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Original article

A non-destructive approach to assess the gut microbiome of honey bee (*Apis mellifera*) queens using fecal samples

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Abstract – Fecal sampling is a widely used, non-invasive method for assessing gut microbiomes across various organisms. However, its suitability for studying the gut microbiome of honey bee (*Apis mellifera*) queens has not been tested. In this study, we evaluated whether fecal microbiomes accurately reflect gut microbiomes in honey bee queens, offering a potential non-destructive approach for microbiome research. We successfully obtained fecal and gut samples from 21 out of 26 test queens. Bacterial communities were analyzed using 16S rRNA amplicon sequencing and qPCR. Our results indicate that queen fecal microbiomes closely resemble gut microbiomes, with no significant differences in alpha diversity and only minor differences in specific bacterial taxa. Beta diversity analyses revealed that within-pair microbiomes (i.e., gut vs. feces from the same queen) were significantly more similar than between-pair comparisons. Additionally, qPCR analyses revealed a strong positive correlation between bacterial abundances in fecal and gut samples, further supporting the use of feces as a proxy for gut microbiome composition. While promising, fecal collection from queens can sometimes be challenging. In our study, we were unable to collect feces from five queens, and those individuals lacked stored fecal material upon inspection of dissected guts. Nonetheless, our findings suggest that fecal sampling can be a useful, non-invasive method for studying honey bee queen microbiomes, enabling longitudinal assessments without compromising colony stability.

microbial diversity / fecal microbiome / gut microbiome / honey bee queen

1. INTRODUCTION

Fecal microbiomes are commonly used to study gut microbiomes due to their ease of collection and non-invasive nature (Vogtmann et al. 2017). Fecal samples often provide a good representation of gut microbial communities, particularly those from the distal gut, making them a practical choice for microbiome research. Their collection is logistically simple, cost effective, and allows for repeated sampling, which is especially beneficial for longitudinal studies (Tedjo et al. 2015). However, fecal samples may not fully capture microbial diversity, particularly for microbes residing in the proximal gut or those attached to mucosal layers (Vogtmann et al. 2017; Ahn et al. 2023). Therefore, direct comparisons between fecal and gut microbiomes should be made whenever possible to better assess the extent to which fecal samples accurately represent gut microbial communities.

In honey bee (*Apis mellifera*) research, microbiome studies typically involve dissecting the gut to obtain DNA for metagenomic analysis,

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providing a detailed view of an individual's microbial composition, including specific gut compartments (Powell et al. 2014; Kwong et al. 2017; Motta et al. 2023; Caesar et al. 2024; Burks et al. 2024). While this method is feasible for studies of worker and drone bees, given that a colony is comprised of thousands of these individuals (Winston 1987), it poses a significant challenge for queens, whose removal can threaten colony stability due to their role as the sole reproductive female in the colony. As a result, queen microbiome studies are often limited to periods of queen rearing or cases where colonies (or their queens) are no longer needed and can be sacrificed (Powell et al. 2018; Caesar et al. 2024).

Previous studies have indicated that queen gut microbiomes are distinct from those of workers and drones (Tarpy et al. 2015; Powell et al. 2018; Caesar et al. 2024), highlighting the need for targeted research. In honey bee workers, the gut microbiome is dominated by five socially acquired core bacterial genera: *Bifidobacterium*, *Bombilactobacillus*, *Gilliamella*, *Lactobacillus*, and *Snodgrassella*. These bacteria are transmitted through social interactions and remain consistent across age (Kwong and Moran 2016). They contribute to digestion, development, and pathogen protection (Motta and Moran 2024). In contrast, the queen's gut microbiome appears to be more influenced by environmental factors and consists primarily of *Apilactobacillus*, *Bombella*, *Commensalibacter*, and *Lactobacillus*, many of which are also found in nectar, larvae, and hive compartments (Kapheim et al. 2015; Tarpy et al. 2015; Powell et al. 2018; Caesar et al. 2024). While some of these bacteria, such as *Bombella*, are known to protect larvae against pathogenic fungi (Miller et al. 2021) and enhance nutrition through lysine supplementation (Parish et al. 2022), their specific roles in queen health remain poorly understood. Understanding the queen's microbiome is essential, as it may influence her health and, consequently, the health of the entire colony.

Recent studies have explored the use of fecal material for queen genotyping (Bubnič et al. 2020) and worker gut microbiome analysis (Cabirol et al. 2024), providing a non-invasive alternative for longitudinal studies. However, its applicability for assessing the queen's gut microbiome remains untested. Since fecal microbiomes may not fully reflect a gut's microbial composition, comparative studies are necessary before implementing this method in large-scale experiments. Here, we tested whether queen fecal sampling can serve as a non-destructive approach to investigate the queen's gut microbiome. We collected fecal samples and dissected the guts of honey bee queens to compare their microbiomes using high-throughput sequencing and qPCR. This approach allowed us to determine whether fecal microbiome profiles reliably represent those of gut microbiomes, potentially providing a valuable non-destructive assessment method for longitudinal studies of honey bee queen microbiomes.

