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ANIMAL HOST–MICROBE INTERACTIONS

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Host effects on microbiota community assembly

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Abstract

1. To what extent host-associated microbiota assembly is driven by host selection or simply by happenstance remains an open question in microbiome research.
2. Here, we take a first step towards elucidating the relative importance of host selection on the establishing gut microbial community in an ecologically relevant organism.
3. We presented germ-free bumblebee, *Bombus terrestris*, workers from 10 colonies with a "global" microbial species pool comprised of an equal mixture of the gut microbiota of all colonies.
4. By means of 16S amplicon sequencing, we found that overall microbiota community composition was generally shifted between pool-exposed workers compared to workers that naturally acquired their gut microbiota, but that the specific composition of the established microbiota also depended on colony identity (e.g. genetic background).
5. Because the microbiota is protective against parasite infection in this system, variation in the filtering of a beneficial microbial community can have important consequences for host resistance and eventual co-evolution with parasites.

KEYWORDS
16S, community assembly, community ecology, host–microbiota, host–parasite, insects, microbiome

1 | INTRODUCTION

According to community ecology, the species composition of communities is determined by four main processes: local selection, local speciation and extinction, global dispersal and random effects (drift) (Vellend, 2010). However, these processes can vary in their relative importance to one another and interact on different spatial scales, complicating the understanding of the assembly and the eventual configuration of different organisms into communities (Götzenberger et al., 2012). While these concepts of community assembly have primarily been developed and applied in studies on plants and animals, there is currently an interest to translate these eco-evolutionary theories to the study of microbial communities (Martiny et al., 2006; Prosser et al., 2007). In particular, determining and dissecting the processes of assembly, structure and function of host-associated microbial communities is rapidly becoming a major research objective (Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012; Foxman, Goldberg, Murdock, Xi, & Gilsdorf, 2008; Mihaljevic, 2012; Robinson, Bohannan, & Young, 2010; Zilber-Rosenberg & Rosenberg, 2008).

The gut microbial community of insects, and of bees in particular, is especially amenable for studies (Dillon & Dillon, 2004), as it is typically much less complex than that of mammals, for instance. Studies have identified that environmental factors, such as diet, are major determinants of microbiota composition (Chandler, Morgan Lang, Bhatnagar, Eisen, & Kopp, 2011; Colman, Toolson, & Takacs-Vesbach, 2012; Engel & Moran, 2013; Graystock, Rehan, & McFrederick, 2017; Kwong et al., 2017; McFrederick et al., 2012, 2017). However, gut microbial communities of hymenopterans and termites, and, to some degree, in beetles showed signals of host phylogeny too (Brucker & Bordenstein, 2012; Colman et al., 2012; Graystock et al., 2017; Koch, Abrol, Li, & Schmid-Hempel, 2013; Kwong et al., 2017; McFrederick et al., 2012; Sanders et al., 2014).

Thus, in the light of community ecology, gut microbiota composition is not randomly assembled and the factors shaping it are of great interest. The observation that certain microbiota species associate with certain hosts suggests the possibility of an "active" involvement of the host on the community assembly process, for example, via selection ("filtering") from the total available microbial species pool. Note that selection on the microbiome by the host could occur via a variety of mechanisms that not necessarily are "active" in the sense of displaying different behaviours or require a conscious decision. Examples are variation in gene expression (Buchon, Silverman, & Cherry, 2014; Näpflin & Schmid-Hempel, 2016) or differential adhesion to mucosal surfaces (McLoughlin, Schluter, Rakoff-Nahoum, Smith, & Foster, 2016). Yet, variation in the potential for host microbiome selection is particularly intriguing when the microbiota is beneficial to the host, as is generally the case: the host may then coordinate the assembly process of the microbiota such that it maximizes its benefit. Such host selection potential on the microbial gut community is plausible in honeybees and bumblebees where microbiota community composition is simple, distinctive, species-specific to some degree, and also functions in the defence against parasites (Koch & Schmid-Hempel, 2011a; Koch et al., 2013; Lim, Chu, Seufferheld, & Cameron, 2015; Martinson et al., 2011). Most importantly, because adults of honeybees and bumblebees emerge from the pupa essentially germ-free (Hakim, Baldwin, & Smagghe, 2010; Koch & Schmid-Hempel, 2011b; Martinson et al., 2011), and the gut microbiota is subsequently acquired within the social environment (the nest) likely via faeces-contaminated nest material, and coprophagy (Koch & Schmid-Hempel, 2011b; Martinson, Moy, & Moran, 2012; Powell, Martinson, Urban-Mead, & Moran, 2014), there are ample possibilities for the host to select on the establishing microbiota.

In the bumblebee, *Bombus terrestris*, the gut microbiota provides a protective function against its natural gut parasite, the trypanosome *Crithidia bombi* (Koch & Schmid-Hempel, 2011b). A recent study (Cariveau, Elijah Powell, Koch, Winfree, & Moran, 2014) shows that the presence of *Gilliamella*, a bacterium belonging to the core species of the *B. terrestris* microbiome, negatively correlates with infection by *C. bombi*, yet, the richness of the remaining components of the microbiota also played a role. Hence, the protective function against the parasite may not be provided solely by any particular species of the gut microbial community alone, but rather by several elements of the gut microbiota as a whole (Koch & Schmid-Hempel, 2011b; Näpflin & Schmid-Hempel, 2016). Furthermore, the level of protective function of the microbiota also differs across colonies (i.e. host backgrounds/genotypes) and varies among different parasite strains (Koch & Schmid-Hempel, 2012).

