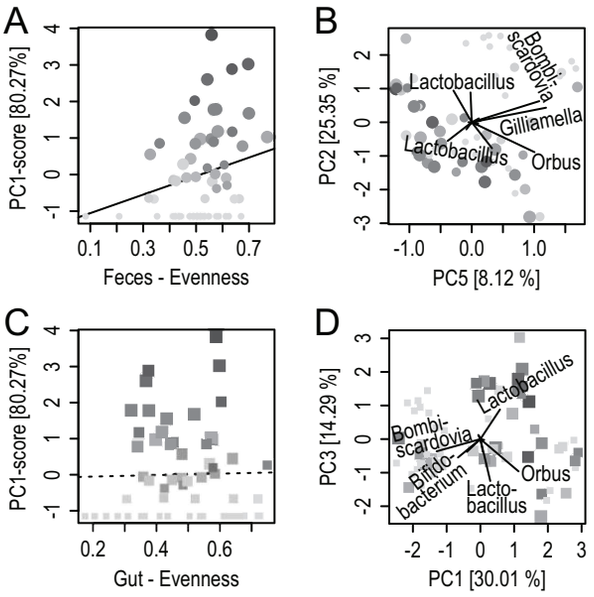

Figure 3: Microbiota alpha diversity before parasite exposure, but not after exposure, explains infection outcome. *A*, *C*, Relationship between variation in infection outcome (first principal component [PC1] score) and alpha diversity (evenness) before parasite exposure (*A*) and 7 days after parasite exposure (*C*; also see fig. A7). Infection intensity and infection richness summarized by the PC1 score are visualized with point size and fill shading, respectively (size and shading intensity increase with susceptibility). Circles are fecal samples, and squares are gut samples. Lines are the fitted regression lines from the linear mixed effects model accounting for colony identity as a random factor. Solid lines represent statistically significant relationships ($P < .05$). *B*, *D*, Principal component analysis biplot of core operational taxonomic units. Axes are the principal components (PCs) that significantly predicted infection outcome before (*B*) and after (*D*) parasite exposure.

outcome as those with the largest eigenvector values in the PC5 and PC2 direction (fig. 3*B*) of a principal component analysis of core OTU abundances.

Gut Microbiota after Parasite Exposure: Does the Microbiota Change with Infection?

First, we checked whether gut microbial composition was significantly altered 7 days after parasite exposure compared with that in unexposed individuals (control-delay vs. parasite-exposed treatment). We found that neither alpha diversity measures (Welch's *t*-test; *S*: $t_{69.1} = -0.47$, $P = .642$; *H*′: $t_{59.9} = 0.70$, $P = .490$; *E*: $t_{59.8} = 0.75$, $P = .455$) nor beta diversity (vegan::adonis; $F_{1,87} = 0.19$, $P = .923$; fig. A8) was significantly altered by parasite exposure. Furthermore, variation in infection outcome (PC1 score) could not be explained as a function of the gut microbiota alpha diversity (i.e., evenness) within the parasite-exposed treatment (LMM, full OTU data set: $\chi^2_1 = 0.03$, $P_{\text{adjusted}} = .872$, $\beta = 0.22 \pm 1.33$ [SE], AICc = 202.6, $R^2_m < 0.01$, $R^2_c = 0.30$; fig. 3*C*; core OTU data set: $\chi^2_1 = 1.35$, $P_{\text{adjusted}} = .491$, $\beta = 1.36 \pm 1.16$ [SE], AICc = 201.2, $R^2_m = 0.03$, $R^2_c = 0.32$; fig. A6). Hence, while we find that the characteristics of the microbiota before parasite exposure correlate with a later infection outcome (e.g., higher diversity microbiota is associated with more infection), the microbiota after parasite exposure does not differ anymore among bees that became infected. This suggests that a subtle change in the microbiota composition of at least some of the individuals must have occurred. This is further suggested by a nonsignificant correlation between diversity measures of fecal versus gut microbiota (corresponding to pre- and postexposure samples) in parasite-challenged individuals (*S*: $r = -011$, $P = .410$; *H*′: $r = 0.13$, $P = .316$; *E*: $r = 0.06$, $P = .659$; fig. 2*A*–2*C*) in contrast to the control individuals (fig. 2*A*–2*C*). We identified the axes of the principal component analysis of core OTU abundances that showed a significant effect on the PC1 score of infection outcome. Interestingly, PC1 of the OTU abundances varied with the PC1 score of infection outcome, suggesting that compositional variation is linked to parasite infection. Inspection of the OTUs with the largest eigenvector components in the PC1 and PC3 direction revealed similar candidates as identified before infection that prove most influential with respect to parasite infection (fig. 3*D*).

