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INFECTION, MICROBIOTA AND EVOLUTIONARY PARASITOLOGY

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…to all the people that inspired me along the way with their curiosity and knowledge.

Contents

Zusammenfassung

Es ist weitgehend anerkannt, dass wirtsverbundene Darmbakterien wichtige Modulatoren der Interaktion zwischen Wirten und ihren Parasiten sind. Diese Dissertation untersucht die Dreifach-Interaktion zwischen Wirt, Parasit und der Mikrobiota in einem gut untersuchten, natürlichen Wirt-Parasitensystem bestehend aus der Hummel (*Bombus terrestris*), und Trypanosomen (*Crithidia bombi*), einem Darmparasiten. Um die komplexe Interaktionen in diesem System zu entflechten, habe ich mehrere Faeces-Transplantationen und experimentelle Infektionen durchgeführt. Zu Beginn habe ich die Wirt-Mikrobiota-Interaktion aus zwei Blickwinkeln betrachtet. Als Erstes fragte ich, ob Wirte, die entweder resistent oder anfällig gegenüber *C. bombi*-Infektionen sind, unterschiedlich auf Faeces-Transplantate mit ihrerseits unterschiedlichem Schutzwert reagieren. Durch Messungen der Immungenexpression des Wirtes war es mir möglich zu zeigen, dass Wirte eine solche Mikrobiota selektieren könnten, welche sie vermutlich bestmöglich von einer Infektion schützt, aber auch, dass die in der Mikrobiota vorkommenden Arten die Immunantwort des Wirtes zu ihren Gunsten beeinflussen könnten. Als Zweites habe ich diese "Selektionsfähigkeit" des Wirtes auf den angebotenen, bakteriellen Artenpool beurteilt, indem ich die Ähnlichkeit zwischen der etablierten Darmmikrobiota von Empfängern eines Faeces-Transplantates mit Arbeiterinnen verglich, welche ihre Mikrobiota auf naürlichem Weg erworben haben. In einem weiteren Schritt habe ich die Beziehung zwischen Mikrobiota und Parasit analysiert. Über Vergleiche der Darmmikrobiota-Komposition vor- und nach einer Parasitenstimulation, habe ich entdeckt, dass die Mikrobiota eine hohe Belastbarkeit gegenüber der Parasiteninvasion und eine erstaunliche Korrelation zwischen Mikrobiotadiversität und Infektionsausgang aufweist. Allgemeiner habe ich die Unterschiede und Ähnlichkeiten zwischen Arbeiterinnen- und Königinnen-Mikrobiota, wovon das letztere nur spärlich erforscht ist, beschrieben. Meine Resultate bekräftigen vorhergehende Erkenntnisse, dass Wirte in einigen Fällen ihre Mikrobiota möglicherweise beeinflussen, aber in anderen Fällen der Prozess durch die sich etablierende Mikrobengesellschaft angetrieben zu sein scheint. Zusätzlich konnte ich die positive Korrelation zwischen der Diversität der Königinnen-Mikrobiota zu einem Zeitpunkt spät im Koloniezyklus und dem Resistenzprofil der Kolonie (sprich Infektionserfolg) wiederfinden. Schlussendlich präsentiere ich eine Zusammenfassung einer Pilotstudie, in der ich die Struktur und Diversität von Mikrobiota in mehreren Hummelarten aus zwei Regionen in Eurasien, dem evolutionären Ursprung der Hummeln, unter-

suche. Zusammenfassend zeigen meine Resultate, dass in der Hummel sowohl der Etablierungsvorgang als auch die Mikrobiotazusammensetzung, welche sich schlussendlich etabliert, dynamisch, einerseits durch den Wirt, andererseits durch einzelne Angehörige der Mikrobitota sowie deren Konstellation, beeinflusst wird. Diese Dissertation legt ein wichtiges Fundament vor, um den komplexen Einfluss der Wirtsmikrobiota auf die Wirts-Parasiteninteraktion weiter zu untersuchen.

Summary

The host-associated gut microbiota is considered to be an important modulator of the interaction between hosts and their parasites. This thesis presents an investigation into the three-way interaction between host, parasite, and microbiota in a well-researched, natural host-parasite system of bumblebees (*Bombus terrestris*) and their trypanosome gut parasites (*Crithidia bombi*). To disentangle the complex interactions in this system, I performed several faecal microbiota transplants as well as experimental infections. To start with, the host-microbiota interaction was analysed from two angles. First, I asked whether hosts resistant and susceptible to *C. bombi* differentially respond to faecal microbiota transplants that are themselves of different protective values. By measuring the host's response in terms of immune gene expression, I was able to show that hosts may select a microbiota that best protects them from infections, but also, that the members of the microbiota may influence the host immune response to create a more favourable environment for themselves. Second, I assessed this potential "selection ability" of the host on the bacterial species pool by comparing the resemblance of the established gut microbiota community from recipients of faecal transplants to workers that acquired their microbiota naturally. In a further step, I analysed the microbiota-parasite interaction. By comparing the gut microbiota composition before and after a parasite challenge I discovered a high resilience of the microbiota to invasion by the trypanosome gut parasite and a surprising positive correlation between microbiota diversity and infection success. More generally, I describe differences and similarities between worker microbiota and the, poorly investigated, queen microbiota composition. My results corroborate previous findings that host selection may influence microbiota composition in some cases, whereas in others, the process seems to be driven by the colonisation ability of the establishing microbiota community. Furthermore, I recovered a positive relationship between queen microbiota diversity late in the colony's life cycle and the colony resistance profile (i.e. infection outcome). Finally, I present a summary of results from a pilot study that investigates the structure and diversity of bumblebee microbiota across several bumblebee species in two populations in Eurasia, the centre of origin of bumblebees. Overall, my results indicate that hosts, as well as particular members or certain constellation of the microbiota, dynamically influence the establishment and eventual composition of a bumblebee gut microbiota. This thesis presents the groundwork to further disentangle the complex influence of host-associated microbiota on host-parasite

interactions.

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General Introduction

Parasites are a major threat to the health of humans, livestock and wildlife, and also to many conservation efforts. In order to cure, control or prevent disease, or mitigate the detrimental effects caused by a parasite to its host population, or the ecosystem, a good understanding of the particular host-parasite system is required. To accomplish this goal sustainably, the knowledge of the conditions and constraints under which hosts and their parasites evolve is needed. Changes in either one of the interacting partners will influence selection on the other, and thus the evolutionary trajectories of both (Schmid-Hempel, 2011; Thompson, 2009).

These reciprocal interactions are an important driver of one of the most ubiquitous feature of any host-parasite system, that is, not every parasite is equally able to infect every host, and not every host is equally resistant against every parasite (Schmid-Hempel, 2011). Such specificities between hosts and parasites (i.e. variation in host resistance or infectivity) are thought to have evolved either as a by-product of the specific parasite adaptations to different host species, or as the result of parasite adaptation to the current host (Antonovics et al., 2012). However, most hosts are not simple and unitary bodies, as they harbour a diverse community of associated microorganisms, the microbiota. Thus, the interaction between a host and a parasite also affects the microbiota, and, vice versa, the interaction is bound to be modulated by these host-associated microbes, ultimately affecting infection outcome. Uncovering and investigating this three-way interaction between hosts, parasites and host-associated microbiota is the topic of this thesis.

Microbiota and health

A number of examples exist that show the ability of host-associated microorganisms to modulate the infection outcome of various parasites in different hosts. In aphids, a single symbiont species increases host resistance against infection by a parasitoid wasp, though different isolates vary in their degree of the protection they confer (Oliver, Moran, and Hunter, 2005). As a further example, certain bacteria that co-infect with *Plasmodium* alter the expression of the immune system in the *Anopheles* host (Dong, Manfredini, and Dimopoulos, 2009), which influences the parasite life cycle and ultimately the competence of the *Anopheles* vector (Boissiere et al., 2011; Cirimotich et al., 2011; Gonzalez-Ceron et al.,

2003; Pumpuni et al., 1993). In locusts, a more diverse gut microbial community increases resistance against the invasion of a pathogenic bacteria (Dillon et al., 2005). Finally in bumblebees, the whole gut-associated microbiota reduces the overall parasite load, whereas a single tested microbial isolate does not (Koch and Schmid-Hempel, 2011b). Furthermore, the protective value of the gut microbiota in bumblebees varies among colonies and genetically distinct strains of the same gut parasite (Koch and Schmid-Hempel, 2012). While this list completely ignores the large vertebrate literature (Kamada et al., 2013; Kreisinger et al., 2015; Sekirov et al., 2010), these few examples already illustrate the diversity by which either single microorganisms or whole communities of microorganisms (i.e. microbiota) can modulate the relationship between parasites and their hosts in insects.

The particular mechanisms, by which the protective function of the microbiota is provided to the host in these examples, remain, as yet, largely unknown. In general, two possible mechanisms have been discussed: First, microbiota can alter infection outcome either directly, through interaction with the pathogen, or second, indirectly, through interaction with the host immune system (Hooper, Littman, and Macpherson, 2012; Kamada et al., 2012, 2013; Stecher and Hardt, 2011). By here viewing the problem in the light of community ecology, we can identify and understand the ramifications of these mechanisms on the evolutionary dynamics in a particular system of microbiota, hosts, and parasites.

Microbiota and community ecology

Community ecology provides a useful conceptual framework for the study of microbiota (Costello et al., 2012; Foxman et al., 2008; Mihaljevic, 2012). From this point of view, the microbiota is a dynamic ecological community, where the interactions among the individual members of the community matter, in addition to their interactions with the host, the environment and the parasite (Foxman et al., 2008). Alternatively, the microbiota can be viewed as a single entity, i.e. an "organ", that provides a certain function but only interacts with the host, environment and parasite as an entire whole (Foxman et al., 2008). The examples discussed above already suggest that the former is the more adequate description of the patterns.

The framework of community ecology also allows for the prediction of the expected microbiota composition, assuming that certain forces are acting: local selection, local speciation and extinction, dispersal across regions and random effects (i.e. drift) (Vellend, 2010). Vice versa, given a specific observed pattern of microbiota composition, the theory enables the inference of the underlying forces that gave rise to this pattern (Costello et al., 2012; Foxman et al., 2008; Mihaljevic, 2012). In the context of host-parasite interactions, the host as well as the parasite likely represent a selective force on the composition of the microbiota. For the host, manipulating the composition of the microbiota such that it conveys a larger benefit or improved function would be advantageous. For the parasite, direct interaction with members of the microbiota — such as

through competition for niches — might alter the microbiota composition. Unfortunately, investigating the three-way interaction between host, parasite and microbiota simultaneously is inherently difficult. However, by teasing apart this triangle into pairwise interactions in a single system, we can assemble the different pieces of the puzzle to shed light on the evolutionary dynamics of parasites, their hosts and the host-associated microbial communities.

Parasites and microbiota in bumblebees

In the field, *Bombus* spp. are commonly infected with the trypanosome gut parasite, *Crithidia bombi* (Lipa and Triggiani, 1980). In the bumblebee, *Bombus terrestris*, infection with *C. bombi* has previously been found to negatively affect several aspects host fitness: reduced colony founding success of queens after hibernation (Brown, Schmid-Hempel, and Schmid-Hempel, 2003), reduced early colony growth (Shykoff and Schmid-Hempel, 1991), increased worker mortality under stressful environmental conditions (Brown, Loosli, and Schmid-Hempel, 2000), and delayed reproduction (Shykoff and Schmid-Hempel, 1991). A general infection prevalence of between 20 and 80% is observed in different natural populations, with common multiple infections by genetically distinct parasite strains (Salathé and Schmid-Hempel, 2011). Most interestingly, however, the host-parasite system shows a striking pattern of variation in colony susceptibilities to various parasite strains and, vice versa, variation in parasite infectivity (Schmid-Hempel, 2001). These specific host-parasite interactions are further reflected in specific gene expression patterns (Barribeau et al., 2014; Brunner, Schmid-Hempel, and Barribeau, 2013, 2014; Riddell et al., 2009). However, in addition to the gene expression patterns that underlie the specificity of the host-parasite system, the gut microbiota also modulates the infection outcome (Koch and Schmid-Hempel, 2012).

In the bumblebee, workers emerge germ-free (Hakim, Baldwin, and Smagghe, 2010; Koch and Schmid-Hempel, 2011b), and the microbiota is subsequently acquired within their social environment, likely via faeces-contaminated nest material and coprophagy (Koch and Schmid-Hempel, 2011b; Martinson, Moy, and Moran, 2012; Powell et al., 2014). This process therefore contributes to the specialized and well-defined species-specific microbiota composition that is observed in *Apis* and *Bombus* species (Colman, Toolson, and Takacs-Vesbach, 2012; Engel and Moran, 2013a; Koch and Schmid-Hempel, 2011a; Koch et al., 2013; Lim et al., 2015; Martinson et al., 2011).

Most important for this thesis is the protective function of the bumblebee microbiota against parasite infection: the microbiota modulates the infection probability and intensity of the trypanosome gut parasite *Crithidia bombi* (Koch and Schmid-Hempel, 2011b). In addition, different microbiota originating from different colonies, display variation in the level of protection against the parasite (Koch and Schmid-Hempel, 2012), indicating that a certain degree of specificity is mediated by the microbiota-parasite interaction (Koch and Schmid-Hempel, 2012). However, the mechanisms that underlie this modulation of the

host-parasite interaction are so far unknown.

Thesis Outline

Chapter 1: Immune response and gut microbial community structure in bumblebees after microbiota transplant

The microbiota is important for host health and provides protection against parasitic infection. But the microbiota consists of bacteria organisms "foreign" to the host and should thus be controlled by the host's immune system. We show that not only do resistant and susceptible bumblebee hosts differentially respond to microbiota transplants, but also that microbiota from susceptible and resistant hosts elicit differential responses in bees. This suggests that the host's immune system differentiates between microbiota of different protective value and might be able to modulate it, emphasizing the importance of microbiota for host-parasite interactions.

Chapter 2: Microbiota community assembly: colonization versus host selection

In bumblebees, where the gut microbiota provides an important protective function against a common gut parasite, hosts should posses the ability to influence the establishing microbial community composition to their advantage. We presented a "global" gut microbial species pool to bumblebee workers and found that individual colonies differ in their ability to modulate the microbiota composition that eventually established. Only some colonies filter out their own specific microbiota composition from the this "global" pool. Due to a lack of significant colony resistance variation, we were not able to investigate the differential filtering ability in the context of infection outcome. However, we hypothesise that the variation in this filtering ability presents one mechanism by which complex evolutionary host-parasite interaction pattern arise.

Chapter 3: Robustness of bumblebee gut microbiota in response to a parasite challenge

Host-associated microbiotas modulate the interaction between hosts and their parasites. On mechanism by which the microbiota can influence the host-parasite

interaction is by providing colonization resistance to the host. We found that the gut microbiota of bumblebees was surprisingly resilient to parasite exposure. We show that microbiota diversity prior to parasite infection is predictive of infection outcome and that this relationship disappears once the parasite had the opportunity to establish. While this result suggests changes in composition of some individuals, we were not able to uncover the exact changes. We conclude that microbiota-host interactions prior to parasite exposure are thus key mechanism modulating infection outcome.

Chapter 4: The significance of vertical transmission: From the queen to the worker gut microbiota

In bumblebees, queens are hypothesised to play an essential role in the gut microbiota transmission from on generation to the next, implying vertical transmission patterns. However, investigations of queen microbiota composition are virtually absent, despite a well studied worker gut microbiota. We show differences, as well as similarities, between queen and worker microbiota composition. We recover a dynamic component in the microbiota composition of queens over the course of a colony's life cycle and thus only weak support for a solely vertically mediated transmission of the gut microbiota composition in bumblebees.

Chapter 5: From Eurasia to North America: Gut microbiota composition across species along the dispersal route of bumblebee species — a pilot study

A prominent idea is that diversity is particularly high at the evolutionary centre of origin of a species. Indeed, Eurasia, the centre of origin of bumblebees shows high species richness. We investigated, in a pilot study, the patterns of diversity in gut microbiota of several bumblebee species in two populations in Eurasia. We found preliminary results in line with previous studies on species-specific microbiota structure and enterotype structure. Once completed, the study will provide valuable insights into the global phylogeography of bumblebee microbiota composition.

Infection, microbiota and evolutionary parasitology

Chapter 1

Immune response and gut microbial community structure in bumblebees after microbiota transplants

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Abstract

Microbial communities are a key component of host health. As the microbiota is initially "foreign" to a host, the host?s immune system should respond to its acquisition. Such variation in the response should relate not only to host genetic background, but also to differences in the beneficial properties of the microbiota. However, little is known about such interactions. Here, we investigate the gut microbiota of the bumblebee, *Bombus terrestris*, which has a protective function against the bee?s natural trypanosome gut parasite, *Crithidia bombi*. We transplanted "resistant" and "susceptible" microbiota into"resistant? and "susceptible? host backgrounds and studied the activity of the host immune system. We found that bees from different resistance backgrounds receiving a microbiota differed in aspects of their immune response. At the same time, the elicited immune response also depended on the received microbiota's resistance phenotype. Furthermore, the microbial community composition differed between microbiota resistance phenotypes (resistant vs. susceptible). Our results underline the complex feedback between the host?s ability to potentially exert selection on the establishment of a microbial community and the influence of the microbial community on the host immune response in turn.

Introduction

Virtually all organisms have symbionts or host-associated bacterial communities, which are known as the microbiota. In the context of general host health, the importance of the microbiota, especially the one in the gut, has long been recognized (Sekirov et al., 2010). Microbiota can benefit the health of the host in various ways, for example, through effects on nutrition (Nicholson et al., 2012), or by increasing resistance towards parasitic infections. The latter can be either through direct interaction with the pathogen (Kamada et al., 2012, 2013) or indirectly, through interaction with the host immune system (Hooper, Littman, and Macpherson, 2012). However, even though the protective function of microbiota has been observed in various organisms (Dheilly, Poulin, and Thomas, 2015; Dillon and Dillon, 2004; Feldhaar, 2011), clear examples of how symbionts affect resistance against parasitism are more recent, as for example in the aphid-parasitoid wasp system (Oliver, Moran, and Hunter, 2005; Rouchet and Vorburger, 2012).

Koch and Schmid-Hempel (2012) demonstrated a microbiota mediated specific infection outcome: In the bumblebee, *Bombus terrestris*, the transplanted microbiota had a similar influence on specificity (i.e. differences in resistance to various infecting strains of the trypanosome parasite, *Crithidia bombi*) as the host genotype did. More importantly, different microbiota conveyed varying levels of overall protectiveness to the host. Bumblebee larvae shed their gut and its content (as the meconium) during metamorphosis (Hakim, Baldwin, and Smagghe, 2010), and thus emerge as microbiota-free adults from the puparium (Hakim, Baldwin, and Smagghe, 2010; Koch and Schmid-Hempel, 2011b). Within the first few day upon emergence, bees acquire a typical gut microbiota within their social environment, likely via faeces contaminated nest material and coprophagy (Koch and Schmid-Hempel, 2011b; Martinson, Moy, and Moran, 2012; Powell et al., 2014). Therefore, it is reasonable to think of the host as the provider of the environment for the establishing microbial community. In fact, as previously shown, the individual microbiota is taken up within and from the social environment of the colony (Koch and Schmid-Hempel, 2011b). Because many bacteria are circulating in such an environment, the host could and should "select" what bacteria it will acquire; especially it has to differentiate between benign and harmful microorganisms. The microbes, in turn, will be selected to follow their own interests of being taken up and propagated. They may also signal to the host whether they are benign or harmful, and even send dishonest signals, too. It is as yet unknown whether these signals exist and how these potential processes exactly unfold. In a simple manner, hosts may, for example, actively modify their gut environment in order to accommodate and select for the establishment of a beneficial microbiota and keep out unwanted microorganisms. If so, interactions of the microorganisms with the immune system are to be expected during establishment, as bacteria invading the gut are initially "foreign" to the host (Buchon, Broderick, and Lemaitre, 2013). Whereas the immune response upon parasite exposure in bumblebees is partly understood (Barribeau

and Schmid-Hempel, 2013; Brunner, Schmid-Hempel, and Barribeau, 2013, 2014; Riddell et al., 2009), the nature of the immune response to potentially mutualistic or commensal microbes is still a matter of considerable debate (Buchon, Broderick, and Lemaitre, 2013). Nevertheless, if hosts have the ability to influence the establishment of the microbiota via the host immune system, we expect to find variation in the immune response towards different classes of microorganisms, i.e. beneficial and non-beneficial, and among the hosts themselves (Sleiman et al., 2015; Vance, Isberg, and Portnoy, 2009), similar to the variation in gene expression that underlies the specificity of host-parasite interactions (Barribeau et al., 2014; Riddell et al., 2009). Additionally, in different environments (i.e. the background set by the different colonies) there may be different kinds of microbiota available to be taken up by newly emerged workers (i.e. the hosts). If, for example, a "protective" microbiota is available (i.e. a bacterial community that provides protection against infections), we would expect any host's immune system to respond less intensively than when confronted with a "non-beneficial", non-protective microbiota. Here, a reasonable but as yet untested assumption is that milder responses would facilitate the uptake of the bacterial community. In this hypothetical scenario, the microbiota that eventually establishes is results from the host's immune response selecting from the available bacterial community in the environment.

While this hypothesis is likely too simplistic, it has the advantage of being testable as a first step towards understanding the functional significance of microbiota establishment. In this study we therefore explored this hypothesis in three stages. (a) We investigated whether there is variation in how bumblebee colonies respond to infections by the trypanosome gut parasite, *Crithidia bombi*, and define "resistant" or "susceptible" colonies. At the same time, we define the microbiota contained in these colonies, respectively, as "resistant" or "susceptible", too. (b) We tested for the (early) immune gene expression response of microbe-free recipient workers from a resistant or susceptible colony background to faecal transplants of microbiota from donors of either resistant or susceptible colony origin. This allowed us to disentangle effects of host phenotype, i.e. the recipient's resistance background, from effects due to differences between microbiota resistance phenotypes (i.e. from donor transplants). (c) We asked whether the different recipient and donor resistance phenotypes are associated with different compositions of microbiota, that is, differences in the bacterial community structure at the time of the early response.

Materials & Methods

Thirty colonies were raised in the laboratory from queens of *Bombus terrestris*, that were field-caught in spring 2013 in Northern Switzerland (Neunforn). We assayed the resistance profile of five workers for each colony 7 d post infection towards a cocktail of five genetically distinct parasite strains of *Crithidia bombi*, mixed in equal proportions. For this, parasite load (number of cells) was quantified by means of qPCR (Ulrich, Sadd, and Schmid-Hempel, 2011), and in-

fection diversity (number of strains, i.e. different genotypes present) through microsatellite genotyping (Schmid-Hempel and Reber Funk, 2004; see SI Section 1.A). The obtained resistance profiles (Figure 1.1) were then used to select four of the most susceptible, and three of the most resistant colonies. An additional selection criterion for the microbiota transplant experiment was good colony development (i.e. having enough workers). From each of these colonies, brood was isolated and surface-sterilized. Workers, which emerge germ-free (Hakim, Baldwin, and Smagghe, 2010; Koch and Schmid-Hempel, 2011b) were taken from the isolated brood and served as recipients in the transplant experiment. Brood and recipients were kept sterile at all times. To transplant the microbiota, we collected faeces from workers (referred to as donors), which emerged and remained in their source colonies and thus had naturally acquired their microbiota (see SI for experimental details). Note that the resistance phenotype of donors and recipients was always defined as either "resistant" or "susceptible" and refers to the measured resistance profile (i.e. infection outcome) for a given colony (as in Figure 1.1). Thus, each colony represents a certain (genotypic) background that carries at same time a certain microbiota type. Note that the defined resistance phenotype relates to, both, a colony (as the recipient) and its microbiota (as the donor).

We performed crosswise faecal microbiota transplants of resistant and susceptible donors into recipients of resistant and susceptible backgrounds (see Supp. Figure S1.1 for experimental design). Standardized microbiota transplants were orally administered one to three days after emergence of the recipients (see SI Section 1.B). These bees were snap-frozen 18 h post-transplant in liquid nitrogen and stored at $-80°C$. This time point was chosen to make the results comparable to earlier studies of gene expression upon infection by *C. bombi* in this system (Barribeau et al., 2014). Later, from these samples, we simultaneously extracted RNA and DNA from whole abdomen (SI Section 1.C). The host gene expression response to the microbiota transplant was measured in triplicates by quantitative PCR for 31 candidate genes belonging to six functional gene classes, and normalized against the most stable combination of housekeeping genes (Hellemans et al., 2007; SI Section 1.D, SI Table S1.1). In order to assess the microbial community present at the time the variable regions V3–V4 of the 16S rRNA gene were amplified using the region-specific universal primers (314F, 806R, SI Table S1.5) and subjected to sequencing on the Illumina MiSeq® platform (SI Section 1.F). Amplicons passing quality control were, merged, chimeras were eliminated and operational taxonomic units (OTUs) were generated based on 97% sequence identity (SI Section 1.G). Statistical analyses of microbiota community composition were performed either on non-rarefied OTU count data (McMurdie and Holmes, 2014) or for comparability on the count data rarefied to the smallest library size, the results of the latter are reported in SI Section 1.H. For both, the analysis of the recipient immune response to the faecal microbiota transplants (SI Section 1.E) and the analysis of the microbiota community structure (SI Section 1.H) we tested for a recipient resistance phenotype and donor resistance phenotype effect, as well as

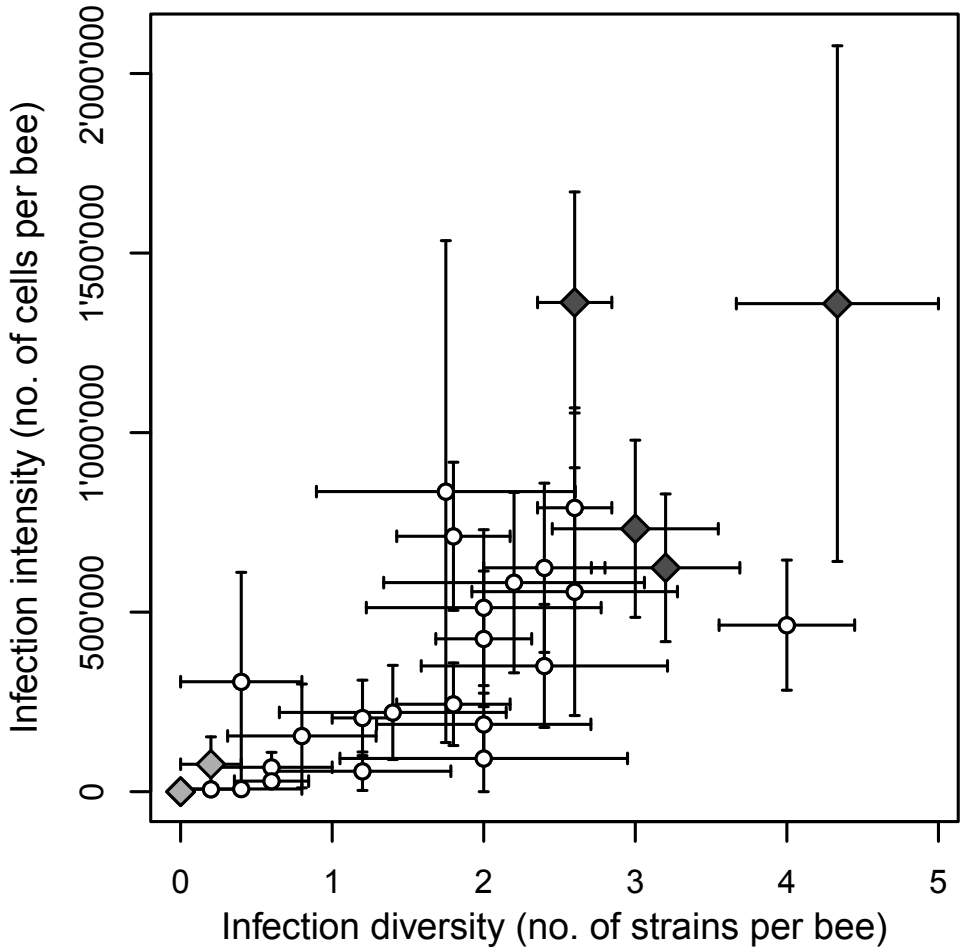

Figure 1.1: Variation in resistance among colonies. The graph plots colony mean infection intensity against colony mean infection diversity 7 d post infection (Spearman's $\rho = 0.74, p < 0.001, n = 30$, bars represent SEM). Diamonds highlight colonies used in the microbiota transplant experiment (light grey = "resistant" phenotype, dark grey = "susceptible" phenotype; two resistant colonies overlay each other at zero). Note that infection intensity represents total number of parasite cells present in a worker as assessed by quantitative PCR.

their interaction.

Results

Colony resistance profiles

To assess the resistance profile for each colony, we exploited the natural variation in the infection outcome, that is, when the workers were exposed to a "cocktail" infection that initially contained five genetically distinct strains of *C. bombi* at equal amounts. As Figure 1.1 shows, we found variation and a positive association between infection intensity (number of parasite cells) and infection diversity (number of parasite strains) that had established 7 d post infection. Hence, infection intensity builds up as more strains are able to establish. We therefore defined "susceptible" and "resistant" to be associated with higher and lower infection intensity and higher and lower infection diversity, respectively.

For the second part of this study, we then selected, out of this pool, four "susceptible" and three "resistant" colonies from the extreme ends of the distribution (Figure 1.1). Workers from the selected colonies of the "susceptible" type showed a mean infection intensity of 981'554 cells per worker (SE = 172'986, n = 18) and an infection diversity of 3.17 strains per worker (SE = 0.26, n = 18). Colonies of the resistant group showed virtually no infection, with only one worker (from colony 13.094) infected with an estimated 381'846 cells of one parasite strain (strain tag: 08.192).

Gene expression upon faecal microbiota transplant

To measure the response of workers upon receiving a microbiota transplant, we analysed the expression of a set of candidate genes, representing six immunologically relevant functional gene classes (SI Table S1.1). We performed MANOVA analyses separately for each functional gene class to test for both differential effects of donor and recipient phenotypes (resistant vs. susceptible) and their interaction effects on the gene expression response. Minimal adequate models are reported in Table 1.1 (see SI Section 1.E). Contrary to expectation, neither one of the gene classes showed a statistical interaction between recipient phenotype and donor phenotype (Table 1.1).

However, we found that all gene classes, except for the recognition genes, were significantly affected by either donor phenotype (the microbiota received) or by recipient phenotype (the resistance background of the recipient). In particular, the donor phenotype elicited differential expression responses in four out of six gene classes (signalling, reactive oxygen species (ROS), metabolism, melanisation), as well as having a weak influence on the expression pattern of the effector class. By contrast, the recipient phenotype only influenced expression in the effector and melanisation classes, with some evidence for an effect in the recognition class. Only the melanisation class showed an effect of both donor and recipient phenotype (but not their interaction; Table 1.1).

Table 1.1: MANOVA results for all gene classes and summary of linear discriminant analysis (LDA).

Functional gene class	Factor[1]	Df, Residuals	Pillai	Approx F	Num Df, Den Df	P-value	LDA accuracy[2]	Two genes with highest coefficient[2]
Recognition	Recipient phenotype	1,28	0.419	2.266	7,22	0.068	65.52%	*BGRP2, PGRP-LC*
Signaling	Donor phenotype	1,27	0.438	4.683	4,24	0.017	68.97%	*relish, hopscotch*
Effectors	Recipient phenotype	1,28	0.726	6.609	8,20	0.0003	79.31%	*TEPA, defensin*
	Donor phenotype	1,28	0.461	2.141	8,20	0.080	60.00%	*TEPA, ferritin*
Metabolism	Donor phenotype	1,29	0.346	4.750	3,27	0.009	61.29%	*vitellogenin, apolipophorin.III*
ROS	Donor phenotype	1,28	0.377	8.175	2,25	0.003	76.67%	*peroxiredoxin5, jafrac*
Melanisation	Donor phenotype	1,27	0.366	3.315	4,23	0.028	79.31%	*catsup, serpin27a*
	Recipient phenotype	1,27	0.390	3.674	4,23	0.019	59.26%	*serpin27a, PPO*

[1]Statistics of the minimal model (i.e. retaining only independent variables with a p-value $\leqslant 0.1$) for the MANOVA results are reported.

[2]Summary values for LDA classification function are given (i.e. accuracy), as well as the two genes contributing most to the discriminant function.

See SI Tables S3–S5 for detailed LDA report and MANOVA results with outliers excluded.

To tease apart the MANOVA results, we performed linear discriminant analysis to identify the gene classes that best discriminate between the two resistance phenotypes of donors and recipients, respectively, as well as to deduce genes contributing most to group separation (Table 1.1). Depending on gene class, leave-one-out (jack-knifed) cross-validation resulted in correct classification of donor phenotype in 61–79% of the cases and recipient phenotype in 59–79% of the cases. This is higher than expected by chance (50%) for all cases (SI Table S1.2).

Figure 1.2 shows the univariate responses of gene expression within each class as the fold-change relative to the resistant phenotype for both the recipient (Figure 1.2A) and the donor (Figure 1.2B) effects. Genes that were significantly differentially expressed (with asterisks in Figure 1.2) were not necessarily those genes that contributed most to the phenotype separation (Table 1.1) and thus had the highest impact on the discriminant function. This discrepancy is most pronounced for the effector gene class, where the antimicrobial peptide *apidaecin* is differentially expressed between the recipient phenotypes, but the gene's contribution to group separation measured by the partial coefficient is relatively small (coef = -1.30; for all coefficients see SI Table S1.3). This is not nearly as influential for separation as the genes not differentially expressed, such as the effector gene of the JAK/STAT pathway *TEPA* (coef = 99.88). Also, the signalling gene of the JNK pathway, *basket* (coef = -14.78), is differentially expressed, but *relish* (coef = 471.9), of the Imd pathway, which contributes more to the resistance phenotype separation, is not differentially expressed. Interestingly, a permutation test for homogeneity of multivariate variance revealed that the gene expression response to a susceptible donor transplant was less variable in several gene classes compared to the resistant transplant phenotype (recognition: $F_{1,29} = 8.19$, $p = 0.004$; metabolism: $F_{1,29} = 11.71$, $p = 0.003$; ROS: $F_{1,29} = 8.40$, $p = 0.002$). Similar, the gene expression response was less variable in susceptible recipients compared to the response of the resistant recipients. However, this is only true for the gene class of ROS ($F_{1,29} = 14.19$, $p = 0.001$).