Rather than considering the effects of single bacterial species in isolation, we here take a community-based approach by analysing the community structure of the entire microbiota. The two central open questions therefore are: first, what is the structure and composition of the microbiota that is most protective for the host, and second, how much influence does the host have on determining the composition of the microbiota it acquires? In this paper, we specifically address the latter question. A first step towards an answer to this is to determine whether colonies differ in the assembly and establishment of the gut microbiota. Therefore, here we assess whether the microbiota composition that eventually establishes from a common microbial species pool varies across colonies. Such variation would provide evidence that colonies can select their own bacterial flora, at least to a certain degree. For this, we presented the same inoculum of faecal microbiota (later referred to as "Pools"), to germ-free workers from 10 different colonies and allowed the microbiota to establish. The offered inoculum represented the "global" microbial species pool as each of the test colonies contributed equally to the administered microbiota inoculum. In addition to studying whether colonies differed in their ability to impose "selection" on the offered species pool, we could also compare the resemblance of the established gut microbiota in these test workers (treatment: "Pool exposed"), to their nest mates. These nest mates had acquired their microbiota naturally (treatment: "Naturals") within their colony environment, and were thus only exposed to their respective colony-specific bacterial species pool. Hence, we could ask whether the composition of the microbiota in a given colony not only deviates from the overall available pool but also whether the selected subset is consistent among workers of the same colony.

2 | MATERIALS AND METHODS

2.1 | Bee colonies and sample collection

Bombus terrestris queens were field-caught in spring of 2014 from two populations in Switzerland (Aesch and Neunforn). Unrelated F1-offspring of these queens (i.e. the virgin daughter queens) were allowed to mate in flying tents with F1-males (i.e. drones) that were unrelated to their mating partner. The newly mated queens were then allowed to establish colonies after an artificial hibernation period in the laboratory. Of these, 10 well-established colonies were selected for the purpose of this study (Table S1). At all times, colonies and isolated workers were kept in the same facility and under standardized conditions (28 ± 2°C, 60% RH, constant red-light illumination), and provided with pollen and sugar water (ApiInvert®) ad libitum. All handling and housing material involved in raising germ-free workers and performing faecal transplant experiments were either autoclaved or washed in 80% ethanol before use. To further prevent bacterial contamination, a diet of X-ray-radiated pollen (dose: 26.7 kGy) and filter-sterilized sugar water [50%] (pore size 0.2 μm) was provided ad libitum to bees kept in the sterile environment. A schematic of the experimental design and procedure is shown in Figure 1.

For each of the 10 colonies, we raised germ-free workers in a sterile environment, which later were exposed to the pool or sham inoculum of microbiota (treatment: "Pool exposed" or "Steriles" respectively) to study effects of host selection on microbiota composition and the effectiveness of the sterility treatment respectively. For this, we followed the experimental faecal transplant protocol described in Koch and Schmid-Hempel (2011b) with one modification: the isolated cocoons of each colony were surface-sterilized

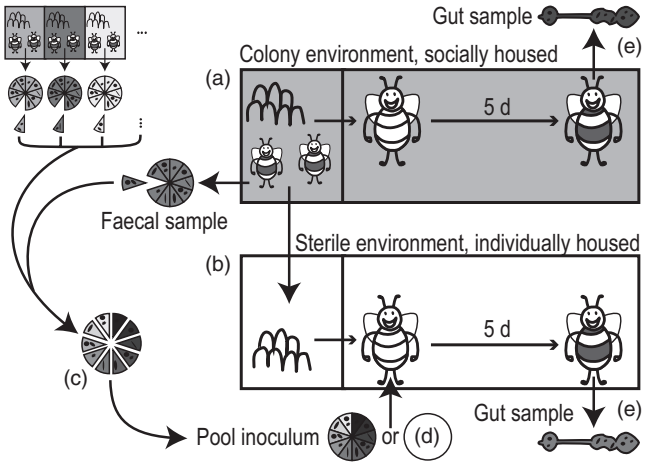

FIGURE 1 Experimental design. A gut microbiota was allowed to establish in workers of 10 colonies in two ways: (a) naturally, within the social environment of the colony (Naturals; grey zone), or (b) experimentally, by exposing germ-free workers in a sterile environment with a pool inoculum of microbiota (Pool exposed). The transplant was composed, in equal parts, of faecal matter collected from each of the 10 colonies (Pools; c). Additionally, as a technical control of the sterility treatment, some bees received a microbiota-free "sham" inoculum (Steriles; d). The microbiota was allowed to establish for 5 days before the bees were sacrificed and the gut microbiota community was analysed (e)

by submerging the cocoons for 90 s in a 3% sodium hypochlorite (Murrell & Goerzen, 1994) and air-dried before being placed in sterile box. Once a day, callows that naturally emerged from the surface-sterilized brood clumps were transferred to individual housing boxes and kept for 1–2 days before they were fed either 15 μl of faecal microbiota mix (Pool exposed) or 15 μl sugar water only (Steriles) after a 30-min starvation period. Any bee that did not take up the inoculum was excluded from the experiment. The faecal microbiota mix (Pools) consisted of equal volumes of freshly collected faeces from each colony and of at least five donor workers diluted with sugar water (1:2 (v/v) ratio). A sample of each of the freshly prepared pool microbiota inocula was set aside and frozen for later analysis (Pools). Vice versa, to study the natural, colony-specific, acquisition of microbiota (treatment: "Naturals"), around the same time, each day, and in all colonies, emerging workers (i.e. within 24-hr post-hatching, "callows") were collected and marked. The marked callows were returned to their colony and allowed to naturally acquire their gut microbiota. Because a sufficient number of age-controlled workers could not be collected from all colonies, we picked at random some additional, older workers to compensate for the missing workers in Naturals (see Table S2 for sample size per colony and treatment). The microbiota of Pool exposed, Steriles and Naturals was allowed to establish for 5 days [a period suggested by Martinson et al. (2012)] before bees were sacrificed and frozen at −20°C. We consider this as an adequate protocol for the natural and experimental acquisition of microbiota, as the exact conditions (time interval, social surrounding, exact inoculum amount) are not yet known in detail.

