The Bee Microbiome: Impact on Bee Health and Model for Evolution and Ecology of Host-Microbe Interactions

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ABSTRACT

As pollinators, bees are cornerstones for terrestrial ecosystem stability and key components in agricultural productivity. All animals, including bees, are associated with a diverse community of microbes, commonly referred to as the microbiome. The bee microbiome is likely to be a crucial factor affecting host health. However, with the exception of a few pathogens, the impacts of most members of the bee microbiome on host health are poorly understood. Further, the evolutionary and ecological forces that shape and change the microbiome are unclear. Here, we discuss recent progress in our understanding of the bee microbiome, and we present challenges associated with its investigation. We conclude that global coordination of research efforts is needed to fully understand the complex and highly dynamic nature of the interplay between the bee microbiome, its host, and the environment. High-throughput sequencing technologies are ideal for exploring complex biological systems, including host-microbe interactions. To maximize their value and to improve assessment of the factors affecting bee health, sequence data should be archived, curated, and analyzed in ways that promote the synthesis of different studies. To this end, the BeeBiome consortium aims to develop an online database which would provide reference sequences, archive metadata, and host analytical resources. The goal would be to support applied and fundamental research on bees and their associated microbes and to provide a collaborative framework for sharing primary data from different research programs, thus furthering our understanding of the bee microbiome and its impact on pollinator health.

Bees, ranging from wild solitary species to highly social and managed species like honey bees, play key roles in natural and agricultural ecosystems worldwide (1–3). Recent losses of honey bees and wild bees have been attributed to pesticide exposure, poor nutrition, increased parasite loads, habitat degradation, and reduced genetic diversity (4, 5). While the latter cause has been challenged by genome-wide surveys of honey bee populations (6), pesticides, parasites and malnutrition have been experimentally linked to poor bee health (5, 7–11).

Bee-associated microorganisms include a diverse set of viruses, bacteria, fungi, and protists, some of which are important pathogens of bees (12). For example, the two bacterial pathogens Paenibacillus larvae and Melissococcus plutonius are the causative agents of American and European foulbrood, respectively (13, 14). They are spread easily by beekeeping practices, and if left untreated,


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they are usually lethal to the colony. Their treatment is correspondingly rigorous and expensive, involving the sacrifice of both the colony and hive materials, and, in some countries, quarantine procedures for the affected beekeeping operation and surrounding apiaries.

However, for many pathogens, the precise impacts on honey bee health and colony fitness remain elusive (12). For example, the respective effects on honey bee health of two common microsporidian pathogens, Nosema apis and Nosema ceranae, remain controversial. Some studies suggest that N. ceranae is more virulent than N. apis and may be in part responsible for recent colony losses (15–17). Other studies have failed to show such links, and it has been argued that additional factors might contribute to the virulence of Nosema (5, 18–22).

In addition to microsporidian pathogens, over twenty different bee viruses have been described to date (23, 24). Most can cause detrimental effects, ranging from physiological changes to gross physical deformities, behavioral alterations, and reduced longevity. The degree of pathology may differ between individual hosts, and most viruses can persist chronically and asymptptomatically within bee colonies (25). There is accumulating evidence that simultaneous infection with multiple pathogens or the combined exposure to pathogens and pesticides can have synergistic negative effects on bee health (12, 26–32). Furthermore, many honey bee viral pathogens can cross-infect bumblebees, solitary bees, and wasps, suggesting that the pool of pathogens in a particular host, such as the honey bee, must be viewed in the context of a larger set of host species with overlapping geographic ranges (33, 34).

In addition to the plethora of pathogens, honey bees harbor a relatively simple but remarkably specialized and consistent intestinal microbial community, consisting of 8 to 10 bacterial species or phyotypes (i.e., bacteria with ≥97% 16S rRNA sequence identity) (35), belonging to three different phyla: Proteobacteria, Actinobacteria, and Firmicutes. The precise functions of these gut bacteria and the nature of their symbiotic relationships with the host have so far remained largely elusive. Nevertheless, analogous symbionts found in vertebrate gut communities play key roles in host health and assist the host in the face of environmental stress (36, 37).