Microbiota community composition

In order to link gene expression patterns with features of the gut microbiota community composition, we assessed the microbial community structure present at the time of the gene expression measurement. Overall, after the generated 16S amplicon sequences were processed and quality controlled (see SI Section 1.G), the microbiota composition was described by 159 OTUs (operational taxonomic units). We defined "ecologically" abundant OTUs as OTUs that occur in at least 85% of all samples within a data set. This resulted in 19 common OTUs (out of 159) still representing 99.2% of the total number of reads. Rarefaction analysis revealed that achieved sequencing depth adequately reflected microbiota complexity (SI Figure S1.2). As sequencing depth among individual samples differed, we present the results of the statistical analysis from the non-rarefied datasets, where we use methods that statistically account for unequal sequencing

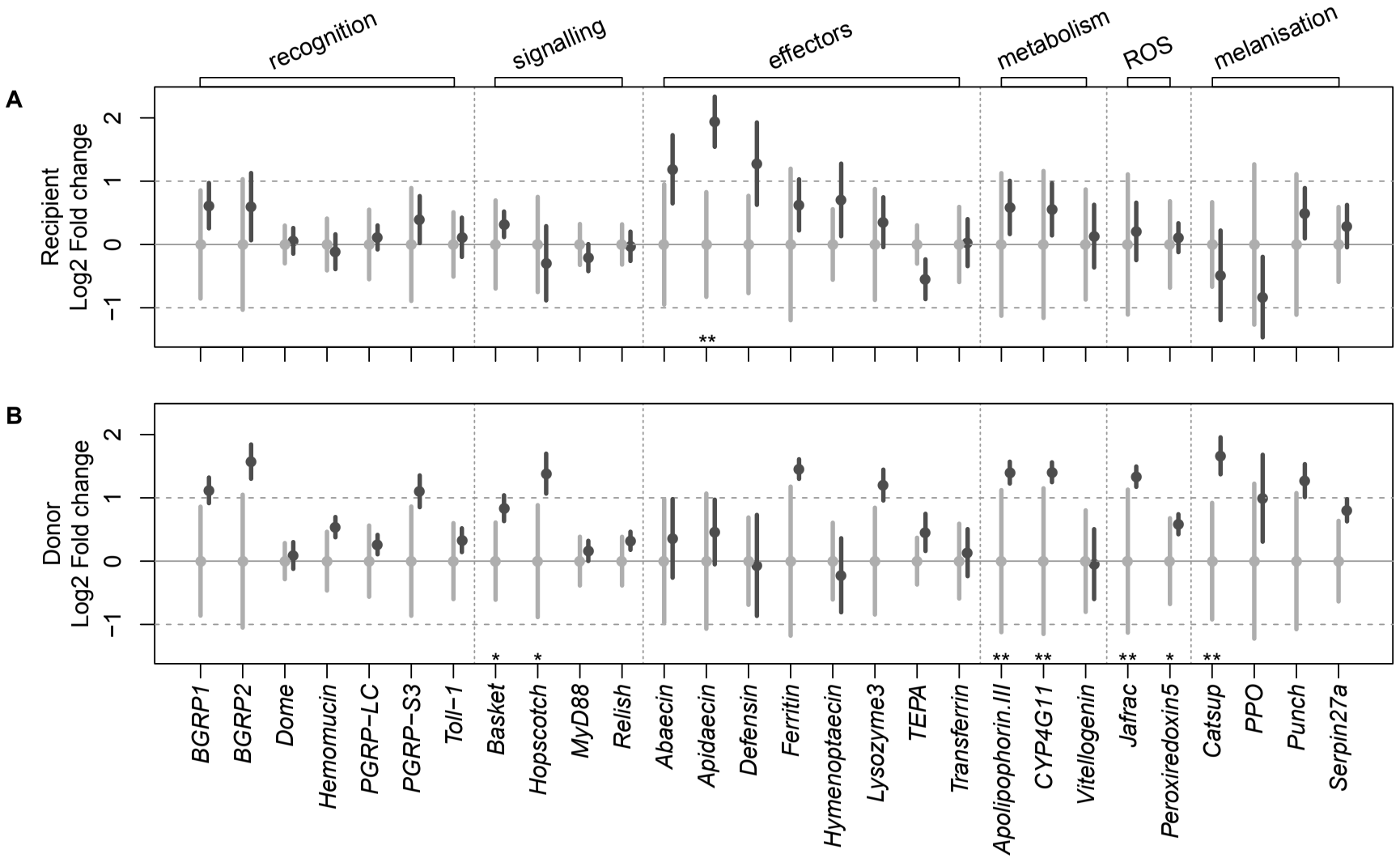

Figure 1.2: Gene expression in different phenotypes. Shown are gene expression fold changes of the susceptible phenotype (dark grey) relative to the resistant phenotype (light grey) for (A) recipients and (B) donors upon microbiota transplant. Plotted are the group means of the $\log_2(-\text{ddCt values})$ with 95% CI including error propagation after Hellemans et al. (2007) (SI Section 1.E). A $\log_2$-fold change of zero equals no change in expression between resistance phenotypes; a $\log_2$-fold change of one translates to a two-fold absolute difference. Asterisks mark individual genes significantly differentially expressed between phenotypes after correction for multiple testing (* $p < 0.05$; ** $p < 0.01$).

depth among samples (McMurdie and Holmes, 2014). Alternatively, random subsampling of the OTU table of each sample to the lowest sequencing depth is common practice. The repeated ($n = 100$) rarefaction to the smallest sequencing depth ($n = 50?792$) retained, on average, 134.6 OTUs (SD = 3.3). Applying the same OTU classification criteria to these rarefied data sets, we identified on average in 12.1 ecologically common OTUs (SD = 1.1, $n = 100$). The report of further statistical analysis of the rarefied data can be found in the SI Fig. S1.3.

We investigated whether differences in microbiota community structure could be attributed to the resistance phenotype (resistant vs. susceptible) of the donor and/or the recipient, or to the interaction of the two. First, we looked at the total proportion of reads from ecologically common OTUs in samples and found that the proportion of common reads was only affected by donor resistance phenotype (glm: $F_{1,29} = 12.44$, $p = 0.001$), where microbial communities of the susceptible phenotype contained on average 1.8% (SE = 0.6%) more common reads compared to the resistant donor phenotype. Second, we tested ecologically common OTUs for differential abundances between donor and recipient phenotypes. Figure 1.3 shows the $\log_2$-fold change in abundance of OTUs relative to the abundance in the resistant recipient phenotype (Figure 1.3A) and to the resistant donor phenotype (Figure 1.3B), respectively. Donor resistance phenotypes differed significantly from zero in the $\log_2$-fold changes of OTU abundance (Wald test: OTU_6: $\chi^2_1 = -3.78$, $p < 0.001$; OTU_9: $\chi^2_1 = -5.41$, $p < 0.001$; OTU_44: $\chi^2_1 = -2.66$, $p = 0.008$; Figure 1.3B with asterisk). OTU_6 belongs to the Class of Gammaproteobacteria, OTU_9 is ascribed to the Order *Rhizobiales* of the Class Alphaproteobacteria, and OTU_44 was classified to the *Lactobacillus* genus (see SI Table S1.6 for taxonomy of ecologically common OTUs). We detected neither a difference in OTU abundance among the recipient phenotypes (Figure 1.3A), nor any statistical interaction between recipient and donor phenotypes.

Surprisingly, despite the absence of differentially abundant OTUs between recipient resistance phenotypes, leave-one-out (jack-knifed) cross-validation of the linear discriminant analysis of the common OTUs performed on average equally well for both donor and recipient phenotype discrimination (74.19% correctly classified compared to 50% expected by chance, $n = 31$). Table 1.2 lists the top six OTUs that contributed most to the separation of donor and recipient resistance phenotypes. It indicates, similarly to the results from the gene expression analysis, that differential abundance is not predictive of an OTU's relative contribution to the discriminant function.

Discussion

The specificity of host-parasite interactions has been shown to be mediated by the presence of symbionts, or by the entire host-associated bacterial community, i.e. the microbiota (Koch and Schmid-Hempel, 2012; Oliver, Moran, and Hunter, 2005; Rouchet and Vorburger, 2012). As such, the establishment of a community that is beneficial to the host becomes an important element of host

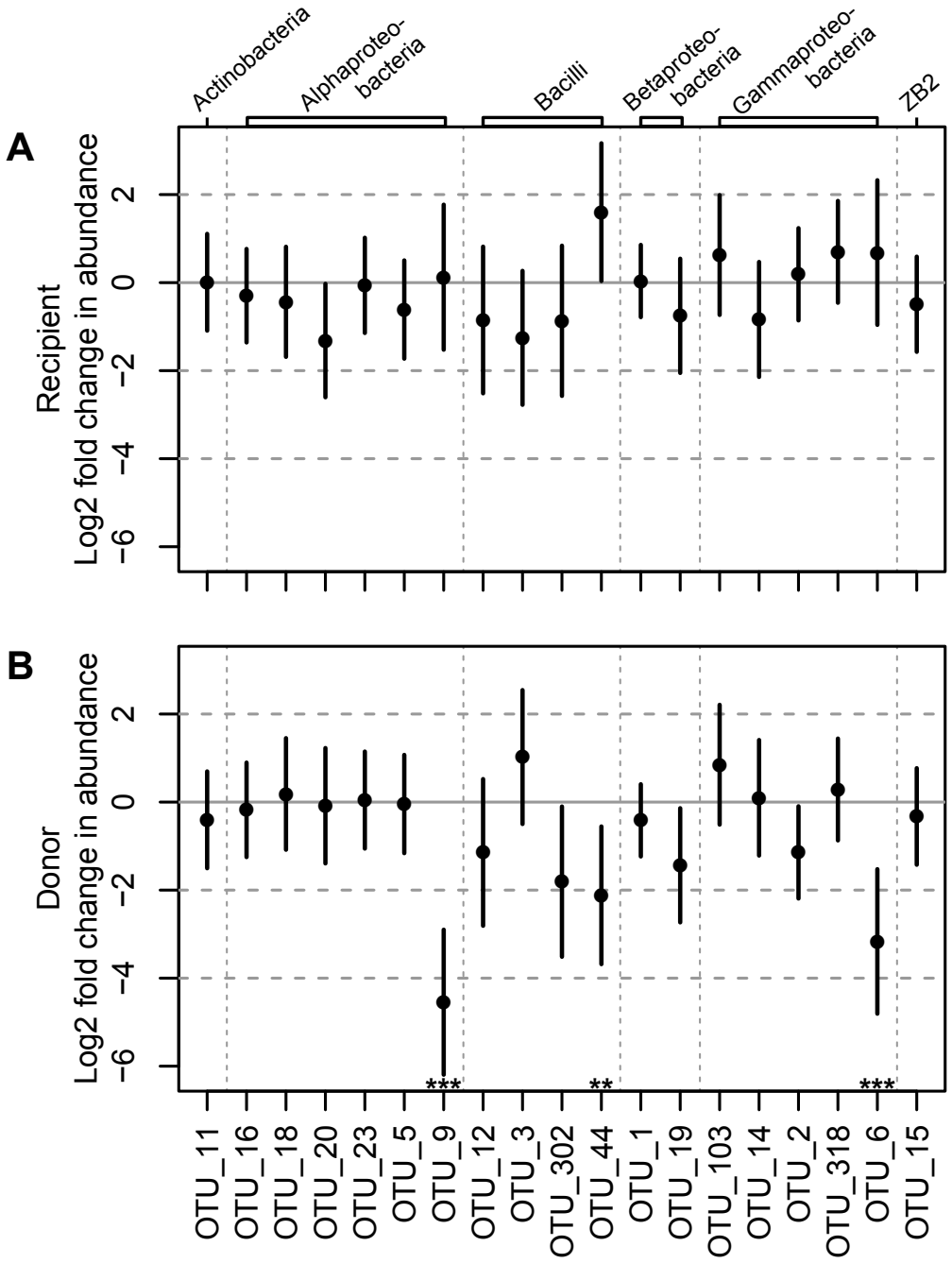

Figure 1.3: Abundance fold change in microbiota "species" (OTU) composition. $\log_2$-fold changes in abundance are shown for ecologically common OTUs of the resistant phenotype (black) relative to (A) the susceptible recipient phenotype, and (B) the susceptible donor phenotype. Plotted are mean "moderated"-fold changes (Love, Huber, and Anders, 2014) for each OTU with 95% CI. Asterisks indicate significant abundance fold changes after accounting for multiple testing (** $p < 0.01$; *** $p < 0.001$). OTUs with a $\log_2$-fold change value >0 are more abundant (<0 less abundant) in the resistant phenotype relative to the susceptible phenotype.

Table 1.2: Top six partial correlation coefficient of linear discriminant analysis of ecologically common OTUs for phenotype discrimination.

Recipient phenotypes		Donor phenotypes	
OTU	LD coefficient	OTU	LD coefficient
OTU_318	-2.04	OTU_5	-4.51
OTU_44	-1.39	OTU_16	2.31
OTU_11	-1.29	OTU_1	-1.40
OTU_2	-0.95	OTU_11	1.08
OTU_5	0.94	OTU_9	1.07
OTU_9	0.91	OTU_15	1.00

defence.

Screening host colonies for their resistance profile provided us with the prerequisite to separate both the effects of among-host background variation and the variation among the gut microbiota of different host backgrounds with respect to the outcome of the host-parasite interaction. On the one hand, the positive correlation between infection intensity and infection diversity (number of strains that established; Figure 1.1) confirmed previous studies (Schmid-Hempel et al., 1999; Ulrich, Sadd, and Schmid-Hempel, 2011). This pattern is suggestive of a situation where each parasite strain occupies its own niche within the host (May and Nowak, 1995). On the other hand, and for the purpose of our study, the resistance variation among host colonies enabled us to choose specific resistance phenotypes for the successive experiment, based on the natural infection outcome for a given host colony (Figure 1.1). Thus, recipient hosts are assumed to have their colony's (genetic) resistance background (i.e. the presumed selection potential for allowing a protective microbial community to establish). Donor transplants from colonies represent the naturally established microbial gut communities with the presumed protective ability (resistant vs. susceptible). The microbiota's phenotype would thus modulate the observed resistance (here immune response) of the receiving host — either independently or in combination with the pre-existing background resistance of the receiving host.

The early gene expression response upon faecal transplants (Figure 1.2, Table 1.1) suggests that host genetics as well as the transplanted microbiota affect the host immune response. Surprisingly, we did not detect a statistical interaction between donor and recipient phenotype, which suggests that the specificity observed for the infection outcome is not reflected in a specific host-microbiota interaction as tentatively suggested before (Koch and Schmid-Hempel, 2012). We also found differential gene expression patterns among susceptible and resistant colony phenotypes, comparable to recent studies in *Drosophila* (Broderick, Buchon, and Lemaitre, 2014; Sleiman et al., 2015).

The recipient effect on the immune response may indicate inherent differences between the two genetic host resistance backgrounds in their ability to impose selection on the establishing community. In particular, effector and

melanisation gene classes emerged as potential candidates to mediate such selection. Interestingly, within the effector gene class, the anti-microbial peptides (AMPs) showed a strong signal of differential expression. This highlights a surprising similarity to the differential response in AMPs observed upon infection by a parasite, where more susceptible colonies show a stronger up-regulation of AMPs as compared to more resistant colonies (Barribeau et al., 2014). In our experiment, recipients from a susceptible resistance background also up-regulate AMPs compared to recipients from a resistant background. AMPs have previously been suggested to play an important role in shaping host-associated communities in various other organisms (Franzenburg et al., 2013; Salzman et al., 2009). Yet, in *Drosophila* at least, it remains unclear how host genotype eventually maps to host resistance phenotype (Lazzaro, Sceurman, and Clark, 2004; Sleiman et al., 2015).

In turn, we found that the microbiota phenotypes differed in their ability to modulate the receiving host's immune response. Donor microbiota from susceptible colonies elicited a stronger gene expression response in their recipients compared to resistant microbiota, supporting our simple hypothesis for best assembly. Indeed, differential responses could potentially result in a differential effect on community assembly — an effect that remains to be tested in this system. Such effects have previously been observed in *Lepidoptera*, where immune suppression by the baculovirus in larvae resulted in a less stringent regulation of the microbiota and thus an increased gut microbial load (Jakubowska, Vogel, and Herrero, 2013). We also found that signalling and ROS gene classes discriminated best between the two donor phenotypes. Within the signalling gene class, *relish* contributed most to the linear discriminant function in our analysis. *Relish* is a key factor in the induction of the humoral immune response, such as the production of AMPs. *Relish* has previously been shown to be of great importance in the *Drosophila* host-microbiota association, where $Relish^{E20}$-deficient flies show altered gut microbiota composition and gene expression patterns, especially genes of the signalling pathways are thereby disproportionally affected (Broderick, Buchon, and Lemaitre, 2014). While the finding of a more variable gene expression response to a susceptible microbiota transplant is intriguing, the causes and consequences remain elusive. Overall, the independent influence of donor and recipient phenotypes should result in complex feedbacks between the host and the microbiota during the process of community establishment. The particular effects of these feedbacks on the infection outcome would need to be assessed in further studies.

Based on the gene expression result we expected to find the microbial community to be affected by both recipient and donor phenotypes. We checked for both main effects of donor and recipient phenotypes and their interaction on microbiota community structure, analogous to the gene expression response. In the univariate analysis, by looking for differentially abundant OTUs, we detected only significant differentially abundant OTUs between donor phenotypes (Figure 1.3). Although not directly assessed before the experimental microbiota transplant was administered, our results clearly indicated differences in the mi-

crobiota community structure among resistant and susceptible donors that persisted at least until 18 h after the transplant. However, in the multivariate analysis, by leave-one-out cross-validation of the linear discriminant function, both recipient and donor phenotypes performed equally well. The absence of any differentially abundant OTUs between recipient phenotypes might suggest that at our chosen time of community assessment eighteen hours after the transplant — an interval chosen for compatibility with earlier studies (Barribeau et al., 2014) — is probably too early for any recipient effect to strongly manifest itself. A study in honey bees (*Apis mellifera*) showed that bacterial abundance in newly emerged workers increases until an age of nine days before it remains more or less stable (Martinson, Moy, and Moran, 2012). Thus, differential selection pressure of recipient phenotype via gene expression variation on the microbiota community structure would most likely only become more strongly visible at a later time point.

Despite this, the differentially abundant OTUs between resistant and susceptible donor phenotypes indicated three candidate species that might predominantly be involved in mediating the protective function of the microbiota. The three OTUs belong to three major taxonomic orders typically associated with honey bee and bumblebee gut microbiotas (Koch and Schmid-Hempel, 2011a; Martinson et al., 2011). In particular, OTU_9 matches the recently described *Bartonella apis* species isolated from the honey bee gut (Kešnerová, Moritz, and Engel, 2016). While this Alphaprotebacteria occurs in honey bees, the presence has rarely been reported in bumblebees (Cariveau et al., 2014; Lim et al., 2015; Martinson et al., 2011). This could either reflect sensitivity differences in the detection methods used or potential non-exclusivity to the gut, because we analyzed 16S sequences from whole abdomen. However, Cariveau et al. (2014) report potential relationships between certain Alphaproteobacteria species in *B. bimaculatus* and *Crithidia* infections. There is some evidence for Gammaproteobacteria to be an indicator species for a negative infection status (Cariveau et al., 2014). Associations between *Lactobacillaceae* and *Crithidia* infection have, to our knowledge, so far not been reported.

The differentially abundant OTUs could also be viewed in the light of within-host competition and multiple infections (Read and Taylor, 2001; Tognazzo, Schmid-Hempel, and Schmid-Hempel, 2012). In this context, several possible modes of interaction between the microbiota and the parasite need to be considered, such as exploitation (passive, through resource limitation), interference (direct attack or mechanical/chemical exclusion) or apparent competition (mediated through differential sensitivity to elicited immune response). Here, we cannot distinguish between these modes of interactions, but the differentially abundant OTUs are potential candidate species for such interactions. *Crithidia* likely attaches to the gut wall, as does the microbiota (Martinson, Moy, and Moran, 2012), and therefore it is likely that direct interactions between *Crithidia* and OTUs occur, and that specifically, differentially abundant OTUs are involved in the dense colonization of the hindgut epithelium as observed in the honey bees (Martinson, Moy, and Moran, 2012).

In short, this study highlights both host resistance background effects on the immune response to microbiota transplants, as well as the potential capacity of the received microbiota to modulate the host immune response. This certainly emphasizes the role of microbiota in the context of host-parasite interactions (Cirimotich et al., 2011; Koch and Schmid-Hempel, 2012). We furthermore provide evidence for potential complex feedbacks between the host's immune response and the establishing microbial community, which in turn affects a possible future parasite encounter, either through mechanism of within-host competitions (Read and Taylor, 2001) or in the light of immune priming and immune maturation (Chambers and Schneider, 2012).

Supplementary Information

1.A Colony resistance profile

We set up laboratory colonies with field caught *Bombus terrestris* queens from Neunforn, Switzerland in spring 2013. The resistance profile of thirty parasite-free colonies was determined by administering a "cocktail" infection to five workers per colony. The cocktail contained 2'000 parasite cells each of five genetically distinct *Crithida bomi* strains (project tags: 08.068, 08.075, 08.091, 08.161, 08.192) in 10 μl of 50% sugar water and was presented to the worker after a starvation period of 2 h. Seven-days post infection (Korner and Schmid-Hempel, 2004; Schmid-Hempel and Schmid-Hempel, 1993) bees were frozen and DNA was extracted from dissected guts with Qiagen DNeasy Blood & Tissue Kit following the manufacturer's protocol.

Infection intensity was assessed by quantitative real-time PCR (see (Ulrich, Sadd, and Schmid-Hempel, 2011) for primers and cycling protocol). The total number of parasite cells in a sample was determined by absolute quantification using the standard curve method. For this, we extracted DNA from a known number of *C. bombi* cells to generate the standard curves. Each biological sample was run in triplicates in a total reaction volume of 10 μl containing: 0.2 μl [10 μM] of each forward and reverse primer, 2 μl of 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne) and 1 μl DNA template.

Infection diversity was determined by genotyping the samples for three *C. bombi* microsatellite markers Cri4G9, Cri4 and Cri2F10 (Schmid-Hempel and Reber Funk, 2004). Microsatellite were amplified in one multiplexed reaction in a total reaction volume of 10 μl containing: 2 μl of 5x Colorless GoTaq® Reaction Buffer (Promega), 0.5 μl of dNTPs [2.5 mM each], 0.1 μl (0.15 μl Cri4G9) [10 μM] of each forward and reverse primer, 0.05 μl GoTaq DNA polymerase (Promega, 5 U/μl) and 2 μl DNA template. A total of 40 PCR cycles were performed with the following steps: denaturation (94°C, 30 s), annealing (48°C, 30 s) and extension (72°C, 30 s). PCR products were run on a 3730xl DNA Analyzer (Applied Biosystems).

1.B Microbiota faecal transplant experiment

To transplant the microbiota, we followed the faecal transplant protocol by Koch and Schmid-Hempel (2011b) with a few modifications. Briefly, all materials

used in the context of the microbiota transplant experiment were either autoclaved or washed in 80% alcohol before use. In order to raise germ-free workers (referred to as recipients), we removed and surface sterilized brood from each of the selected colonies by submerging the brood in a 3% bleach solution for 90 s. Subsequently, the brood was placed, for each colony individually, in sterilized housing boxes.

Daily, newly emerged recipients were transferred into individual housing boxes and kept for 1–3 days before a faecal transplant was offered to them. Upon emergence, recipients were provided with *ad libitum* pollen (gamma-irradiated, LEONI Studer Hard AG, Switzerland, dose: 35.5 kGy (WHO, 1999), kept frozen at −20°C until use) and 50% sugar water (autoclaved).

Faeces used for the microbiota transplants were collected from workers (referred to as donors), which emerged and remained in their source colonies (i.e. naturally acquired their microbiota). A single transplant consisted of faeces collected from at least three source colony workers; faeces collected from different colonies were never mixed. A microbiota transplant consisted of 5 μl faeces mixed with 10 μl 50% sugar water and was offered to a recipient after a 30 min starvation period. Once recipients were observed to have fed on the inoculum, they were put back in their housing boxed and kept for 18 h before being snap frozen in liquid nitrogen and stored at −80°C. Any recipient bee that did not feed or only partially fed on the inoculum was excluded from the experiment.

1.C RNA and DNA extraction

RNA and DNA were simultaneously extracted from whole abdomen. For this, we disrupted the abdomen with 0.5 g 1.4 mm Zirconium Oxide beads in 1 ml peqGold TriFast^{TM} reagent (peQlab) at room temperature (Omni Bead Ruptor 24 Homogenizer). After a 10 min centrifuging step (12′ 000 g, at 4 °C) 450 μl of the supernatant was transferred to a new tube containing 50 μl BCP (1-Bromo-3-chloropropane), mixed well, incubated for 10 min at room temperature and then centrifuged for 10 min (12 000 g, at 4 °C). The result was a phase separation into three phases, an aqueous phase, an interphase and a phenol phase. For RNA extraction, 180 μl of the upper aqueous phase was transferred into a RNAse-free tube containing 225 μl Isopropanol, vortexed for $5 - 10$ s, incubated for 10 min at room temperature and centrifuged for 10 min (17′ 785 g, 4 °C). The supernatant was carefully discarded and 300 μl 75% EtOH (prepared with DEPC treated H_2O) was added, vortexed and centrifuged for 5 min (17′ 785 g). To improve RNA quality, this washing step was repeated. Finally, the supernatant was removed and the pellet was allowed to air dry for $3 - 5$ min before RNA was resolved in 50 μl DEPC treated H_2O and stored at −80 °C. Samples were extracted in four randomly selected extraction batches.

For DNA extraction 100 μl of the phenol phase was removed and discarded before 300 μl 100% EtOH was added to the sample, vortexed, incubated at room temperature for 5 min and centrifuged for 15 min (17′ 785 g, 4 °C) to precipitate DNA. The supernatant was discarded, and the DNA pellet was washed in 200 μl

100% EtOH and centrifuged for 5 min (17′ 785 g, 4 °C) before the supernatant was carefully discarded. The pellet was resuspended in 50 μl H_2O and incubated for 1 h at 56 °C. Following the incubation period, 150 μl PBS were added before DNA was extracted with Qiagen DNeasy Blood & Tissue Kit following manufacturer's protocol, however the final elution volume was reduced to 70 μl.

1.D Gene expression protocol

DNA quality and purity was checked for 12 randomly selected samples on a 2100 Bioanalyzer (Agilent Technologies) with the RNA 600 Nano Kit and all samples were quantified and normalized based on Nanodrop 8000 (ThermoScientific) measurements. Even though no genomic DNA contamination was indicated, we used the Turbo DNA-free kit (Ambion) to purify 1.52 μg RNA before reverse transcribing 0.5 μg RNA using the QuatiTecR Reverse Transcription Kit (Qiagen). Manufactures protocol (Advanced Development Protocol 14, PN 100-1208B) was followed to measure gene expression with a Fluidigm 96.96 dynamic array IFCs (BioMark) using EvaGreen DNA Binding Dye (Biotium) as a reporter.

Each biological sample was run in triplicates for 31 genes (SI Table S1.1). Samples with a Ct standard deviation >0.2 were checked for quantification outliers among the technical replicates. The technical replicate with the largest deviation to the mean was excluded given three replicates (i.e. no failed quantification) otherwise all measurements were retained.

The most stable combination of housekeeping genes was identified by the geNorm algorithm within qbase+ (Biogazelle). Thus, we excluded the housekeeping gene PLA2 (M = 0.909, CV = 0.284) and used the arithmetic mean of the measured Ct values for the genes ef1α (M = 0.556, CV = 0.184) and RPL13 (M = 0.556, CV = 0.204) as the normalization factor within samples (Hellemans et al., 2007). Measurements from transplants of the same donor-recipient colony combinations were averaged (SI Figure S1.1B). Every recipient colony received transplants from both donor phenotypes; as well as every donor colony was transplanted into both recipient phenotypes (SI Figure S1.1A).

1.E Statistical analyses of gene expression data

All statistical analyses for gene expression data were performed on dCt-values. Only for illustration purposes do we show fold changes values of gene expression relative to the resistant phenotype for both recipient and donor effects (ddCt). We applied MANOVAs to test for effects of recipients or donor resistance phenotype (SI Figure S1.1A) on the gene expression response of the functional gene groups. The dCt-values within each gene class were subjected to Yeo-Johnson power transformation to achieve multivariate normality. Backwards model selection was performed for each gene class on the initial full model: donor phenotype (resistant, susceptible) * recipient phenotype (resistant, susceptible) retain-

Table S1.1: Genes and primers used for the quantification of the expression response.

Functional gene class	Gene	Putative gene function (pathway)	NCBI accession	Forward primer	Reverse primer	Product length	Primer ref[1]
Recognition	BGRP1	Recognition receptor (Toll)	XM_003397996	AACGTGGAAGTCAAAGATGG	GCGAACGATGACTTGGTATT	206	[1]
	BGRP2	Recognition receptor (Toll)	XM_003394713	TAACTCCCTTTGGAAACACG	GGCGGTAAAATACTGAACGA	249	[1]
	Dome	Recognition receptor (JAk/STAT)	XM_012310386	AAAGCCGTTCACTCTAAGCA	GACTTGCGAAAGAAGAAACG	116	
	Hemomucin	Surface glycoprotein, potential recognition receptor	XR_131963	AGCATTCCCAGATTTAGCACT	TAACAGTTGATTTCGGAGGTA	173	[2]
	PGRP-LC	Recognition receptor (Imd)	XM_003396463	CAGCCACCTACGACAGATTT	GTACATTCCGCTTGTGTCCT	101	[1]
	PGRP-S3	Recognition receptor (Toll)	XM_003401893	CGTGAAGGAGCTCATACCAT	CCAGGACTCATAGTGGCTGT	200	[1]
	Toll-1	Recognintion receptor (Toll)	XM_012307988	CGAATGGAGTTTAGAGCAGC	ATTTATCCCAGAACCAAGGG	172	
Signaling	Basket	Signal molecule (JNK)	XM_003402794	GGAACAAGATAATCGAGCAACTG	CTGGCTTTCAATCGGTTGTG	177	[1]
	Hopscotch	Signal molecule (JAK/STAT)	XM_003401903	CACAGACTGAAGCAGGTTGA	CATATGGGTAATTTGGTGCC	353	[1]
	MyD88	Signal molecule (Toll)	XM_003394153	GCATTAGGCATTGACAAACGAC	CAGAAGTCATACAAACCCACTCTG	125	
	Relish	Signal molecule (Imd)	XM_003399472	CAGCAGTAAAAATCCCCGAC	CAGCACGAATAAGTGAACATA	156	[2]
Effectors	Abaecin	Antimicrobial peptide	XM_003394653	GCCACAATATGTGGAATCCT	ATGACCAGGGTTTGGTAATG	141	[1]
	Apidaecin	Antimicrobial peptide	XM_003402966	CCCGACTAATGTACCTGCCA	GAAGGTGCGAATGTGTTGGA	131	[1]
	Defensin	Antimicrobial peptide	XM_003395924	GTCTGCCTTTGTCGCAAGAC	GACATTAGTCGCGTCTTCTTCG	139	[1]
	Ferritin	Iron transportation	XM_003393332	AAAGAATTGGACGCAAATGG	CAGCGAACTGATGTCCAAGA	259	[1]
	Hymenoptaecin	Antimicrobial peptide	XR_132450	TTCATCGTACTGGCTCTCTTCTG	AGCCGTAGTATTCTTCCACAGC	85	[1]
	Lysozyme3	Bacteriolytic effector	XM_003394052	TATGGGCAAGAAGATTCGAC	GTGTACATCGTTCACGCATC	219	[1]
	TEPA	Effector molecule (JAK/STAT)	XM_003399699	GCGTTCTATGACCACCTGTT	TACAGGTTACTCCACAGCCC	212	[1]
	Transferrin	Iron-binding, antibacterial	XM_003401163	CAATTTCTTCACCGCATCCT	CCTCGTTATTTGGCTTGCAT	131	[1]
Metabolism	Apolipophorin.III	Lipid transport	XM_003402572	ATCAGGCTCAAACGAACATC	TTCGTTCACTTGTTGCTGAG	269	[3]
	CYP4G11	Stress response	XM_003399563	GAATGCGCAAAGAAGGTAGC	CGCTTTCCGCTCTTGTAATC	313	[3]
	Vitellogenin	Metabolic, endorinological	XM_003402655, XM_003402656	GTGACAAGCGAAGAGACTATTATG	CCGTGTTATCTGGCGTGAC	154	[4]
ROS	Jafrac	Peroxiredoxin, ROS regulation	XM_003401245	CTCACTTCAGTCACTTGGCA	GCCAGCAGGACATACTTCTC	290	[1]
	Peroxiredoxin5	Peroxiredoxin, ROS regulation	XM_003394777	TCACACCAGGATGTTCCAAGAC	TTCTGCTCCGTGTTCTTTACCC	146	[1]
Melanisation	Catsup	Enzyme, melanin synthesis	XM_003398173	TTACCATGACGAGTCACCAA	ATGAGGAACCAAAGCATGAG	355	[1]
	PPO	Prophenoloxidase, melanin synthesis	HM142999	AGCGGCATAATACGTTGTGT	CCGAGGGATAGAAAGTCTCC	329	[3]
	Punch	Enzyme, melanin synthesis	XM_012320347	ATTGCCAGGACACTTTCAAC	TACAAGCTGGAAACGGAAAC	212	[1]
	Serpin27a	Serine protease inhibition (PPO)	XM_003392985	CCGATCATCCATTCGTATTC	ACCTGCACTTGATATCCCTG	164	[1]
Housekeeping	ef1α	Elongation factor 1 α	XM_003401944	GCTGGTGACTCGAAGAACAATC	GGGTGGTTCAACACAATAACCTG	74	[3]
	PLA2	Phospholipase A2	FN391388	TATCTTTCAATGCCCAGGAG	GTCGTAACAAATGTCATGCG	129	[5]
	RPL13	Ribosomal protein L13	FN391387	GGTTTAACCAGCCAGCTAGAAA	CTTCACAGGTCTTGGTGCAA	83	[5]

[1] Primers with no reference were designed with either Primer3 [6] or Quantprime [7] with the following specifics: primer length 20±2 bp; melting temperature 60±1°C with a maximum temperature difference between forward and reverse primer of 0.5°C. Primers were evaluated with an annealing temperature of 60°C and found to be specific with an amplification efficiency of 1.9–2.1.