2.2 | DNA extraction

We extracted DNA from all samples using the Qiagen DNeasy Blood & Tissue Kit and followed the manufacturer's protocol (Animal Tissue DNeasy 96 Protocol), with a few modifications depending on sample types. DNA extraction of samples for later microbiota analysis occurred from aseptically dissected whole guts (Engel et al., 2013). Before extraction, a sterile zirconium oxide bead (2.8 mm) was added to the dissected gut and the sample was shredded three times for 1 min at 30 Hz on a Mixer Mill MM 301 (Retsch GmbH). Also, we extracted 35 μl of each of the microbiota pool inocula. Both sample types were incubated for 1 hr at 56°C, but extracted DNA of the Pools were eluted in only 100 μl AE buffer. For the analysis of a colony's resistance profile, DNA extraction occurred from whole abdomen of the parasite-infected bees (see next section). For this, we shredded the abdomen on a Bead Ruptor 24 Homogenizer (Omni) with 0.5 g added zirconium oxide beads (1.4 mm). The disrupted abdomen was lysated in 600 μl lysis buffer (540 μl ATL buffer and 60 μl proteinase K) for 2 hr at 56°C, but only 200 μl of the lysate was used for DNA extraction.

2.3 | Colony resistance profiles

Additionally, we assessed the resistance profile of each colony to the trypanosome gut parasite *Crithidia bombi*. For this, we haphazardly selected six workers per colony and administered to each an infective dose of 10,000 parasite cells in 10 μl [50%] sugar water after a 2-hr starvation period. The parasite inoculum contained equal numbers (i.e. 2,000 cells) of five genetically distinct *C. bombi* strains (strain IDs 08.068, 08.075, 08.091, 08.161, 08.192; Ulrich, Sadd, & Schmid-Hempel, 2011). Infected bees were kept in individual housing boxes for 7 days, allowing enough time for the parasites to establish (Schmid-Hempel & Schmid-Hempel, 1993), before bees were killed and frozen at −20°C.

Infection intensity for each bee was determined by quantifying the total number of parasite cells present in a sample using quantitative real-time PCR on a 7500 FAST RT-PCR System (Applied Biosystems). Total numbers of parasite cells were estimated by preparing a calibration curve by serial dilution of DNA extracted from a known number of *C. bombi* cells. Each PCR reaction was run in triplicates and simultaneously in the presence of the prepared calibration curve following the cycling protocol described in Ulrich et al. (2011) in a total reaction volume of 10 μl containing: 0.2 μl [10 μM] of each forward and reverse primer (Ulrich et al., 2011), 2 μl of 5× HOT FIREPol© EvaGreen© qPCR Mix Plus (ROX) (Solis BioDyne) and 2 μl of DNA template (1:10 diluted).

Simultaneously, parasite infection richness was identified by genotyping the samples at five *C. bombi* microsatellite markers amplified in two multiplexed PCR reactions with the primer set Cri4G9, Cri4, Cri2F10 and the primer set Cri16, Cri1B6, respectively (Schmid-Hempel & Reber Funk, 2004), allowing us to determine the number of parasite strains remaining after 7 days of infection. A 10 μl PCR reaction volume contained: 2 μl of 5× Colorless GoTaq® Reaction Buffer

(Promega), 0.5 μl of dNTPs [2.5 mM each], 0.05 μl GoTaq DNA polymerase (Promega, 5 U/μl) and 2 μl DNA template and either 0.2 μl Cri4, 0.2 μl Cri2F10 and 0.25 μl Cri4G9) [10 μM] of each forward and reverse primer, or 0.2 μl Cri 16 and 0.1 μl Cri1B6 [10 μM] of each forward and reverse primer respectively. A total of 40 PCR cycles were performed with the following steps: denaturation (94°C, 30 s), annealing (48°C or 53°C, respectively, 30 s) and extension (72°C, 30 s). PCR products were run on a 3730xl DNA Analyzer (Applied Biosystems), and the results scored twice independently using Peak Scanner™ software v1.0 (Applied Biosystems).

2.4 | Microbiota: 16S amplicon library preparation and sequencing

We prepared two multiplexed (96 samples each) amplicon libraries for paired-end sequencing on the MiSeq Illumina platform in order to assess the composition of the microbiota. For this, we amplified the variable region V3-V4 of the 16S rRNA gene with universal primers (Klindworth et al., 2013; Liu et al., 2011). To increase overall sequencing performance on the MiSeq platform, we introduced frame-shifting nucleotides in 1-nt increments (Lundberg, Yourstone, Mieczkowski, Jones, & Dangl, 2013) between the region-specific part of the primer and the Illumina overhang adapter (Table S3). We generated the amplicon libraries by following the manufacturer's suggested two-step amplification workflow (see SI for detailed protocol). Briefly, we amplified the desired region with an initial PCR of 25 cycles for each sample (including two blank extraction controls; "Negatives") in four independent reactions. Following clean up, we attached in a 10-cycle amplification step, a unique sequence identifier to the pooled amplicon product of each sample using the Nextera® XT Index Kit v2 Set D (Illumina). Quality control for library preparation was checked for 20 randomly selected samples on a Bioanalyzer (Aligent Technologies) DNA HS 1000 chip. All libraries were quantified in triplicates by quantitative PCR in relation to the Library Quant Illumina Kit (KAPA Biosystems) DNA standards. Following quantification, equimolar amounts of all libraries were pooled (i.e. twice 96). After final quantification, each library was loaded with a 5% PhiX spike-in with MiSeq® Reagent Kit v3.