2. MATERIAL AND METHODS

2.1. Honey bee queen rearing

The colonies used in this study were maintained at the Janice and John G. Thomas Honey Bee Facility of Texas A&M University in Bryan, TX (N 30° 38′ 31.037″ W 96° 27′ 39.495″). All colonies were headed by queens of Italian descent. Experimental queens were reared by using the standard queen-rearing procedure known as "grafting" (Laidlaw and Eckert 1964; Rangel et al. 2013), which entailed selecting a frame of developing brood, and transferring first instar worker larvae from their cells into plastic cups using a grafting tool (JZ's BZ's Honey Co., Santa Cruz, CA). We prepared the plastic cell cups by priming them with a small amount of royal jelly to encourage larval acceptance and grafted a total of 30 larvae per each of two grafting sessions, as shown in Figure 1. The grafted cell cups were then attached to a queen-rearing frame and placed in a queenless unit of bees known as a "cell builder" (Laidlaw and Eckert 1964), where nurse bees cared for queens during larval and pupal development. Larvae were grafted during two different sessions, one on

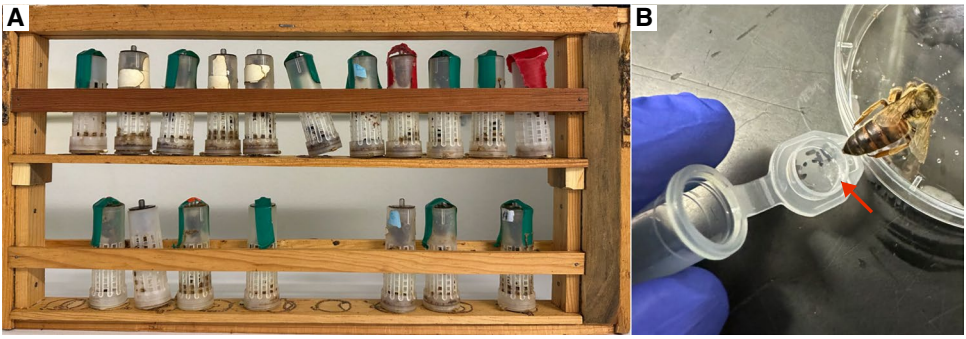


Figure 1. Honey bee queen grafting and feces sampling. **A** Individual queens were raised in plastic cups via grafting. Once the cells were capped, they were individually placed in plastic "roller" cages and placed in a queen "bank" where they were taken care of by tending bees until collection for analysis. **B** Feces were sampled from chilled queens by positioning the distal region of the abdomen toward the cap of a sterile microcentrifuge tube and gently pressing the abdomen with the investigator's index and thumb fingers until feces were released. The queen feces were mostly clear, as indicated by the red arrow.

7 July 2024 (first session) and the other on 11 July 2024 (second session). All emerged queens were placed in a "bank," where they were taken care of by tending workers until they were used. Banked queens were brought to the laboratory for analysis on two different dates: eight queens from the second grafting session (approximately 38 days old) were analyzed on 29 August 2024, and 18 queens (13 from the first grafting session and five from the second grafting session, approximately 80–85 days old) were analyzed on 8 October 2024.

2.2. Feces sampling and gut dissections

We used 26 queens for fecal sample collection and gut dissections. Each queen was individually immobilized in a clean, sterile Petri dish on ice for about 10 min. Then, queens were carefully handled by directing the postal region of the abdomen toward the cap of a sterile 1.5-mL microcentrifuge tube, after which light pressure was applied to their abdomen with the researcher's index and thumb fingers (Figure 1). The total volume of feces collected from queens varied considerably, ranging from 0 to 10 µL. For the queens from which we did not obtain feces ($n = 5$), a stronger pressure was applied to the abdomen, which may have caused damage to the queen. As these queens were going to be dissected for gut extractions, we were not worried about such damage, but if these queens were to be transferred back to a hive, this strong pressure should be avoided. For the queens from which we were able to collect at least 1 µL of feces ($n = 21$), we proceeded with the gut dissections. Guts were removed by grabbing the last abdominal segment with sterilized forceps and gently pulling out the entire gut. Each gut was transferred to an individual sterile 1.5-mL microcentrifuge tube. Guts of three workers collected from the same queen bank were also processed to be used as controls, as worker microbiomes are well characterized and vary significantly from those of queens (Powell et al. 2018). All feces and gut samples were obtained under sterile conditions and immediately stored at − 80 °C until further processing.

