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Development of the Honey Bee Gut Microbiome throughout the Queen-Rearing Process

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The European honey bee (*Apis mellifera*) is used extensively to produce hive products and for crop pollination, but pervasive concerns about colony health and population decline have sparked an interest in the microbial communities that are associated with these important insects. Currently, only the microbiome of workers has been characterized, while little to nothing is known about the bacterial communities that are associated with queens, even though their health and proper function are central to colony productivity. Here, we provide a large-scale analysis of the gut microbiome of honey bee queens during their developmental trajectory and through the multiple colonies that host them as part of modern queen-rearing practices. We found that queen microbiomes underwent a dramatic shift in size and composition as they aged and encountered different worker populations and colony environments. Queen microbiomes were dominated by enteric bacteria in early life but were comprised primarily of alphaproteobacteria at maturity. Furthermore, queen gut microbiomes did not reflect those of the workers who tended them and, indeed, they lacked many of the bacteria that are considered vital to workers. While worker gut microbiotas were consistent across the unrelated colony populations sampled, the microbiotas of the related queens were highly variable. Bacterial communities in mature queen guts were similar in size to those of mature workers and were characterized by dominant and specific alphaproteobacterial strains known to be associated with worker hypopharyngeal glands. Our results suggest a model in which queen guts are colonized by bacteria from workers' glands, in contrast to routes of maternal inoculation for other animal microbiomes.

Honey bees (*Apis* spp.) are characterized by a highly partitioned reproductive division of labor, where a single queen lays the eggs that give rise to virtually all members of her colony, and her daughters, the workers, execute all other laborious jobs, including that of caring for her offspring (1). As the sole caregivers in the colony, workers share food extensively with one another, consuming their colony's food reserves and then distributing nutrients in various forms to their queen, other workers, and reproductive males (drones) (2). For all three of these castes, workers share food with adults through trophallaxis (mouth-to-mouth food transfer of liquids from one worker's gut to another's) or by feeding developing larvae brood food (glandular secretions derived from consumed nutrients). Because of these mechanisms of food distribution, a honey bee colony often is considered to have a "social stomach." Studies with tracers show rapid distribution (<24 h) of food from small numbers of individuals to many, if not the majority, of colony members across all ages and castes (3–7). Nurse-age bees (typically less than 10 days old) are the primary consumers of pollen (8); thus, they are the main distributors of pollen-based nutrients to adults and the brood that they rear (3, 5). Nectar also is distributed among colony members; foragers bring it back to the hive in their crops (foregut), and then it is handled by bees of various ages, either to feed adults and larvae or to store in wax combs and ripen into honey (7). This continual exchange of food among nestmates during all stages of their lives makes the establishment and maintenance of gut microbiomes particularly complex to understand across castes and for individuals as they age. However, the microbial communities that are associated with honey bees are considered to have an important influence on nutrient availability in colonies (although the specific role of microbes is uncertain) (9). As such, there is growing interest in elucidating the microbial communities within insect societies using culture-independent techniques.

Presently, gut microbiomes have been best explored in workers of the European honey bee (*Apis mellifera*) (10). Workers have a characteristic microbial community that is composed predominantly of three major bacterial phyla (*Firmicutes*, *Proteobacteria*, and *Actinobacteria*) that are transcriptionally active within the honey bee gut (11). Within these larger taxonomic groupings, several honey bee-specific families and genera have been identified (12–14). The core microbiome of the adult worker has been characterized as being comprised of a small number of bacterial clades, some with new genus and species designations (12, 15, 16). These core clades have been referred to as Firm-4, Firm-5 (within the *Firmicutes*), Bifido (within the *Actinobacteria*), and Alpha-2.1, Alpha-2.2, Alpha-1, Beta, Gamma-1, and Gamma-2 (within the *Proteobacteria*) (12). Although several other bacterial species are found to be associated with honey bee workers, these specific clades are consistently found across different geographic regions and throughout different seasonal samplings (12, 13, 17). A number of beneficial interactions among these microbes and the honey

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bee have been suggested, including increased metabolic functionality; these microbes may degrade complex polysaccharides that otherwise are inaccessible to the host organism (10). Indeed, the bacterial community encodes and expresses β-glucosidase genes specific to the breakdown of cellulose (10, 11). Another potential benefit of the honey bee microbiome includes protection from invading pathogens through facilitation of the immune response (18) or exclusionary effects (19–21).

In contrast, virtually nothing is known about the microbial communities that are associated with queens, even though their health and proper function are central to the productivity of their colonies. Complicating such studies are the realities of queen production in modern apiculture, where colonies are managed intensively to yield hive products (e.g., honey) and particularly for crop pollination. In managed colonies, the natural process of queen replacement, where an aging queen is superseded by a daughter queen, raised by her worker sisters from within the larval ranks, typically is prevented by beekeepers. Instead, intentionally bred queens are artificially introduced to a colony by beekeepers only after taking a circuitous path through several other related and unrelated host colonies (22, 23).

Queens from different genetic sources (known as grafting sources) are reared as larvae in cell builders, which are unrelated colonies whose queenless workers nurse larval queens until they are sealed into their cells (~1 week). Each sealed cell then is transferred to a small nucleus colony, or mating nuc, where another set of unrelated workers support the queen through the first week or more of her adult life. Once she has mated and started laying eggs (typically 2 to 3 weeks), eventually she is transferred into a final host colony of queenless, unrelated workers who care for her as she begins to lay eggs for the new colony. Eventually her worker progeny replace the resident, unrelated worker population as the initial colony population dies. Queens do not feed themselves at any point during this process; both larval and adult queens receive food products from many different workers who care for them. Larval queens are reared by nurse-aged workers and, as adults, spend most of their time in the brood area of the nest, where they continue to be fed by nurse-aged bees from the resident worker population (24, 25). Thus, in addition to understanding the general characteristics of queen microbiomes relative to the worker caste, we also must understand how their exposure in managed colonies to different populations of workers shapes their microbiomes over the course of their lives.