2.5 | Amplicon processing and OTU clustering

All paired-end raw reads were quality controlled with FastQG (v0.11.2), end-trimmed with PRINSEQ-lite (v0.20.4), merged (FLASH v1.2.9), primers-trimmed (cutadapt v1.5) and quality filtered (PRINSEQ-lite). We generated operational taxonomic units (OTUs) based on 97% sequence identity from all reads combined using UPARSE in USEARCH 8.0.1623 (64 bit), and assigned taxonomic information to each identified OTU using SINTAX within USEARCH 9.2.64 (32 bit) based on the RDP v16 database (Cole et al., 2014; Edgar, 2010, 2013, 2016). We verified the taxonomic assignment by also aligning against the SILVA database v128 (Pruesse, Peplies, & Glöckner, 2012). For phylogenetic analysis, OTUs were aligned using PyNAST (Caporaso, Bittinger, et al., 2010) implemented in Qiime (v1.8.0) (Caporaso, Kuczynski, et al., 2010). Any OTU classified as chloroplast or mitochondria was excluded from further analysis. We assessed sequencing depth by constructing rarefaction curves (number of OTUs as a function of sample size, Figure S1) as the number of reads generated among libraries (i.e. samples) differed. Detailed specifications on the number of reads processed and the amplicon processing workflow, can be found in the Table S4.

2.6 | Analysis and statistics

After the standard purging protocols, we could analyse a total of 17,325,117 reads. First, we tested for any effect of the four treatment groups on the microbiota composition that had established in the workers; this is, when the microbiota was acquired (1) naturally (Naturals), (2) from pool inoculum (Pool exposed), (3) from sterility control treatments (Steriles) and (4) the negative sequencing controls (Negatives). Negatives and Steriles, in particular, showed overall low read quality, a low proportion of merged reads (Figure S2a), a high proportion of chloroplast and mitochondrial sequences (Figure S2b) and two orders of magnitude fewer clean reads (Figure S2c). This leads us to conclude that Steriles are indeed sterile (Figure S2d). The remnant sequences in these samples did not allow us to deduce meaningful biological signals; the distribution of OTUs in these samples rather reflected the OTU distribution across all samples and hence these sequences seem to mostly reflect technical noise (Figures S2–S4). Thus, Steriles and Negatives were excluded from further analyses. This left a total of 17,038,930 reads mapping to 142 different OTUs for analysis. We collapsed age-controlled Naturals together with the randomly picked non-age-controlled Naturals into the same treatment group, as their microbiotas did not differ in any obvious way from each other (Figure S2d). We accounted for different sequencing depths by rarefying all libraries to the smallest library size (which was $n = 6{,}850$ reads). We repeated the rarefaction one hundred times to account for the effects of randomly subsampling the libraries. To investigate diversity differences among treatments, we calculated the ecological alpha-diversity measures (taking OTUs as "species"): Shannon–Wiener index (H), species richness (S) (i.e. number of different OTUs) and species evenness ($E = H/\ln(S)$) for each sample, and then used the respective averages over all rarefied replicate datasets ($n = 100$) for further analysis. Similarly, to analyse microbiota composition differences (beta-diversity), we calculated Bray–Curtis pairwise dissimilarity between all samples averaged over all rarefaction replicates.

We used liner mixed effect models (GLMM; lme4 v1.1.12; Bates, Maechler, Bolker, & Walker, 2015) and subsequent simultaneous test for general linear hypotheses, using Tukey contrasts (glht::multcomp v1.4.6; Hothorn, Bretz, & Westfall, 2008), to determine differences in microbiota alpha-diversity measures across the three groups of interest: (a) microbiota naturally acquired (Naturals), (b) microbiota established from the pool inoculum (Pool exposed) and (c) the microbiota in the pool inoculum itself (Pools). Colony identity was modelled as a random effect to accounted for shared natal colony identity (one dummy colony id was assigned to Pools samples). Significance of the main effect was determined by comparing the statistical model with a fixed

treatment effect to an intercept-only model with the same random effect structure, using likelihood ratio test. Similarly, we used GLMMs to test for effects of the primary treatment of interest Naturals vs. Pool exposed and the effects of colony. For this, we considered treatment as a fixed effect and included colony as random effect nested in population of origin (Aesch vs. Neunforn). To assess whether the treatment effect differed between colonies (i.e. interaction), we fit a random slope model for colonies and populations. The effect sizes for populations were estimated to be zero in the full analysis, such that we dropped the population effect and only retained the colony effect in subsequent analyses. Similarly, we tested for the clustering of microbiota composition, as quantified by Bray–Curtis dissimilarity, due to effects attributed to treatment and colony (including interaction) using permutational multivariate analysis of variance (vegan::adonis; Oksanen et al., 2015). For this particular analysis, we ignored the population-level grouping as a source of variation (for the same reasons mentioned above) and just considered colony and treatment, as well as their interaction as fixed effects. We tested for multivariate homogeneity of group dispersion before applying the adonis variance analysis, ensuring that model assumption is not violated. Furthermore, we computed empirical p-values for within- and between-colony distance differences of Pool and Naturals by randomizing colony identity of bees within treatments and comparing the observed distance differences to this null distribution. Enterotype analysis was performed after (Arumugam et al., 2011) using a Jensen–Shannon distance. Heuristic exploration of the optimal number of clusters based on the Calinski–Harabasz (CH) index resulted in an optimal number of seven cluster with a silhouette value of 0.27 (i.e. cluster cohesion). We performed principal component analysis that separates between class variance (i.e. PCA on cluster centroids) on log-transformed mean abundances and used the eigenvectors to investigate the genera that drive cluster separation. All analyses were performed in R v3.3.3 (R Core Team, 2017).