Genomic and metagenomic analyses suggest that bee gut bacteria contribute to the digestion of macromolecules, nutrient provisioning, neutralization of dietary toxins, and defense against parasites (38–40). Whether and to what extent the bacterial gut communities of bees influence pathogen loads, through modulation of the immune response, by providing barriers against invasion, or through competition for nutrients, is still unclear. However, there is evidence that some of the Firmicutes bacteria can inhibit the growth of the two principal honey bee bacterial pathogens, Paenibacillus larvae and Melissococcus plutonius (41, 42). Moreover, in bumble bees, gut communities have been associated with reduced levels of the parasite Crithidia bombi, both in experiments and wild populations (43, 44). In honey bees, a common member of the gut microbiome, Frischella perrara, is responsible for causing the widespread scab phenotype in the gut, likely due to the local induction of a melanization immune response (45). This example illustrates that microbes can be the hidden cause of host phenotypes and raises the possibility that widespread gut symbionts may be detrimental to their hosts.

In this paper, we collectively refer to the microbes associated with bees as the ‘bee microbiome,’ regardless of whether these symbionts engage in mutualistic, commensal, or parasitic relationships with the host. In the following sections, we discuss the most relevant research questions to be addressed with respect to the evolution of the bee microbiome and its relevance to the health of the host (Table 1). We argue that, to achieve these goals, standardized research methods and analyses are required, as well as communal resources that integrate information from disparate studies.

We also propose that bees present an excellent model to study fundamental aspects of the ecology and evolution of microbe-host interactions in multispecies relationships. Bees constitute a diverse group of insects that have evolved remarkably different lifestyles and social behaviors (46, 47), and yet they share a large number of microbial commensals and pathogens (33, 48, 49). Knowing how such lifestyle differences affect microbial transmission and thereby shape the composition and ecology of the microbiome in different species will be invaluable for a better understanding of bee biology and microbiome evolution.

**MICROBIOME AS A COMPONENT OF BEE HEALTH**

As with the human microbiome, the bee gut microbiome likely consists of a mix of beneficial, commensal, and pathogenic microbes. The relative and absolute abundances of the community members and their interactions with one another will determine the microbiome’s overall contribution to host health. Bees may therefore be colonized by different microbial communities, depending on individual host circumstances and environmental

### TABLE 1 Outstanding questions in bee microbiome research

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<thead>
<tr>
<th>Field of knowledge</th>
<th>Research question</th>
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<tbody>
<tr>
<td>Bee health</td>
<td>How do different microbes impact bee health?</td>
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<td></td>
<td>Nutritional versus defensive symbioses?</td>
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<td>Host range and fitness effects of pathogens?</td>
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<td></td>
<td>Which combinations of microbes are most detrimental or beneficial?</td>
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<td></td>
<td>Do microbes in wild and managed bee populations influence health in similar ways?</td>
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<td></td>
<td>How do environmental factors influence host-microbiome interactions?</td>
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<td></td>
<td>How do the interactions among microbiome members modulate the impact of individual members, to the benefit or cost to the host?</td>
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<tr>
<td>Evolution and ecology</td>
<td>What are the functions of the different microbes in the bee gut?</td>
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<td></td>
<td>Which factors drive the composition and evolution of the bee microbiome?</td>
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<td>How does the social/solitary lifestyle of bees influence microbiome evolution?</td>
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<td>How has domestication changed the bee microbiome?</td>
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TABLE 2 Major challenges for bee microbiome studies