2.3. DNA extraction

DNA was extracted from queen feces ($n = 21$), queen guts ($n = 21$), and worker guts ($n = 3$) using a previously described protocol (Motta et al. 2023), with some adaptations. Briefly, guts were homogenized with 100 µL of CTAB

buffer (0.1 M Tris–HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, and 2% (w/v) cetrimonium bromide), resuspended in an additional 600 µL of CTAB buffer and 20 µL of proteinase K solution (0.1 M tris–HCl, 26 mM $CaCl_2$, 50% glycerol, and 20 mg/mL proteinase K), and transferred to a capped vial with 0.5 mL of 0.1-mm Zirconia beads (BioSpec Products). After adding 2 µL of 2-betamercaptoethanol, samples were bead-beated twice for 2 min each. Samples were digested at 56 °C overnight, transferred to a clean 2-mL vial and mixed with 500 µL of phenol–chloroform–isoamyl alcohol (25:24:1, pH 8.0). Samples were inverted five times and centrifuged for 15 min at 4 °C and 18,000 RCF. The aqueous layer was transferred to a new vial, and DNA was precipitated at − 20 °C overnight with 500 µL of isopropanol and 50 µL of 3 M NaOAc (pH 5.4). Precipitated samples were centrifuged for 30 min at 4 °C and 18,000 RCF, and the supernatant was removed. DNA pellets were washed with 1 mL of ice cold 75% ethanol and centrifuged for an additional 3 min at 4 °C. After removing the ethanol wash, the DNA pellets were dried at room temperature for 30 min and then resuspended in 50 µL of water. DNA samples were stored at − 20 °C.

2.4. Quantitative PCR analysis

DNA samples from feces were used directly as templates for qPCR, while DNA samples from guts were diluted ten-fold. Triplicate reactions were prepared in 96-well plates (one for feces, one for gut samples) and run on a Bio-Rad CFX96 Touch Real-Time PCR instrument. Each reaction contained 5 µL SYBR Green Universal Master Mix (Applied Biosystems), 0.05 µL of 100 µM forward and reverse primers (27F: 5′-agagtttgatcctggctcag-3′ and 355R: 5′-ctgctgcctcccgtaggagt-3′), 3.9 µL H_2O, and 1.0 µL template DNA. Cycling conditions included an initial step at 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Total bacterial 16S rRNA gene copies were quantified using standard curves generated from a synthesized 347-bp 16S rRNA gene fragment (Twist Bioscience). The fragment was quantified (Qubit) and adjusted to 10^{10} copies/µL. Serial dilutions from 10^8 to 10^2 copies/µL were prepared as standards and used in each plate. 16S rRNA gene copy numbers were calculated as $10^{(Ct-b)/m}$, where "*b*" and "*m*" are the *y*-intercept and slope of the standard curve, respectively, and Ct (cycle threshold) is the average of triplicates. Values were corrected for the dilution factor.

2.5. 16S rRNA amplicon sequencing

DNA samples from feces and DNA samples from guts (diluted ten-fold) were used as templates for 16S rRNA library preparation, involving two PCR reactions. PCR 1 amplified the V4 region of the 16S rRNA gene in 25 µL single reactions, including 1 µL of 10 µM forward and reverse primers (Hyb515F: 5′-tcgtcggcagcgtcagatgtgtataagagacaggtgycagcmgccgcggta-3′ and Hyb806R: 5′-gtctcgtgggctcggagatgtgtataagagacagggactachvgggtwtctaat-3′), 12.5 µL of 2 × AccuStart™ II PCR SuperMix (Quantabio), and 1 µL of template DNA. Cycling conditions were 94 °C for 3 min; 30 cycles of 94 °C for 20 s, 50 °C for 15 s, and 72 °C for 30 s; followed by 72 °C for 10 min. PCR 2 attached dual indices and Illumina sequencing adapters to PCR 1 products in 25 µL single reactions, including a unique combination of 2 µL of 5 µM index primers (Hyb-F*nn*-i5: 5′-aatgatacggcgaccaccgagatctacacnnnnnntcgtcggcagcgtc-3′, and Hyb-R*nn*-i7: 5′-caagcagaagacggcatacgagatnnnnnngtctcgtgggctcgg-3′), 12.5 µL of 2 × AccuStart™ II PCR SuperMix, and 5 µL of PCR 1 product. Cycling conditions were 94 °C for 3 min; 10 cycles of 94 °C for 20 s, 50 °C for 15 s, and 72 °C for 60 s; followed by 72 °C for 10 min.