Here, we present the first description of the bacterial communities that are associated with honey bee queens that have navigated the complicated queen-rearing process typically employed by beekeepers. We used 16S rRNA gene amplicon sequencing to identify milestones in the development of their microbiome and to evaluate the relative impact on their bacterial communities of the unrelated and related worker populations that they encounter over their lifetimes. In addition, we use quantitative PCR to elucidate the relative numbers of 16S rRNA gene copies in queen microbiomes as they are hosted by different populations of workers during their larval and adult development. Our data reveal many intriguing aspects of the development of the queen gut microbiome. Most notably, queens experience a dramatic shift in the composition of their bacterial communities as they age, especially between larvae and adults; interestingly, these communities do not reflect the microbial profiles of the workers who cared for them. Moreover, while unrelated populations of host workers had relatively similar gut microbiomes, the gut microbiomes of the related queens that they tended were far more variable across individuals. Queen microbiota were dominated by honey bee-specific alphaproteobacteria and lacked the canonical core gut microbiome that is associated with honey bee workers. Because queens are fed from worker hypopharyngeal glands, and because bee-specific alphaproteobacteria are heavily associated with this gland, our results suggest a model in which queen guts are colonized by bacteria from a specific worker organ. This result contrasts with routes of inoculation for mammalian microbiomes, where the maternal environment is the source of bacteria for the next generation (26–28).

MATERIALS AND METHODS

Queen rearing and sample collection. The field research was conducted at the North Carolina State University Lake Wheeler Honey Bee Research Facility (Raleigh, North Carolina). A single honey bee colony was chosen as the sole source of all reared queens to minimize genetic variance among individuals within the focal caste. Our general approach was to track these queens throughout their larval and adult development, sampling a subset of queens and host workers each time queens encountered a new colony in order to characterize and compare microbiomes across castes and host populations.

The experiment began by first marking with paint a single cohort of newly emerged workers from the single queen source colony (here referred to as the grafting source). These workers emerged from sealed cells over a 24-h period in an incubator set under broodnest conditions (34°C and ~50% relative humidity), and then they were paint marked as 1-day-old adults and reintroduced within hours to their natal hive ($n > 200$ marked workers). Five days later, 45 female larvae were transferred (grafted) from their cells into plastic queen cells by following standard queen-rearing techniques (e.g., see reference 23). All worker larvae were <24 h old to ensure that the resultant queens were of high reproductive potential (see reference 29). Larval transfer was accomplished without priming each queen cell with royal jelly (known as dry grafting) to avoid potential microbial cross-contamination, and the grafting needle also was dipped in 95% ethanol and flamed with a lighter in between larval transfers for the same reason. An additional 24 larvae of the same age were sampled from the grafting source directly into cryopreservation tubes, which were immediately submerged in liquid nitrogen. The tubes were later decanted from the liquid nitrogen and stored in a −80°C freezer until further processing. On the same day as grafting and continuing into the next, marked workers (now 5 days old) were individually collected from the inner combs of the grafting source colony, placed into separate glass vials, and cooled on ice until they were immobile. Once chilled, the mid- and hindguts were removed from each worker by gently pulling on the stinger with sterilized metal tweezers until the lower organs of the gastrointestinal (GI) tract were extracted ($n = 100$ grafting-source workers). The gut from each worker bee then was placed into an individually labeled cryotube, immediately flash frozen in liquid nitrogen, and later stored in a −80°C freezer until further processing (21). Thus, 5-day-old workers were sampled from the grafting-source colony, which is an age when workers are likely to be nurse bees that provide larvae with brood food, including the young focal queen larvae (25). After all larvae were grafted, the queen cells were placed into a queenless cell builder, where its resident workers reared the unrelated queen larvae to pupation over the next 7 days. Two days prior to the transfer of queen cells to the cell builder, a cohort of same-aged, newly emerged workers from the cell builder were paint marked as described previously.

Five days later, mid- and hindguts were collected from marked workers in the cell builder, as described already ($n = 100$ cell-builder workers). Therefore, these 5-day-old workers in the common cell builder were the most likely to have been actively provisioning the developing queen larvae with royal jelly over the majority of their larval period. Three days later,

after the queen cells were sealed by workers so that the queens could pupate, the cells were moved into an incubator to complete their development.

The day prior to their emergence of queens from sealed cells, 30 queen cells were transferred into 30 separate mating nucleus colonies that had been established from six unrelated colonies (5 mating nuclei per source colony). Each mating nucleus contained 500 to 2,000 adult worker bees of unknown age and three miniframes of brood and stored food (honey and pollen); these workers cared for the queen during the early period of her adult life, while she mated, and when she began to lay eggs. The remaining queen cells were placed into separate glass test tubes so that the queens could be captured upon their emergence in the incubator. Therefore, these queens had no contact with worker populations after leaving the cell builder. The following day, 13 newly emerged cell builder queens were collected from the incubator, and their mid- and hindguts were removed. Unlike workers, the guts of which could be easily extracted by gently pulling on the sting shaft, each queen was separately dissected with sterilized microscissors and forceps in order to obtain their GI tracts, which were immediately flash-frozen as described above.

Between 10 and 20 days following queen emergence in their respective mating nucleus colonies, adult worker bees were sampled from each mating nucleus for their gut contents by following the procedure outlined above. Unlike the workers sampled from the grafting source and cell builder, these workers were of unknown age, because sufficient numbers of emerging workers from each small unit could not be obtained. Of the 30 mating nuclei that were established, several were not successful because the queens did not emerge from their cells properly, were not accepted by the workers upon emergence, or failed to successfully mate on their mating flights and begin oviposition. A total of 13 mating nuclei had samples for 100 mating nucleus workers collected per colony (one unit yielded only 81 workers because of a limited worker population at the time of sampling). At this time, five queens also were destructively sampled from their mating colonies as outlined above (here referred to as the mating nuc queens), each from a different source of the six source colonies originally used to create the nuclei.