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<thead>
<tr>
<th>Area of study</th>
<th>Questions and considerations</th>
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<tr>
<td>1. Defining bee health</td>
<td>What are the best measures of fitness at the individual and colony levels? In social insects, fitness experiments with individual bees may not reflect the fitness of a colony.</td>
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<td>2. Microbiome composition</td>
<td>How do distinct microbes interact to affect hosts? Microbes likely influence one another’s effect on the host. Coinfections pose a challenge for disentangling individual roles, and certain experiments may need to be conducted in microbiome-free hosts.</td>
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<td>3. Host genetics</td>
<td>Bees are genetically diverse, which may affect microbiome-host interactions. Can the influence of genetic differences among hosts be controlled for?</td>
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<td>4. Environmental factors</td>
<td>These are likely to contribute to microbiome composition and function. How can such environmental factors be reliably measured and tested?</td>
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<td>5. Physiological variables</td>
<td>The physiology and development of bees can differ substantially according to season, age, housing, and nutrition. Do these differences influence microbiome relationships?</td>
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<td>6. Microbiome quantification</td>
<td>Different diagnostic tools (quantitative and qualitative) exist to study microbiome compositions. How comparable are these tools?</td>
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<td>7. Wild pollinators</td>
<td>For the majority of the ~20,000 wild bee species, the microbiome composition and functions are unknown. Systematic and standardized sampling approaches are needed.</td>
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<td>8. Data accessibility</td>
<td>Systematic archiving of large sequencing datasets with accompanying metadata information is crucial so that cross-study analyses can be conducted.</td>
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conditions. A recent study of the gut microbiome of bumble bees supports this hypothesis. Two distinct gut microbial communities, so-called enterotypes, were found to occur in individuals of various bumble bee species in China (50). One enterotype was dominated by core bacterial species that are typical for honey bees and bumble bees, while the other was dominated by species often found to be pathogenic for insects. How these dramatic differences in microbiota occurring within host species affect host health is not known. An important future research avenue is to disentangle the various individual contributions of gut symbionts so as to reconstruct and understand their combined contribution to host fitness, in managed as well as wild pollinators.

The impact of most microbial parasites on bee health has likewise remained elusive. *Microsporidia*, trypanosomatids and viruses are frequently detected in honey bees, without any obvious detrimental effects (51–53). These asymptomatic infections render the definition of health more complex: particularly in social bees, in which individuals of different castes, ages, and genetic backgrounds (i.e., varying in relatedness due to polyandry) form a “superorganism,” it is crucial to define measurable health criteria instead of focusing on the somewhat nebulous idea of “bee health” or “bee vitality” (Table 2). For example, to study the effects of pathogen transmission or replication in host tissues, the measurable criteria for individual bees could include longevity, adult weight and the ability to collect resources for nest mates. Measurable traits at the colony level include colony growth and strength (e.g., the Liebefeld method [54] or frame counts for honey bees), honey production, queen production for bumble bees, and survival during winter. Independent pathogen-specific criteria include prevalence, abundance, replication rate, transmissibility, and infectivity. Additionally, in highly social species, such as the honey bee, it remains to be determined how individual health and infection frequency translate into colony fitness measurements.

The effects of specific microorganisms on bee health may be modulated by the context of infection, including cointfection or colonization by different microorganisms (41, 42, 55, 56). Some microbes, such as the gut symbionts in bumble bees (44), might provide protection against pathogenic agents. Others could have synergistic effects with pathogens and, thus, have negative effects even if individually they have a neutral or positive impact on bee health. Thus, interactions between microorganisms can influence the results or interpretation of infection experiments. Cocolonization studies in sterile host backgrounds are one means to initially characterize these interactions precisely and elucidate their effects on the host.

The contribution of the hosts’ genetic background has largely been ignored in bee microbiome studies. Honey bee queens typically mate with 10 to 20 male bees, whereas queens of many other bee species mate with only one male. Consequently, the genetic structure and diversity within and across colonies and between species vary substantially. This genotypic variation may modulate interactions with the microbiome, as shown for other insects (57, 58) and as indicated by reduced pathogenic pressure in polyandrous versus monandrous honey bee queens (59, 60). For example, genotypes may vary in immune response patterns that influence the susceptibility to colonization by particular microbes.

Another challenging question is the extent to which the environment contributes to bee microbiome composition and dynamics, and how this in turn affects host fitness. The honey bee and bumble bee gut microbiomes appear to be highly host specific and most likely acquired through social activity, while the microbiomes of other bees (both solitary and primitively eusocial) appear to mostly consist of environmentally acquired microbes (39, 61, 62). Therefore, environmental context may be more important to symbiont acquisition in these bee species than in Bombus and *Apis* species. Although little is known about microbiome host specificity in wild bees, their microbial compositions may be reflected by transmission/acquisition parameters that could include the number and species of flowers visited and the rates at which those flowers are visited by other pollinators.

Studies aimed at determining the diversity and overlap of pollinator microbes and pathogens throughout pollinator ecosystems are needed. Several recent studies demonstrate that potential pathogens are shared between different pollinator species, most likely via horizontal transmission at common floral resources (34, 49, 63).