Both PCR product sets were purified using 0.8 × HighPrep PCR magnetic beads (MagBio) and quantified with a Qubit Fluorometer (Thermo Fisher Scientific). Samples (200 ng each) were pooled and submitted for Illumina sequencing on the MiSeq platform (2 × 250 bp run) at Texas A&M University's Genomics & Bioinformatics Service (College Station, TX).

During library preparation, we included one negative control ($n = 1$) to check for contamination, which consisted of adding molecular biology grade water instead of a DNA sample for the first PCR reaction.

2.6. 16S rRNA amplicon analysis

Illumina sequence reads were demultiplexed based on barcode sequences using MiSeq software. Read quality was assessed using FastQC (Andrews 2010) and MultiQC (Ewels et al. 2016). Reads were then processed in QIIME 2 version 2024.5 (Bolyen et al. 2019) on the Grace cluster at Texas A&M University's High-Performance Research Computing (HPRC) facility. Paired-end FASTQ files were imported into QIIME 2 using the Casava 1.8 paired-end demultiplexed FASTQ format. Primer sequences (V4 region: gtgycagcmgccgcggta and ggactachvgggtwtctaat) were removed using the cutadapt plugin (Martin 2011). Reads were filtered for quality, truncated to 220 bp, denoised, merged, and chimeric reads were removed, all using the DADA2 plugin (Callahan et al. 2016).

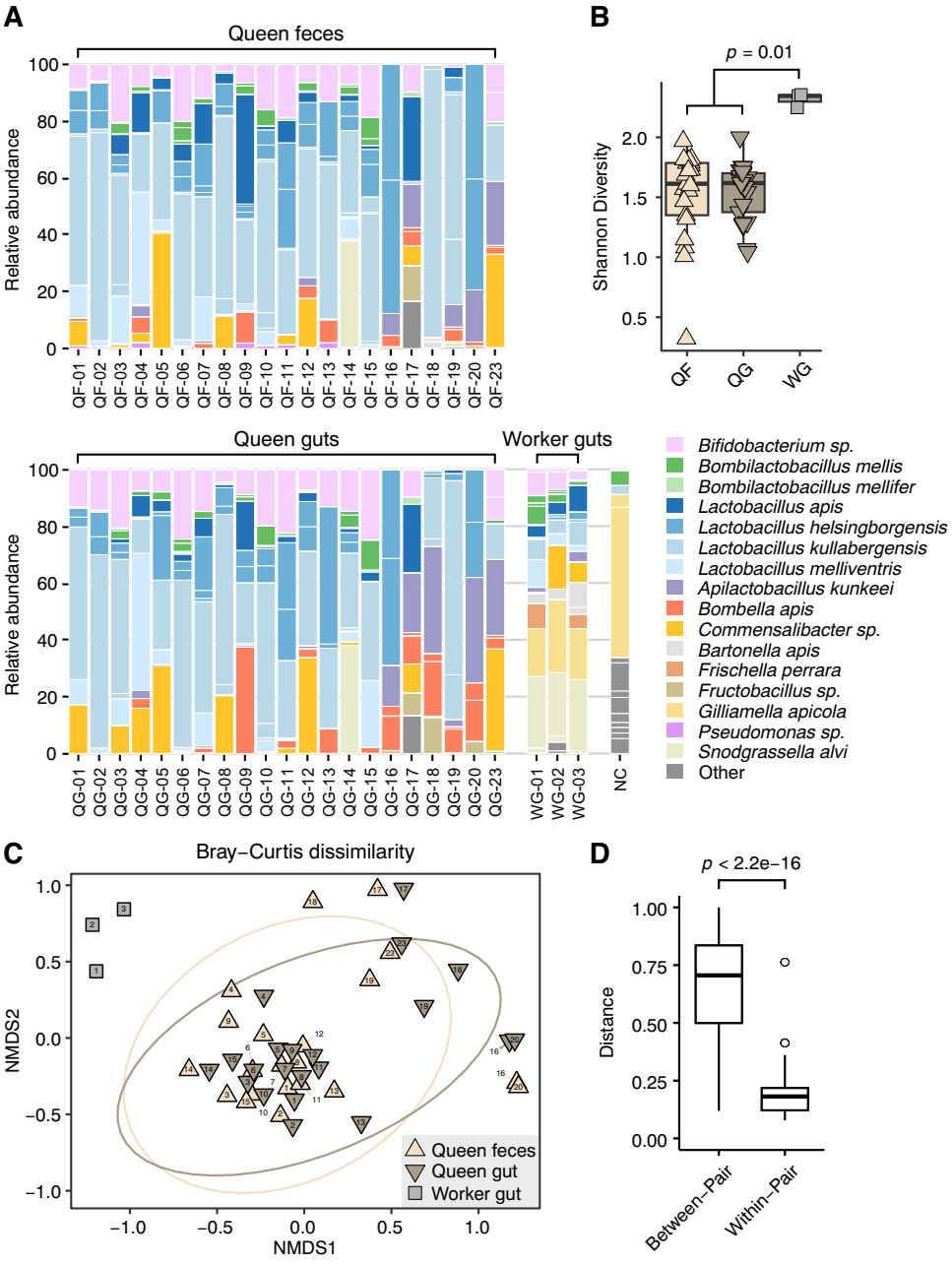
Taxonomy was assigned to amplicon sequence variants (ASVs) using the SILVA 138 reference database in the feature-classifier plugin (Bokulich et al. 2018). To validate taxonomic assignments when needed, representative sequences were examined using BLASTn against the NCBI database. Low-abundance reads (< 0.1% relative abundance) and non-bacterial reads (mitochondrial and chloroplast) were filtered using the feature-table and taxa filter-table plugins. An ASV table (Supplementary File 1) was generated to investigate microbial composition across all samples and exported for data visualization and statistical analysis in R version 4.3.2 (R Core Team 2023).