[1] Brunner, Schmid-Hempel, and Barribeau (2014); [2] Schlüns et al. (2010); [3] Barribeau and Schmid-Hempel (2013); [4] Li et al. (2009); [5] Horňáková et al. (2010); [6] Rozen and Skaletsky (2000); [7] Arvidsson et al. (2007)

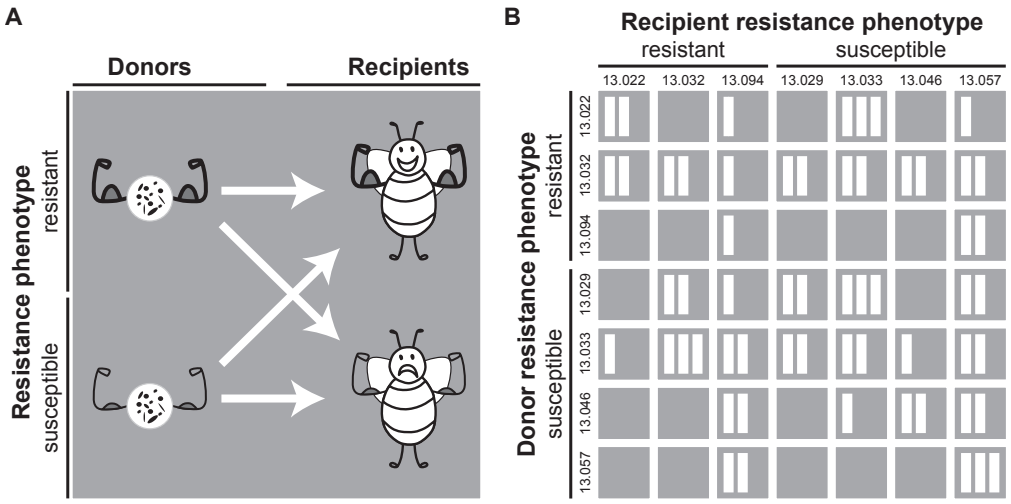

Figure S1.1: Schematic of the experimental design. Panel (A) visualizes all possible faecal transplant crosses of donor and recipient resistance phenotypes (arrows). Panel (B) shows all possible donor-recipient colony combinations as grey squares; each bar within a square represents a successful independent faecal microbiota transplant (n = 58) of a particular donor-recipient colony combination (n = 31). Axes are colony ID numbers.

ing only effects with a p-value < 0.1. We checked for outliers using Mahalanobis distances. However, results were robust to outlier exclusion and outliers were therefore not excluded for the reported results (see SI Table S1.4 for results with outliers excluded).

To further evaluate the MANOVA results, we used linear discriminant analysis. We identified genes that contributed most to the separation of resistance phenotypes in the multivariate setting based on partial correlation coefficients of the linear discriminant function. We used leave-one-out (jack-knifed) cross-validation of the linear discriminant analysis to assess the accuracy of the phenotype discrimination by the linear function within a gene class for either donor or recipient effects (SI Table S1.2,S1.3). If MANOVA results indicated a significant effect, we checked the univariate output of the MANOVA to investigate differential expression between phenotypes for each gene individually and we used the Benjamin and Hochberg method (Benjamini and Hochberg, 1995) to control the false discovery rate.

To test for a difference in gene expression variation among the resistant phenotypes of either recipient or donor effect we calculated Euclidean distances between samples, and used a permutation test on distances to the centroid to compare to the null hypothesis of equal variance among the resistant phenotypes (Oksanen et al., 2015).

1.F 16S amplicon library preparation and sequencing

We generated a multiplexed 16S amplicon paired-end library for sequencing on the MiSeq® Illumina platform in order to investigate microbial gut communities of recipients that received a microbiota transplant. We amplified the variable re-

Table S1.2: Percentage of correctly classified cases of the cross-validation of the linear discriminant function for each functional gene class.

		Recipient effect *Predicted phenotype*		Donor effect *Predicted phenotype*	
		resistant	susceptible	resistant	susceptible
Melanisation					
True phenotype	Resistant	54.55	45.45	81.82	18.18
	Susceptible	37.50	62.50	22.22	77.78
Proportion[1]		40.74	59.26	37.93	62.07
Effctors					
True phenotype	Resistant	81.82	18.18	58.33	41.67
	Susceptible	22.22	77.78	38.89	61.11
Proportion[1]		37.93	62.07	40.00	60.00
Recognition					
True phenotype	Resistant	63.64	36.36	66.67	33.33
	Susceptible	33.33	66.67	33.33	66.67
Proportion[1]		37.93	62.07	40.00	60.00
Metabolism					
True phenotype	Resistant	0	100	38.46	61.54
	Susceptible	72.22	27.78	22.22	77.78
Proportion[1]		41.94	58.06	41.94	58.06
ROS					
True phenotype	Resistant	27.27	72.73	83.33	16.67
	Susceptible	50.00	50.00	27.78	72.22
Proportion[1]		37.93	62.07	40.00	60.00
Signaling					
True phenotype	Resistant	36.36	63.64	63.64	36.36
	Susceptible	56.25	43.75	27.78	72.22
Proportion[1]		40.74	59.26	37.93	62.07

[1] Proportions gives percentage of phenotypes in the data set. Proportions vary for some gene classes due to gene expression failure for some samples and genes. However note, the prior classification probability for the cross-validation of the linear discriminant function was set to 0.5.

Table S1.3: Partial correlation coefficient of linear discriminant functions for either recipient or donor phenotypes.

	Recipient phenotype	Donor phenotype
Melanisation		
Catsup	4.130598	−5.824617
PPO	7.484474	1.427122
Punch	−2.967775	−1.082558
Serpin27a	−28.734568	2.721916
ROS		
Jafrac	−1.293329	−2.512490
Peroxiredoxin5	8.873714	5.542687
Metabolism		
Apolipophorin.III	−0.4304080	−0.3563731
CYP4G11	0.0173596	−0.1947528
Vitellogenin	0.3259856	0.7014135
Effectors		
Abaecin	−0.1818116	0.1281196
Ferritin	−0.3394093	−1.7669270
Apidaecin	−1.3002121	1.2680009
Defensin	0.2550820	−0.5152214
Hymenoptaecin	−0.5381095	−0.2040488
TEPA	99.8821058	32.3809248
Lysozyme3	−1.8080832	−0.8738062
Transferrin	0.8915005	1.4742006
Signalling		
Basket	−50.42431	−14.78089
MyD88	50.35166	−16.51957
Hopscotch	73.90705	−73.37020
Relisch	−196.73343	471.90052
Recognition		
PGRP-S3	−11.928997	20.5579004
BGRP1	−3.574761	−1.4058511
BGRP2	−16.837104	−12.6516326
Dome	−1.135818	6.5397240
PGRP-S3	6.236538	−1.6064176
Toll−1	−11.435283	1.4474364
Hemomucin	9.321592	0.7595999

Table S1.4: MANOVA results for all gene classes and summary of linear discriminant analysis (LDA) with outliers excluded.

Functional gene class	Factor[1]	Df, Residuals	Pillai	Approx F	Num Df, Den Df	p-value	LDA accuracy[2]	Two genes with highest coefficient
Recognition (–10 outliers)	Recipient phenotype	1,18	0.641	3.062	7,12	0.043	60.00 %	PGRP-LC, dome
Signalling (–6 outliers)	Donor phenotype	1,22	0.435	3.653	4,19	0.023	73.91%	Relish, hopscotch
Effectors (–10 outliers)	Recipient phenotype	1,16	0.786	4.584	8,10	0.014	90.91%	TEPA, defensin
Metabolism (–5 outliers)	Donor phenotype	1,24	0.261	2.583	3,22	0.079	69.23%	Vitellogenin, apolipophorin.III
ROS (–3 outliers)	Donor phenotype	1,25	0.334	6.019	2,24	0.008	74.07%	Peroxiredoxin5, jafrac
Melanisation (–6 outliers)	Recipient phenotype	1,22	0.471	4.007	4,18	0.017	95.65%	Serpin27a, PPO
	Donor phenotype	1,22	0.528	5.036	4,18	0.007	69.57%	Catusp, punch

[1] Statistics of the minimal model (i.e. retaining only independent variables with a p-value $\leqslant 0.1$) for the MANOVA results are reported.

[2] Summary values for LDA classification function are given (i.e. accuracy), as well as the two genes contributing most to the discriminant function.

Table S1.5: Amplicon Primer to generate V3-V4 16S template library.

Primer Name	Sequence 5′–3′
U341F_nex0	tcgtcggcagcgtcagatgtgtataagagacagCCTACGGGDGGCWGCA
U341F_nex1	tcgtcggcagcgtcagatgtgtataagagacag**N**CCTACGGGDGGCWGCA
U341F_nex2	tcgtcggcagcgtcagatgtgtataagagacag**NN**CCTACGGGDGGCWGCA
U341F_nex3	tcgtcggcagcgtcagatgtgtataagagacag**NNN**CCTACGGGDGGCWGCA
U806R_nex0	gtctcgtgggctcggagatgtgtataagagacagGGACTACHVGGGTMTCTATTC
U806R_nex1	gtctcgtgggctcggagatgtgtataagagacag**N**GGACTACHVGGGTMTCTAATC
U806R_nex2	gtctcgtgggctcggagatgtgtataagagacag**NN**GGACTACHVGGGTMTCTAATC
U806R_nex3	gtctcgtgggctcggagatgtgtataagagacag**NNN**GGACTACHVGGGTMTCTAATC

Lower case letters represent Illumina-specific overhang adapters, to which in a subsequent limited-cycle amplification step the sample specific indices and sequencing adapter attach. Frameshifting nucleotides are indicated in bold capital letters. The gene-specific sequences targeting 16S V3 and V4 region are shown in capital letters.

gion V3–V4 of the 16S rRNA gene with universal primers (Klindworth et al., 2013; Liu et al., 2011) (SI Table S1.5). Primers were designed after principles described in (Lundberg et al., 2013). We thus had three primer pairs containing frame-shifting nucleotides between the region-specific part and the Illumina overhang adapter in order to increase general sequence diversity in the generated 16S amplicon libraries (SI Table S1.5). We followed the manufacturer's suggested two-step amplification work flow.

Thus an initial PCR was set up for each sample and each primer pair as followed: 1.5 μl [10 μM] forward and reverse primer mix, 12.5 μl 2 x KAPA HiFi Hot Start Ready Mix and 2 μl DNA template in a total reaction volume of 25 μl. The cycling protocol used a 5 min initial degradation step at 95 °C, followed by 22 cycles of 98 °C for 20 s, 58 °C for 15 s and 72 °C for 15 s and finished with a final elongation step for 5 min at 72 °C. All four independent PCR reaction products were pooled and purified using Agencourt AMPure XP beads (Beckman Colter) with a ratio of 0.8:1 (beads to PCR product) and eluted in 30 μl [10 mM] TrisBuffer.

A second limited-cycle PCR was performed in order to attach indices and sequencing adapters to the template libraries. A total reaction volume of 50 μl contained 10 μl of the purified PCR product, 25 μl 2x KAPA HiFi Hot Start Ready Mix and 5 μl of each forward and reverse primers of the Nextera® XT Index Kit v2 Set A (Illumina). The indexing cycle protocol used a 3 min initial denaturation step at 95 °C followed by 10 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C and 5 min final extension step at 72 °C. The indexed amplicon libraries were purified using Agencourt AMPure XP beads (Beckman Colter) with a ration of 1:1 (beads to amplicon product) and eluted in 30 μl [10 nM] TrisBuffer.

Amplicon library quality and library fragment size were checked for 10 randomly selected samples on a Bioanalyzer (Aligent Technologies) DNA HS 1000 chip. All libraries were 1:10'000 diluted and quantified in duplicates by quantitative PCR using the 2x KAPA SYBER qPCR Ready Mix on an ABI 7500 Real-

time PCR System (Applied Biosystems). We used the Library Quant Illumina Kit (KAPA Biosystems) with quantification standards ranging from 0.002 pM to 20 pM for absolute quantification. Following quantification, equimolar amounts of all libraries were pooled. The final, multiplexed pool was quantified by qPCR as previously described. We followed the manufacturer's manual (Preparing Libraries for Sequencing on the MiSeq® #15039740 Rev. D) to prepare the library for paired-end sequencing. We loaded a final library concentration of 17.5 pM and a 15% PhiX spike-in with MiSeq® Reagent Kit v3.

1.G Amplicon processing and OTU clustering

A total of 18'122'556 paired-end raw reads were generated in a single 600-cycle MiSeq run. Qualities of reads were checked with FastGQ (v0.11.2). 96% (17'432'405) of the paired reads successfully merged using FLASH (v1.2.9) with the following specifications: minimum overlap of 15 bp, maximum overlap of 250 bp and maximum mismatch density of 0.25. Primer sequences were trimmed from the merged reads using cutadapt (v1.5) requiring a full-length error-free overlap but allowing wildcards.

Merged and primer trimmed reads were quality filtered with PRINSEQ-lite (v0.20.4) and 13'878'054 reads (76.6%) passed filtering, given a fragment range of 350–550 bp, GC range of $20 - 80$ and a minimum quality mean of 30, and allowing no ambiguous nucleotides.

OTU clustering was performed with the pooled merged and trimmed reads using UPARSE-OTU algorithm (usearch v7.0.1090_i86linux64) (Edgar, 2010, 2013). For this the dataset was sorted (sortbylength), de-replicated (derep_fulllength), abundance sorted (sortbysize; size = 2), and clustered (cluster_otus, minimum identity = 97%).

The clustering step detects and removes chimera sequences. In addition we also applied a reference based chimera removal step using the Green Genes database (version May 2013, http://greengenes.secondgenome.com). The UPARSE-OTU workflow resulted in 352 OTUs. 98.7% of the reads could be successfully mapped back to OTU reference centroid sequences (usearch_global; id = 97%). The OTUs were blasted (blastn (Altschul et al., 1990)) against the GreenGenes database (v13_5) (McDonald et al., 2011) to assign taxonomic information with a bit-score cut-off of equal or bigger 100. Additionally, we checked taxonomic assignment of the best hit using MEGABLAST search against the NCBI nucleotide collection database. The phylogenetic tree for the OTUs was built using PyNAST (Caporaso et al., 2010a) and FastTree (Price, Dehal, and Arkin, 2010) as implemented in Qiime (v1.7) (Caporaso et al., 2010b).

In total 178 OTUs were excluded because assigned taxonomy was either of non-bacterial origin, classified as mitochondria or chloroplast, unique to negative probes run in parallel through whole extraction and sequencing process, or unique to samples that do not belong to this data set, but were included in the previous data processing. Read counts for the same donor-recipient colony combinations were averaged (SI Figure S1.1B) and rounded, thus the final data

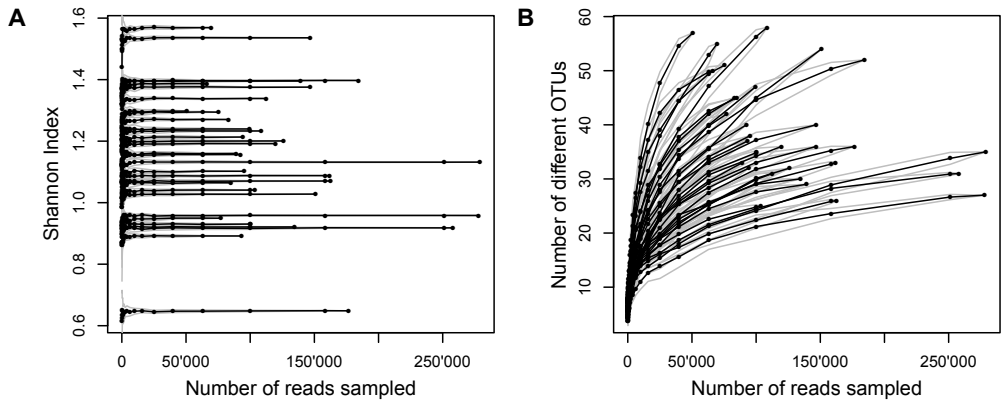

Figure S1.2: Rarefaction curves each sample. (A) shows the Shannon-Wiener diversity index, and (B) number of different OTUs (richness) at given sampling depth (x-axis). Lines connect means and SD (grey lines) of 20 independent draws at a given sampling depth for a given library.

set contained a total of 3'961'051 read counts mapping to 159 OTUs. The mean read count for the unique donor-recipient colony combinations was 127'776 (n = 31, SD = 58'551).

To show that achieved sequencing depth was adequate to reflect sample complexity we created rarefaction curves by randomly resampling the sequencing pool of each sample without replacement twenty times at different sampling depths. SI Figure S1.2A shows that the alpha diversity index (Shannon Index) of all samples reached the plateau well below the smallest library size (50'792 reads). SI Figure S1.2B plots sample richness (i.e. number of different OTUs discovered) at given subsampling depth and indicates that for most samples the majority of different OTUs were sampled.

1.H Statistical analyses of microbiota community composition

We performed statistical analyses on both the non-rarefied data set, as suggested by McMurdie and Holmes (2014) and on data sets rarefied to the smallest library size for comparison. Rarefaction to the smallest library size was repeated a 100 times in order to evaluate chance effects of the sampling process. For all datasets, we defined "ecologically" common and rare OTUs. Common OTUs were observed in at least 85% of the samples. We tested, similar to gene expression analysis, for effects of recipient and donor resistance phenotypes on the microbiota community structure.

To investigate potential effects of donor and/or recipient resistant phenotypes on the total proportion of reads defined as common OTUs, we fitted a generalized linear model (glm) with a quasibinomial error distribution using a logit link function to the count data. The model weights the proportions according to sample size, thus controlling for differences in sequencing depth. We performed backwards model selection (i.e. retaining only independent variables with a p-value $\leqslant 0.1$) on the full model: recipient phenotype (resistant, susceptible) * donor phenotype (resistant, susceptible), using F-tests to find the

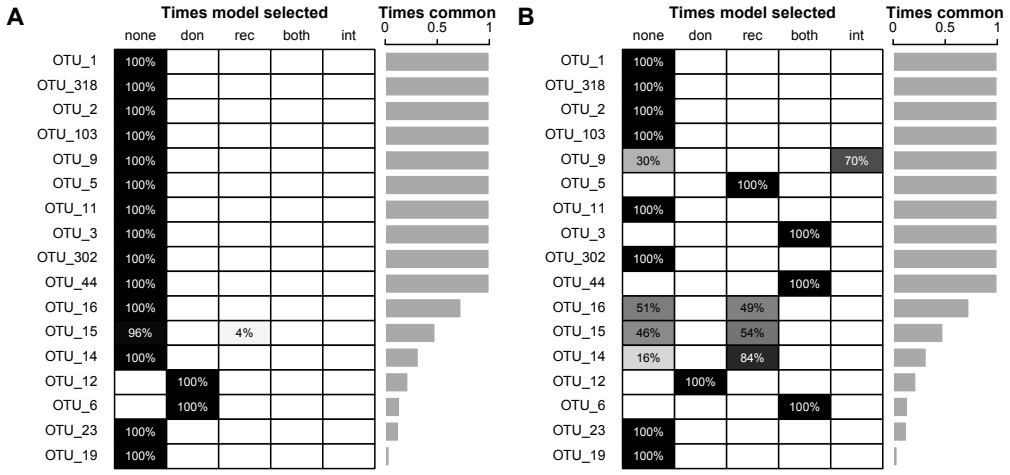

Figure S1.3: Differential abundance in microbiota – analysis of rarefied data sets. Panel (A) shows a result summary with correction for false discovery rate, panel (B) shows a result summary without correction. Shown are the percentage of times (grey-scaled) a particular model was selected when it explained significantly more variation with the addition of an explanatory variable (none = model with intercept only; don = model wit donor main effect; rec = model with recipient main effect; both = donor and recipient main effects; int = full model with main effects and interaction). Grey bars to the right illustrate how often a OTU was defined as common OTU after the rarefaction process (1 = always common, 0 = never common; n = 100).

minimal adequate model.

To test for differential abundance of common OTUs, we fitted a negative binomial generalized linear model of the form: recipient phenotype (resistant, susceptible) * donor phenotype (resistant, susceptible), as described in Love, Huber, and Anders (2014) to the non-rarefied dataset. Briefly, the modelling process statistically accounts for differences in sequencing depth, applies independent filtering to increase detection power, and uses Wald-tests to test for significant coefficients (i.e. $\log_2$-fold changes) in the negative binomial generalized linear model. We accounted for false discovery rate (Benjamini and Hochberg, 1995) at a cutoff of α = 0.05.

We use linear discriminant analysis on regularized logarithm transformed count data to find the linear combination of common OTUs that best discriminates between phenotypes. Influential OTUs were identified based on partial correlation coefficients of the linear discriminant function and leave-one-out cross-validation (jack-knifed) assessed accuracy of phenotype discrimination by the discriminant function.

Complementary to the above analysis, we applied to each rarefied data set a negative binomial generalized linear model and performed backwards model selection from the initial full model: recipient phenotype (resistant, susceptible) * donor phenotype (resistant, susceptible), using chi-square tests to identify the minimal adequate model for each common OTU within a rarefied data set. We accounted for false discovery rate (Benjamini and Hochberg, 1995) at a cut-off of α = 0.05 (SI Figure S1.3).

Table S1.6: Taxonomic information for ecologically common OTUs

OTU	Kingdom	Phylum	Class	Order	Family	Genus	Species
OTU_15	Bacteria	OD1	ZB2	NA	NA	NA	NA
OTU_14	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	NA
OTU_19	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	NA	NA
OTU_1	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	NA	NA
OTU_318	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	NA	NA	NA
OTU_2	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	NA	NA	NA
OTU_103	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	NA	NA	NA
OTU_6	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	NA	NA	NA
OTU_9	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bartonellaceae	NA	NA
OTU_16	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	NA	NA
OTU_20	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	NA	NA
OTU_23	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	*Methylobacterium*	NA
OTU_18	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	NA	NA
OTU_5	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	NA
OTU_11	Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bombiscardovia*	NA
OTU_12	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
OTU_3	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
OTU_302	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
OTU_44	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA

Table S1.7: Taxonomic classification of best hit of BLAST search against NCBI database for ecologically common OTUs

OTU	Kingdom	Phylum	Class	Order	Family	Genus	Species	Accession[1]	Bit score[1]	Id. %[1]	Source[1,2]
OTU_15	**uncult. bact.**	NA	NA	NA	NA	NA	NA	JX222661.1	699	98	NA
OTU_14	**Bacteria**	**Proteobacteria**	**Gammaproteobacteria**	**Pseudomonadales**	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas libanesis*	KT382242	793	100	compost
OTU_19	**Bacteria**	**Proteobacteria**	**Betaproteobacteria**	**Burkholderiales**	Oxalobacteraceae	*Massilia*	*Massilia suwonensis*	LN774642	793	100	air sample
OTU_1	**Bacteria**	**Proteobacteria**	**Betaproteobacteria**	**Neisseriales**	Neisseriaceae	*Snodgrassella*	*Snodgrasella alvi*	JQ746646	793	100	*Bombus bimaculatus* / bee gut
OTU_318	**Bacteria**	**Proteobacteria**	**Gammaproteobacteria**	**Pasteurellales**	Pasteurellaceae	NA	NA	JQ363618	734	100	*Pyrobombus hypnorum* / digestive tract
OTU_2	**Bacteria**	**Proteobacteria**	**Gammaproteobacteria**	Orbales	Orbaceae	*Gilliamella*	*Gilliamella apicola*	JQ936676	793	100	*Bombus vagans* / bee gut
OTU_103	**Bacteria**	**Proteobacteria**	**Gammaproteobacteria**	**Pasteurellales**	Pasteurellaceae	NA	NA	JQ363618	760	99	*Pyrobombus hypnorum* / digestive tract
OTU_6	**Bacteria**	**Proteobacteria**	**Gammaproteobacteria**	NA	NA	NA	NA	HM215025	793	100	Bumblebee / gut
OTU_9	**Bacteria**	**Proteobacteria**	**Alphaproteobacteria**	**Rhizobiales**	**Bartonellaceae**	*Bartonella*	*Bartonella apis sp. nov.*	KP987884	747	100	*Bartonella apis*
OTU_16	**Bacteria**	**Proteobacteria**	**Alphaproteobacteria**	**Rhizobiales**	**Bradyrhizobiaceae**	*Bradyrhizobium*	*Bradyrhizobium erythrophlei*	NR_135877	747	100	*Erythrophloeum fordii*
OTU_20	**Bacteria**	**Proteobacteria**	**Alphaproteobacteria**	**Rhizobiales**	**Methylobacteriaceae**	***Methylobacterium***	*Methylobacterium aquaticum*	AP014704	747	100	*Methylobacterium aquaticum*
OTU_23	**Bacteria**	**Proteobacteria**	**Alphaproteobacteria**	Rhizobiales	Methylobacteriaceae	*Methylobacterium*	*Methylobacterium oryzae* CBMB20	CP003811	747	100	Rice
OTU_18	**Bacteria**	**Proteobacteria**	**Alphaproteobacteria**	**Caulobacterales**	**Caulobacteraceae**	*Brevundimonas*	*Brevundimonas sp.* ADMK76	KU851032	747	100	soil
OTU_5	**Bacteria**	**Proteobacteria**	**Alphaproteobacteria**	**Sphingomonadales**	**Sphingomonadaceae**	***Sphingomonas***	*Sphingomonas sp.* Sph10	KP866800	747	100	membrane biofilm
OTU_11	**Bacteria**	**Actinobacteria**	**Actinobacteria**	**Bifidobacteriales**	**Bifidobacteriaceae**	***Bifidobacterium***	NA	KC477410	756	100	*Bombus terrestris* / gut
OTU_12	**Bacteria**	**Firmicutes**	**Bacilli**	**Lactobacillales**	**Lactobacillaceae**	***Lactobacillus***	*Lactobacillus sp.* G5_12_5MO2	KF600199	793	100	*Apis mellifera* / hindgut
OTU_3	**Bacteria**	**Firmicutes**	**Bacilli**	**Lactobacillales**	**Lactobacillaceae**	***Lactobacillus***	*Lactobacillus bombicola*	LK054485	793	100	Bumblebee / gut
OTU_302	**Bacteria**	**Firmicutes**	**Bacilli**	**Lactobacillales**	**Lactobacillaceae**	***Lactobacillus***	*Lactobacillus melliventris*	KM068135	726	97	*Apis mellifera* / digestive tract
OTU_44	**Bacteria**	**Firmicutes**	**Bacilli**	**Lactobacillales**	**Lactobacillaceae**	***Lactobacillus***	*Lactobacillus apis*	NR_125702	787	99	*Apis mellifera* / digestive tract

Taxonomic classification in bold corroborates blastn results against the currated 16S GreenGenes database

[1] Top hit of the first one hundred BLASTN (2.3.1+) hits against NCBI nucleotide collection database (nr/nt; accessed 5 April 2016); given the same hit quality, preference was given to full lenght 16S sequences

[2] Indicates host and/or isolation source

Table S1.8: Representative sequence for each ecologically common OTU

OTU	Sequence[1]
OTU_15	GTCGAGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCGACGCCGCGTGGTGGATGAAGTCCTTAGGGACGTAAACACCTTTTATGAGGGAGAAAGTATATTGATGTTACCTCATGAATAAGGGGCTCCCAACTCTGTGCCAGCAGGAGCCGGTAATACA GAGGCCCCAAGCATTATCCGGATTTACTGGGCGTAAAGGGTGCGTAGGCGGTTATATTAGTCGGGTGTTAAATCCTGAGGCTCAACCTCAGGCTCGCATTCGAAACGGTATAACTAGAAGGAGTCAGAGGCAAGCAGAACTCATGGTGTAGGGGTGAAAT CCGTTGATATCATGGGGAATACCAAATGCGAAGGCAGCTTGCTGGGACTTTCTTGACGCTGAGGCACGAAAGCGTGGGTAGCGAATCG
OTU_14	GTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGT GCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACGAGCTAGAGTATGGTAGAGGGTGGTGGAA TTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
OTU_19	GTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTGTCAGGGAAGAAACGGTAGAGGCTAATATCCTTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGT GCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGTCGTGAAATCCCCGGGCTCAACCTGGGAATTGCGATGGAGACTGCAAGGCTAGAATCTGGCAGAGGGGGGTAGAA TTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAG
OTU_1	GTGGGGAATTTTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTCTGAAGAAGGCCTTCGGGTTGTAAAGGACTTTGTTAGGGAGAAAAAGCTGGGTGCTAATACCATCTAGTGCTGACGGTACCTAAAGAATAAGCACCGGCTAACTACGT GCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCGCAGACGGTTAAATAAGTCAGATGTGAAATCCCCGAGCTCAACTTGGGACGTGCATTTGAAACTGTGTAACTAGAGTGTGTCAGAGGGAGGTAGAA TTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGTTCATGCTCGAAAGCGTGGGTAGCAAACAG
OTU_318	GTGGGGAATATTGCATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCGGTGATGAGGAAGGCGGTGTATCTAATAGGTGCATCGATTGACGTTAATCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT ACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATTGTAGAGGAAGGTAGAATTCCACGTGTAGCGGTGA AATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAG
OTU_2	GTGGGGAATATTGCACAATGGGGGGAACCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCGGTGATGAGGAAGGTGATGAATCTAATAGGTTTATTAATTGACGTTAATCACAGAAGAAGCACCGGCTAACTCCGT GCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATTGTAGAGGAAGGTAGAA TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAG
OTU_103	GTGGGGAATATTGCACAATGGGGGGAACCCTGATTCCTCCCTTCCTCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCGGTGATGAGGAAGGCGGTGTATCTAATAGGTGCATCGATTGACGTTAATCACAGAAGAAGCACCGGCTAACTCCGT GCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATAATTAAGTTAGGTGTGAAAGCCCTGGGCTCAACCTAGGAATTGCACTTAAAACTGGTTAACTAGAGTATTGTAGAGGAAGGTAGAA TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAG
OTU_6	GTGGGGAATATTGCACAATGGGGGGAACCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCGGTGATGAGGAAGGTAGTGTATTTAATAGATGCACTAATTGACGTTAATTACAGAAGAAGCACCGGCTAACTCCGT GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGGGCATGTAGGCGGATGATTAAGTTAGGTGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGGTCGTCTGGAGTATTGTAGAGGAAGGTAGAA TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCTTCTGGACAGATACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAG
OTU_9	GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG GGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTGGATATCTTGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAATTCGT AGATATTGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAG
OTU_16	GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTGTGCGGGAAGATAATGACGGTACCGCAAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG GGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGGTCTTTAAGTCAGGGGTGAAATCCTGGAGCTCAACTCCAGAACTGCCTTTGATACTGAAGATCTTGAGTTCGGGAGAGGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTCGT AGATATTGGCAAGAACACCAGTGGCGAAGGCGGCTCACTGGCCCGATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAG
OTU_20	GTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAGTGATGACGGCCTTAGGGTTGTAAAGCTCTTTTCTCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG GGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGCTGATTTAGTCGAGGGTGAAAGCCCGTGGCTCAACCACGGAATGGCCTTCGATACTGATTGGCTTGAGACCGGAAGAGGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGT AGATATTGGCAAGAACACCGGTGGCGAAGGCGGCCAACTGGACCATTACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAG
OTU_23	GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTATCCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG GGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGCGTTTAAGTCGGGGGTGAAAGCCTGTGGCTCAACCACAGAATGGCCTTCGATACTGGGACGCTTGAGTATGGTAGAGGTTGGTGGAACTGCGAGTGTAGAGGTGAAATTCGT AGATATTGGCAAGAACACCGGTGGCGAAGGCGGCCAACTGGACCATTACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAG
OTU_18	GTGGGGAATCTTGCGCAATGGGGGAAAGCCTGACGCAGCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTAAAATTCTTTCACCGGGGACGATAATGACGGTACCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG GGGCTAGCGTTGCTCGGAATTACTGGGCGTAAAGGGAGCGTAGGCGGACATTTAAGTCAGGGGTGAAATCCGGGGCTCAACCTCGGAATTGCCTTTGATACTGGGTGTCTTGAGTATGAGAGAGGTGTGTGGAACTCCGAGTGTAGAGGTGAAATTCGT AGATATTCGGAAGAACACCAGTGGCGAAGGCGACACACTGGCTCATTACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAG
OTU_5	GTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTACCCGGGATGATAATGACAGTACCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACG GAGG GAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGCTTTGTAAGTTAGAGGTGAAAGCCTGGAGCTCAACTCCAGAACTGCCTTTAAGACTGCATCGCTTGAATCCAGGAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGT AGATATTCGGAAGAACACCAGTGGCGAAGGCGGCTCACTGGACTGGTATTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAG
OTU_11	GTGGGGAATATTGCACAATGGGGCGAAAGCCTGATGCAGCGACGCCGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTTAGCAAGGAGCAAGCGAGAGTGAGTGTACTTGTTGAATAAGGCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC GTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGGTGTGAAAGTCCACTGCTTAACGGTGGATTGGCGCCGGGTACGGGCAGGCTAGAGTGCAGTAGGGGAGACTGGAATTCCCGGTGTAACGGTGGAA TGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCTGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAG
OTU_12	GTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGGGATAGTAACTGATTCTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAACGCAGGCGGGAAGAACAAGTCAGCTGTGAAAGCCCTCGGCTTAACCGGGGAAGTGCAGCTGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAA CTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGTTCGAAAGCATGGGTAGCGAACAG
OTU_3	GTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAAGACAAGGATAGTAACTGATTCATGTTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAACGCAGGCGGGAAGATAAGTCAGCTGTGAAAGCCCTCGGCTCAACCGGGGAACGGCAACTGAAACTATTTTTCTTGAGTGCAGAAGAGGAGAGTGGAA CTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGTTCGAAAGCATGGGTAGCGAACAG
OTU_302	GTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGACAAGGATAGTAACTGATTTTTGTTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAACGCAGGCGGGAAGATAAGTCAGCTGTGAAAGCCCTCGGCTCAACCGGGGAAGTGCAGCTGAAACTATATTTCTTGAGTGCAGAAGAGGAGAGTGGAA CTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGATGCGAAAGCGTGGGGAGCAAACAG
OTU_44	GTCGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGACATGGGTAGTAACTGATCTATGTTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGT GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAACGCAGGCGGGAGAACAAGTCAGCTGTGAAAGCCCTCGGCTTAACCGAGGAACGGCAACTGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAA CTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGTTCGAAAGCATGGGTAGCGAACAG

[1] centroid sequences

Chapter 2

Microbiota community assembly: colonization *versus* host selection

Abstract

Understanding what determines the composition and function of a host-associated microbiota is currently one of the central questions in microbiota research. This requires an understanding of the forces that shape the community assembly. One important element in determining the composition of the microbiota is the host itself. Here, we take a first step to elucidating whether hosts have the ability to directly influence the assembly and establishment of the gut microbiota. To this end we presented bumblebee, *Bombus terrestris*, workers of ten colonies with a global microbial species pool transplant, comprised of the equal mixture of the microbiota of all colonies. We hypothesised that if hosts were able to select on this "global" species pool, then the resulting microbiota composition should show colony-specific effects. We found that overall transplants reduce species Richness and diversity, but that workers from only some colonies filter out their own specific microbiota composition from this "global" pool. Because the microbiota is protective against a common gut parasite, *Crithidia bombi*, variation in this filtering ability, i.e. the ability to assemble a beneficial microbial gut community, might be another manifestation of complex evolutionary host-parasite interaction patterns.