Human activity is the major disruptive influence in many ecosystems around the world (64–67). Habitat fragmentation, agricultural intensification and monocultures, climate change, the globalization of trade, and the accidental or deliberate introduc-
tion of invasive species all conspire to threaten endemic or other valued species around the world. One open question concerns the effects of environmental anthropogenic toxins (including pesticides) on the structure and composition of the bee microbiome, especially in the gut. For honey bees, recent evidence that beekeeping practices can shape the gut microbiome is provided by the emergence of increased antibiotic resistance in honey bee gut symbionts in the United States (68). This is likely due to extensive prophylactic use of oxytetracycline to fight Paenibacillus larvae, the causative agent of American foulbrood. In addition, pesticide exposure can modulate the immune response of bees, thereby increasing susceptibility to opportunistic viral infections (69). These examples show the importance of considering environmental factors when studying microbiome-host interactions.

Relative to the studies on managed pollinator species, there are fewer in-depth studies of anthropogenic environmental influences on the microbiomes of wild pollinators, including solitary bees and bumble bees. However, the human impact may be substantial; for example, the loss of two once-common bumble bee species in North America may be due to the introduction of Nosema bombi by human activity (70). The introduction of non-native solitary bees may also introduce disease (71). Given global trade and extended pollinator movement through accidental introductions or commercial beekeeping, the potential for the spread of microbes affecting pollinators should not be underestimated (72–74).

A comprehensive catalogue of Apis and non-Apis diseases, microbes, and supporting literature, providing an overview of most of the microorganisms known to date, can be found in the supplemental material (see Tables S1, S2, and S3 and Texts S1, S2, and S3).

THE BEE MICROBIOME AS AN EVOLUTIONARY MODEL FOR SYMBIOSIS

The multitude of microbial interactions in bees, both beneficial and parasitic, offers an excellent opportunity for investigating the evolution of different symbiotic strategies. Of particular interest is the question of how host lifestyle influences these processes. The effect of sociality on microbiome evolution and the potential reciprocal impacts on the host remain largely unstudied. Bees represent an excellent model to address such questions, because related species exhibit marked differences in the degree of sociality, ranging from solitary through facultative social to highly eusocial. We hypothesize that the transmission of microbes is facilitated by specialized social contacts among host individuals, favoring the maintenance of microbial associations. Sociality does not always correlate with host specificity (61, 75), but social transmission has been shown to be important for establishing the honey bee and bumble bee gut microbiome (76–78). Perhaps specialized behaviors like trophallaxis (transfer of food among members of a community) allow the maintenance of characteristic associations, such as those found with Apis and Bombus spp. In this respect, the gut microbiome is of particular interest.

The honey bee gut community represents a complex ecosystem involving multiple species of bacterial symbionts interacting within a dynamic host environment, but one that is nonetheless simpler than those of mammalian models (79). How stable heritable microbial gut communities arise and evolve is a central question in microbiome research, and resolving the extent to which such communities exist in different bee species will be invaluable for identifying general principles of gut microbiome evolution that are more broadly applicable across social animals.

The evolutionary forces that shape the animal microbiome remain unclear. One hypothesis is that the dominant forces driving microbiome evolution are microbe-microbe interactions, with host effects being relatively minor, consisting primarily of providing the physical substrate and raw nutrients for the development of the microbiome. Microbial cooperation and competition may give rise to traits such as resource partitioning and spatial organization. An alternative and perhaps more appealing hypothesis is that microbiome evolution is driven by coevolutionary dynamics occurring between gut microbiomes and their hosts. To date, evidence suggests that only the social corbiculate bees possess distinctive gut communities, while most other bee species and wasps possess transient or highly variable microbiomes (62, 79). This is consistent with the hypothesis that the evolution of intimate microbial associations is favored in social hosts (80, 81). Continuous close social contacts likely aid transmission of beneficial microbes from parent to offspring and between colony members (77, 78), facilitating the evolution of mutualistic interactions.

Social contacts also provide opportunities for the emergence of cheaters: symbionts that evolve to spread rapidly at the expense of the host and other gut community members, by hijacking efficient routes of social transmission. How these selfish tendencies are kept in check is mostly unknown for gut symbionts, and the bee gut is a tractable ecological system in which to examine this issue. Social contact will facilitate the spread not only of beneficial microbes but also pathogenic ones (82). The large population size of some social bee species could favor the emergence and evolutionary persistence of pathogens causing acute diseases that spread rapidly through the host population (e.g., Paenibacillus larvae or Melissococcus plutonius). Conversely, a solitary lifestyle may select for pathogens causing chronic infections, because host transmission is less frequent and persistence strategies increase the chance of pathogens being maintained, to allow their eventual transmission. The bee microbiome presents an excellent model to study such coevolutionary dynamics of microbe-host relationships. However, additional data on the microbiome composition, especially for wild bee species, is needed for a more comprehensive picture of these processes.