16S rRNA amplicon sequencing data were correlated with qPCR data to estimate the absolute abundance of individual ASVs. To that end, the read count of each ASV (estimated by 16S rRNA amplicon sequencing; Supplementary File 1) was multiplied by the total bacterial load in the corresponding sample (estimated by qPCR; Supplementary File 2). This product was then divided by the total read count of the entire sample (i.e., all ASV read counts), yielding an estimated absolute abundance value for each ASV.

2.7. Statistical analysis

Microbial diversity analyses were performed using the R package phyloseq (McMurdie and Holmes 2013). An ASV table, phylogenetic tree, and mapping file were imported and merged into a single phyloseq object. Taxonomy rank columns were assigned, and read counts were scaled to an even depth (10,538 reads per sample) using a custom function (Denef 2015). For alpha diversity, which measures taxonomic diversity within samples, Shannon index was calculated and differences between groups were assessed by performing the Kruskal–Wallis test followed by Dunn's test using the R package FSA (Ogle et al. 2025). For beta diversity, which measures taxonomic diversity between samples, ordinations and distance matrices were calculated using Bray–Curtis and Weighted UniFrac metrics and visualized as Non-Metric Multidimensional Scaling (NMDS) plots. PERMANOVA was conducted to analyze differences between groups using the adonis2 function from the R package vegan (Oksanen et al. 2025), and pairwise comparisons were performed with the pairwise.adonis function from the same package. To investigate whether each pair of gut and fecal samples from the same individual were more similar than those from different individuals, we extracted within-pair and between-pair distances from both beta diversity metrics using the R package phyloseq (McMurdie and Holmes 2013), and then performed *t*-tests using the R package ggpubr (Kassambara 2023).

For differential abundance analysis, we used the R package DESeq2 (Love et al. 2014). The phyloseq object was converted to a DESeq2 object, and size factors were estimated to normalize for sequencing depth. We ran the



DESeq2 analysis to test for differential abundance between groups and extracted significant ASVs. Results were visualized using the R package ggplot2 (Wickham 2016), creating a volcano plot and boxplots for significant ASVs. We set the level of statistical significance for all tests at $\alpha = 0.05$.

3. RESULTS

3.1. Collection of fecal samples from honey bee queens

To determine whether honey bee queen feces harbor bacterial communities similar to those

◂**Figure 2.** Comparisons of the relative abundance and microbial diversity of honey bee queen fecal and gut microbiomes. **A** Stacked column graphs showing the relative abundance of gut bacterial species in queen feces (QF, $n=21$), queen guts (QG, $n=21$), worker guts (WG, $n=3$), and a negative control (NC, $n=1$) to check for contamination during library preparation. Fecal and gut samples with the same number originated from the same queen. **B** Shannon diversity index comparing alpha diversity across groups. Statistical analysis was performed using the Kruskal–Wallis test ($\chi^2=8.58$, df = 2, p-value = 0.01, $n=45$), followed by Dunn's pairwise comparisons with p-values adjusted using the Benjamini–Hochberg method (p-value adj < 0.05 shown). NC was excluded from the analysis. **C** Non-Metric Multidimensional Scaling (NMDS) based on Bray–Curtis dissimilarity of gut community compositions. Statistical analysis was performed using PERMANOVA ($F_{3,42}=3.52$, permutations = 999, p-value = 0.01), followed by pairwise comparisons. Significant differences were observed between QF vs. WG (p-value adj = 0.012) and QG vs. WG (p-value adj = 0.006). **D** Pairwise distance analysis using Bray–Curtis dissimilarity to compare within-pair (gut vs. feces from the same queen) and between-pair (samples from different queens) comparisons (t-test, p-value < 2.2e − 16).

found in their guts, we collected fecal samples before performing gut dissections (Figure 1). Collection was easy for some queens, but challenging for others. Of the 26 queens sampled, we obtained at least 1 µL of feces from 21 queens (81% of the total samples; Table S1). We then dissected the guts of the queens from which we successfully collected feces, yielding 21 paired feces/gut samples. These were processed for 16S rRNA amplicon sequencing and qPCR analysis alongside three worker gut samples and a negative control, which contained all components of the library preparation protocol except for the DNA template.