Introduction

Community ecology proposes that the species composition of communities is determined by four main processes: local selection, local speciation and extinction, dispersal across regions and random effects (drift) (Vellend, 2010). However, these processes can vary in their relative importance to one another and interact on varying spatial scales, which makes disentangling the processes difficult. This complicates 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 are still major research objectives (Costello et al., 2012; Foxman et al., 2008; Mihaljevic, 2012; Robinson, Bohannan, and Young, 2010; Zilber-Rosenberg and Rosenberg, 2008).

A particularly well studied example of a host-associated microbial community is the gut microbiota in insects (Dillon and Dillon, 2004). Studies that analysed the determinants of microbiota composition showed for example that environmental factors such as diet can have a major influence (Chandler et al., 2011; Colman, Toolson, and Takacs-Vesbach, 2012; Engel and Moran, 2013b). However, signals of host phylogeny have also been detected in the composition of hymenopteran and termite gut microbial communities (Brucker and Bordenstein, 2012; Colman, Toolson, and Takacs-Vesbach, 2012; Koch et al., 2013; Sanders et al., 2014), but less so in beetles (Colman, Toolson, and Takacs-Vesbach, 2012). Thus, in the light of ecological theory, gut microbiota composition seems not to be randomly assembled. Whereas the external determinants of microbiota composition such as diet are certainly relevant, 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 from the total available microbial species pool. Such an active involvement of the host is particularly intriguing when the microbiota performs functions or services that are beneficial to the host: the host may then actively coordinate the assembly process of the microbiota such that it maximises the benefit to the host. Such an "active" selection potential on the microbial gut community is especially plausible in honeybees and bumblebees, where microbiota community composition is simple, distinctive and highly species specific, and also functions in the defence against parasites (Koch and Schmid-Hempel, 2011a; Koch et al., 2013; Lim et al., 2015; Martinson et al., 2011).

Adults of honeybees and bumblebees emerge from the pupa essentially germ-free (Hakim, Baldwin, and Smagghe, 2010; Koch and Schmid-Hempel, 2011b; Martinson et al., 2011). The gut microbiota is subsequently acquired within their social environment, likely via faeces contaminated nest material, and coprophagy (Koch and Schmid-Hempel, 2011b; Martinson, Moy, and Moran,

2012; Powell et al., 2014). In particular, in the bumblebee, *Bombus terrestris*, the gut microbiota provides a protective function against its natural gut parasite, the trypanosome *Crithidia bombi* (Koch and Schmid-Hempel, 2011b). Importantly, the protective function against the parasite is not provided solely any particular species of the gut microbial community alone, but rather by the gut microbiota as a whole (Koch and Schmid-Hempel, 2011b). The level of protective function of the microbiota also differs across colonies (i.e. host backgrounds/genotypes) and varies among different parasite strains (Koch and Schmid-Hempel, 2012). The two central, yet complex open questions are, first, what the precise structure and composition of the microbiota must be, such that the microbiota is optimally protective for the host; and, second, how much influence the host has on determining the composition of the microbiota. A first step towards answering the important second question is to determine weather colonies have the ability to directly influence the assembly and establishment of the gut microbiota.

Here we assess whether the microbiota that eventually establishes from a common pool varies among colonies, a pattern that would provide evidence that colonies — to a degree — can select their own bacterial flora. For this, we presented the same faecal microbiota transplant to germ-free workers from each of a number of colonies and allowed the microbiota to establish. The offered transplant represented the "global" microbial species pool, as each of the test colonies contributed equally to the administered microbiota inoculum. Thus, 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 to their nest mates that had acquired their microbiota naturally within their colony environment, and were thus only exposed to their respective colony specific bacterial species pool.

Material and Methods

Bee colonies and sample collection

Ten colonies were raised in the laboratory from mated F1-offspring of *Bombus terrestris* queens field-caught in spring 2014, from two populations in Switzerland (Aesch and Neunforn; Table S2.1). At all times, colonies and isolated workers were kept under standardized conditions ($28 \pm 2\,^{\circ}\mathrm{C}$, 60% RH, constant red-light illumination) and provided with pollen and sugar water (ApiInvert®) *ad libitum*.

The experimental design and procedure is shown in Figure 2.1. Once the colonies had grown in size to sustain the removal of brood, we removed a part of the brood from each of the ten colonies in order to raise germ-free workers in a sterile environment for the faecal microbiota transplant experiment. Around the same time, each day and in all colonies, emerging workers (i.e. within 24 h post-hatching, "callows") were collected and marked. Subsequently, the callows were returned to their colony and allowed to naturally acquire their gut microbiota for five days before the bees were sacrificed and frozen at $-20\,^{\circ}\mathrm{C}$. Because a suffi-

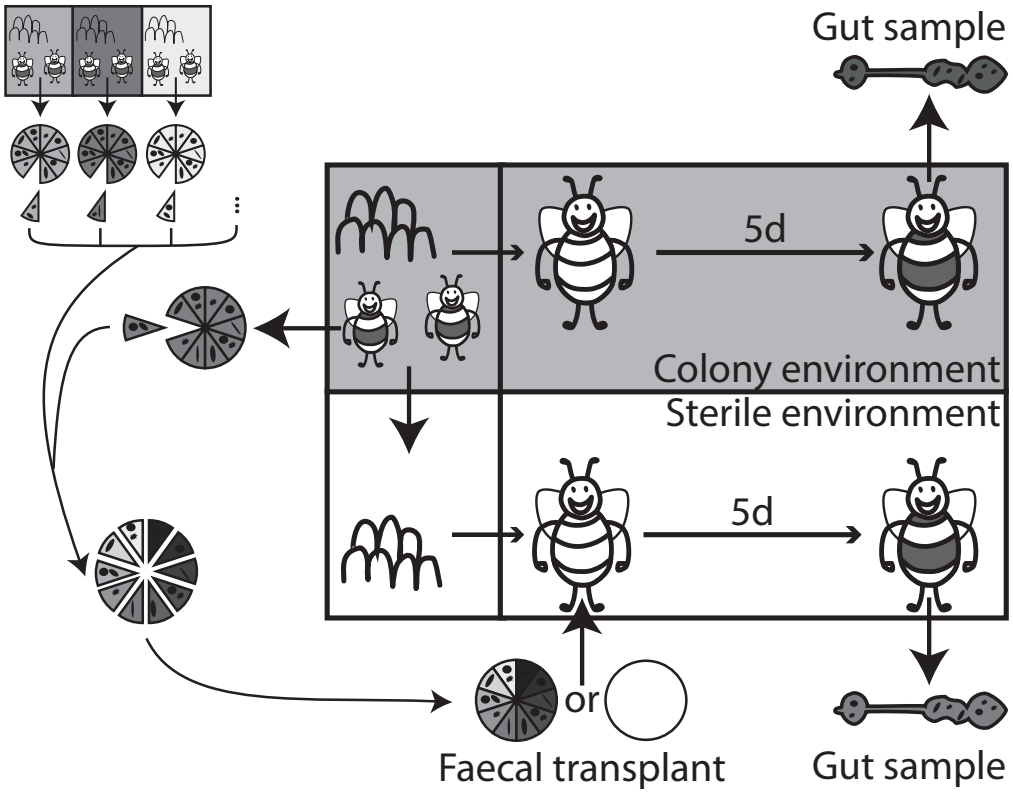

Figure 2.1: Experimental design. A gut microbiota was allowed to establish in workers of ten colonies in two ways: naturally, within the social environment of the colony (grey zone), or experimentally, by inoculating germ-free colony workers in a sterile environment with a faecal microbiota transplant. The transplant was composed, in equal parts, of faecal matter collected from each of the ten colonies. Additionally, as a technical control of the sterility treatment, some bees received a microbiota-free "sham" inoculum. The microbiota was allowed to establish for five days before the bees were sacrificed and the gut microbiota community analysed.

cient number of age-controlled workers could not be collected for all colonies, we picked additional older workers at random to compensate for the missing workers from older workers (see details in Table S2.2).

For the faecal transplant experiment, we followed the experimental protocol described in Koch & Schmid-Hempel (Koch and Schmid-Hempel, 2011b) with a few modifications. Briefly, the isolated brood of each colony was surface-sterilized by submerging the brood for 90 s in a 3% bleach solution (Murrell and Goerzen, 1994). Daily, the new workers emerging from the brood clump were transferred to individual housing boxes and kept for 1–2 days before we offered them a faecal microbiota transplant. For this, we collected faeces from each colony and from a minimum of five donor workers. To inoculate the germ-free workers and to mimic natural transmission, equal volumes of freshly collected faeces from each colony were pooled and diluted with sugar water (1:2 ratio). This faecal microbiota mix was offered as an inoculum of 15 μl to eight workers per colony. Another three test workers of the same colony received a sham treatment with a sugar water only inoculum of 15 μl. Workers were starved for 30 min before the inoculum was given; bees that did not take up the inoculum

were excluded from the experiment. Five days post-exposure, bees were killed and frozen at $-20\,^{\circ}\mathrm{C}$. Also, a sample of each of the freshly prepared microbiota transplant inocula was set aside and frozen for later analysis. All handling and housing material used in this part of the experiment were either autoclaved or washed in 80% ethanol before use. To further prevent bacterial contamination, a diet of X-ray radiated pollen (dose: $26.7\,\mathrm{kGy}$) and filter-sterilized sugar water [50%] (pore size $0.2\,\mu\mathrm{m}$) was provided *ad libitum* to these experimental bees.

DNA extraction

We extracted DNA from all samples using the Qiagen DNeasy Blood & Tissue Kit and followed the manufacturer's protocol (Animal Tissue DNaey 96 Protocol), with a few modifications depending on sample types. DNA extraction of samples for later microbiota analysis occurred from aseptically dissected guts. Before extraction, a sterile zirconium oxide bead ($2.8\,\mathrm{mm}$) was added to the dissected gut and the sample was shredded three times for one minute at $30\,\mathrm{Hz}$ on a Mixer Mill MM 301 (Retsch GmbH). Also, we extracted $35\,\mu\mathrm{l}$ of each of the microbiota inoculums. Both sample types were incubated for $1\,\mathrm{h}$ at $56\,^{\circ}\mathrm{C}$, but extracted DNA of the microbiota inoculums were eluted in only $100\,\mu\mathrm{l}$ AE buffer. For the analysis of a colony's resistance profile, DNA extraction occurred from whole abdomen samples. For this, we shredded the abdomen on a Bead Ruptor 24 Homogenizer (Omni) with $0.5\,\mathrm{g}$ added zirconium oxide beads ($1.4\,\mathrm{mm}$). The disrupted abdomen was lysated in $600\,\mu\mathrm{l}$ lysis buffer ($540\,\mu\mathrm{l}$ ATL buffer and $60\,\mu\mathrm{l}$ proteinase K) for $2\,\mathrm{h}$ at $56\,^{\circ}\mathrm{C}$, but only $200\,\mu\mathrm{l}$ of the lysate was used for DNA extraction.

Colony resistance profiles

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 $10\,\mu\mathrm{l}$ [50%] sugar water after a $2\,\mathrm{h}$ starvation period. The 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). After infection, infected bees were kept in individual housing boxes. Infection were allowed to establish for seven days (Schmid-Hempel and Schmid-Hempel, 1993), before bees were killed and frozen at $-20\,^{\circ}\mathrm{C}$.

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 number of parasite cells was 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, Sadd, and Schmid-Hempel (2011) in a total reaction volume of $10\,\mu\mathrm{l}$ containing: $0.2\,\mu\mathrm{l}$ [$10\,\mathrm{M}$] of each forward and reverse primer (Ulrich, Sadd, and Schmid-Hempel, 2011), $2\,\mu\mathrm{l}$ of 5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne) and $2\,\mu\mathrm{l}$ of

DNA template (1:10 diluted).

Infection diversity was identified by genotyping the samples at five *C. bombi* microsatellite markers amplified in two multiplexed PCR reactions with the primers Cri4G9, Cri4, Cri2F10 and Cri16, Cri1B6, respectively (Schmid-Hempel and Reber Funk, 2004). This allowed us to determine the number of parasite strains present seven days post-infection. A 10 μl PCR reaction volume contained: 2 μl of 5x 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).

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 et al., 2013) between the region-specific part of the primer and the illumine overhang adapter (SI Table S2.3). We generated the amplicon libraries by following 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 in four independent reaction. 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 ten 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.

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 com-

bined, and assigned taxonomic information to each identified OTU using the UPARSE (usearch v8.0.1623_i86linux64) workflow (Edgar, 2010, 2013). A phylogenetic tree of the OTUs was built with PyNAST Caporaso:2010iq implemented in Qiime (v1.8.0) (Caporaso et al., 2010b). We assessed sequencing depth by constructing rarefaction curves (number of OTUs as a function of sample size, SI Figure S2.1) 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 SI Table S2.4.

Analysis and statistics

After the standard purging protocols, we could analyse a total of 19'520'956 reads. We tested for any effect of the four "treatments" on the microbiota composition that established in the workers. This is, the microbiota composition that established when the microbiota was acquired (1) naturally, (2) from transplant, (3) from sterility control treatment, and (4) from the negative sequencing controls (SI Table S2.2). The result showed that the microbiota of the sterility controls and negative sequencing controls clearly differed from all other treatments; they were thus excluded from any further analysis (SI Figures S2.2, S2.3). This left a total of 18'842'607 reads mapping to 205 different OTUs for analysis. We collapsed age-controlled workers and randomly picked older workers that acquired their microbiota naturally into the same treatment group, as their microbiotas did not differ in any obvious way from each other (SI Figure S2.3). We accounted for different sequencing depth by rarefying all libraries to the smallest library size (n = 20'554 reads). We repeated this process one hundred times to account for the effects of randomly subsampling the libraries. To investigate diversity differences among treatment we calculated the ecological measures of the 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 datasets (n = 100) for further analysis.

To analyse microbiota composition differences, we calculated Euclidean and Bray-Curtis pairwise distances between the OTUs of all samples. We used one-way ANOVAs and subsequent Tukey's HSD tests to determine differences in microbiota diversity measures across the three groups of interest: (a) microbiota naturally acquired, (b) microbiota established from the transplants, and (c) the microbiota in the inocula themselves. Further, we used linear mixed effect models (Bates et al., 2015) to test for effects of treatment (microbiota naturally acquired, microbiota from transplant) and colony. For this, we considered treatment as a fixed effect and included colony as random effect and nested in populations. To assess whether the treatment effect differed between colonies, we fit a random slope model for colonies and populations. The effect sizes for populations were always estimated to be zero, such that we dropped the population effect and only retained the colony effect. We assessed the significance of the mean treatment effect by comparing the statistical model with a fixed treatment effect to one without treatment effect, using a likelihood ratio test. Similarly, we

tested for the clustering of microbiota composition, as quantified by Euclidean and Bray-Curtis distances, due to effects attributed to treatment and colony 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 and just considered colony and treatment, as well as their interaction as fixed effects.

Results

Colony resistance profile

Infection intensity (i.e. the number of parasite cells) and infection diversity (i.e. the number of established strains) of the gut parasite *C. bombi* in workers did not correlate (Figure 2.2), contrary to earlier reports (Schmid-Hempel et al., 1999; Ulrich, Sadd, and Schmid-Hempel, 2011). The absence of the correlation is likely due to the breeding and colony rearing process that sampled colonies in "good condition". Also, the number of *C. bombi* strains that were able to establish did not significantly differ among the two populations (Aesch vs. Neunforn: Welch two sample t-test; $t(50.97) = -0.53$, $p = 0.601$). However, for the same average number of parasite strains that were able to establish, there is a tendency for higher infection intensities in colonies from Aesch compared to colonies from Neunforn (Welch two sample t-test; $t(47.59) = 1.97$, $p = 0.054$).

Microbiota 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 "5dC"); (2) Samples of the global species pool that was composed of equal parts of faecal matter collected from each colony (Inoculum); and (3) Gut samples from germ-free workers that acquired their gut microbiota from the inoculation with the global species pool (T). We found that the microbiota diversity as measured by the Shannon Index differed significantly among these groups (ANOVA; $F_{2,157} = 10.99$, $p < 0.001$), as did species Evenness (ANOVA; $F_{2,157} = 8.06$, $p < 0.001$) and species Richness (ANOVA; $F_{2,157} = 15.09$, $p < 0.001$; Figure 2.3).

Because the aim of this study was to determine the selection potential of colonies on the microbiota, we analysed the route of microbiota acquisition (naturally vs. transplanted) more closely. Overall, the gut microbiota that established from the experimental transplant showed a reduced diversity as measured by the Shannon Index compared to the naturally acquired microbiota (GLMM; $\chi^2_1 = 5.82$, $p = 0.016$, effect size = $-0.13[-0.23, -0.04]$ 95% CI). Similarly, species Richness was reduced in the gut microbiota acquired from transplants (GLMM; $\chi^2_1 = 8.83$, $p = 0.003$, effect size = $-4.72[-7.19, -2.25]$ 95% CI). By contrast, species Evenness was not significantly affected by the route of microbiota acquisition (GLMM; $\chi^2_1 = 0.05$, $p = 0.831$, effect size = $0.00[-0.03, 0.02]$ 95% CI).

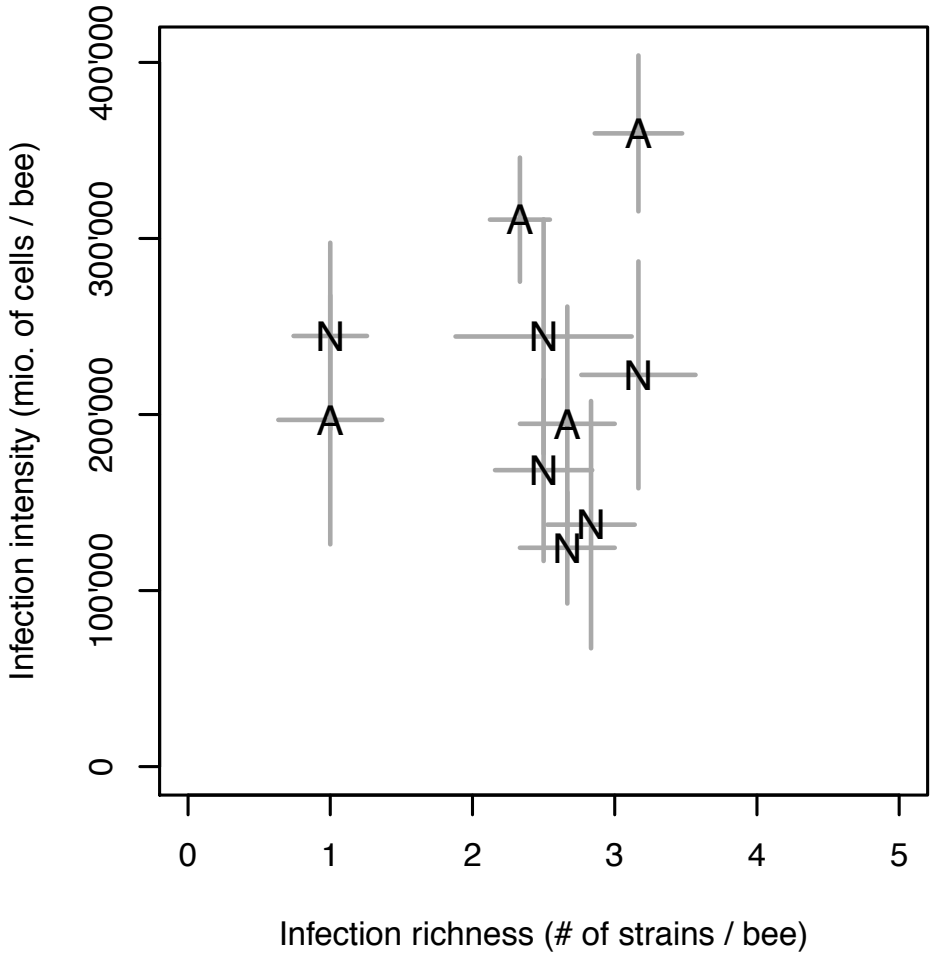

Figure 2.2: Variation in resistance profiles of colonies. Infection outcome by colony seven days post infection. Plotted are colony mean infection intensity against colony mean infection Richness (Spearman's $\rho = -0.12$, $p = 0.723$, n = 10, bars are SEM). Letters denote colony population origin (A: Aesch, N: Neunforn).

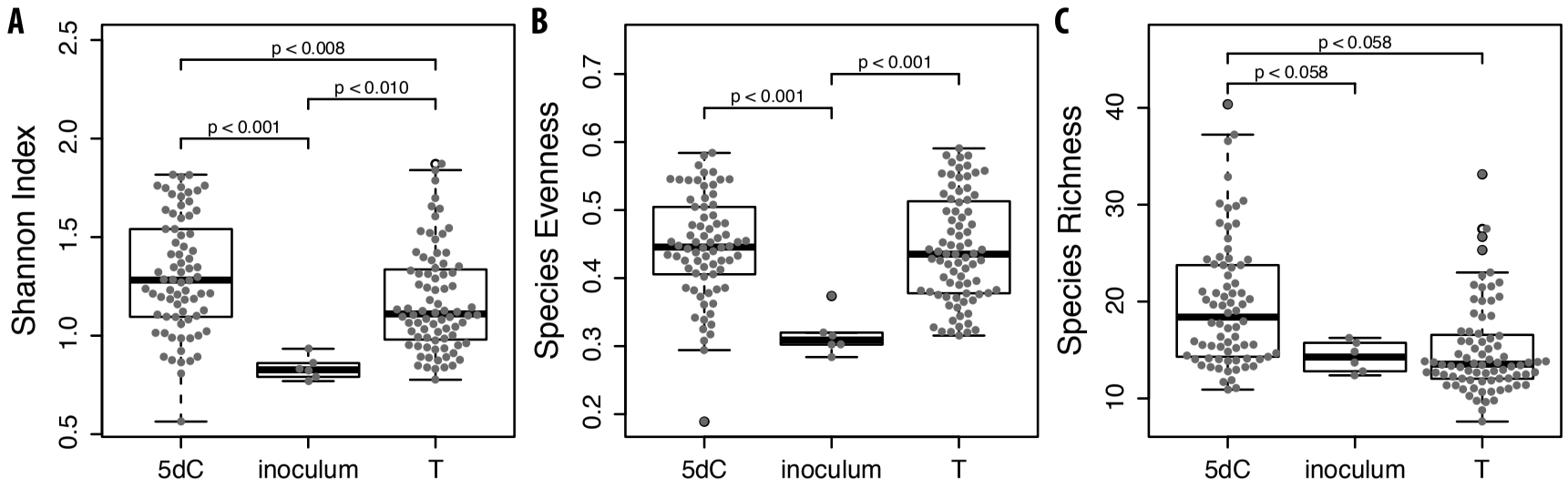

Figure 2.3: Microbiota diversity of three microbiota groups. Boxplots of the Shannon Index (A), species Evenness (B), and species Richness (C); for naturally acquired microbiota (5dC), microbiota acquired from transplants (T), and the "global species pool" (inoculum). The boxes show median and interquartile range (IQR), the hinges extend 1.5x IQR from the box. Bars show significant differences between group means as identified by Tukeys HSD test.

Table 2.1: Permutational multivariate analysis of variance using distance matrices.

Model term	Df	F	R^2	p-value
Bray-Curtis distances				
Treatment	1	25.69	0.13	0.001
Colony	9	2.44	0.11	0.001
Treatment:colony	9	2.63	0.12	0.001
Residuals	134		0.65	
Euclidean distances				
Treatment	1	24.96	0.12	0.001
Colony	9	2.51	0.11	0.001
Treatment:colony	9	2.80	0.12	0.001
Residuals	134		0.65	

Our interest, however, was to also test whether, in different colonies, the route of microbiota acquisition affected the diversity measures differently (i.e. the acquisition x colony interaction). Overall, colonies responded in the same direction (Figure 2.4). However, the inspection of the colony-level random slopes (see Material & Methods) showed that Shannon diversity and species Richness was affected in a colony-specific manner, as indicated by the non-overlap of the 95% CI with the main treatment effect (Figure 2.4).

Microbiota composition

Similar to the effects for the diversity measures, differences in the microbiota composition — measured by pairwise Euclidean and Bray-Curtis distances — showed consistent effects that could be attributed to both colony and treatment effects, and imply significant clustering in microbiota community composition (Table 2.1). The direction of this clustering can be visualised by comparing the distances of the gut community between workers that established their microbiota from the transplanted "global" species pool to workers that acquired their microbiota naturally, from either the same or different colonies (Figure 2.5). For some colonies, the microbiota was more similar to the workers from their own colony. For other colonies, the established microbiota was more dissimilar to their own colony.

Discussion

The structure of communities should become understood once the main processes governing their assembly are known (Costello et al., 2012; Vellend, 2010). Such insights might be especially valuable for host-associated microbial communities that are involved in providing important function to the host, such as host health (Sekirov et al., 2010). By employing an experimental approach, we were able to specifically focus on the role of selection as exerted by the host on the

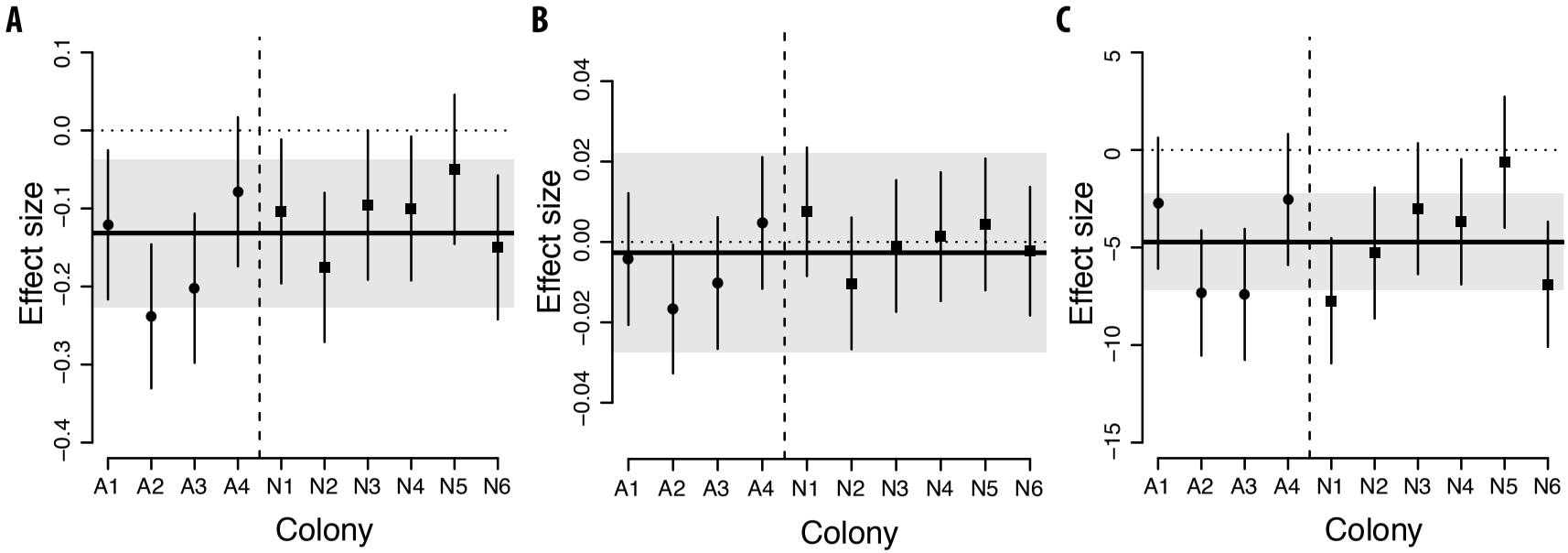

Figure 2.4: Effects of route of acquisition on the individual colonies. Shown is the effect of route of acquisition (fixed effect) on Shannon Index (A), species Evenness (B), and species Richness (C). The mean across all colonies is shown as the bold black line with the 95% CI in grey. Points (with 95% CI error bars) indicate the combined overall effect for each colony including their random slopes. 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).

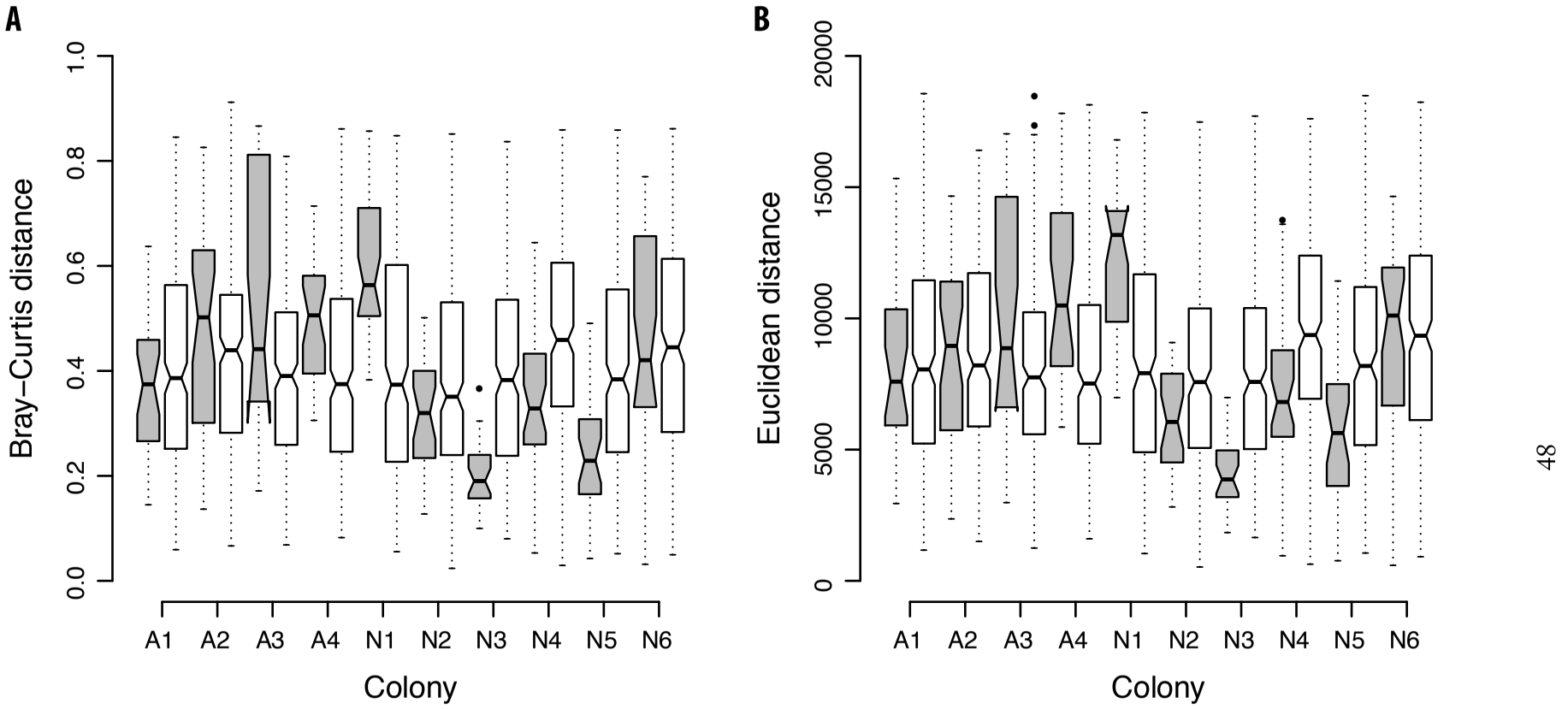

Figure 2.5: Similarity of gut microbiota composition. Grey shaded boxes show Bray-Curtis distance (A) and Euclidean distances (B) of each individual that acquired its microbiota from the "global" species pool to all individuals that naturally acquired their microbiota from the same colony. Empty boxes show the distances from an individual that acquired the microbiota from the "global" species pool to all workers from other colonies that naturally acquired their microbiota. Each box shows the median and interquartile range (IQR), hinges extend 1.5x IQR from the box and notches extend $\pm 1.58\mathrm{IQR}/\sqrt{n}$. Non overlap of notches indicates strong support for differences in medians.

assembly process of a gut microbial community, i.e. a process by which only certain microbes are allowed to establish (Koch and 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 various microbiota members, the "global species pool". Such a filtering ability would imply that the host actively influences the microbiota community assembly in the gut, regardless of the actual process. If so, this has important implications, for instance, for the understanding of the protective function of the assembled gut microbiota in the context of host-parasite interactions (Kamada et al., 2013; Koch and Schmid-Hempel, 2012).

Our global species 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 colonies was thus eliminated. Our analysis showed that species Richness, Shannon diversity, and species Evenness were all reduced in the inoculum representing the global species pool as compared to natural control workers. Note that even though we may have "lost" some species when composing the inoculum, we would still be able to detect a filtering by the host of this incomplete global species pool.

Such a decrease in species Richness during mixing is somewhat unexpected, but could have various explanations. First, despite being similar in composition (Koch and Schmid-Hempel, 2011b), the OTUs that are shed in faeces might not be an exact representation of the gut, although this seems unlikely in the context of our own analyses (see Chapter 3). Second, some of the less abundant species may have been lost during the sampling procedure. But this also appears unlikely when looking at species Richness in transplants, and as further discussed in the following paragraph. Finally, the abundances of some OTUs might fall below the technical detection limit thus produce false negatives, an explanation we favour. The decrease in Evenness and Shannon index in the inoculum would then reflect 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 composed inoculum. Rare OTUs, by contrast, would not be detected in the inoculum yet recover in abundance inside the receiving bee. In fact, the increase in species Richness in some of the individual workers that received a transplant suggests that many of the "missing species" in the inoculum were not detected due to these technical issues, rather than being absent altogether from the global species pool. This would also mean that the skewed abundance distribution in the inoculum is rectified during the community assembly process. The mechanisms of reorganisation are as yet unknown. Competitive or mutualistic interactions among members of the microbiota may both play a role. Alternatively, "direct" selection by the host could be important. The latter is suggested by the fact that we observed differences in the change of diversity measures that are expressed in a colony specific manner. The general decrease in species Richness between natural controls and transplant workers is somewhat

expected from the decreased Richness in the inoculum, but we nevertheless observed large differences between colonies, some of which managed to restore Richness to almost natural levels. Although characterizing community diversity using summary statistics is rather crude, our conclusions are further supported by more fine-grained multivariate analyses.