The remaining 8 mated and laying queens then were removed from their respective mating nuclei, placed into separate queen cages, and introduced into new field colonies by following standard techniques (23). All of the final colonies were unrelated to each other and all other colonies in the experiment. After 2 days of acclimation, the five surviving queens were released from their cages into their final colonies and visually verified as accepted by the resident unrelated workers (three of the queens died in their cages; thus, they were not accepted by the workers). Two weeks after introduction, frames of emerging workers were collected from each hive and emerged in the incubator to paint mark and reintroduce a cohort of age-matched workers in each, as described above. The mid- and hindgut were removed from a sample of these workers 5 days later, as described previously ($n = 100$ final-colony-prior-to-offspring-emergence workers per colony). Several of the remaining queens also were sampled at this time by following the methods described above ($n = 3$ final-colony-before-offspring-emergence queens). Two weeks later, a second cohort of newly emerged resident worker bees was similarly marked in each of the remaining colonies, when emerging brood were the genetic offspring of the 2 remaining focal queens. These marked workers were sampled and their guts removed 5 days later, as described above ($n = 2$ final-colony-after-offspring-emergence workers per colony). At this time, the final remaining two queens also were sampled for their gut contents (here referred to as final-colony-after-offspring-emergence queens).

DNA extraction and amplicon library generation. Queen and larvae were processed individually, and workers from each colony were processed in pools of 10. In addition, we also processed and sequenced individual workers (10 each) in order to account for sequencing depth for comparisons to individual queens. DNA was extracted from all samples using a modified liquid nitrogen protocol. Briefly, sterilized, ceramic mortar and pestles were precooled in liquid nitrogen, and samples were ground into a fine powder. This powder was added to Tris-EDTA with added proteinase K (at 0.025 μg/ml final concentration) and incubated for 1 h at 50°C. After this incubation, a phenol-chloroform extraction was performed twice before ethanol precipitation. DNAs were resuspended in Tris-EDTA and cleaned using a column-based genomic cleanup kit (Zymo) according to the provided instructions. DNAs then were quantified (using an Epoch Take3 plate) and stored at −80°C before use in PCR.

PCR using barcoded Illumina primers was performed by following the Earth Microbiome protocols (30), with the following differences. HF Phusion polymerase mix (New England BioLabs, Ipswich, MA) was used and 3% dimethylsulfoxide (DMSO) was added to the reaction mixtures before cycling at 98°C for 45 s, 50°C for 60 s, and 72°C for 90 s. Amplifications were performed in triplicate and pooled before normalization based on PicoGreen quantification.

Sequencing and bioinformatics analysis. Pooled amplicons were sequenced at the Indiana Center for Genomics and Bioinformatics core facility (Bloomington, Indiana) using an Illumina MiSeq and 250 paired-end (PE) cycles. Adapter sequences were removed from all reads before raw processing of data (using the program suite Mothur). We utilized the Schloss SOP (31), accessed September 2014, for MiSeq data, utilizing the previously described honey bee-specific training set combined with the current Greengenes release for classification of operational taxonomic units (OTUs) (14). Briefly, contigs were generated using the make.contigs command, and sequences were screened for ambiguous base pairs and length using screen.seqs. Unique sequences were preclustered based on 2 nucleotide differences. Chimeras were detected and removed using the chimera.uchime command, and lineages found in blank water samples (*Halomonas* and *Shewanella*) were removed, as were sequences classified as chloroplasts, mitochondria, *Archaea*, or eukaryotes. All samples were rarified to 5,000 sequences (the size of the smallest library). All OTU-based analyses also were performed in Mothur, including rarefaction, heatmap creation, analyses of molecular variance, UniFrac analyses, and principal component analyses. Statistical tests beyond those performed in Mothur were implemented using SPSS (IBM, Armonk, New York).

Quantitative PCR analysis. Quantitative PCR was performed on the DNA extracted from each sample to detect the bacterial titer (using standardized calibration curves) using an Applied Biosystems StepOne real-time PCR system and Sybr green chemistry (Thermo Fisher Scientific, Waltham, Massachusetts). Calibration curves were generated using cloned 16S rRNA gene fragments (in pPCR-TOPO vectors) that were amplified, cleaned, and PicoGreen quantified. We used 16S rRNA primers for the bacterial fraction (331F, TCCTACGGGAGGCAGCAGT; 797R, GGACTACCAGGGTATCTAATCCTGTT) (32) with the following cycling temperatures: 95°C for 10 min, 40 cycles of 95°C for 15 s, and then 60°C for 1 min. Reactions were performed in triplicate, and any biological replicates with threshold cycle (C_T) standard deviations above 0.5 were removed from the analysis.

Microbiome sequence accession number. Raw sequencing reads have been deposited in the DDBJ under project identification (bioproject_id) number PRJDB3520.

RESULTS

Sequencing statistics and overall diversity metrics. Our sampling regimen resulted in a total of 59 distinct barcoded amplicons across two castes (queens and nurse-aged workers) and from each of five different hive environments (grafting source, cell builder, mating nucleus, and final colonies before and after queens began laying), corresponding to five different developmental stages for queens (larvae, newly emerged queens, maturing queens in the mating nucleus, and laying queens before and after their offspring emerged). A single MiSeq PE run of 250 cycles resulted in 11,167,225 reads, of which 7,053,677 (63%) passed stringent quality thresholds (see Materials and Methods). The data set was rarefied (i.e., subsampled to the size of the smallest library) to 5,000

sequences, and after alignment and clustering, we identified a total of 264,865 unique sequences and 897 OTUs (at 97% identity) across the entire data set. Of these OTUs, the top 20 comprised >96% of the total data set, so the rest of our analyses focused on these top 20 OTUs. Below, we explore the bacterial composition and diversity found in each of the castes and at each developmental time point, focusing on differences between queens and workers.