3.2. Processing of bacterial sequencing data

A total of 1,026,596 paired-end reads were obtained from the MiSeq run, with individual samples ranging from 14,778 to 29,183 reads, and 1,673 reads for the negative control (Supplementary File 3). After primer removal, 1,000,196 reads remained (range 14,369–28,515; 1,629 for the negative control) (Supplementary File 4). DADA2 merging yielded 787,726 reads (range 10,541–25,074; 1,425 for the negative control) (Supplementary File 5 and Supplementary File 6), and after filtering low-abundance ASVs (< 0.1%) and non-bacterial reads, 786,882 reads (~ 77% of the original raw reads) were retained for downstream diversity analyses (Supplementary File 7 and Supplementary File 8). The final sample read counts ranged from 10,538 to 25,069 reads, and 1,404 reads were retained in the negative control. In total, 65 ASVs were detected, and their representative sequences are provided in Supplementary File 9. Supplementary files 3 to 9 were generated using QIIME 2 version 2024.5 (Bolyen et al. 2019) and can be visualized by uploading them to https://view.qiime2.org/.

Before performing the microbial diversity analysis, rarefaction curves were generated to evaluate whether the sequencing depth was sufficient to capture the observed bacterial richness in each sample. Based on those curves, all samples were subsampled to 10,538 reads to normalize sampling effort across samples. This sequencing depth was sufficient to capture the full bacterial richness in all samples (see Figure S1 for rarefaction curves by sample).

3.3. Bacterial diversity in fecal and gut samples

Based on 16S rRNA amplicon sequencing, we found that the bacterial communities in queen feces were similar to those detected in their respective guts (Figure 2A, Figure S2). The main bacterial genera detected in both types of samples included species of *Apilactobacillus*, *Bifidobacterium*, *Bombella*, *Bombilactobacillus*, *Commensalibacter*, and *Lactobacillus*, which is consistent with findings from other studies on queen gut microbiome (Tarpy et al. 2015; Powell et al. 2018; Caesar et al. 2024).

Comparisons of microbial diversity (both alpha and beta) between queen feces and guts demonstrated a high degree of similarity, but both differed significantly from those of worker

guts. For alpha diversity, we examined the Shannon diversity index (accounting for both species richness and evenness) and found a significant difference among the three sample types: queen feces, queen guts, and worker guts (Kruskal–Wallis test, $\chi^2 = 8.58$, df = 2, $n = 45$, p-value = 0.01). Post hoc analysis indicated that both queen feces and queen gut samples exhibited significantly lower Shannon index values compared to worker gut samples (Dunn's test, queen feces vs. worker gut, $Z = -2.63$, p-value adj = 0.01; queen gut vs. worker gut, $Z = -2.93$, p-value adj = 0.01, Figure 2B); however, there was no significant difference between queen feces and gut bacterial communities (Dunn's test, $Z = 0.59$, p-value adj = 0.55, Figure 2B). Additionally, a differential abundance analysis between queen gut and fecal microbiomes revealed that only two amplicon sequence variants (ASVs) belonging to the genera *Pseudomonas* and *Lactobacillus* were significantly enriched in queen feces compared to queen guts (DESeq2, p-value adj < 0.05, fold change > |2|, $n = 42$, Figure S3).

Regarding beta diversity, we used two metrics: Bray–Curtis dissimilarity (measuring differences in species abundances) and Weighted UniFrac (accounting for species abundances and their evolutionary relationships) (Lozupone and Knight 2005; Xia and Sun 2023). NMDS plots based on these metrics were used for data visualization (Figure 2C and Figure S4A). PERMANOVA analyses indicated significant differences between groups for both metrics (Bray–Curtis, $F_{2,42} = 3.46$, permutations = 999, p-value = 0.002; weighted UniFrac, $F_{2,42} = 13.21$, permutations = 999, p-value = 0.001). Pairwise comparisons revealed that these differences were primarily driven by comparisons between worker gut samples and queen samples, for both gut and feces (p-value adj < 0.01).