The bee immune system (83, 84) almost certainly plays a substantial role in mediating symbioses. In the gut microbiome, evidence for co-diversification (77, 85, 86) suggests that the association between the corbiculate bees and their symbionts is ancient. An understanding of how the microbiome can successfully colonize the gut without rejection by the host immune system may help to reveal the evolutionary processes responsible for the development of specialized and heritable gut communities. Host immunity may act as a mechanism of partner choice (87), permitting only certain strains to colonize, resulting in host specificity and driving divergence of the microbiomes between separate bee lineages (39, 77). The microbiome itself may be a critical component of bee immunity; there is evidence for defensive functions by some gut bacterial species and for potential pathogen-specific interactions (44, 88).

The recent discovery of high strain-level diversity within many of the gut bacterial species of honey bees is intriguing (39, 85, 89, 90). Several explanations for this fine-scale diversity have been proposed; for example, functional diversification due to niche partitioning (38) and co-divergence with, and adaptation to, host...
lineages (77). The pattern of diversification in the honey bee gut microbiome parallels that in mammalian gut microbiomes, in which a large number of strains have emerged from a relatively few founder lineages (91, 92). Whether such microbiome diversity benefits the host is debated (93, 94). To understand functionally relevant diversity, we need more information on how divergent functions are distributed across the members of the gut community and how they affect hosts.

The consequences of diversity for microbiome stability are also unknown. Dynamic interaction networks between gut species and strains could give rise to stable and distinct states (enterotypes) within host species (95). Identifying how different factors, such as diet, sociality, and host physiology, drive microbiome composition will be an important step forward. The effects of beekeeping practices on the bee microbiome should also be assessed. For example, routine mixing of bees from multiple colonies or the importation of nonnative (sub)species into new geographic regions may irrevocably alter gut communities in an artificial and negative manner. Even the distance between honey bee colonies may play a role in promoting the exchange of symbionts and potentially affecting the strains that replicate at the host’s expense, versus mutualistic genotypes that benefit or are benign to hosts.

PERSPECTIVE: CHALLENGES AND NOVEL APPROACHES

Measuring the effects of microbes on bee health is a challenging undertaking that requires consideration of many abiotic and biotic factors and their interactions (Table 2). Approaches to assess variation beyond the standard markers, such as the 16S rRNA gene, are needed in order to uncover intraspecific variation that may affect bee ecology. Elucidating the central processes affecting bee microbiomes will require sequencing of genomes and transcriptomes of cultured strains, as well as metagenomes and metatranscriptomes, which can provide snapshots of whole communities. These data could be used to reveal patterns of microbial gene flow within and between host species and may define microbial pangenomes, thus providing insight into microbiome evolution. Genomic characterization should not be confined to members of the gut microbiome but should also extend to other microbes, for example by targeted sequencing of virus-derived small RNAs (96) and species found on flowers and hive material (97, 98). Such broader sampling would provide a more complete picture of the microbial environment bees inhabit.

Correlative studies based on high-throughput sequencing of targeted samples can be extremely powerful for formulating hypotheses about the contributions of different factors to bee health. Sequencing data can be linked to metadata information to suggest how factors such as colony health, host genotype, season, and environmental stressors correlate with the composition of the microbiome (e.g., Cornman et al. [29]). Such correlative patterns can narrow the set of hypotheses to be tested by confirmatory experimental studies of causal relationships.

A strength of the bee microbiome system is that it is being addressed by a worldwide community of researchers using diverse approaches. However, the lack of coordination across studies also presents an obstacle. By combining data from different sequence-based studies, global patterns of bee microbiome evolution might become evident. To promote better integration, we propose the establishment of a curated online database (Fig. 1) dedicated to bee-associated microbes with the overall aims to (i) facilitate standardization, data integration, and collaboration between researchers in the field and (ii) facilitate transparent scientific exchange and communication with the public, research councils, and policy makers.