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 et al., 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 et al., 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 is highly relevant for the establishment of a specific gut community.

In social insects like the bumblebees the social environment is the colony, which can explain why the microbiota composition in social insects is highly host species-specific (Koch and Schmid-Hempel, 2011a; Koch et al., 2013; Kwong and Moran, 2015; Lim et al., 2015; Martinson et al., 2011). This environment of social transmission might also 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 five days until they were screened, whereas in the latter, the bees were inoculated at a single point in time and screened five days later.

While we can only speculate about the mechanism leading to the species filtering in our experiment, it is very likely that the host's immune system plays an important role (Buchon, Silverman, and Cherry, 2014). 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 (Chapter 1). However, because colony resistance profiles did not show considerable variation in this chapter, 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 are needed to elu-

cidate exact interactions between immune system and gut microbiota community assembly.

Supplementary Information

Table S2.1: Mating information and colony development data of experimental colonies.

ProjectID	Population	ColonyID	Gyne	Male	First eggs[1]	First worker[1]	Transfer[1]	Brood removed[1]
N1	Neunforn	14.651	14.015	14.203	10.10	04.11	07.11	30.11
N2	Neunforn	14.1076	14.059	14.038	10.10	30.10	31.10	30.11
N3	Neunforn	14.429	14.004	14.025	10.10	28.10	31.10	30.11
N4	Neunforn	14.1010	14.036	14.152	10.10	30.10	31.10	30.11
N5	Neunforn	14.619	14.141	14.082	07.10	30.10	31.10	30.10
N6	Neunforn	14.1066	14.124	14.055	17.10	07.11	11.11	02.12
A1	Aesch	14.727	14.259	14.250	07.10	28.10	28.10	30.11
A2	Aesch	14.939	14.332	14.250	07.10	04.11	04.11	01.12
A3	Aesch	14.788	14.269	14.258	07.10	04.11	04.11	01.12
A4	Aesch	14.539	14.267	14.250	07.10	30.10	04.11	30.11

[1] Dates in 2014

Table S2.2: Number of samples per treatment group.

Treatment group	Colony									
	A1	A2	A3	A4	N1	N2	N3	N4	N5	N6
Microbiota naturally acquired age-controlled (5dC)	6	8	6	5	8	7	7	8	4	8
Microbiota naturally acquired not age-controlled (W)	1	0	1	2	0	0	0	0	3	0
Microbiota acquired from transplant (T)	8	8	8	8	8	8	8	8	8	8
Sterile controls sham inoculum (S)	3	3	3	3	3	3	3	3	3	3
Microbiota inoculum	6									

Table S2.3: Amplicon Primer to generate V3–V4 16S template library.

Primer Name	Sequence 5′–3′
U341F_nex0	tcgtcggcagcgtcagatgtgtataagagacag**ga**CCTACGGGDGGCWGCA
U341F_nex1	tcgtcggcagcgtcagatgtgtataagagacag**Nga**CCTACGGGDGGCWGCA
U341F_nex2	tcgtcggcagcgtcagatgtgtataagagacag**NNga**CCTACGGGDGGCWGCA
U341F_nex3	tcgtcggcagcgtcagatgtgtataagagacag**NNNga**CCTACGGGDGGCWGCA
U806R_nex0	gtctcgtgggctcggagatgtgtataagagacag**ca**GGACTACHVGGGTMTCTATTC
U806R_nex1	gtctcgtgggctcggagatgtgtataagagacag**Nca**GGACTACHVGGGTMTCTAATC
U806R_nex2	gtctcgtgggctcggagatgtgtataagagacag**NNca**GGACTACHVGGGTMTCTAATC
U806R_nex3	gtctcgtgggctcggagatgtgtataagagacag**NNNca**GGACTACHVGGGTMTCTAATC

Lower case letters represent Illumina-specific overhang adapters, to which in a subsequent limited-cycle amplification step the sample specific indices and sequencing adapter attach. Frame-shifting nucleotides are indicated in bold uppercase and linker sequence in bold lowercase. The gene-specific sequences targeting 16S V3 and V4 region are shown in uppercase.

2.A Two-step amplification 16S MiSeq library preparation protocol

1. Initial PCR for each sample with each primer pairs of Table S2.3 independently:
   Total reaction volume of 25 µl:
   1.5 μl [10 μM] forward and reverse primer mix, 12.5 μl 2x KAPA HiFi Hot Start Ready Mix, 2 μl DNA template (1:2 diluted)
   Cycling protocol:
   initial degradation: 5 min, 95 °C
   25 cycles of 98 °C for 20 s, 58 °C for 15 s and 72 °C for 15 s
   final elongation: 5 min, 72 °C

2. Agencourt AMPure XP beads (Beckman Colter) PCR product clean-up:
   22.5 μl of each of the four independent PCR pooled, ratio of 0.8:1 (beads to PCR product), eluted in 27.5 μl [10 mM, 8.5 pH] TrisBuffer

3. Indexing PCR reaction:
   Total reaction volume of 50 μl:
   25 μl 2x KAPA HiFi Hot Start Ready Mix, 15 μl of the purified PCR product, 5 μl of each forward and reverse primers of the Nextera®XT Index Kit v2 Set D (Illumina)
   Cycling protocol:
   initial denaturation: 3 min, 95 °C
   10 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C
   final extension: 5 min, 72 °C

4. Agencourt AMPure XP beads (Beckman Colter) PCR product clean-up:
   0.8:1 (beads to amplicon product), eluted in 32.5 μl [10 nM, 8.5 pH] Tris-Buffer.

5. Quality control on Bioanalyzer (Aligent Technologies) DNA HS 1000 chip.

6. Concentration quantification of each library:
   4 μl 1:10'000 diluted amplicon library template
   6 μl 2x KAPA SYBER qPCR Ready Mix
   Calibration curves: Library Quant Illumina Kit (KAPA Biosystems)

7. Final Agencourt AMPure XP beads (Beckman Colter) clean-up of equimolar pooled libraries.

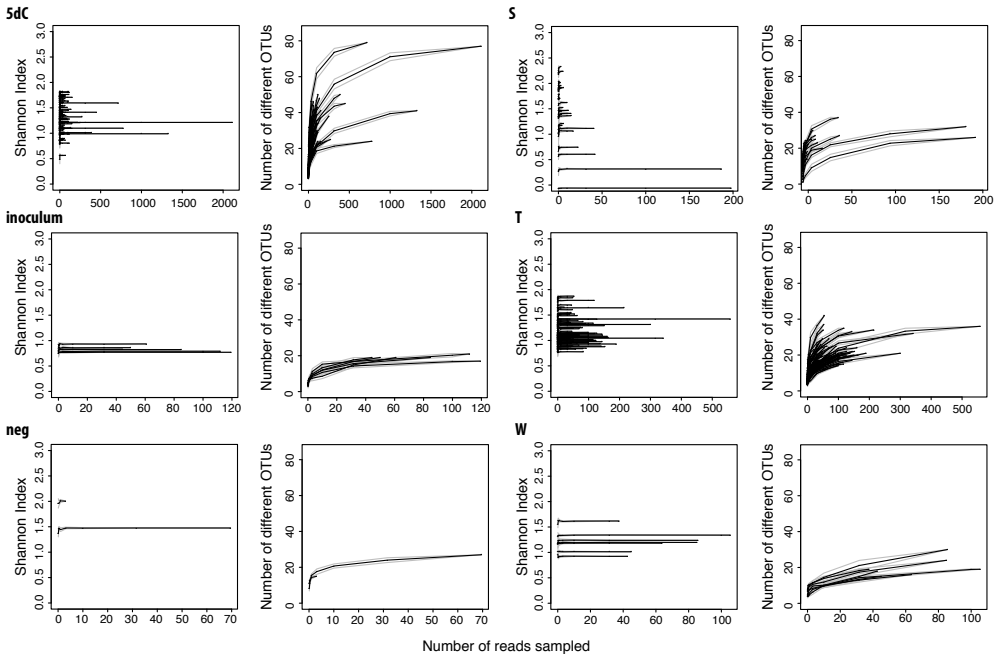

Figure S2.1: Rarefaction curves of each sample. The panels to the left show Shannon-Index, and panels to the right show number of different OTUs (i.e. Richness) at given sub-sampling depth (x-axis in 1000s) of the sequencing pool. Lines connect means (black) and SD (grey) of 10 independent draws at a given sampling depth from the total sequencing pool, i.e. the total number of sequence reads of a particular amplicon library. (5dC) Microbiota naturally acquired, age-controlled; (W) Microbiota naturally acquired, not age controlled; (T) Microbiota acquired from faecal microbiota transplant; (S) Sterility control, sham transplant inoculum; (neg) Sequencing negative conrols.

Table S2.4: Amplicon processing and read counts.

Processing step	Specifications
Raw reads quality control: FastQG (v0.11.2)	
	Run 1: 14976991
	Run 2: 18906672
End-trimming: PRINSEQ-lite (v0.20.4)	
	Min quality mean: 15
	Trim Run 1: 15 nt
	Trim Run 2: 40 nt
	Min length: 150 nt
Merge reads: FLASH (v1.2.9)	
Run 1: 12808084	Min overlap: 15
Run 2: 16285142	Max overlap: 300
	Max mismatch density: 0.25
Primer-trimming: cudadapt (v.1.5)	
Run 1: 10988058	Overlap: full length
Run 2: 14146357	Error rate: 0.01
	Wildcards allowed
Quality filtering: PRINSEQ-lite (v0.20.4)	
Run 1: 10945334	Size selection: 326 nt–465 nt
Run 2: 9247248	Min quality mean: 20
	No amibuous nucleotides
	Low-complexity filter: dust
	Low-complexity threshold: 10
OTU clustering: UPARSE (usearch v8.0.1623_i86linux64)	
Number of OTUs: 231	sortbylength
Reads mapped: 19521513	derep_fullength
Reads not mapped: 671069	sortbysize: size = 2
	cluster_otus
	uchime_ref
	userch_global: id = 97%
Taxonomic assignment: Qiime (v1.8.0)	
	UTAX: Reference: rdp_16s.fa
Phylogenetic analysis: PyNAST	
557 reads mapped to 9 OTUs that failed to align, excluded	Min identity 55 %

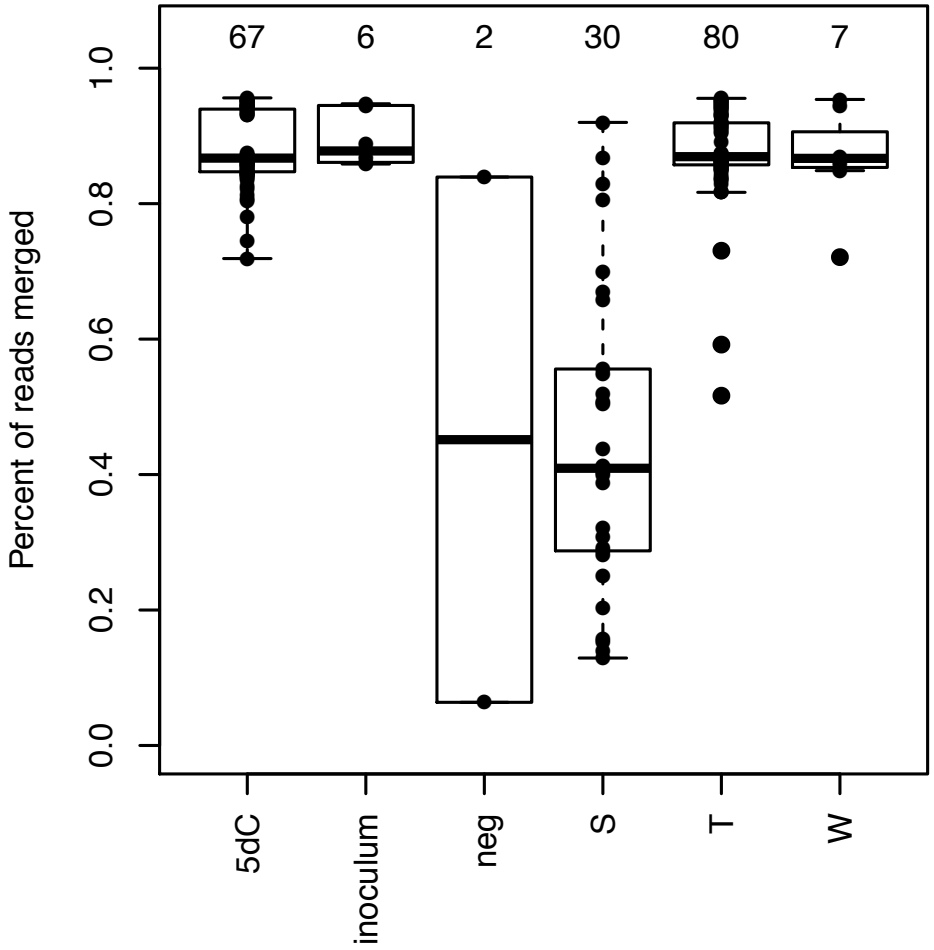

Figure S2.2: Percentage of reads merged. Boxplots show percentages of reads successfully merged, grouped by treatment. Abbreviation of treatments as follows: 5dC: Microbiota naturally acquired/age-controlled; inoculum: administered faecal transplant mix; neg: negative controls library preparation; S: sterility control/sham inoculum; T: microbiota acquired from transplant; W: microbiota naturally acquired/not age-controlled. Numbers indicate sample size of each treatment group.

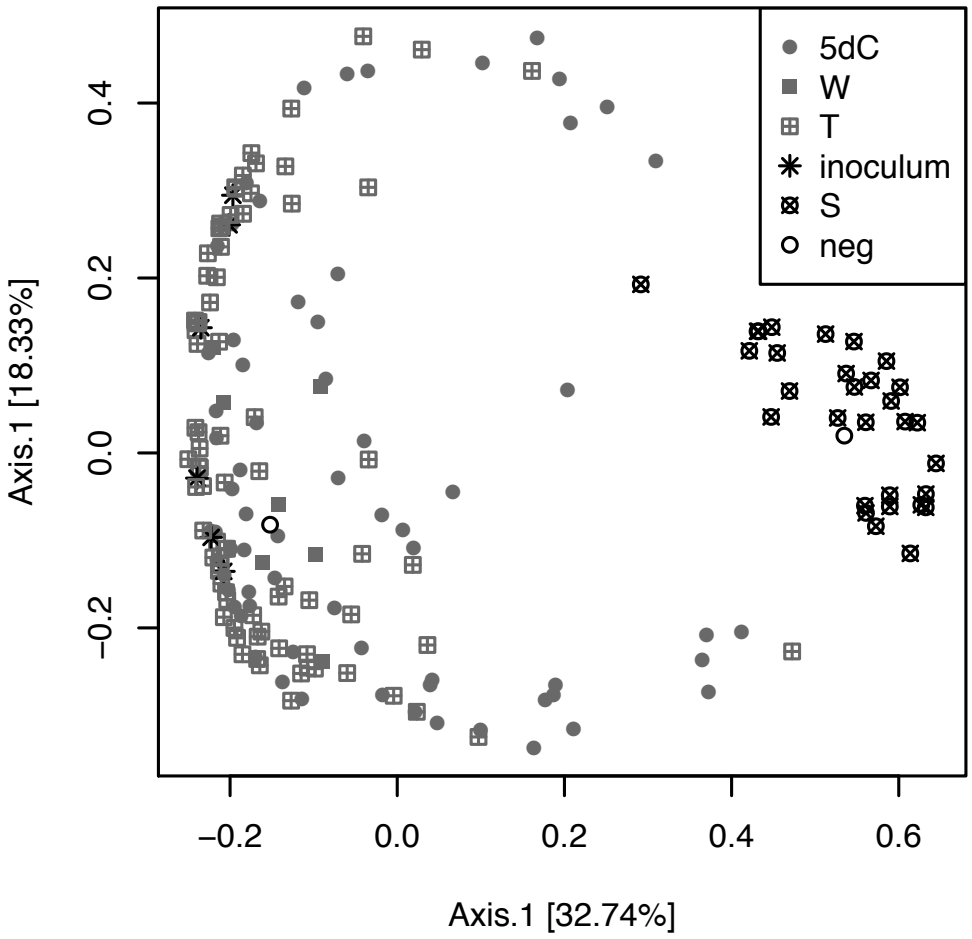

Figure S2.3: Principal Component Analysis of Bray-Curtis distances of non-rarefied data. Each point represents a sample and colour in combination with symbols represent treatment groups. Abbreviation of treatments as follows: 5dC: Microbiota naturally acquired/age-controlled; inoculum: administered faecal transplant mix; neg: negative controls library preparation; S: sterility control/sham inoculum; T: microbiota acquired from transplant; W: microbiota naturally acquired/not age-controlled.

Chapter 3

Robustness of bumblebee gut microbiota in response to a parasite challenge

Abstract

The microbiota of a host organism can modulate the interaction between hosts and their parasites. Given that the host-associated microbiota is already established at the time of parasite encounter, then from the standpoint of ecological theory, an infection by a parasite is an invasion process into the pre-existing microbial community. Hence, in addition to the intrinsic defence characteristics of the host itself, infection success depends on the colonization resistance of the microbiota. In *Bombus terrestris* resistance to the intestinal parasite, *Crithidia bombi*, is known to be affected by the gut microbiota, yet it is unclear precisely how microbiota and parasite interact. Here, we show that the gut microbial community structure prior to parasite exposure can be informative regarding infection outcome. By contrast, we found that the microbial community structure post-parasite exposure is uninformative for the infection status. This suggests that parasite infection alters microbiota composition, although the exact alteration that occur remain unclear. In conclusion, the bumblebee microbiota is surprisingly unaffected by parasite exposure and infection. Rather, it appears that microbiota-host interactions prior to parasite exposure are a key mechanism regulating resistance to infection.

Introduction

There is an increasing awareness of the importance of host-associated microbial communities in health and disease of an organism (Kamada et al., 2013; Sekirov et al., 2010). In particular, the intestinal gut microbiota not only interacts closely with the host itself (Chu and Mazmanian, 2013; Hooper, Littman, and Macpherson, 2012; Thaiss et al., 2014), but also interferes directly or indirectly with enteric infections (Kamada et al., 2013; Stecher and Hardt, 2011). If, at the time of the parasite encounter, the microbial community is fully established, infection by a parasite can be viewed as an invasion process into this community — a process that can be considered in the light of ecological theory (Costello et al., 2012; Foster, Krone, and Forney, 2008; Shea and Chesson, 2002; 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, as, for instance, those represented by different sites within the host (Stacy et al., 2016). On the other hand, infection success also depends on the parasite's ability to overcome the colonization resistance of the microbiota (Buffie and Pamer, 2013; Sekirov and Finlay, 2009; Stecher and Hardt, 2011). Colonization resistance exerted by the gut microbiota can occur through various means: For example, through direct interactions between the parasite and gut microbes, through competition 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 colonization resistance are known across many different insect species, but the specific underlying microbial sub-constellation that mediates this resistance is generally difficult to uncover and has only rarely been described. In *Anopheles* mosquitos, infection success by the *Plasmodium* parasite is not directly affected by the microbiota; however, the expression of the host immune system (Boissiere et al., 2011; Dong, Manfredini, and Dimopoulos, 2009), the parasite life cycle, and thus ultimately *Anopheles* vector competence, is influenced by co-infecting bacteria (Gonzalez-Ceron et al., 2003; Pumpuni et al., 1993). Experimental evidence shows 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* parasasite to establish (Sant'Anna et al., 2014). While these examples illustrate that certain microbial species can modulate the establishment success of the parasite, in locusts, the microbiota community acts as a unit, as increased gut microbiota diversity is more protective and reduces the colonization ability of the bacterial pathogen *Serratia marcencens* (Dillon et al., 2005). In order to understand how changes in microbiota composition affect infection outcome, it is necessary to understand the interaction pattern of parasite and microbiota.

In the bumblebee, *Bombus terrestris*, a primitively social insect, the microbiota reduces infection probability and intensity of the trypanosome gut parasite *Crithidia bombi* (Koch and Schmid-Hempel, 2011b). Generally, the mi-

crobiota does not only protect the bee, 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. However, in which way microbiota organization underlies this resistance has not yet been established. To resolve this, we first need to determine whether the interaction between the parasite and the microbiota takes place between the parasite and specific members of the microbiota, or with the microbiota as a whole, e.g. by variation in community diversity.

We aimed at uncovering possible structural properties of the microbiota that can prevent establishment of the infection by the parasite. Even though variation in infection outcome will ultimately be determined by an interaction of several components, such as the actions of host, microbiota, and parasite, we focus here on the changes in microbiota composition once perturbed by the parasite and how this relates to colonization resistance. Specifically, we investigated whether there are any properties of the microbiota composition prior to parasite exposure that are predictive of infection outcome. Complementary to this, we assessed whether the gut microbiota composition post-exposure could provide information on the infection outcome. Finally, we assessed the robustness of the microbiota composition, which in ecology is sometimes known as resilience (Shade et al., 2012), by analysing the change from before parasite exposure to after infection establishment.

Materials & Methods

Bee colonies and parasite infections

Thirty-three laboratory colonies were raised from healthy *Bombus terrestris* queens field-caught in spring 2014, from two populations in Switzerland (Neunforn and Aesch). Prior to being selected for the experiment, all queens were thoroughly inspected under the microscope for infections with parasites and only healthy colonies were used in the experiment. Throughout the experiment the bees were kept under standardized conditions ($28 \pm 2\,^{\circ}\mathrm{C}$, 60% RH, constant red-light illumination) and provided with *ad libitum* pollen and sugar water (ApiInvert). An illustration of the experimental design can be seen in Figure 3.1. Twelve mature workers per colony were selected at random for treatment. From this time-point onwards, all handling materials were either autoclaved or sterilised by washing with 80% ethanol before use. Also, only filter-sterilised sugar water (pore size $0.2\,\mu\mathrm{m}$) and X-ray radiated pollen (dose: $26.7\,\mathrm{kGy}$) was given to the experimental bees. Before bees were placed into the sterile housing environment, a faecal sample was collected from each bee. The following day, after a $2\,\mathrm{h}$ starvation period, eight arbitrarily chosen workers per colony received an invective dose of 10'000 parasite cells in $10\,\mu\mathrm{l}$ sugar water [50%]. This inoculum consisted of a parasite "cocktail" made up of five genetically distinct *Crithidia*

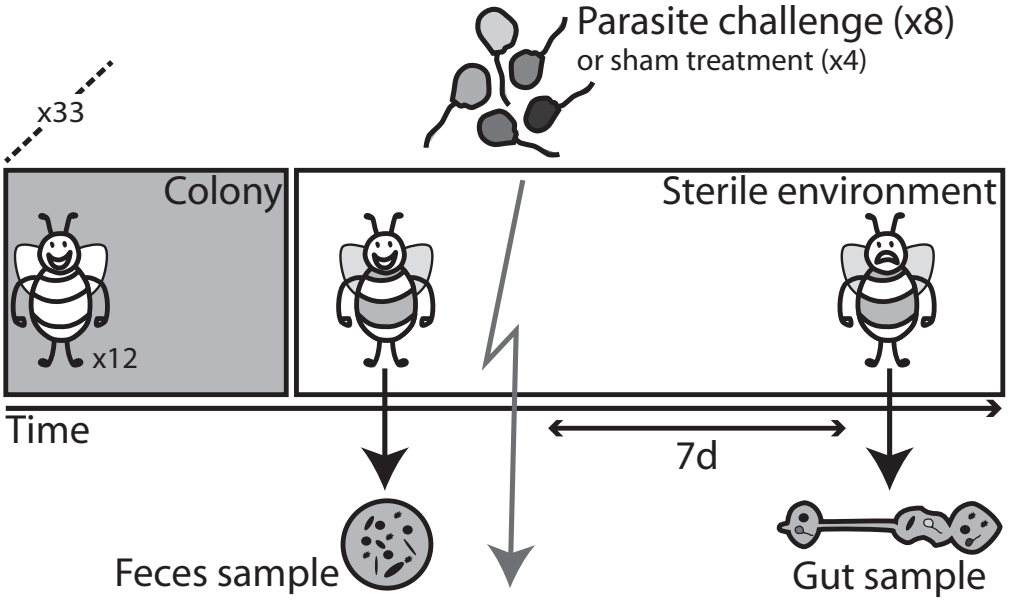

Figure 3.1: Schematic of the experimental design. Shown is the workflow over time for one focal bee, with an indication of the number of replicates per colony (x8, x4). The entire setting was repeated in 33 colonies (x33). The focal worker matures within its natural social environment (colony, grey zone), before it is removed and kept individually in a sterile environment (clear zone). A faecal sample is taken before the parasite challenge or sham treatment. Bees were sacrificed seven days post-infection and the gut dissected for analysis of the microbiota.

bombi strains (strain IDs 08.068, 08.075, 08.091, 08.161, 08.192) with 2'000 cells each. At the same time, the remaining four workers per colony received a "sham" treatment, i.e. an inoculum prepared in the same way but without parasite cells. Seven days post infection, long enough for infections of *C. bombi* to establish (Schmid-Hempel and Schmid-Hempel, 1993), the bees were killed and kept frozen at $-20\,^{\circ}\mathrm{C}$ (Figure 3.1). DNA was extracted from both the faecal samples and aseptically dissected guts with the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's protocol, apart from DNA, which was eluted in 100 μl AE buffer. Note that, for technical reasons, the microbiota was assessed from faeces before infection (or "sham" treatment), but from the gut after infection. This will be discussed below.

Colony resistance profiles

The infection intensities in the gut sample of a given bee, i.e. total number of parasite cells present seven days post-infection, was determined by measuring the parameters from quantitative real-time PCR on a 7500 FAST RT-PCR System (Applied Biosystems). Quantification to estimate the total number of parasite cells was achieved using a calibration curve method, where samples were prepared in the same manner from DNA extractions but with a known number of *C. bombi* cells. All PCR-samples were run in triplicates, following the cycling protocol described in Ulrich, Sadd, and Schmid-Hempel (2011) in a total reaction volume of 10 μl containing: 0.2 μl [10 μM] of each forward and reverse

primer, 2 μl of 5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne) and 2 μl of DNA template (1:10 diluted).

We genotyped the gut samples at five *C. bombi* microsatellite markers amplified in two multiplexed PCR reactions with the primers Cri4G9, Cri4, Cri2F10 and Cri16, Cri1B6, respectively (Schmid-Hempel and Reber Funk, 2004) in order to evaluate infection richness defined as the number of different strains present seven days post-infection. A 10 μl PCR reaction volume contained: 2 μl of 5x Colorless GoTaq®Reaction Buffer (Promega), 0.5 μl of dNTPs [2.5 mM each], 0.2 μl (Cri4, Cri2F10) and 0.25 μl Cri4G9 [10 $\mu\mathrm{M}$] or 0.2 μl (Cri16) and 0.1 μl (Cri1B6) [10 $\mu\mathrm{M}$] of each forward and reverse primer, respectively, 0.05 μl GoTaq DNA polymerase (Promega, 5 $\mathrm{U}/\mu\mathrm{l}$) and 2 μl DNA template. A total of 38 PCR cycles were performed with the following steps: denaturation (94 $^\circ\mathrm{C}$, 30 s), annealing (48 or 58 $^\circ\mathrm{C}$, respectively, 30 s) and extension (72 $^\circ\mathrm{C}$, 30 s). An initial denaturation step of 5 min at 94 $^\circ\mathrm{C}$ proceeded the 38 cycles and a final extension step for 10 min at 72 $^\circ\mathrm{C}$ concluded the cycling protocol. PCR products were run on a 3730xl DNA Analyzer (Applied Biosystems), the results scored twice independently and blind to the treatment using Peak ScannerTM software v1.0 (Applied Biosystems).

16S amplicon library preparation and sequencing

For deep sequencing, we amplified the variable region V3–V4 of the 16S rRNA gene with universal primers (Klindworth et al., 2013; Liu et al., 2011) (Table S3.1) and generated a multiplexed amplicon paired-end library for sequencing on the MiSeq®Illumina platform. From this region, we used three primer pairs containing frame-shifting nucleotides (in 1 nt increments) between the region-specific part and the Illumina overhang adapter; this was to increase the general sequence diversity of the so generated 16S amplicon libraries (Table S3.1) (Lundberg et al., 2013). We followed the manufacturer's suggested two-step amplification workflow.

An initial PCR was set up for each sample and each primer pair as follows: 1.5 μl [10 $\mu\mathrm{M}$] forward and reverse primer mix, 12.5 μl 2x KAPA HiFi Hot Start Ready Mix and 2 μl DNA template in a total reaction volume of 25 μl. The cycling protocol used a 5 min initial degradation step at 95 $^\circ\mathrm{C}$, followed by 22 cycles (for faecal samples) or 25 cycles (for gut samples) of 98 $^\circ\mathrm{C}$ for 20 s, 58 $^\circ\mathrm{C}$ for 15 s and 72 $^\circ\mathrm{C}$ for 15 s and finished with a final elongation step for 5 min at 72 $^\circ\mathrm{C}$. 22.5 μl of each of the four independent PCR reaction products were pooled and purified using Agencourt AMPure XP beads (Beckman Colter) with a ratio of 0.8:1 (beads to PCR product) and eluted in 27.5 μl [10 mM, 8.5 pH] TrisBuffer.

In a second limited-cycle, PCR indices and sequencing adapters were attached to the template libraries. A total reaction volume of 50 μl contained 15 μl of the purified PCR product, 25 μl 2x KAPA HiFi Hot Start Ready Mix and 5 μl of each forward and reverse primers of the Nextera®XT Index Kit v2 (Illumina). The indexing cycle protocol used a 3 min initial denaturation step at

95 °C followed by 10 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C and 5 min final extension step at 72 °C. Again, the now indexed amplicon libraries were purified using Agencourt AMPure XP beads (Beckman Colter) with a ration of 0.8:1 (beads to amplicon product) and eluted in 32.5 μl [10 nM, 8.5 pH] TrisBuffer.

Amplicon library quality and library fragment size were checked for 10 randomly selected samples on a Bioanalyzer (Aligent Technologies) DNA HS 1000 chip. All libraries were 1:10'000 diluted and quantified in triplicates by quantitative PCR using the 2x KAPA SYBER qPCR Ready Mix on an ABI 7500 Real-time PCR System (Applied Biosystems). We used the Library Quant Illumina Kit (KAPA Biosystems) with quantification standards ranging from 0.002 pM to 20 pM for absolute quantification. Following quantification, equimolar amounts of all libraries were pooled. One gut sample was excluded from pooling, as the concentration of this amplicon library was lower than the negative control. We purified our pooled library using Agencourt AMPure XP beads (Beckman Colter) with a ratio of 0.8:1 (beads to library pool). The final, multiplexed pool was quantified by qPCR as previously described. We followed the manufacturer's instructions (Preparing Libraries for Sequencing on the MiSeq® #15039740 Rev. D) to prepare the library for paired-end sequencing. We loaded a final library concentration of 17.5 pM and a 5% PhiX spike-in with MiSeq® Reagent Kit v3.

Amplicon processing and OTU clustering

A total of 16'234'006 (from faeces) and 19'302'370 (from guts) paired-end raw reads, respectively, were generated by two 600-cycle MiSeq® runs. The quality of reads was checked with FastGQ (v0.11.2). We found that 92.7% of the faeces and 89% of the guts paired reads successfully merged using FLASH (v1.2.9) with the following specifications: minimum overlap of 20 bp, maximum overlap of 280 bp and maximum mismatch density of 0.25. Primer sequences were trimmed from the merged reads using cutadapt (v1.5) requiring a full-length error-free overlap but allowing for wildcards.

Merged and primer trimmed reads were quality filtered with PRINSEQ-lite (v0.20.4). In total 88.9% (faeces) and 90% (guts) of the reads, respectively, passed filtering, given a minimal fragment length of 200 bp, a minimum quality mean of 20, maximally one ambiguous nucleotide, and low complexity sequences were filtered at a threshold of 10 (dust).

Operational taxonomic unit (OTU) clustering was performed with the pooled merged and trimmed reads using the UPARSE-OTU algorithm (usearch v7.0.1090_i86linux64) (Edgar, 2010, 2013). For this, the dataset was sorted (option: sortbylength), de-replicated (derep_fulllength), abundance sorted (sortbysize; size = 2), and clustered (cluster_otus, minimum identity = 97%). The clustering step detects and removes chimera sequences. In addition, we also applied a reference based chimera removal step using the Green Genes database (version May 2013, `http://greengenes.secondgenome.com`).

The usearch/UPARSE (v8.0.1623) OTU clustering workflow resulted in 173

OTUs. In all, 98.3% of the reads could be successfully mapped back to OTU reference centroid sequences (usearch_global; id = 97%). Taxonomic information was assigned to OTUs using usearch/utax (v8.0.1623). Four OTUs could not be classified and were thus discarded from further analysis (922 reads). The phylogenetic tree for the OTUs was built using PyNAST (Caporaso et al., 2010a) and FastTree (Price, Dehal, and Arkin, 2010) as implemented in Qiime (v1.8.0) (Caporaso et al., 2010b). Control samples from sequencing reactions and one faecal sample missing a matching gut sample were excluded from further analysis, as well as one individual from the control treatment group, where an infection was detected of unidentified origin. Thus, the raw dataset contained 186 samples in total with a total of 28'124'906 reads mapping to a total of 165 OTUs.

Evaluation of sequence data

The number of OTUs detected in a sample generally increases the number of reads included in the analysis. To estimate the effects of differing sequencing depths, we constructed rarefaction curves (number of OTUs as a function of number of reads) from our data. For this, and for each sample, we randomly resampled the pool of reads without replacement ten times at different sampling depths, which reflects different sample sizes. The rarefaction curves generated in this way suggested that we had achieved adequate sequencing depth to reflect sample complexity. As shown in SI Figure S3.1, the alpha diversity (Shannon Index) of OTUs identified for a given sampling depth quickly reached a plateau. Also, the majority of different OTUs within most samples were discovered, as indicated by plotting sample species richness (number of different OTUs) as a function of subsampling depth. Based on the rarefaction analysis, two samples with small sequencing depths (n = 801 and n = 1429 reads) were excluded from further analysis. All samples were rarefied to the smallest library size (n = 26'452 reads) and this process was replicated one hundred times to assess effects of the rarefaction process. As such, a rarefied dataset contained 134.61 different OTUs (SD = 3.99) on average. All statistical analyses were performed on these rarefied datasets in order to ensure equal sample sizes across samples.