To investigate whether gut and fecal samples from the same individual were more similar compared to those from different individuals, we performed an additional pairwise distance analysis using Bray–Curtis and weighted UniFrac distances. The analyses included both within-pair comparisons (gut vs. feces from the same individual) and between-pair comparisons (gut vs. feces from different individuals). Both the Bray–Curtis and weighted UniFrac distances between gut and fecal samples from the same individual were significantly smaller than the distances between samples from different individuals, suggesting that gut and fecal microbiomes within the same individual (within-pair comparisons) were more similar to each other compared to those from different individuals (between-pair comparisons) (t-test, p-value < 2.2e − 16, Figure 2D, Figure S4B).

3.4. Bacterial loads in fecal and gut samples

When we quantified bacterial loads in our samples, we found that queen feces exhibited average bacterial loads of 1.08×10^8 16S rRNA amplicon copies per µL (95% CI, 4.07×10^7 to 1.76×10^8, $n = 21$), while queen gut samples exhibited average bacterial loads of 1.16×10^{10} 16S rRNA amplicon copies per gut (95% CI, 6.24×10^9 to 1.69×10^{10}, $n = 21$). Compared to worker gut samples, whose average bacterial loads were 2.86×10^9 16S rRNA amplicon copies per gut (95% CI, 6.79×10^8 to 5.04×10^9, $n = 3$), our findings also suggest that queen guts can harbor similar bacterial loads than worker guts (Figure 3A). A negative control sample indicated almost no external contamination of our processed samples (17.27 16S rRNA amplicon copies per µL).

Finally, when we combined information from the qPCR analysis with the 16S rRNA amplicon sequencing data, we found a positive correlation between the absolute abundance of the most common bacterial taxa found in queen guts and feces, indicating that the level of gut colonization by a specific taxon was mirrored by its abundance in the feces (Pearson correlation coefficient $r = 0.75$, $n = 42$, p-value = 0.008, Figure 3B, Figure S5). Overall, our findings suggest that fecal samples can be used for the study of queen gut microbiome composition, as they enabled us to determine the presence or absence

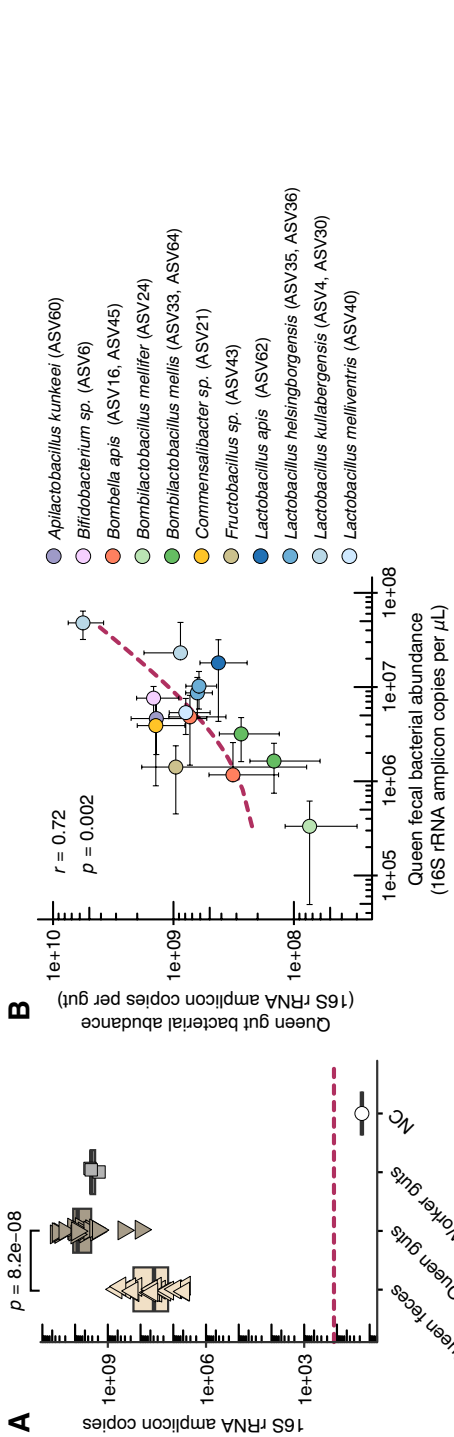


Figure 3. Comparisons of bacterial loads in honey bee queen fecal and gut microbiomes. **A** Box plots showing estimates of total bacterial abundance in queen feces (QF, $n = 21$), queen guts (QG, $n = 21$), worker guts (WG, $n = 3$), and a negative control (NC, $n = 1$). Statistical analysis was performed using Kruskal–Wallis test ($\chi^2 = 31.23$, df = 2, p-value = 1.65e−07, $n = 45$), followed by Dunn's pairwise comparisons with p-values adjusted using the Benjamini–Hochberg method (p-value adj < 0.05 shown). The maroon dashed line indicates the limit of detection of the qPCR analysis. **B** Scatter plot showing the correlation between the absolute abundances of bacterial genera in feces and gut samples (Pearson correlation coefficient r and p-value shown). Only ASVs with absolute abundance above 0.1% in at least three samples are displayed for clarity. Each circle represents a single amplicon sequence variant (ASV), colored by bacterial species. Vertical and horizontal error bars indicate the 95% confidence intervals of the mean abundance in the gut and feces, respectively; error bars extending into non-positive values were omitted due to the log scale. The maroon dashed line represents the linear regression curve, which appears as non-linear due to log axes.

of bacteria (community membership), as well as estimate bacterial loads (colonization levels) in the gut of individual queens in a non-destructive manner.