The honey bee queen gut microbiome shifted substantially during development. We sought to determine microbial signatures of queen development; therefore, we began by characterizing the communities that were found across our biological replicates for each developmental stage. Honey bee workers are known to harbor distinctive populations of bacteria (13), including the following core bacteria: Firm-5, Firm-4, Beta, Gamma-1, *Bifidobacterium*, Alpha-2.1, Alpha-2.2, and Alpha-1. These same phylotypes were found to be associated with queens throughout the rearing process. However, we observed that their microbiome changed dramatically during development and as they moved between host colonies. Specifically, larval queen microbiome libraries were dominated (~78% abundance) by enteric bacteria such as *Escherichia* and Gamma-1 (*Gilliamella*), with relatively small proportions (~19%) of other core bacteria (such as Firm-5, Firm-4, *Bifidobacterium*, Beta, and the alphaproteobacterial groups Alpha-2.1, Alpha-2.2, and Alpha-1) (Fig. 1). Amplicons generated from newly emerged queens also contained large proportions of enteric bacteria (such as *Escherichia*; 1,837.7 reads/sample), and although we were able to detect in all queens some members of the characteristic microbiome of honey bee workers (such as Firm-5, Firm-4, Gamma-1, Alpha-2.1, and Alpha-2.2), these sequences appeared at much lower frequencies than those of *Escherichia* (minimum and maximum sequences seen in rarified libraries were the following: Firm-5, range of sequences 30 to 366; Firm-4, 9 to 97; Gamma-1, 9 to 122; Alpha-2.1, 7 to 530; and Alpha-2.2, 4 to 237) (Fig. 1). Amplicons generated from newly emerged queens had small proportions of alphaproteobacteria (means for Alpha-2.1 and -2.2, 120.5 and 49.4 sequences per sample, respectively). Therefore, newly emerged queen gut microbiomes from the cell builder colony greatly resembled those generated for larval queens in the grafting source.

In contrast to microbiomes found associated with larval and newly emerged queens, amplicon libraries from mature queens (both from the mating nucleus and the final colonies, before and after the queens' own genetic offspring were present in colonies) were characterized by a large proportion of alphaproteobacterial sequences (~46% Alpha-2.1 and ~25% Alpha-2.2) (Fig. 1). Specifically, two OTUs dominated the mature queen amplicon libraries and were classified as well-known honey bee-associated alphaproteobacteria, the Alpha-2.2 and Alpha-2.1 clades (means and standard errors [SE] for each were 2,252 ± 604 reads and 1,219 ± 520 reads, respectively) (Fig. 1). Queens were much less likely to be colonized by the related Alpha-1 clade (found in low quantities in only 5 of the 10 mature queens that were sampled; 1.4 ± 0.4 reads, or 0.03% of their bacterial community). We saw no significant difference in microbiome composition between queens from the mating nucleus or from the final colonies ($P > 0.05$ by *t* test; Kruskal-Wallis test results were not significant). Therefore, the subsequent analyses pooled queen samples from the mating nucleus and the final colonies (here referred to as mature queens).

Gut microbiomes differed between queens and workers. In order to contextualize gut microbiomes of the queens, we also analyzed gut microbiomes of the workers who were of nursing age and may have participated in the rearing of these queens. All of the workers sampled in this study harbored well-known bacterial community members in relative proportions expected based on previous work (12, 13, 17). Of the top 20 OTUs that were classified and identified from workers in our study, 11 are known members of the honey bee-associated community and are included in the clades Firm-5, Firm-4, Beta, Gamma-1, *Bifidobacterium*, Alpha-2.1, Alpha-2.2, and Alpha-1. These core members represented 84% of the classified reads in this subsampled worker data set, and all of the libraries generated for workers sampled in this study contained sequences that were classified as Firm-5, *Bifidobacterium*, Gamma-1, Alpha-2.1, and Beta. Additionally, we also identified several unclassified *Enterobacteriales*, *Enterobacteriaceae*, and *Lactobacillales* that were present at lower frequencies and more sporadically than the core microbiome. One of these OTUs was found consistently across all sampled colonies (*Lactobacillaceae incertae sedis*; average number of sequences per queen, 103.6; maximum, 1,926).

Worker microbiomes were significantly different from those that were found in the queens for which they cared; Kruskal-Wallis tests determined nearly all top 20 OTUs differed between workers, larvae, newly emerged queens, and mature queens (degrees of freedom [df] = 3; $P < 0.05$; except for *Lactobacillales incertae sedis*) (Fig. 2). To visualize similarities between microbial communities from each of these sampled environments, we performed both weighted and unweighted UniFrac analyses, which compares bacterial community composition between environments (based on phylogenetic relatedness of these microbes, weighting relative abundance or leaving it unweighted). Amplicon libraries sequenced from worker bee digestive tracts clustered to the exclusion of those from larvae and queens (Fig. 3) ($P < 0.001$ by Unifrac weighted analysis for each pairwise comparison). This means that worker microbiomes were more similar across unrelated colonies than they were to the queens that they hosted (which were unrelated or related to them, depending on the sampling point). Interestingly, within the queens, we saw a clear developmental progression in microbiome composition; mature queens clustered with some of the newly emerged adult queens, and some newly emerged queens clustered with larvae (Fig. 2). Therefore, development seemed to have a strong effect on the microbial communities that were seen in queens.