3 | RESULTS

3.1 | Colony resistance profile

We phenotyped the colonies for their susceptibility to parasite infection: this phenotype is defined by the combined effect of infection intensity (i.e. the number of parasite cells in the bee's gut) and infection richness (i.e. the number of established strains) of the infection outcome. In our study, infection intensity and infection diversity of the parasite *C. bombi* in workers did not correlate (Figure 2) despite variation among colonies, which, to a degree, contrasts with earlier reports (Näpflin & Schmid-Hempel, 2016; Schmid-Hempel, Puhr, Kruger, Reber, & Schmid-Hempel, 1999; Ulrich et al., 2011). The absence of the correlation may be due to the breeding and colony rearing process used here, which only sampled colonies in "good condition". Also, the number of *C. bombi* strains that were able to establish did not significantly differ between the two populations (Aesch vs. Neunforn: Welch two sample *t* test; t (50.97) = −0.53, p = .601). However, for the same average number of parasite strains that were able to establish, there is a marginally significantly higher infection intensity in colonies from Aesch compared to colonies from Neunforn (Welch two sample *t* test; t (47.59) = 1.97, p = .054). The lack of correlation between infection intensity and infection richness impedes a differentiation of resistance phenotypes among colonies and hence hampers relating the colony resistance phenotype to microbiota selection ability (analysed below).

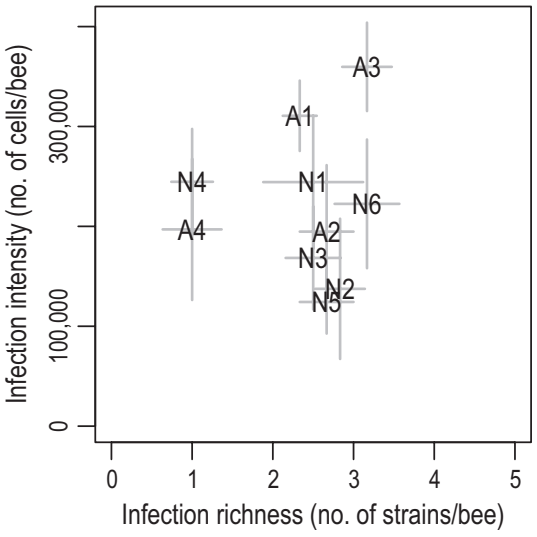

FIGURE 2 Variation in resistance profiles of colonies. Infection outcome by colony 7 days post parasite infection. Plotted are colony mean infection intensity against colony mean infection richness (Spearman's ρ = −0.13, p = .723, n = 10, bars are SEM). Characters denote colony identity whereas the first letter indicates the population origin (A: Aesch, N: Neunforn)

3.2 | Microbiota alpha-diversity

We compared the overall diversity between three groups: (1) Gut samples from workers that acquired their gut microbiota in the social environment of their respective colonies (treatment: Naturals); (2) Samples of the inoculum representing the global species pool that were composed of equal parts of faecal matter collected from each colony (Pools); and (3) gut samples from germ-free workers that acquired their gut microbiota from the inoculation with the global species pool (Pool exposed). We found that microbiota diversity—as measured by OTU species richness—significantly differed among these groups (GLMM; $\chi^2(2) = 15.76$, $p < .001$, Figure 3a). Similar differences were found for the Shannon index (GLMM; $\chi^2(2) = 9.59$, $p = .008$, Figure 3b) and for evenness (GLMM; $\chi^2(2) = 23.85$, $p < .001$, Figure 3c). Importantly, the number of OTUs (richness) did not differ between our control ("Naturals") and the assembled microbiota "Pools".

Because the aim of this study was to determine the selection potential of colonies on the microbiota, we analysed the route of microbiota acquisition (Naturals vs. Pool exposed) more closely. Overall, the gut microbiota that established from the pool-exposed bees showed a reduced diversity, as measured by the OTU richness, compared to the naturally acquired microbiota (GLMM; $\chi^2(1) = 6.86$, $p = .009$, effect size = −1.38 [−2.26, −0.49] 95% CI, Figure 3d), while OTU diversity summarized by the Shannon index did not change (GLMM; $\chi^2(1) = 1.11$, $p = .293$,