Microbiota Resilience: Quantification of Microbiota Change in Response to Parasite Exposure

We assessed the robustness of microbiota to parasite invasion by analyzing the change in microbiota composition from before exposure to after parasite infection. For this, we analyzed the difference in alpha diversity (fig. 4*A*–4*C*) and beta diversity (fig. 4*D*) from before and after infection as a function of infection outcome. However, no measure of change in the microbiota composition was explained by infection outcome (table 1; fig. 4).

Discussion

The potential ability of host-associated microbial communities to provide colonization resistance against the invasion of a pathogen is an important component in mediating the infection outcome of host-parasite interactions (Sekirov and Finlay 2009; Stecher and Hardt 2011; Buffie and Pamer 2013). The current study allowed us to quantify and identify elements of the microbiota community that explained variation in infection resistance (i.e., colonization resistance) and the impact of parasite exposure on the microbiota composition (i.e., the resilience of the microbial community toward perturbation).

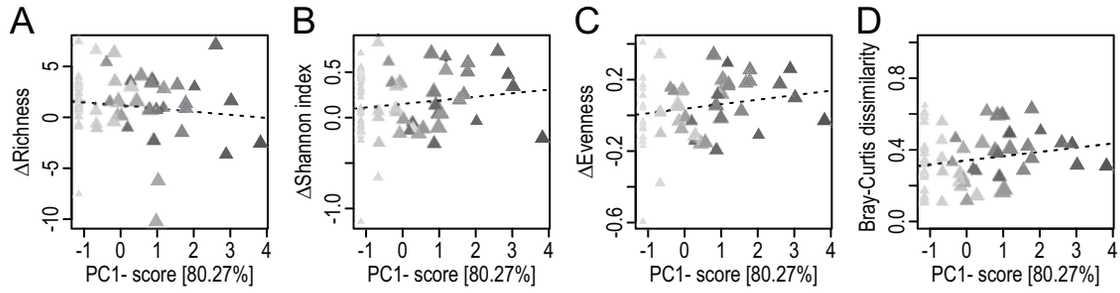

Figure 4: Infection outcome does not explain microbiota composition change from before to after parasite exposure. Panels show the difference in alpha diversity measures (*A*–*C*) and beta diversity (*D*), quantified by the Bray-Curtis dissimilarity, from before infection to 7 days after parasite exposure as a function of infection outcome. Lines are the fitted nonsignificant ($P < .05$) regression lines estimated from a linear mixed effects model. Point size is proportional to infection intensity, and darker fill shading indicates a higher number of parasite strains. The corresponding statistics are reported in table 1. PC1 = first principal component.

We found that variation in diversity of the microbiota community composition before infection correlated with infection outcome (fig. 3*A*). In particular, individuals more resistant to parasite exposure had a less diverse microbiota, as quantified by evenness. At first sight, this is somewhat contrary to the findings of Cariveau et al. (2014), who found the opposite effect when considering the noncore microbiota only. However, together the results seem to suggest that bees with a skewed microbiota abundance distribution—composed of a few highly abundant OTUs ("species"; typically included in the core microbiota) and many rare species (typically included in the noncore microbiota)—may be more protected against parasite invasion (i.e., have a stronger colonization resistance potential) than bees with a more even or more diverse microbiota abundance composition. Overall, microbiota diversity explained between 6% and 10% of the variation in infection outcome (PC1 score). Obviously, diversity is only a crude measure for the relevant properties of the microbiota, and many factors other than the microbiota are known to influence infection outcome as well—for example, general stress (Brown et al. 2000, 2003), environment (Sadd 2011), genetics (Schmid-Hempel and Reber Funk 2004; Barribeau et al. 2014), and diet (Brunner et al. 2014). Against this background, it is quite remarkable that a sizeable fraction of variance is explained by microbiota diversity alone. Furthermore, that alpha diversity measures explain a significant amount of variation in infection outcome even when colony identity is accounted for suggests that this alpha diversity might be directly influencing infection outcome rather than only being a property that varies across colonies.