To further visualize similarities and also to statistically determine which microbial community members contributed to the differences observed across our samples, we performed a principal component analysis (Fig. 4). Again, we observed that clustering of our amplicon libraries was dependent on developmental stage and was strongly influenced by caste, such that worker samples clustered to the near exclusion of mature queen samples (the first component accounted for 29.7% of the total variance, while the second was 11.2%). We further identified the microbiome members that might be contributing to the specific clustering of these two communities, based on caste and developmental stage, by identifying statistically significant differences in pairwise comparisons of microbiome composition. We identified the following classified OTUs to be significantly different between workers and mature queens: Firm-4, *Enterobacteriaceae*, Beta, Gamma-1, *Bifidobacteriaceae*, Alpha-2.1, Alpha-1, *Enterobacteriales*, unclassified

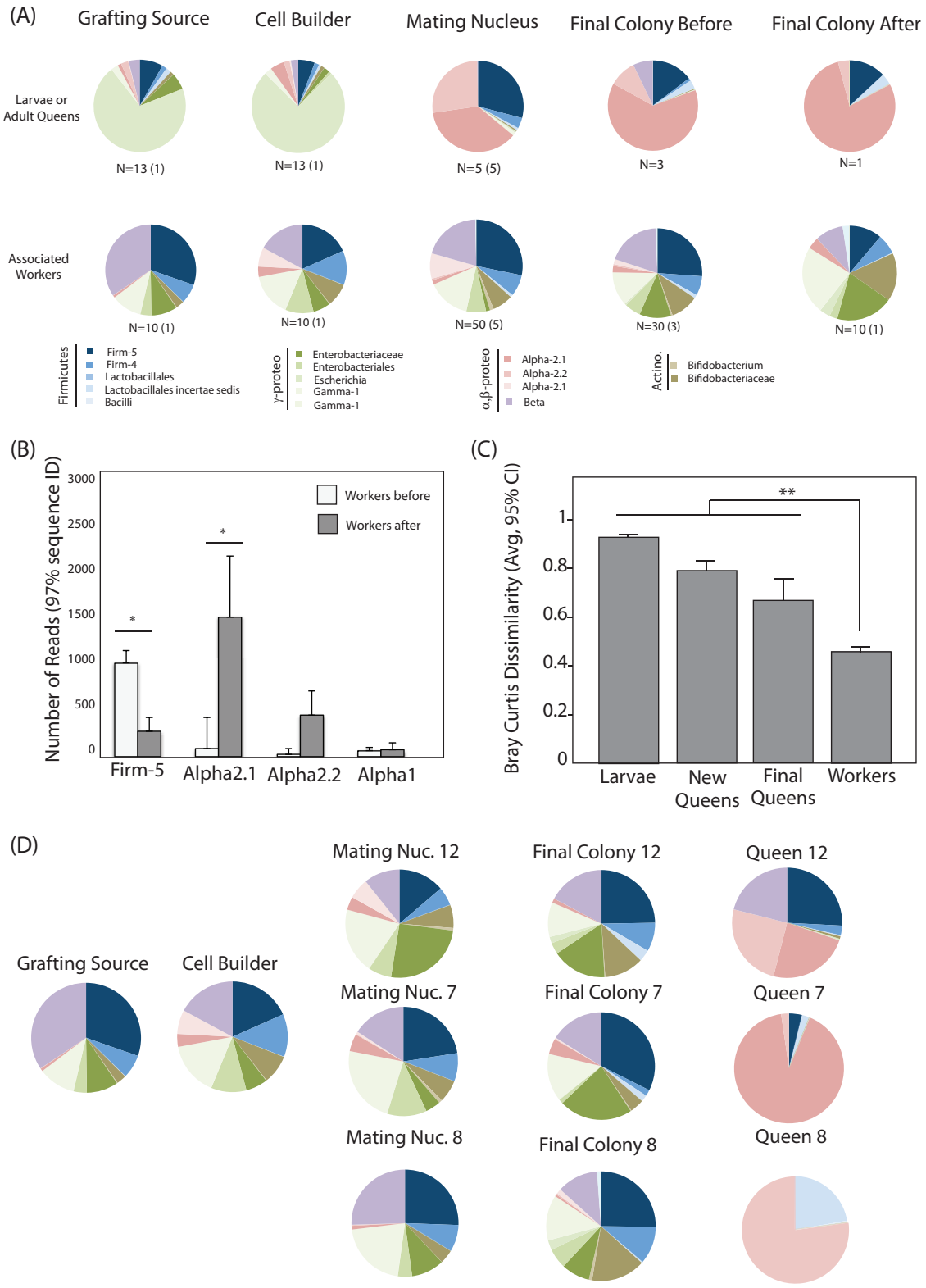

FIG 1 (A) Microbial composition of young larvae (from the grafting source colony, destined to be reared into queens) and queens (newly emerged and mature), and their associated workers, at each stage of queen development. N, number of individuals; the number of colonies is in parentheses. (B) Workers exhibited a relatively consistent microbiome profile, although workers that were the offspring of laying adult queens (workers after) exhibited larger proportions of Alpha-2.1 and smaller proportions of Firm-5 than workers present in final host colonies before queen progeny emerged (workers before). ID, identity. (C) Bray-Curtis dissimilarity metrics for pairwise comparisons between sampled communities were significantly different between workers and all other sampled castes ($P < 0.01$ by pairwise *t* tests with Bonferroni correction), supporting the assertion that microbiomes of unrelated worker populations were more consistent across the host colonies than were the microbiomes of the related queens over the course of their development. CI, confidence intervals. (D) Visual depiction of consistency in microbiome composition between workers across eight different colonies compared to those of three mature queens. In this series, all individual queens interacted with the same populations of grafting-source and cell-builder workers, but each was moved to its own mating nucleus and final host colony (sampled before emergence of queens' genetic offspring).