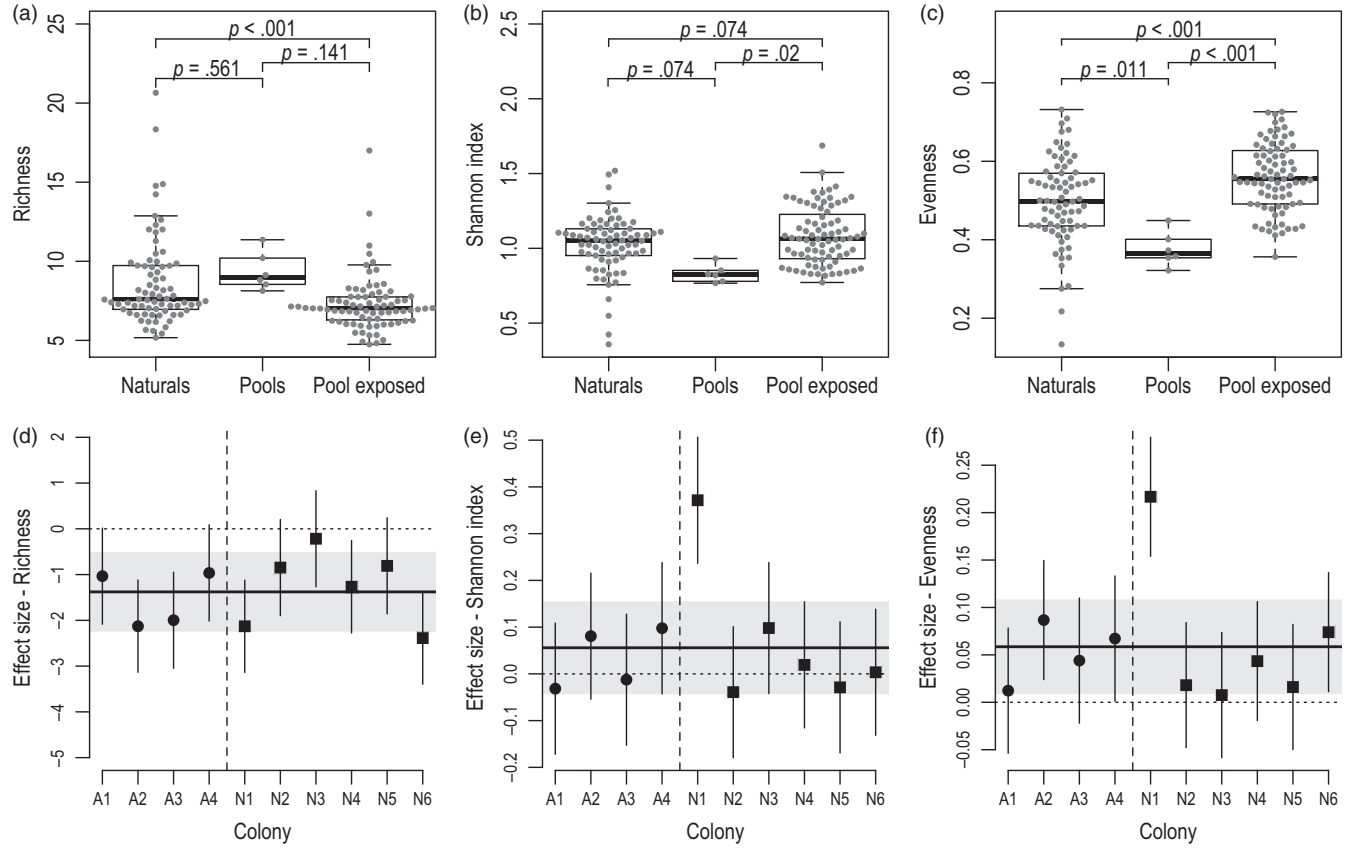

FIGURE 3 Effect of route of acquisition of microbiota on alpha-diversity measures. (a–c) Boxplots for the distribution of the species richness (a), Shannon index (b) and evenness (c) for naturally acquired microbiota (Naturals), microbiota acquired from the global species pool (Pool exposed) and the experimentally assembled global species pool inocula (Pools). The boxes show median and interquartile range (IQR), the hinges extend 1.5 × IQR from the box. Significance between group means, as identified by Tukey's test, is highlighted. (d–f) Visualize the effect sizes and random slope effects of species richness (d), Shannon index (e) and evenness (f) as estimated by the GLMM. The bold black line with the 95% CI in grey shade represents the overall effect size of the fixed effect of Pool exposed relative to Naturals. Points (with 95% CI error bars) show the combined overall effect for each colony including their random slopes (i.e. the interaction term of colony × treatment). Non-overlap of the confidence interval with the bold black line indicates a significant deviation of the colony-specific treatment effect from the mean. Symbols indicate colony origin (circles: Aesch, squares: Neunforn)

effect size = 0.06 [−0.05, 0.16] 95% CI, Figure 3e). Together, this translates into a significant difference in evenness between natural controls vs. pool-exposed workers (GLMM; $\chi^2(1) = 4.15$, $p = .042$, effect size = 0.06 [0.01, 0.11] 95% CI, Figure 3f).

To test for a colony-dependent selection ability on alpha-diversity, we investigated whether the route of microbiota acquisition differs in different colonies (i.e. treatment × colony interaction). Overall, however, colonies responded in the same direction (Figure 3d,f). The inspection of the colony-level random slopes showed that essentially none of the alpha-diversity measures are modified in a colony-specific manner, as indicated by the non-overlap of the 95% CIs with the main treatment effect (see Section 2, Figure 3d–f). Thus, there is little support that alpha-diversity is influenced in a host-genotype-dependent manner.

3.3 | Microbiota composition: Beta-diversity

Similar to the effects for the alpha-diversity measures, pairwise Bray–Curtis dissimilarities (beta-diversity) revealed differences in the microbiota composition. On the one hand, Pool exposed individuals had a shifted microbiota composition compared to Naturals (Figure 4a and Table 1). A genus-level enterotype analysis produced seven clusters that corroborated the shift in microbial composition due to treatment (Figure S5a and Table S5). In a PCA analysis on the enterotype cluster centroids, the eigenvectors corresponding to genera *Snodgrasella* and *Lactobacillus* show opposite effects from *Bombiscardovia* (*Bifidobacteria*) and *Gilliamella*. This suggests that these genera might describe finer scale separations (Figure S5b). Specifically, Pool exposed individuals were characterized by a high abundance of a bifidobacterium that was only present in a subset of Naturals (Figure S3). On the other hand, colony identity also significantly affected clustering of the microbiota community (Table 1). Importantly, colony identity also interacted significantly with treatment, indicating a possible differential selection ability on the administered Pools across colonies (Table 1). The direction of this colony-dependent clustering can be visualized by comparing the dissimilarity of the gut microbial community of Pool exposed to Naturals, from either the same (within) or different colonies (between; Figure 4b). For some colonies, the microbiota composition was more similar among workers from their own