Comparing faeces and gut microbial samples

For technical reasons, the microbiota was sampled non-destructively from faeces before exposure to parasites, but from the guts (destructively) after exposure. The main questions analysed in this study were tested within each sample type — this is, the microbiotas were compared within the faeces samples, or within the gut samples. The investigation of robustness of the microbial community to parasite exposure, as assessed by faecal samples from before the challenge to gut samples from after the challenge, required a general assessment of the comparability of microbiota communities of faecal samples and gut samples. We formally investigated this by looking at the number of OTUs within an individual that occurred in both the faecal and gut samples of individuals that received the "sham" treatment (control). From this analysis, we identified a "core" set of OTUs (i.e.

shared OTUs) that included all OTUs that were detected at least once in both the faecal and gut sample of any individual irrespective of the treatment group.

Statistical analyses

We performed all statistical analyses on the rarefied datasets (n = 100 sets). Thus, if not otherwise indicated, statistical reports are the averages across these replicates. We investigated microbial community structures of faecal samples (before infection) and gut samples (after infection). The aim of the former was to identify differences in microbiota structures indicative of infection outcome. The aim of the latter was to identify microbiota structures of non-infected (healthy) and infected individuals. For this, we calculated the Shannon Index and species (OTU) richness (number of different OTUs) for each sample — once with the complete OTU set, and once only with the "core" set. To test if microbiota diversity or richness affects infection outcome, we fitted a linear regression model to the diversity indices. Because infection outcome was not a binary response but also measured as the resulting infection intensity and infection richness (Figure 3.1), classifying individuals as susceptible or resistant did not capture the complexity of the full resistance profile. We thus collapsed, by principle component analysis, the two-dimensional resistance profile (i.e. infection intensity and infection richness) into a one-dimensional variable (principle component 1), where the PC1 represented 80.27% of the total variation in resistance. We then subsequently used PC1 as our continuous variable describing infection outcome. If a significant effect of microbiota diversity on PC1 was found, we further looked whether infection outcome was explained by a function of OTU abundance for each OTU of the "core" set. For this we fitted a linear regression model to the PC1 and the log transformed abundance + 1 of an OTU. We then compared this model to a model with only an intercept using a chi-squared likelihood ratio test. We applied this to each OTU in each rarefied dataset and counted the number of times a significant amount of variation in infection outcome was explained by abundance variation of a particular OTU.

To assess the robustness of microbiota community composition, we investigated three measures using linear regression models. First, we analysed the change in Shannon Index from before infection (faecal sample) to after infection (gut sample) in order to quantify changes in the complexity of microbiota structures attributed to infection outcome. Second, we calculated Euclidean distances between the faecal microbiota composition to the gut microbiota composition of an individual that captures the overall amount of change. Third, we calculated Bray-Curtis distances that quantify the compositional dissimilarity of the microbial community before and after infection of an individual.

Results

Colony resistance profile

When workers were exposed to the "cocktail" of five genetically distinct *C. bombi* parasite strains (i.e. genotypes), we observed varying levels of resistance among the colonies. In particular, the combined increase in infection intensity (i.e. the total number of parasite cells in the host) and infection richness (i.e. the number of different parasite strains that had established and were recovered seven days post infection) was considered to reflect an increased susceptibility towards *C. bombi* (Figure 3.2). Given this natural variation in infection outcomes, we arbitrarily selected eight colonies for subsequent microbiota sequencing (four from each of the populations in Aesch and Neunforn, respectively) covering the whole range of susceptibilities (Figure 3.2).

Comparing faecal and gut microbial communities

To assess how the microbial communities from faecal and gut sample of the same individual compare to one another, we first analysed the rarefied microbiota of the control individuals (n = 29 workers; Figure 3.3). There was no difference in the average number of OTUs (richness) found in the faecal (14.49 ± 3.33 SD OTUs) vs. the gut (13.30 ± 4.06) microbial community of the same individual (paired t-test: $t(28) = 1.32$, $p = 0.197$). On average, 7.37 ± 1.53 (SD) OTUs were shared between the two sample types within one individual. Interestingly, the same was true for the individuals that had been exposed to the parasites. In those, a similar number of OTUs (7.41 ± 1.77 SD) were shared, and there was also no difference between the average number of OTUs found in the faecal (14.40 ± 3.93 SD) and gut microbiota (13.95 ± 3.78 SD) of the same individual (paired t-test: $t(61) = 0.61$, $p = 0.548$; Figure S3.2).

In all, we identified a total of 165 OTUs that could potentially be shared between the faecal and the gut microbial community of an individual. Of those, only a subset of 31 OTUs were identified in the control individuals. Only seven OTUs were shared in more than 50% of the control bees. Similarly, 35 OTUs were shared in individuals exposed to the parasite. Overall this resulted in 37 OTUs that were identified as shared in individuals irrespective of their treatment. This pattern, and whether an OTU was observed in both sample types, reflects the distribution of reads among the OTUs within a sample type (Figure 3.4). Based on this, we identified the 37 shared OTUs as the "core" species of the microbial community, which together represented 99.94% (CI = 0.001%, n = 100) of the total number of reads. The majority of these OTUs are ascribed to the class of *Bacilli* (n = 14) or *Alphaproteobacteria* (n = 9). A minority of them classify as *Gammaproteobacteria* (n = 7), *Betaproteobacteria* (n = 3) and *Actinobateria* (n = 2). Finally, there was one representative each of the Phylum of *Bacteroidetes* (n = 1) and *Acidobacteria* (n = 1) (Table S3.2).

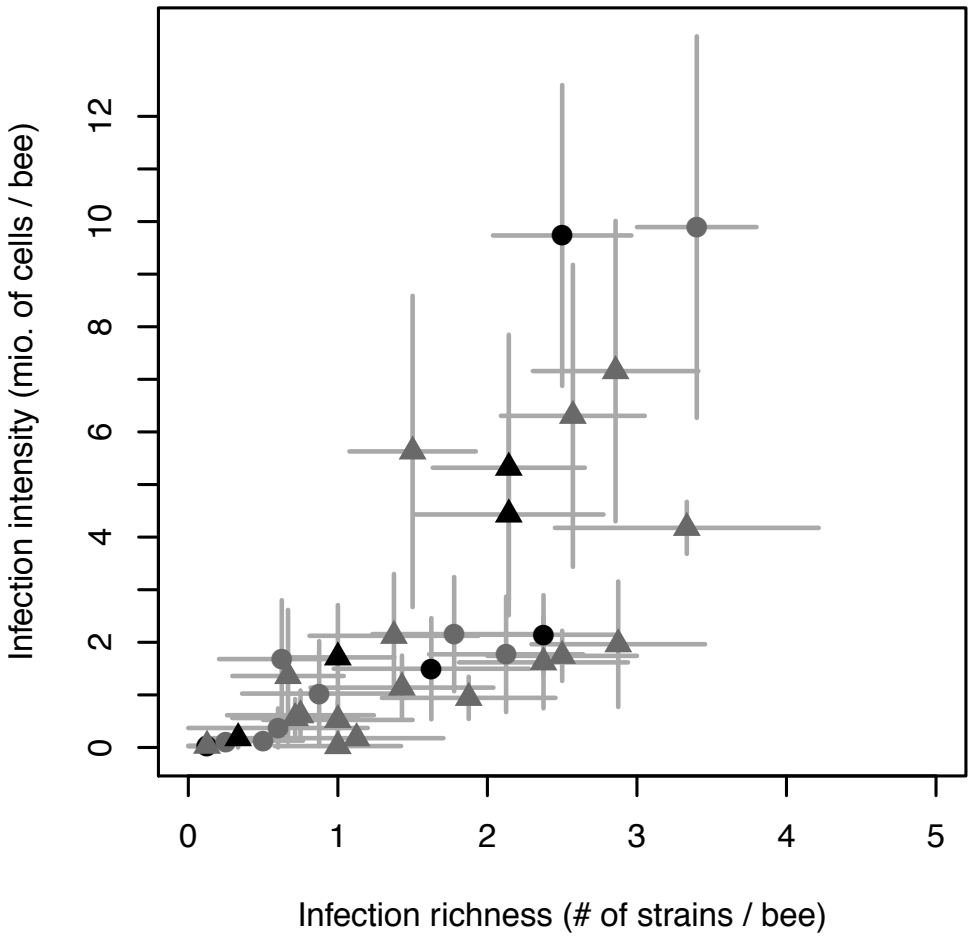

Figure 3.2: Natural variation in resistance profiles of colonies. Mean infection intensity per colony plotted against a mean infection richness, seven days post infection (Spearman's $\rho = 0.82$, $p < 0.001$, $n = 33$ colonies, bars are SEM). The inoculum contained five genetically distinct strains. Black filled symbols ($n = 8$) highlight the colonies selected for the analysis of the microbial community of faecal and gut samples (circles: colonies from Aesch; triangles: colonies from Neunforn).

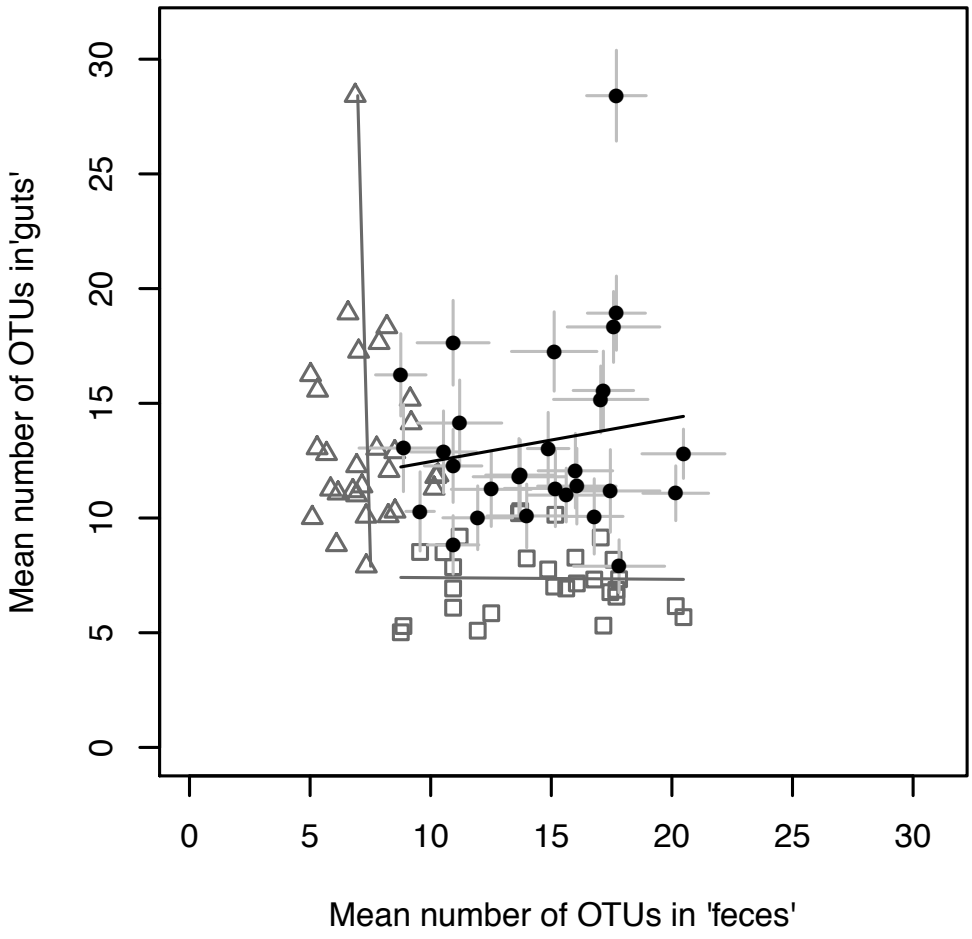

Figure 3.3: Number of OTUs in the faecal and gut microbial communities. Each filled circle represents an individual bee (n = 29) and plots the average number of different OTUs found in the gut sample against the average number of different OTUs in the faecal sample. Point values are the mean over the 100 independently rarefied datasets. Error bars show the SD. Grey symbols show the same individuals, but plot the number of OTUs shared between the microbial community of faeces and guts against the total number of OTUs found in either faeces (diamonds) or guts (triangles). The drawn regression lines all show statistically non-significant relationships.

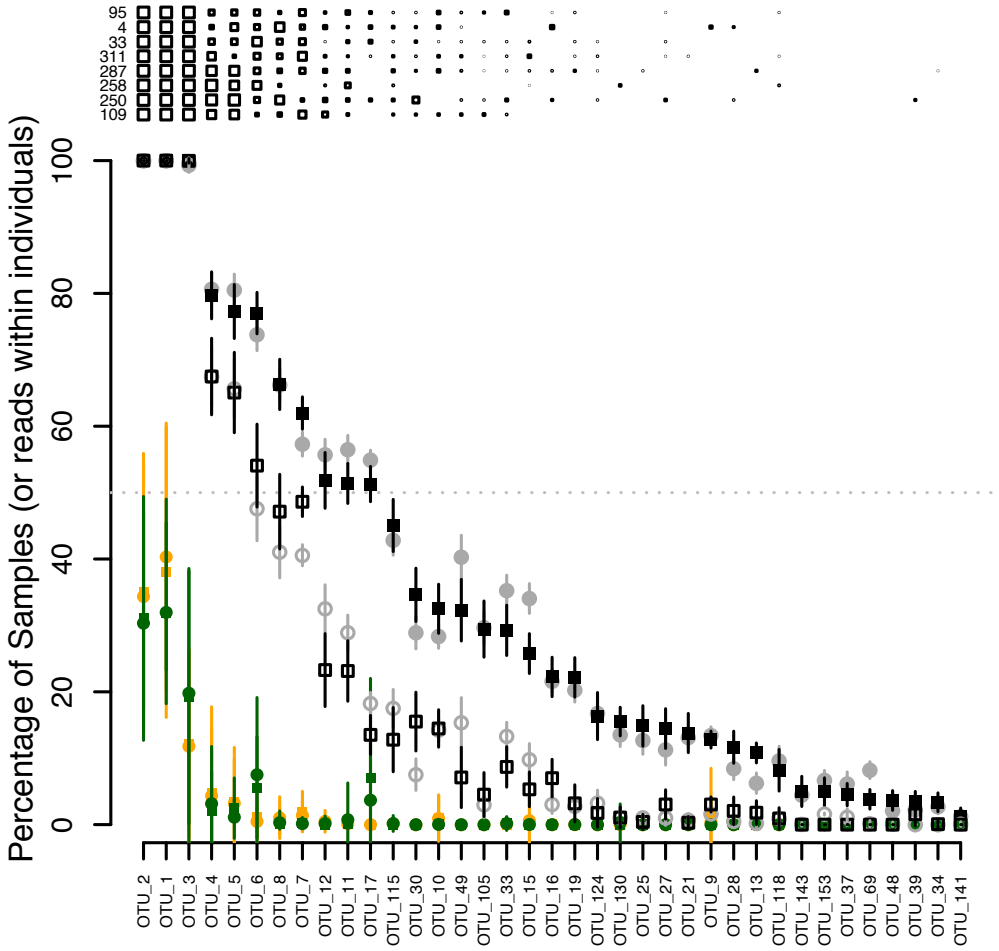

Figure 3.4: Shared OTUs between faecal and gut microbial communities. The graph shows the average percentage of individuals that shared a particular OTU in their faecal and gut microbial communities. Individuals are split by treatment groups (circles: parasite challenged, n = 62; diamonds: controls, n = 29). Values are the means of the average percentages across the rarefied datasets (mean of n = 100). Empty symbols show the percentage of samples where OTUs were shared within an individual. Filled symbols show the percentage of samples where the OTU was found in either sample type. The size of the squares at the top represents the percentage of bees within the eight colonies (rows) where an OTU was shared in unexposed individuals. The average abundance of the OTUs within a sample is plotted in colour and split by sample type (yellow: guts, green: faeces) and treatment (circles: exposed, squares: control). Error bars represent SD.

Faecal microbiota prior to infection: does it predict infection outcome?

We analysed the faecal microbiota community of individual workers prior to parasite exposure in order to identify any properties of the microbiota that are predictive of the infection outcome. Resistance (represented by the PC1 of infection intensity and infection richness) did not vary as a function of total OTU richness (linear regression model (lm); $F_{1,59} = 0.18$, $p = 0.748$, $\beta = 0.006 \pm 0.040$ SE, adj. $R^2 = -0.01$) or number of "core" OTUs (lm; $F_{1,59} = 0.84$, $p = 0.452$, $\beta = -0.07 \pm 0.08$ SE, adj. $R^2 = -0.003$). However, individuals with a faecal microbiota community that was more diverse (i.e. had a higher Shannon Index) had higher susceptibility to the parasites (i.e. higher PC1-score, lm; $F_{1,59} = 6.53$, $p = 0.013$, $\beta = 1.48 \pm 0.58$, adj. $R^2 = 0.08$). This effect is more pronounced when only "core" OTUs are considered (Figure 3.5), suggesting that "non-core" OTUs introduce noise and obscure the relationship. Candidate OTUs that may be responsible for mediating this effect were identified by fitting a linear model to OTU abundance data and PC1 (i.e. infection outcome). Only two OTUs showed a consistent effect of infection outcome on the variation in OTU abundance across all rarefied datasets (Figure 3.6).

Microbiota of infected vs. non-infected individuals

To identify any properties of the microbiota that can distinguish uninfected (resistant) from infected workers (susceptible), we analysed the gut microbiota community of individual workers at a time when an infection would have been established (7 d post-exposure (Schmid-Hempel and Schmid-Hempel, 1993)). Overall, no differences could be detected. Neither total OTU richness varied with variation in resistance (lm; $F_{1,60} = 0.43$, $p = 0.609$, $\beta = 0.02 \pm 0.04$ SE, adj. $R^2 = -0.01$), nor did the number of "core" OTUs (lm; $F_{1,59} = 0.84$, $p = 0.452$, $\beta = -0.07 \pm 0.08$ SE, adj. $R^2 = -0.003$). Furthermore, infection outcome did not differ as a function of the microbiota diversity (Shannon-Wiener Index), irrespective of whether diversity was calculated using complete OTU information (lm; $F_{1,60} = 0.34$, $p = 0.563$, $\beta = 0.29 \pm 0.51$, adj. $R^2 = -0.01$), or whether the Shannon Index was calculated only from "core" OTUs (Figure 3.7). Even though relationships were not significant, the trend was in the same direction as with the microbiota analysed prior to infection in that microbiota diversity correlated with higher susceptibility to infection (Figure 3.7). The fact that the prior characteristics of the microbiota correlate with later infection outcome, but that the microbiotas did not differ anymore once an infection has established, suggests that a change in the microbiota composition of at least some of the individuals had occurred.

Microbiota robustness: quantify microbiota change in response to parasite exposure

We assessed the robustness of microbiota to parasite invasion by analysing the change in microbiota composition from before exposure to after parasite infec-

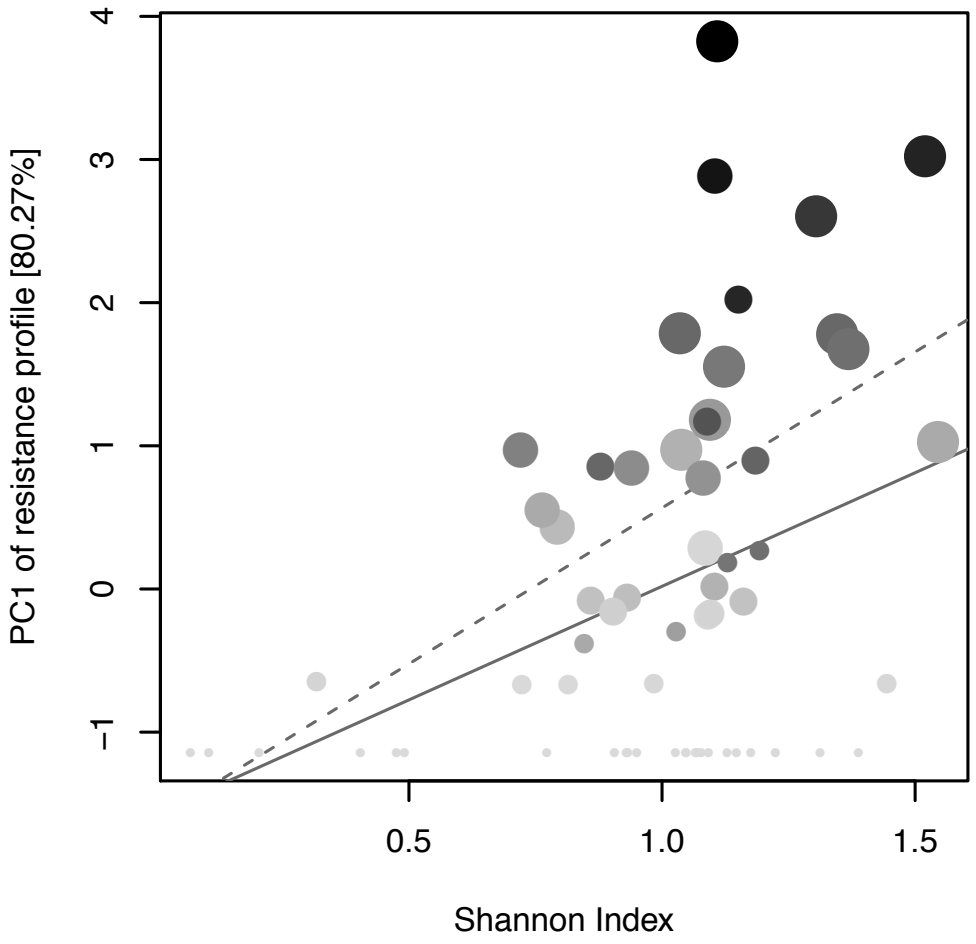

Figure 3.5: Diversity of the "core" faecal microbiota correlates with infection outcome. The variation in resistance, shown as the PC1-score, increases with the Shannon Index of the "core" faecal microbiota for each sample before the parasite challenge (lm; $F_{1,59} = 8.94$, $p = 0.007$, $\beta = 1.53 \pm 0.52$, adj. $R^2 = 0.12$). Point size is proportional to infection intensity, and darker fill colour indicates a higher number of parasite strains (data measured seven days post-infection). The solid line is the fitted linear regression for all individuals, the dashed line excluding resistant individuals.

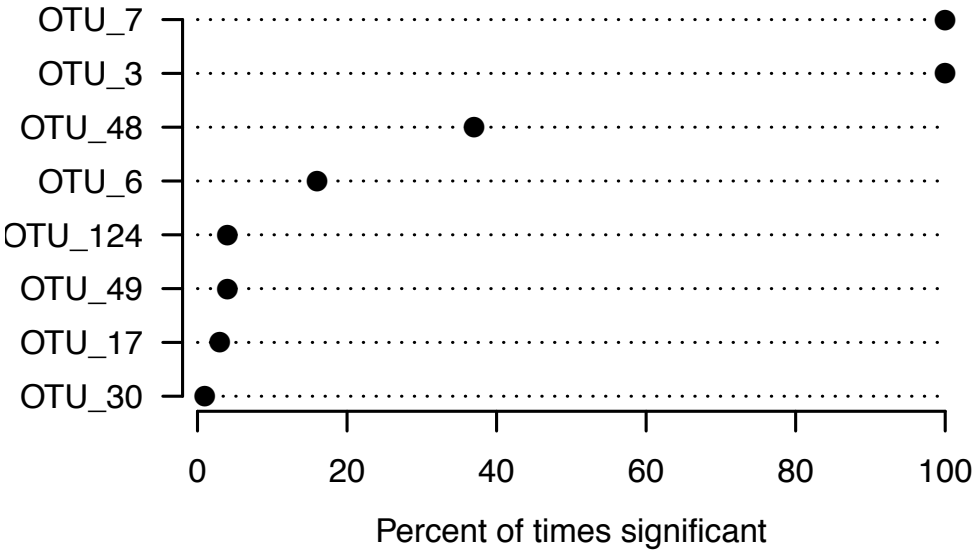

Figure 3.6: OTU abundances affected by infection outcome. Shown is the fraction of rarefied datasets where infection outcome (i.e. PC1-score) explained a significant amount of variation in individual OTU abundances.

tion. We used two distance measures and a diversity index to test for an effect of infection outcome. However, no measure of change in the microbiota composition was explained by infection outcome (Table 3.1). On the one hand, changes in the complexity of microbiota, i.e. difference in Shannon Index from before to after infection, did not vary as a function of infection outcome (Table 3.1). On the other hand, neither did the overall distance of the microbiota composition (Eucledian), nor the compositional dissimilarity of microbiota (Bray-Curtis) from before infection to after infection (Table 3.1).

Table 3.1: Linear regression models quantifying change in microbiota as a function of infection outcome.

Model	$F_{1,59}$	p-value	SE	adj. R^2
Shannon Index				
complete OTU set	1.51	0.224	-0.05 ± 0.04	0.008
"core" OTU set	1.75	0.223	-0.04 ± 0.04	0.01
Euclidian distance				
complete OUT set	1.3	0.25	548.08 ± 475.84	0.005
"core" OTU set	2.54	0.124	724.53 ± 458.08	0.02
Bray Curtis distance				
complete OTU set	1.31	0.257	0.02 ± 0.02	0.005
"core" OTU set	2.09	0.160	0.02 ± 0.02	0.02

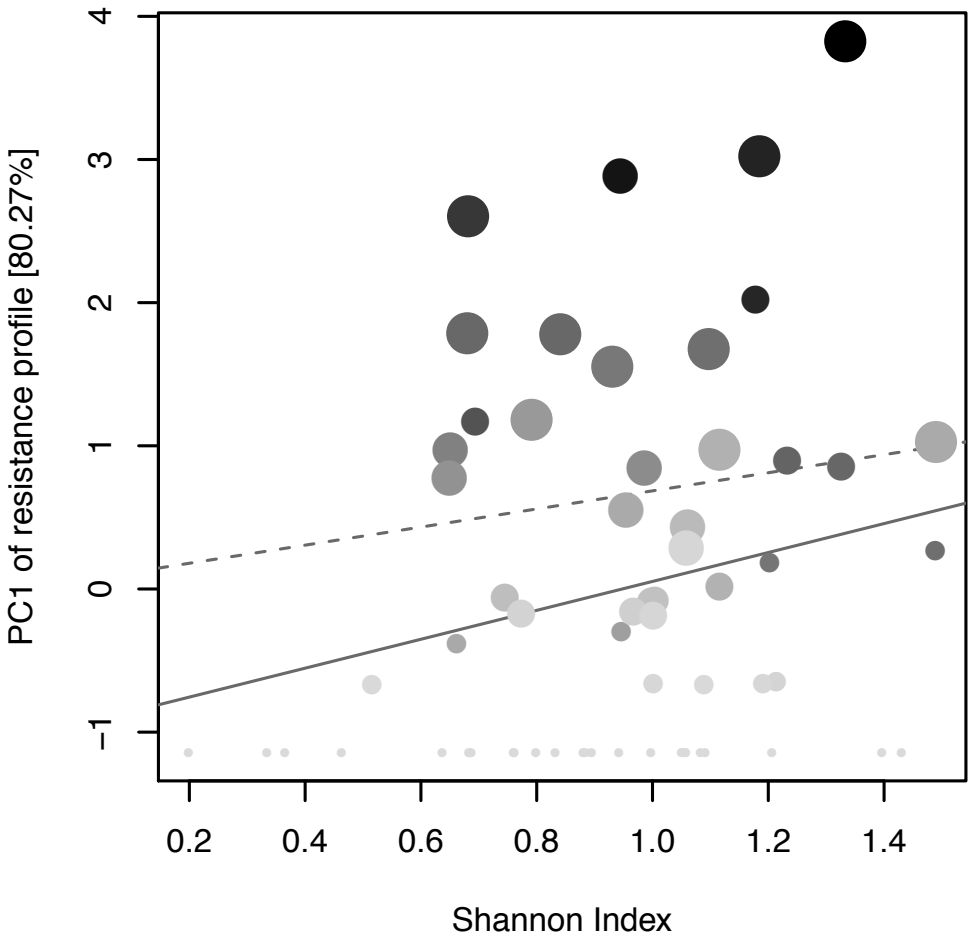

Figure 3.7: Infection outcome as a function of Shannon Index of "core" gut microbiota. The variation in resistance is shown as a function of the Shannon Index of the "core" gut microbiota for each sample after the parasite challenge. The PC1-score, i.e. susceptibility, shows no significant dependence on the Shannon Index (lm; $F_{1,59} = 2.15$, $p = 0.172$, $\beta = 0.85 \pm 0.59$ SE, adj. $R^2 = 0.02$). Larger points represent higher infection intensity and more intense fill colours indicate a higher number of parasite strains found seven days post-infection. The solid line is the fitted linear regression for all individuals, the dashed line excluding resistant individuals.

Discussion

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

We found that variation in diversity of the microbiota community composition prior to infection correlated with infection outcome. In particular, individuals more resistant to parasite exposure harboured a less diverse microbiota, as quantified by the Shannon Index. This suggests that bees with a skewed microbiota abundance distribution — composed of a few highly abundant OTUs ("species") and many rare species — may be more protected against parasite invasion (i.e. harbour a stronger colonisation resistance potential) than bees with a more even microbiota abundance composition. Overall, microbiota diversity explained a significant 12% of the variation in infection outcome. 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, for example, general stress (Brown, Loosli, and Schmid-Hempel, 2000; Brown, Schmid-Hempel, and Schmid-Hempel, 2003), environment (Sadd, 2011), genetics (Barribeau et al., 2014; Schmid-Hempel and Reber Funk, 2004), or diet (Brunner, Schmid-Hempel, and Barribeau, 2014). Thus, the observed fraction of explained variance is quite remarkable.

The finding that a less, rather than a more diverse microbiota is protective might seem counter-intuitive at first, given that community diversity is generally considered to increase robustness against perturbations or invasions (Girvan et al., 2005; Shea and Chesson, 2002). Nevertheless, some studies showed that a high species diversity in plant communities might simply reflect abiotic conditions, which are more rather than less, favourable for invasion (Levine, 2000; Levine and D'Antonio, 1999; Shea and Chesson, 2002). While it is as yet unknown what the corresponding factors might be in bumblebees, or other social insects, the positive relationship between diversity and resistance to invasion we find here may also be due to other factors co-varying consistently with diversity. The protective function of the microbiota seems thus to 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 mostly driven by OTUs that occur both in the faecal and gut sample of the same individual ("core" set). Consequentially, non-core OTUs do not appear important for colonization resistance. Because faecal and gut samples were collected with a time delay, these non-core OTUs may represent transient members of the microbiota that are less intimately linked to the host-parasite-microbiota interaction.

Furthermore, infection outcome co-varied significantly with the abundances of specific set of OTUs (Figure 3.6) and might be mediated in particular by representatives of the genera *Bifidobacerium* and *Lactobacillus*, and to a lesser extent by members of the classes of *Alpha-* and *Betaproteobacteria*. These candidate OTUs have appeared in previous studies in bees. They belong to the major taxa that are strongly associated with bee microbiota (Engel and Moran, 2013a; Koch and Schmid-Hempel, 2011a; Martinson et al., 2011). Moreover, they have previously been associated with a protective function against disease and usually occur at high abundances in bees (Cariveau et al., 2014; Koch and Schmid-Hempel, 2011a; McFrederick et al., 2012). Furthermore, a recent study on the variation of microbiota in wild bumblebees showed that an increased probability of harbouring a *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 (Cariveau et al., 2014). 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 the function of 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, suggesting 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-sensitive 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 the non-infected 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. However, statistically, no effect of infection outcome could be detected on the change of the microbiota composition within individual workers before and after an infection.

Our results suggest that characteristics of the microbiota associating with resilience in response to parasite exposure in bumblebees appear to be subtle, although our analysis based on two time points limits resolution to extract functional information. However, gut microbiota resilience has previously been observed in other systems. For example, in humans, 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 pre-treatment state in the long-term (Dethlefsen and Relman, 2011; Dethlefsen et al., 2008). The samples in our study were collected seven 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 and Relman, 2011; Dethlefsen et

al., 2008). Nevertheless, these and other results (Costello et al., 2012) suggest that gut communities are surprisingly resilient and that mechanism are in place that help the microbial community regain its initial equilibrium (Lozupone et al., 2012).

Given the statistical associations between the microbiota composition and colonization resistance, the question arises how and where the interaction may take place within a bee. The parasite, *C. bombi*, likely attaches to the gut wall (Lipa and Triggiani, 1980; Schmid-Hempel, 2001), which is also the habitat of many of the bacterial species of the microbiota (Martinson, Moy, and Moran, 2012). Hence competition for the gut wall niche could be one of the mechanisms of how infection outcome is influenced (Ulrich, Sadd, and Schmid-Hempel, 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 between the host, the parasite and the microbiota is organized (Foxman et al., 2008). Alternative 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 (Chapter 1). Such an effect could potentially even persist across generations (Chambers and Schneider, 2012; Sadd and Schmid-Hempel, 2009). Overall, the protective effects of a microbiota composition prior to parasite exposure suggest that, in principle, it should be possible to engineer more protective microbiota in bees.

Supplementary Information

Table S3.1: Amplicon Primers to generate V3–V4 16S template library.

Primer Name	Sequence 5′–3′
U341F_nex0	tcgtcggcagcgtcagatgtgtataagagacag**ga**CCTACGGGDGGCWGCA
U341F_nex1	tcgtcggcagcgtcagatgtgtataagagacag**Nga**CCTACGGGDGGCWGCA
U341F_nex2	tcgtcggcagcgtcagatgtgtataagagacag**NNga**CCTACGGGDGGCWGCA
U341F_nex3	tcgtcggcagcgtcagatgtgtataagagacag**NNNga**CCTACGGGDGGCWGCA
U806R_nex0	gtctcgtgggctcggagatgtgtataagagacag**ca**GGACTACHVGGGTMTCTATTC
U806R_nex1	gtctcgtgggctcggagatgtgtataagagacag**Nca**GGACTACHVGGGTMTCTAATC
U806R_nex2	gtctcgtgggctcggagatgtgtataagagacag**NNca**GGACTACHVGGGTMTCTAATC
U806R_nex3	gtctcgtgggctcggagatgtgtataagagacag**NNNca**GGACTACHVGGGTMTCTAATC

Lower case letters represent Illumina-specific overhang adapters, to which in a subsequent limited-cycle amplification step the sample specific indices and sequencing adapter attach. Frame-shifting nucleotides are indicated in bold uppercase and linker sequence in bold lowercase letters. The gene-specific sequences targeting 16S V3 and V4 region are shown in uppercase letters.