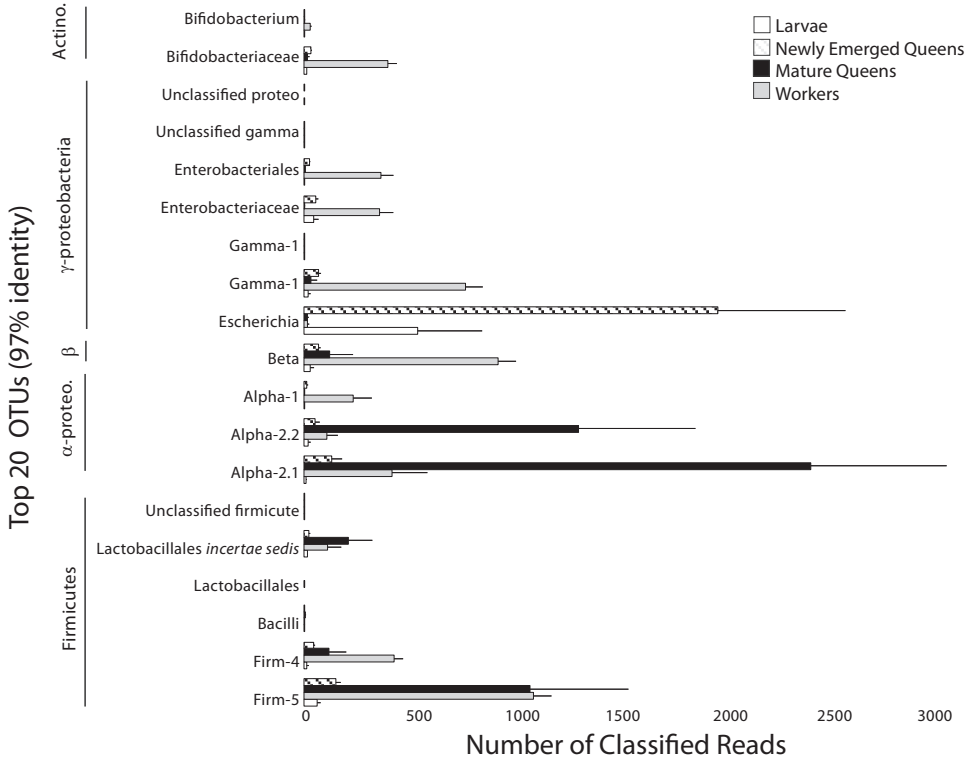

FIG 2 Distribution of the top 20 bacterial operational taxonomic units (OTUs) found across all samples and comprising >96% of the data. The prevalence of 19 of these top 20 OTUs (excluding *Lactobacillales incertae sedis*) was found to distinguish worker and queen microbiomes (df = 3; $P < 0.05$ by Kruskal-Wallis test). Libraries from mature queens are characterized by a bacterial community in which alphaproteobacteria predominate. Error bars indicate SE of the means across all sampled libraries.

bacteria, unclassified proteo, bacilli, *Lactobacillales*, and *Bifidobacterium* (based on Kruskal-Wallis and subsequent Mann Whitney U tests producing statistically significant differences between workers and mature queens; $P < 0.05$). To test the hypothesis that developmental stage and caste contributed to a distinguishing microbiome profile, we performed a stepwise discriminant function analysis utilizing abundance data from the top 20 OTUs in the data set. This analysis showed that microbiome composition could readily categorize each sample as a larva, a newly emerged queen, a mature queen, or a worker (Wilks's lambda coefficient, 0.001; $\chi^2 = 404.8$; df = 36; $P < 0.001$).

Queen replacement may affect gut microbiome profiles of workers. We expected to observe a microbial signature in the queens that reflected in some way their interactions with the workers who tended to them throughout the rearing process. Interestingly, we did not see a resemblance between the queen microbiomes and the worker populations that reared them at any stage of their development. In contrast, we saw a statistically significant change only in the worker microbiome composition in the final colonies, when colony populations shifted from workers who were unrelated to their queen to the queen's offspring (Fig. 1B). Specifically, we saw a statistically significant reduction in the proportion of Firm-5 found in these worker bees ($P < 0.02$ by *t* test) and a qualitative increase in the proportion of Alpha-2.1 (with the removal of one outlier; $P < 0.04$) for four colonies sampled after the emergence of queen-produced workers. Importantly, in one colony where we sampled workers before and after the emergence of the offspring of the queen, we also were able to sample the associated mature, laying queen. For this one colony, we did not see a significant shift in worker microbiome composition between unrelated host workers and subsequent offspring of the queen (Fig. 1A). Because of the nature of destructive sampling, which was necessary to complete this study, we were unable to increase the sample size for this last time point (only 2 queens were sampled after they began laying, and only 4 worker colonies were sampled after queens began laying). Therefore, although this result is interesting and deserves future study, it should be interpreted cautiously.

Queen gut microbiomes were variable in size and in diversity. In order to more deeply characterize microbiome variability across queens, we calculated pairwise Bray-Curtis dissimilarity values within each of our sampled developmental stages (Fig. 1C). Based on this metric, we found that microbiome composition was significantly more consistent across the unrelated populations of adult workers than the related queens at each developmental stage in which they were sampled ($P < 0.01$ by pairwise *t* tests using Bonferroni correction). Amplicons from queen larvae were the most variable across individuals with regard to composition, showing the largest Bray-Curtis similarity distributions and highest means (Fig. 1C). In contrast, as the queens matured, their microbial compositions became more consistent across individuals, with pairwise differences between samples settling on a Bray-Curtis similarity of 0.67. Importantly, mature queens did not differ from workers with regard to the average number of OTUs found within their digestive tracts (Table 1).

We wondered if the variability in microbiome composition

FIG 3 UniFrac analysis of bacterial communities found across sampled environments, including larvae transferred from the grafting source (GS), newly emerged queens from the cell builder (CB), and mature queens in their mating nucs (MN) or final colonies (FC). Communities from workers interacting with queens at each stage of development did not cluster with their respective queens but instead claded separately ($P < 0.001$ in all pairwise comparisons between queens, workers, and larvae by Unifrac weighted analysis). Outliers are denoted with an asterisk.