The finding that a less diverse, rather than a more diverse, microbiota is protective might seem counterintuitive at first, given that community diversity is generally considered to increase robustness against perturbations or invasions (Shea and Chesson 2002; Girvan et al. 2005) and also more specifically against parasite infections in insects. Nevertheless, some studies in plant systems, for example, showed that a high species diversity in plant communities might simply reflect abiotic conditions, which are more, rather than less, favorable for invasion (Levine and D'Antonio 1999; Levine 2000; Shea and Chesson 2002). Similarly, it is plausible that those conditions that allow for a more diverse microbiota (i.e., more niches) are the same as those that facilitate parasite invasion. What the corresponding conditions that underlie bee—or other social insect—microbiota diversity are remain unknown, but there is evidence that microbiota composition depends on more than just the intrinsic interactions between community members (Näpflin and Schmid-Hempel 2017). Importantly, such a covariation of parasite susceptibility and microbiota diversity might be apparent only when the microbiota is allowed to assemble nat-

Table 1: Statistical report of analysis of infection outcome as a predictor of microbiota change from before to after parasite exposure for the full data set

Response variable	χ^2_1 (LMM)	P	$\beta \pm$ SE	R^{2a}
ΔS	1.00	.319	$-.31 \pm .31$	.02, .02
$\Delta H'$	1.09	.297	$.04 \pm .04$	.02, .20
ΔE	1.90	.168	$.03 \pm .02$	.04, .14
Bray-Curtis dissimilarity	2.53	.111	$.02 \pm .02$	.04, .04

[a] Marginal R^2 followed by conditional R^2 reported for the linear mixed effects model (LMM).

urally and hence depends on both intrinsic interactions and extrinsic conditions. When microbiota composition is enforced experimentally, diversity does not necessarily reflect external conditions, and hence higher diversity might decrease parasite infection (Dillon et al. 2005), through for example direct interactions between the microbiota members and the parasite.

Alternatively, the protective function of the microbiota might be modulated by a few specific OTUs and not by diversity per se. This is supported by the observation that the relationship between microbiota composition and colonization resistance is more strongly influenced by OTUs that occur in both the fecal and the gut sample of the same individual (core set; figs. 2*D*, 3*B*). Consequently, noncore OTUs appear to be less important for colonization resistance. Likely candidates mediating colonization resistance belong primarily to the family of Orbaceae and the bee-specific *Gilliamella apicola* species (Cariveau et al. 2014; Mockler et al. 2018; see also fig. 3*B*). In addition, Bifidobaceriacea (*Bombiscardovia*) and *Lactobacillus* species are likely to be of importance (fig. 3*B*, 3*D*; Mockler et al. 2018). These candidate OTUs have appeared in previous studies of bees. They belong to the major taxa that are strongly associated with bee microbiota and might reflect the different compartments in the gut (ileum vs. rectum; Martinson et al. 2011; Koch and Schmid-Hempel 2011*a*; Engel and Moran 2013; Kwong and Moran 2016). Moreover, they have previously been associated with a protective function against disease and usually occur at high abundances in bees (Koch and Schmid-Hempel 2011*a*; McFrederick et al. 2012; Cariveau et al. 2014). Furthermore, Cariveau et al. (2014) showed that *Crithidia* infection correlated with an increased richness of bacterial taxa that are not monophyletically related to those taxa primarily associated with *Apis* or *Bombus* species. More generally, this pattern is in line with concepts from community ecology, where common and abundant taxa are thought to be the major provider of services by a particular ecosystem (Gaston 2010; Winfree et al. 2015).

Interestingly, the observed relationship between microbiota diversity and infection outcome disappeared once the parasites were established (fig. 3*C*). One possibility is that colonization by the parasite evens the abundance distribution among taxa of the microbiota of infected individuals. We could not firmly deduce from our results whether diversity increases in infection-resistant individuals or decreases in infection-susceptible individuals after a parasite challenge. However, because parasite establishment means a successful colonization by an additional microorganism in the same habitat, it is plausible that the microbiota composition in the infected individuals was altered and that in the noninfected individuals remained unchanged. If so, parasite infection would decrease the diversity of a microbiota because higher diversity "invites" infection, and this makes the microbiota eventually indistinguishable from the presumably unchanged resistant and low-diversity microbiota. Yet statistically no effect of infection outcome could be detected on the change in microbiota composition within individual workers before and after an infection (fig. 4; but see fig. 2*A*–2*C*). Obviously, individuals could be resistant to the parasite for reasons other than the microbiota, and this background variation could overshadow any real differences. Yet this must remain speculative for the time being and requires more study.