One planned element of the database would be an organized encyclopedia for bee-associated microbes (bees ranging from solitary bees to social bees), providing a general description of each microbe, listing relevant publications and methods for detection, and linking available genomic data. Full genome sequences for the major viruses, bacterial disease agents, fungi, and eukaryotic gut
parasites are available in general public sequence repositories (e.g., GenBank and ENA) with new ones added continuously to encompass the breadth of global bee microbiome species and strains. For microbes currently lacking genome assemblies, sequences of marker genes, such as the 16S rRNA gene, are available. As an example, a nonredundant sequence set from honey bee-associated microbes has been in development (99, 100). An expanded version of this could serve, alongside other datasets, in the BeeBiome database.

A second element in the database envisioned can serve as a centralized place for information from bee microbiome projects. The database can link to existing high-throughput sequencing datasets in repositories suitable for long-term storage, such as the Sequence Read Archive (at NCBI) or the European Nucleotide Archive (at EMBL-EBI). Datasets will include metadata (e.g., sample type, sampling date and location, host genotype, host age/caste, sequencing platform, sequencing depth, sample processing methods, and metagenomic assembly and analysis routines). These will enable common practices in the future and promote the discussion of what those practices should entail. Finally, as a third element we propose the development of bioinformatic processing pipelines to promote common analysis protocols. These data and pipelines could be distributed via dedicated Web portals along with public resources for genomic analysis. Existing portals that minimize technical burdens while retaining transparency and flexibility include Galaxy, Embassy Cloud, CyVerse (previously iPlant), and various cloud-based environments (e.g., Amazon-Quime).

Gathering high-throughput sequencing projects and making them accessible from one location can aid the design of future studies so that they are compatible with, and complementary to, existing datasets, facilitating data integration and filling knowledge gaps. Finally, the database can enable the monitoring of changes in the bee microbiome across time and space, to allow detection of large-scale shifts in pathogen distributions and numbers or in genomic composition and to link such changes to variables representing the environment, climate, parasite load, or composition of the gut microbiome.

The first step toward the establishment of such a database occurred during the first bee microbiome workshop funded through the National Evolutionary Synthesis Center (NESCent), United States. A second meeting will focus on practical issues of financing and maintaining such a database, a major challenge for providing high-quality community resources. Ideally, the database should be curated by a dedicated project manager and financially supported by international public funding agencies to guarantee the continuity and stability of the resources.

CONCLUSIONS
The bee microbiome (i) is an important factor in bee health and (ii) can serve as an excellent model to study the evolution and ecology of microbial symbioses. However, a large number of important questions remain unsolved (Table 1). Co-infections of pathogens are frequent, the contribution of gut microbial communities to bee health is not yet understood, and the effects of environmental parameters on host-microbe interactions are unclear. Investigating possible synergistic and antagonistic interactions among the microbes, their environment, and the bee host will be crucial for the evaluation of host impact. This requires comprehensive characterization and systematic, functional analysis of all microbes associated with bees, i.e., the bee microbiome. High-throughput sequencing methods provide means to conduct large-scale surveys of bee microbes in nature and to identify correlations, for which causative processes can then be tested in laboratory experiments. However, formidable challenges are linked to such systematic approaches, including the sheer diversity of bee species, the variability of environmental parameters, the complex lifestyle of social bee species, and the lack of standardization of methods to allow cross-study comparison (Table 2). A number of relatively simple steps for advancements are proposed in Table 3. In addition, a database dedicated to bee microbiome research may help to overcome these challenges and allow scientists to join forces to study fundamental aspects of bee microbiome evolution, ecology and bee health. We envision such a database as providing guidelines for standard operational procedures, archiving capabilities for sequencing data and metadata information, and cross-study analysis tools. These resources will facilitate research, add transparency to data analysis, and improve the comparability of results across studies, approaches, and study systems. Such resources would evolve to keep track of new data, as well as improved analytical or laboratory techniques. A working group has been created to strive toward those objectives.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/supp/doi:10.1128/mBio.02164-15/-/DCSupplemental.

Table S1, XLSX file, 0.02 MB.
Table S2, XLSX file, 0.01 MB.
Table S3, XLSX file, 0.02 MB.
Text S1, DOCX file, 0.02 MB.
Text S2, PDF file, 0.1 MB.
Text S3, DOCX file, 0.02 MB.

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