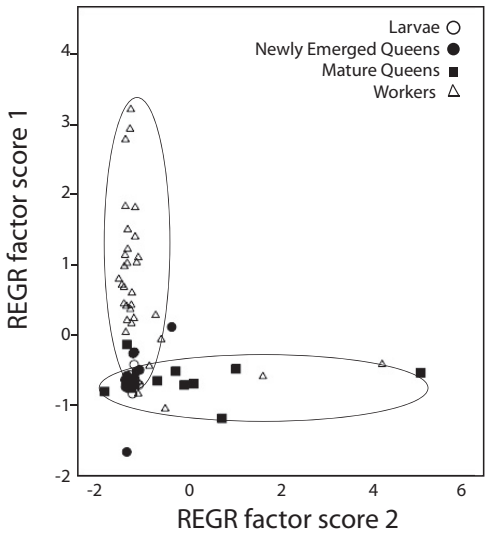

FIG 4 Principal component analysis clustered the microbial communities of workers separately from those of mature queens, while newly emerged queens and larvae clustered together. Ellipses are presented to highlight the visual pattern only. REGR, regression.

between individual mature queens reflected stochastic sampling of transient and rare members; if queen microbiomes have few bacteria, the amplicons resulting from these environments might increase the variability observed across sampled queens. Sequencing libraries generated from few bacteria would result in deeper sequencing of rare members compared to libraries generated from environments dominated by a single species. In order to investigate the number of microbes found in adult workers and queens

TABLE 1 Diversity metrics for microbial communities found for queens across the developmental stages of queen rearing and for the workers that were associated with them at each stage

Bee type	OTUs[a] (97% avg)	Inv Simpson[b]	*P* value[c]
Larvae	48.5	3.67	0.007
Newly emerged queens	110.7	12.41	<0.0001
Mature queens	26.1	1.88	0.9
Workers	26.9	5.45	

[a] Average numbers of OTUs with 97% identity differ dramatically between larvae and newly emerged queens versus workers.
[b] Inverse of the Simpson diversity estimator.
[c] *P* values determined by *t* tests are the results of pairwise comparisons of diversity, i.e., OTUs of worker microbiomes and queen microbiomes at each developmental stage.

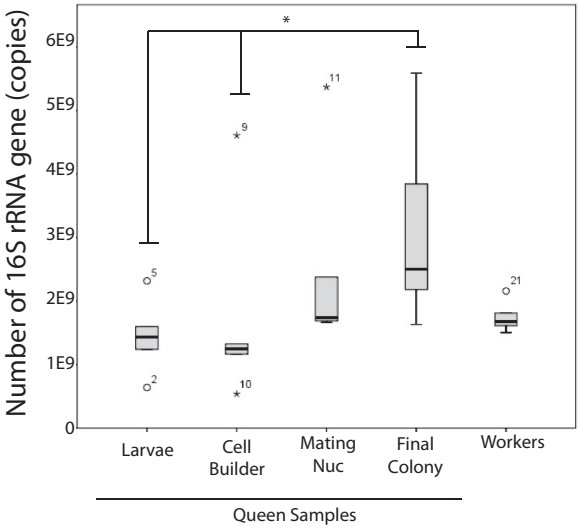

FIG 5 Total number of 16S rRNA gene copies (as detected by quantitative PCR using 16S rRNA gene primers) from queens and workers ($n = 5$ for each caste and developmental stage). Mean total number of bacteria colonizing queens was influenced by age ($\chi^2 = 8.0$; df = 3; $P = 0.046$ by Kruskal-Wallis test); larval queens hosted fewer bacteria than final queens (U = 2, Z = −2.2, and $P = 0.032$ by Mann-Whitney U test). Mean total numbers of bacteria in mature queen digestive tracts did not differ significantly from the mean total number of bacteria colonizing workers (F = 1.5; df = 1.4; $P = 0.24$). Symbols and numbers represent outliers.

during their development, we performed a quantitative PCR analysis using the 16S rRNA gene (Fig. 5). The development of the worker bee microbiome, like that of many animals, follows a well-known trajectory where the number of bacteria colonizing the animal increases over time (33–38). We found a similar increase in community size in queens as they developed from larvae into mature adults (Fig. 5). However, there was no difference in the total number of 16S rRNA gene copies between these same mature queens and the workers in the colonies that hosted them at the final sampling time point. Instead, we saw a broader range of 16S rRNA gene copy numbers associated with mature queens than what was found in workers; queens were less consistent in both composition and total number of microbiome members (Fig. 5).

DISCUSSION

Although the developmental trajectory of honey bee queens in a highly managed setting is complex, some straightforward insights can be gleaned about the establishment of their gut microbiomes throughout this process. First, queen microbiomes changed dramatically as queens aged and they encountered different colony environments, with the greatest shifts in community composition occurring between young queens (larvae and newly emerged adult queens who had not yet contacted more workers) and mature, laying queens. While the microbiomes of the former were dominated by enteric bacteria, the latter were comprised primarily of alphaproteobacteria. Within the boundaries of these broad generalizations, there was greater variability across the related queens that we sampled than among the unrelated workers who hosted them, which showed the core worker microbiota that has been documented previously (12–14), even though queens at maturity had communities that were similar to those of workers in size and diversity. Second, we found little evidence that the bacterial communities of queens reflected those of the workers who were of the age to tend them; queen microbiotas clustered reliably by developmental stage but separately from adult workers. Our study identifies a unique and numerically rich microbial signature for queens that changed as queens aged in a way that was not linked to the gut microbiome of any of the workers that they encountered over their lifetime.