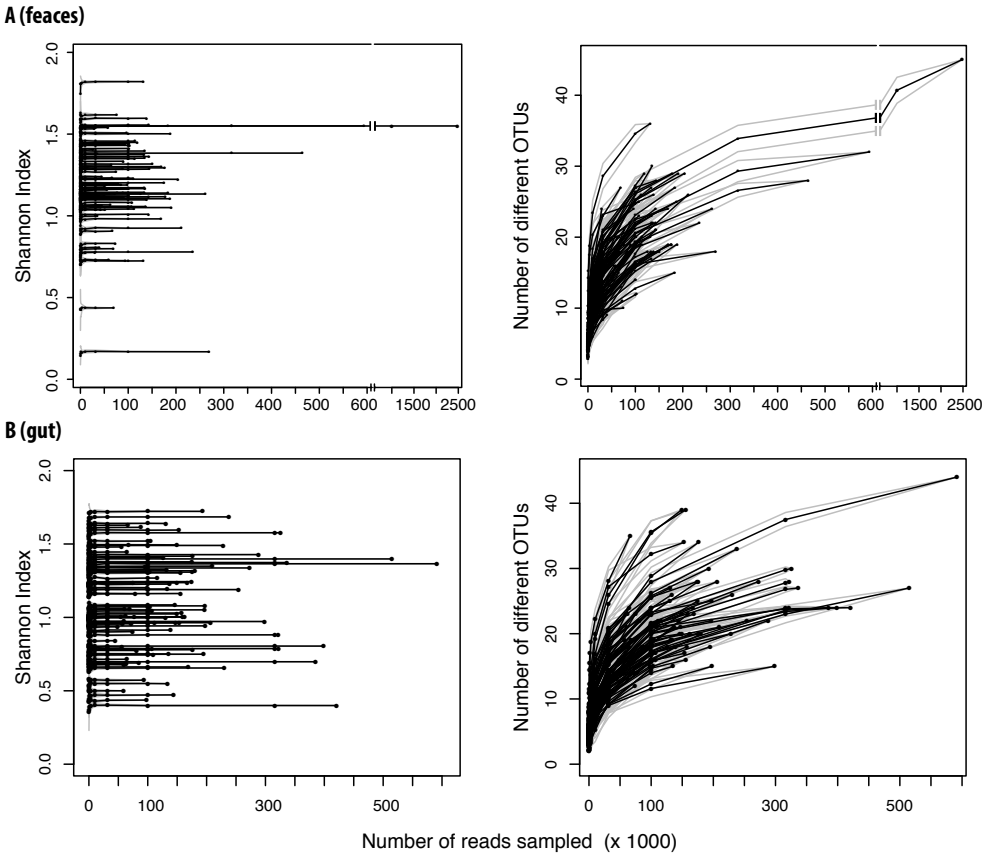

Figure S3.1: Rarefaction curves of each sample. Panels to the left show Shannon-Wiener diversity index, and panels to the right show number of different OTUs (richness) at given sub-sampling depth (x-axis) of the sequencing pool for both (A) faecal microbiota and (B) gut microbiota sequencing. Lines connect means (black) and SD (grey) of 10 independent draws at given sampling depth from the total sequencing pool of a particular amplicon library.

Table S3.2: Taxonomic classification of "core" OTUs

OTU	Phylum	Class	Order	Family	Genus
OTU_69	Acidobacteria	Holophagae	Holophagales	Holophagaceae	*Holophaga*
OTU_7	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*
OTU_10	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*
OTU_9	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Ornithobacterium*
OTU_39	Firmicutes	Bacilli	Bacillales	Bacillaceae_1	*Bacillus*
OTU_27	Firmicutes	Bacilli	Bacillales	Planococcaceae	*Planococcaceae_incertae_sedis*
OTU_130	Firmicutes	Bacilli	Bacillales	Thermoactinomycetaceae_1	*Laceyella*
OTU_15	Firmicutes	Bacilli	Bacillales	Thermoactinomycetaceae_1	*Laceyella*
OTU_4	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_6	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_3	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_33	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_17	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_16	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_124	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_153	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_143	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_141	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU_28	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium*
OTU_13	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Ensifer*
OTU_30	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*
OTU_11	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Stella*
OTU_19	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Stella*
OTU_48	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	*Rickettsia*
OTU_115	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sandaracinobacter*
OTU_12	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sandaracinobacter*
OTU_25	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
OTU_1	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	*Kingella*
OTU_49	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	*Kingella*
OTU_118	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	*Kingella*
OTU_21	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Arsenophonus*
OTU_5	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Xenorhabdus*
OTU_34	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	*Chromohalobacter*
OTU_8	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	*Marinomonas*
OTU_2	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	*Actinobacillus*
OTU_105	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	*Actinobacillus*
OTU_37	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	*Actinobacillus*

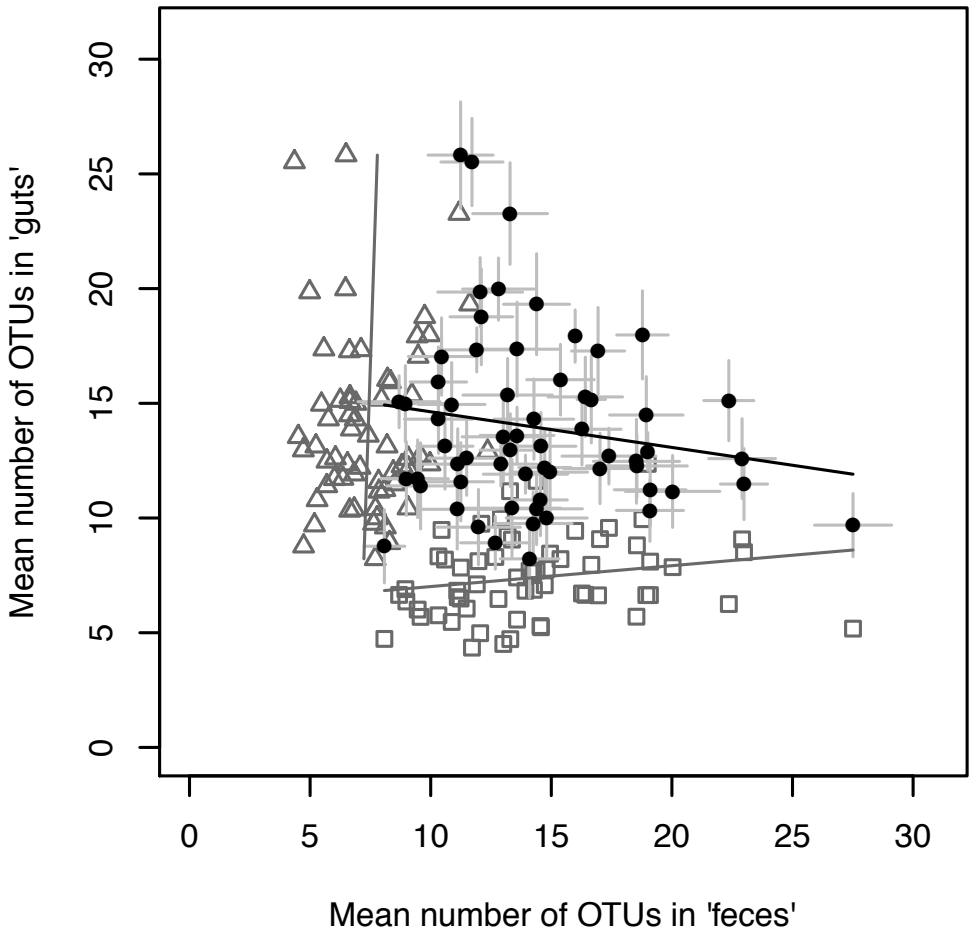

Figure S3.2: Number of OTUs in faecal and gut microbiota within one individual exposed to the parasites. Each filled circle represents an individual bee (n = 62) and plots the average number of different OTUs found in the gut sample against the average number of different OTUs in the faecal sample over the 100 independently rarefied datasets (SD, error bars). Grey symbols plot the number of OTUs shared between the microbial community of faeces and guts against the total number of OTUs found in either faeces (diamonds) or guts (triangles). The regression lines all show statistically non-significant relationships.

Chapter 4

The significance of vertical transmission: from the queen to the worker gut microbiota

Abstract

An understanding of the evolution of the intimate association of hosts and their gut microbiota benefits from insights into the transmission of these microbes from one generation to the next. As the sole survivors of the previous generation, *Bombus terrestris* bumblebee queens are expected to play a key role by vertically transmitting the host-specific gut microbiota to the next generation. Nevertheless, studies on the mode of microbiota transmission and queen microbiota composition are virtually absent. Here we analysed queen microbiota early and late in the colony's life cycle, as well as the microbiota that eventually established in worker offspring. We found that microbiota diversity increased over the course of the colony cycle. By comparing queen microbiota composition to worker microbiota we identified differences in measures of diversity. Furthermore, we found that the queen microbiota composition did not necessarily more closely resemble the microbiota that eventually established in their own worker offspring. However, the association between microbiota diversity and colony's resistance level found in workers could also be recovered in queens, but only late in the colony's life cycle. Our results suggest some dynamic component in microbiota composition in queens over the course of a colony's life cycle and thus do not support a solely vertically mediated transmission of gut microbiota composition.

Introduction

Host-associated microbial species are ubiquitous and often beneficial to host development and health (Dillon and Dillon, 2004; Engel and Moran, 2013b; McFall-Ngai et al., 2013; Robinson, Bohannan, and Young, 2010; Zilber-Rosenberg and Rosenberg, 2008). However, these microbes have to be acquired before they can form a functional community. Two major modes of transmission exist: vertically, that is from the parental line directly to their offspring, or horizontally, from either the environment or from hosts other than immediate ancestors. These two modes of transmission are not mutually exclusive and, in nature, any combination of the two transmission modes can occur (Bright and Bulgheresi, 2010; Ebert, 2013). Nevertheless, the mode of transmission ultimately affects the structure and composition of the microbial community that establishes within a host. Horizontal transmission likely results in a microbial community that is predominantly selected based on symbiont function and is thus taxonomically diverse (Webster, 2014). Vertical transmission favours particular symbiont lineages, microorganisms already established in the parents, and thus results in a more taxonomically well-defined community (Webster, 2014). While most insect species harbour diverse gut microbial communities, in social insects, and especially *Apis* and *Bombus*, the microbiota is relatively specialized and well-defined in composition, advocating the importance of vertical transmission in these systems (Colman, Toolson, and Takacs-Vesbach, 2012; Engel and Moran, 2013b; Koch and Schmid-Hempel, 2011a; Lim et al., 2015; Martinson et al., 2011). Additionally, in bumblebees, the gut microbiota protects against the common parasite *Crithidia bombi* (Koch and Schmid-Hempel, 2011b).

Honeybees and bumblebees emerge essentially germ-free from the pupa (Hakim, Baldwin, and Smagghe, 2010; Koch and Schmid-Hempel, 2011b; Martinson et al., 2011), so that their typical gut microbial community is acquired subsequently within the social environment. Several studies show that microbiota transmission likely happens via faeces-contaminated nest material and coprophagy (Koch and Schmid-Hempel, 2011b; Martinson, Moy, and Moran, 2012; Powell et al., 2014). However, Koch and Schmid-Hempel (2011a) provided the first tentative evidence that the particular mode of transmission across generations can differ between members of the same microbiota: *Snodgrasella* show patterns more consistent with vertical transmission, while *Gilliamella* seem more environmentally influenced (i.e. horizontally transmitted). The sample size in this study, however, was very limited and — more importantly — the knowledge gap concerning how, especially in bumblebees, queen microbiota relates to the microbiota in their worker offspring remains unresolved.

In honeybees, Tarpy, Mattila, and Newton (2015) showed that queen microbiota composition drastically shifted with increasing queen age. Furthermore, the established microbiota in workers and queens was rather different in its composition. Nevertheless, because life histories of honeybees and bumblebees differ in key aspects, conclusions from honeybee studies are only partially transferable to bumblebees. In bumblebees, all members of a colony perish at the end of the

season except for the inseminated daughter queens that enter hibernation and eventually found the next generation in the coming spring (Schmid-Hempel, 2001). Thus, any microbiota that is vertically transmitted should reside in the hibernating daughter queens, as this is the "only" way to get passed on to the next generation. Furthermore, the life history of bumblebees implies that, at least initially, the microbiota must be vertically transmitted from a queen to her first worker offspring.

Here we aim to quantify differences in microbiota composition between the two castes: queen and workers. First, we analysed queen microbiota early in the colony's life cycle and compared it to queen microbiota composition at the end of the season. Second, analogous to the analysis of worker microbiota in Chapter 3, we investigate here whether queen microbiota diversity can predict the whole colony's resistance level. Third, we assessed similarity of microbiota composition in a queen to (a) the microbiota composition in her own worker offspring, and (b) the microbiota composition in workers from other colonies.

Materials and Methods

Queens, workers and parasite infections

In spring 2014, we field-caught *Bombus terrestris* queens on their first foraging flights after hibernation from two different populations in Switzerland (Aesch and Neunforn) and brought them into the lab. Queens were thoroughly inspected for the presence of any parasites, and, if parasite-free, were transferred to individual housing boxes and allowed to initiate their colony. Bees were provided with pollen and sugar water (ApiInvert) *ad libitum* and kept under standardized conditions ($28 \pm 2\,^{\circ}\mathrm{C}$, 60% RH, constant red-light illumination). We sampled the gut microbiota of these queens at two time points: (i) we froze a faecal sample, taken non-destructively from queens, at $-20\,^{\circ}\mathrm{C}$ for each of these queens early in their colony cycle (i.e. less than 5 workers present), and (ii) we aseptically dissected the guts of all queens at the end of the colony cycle. Approximately in the middle of the colony cycle, twelve workers were picked at random from each of a subset of twenty-nine colonies founded by the queens. Similar to the queens, for each worker bee a faecal sample was collected after picking and eight days later their gut was dissected. Holding conditions and experimental protocols for workers are described and illustrated in Figure 3.1 of Chapter 3. Briefly, for each colony, eight of the twelve workers were exposed to five genetically distinct strains of the trypanosome gut parasite, *Crithidia bombi*, one day after the faecal sample was collected. The remaining four bees received a parasite-free "sham" inoculum. In the parasite-exposed workers, infection outcome was assessed seven days after infection, enough time for an infection to establish (Schmid-Hempel and Schmid-Hempel, 1993). For this, we determined the total number of parasites cells (i.e. infection intensity) by quantitative qPCR (Ulrich, Sadd, and Schmid-Hempel, 2011) and the number of parasite strains that were eventually able to establish (i.e. infection richness) by genotyp-

ing infections for five microsatellite markers (Schmid-Hempel and Reber Funk, 2004). We then established an infection profile for each colony using the measured infection intensity and richness (SI Figure S4.2).

Queen DNA extraction and 16S library preparation and sequencing

We analysed the microbiota composition from each of 48 queens early and late in their colony cycle. Specifically, the dissected guts were first shredded twice for 1 min at 30 Hz on on a Mixer Mill MM 301 (Retsch GmbH) together with a (2.8 mm) sterile zirconium oxide bead. DNA was extracted from both the shredded gut and faecal samples using the Qiagen DNeasy Blood & Tissue Kit and following the manufacturer's protocol (Animal Tissue DNaey 96 Protocol). Samples were lysated for 1 h at 56 °C and finally eluted in 100 μl or 200 μl AE buffer for faecal or gut samples, respectively. Finally, we amplified the variable region V3–V4 of the 16S rRNA gene for each sample and prepared a multiplexed library for paired-end sequencing on the MiSeq® Illumina platform as described in the protocol of Chapter 3.

Amplicon processing and OTU clustering

For a subset of seven colonies we had access to microbiota sequence data from workers (Chapter 3). The raw sequence reads from worker faecal and gut microbiota were combined with newly generated queen microbiota sequence data from this study for all amplicon processing and OTU clustering steps. Details on read counts can be found in SI Table S4.1, as well as the specification on the parameters used in the processing steps. Briefly, we checked the quality of the paired-end reads using FastQC (v0.11.2) before they were merged with usearch (v8.1.1812_i86linux64). We trimmed primer using cutadapt v1.5 quality filtered with PRINSEQ-lite (v0.20.4). Sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence identity with UPARSE (usearch v8.0.1623_i86linux64). A phylogenetic tree was built using PyNAST Caporaso:2010iq implemented in Qiime (v1.8.0) (Caporaso et al., 2010b) and taxonomic information were assigned with UTAX (usearch v8.1.1812_i86linux64). We analysed the effect of differences in sequencing depth (i.e. number of reads per sample) on the number of OTUs (i.e. taxa) by generating rarefaction curves: we subsampled the total sequencing pool for each sample at different sampling depth and calculated Shannon Index and species Richness (number of different OTUs; SI Figure S4.1). Based on this, we excluded two samples due to small library sizes. Finally, any sequences matching the Phylum of Cyanobacteria representing chloroplast sequences were excluded.

Statistical Analyses

We accounted for differing sequencing depth by rarefying all samples to equal sequencing depth (n = 12191 reads). We repeated this process one hundred times to account for any sampling effects and used the mean of the measures across all

rarefactions for any further analysis. In a first step, we assessed the microbiota composition in queens and workers. For each bee we checked whether a particular OTU occurred in both its faecal sample and the corresponding gut sample. We broadly defined any OTU occurring at least once in both sample types, i.e. shared, as the "core" OTU set. For each sample, we calculated the Shannon-Wiener Index (H), species Richness (S) and species Evenness ($H/\ln S$) using either the "full" OTU set or the "core" OTU set. We used ANOVAs to test for any effect of population (Aesch, Neunforn), caste (queen, worker) and sample type (faeces, gut) on these diversity measures. We performed a post-hoc analysis using Tukey's HSD tests to elucidate the differences in means of all pairwise comparisons simultaneously. In a second step, we asked whether queen microbiota early in the season (i.e. queen faeces) or the microbiota composition late in the season is indicative of infection outcome. For this, we used a linear regression model with the colony resistance profile (i.e. infection outcome) as an independent variable: that is the combined effect of a colony's mean parasite infection intensity and mean infection richness as captured by the first principal component. As a third step, we assessed whether the microbiota that a queen harboured early in the season more closely resembled either the microbiota that eventually established in workers of the same colony or that of the microbiota of any other worker. For this, we calculated the Euclidean and Bray-Curtis distances between the faecal microbiota composition of queens, on the one hand, to the faecal microbiota composition from each of the workers of their own colony and, on the other hand, to the microbiota composition of any other worker. We then used a linear mixed effects model to assess whether the distances between a queen and its own workers differed from the distances to workers of foreign colonies. We modelled the type of distance (to own vs. foreign workers) as a fixed effect. To investigate whether there was any colony dependent effect on the resemblance of microbiota community composition, we allowed for random slopes within each colony.

Results

Queen and worker microbiota diversity

We first compared species Richness (i.e. number of OTUs) within individual queens early in the season (faecal sample) to species Richness in the corresponding queens late in the colony cycle (gut sample). Similar to the results in Chapter 3 that looked specifically into microbiota composition in workers, we found that species Richness in faecal microbiota did not correlate with species Richness in the gut sample (Figure 4.1). However, the average species Richness was higher late in the season (gut: 16.04 ± 2.74 SD) compared to early in the season (faeces: 12.95 ± 2.67 SD; paired t-test: $t(48) = -5.36$, $p < 0.001$). On average 7.98 ± 1.48 (SD) OTUs were shared between the sample types within an individual queen. We next compared the set of shared OTUs between queens and workers. OTUs most commonly shared within an individual queen were

Table 4.1: ANOVA of microbiota diversity.

Measure	Model terms	df, residual df	F	p-value
Shannon Index[1]	population	1, 145	14.16	< 0.001
	caste	1, 146	0.76	0.384
	sample type	1, 146	7.17	0.008
	caste*sample type	1, 145	17.85	< 0.001
Species Evenness[1]	population	1, 145	9.62	0.002
	caste	1, 146	7.23	0.008
	sample type	1, 146	1.80	0.181
	caste*sample type	1, 145	4.75	0.031
Species Richness[1]	population	1, 145	3.39	0.068
	caste	1, 146	79.03	< 0.001
	sample type	1, 146	18.12	< 0.001
	caste*sample type	1, 145	38.96	< 0.001

[1] Analysis included only workers that were not exposed to the parasite.

also the most commonly shared in workers, with the exception of OTU_4 of the genus *Lactobacillus* and increased variation in less commonly shared OTUs in the workers when compared to the queens (Figure 4.2).

Overall, a total of 199 OTUs were detected in the respective pooled faecal and gut samples and could thus potentially have been shared between the faecal and gut microbial community of an individual. However, only a subset of 44 OTUs were observed at least once in any bee in both sample types (gut, faeces). We used this broadly defined "core" OTU set to investigate differences and similarities in microbiota diversity among queen and worker microbiota using ANOVA.

We found a significant effect of population on microbiota diversity summarised by the Shannon Index and species Evenness (Table 4.1): both measures were increased in Neunforn compared to Aesch. For all three measures (Shannon Index, Evenness and Richness) differences of varying degree were detected among castes (queen, worker; Figure 4.3), depending on sample type.

Can queen microbiota predict infection outcome?

We tested whether microbiota diversity in queens was predictive of their colony's resistance profile (Figure S4.2). The resistance profile of these colonies was characterised by mean infection intensity and mean infection richness as measured in workers seven days post-exposure to the gut parasite *C. bombi*. We found that colony resistance profile, represented by the first principal component of colony mean infection intensity and mean infection richness, varied with Shannon Index of the queen's gut microbiota late in the season (lm; $F_{1,27} = 4.42$, $p = 0.045$, $\beta = 1.98 \pm 0.94$ SE, adj. $R^2 = 0.11$), but not with faecal microbiota diversity early in the season (lm; $F_{1,27} = 0.004$, $p = 0.950$, $\beta = 0.05pm0.77$ SE, adj. $R^2 = -0.04$; Figure 4.4A). Similarly, species Evenness of queen gut microbiota

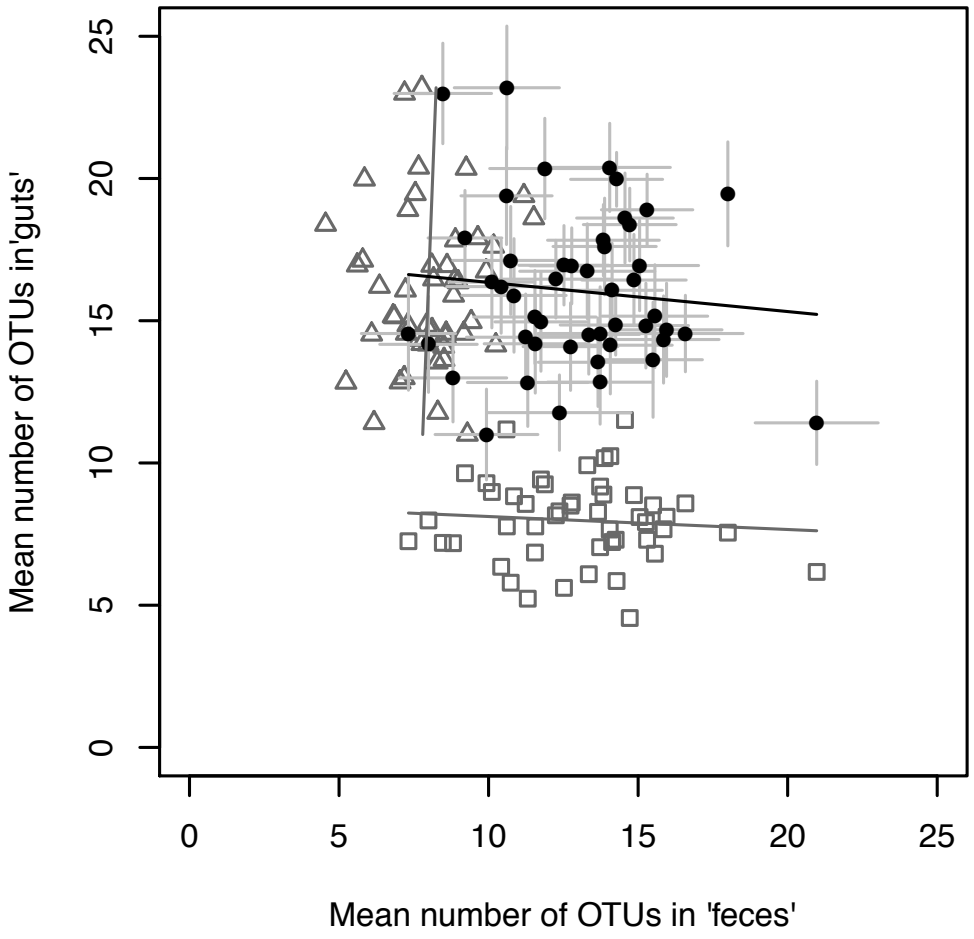

Figure 4.1: Number of OTUs in the faecal and gut microbial communities of queens. Each filled circle represents an individual queen (n = 48) and plots the average number of different OTUs found in the gut sample against the average number of different OTUs in the faecal sample. Point values are the mean over the 100 independently rarefied datasets. Error bars show the SD. Grey symbols show the same individuals, but plot the number of OTUs shared between the microbial community of faeces and guts against the total number of OTUs found in either faeces (diamonds) or guts (triangles). The drawn regression lines all show statistically non-significant relationships.

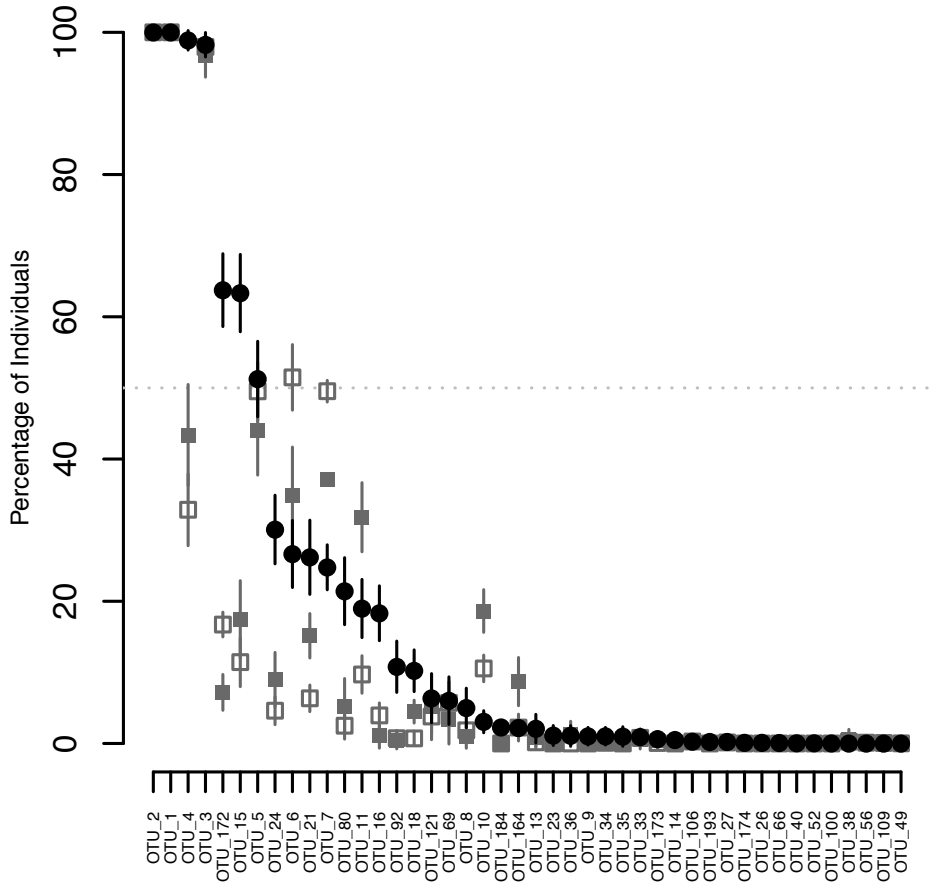

Figure 4.2: Shared OTUs between faecal and gut microbial communities in queens and workers. The graph shows the average percentage of individuals that shared a particular OTU in their faecal and gut microbial communities. Filled symbols show queens, filled grey squares are workers that were not exposed to the parasite and grey empty symbols are workers exposed to the parasite. Values are the means of the average percentages across the rarefied datasets (mean of n = 100). Error bars represent SD.

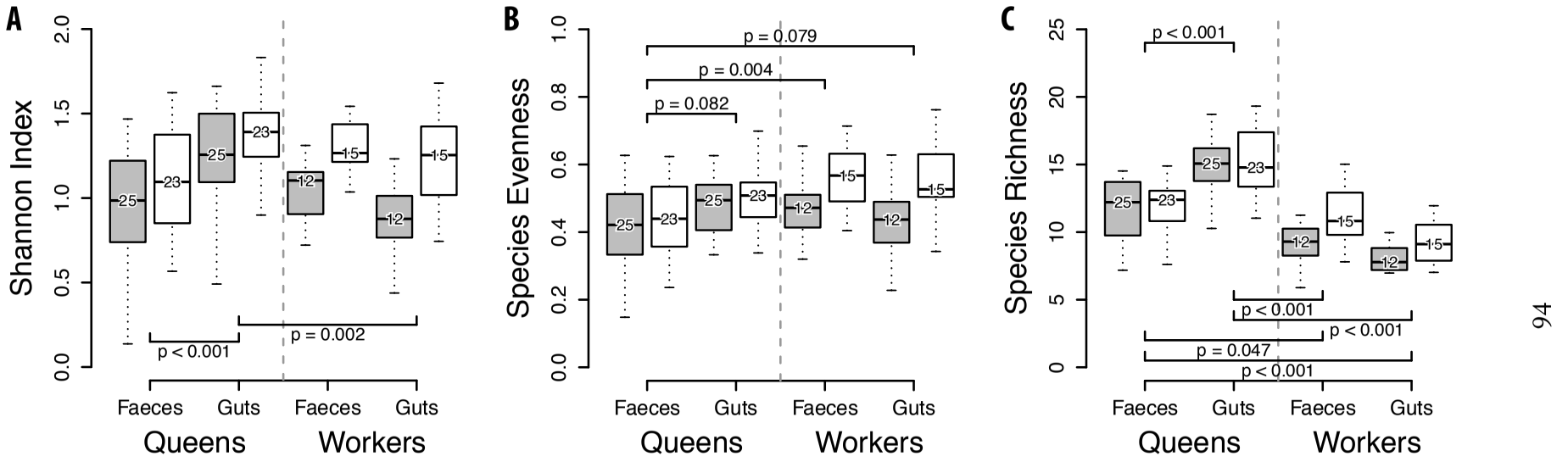

Figure 4.3: Differences in microbiota diversity. Shown are boxplots for Shannon Index (A), species Evenness (B) and species Richness (C) split by caste (queen, worker), sample type (faeces, guts) and population (Aesch: grey shaded, Neunforn: empty). Bars indicate significant differences identified by Tukey's HSD post-hoc test for differences in mean between groups. Each box represents the interquartile range (IQR) and median overlay with the sample size for the particular group. Hinges extend 1.5x IQR from the box. Only workers not exposed to the parasite were included for the analysis.

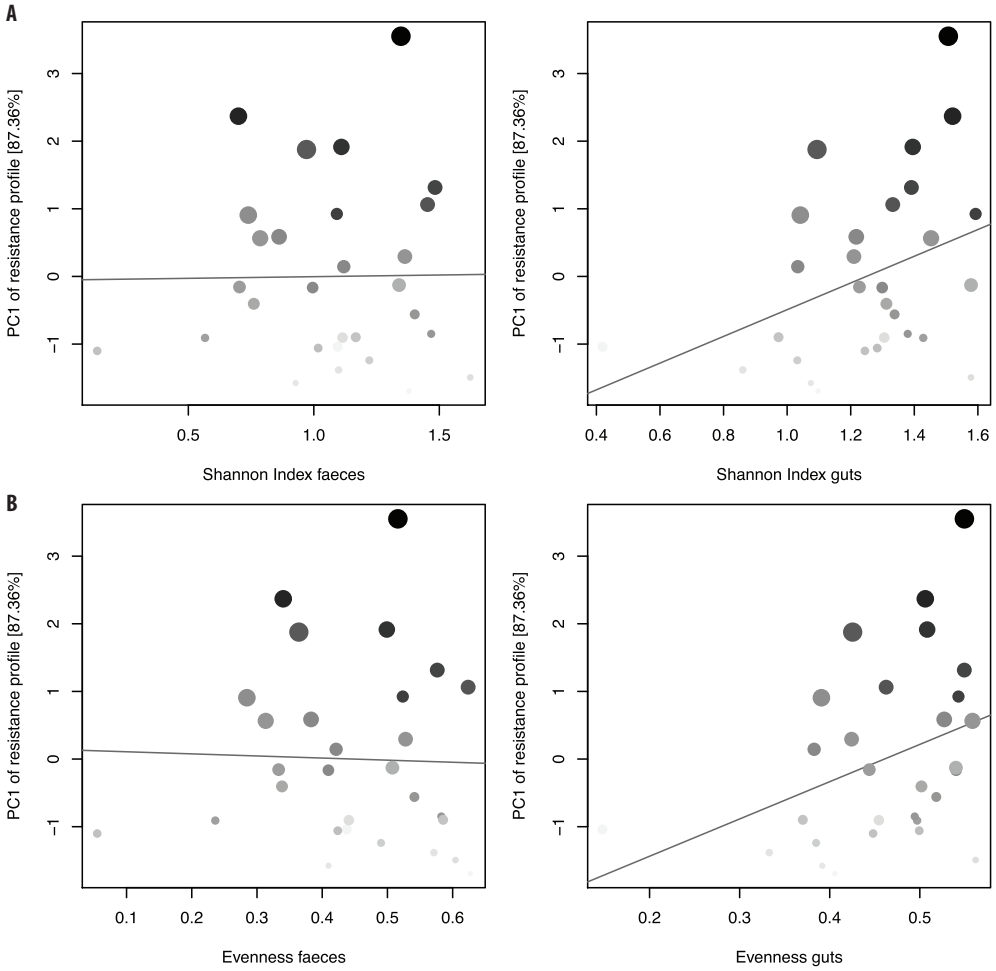

Figure 4.4: Colony resistance profile as a function of queen "core" microbiota diversity. The variation in colony resistance, shown as the PC1-score, correlates with the Shannon Index (A) and species Evenness (B) of the "core" queen gut microbiota (right panels) but not with queen faecal microbiota (left panels). Point size is proportional to mean colony infection intensity, and darker fill colour indicates a higher number of colony mean parasite strains. The solid line is the fitted linear regression.

was predictive of colony resistance (lm; $F_{1,27} = 4.33$, $p = 0.047$,$\beta = 5.51 \pm 2.65$ SE, adj. $R^2 = 0.11$), but not when measured early in the season in faecal microbiota (lm; $F_{1,27} = 0.03$, $p = 0.874$, $\beta = -0.31 \pm 1.93$ SE, adj. $R^2 = -0.04$; Figure 4.4B). Species Richness did not predict colony resistance when analysed in faecal microbiota (lm; $F_{1,27} = 0.30$, $p = 0.587$, $\beta = 0.06 \pm 0.11$ SE, adj. $R^2 = -0.03$), nor did the Richness of the gut microbiota (lm; $F_{1,27} = 0.16$, $p = 0.692$, $\beta = 0.04 \pm 0.11$ SE, adj. $R^2 = -0.03$).