So far, therefore, our results suggest that characteristics of the microbiota that are associated with resilience in response to parasite exposure in bumblebees appear to be subtle, and our analysis based on two time points might not have sufficient resolution to extract functional information. However, gut microbiota resilience has been observed in other systems. In humans, for example, antibiotic perturbation studies showed that perturbations strongly affected community measures, such as species richness and diversity, in the short term, but a significant fraction of the microbial community recovered to its pretreatment state in the long term (Dethlefsen et al. 2008; Dethlefsen and Relman 2011). The samples in our study were collected 7 days apart, which might have given the microbiota enough time to largely recover. Furthermore, in the cited studies the effects of perturbation were subject dependent, and the between-subject variation was the largest contributor to microbiota variability (Dethlefsen et al. 2008; Dethlefsen and Relman 2011). Nevertheless, these and other results (Costello et al. 2012) suggest that gut communities are surprisingly resilient and that mechanisms are in place that help the microbial community regain its initial composition (Lozupone et al. 2012). However, if multiple stressors (such as antibiotics and a parasite) co-occur, dysbiosis of the microbiota might be more persistent, as has been shown in honeybees (Raymann et al. 2017).

Given the statistical associations between microbiota composition and colonization resistance, the question arises how and where the interaction may take place within a bee. The parasite *Crithidia bombi* likely attaches to the hind gut wall (Lipa and Triggiani 1980; Schmid-Hempel 2001), which is also the habitat of many of the bacterial species of the microbiota (Martinson et al. 2012). Hence, competition for the gut wall niche could be one of the mechanisms of how infection outcome is influenced (Ulrich et al. 2011). Other than competition for resources, the close spatial proximity would also allow for other interaction mechanisms, such as apparent competition (Stecher and Hardt 2011). Nevertheless, it is generally not clear how the interaction structure among the host, the parasite, and the microbiota is organized (Foxman et al. 2008). Alternatively to direct competition between the microbiota and the parasite, indirect effects may also be possible. Specifically, the microbiota may influence the host in such a way that makes it

more or less protected against parasite infection, for example, via influences on the immune system (Näpflin and Schmid-Hempel 2016). Such an effect could potentially even persist across generations (Sadd and Schmid-Hempel 2009; Chambers and Schneider 2012).

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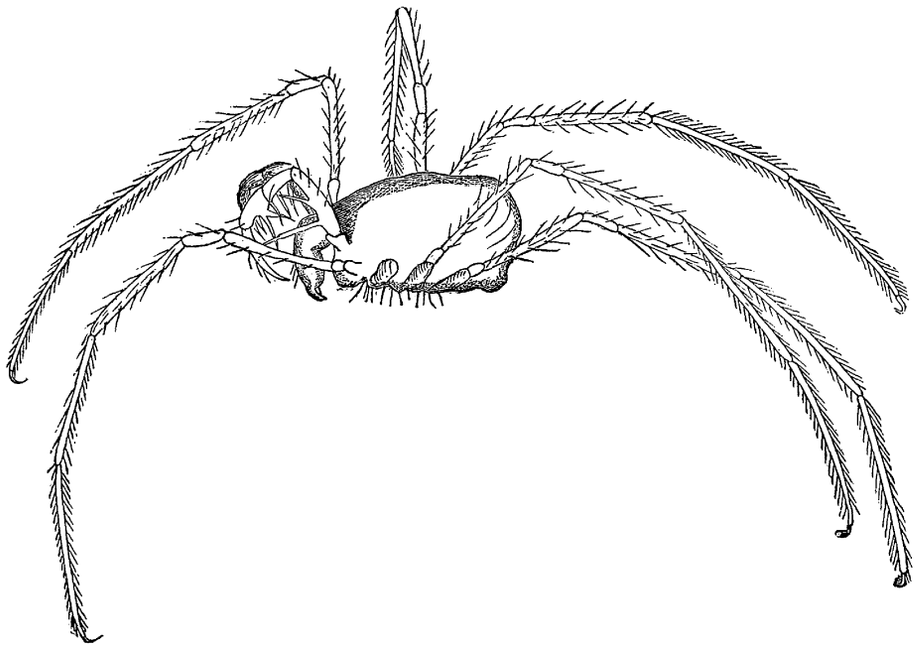

"The family of Harvest men is represented by a small white form, described by Tellkampf under the name of *Phalangodes armata*. . . . The body alone is but half a line long, the legs measuring two lines." From "The Mammoth Cave and Its Inhabitants" by A. S. Packard Jr. (*The American Naturalist*, 1871, 5:739–761).