Several studies now have documented the progressive development of the honey bee worker microbiome from the larval to the adult stage. Initial attempts, based on PCR, showed little amplification overall from larvae and relatively few of the bacterial groups that have previously been associated with the core microbiome of adult workers (17). However, other laboratories have been successful in culturing bacteria from surface-sterilized larvae (19, 39, 40), although even these culture-based studies support the hypothesis that larvae are not colonized consistently with the core microbiome of adults. Similarly, newly eclosed workers also are believed to lack the characteristic microbiome of adults. In a healthy hive, over the span of a few days, young workers are colonized with bacterial phylotypes that are characteristic of adults (33), and interaction with hive components and with fecal material from adult bees facilitates the transmission of these bacterial phylotypes (39). In some ways, the development of the queen microbiome mirrors that of workers. Specifically, larval queens do not host the canonical honey bee-associated bacterial community, and gut microbiomes of newly emerged queens (adults who have not yet had contact with more workers) resemble the depauperate communities of larvae (Fig. 1 to 3). However, by the time queens mature (either in the mating nucleus or in their final colonies), they have developed a queen-specific microbial signature, where libraries are dominated by alphaproteobacteria.

The route of transmission of the queen microbiome likely is quite distinct from that of the worker microbiome. Compared to worker larvae, larvae destined to be queens are fed larger quantities of royal jelly, a protein-rich secretion from worker hypopharyngeal glands containing proteins that may alter DNA methylation (via Dnmt2) (41, 42). This difference in diet is directly responsible for the developmental differences found between workers and queens, and this caste distinction likely is modulated by genome-wide methylation patterns that differ during development (43). After queens mature, they do not feed on bee bread or nectar directly, unlike the nurse-age workers that were sampled here and by others (21). Instead, queens continue to be fed royal jelly by the workers who attend them, who are typically of nurse age (25). Additionally, workers attending the queen dispose of her fecal material, as well as clean and groom the queen, likely diminishing her contact with hive-associated bacteria, as workers would experience. Presumptions about queen rearing and care within the colony fit well with our observation that worker gut microbiomes do not resemble those of the queen based on caste differences in exposure and hive experiences.

There may be another route by which worker-associated microbes influence the queen. Interestingly, one component of worker hypopharyngeal gland secretions that is not well investigated is a specific acetic acid bacterium, "*Candidatus* Parasaccharibacter apium" (within the Alpha-2.2 clade) (40). This bacterial species is associated with young worker larvae (that are fed primarily royal jelly) and also is found in large numbers in the hypopharyngeal glands of nurse bees (~30% of the bacterial community) as well as in royal jelly (~40% of the bacterial community)

(40). Interestingly, Alpha-2.2 often is recovered from libraries constructed from worker guts but usually in low proportions. Indeed, in the libraries from worker digestive tracts that were sampled here, Alpha-2.2 was found to comprise ~2% of the bacterial community, on average. Finally, although royal jelly is known for its antiseptic properties, Alpha-2.2 strains can be cultivated in the presence of royal jelly (10). This result suggests that while Alpha-2.2 can be found in the gut environment, their primary niche within the honey bee is the hypopharyngeal gland. What might "*Candidatus* Parasaccharibacter apium" be doing for queens? The genome of a representative honey bee-associated strain of Alpha-2.2 has been sequenced and annotated recently (44), and it suggests roles for the bacterium within queen bees. For example, based on genomic content, the bacterium is believed to prefer microoxic environments and, under fermentative conditions, to produce lactate (via L-lactate dehydrogenase), acetoin (via acetolactate synthase), and 2,3-butanediol (via acetoin dehydrogenase). These fermentative products, also thought to be produced by other honey bee-associated microbes (11), could impact queen physiology and development through as-of-yet-unknown mechanisms.

The implications of our findings on the commercial-production apiculture industry are reassuring in many ways. Because the queen microbiome does not reflect the workers within a specific colony or even that of the worker caste, the physical movement of queens from one colony environment to another does not seem to have any major effects on either queen-gut or worker-gut communities. Thus, we have no evidence that beekeepers who regularly replace their queens from outside genetic sources detrimentally affect their colonies by disrupting the gut microfauna of a particular genetic line or colony unit. Moreover, our results seem to support several general recommendations for queen rearing, namely, that even the youngest grafted larvae are colonized by the appropriate gut bacteria (i.e., those that are present at later stages of queen development, although we cannot eliminate differences that might be induced by late grafting) and that large populations of nurse-aged worker bees in cell builder colonies are important for adequately provisioning queen larvae with royal jelly, which we speculate is an important source of their early microbiota members.

Maternal (or vertical) transmission is a common, if not nearly universal, mode of acquisition for the host-associated microbiome in mammals (45–49). In humans, for example, the mode of delivery can dramatically impact an infant's microbiome composition; infants born through natural delivery have a microbiome composition that resembles that of the mother's vaginal microbiota, while infants born via Cesarean section harbor communities resembling the skin (26). However, this does not preclude the acquisition of microbes from the environment (or horizontally) during development of the animal host. Indeed, there is evidence that in many animal groups, including insects, individuals acquire their microbiome through horizontal transmission, for example, from their surroundings (38, 50), via coprophagy (51–53), or through interaction with congeners (54). Our data suggest that, unlike the majority of animals, maternal transmission of the microbiome (the passing of microbiota from the queen to her offspring) does not occur in the honey bee; the queen honey bee is not a source of inoculum for her workers' microbiome, because she does not harbor the diversity of bacterial groups that are core to the adult worker bee (Fig. 1 and 2), and she does not participate directly in the rearing of her offspring. In contrast, our data suggest a model in which this single reproductive member of the colony is inoculated by worker bee caregivers, possibly via a specific organ containing a microbial composition quite distinct from that found within worker digestive tracts. Therefore, honey bees, like social bumble bees, likely transmit their microbiome from one generation to the next through horizontal social contact and interaction with the nest (or hive components) (33, 54), although they do so in a way that is heavily mediated by caste membership.

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