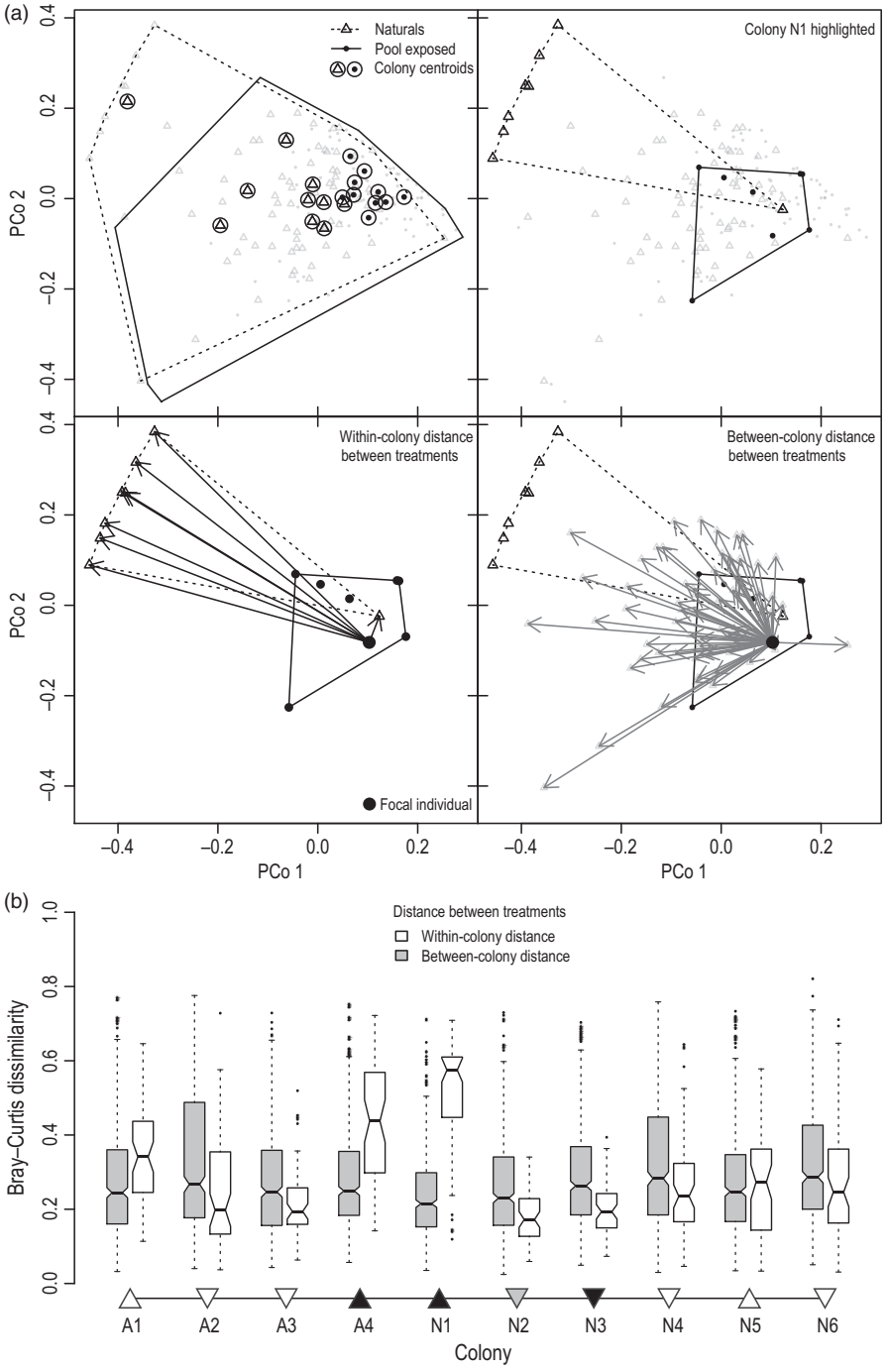

FIGURE 4 Effect of route of acquisition on microbiota composition (beta-diversity). (a) Principal coordinate analysis of the Bray–Curtis dissimilarity and illustration of the within- and between-colony distance calculation in (b). Top left: The shift in colony by treatment centroids of Naturals and Pool exposed show the overall effect of treatment. The data spread is illustrated by polygons. Top right: A specific colony (N1) is highlighted. Bottom left: The within-colony distance for a single focal Pool exposed individual is calculated as the Bray–Curtis dissimilarity between the focal individual to all Naturals of the same colony. Bottom right: The between-colony distance for a single focal Pool exposed individual is calculated as the Bray–Curtis dissimilarity between the focal individual and all Naturals of other colonies. (b) Illustration of the colony by treatment interaction reported in Table 1. The boxplots summarize the distribution of all within- (white boxes) and between- (grey boxes) colony Bray–Curtis dissimilarities, split by colony (see panel a). The direction of effect for each colony is indicated by the triangles on the horizontal axis. Downward (upward) facing triangles show the colonies for which microbiota composition in Pool exposed bees is more (less) similar to Naturals of the same colony than Naturals from other colonies. Significance was determined from empirical p-values through a permutation test and is indicated by triangle fill colour (black: $p < .05$, grey $p < .1$). Each box shows the median and interquartile range (IQR), hinges extend 1.5 × IQR from the box and notches extend ± 1.58 IRR/$\sqrt{n}$

as compared to workers from other colonies. For other colonies, the established microbiota was more dissimilar in distance among workers of their own compared to workers of other colonies. Hence, the colony effect seems to operate in different ways depending on which colony is considered.

4 | DISCUSSION

The prediction of the structure of communities should be possible once the main processes governing their assembly are known

TABLE 1 Permutational multivariate analysis of variance using distance matrices

Model term	*df*	*F*	R^2	*p*-value
Bray–Curtis dissimilarity				
Treatment[a]	1	29.95	0.13	.001
Colony	9	2.74	0.11	.001
Treatment × colony	9	4.36	0.17	.001
Residuals	134		0.59	

[a]Naturals vs. pool exposed.