Vertical transmission: is the queen microbiota more similar to the microbiota of its own workers?

We assessed whether the microbiota composition that the queen harboured early in the season (i.e. faecal microbiota) more closely resembled the microbiota that

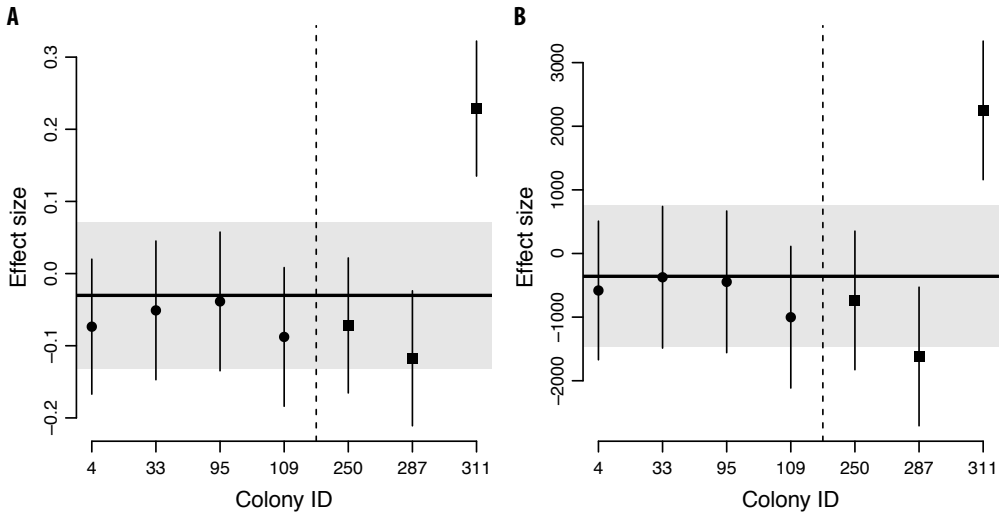

Figure 4.5: Queen microbiota similarity to own worker offspring. Shown is the effect for "type" of distance (from queen to its own worker offspring vs. from queen to any other worker; fixed effect) for Bray-Curtis distance (A) and Euclidean distance (B). The mean across all colonies is shown as the bold black line with the 95% CI in grey. A value < 0 indicates greater similarity between queen microbiota with own offspring worker microbiota. Points (with 95% CI error bars) indicate the combined overall effect for each colony including their random slopes. 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 population origin (circles: Neunforn, squares: Aesch).

eventually established in their own worker offspring compared to microbiota composition found in worker offspring of other colonies. We found that, across all colonies, the Bray-Curtis distance (GLMM; $\chi^2_1 = 0.38$, $p = 0.536$, effect size = $-0.03[-0.13, 0.07]$ 95% CI), as well as the Euclidean distance (GLMM; $\chi^2_1 = 0.45$, $p = 0.502$, effect size = $-358.60[-1470.55, 753.38]$ 95% CI), from queen microbiota to the microbiota of their own worker offspring was overall not smaller (i.e. microbiota more similar) than to workers of other colonies. However, for the majority of the colonies, the direction of the effect showed that queen microbiota tended to more closely resemble their own worker microbiota than that of any other worker (Figure 4.5). A strong colony-specific effect was found for one colony indicating more dissimilarity of queen microbiota to its own worker microbiota composition (Figure 4.5).

Discussion

In bumblebees, queens are the crucial link in the transmission cycle from one generation to the next for the host-associated gut microbiota. Moreover, vertical transmission has been hypothesised as an important factor in shaping microbiota composition (Bright and Bulgheresi, 2010; Ebert, 2013; Koch and Schmid-Hempel, 2011a). However, studies on queen microbiota composition have so far been virtually absent, as studies so far have generally focused on workers.

By quantifying microbiota composition in queens, we were able to relate the

findings for worker microbiota to the queens that, in this respect, have a much more central role in the bumblebee life cycle as compared to the honeybee. We find that species Richness is generally higher in queens compared to workers, which might simply reflect the hierarchy of gut microbiota acquisition if vertical transmission is prevalent. Queens represent the global species pool, and taxa that eventually establish in worker microbiota are a subset of this global pool. Alternatively, the difference in species Richness might reflect differences in the number of "niches" in queens compared to workers, although the nature of these niches are not known so far. Different OTUs might also show different affinities to either queens or workers for reasons not related to the former effects, as tentatively suggested by some of the OTUs in Figure 4.4. These specific OTUs present likely candidates for further investigation into functional differences in microbiota with respect to caste. Interestingly, differences in diversity measures between the two sampled populations (Aesch vs. Neunforn) were more pronounced in workers than in queens, which suggests an influence of the ecological environment. However, further investigation is needed to uncover the causes and consequences that underlie such population differentiation in workers.

Bumblebee colonies only exist for a single season, and the colonies collapse after new daughter-queens have been reared and leave the colony. Thus, when looking at the emerging spring queens next year, the microbiota composition in these daughter-queens from early in the season represent the microbial community that survived this over-winter bottleneck. We found that queen microbiota assessed at an early stage of the colony cycle is less diverse and contains fewer species (OTUs) compared to the microbiota at a later stage of the colony cycle. At first glance, the differences in diversity and species Evenness could potentially indicate acquired changes in the microbiota over the course of a colony cycle. However, the matching pattern found for species Richness leaves room for an alternative hypothesis: when microbiota is assessed in faeces — as was necessary technically to keep the early queens alive, in contrast to the destructive sampling from guts later — some microbial species are either not present in this faecal sample type or fall below the technical detection level. This alternative hypothesis is supported by the findings from Chapter 2, where species Richness was restored in some individuals that received a faecal microbiota transplant, compared to the species Richness measured in the transplanted faecal matter. Future studies should thus look into gut samples at both time points.

Previous studies have shown that the microbiota in bumblebees can modulate the infection outcome of *C. bombi* (Koch and Schmid-Hempel, 2011b, 2012). Here we found that increased queen gut microbiota diversity, as quantified by Shannon Index, and species Evenness was associated with increased colony level susceptibility to the parasite. This strongly supports the findings in Chapter 3, where worker microbiota diversity before parasite exposure explained a similar amount of variation (approx. 12%) in infection outcome, i.e. a more diverse microbiota was also associated with higher susceptibility. This positive association of microbiota diversity and parasite susceptibility might simply reflect abiotic

conditions within the colony favourable to parasite infection that consistently co-vary with microbiota diversity (Levine, 2000; Levine and D'Antonio, 1999; Shea and Chesson, 2002) and is discussed in more detail in Chapter 3. But the surprising and puzzling finding here is that this relationship is only apparent in the gut sample, which was collected late in the colony cycle. If microbiota diversity differences between faecal and gut sample were independent of time (i.e. stage in colony cycle), then a similar association between queen microbiota assessed in faeces early in the colony cycle and a colony's susceptibility would have been expected. The absence of such a correlation may suggest that the microbiota composition in queens indeed changed over time. We thus speculate that the differences in infection outcome may reflect this change in microbiota composition. The causes remain unknown, though, so further investigations are needed. A potentially promising avenue would be to separate time effect from colony growth and study microbiota composition of colonies in a setting where the social structure is experimentally altered (i.e. changes in number of brood, workers etc.). This approach, would allow to elucidate whether the change in colony composition and social structure alters the gut microbiota composition in a way that facilitates infection.

Surprisingly, we were not able to show that queen microbiota composition more closely resembled the microbiota composition in own worker offspring as compared to workers of foreign colonies. One colony even showed a strong colony-specific effect in the opposite direction of our expectations, i.e. the two microbiotas (queen vs. own workers) were more dissimilar than average. While surprising, given our expectation of a strong vertical transmission signal, this lack of association between the microbiota composition of a queen and its own workers is actually in line with results obtained in Chapter 2 (see Ch. 2, Figure 2.5). There, we show that the workers from only a few colonies establish a microbiota that closely resembles their own "colony profile" when presented with a "global" species pool. This illustrates a colony-specific selection potential on the establishment of the gut microbiota community.

A feasible mechanism that could mediate selection by the host on its own microbiota is colony-specific variation in host immune response (Buchon, Silverman, and Cherry, 2014). In Chapter 1, we indeed showed a differential gene expression response upon faecal microbiota transplant, and the associated changes in microbiota composition. Furthermore, host immune gene expression upon parasite infection shows general and consistent, strong colony-specific response patterns (Barribeau et al., 2014; Brunner, Schmid-Hempel, and Barribeau, 2013, 2014; Riddell et al., 2009). Although functional tests, for example, with gene knockouts via RNAi (Deshwal and Mallon, 2014) still need to be carried out, this suggests that host immune response might not only mediate specificity of host-parasite interaction, but also specificity in microbiota composition. Here, we show that microbiota in workers can reflect patterns in queens, which are the major vectors of microbiota into the next generation.

Supplementary Information

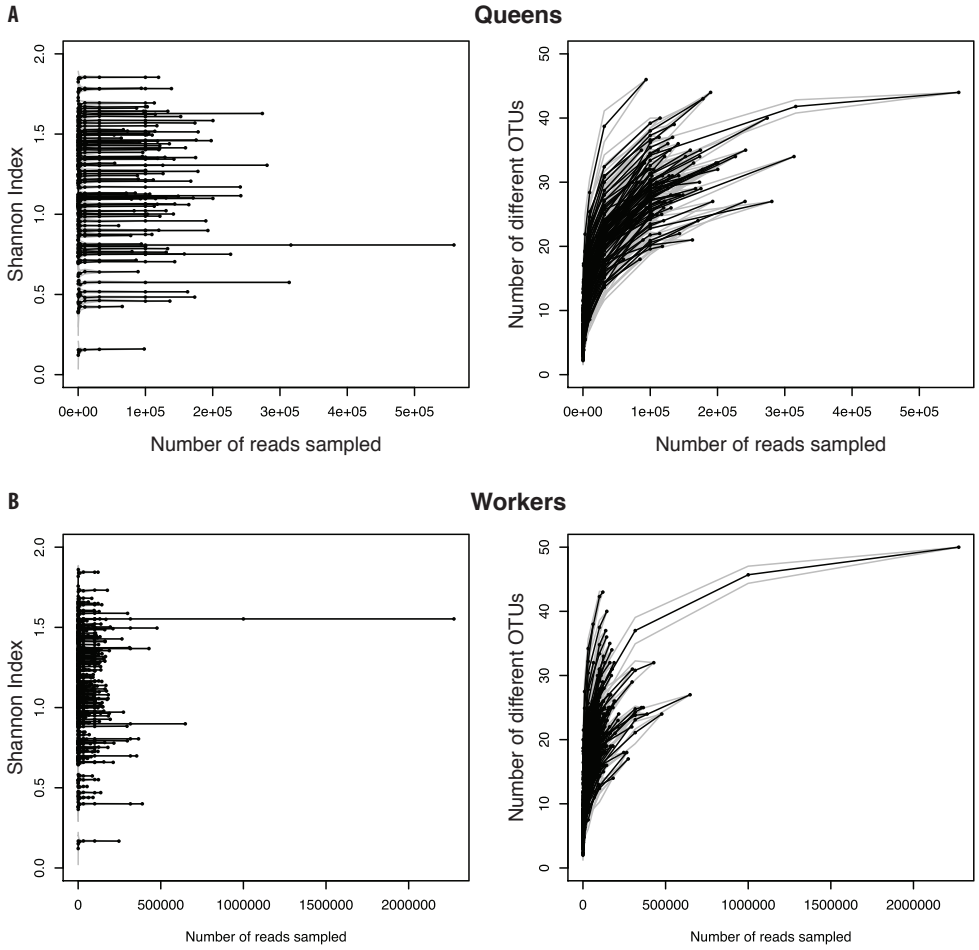

Figure S4.1: Rarefaction curves of each sample. Panels to the left show Shannon-Wiener diversity index, and panels to the right show number of different OTUs (richness) at given sub-sampling depth (x-axis) of the individual sequencing pool for both queen microbiota (A) and worker microbiota (B). Lines connect means (black) and SD (grey) of 10 independent draws at given sampling depth from the total sequencing pool of a particular amplicon library.

Table S4.1: Amplicon processing and read counts.

Processing step	Specifications
Raw reads quality control: FastQG (v0.11.2)	
	Queens: 16042366
	Workers: 30553346
Merge reads: usearch (v8.1.1812_i86linux64)	
Queens: 14110165	Min length: 100
Workers: 26521931	min overlap: 15
	Max error: 5
Primer-trimming: cutadapt (v.1.5)	
Queens: 12714112	Overlap: full length
Workers: 24098020	Error rate: 0
	Wildcards allowed
Quality filtering: PRINSEQ-lite (v0.20.4)	
Queens: 12710390	Size selection: 403 nt–431 nt
Workers: 22468312	Min quality mean: 15
	No ambiguous nucleotides
	Low-complexity filter: dust
	Low-complexity threshold: 10
OTU clustering: UPARSE (usearch v8.0.1623_i86linux64)	
Number of OTUs: 234	sortbylength
Reads mapped: 35037751	derep_fullength
Reads not mapped: 140951	sortbysize: size = 2
	cluster_otus
	uchime_ref
	usearch_global: id = 97%
Taxonomic assignment: Qiime (v1.8.0)	
	UTAX:
	Reference: gg_13_5
	Tax config: 500.tc
Phylogenetic analysis: PyNAST	
	Min identity 55%

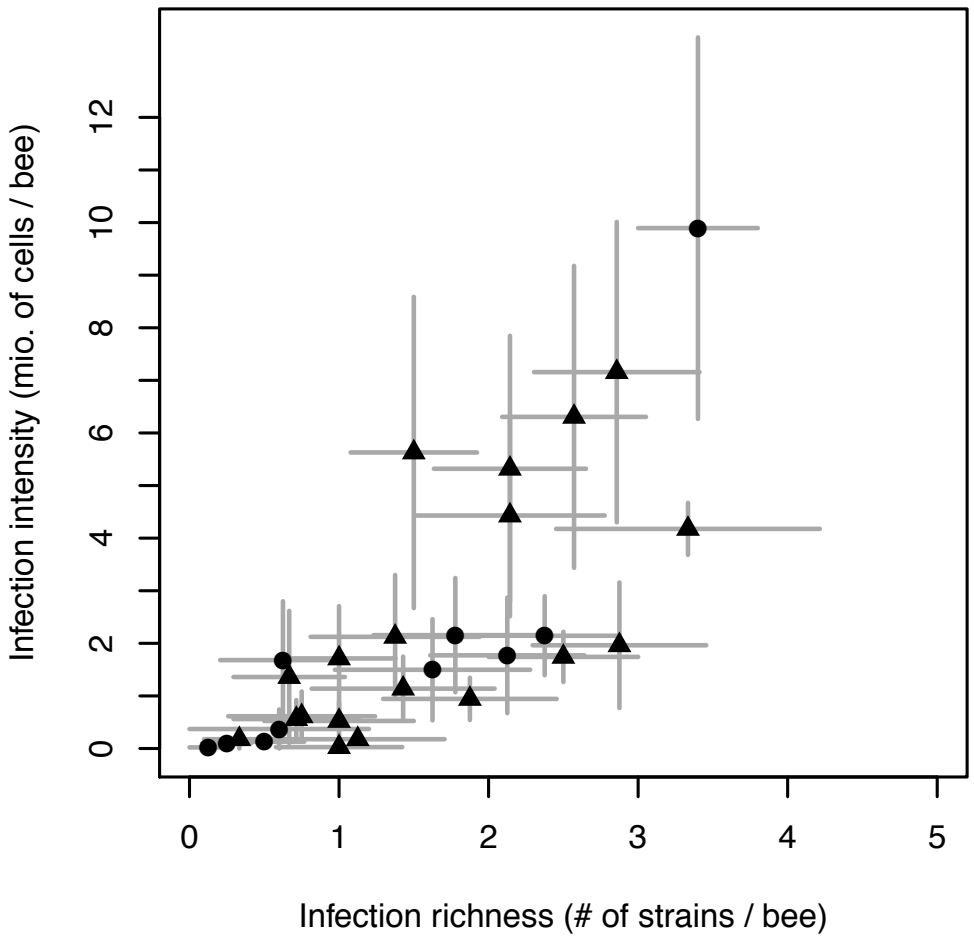

Figure S4.2: Colony resistance profile. Adapted from Chapter 3, but includes only data from colonies analysed in this Chapter. Shown are mean infection intensity per colony plotted against a mean infection richness, seven days post infection (error bars are the SEM). The parasite inoculum contained five genetically distinct strains. Circles: colonies from Aesch; triangles: colonies form Neunforn.

Chapter 5

From Eurasia to North America: gut microbiota composition across species along the dispersal route of bumblebee species — a pilot study

Summary of the supervised MSc thesis:
Britt, Elisabeth (2015) Composition and distribution patterns of microbiota across bumblebee species in China and Kyrgyzstan. ETH Zürich

Abstract

The global diversity of *Bombus spp.* can be traced back to Eurasia. In this pilot study, we aim to quantify the host-associated gut microbiota diversity at the evolutionary origin of bumblebees. We summarize and extend the preliminary results obtained in the context of the MSc thesis of Elisabeth Britt.[1] We found preliminary results in line with previous studies on species-specific microbiota and enterotype structure. Furthermore, a first look at microbiota species shows a potential strong signal of a species-specific effect on microbiota composition. Once completed, the study will provide valuable insights into the global phylogeography of bumblebee microbiota composition.

[1] Please refer to the MSc report for details on the methodology or additional results of this pilot study.

Introduction

Host-associated microbiota communities commonly show species-specific signals, though the relative importance of host versus environmental determinants varies across species. For example, in *Drosophila*, the gut microbiota is mostly shaped by microbes that are taken up with diet (Chandler et al., 2011). Similar patterns have also been observed in beetles, but less so for *Hymenoptera* species (Colman, Toolson, and Takacs-Vesbach, 2012). In social insects, however, the host's influence on the microbiota composition appears to be much more pronounced (Koch and Schmid-Hempel, 2011a; Li et al., 2015; Lim et al., 2015; Martinson et al., 2011). The gut microbiota of bumblebees is relatively simple in its composition and consists of only few taxonomic groups (Koch and Schmid-Hempel, 2011a; Moran, 2015). Microbiota components (i.e. taxa) can be defined as either as "core" or "non-core", depending on whether they they consistently associate with the host or represent transient environmental species. This definition has proven useful to identify potentially beneficial microbiota composition (Cariveau et al., 2014; McFrederick et al., 2012 and Chapters 1, 3 and 4). Furthermore, in the bumblebee, *Bombus terrestris*, the microbiota provides a protective function against the trypanosome gut parasite, *Crithidia bombi* (Koch and Schmid-Hempel, 2011b), and the host may even actively select for certain microbiota compositions (Chapter 1).

Species are generally not randomly distributed across geographical space. Generally, related species can be traced back to a common ancestor at a particular geographical origin. Over evolutionary time, spatial dispersion and evolutionary differentiation resulted in the contemporary global species distribution. Species diversity is then often highest at the location of evolutionary origin. For bumblebees, the evolutionary origin is in eastern Eurasia (Hines, 2008). Given the beneficial role of the microbiota to bumblebee health, the high diversity of bumblebee host species at their evolutionary origin might be reflected in their microbiota composition.

In this pilot study, we investigated the microbiota diversity across several bumblebee species collected at the evolutionary origin of *Bombus*. We assessed microbiota diversity and species Richness in two regions in China and Kyrgyzstan, respectively. This lays the groundwork for a more extensive study microbiota compositional patterns along the diversification route of bumblebees.

Methods Summary

In this pilot study, we investigated the gut microbiota composition from several bumblebee species in Eurasia, the geographic origin of *Bombus* diversification (Hines, 2008). For this, samples from Kyrgyzstan and China were available and analysed. We aimed to analyse a minimum of two and a maximum of five individuals per species. With this strategy, bumblebee species diversity sampled in 2008 could be completely covered, with only one exception in China, and eight out of twelve species from Kyrgyzstan.

Table 5.1: Overview of the number of sampled individuals per *Bombus* spp. by location.

Bombus spp.	China	Kyrgyzstan
B. friseanus	5	
B. impetuosus	5	
B. sibiricus	5	
B. lepidus	5	
B. picipes	5	
B. remotus	5	
B. festivus	5	
B. trifasciatus	3	
B. tichenkoi	3	
B. flavescencs	2	
B. rufofasciatus	5	
B. patagiatus	5	
B. keriensis	3	5
B. lucorum	3	5
B. defector		5
B. melanurus		5
B. biroi		5
B. asiaticus		5
B. bohemicus		2
B. campetris, *B. barbutellus*		4

Data on microbiota composition were generated by amplifying the variable region V3–V4 of the 16S sRNA gene from the bacterial community present in dissected guts and subsequent paired-end sequencing on the MiSeq® Illumina platform. Obtained sequences were processed and operational taxonomic units (OTUs) were identified based on 97% sequence similarity. Due to differences in sequencing depth among samples, three samples with low read counts were excluded from the OTU table and each sample was rarefied to n = 4411.

To identify ecologically relevant taxa (i.e. OTUs), OTUs were defined as "core" if they occurred in at least 75% of the individual within a species. Furthermore, we investigated differences in microbiota diversity as measured by the Shannon Index. Finally, we performed an enterotype analysis (Arumugam et al., 2011) on the OTU abundance data to identify whether any robust clusters of OTU composition could describe gut microbiota composition in bumblebees, independent of species or populations.

Results Summary & Discussion

Generally, a typical bee-like microbiota composition in terms of phyla was recovered, harbouring a low diversity as measured by the Shannon Index, which indicates a highly skewed abundance distribution among the OTUs (Koch and Schmid-Hempel, 2011a; Koch et al., 2013; Moran et al., 2011). We identified three OTUs ubiquitous to all bumblebee species analysed, two of which matched *Gilliamella apicola* sequences and one which matched *Sonodgrasella* species. These candidate species have previously been identified to show contrasting evolutionary dynamics. Comparing the phylogenetic trees of these candidate species to the phylogenetic tree of the bumblebees reveals potential differences in their transmission mode (Koch et al., 2013): *Gilliamella* shows signals of horizontal transmission; *Snodgrasella* shows signals of vertical transmission. Further investigation into these different transmission modes should help tease apart which components of the microbiota might associate with either transmission mode for mixed-mode microbiota transmission (Ebert, 2013).

Preliminary analysis of microbiota composition revealed species Richness differed between *Bombus* spp. from both regions (ANOVA; $F_{19,65} = 2.2166$, $p = 0.009$; see Figure 5.1). It was not possible to test for differences in the regions, as the bumblebee species composition was virtually unique between the two regions: only two species were sampled from both regions. These interesting patterns of species-specific determination of microbiota composition need to be further explored in a more extensive study.

We also investigated whether the OTUs clustered into so-called enterotypes and, in line with (Li et al., 2015), recovered two distinct clusters. We also found significant species-specific enterotype compositions (Fisher's Exact Test; $p < 0.001$), as well as significant subgenera effects (Fisher's Exact Test; $p < 0.001$). The latter was not recovered in (Li et al., 2015), and a closer look at the OTU composition of the two enterotypes revealed further differences. For example, in our data, *Gilliamella* occurred in both enterotypes (OTU_1 and OTU_115),

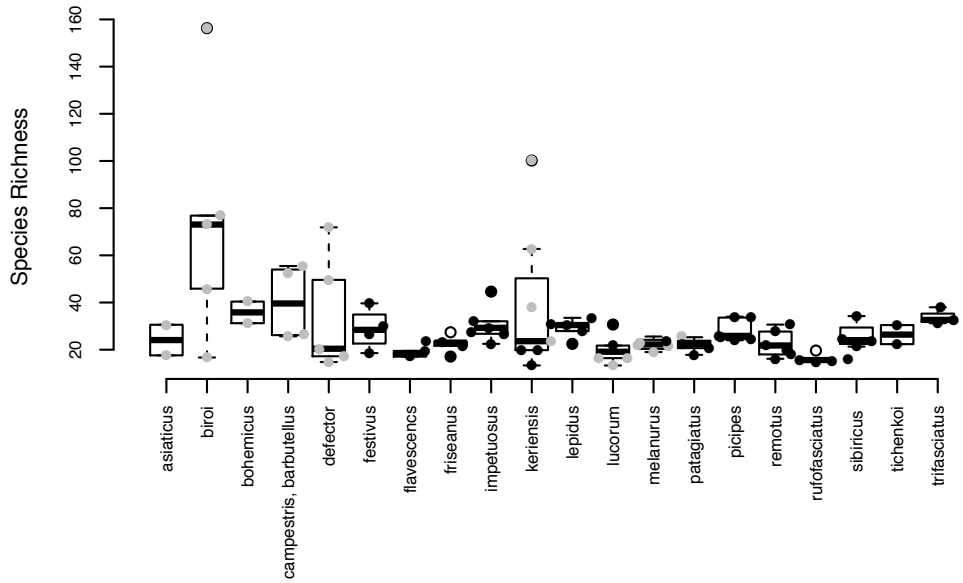

Figure 5.1: Microbiota species Richness in different *Bombus* species. Sampled individuals from China are shown in black, and individuals from Kyrgyzstan in grey. Generally, there is little variation within species, with the expection of *B. biroi*, *B. defector*, *B. keriensis* and *B. campestris*/*B. barbutellus*. For this analysis, samples were rarefied to a sequencing depth of n = 12338. Samples with a library size smaller than the negative control were discarded.

where *Gilliamella* appeared as one of the strongest differentiators of the enterotypes in (Li et al., 2015). Taxonomic assignment in such analysis is not always unambiguous and requires further attention. Yet, the overall pattern of two enterotypes seems consistent across the studies in bumblebees, and such generally low numbers of enterotypes are also found in the more complex human and primate gut (Arumugam et al., 2011).

An understanding of the factors and processes that determine the biogeography of a species can be helpful for the control of invasive species, for diseases prevention and for the conservation of ecosystems (Gaston, 2000). *Bombus* spp. are especially important pollinators, both for natural and agricultural systems (Garibaldi et al., 2013). The use of *Bombus terrestris* as an agricultural pollinator outside of its natural geographical range can have detrimental effects on the local *Bombus* population (Schmid-Hempel et al., 2014). However, the drivers of such invasions are yet to be fully understood. Thus, understanding how the host-associated microbiota associates with the spatial and phylogeographic patterns of *Bombus* is particularly timely and relevant.

General Discussion

The aim of this thesis was to investigate aspects of the three-way interaction between hosts, parasites and microbiota in an ecologically relevant natural host-parasite system, consisting of the bumblebee, *Bombus terrestris*, and its trypanosome gut parasite, *Crithidia bombi*. In several chapters, I reported the results of experiments with faecal microbiota transplants in bees and the outcome of experimental infections.

Microbiota-host interactions

The gut microbiota provides protection against parasitic infections, and is thus important for host health in many organisms (Dillon and Dillon, 2004; Engel and Moran, 2013b; Kamada et al., 2013; Sekirov et al., 2010; Stecher and Hardt, 2011). Because this effect is dependent on a certain type of microbiota rather than just a random assembly of microorganisms, hosts may have evolved mechanisms that select for a beneficial microbiota composition; in particular, the host's immune system is likely to be a key component of this process (Chu and Mazmanian, 2013; Hooper, Littman, and Macpherson, 2012; Thaiss et al., 2014). In a bumblebee colony, bees initially emerge germ-free (Hakim, Baldwin, and Smagghe, 2010; Koch and Schmid-Hempel, 2011b), and subsequently, the whole gut microbial community is acquired and establishes in the first few days of a bee's life (Koch and Schmid-Hempel, 2011b; Martinson, Moy, and Moran, 2012). This presents an ideal opportunity for the host to select on the establishing community to its own advantage. Simultaneously, any of the potential gut microbes might potentially manipulate the host to their own benefits, though this is yet to be fully understood.

In this thesis, we established in Chapter 1, that the receiving host's immune system responds differentially to a faecal microbiota transplant, depending on its resistance phenotype to the parasite. Also, microbiotas originating from hosts of different resistant phenotypes elicit a differential immune response in the receiving host. These results on the variation in immune response were further supported by differences detected in the respective microbiota composition. Surprisingly, however, the pattern of the microbiota-host interaction was not dependent on a particular combination of host resistance phenotype and microbiota resistance phenotype (i.e. donating host). These results indicate independent phenotype differences between hosts and microbiotas, respectively, but a statisti-

cal interaction between hosts and microbiota, which might reflect a specificity in infection outcome (Koch and Schmid-Hempel, 2012), was not detected. Nevertheless, the results demonstrated a possible mechanism by which microbiota and host interact in a context relevant to health in bumblebee — the effect of the host on which microorganisms are "allowed" to form a microbial community.

We further explored this host "selection ability" in Chapter 2 by specifically investigating the filtering ability of different colonies, where each colony represents a different genetic background and thus a different selection potential. We presented workers with a "global" species pool containing the universal microbial source population that potentially initiate the colonisation process in the bee gut. In line with the results from Chapter 1, we found variation among colonies in their ability to reconstruct a microbiota composition that resembled their natural, colony-specific microbiota composition. Despite this overall trend, in certain colonies, a microbiota eventually established that showed a high degree of dissimilarity to the microbiota composition naturally observed in their respective colonies. This lends itself to the speculation, that under some circumstances or in addition to other prime factors, the composition of the microbiota is driven by the microbes themselves. In other words, the host selection potential could be "overridden" by the colonization ability of gut microbes, which then drive the microbiota composition in another direction. Experiments in Chapter 4 further corroborated this possibility, where in a natural setting, the similarities of microbiota composition between queens and worker were investigated. Assuming that the queen microbiota represents the colony's microbiota "template" from which the worker microbiota eventually establishes, we found similar colony specific variation as observed in Chapter 2. Hence, the template of the queen was not enough to explain the microbiota composition found in workers. Nevertheless, a species-specific microbiota composition as identified in Chapter 5 and in other studies (Koch and Schmid-Hempel, 2011a; Li et al., 2015; Lim et al., 2015; Martinson et al., 2011) support, at least at the species level, the importance of vertical transmission within colonies, in addition to host selection, for the composition of the worker microbiota.

Microbiota-parasite interactions

The beneficial contribution of the microbiota to host health and the potential interactions between microbiota and host were outlined before (Chu and Mazmanian, 2013; Hooper, Littman, and Macpherson, 2012; Thaiss et al., 2014). These interactions, between microbiota and host, may have an indirect effect on the infecting parasite. But the microbiota potentially also directly interacts with the pathogen (Buffie and Pamer, 2013; Kamada et al., 2013; Sekirov and Finlay, 2009; Stecher and Hardt, 2011). Such direct interactions might occur through direct inhibition or through efficient competition for niches or nutrients (Buffie and Pamer, 2013; Sekirov and Finlay, 2009; Stecher and Hardt, 2011). Thus, essentially, a parasitic infection, in many cases caused by microbes as well — such as by the protozoans studied here, can be viewed as an invasion process

into the gut microbial community of the host (Costello et al., 2012; Shea and Chesson, 2002; Stacy et al., 2016). From this point of view, the protective function of the microbiota can also be interpreted as colonization resistance (Lawley and Walker, 2013; Stecher and Hardt, 2011; Thaiss et al., 2014); furthermore, robustness and resilience — concepts that have served community ecology well — of the microbiota against parasitic invasion can be quantified (Foster, Krone, and Forney, 2008; Lozupone et al., 2012).

Chapter 3 showed that the colonization resistance of the worker microbiota varied across colonies, and decreased with increasing taxonomic diversity (in terms of OTUs), explaining a remarkable 12% of variation in infection outcome. Furthermore, the correlation between microbiota diversity and infection outcome disappeared in workers once the parasite had the opportunity to establish. This suggests the importance of the composition of the constituent microbiota when an infection process starts, as well as the possibility of effects by the parasite on the microbiota composition as the infection process unfolds. At the same time, it highlights that the gut microbiota composition is quite resilient against perturbation by the parasite. Remarkably, the same pattern (a correlation between microbiota diversity and infection outcome) was recovered in the queen gut microbiota composition analysed late rather than early in the colony's life cycle in Chapter 4. Surprisingly, therefore, at the colony level, the queen microbiota composition early in a colony's life cycle did not show the relationship that was found in the workers, i.e. before the infection starts. This suggests at least a dynamic component to the microbiota community composition. Speculation that this change could be mediated by changes in the social structure over the course a colony's life cycle are close at hand but the evidence so far is not good enough to make any firm statements.

Conclusion and outlook

Overall, in my thesis I showed that the hosts as well as the particular members and constellations of the microbiota, influence — in a dynamic process — the establishment and eventual composition of a bumblebee gut microbiota and its interaction with both host and parasites. Embedding these results in the concepts of community ecology (Costello et al., 2012; Foxman et al., 2008; Mihaljevic, 2012) and comparing them to insights on mechanisms that are governing host-microbiota interaction from vertebrate gut microbiota systems (Kamada et al., 2013; Sekirov et al., 2010; Stecher and Hardt, 2011) proved useful. Uncovering the processes of microbiota establishment and its change in the face of infection can lead to defining a concept of the functional relevant gut microbiota in bumblebees. Clearly, much more work is needed to approach these goals. Thereby, any methodological advances in manipulating specific components of the microbiota, such as via RNAi (Deshwal and Mallon, 2014) or genome engineering of particular microbiota taxa (Cong et al., 2013), will prove invaluable for a complete understanding of functional variations among microbiota taxa, particularly in bees (Engel, Martinson, and Moran, 2012; Engel, Stepanauskas,

and Moran, 2014), and for the understanding of gut microbiota more generally.

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