(Costello et al., 2012; Vellend, 2010), although this task obviously remains challenging. But such insights might be especially valuable for host-associated microbial communities that provide important functions to the host, such as host health (Sekirov, Russell, Antunes, & Finlay, 2010). By employing an experimental approach, we were able to specifically focus on the potential role of the host on the assembly process of a gut microbial community, i.e. a process by which only certain microbes are allowed to establish (Koch & Schmid-Hempel, 2011b, 2012). For this, we assessed whether bumblebee workers have the ability to "filter" specific microbiota community members when presented with a pool of microbiota. This pool represents the "global" species pool that would currently be circulating in the local environment. Such a filtering ability would imply that the host actively influences the microbiota community assembly in the gut, while the actual mechanism might vary (Hooper, Littman, & Macpherson, 2012; Sekirov et al., 2010). If so, this has important implications for the understanding of the protective function of the assembled gut microbiota in the context of host–parasite interactions (Kamada, Chen, Inohara, & Nunez, 2013; Koch & Schmid-Hempel, 2012).

Our global microbiota pool was a mixture of faecal samples from all the colonies, and was therefore expected to reflect the combined overall diversity across all colonies. In the ecological sense, any limitation on the dispersal of microbial species between host colonies was thus eliminated. Our analysis showed that species richness and Shannon diversity was conserved in this pool inoculum as compared to Naturals. Only evenness was diminished in Pools compared to Naturals, likely reflecting an increased skew in the abundance distribution. This is likely due to the repeated sampling of highly abundant species that are shared across all colonies. These species would therefore be strongly overrepresented in the pool inoculum. Rare OTUs, by contrast, would recover in abundance inside the receiving bee (Pool exposed). The mechanisms of such a reorganization might be due to competitive or mutualistic interactions among members of the microbiota. Alternatively, "direct" selection by the host could be important. The latter is suggested by the fact that for some of the colonies, we observe differences in the change of beta-diversity measures that are expressed in a colony-specific manner. We also observed a similar colony-specific effect on alpha-diversity for some of the colonies. This indicates that the more fine-grained multivariate analyses of beta-diversity is preferable to tease apart the subtle differences in host selection ability, compared to the rather crude summary statistics generally used to characterize alpha-diversity. It is likely that these small differences in host selection ability on the community can have important consequences on the establishment of a beneficial microbiome. Hence, the observed variation is of great interest and further studies are needed to uncover the mechanisms that might underlie differential selection ability.

Our results compare to recent findings in other organisms. For example, a recent study in cockroaches showed that, despite being presented with a foreign gut microbiota from a related termite species or even from mice, the gut community that eventually established more closely resembled the gut microbiota composition of natural cockroaches than that of the donor species (Mikaelyan, Thompson, Hofer, & Brune, 2015). Similarly, reciprocal transplants between zebrafish and mice resulted in a composition that more closely reflected that of the natural microbiota of the recipient species (Rawls, Mahowald, Ley, & Gordon, 2006). Similar patterns emerged from a more recent study, where mice were experimentally inoculated with a microbial community from a range of very diverse environments and hosts (Seedorf et al., 2014). Interestingly, when the microbiota was acquired through opportunistic transmission from co-housed mice, primarily non-mouse-associated taxa showed a surprising initial colonization success until a more mice-like microbiota eventually established (Seedorf et al., 2014). This indicates that the social environment where the microbiota is acquired can be highly relevant for the establishment of a specific gut community.

In social insects like bumblebees, microbiota transmission occurs within the social environment of the colony, which can explain why the microbiota composition in social insects is host species-specific (Koch & Schmid-Hempel, 2011a; Koch et al., 2013; Kwong & Moran, 2015; Kwong et al., 2017; Lim et al., 2015; Martinson et al., 2011). This environment of social transmission might explain the slight differences in diversity measures between bees that acquired their gut microbiota naturally, compared to bees that were inoculated experimentally. In the former group, the bees were probably exposed to new microbes throughout the 5 days until they were screened, whereas in the latter, the bees were inoculated at a single point in time and screened 5 days later. As an aside, filtering on the individual level—as measured here—could be less important for a social animal that samples its microbiota from a pre-existing pool that is already filtered to some degree.

While we can only speculate about the mechanism leading to the host effect on microbiota in our experiment, it is very likely that the host's immune system plays an important role (Buchon et al., 2014; Näpflin & Schmid-Hempel, 2016). Experiments with *Hydra* species clearly showed that even a very simplistic immune system using only antimicrobial peptides is capable of selecting species-specific microbiota (Franzenburg et al., 2013). Furthermore, we previously demonstrated experimentally that bees can differ in their immune response to faecal microbiota transplants and that this selection potential might even relate to resistance phenotypes of colonies (Näpflin & Schmid-Hempel, 2016). However, because colony resistance profiles did not show considerable directional variation in this study, we were not able to associate the differential filtering abilities among colonies with their resistance phenotypes. Nevertheless, this hypothesis provides an interesting avenue to further explore the function of the microbiota and connect it with a highly relevant fitness phenotype in bees (i.e. resistance against the parasite). For this, further and more sophisticated experimental manipulation of components of the microbiota is needed to elucidate exact interactions between immune system and gut microbiota community assembly.

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AUTHORS' CONTRIBUTIONS

K.N. and P.S.H. designed the study, wrote the manuscript and approved the final version. K.N. performed the experiment and analysed the data.

DATA ACCESSIBILITY

Raw sequences are available as project PRJEB22577 on the European Nucleotide Archive (ENA). OTU, taxonomic classification and sample data tables used for statistical analysis are deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.v5v3k (Näpflin & Schmid-Hempel, 2017).

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