4. DISCUSSION

Our study demonstrates that fecal samples can serve as a reliable non-destructive method for assessing the gut microbiomes of honey bee queens. We found that bacterial communities in queen feces closely resemble those in their guts, with no significant differences in alpha or beta diversity and only minor differences in specific bacterial taxa. This similarity suggests that fecal samples can provide a representative snapshot of a queen's gut microbiome, enabling potential longitudinal studies with queens, similarly to what has been proposed for workers (Cabirol et al. 2024; Gregory et al. 2025). Additionally, we found a positive correlation between the absolute abundance of bacterial taxa in the feces and guts of queens, further supporting the use of fecal samples for queen microbiome analysis. This positive correlation has also been observed in a study comparing the bacterial loads in the guts and feces of honey bee workers (Cabirol et al. 2024).

Other studies, particularly in insects, have shown that fecal microbiomes can reflect gut microbiomes. For example, research on German cockroaches (Kakumanu et al. 2018), silkworms (Zhang et al. 2021), and spider mites (Zhu et al. 2020) found significant overlap between microbial communities in the gut and feces, suggesting that fecal samples can provide valuable insights into insect gut microbiomes. However, this is not always the case, as studies on mammals indicate that fecal microbiomes may not accurately represent the gut microbiome of specific gut regions. A study on pigs analyzing microbial and metabolic compositions at multiple gastrointestinal sites found significant differences in microbiome diversity and metabolite composition (Ahn et al. 2023), highlighting the need to compare fecal and whole-gut communities when assessing an organism's microbiome.

The fecal sampling process we used in our study presented some challenges. While we successfully collected feces from most of the queens, the volume obtained varied considerably, ranging from 1 µL to several µL. For those queens ($n = 21$), only slight pressure was applied to the abdomen to obtain the feces. However, for a smaller subset of queens ($n = 5$), no feces could be collected, even after applying considerable pressure to the abdomen. This variability in feces collection may be influenced by factors such as hydration, feeding status, or prior defecation and brings an extra level of complexity to consider when designing experiments to assess queen fecal microbiomes. Moreover, our queens remained banked until processed and were never released in the hive. Therefore, it is possible that queens in a regular hive environment may have different levels of fecal material to be collected for these types of studies.

In our study, we immobilized queens by placing them inside clean, sterile Petri dishes and then on ice for several minutes until they were immobilized and processed for feces collection. Bubnič et al. (2020) anesthetized queens using CO_2 gas instead of cold temperature, and then transferred them to a clean, sterile Petri dish, where the queens defecated naturally within minutes after waking up. On the other hand, Cabirol et al. (2024) stunned workers (not queens) using CO_2 and immobilized them on ice to collect feces and guts. In our study, we directly immobilized queens on ice. For the queens from which we could not obtain feces, we allowed them to return to room temperature and stay in the Petri dish for several minutes. However, this did not induce defecation later on. We then chilled them on ice again and dissected their guts for inspection, observing empty rectum sections. This suggests that even if we had exposed the queens to CO_2, those queens would not have defecated as there were no feces stored in their rectums. It is likely that feeding queens prior to sampling could increase the chances of obtaining fecal material in specific cases.

We also did not monitor the queens for the effects that chilling and abdominal squeezing might have caused in their longevity or

reproductive quality. Chilling and abdominal squeezing have been shown to be largely reversible in honey bee workers, with rapid recovery of motor function and survival if the exposure is brief (Tutun et al. 2020; Chhun et al. 2024). However, prolonged or abrupt chilling, as used in the cold storage of whole colonies, can reduce brood levels (Meikle et al. 2023), potentially due to impacts on queen oviposition. While our study focused on brief chilling for fecal sampling, followed by gut dissections for microbiome comparisons, we did not assess longer-term parameters such as survival post-treatment. Given the sensitivity of queen physiology, we acknowledge this limitation and propose that future work should measure post-sampling reproductive performance of queens (e.g., egg-laying rate and long-term survival).

In conclusion, fecal collection is a promising, non-destructive approach for the study of honey bee queen gut microbiomes. This method can facilitate longitudinal research to assess the impacts of environmental stressors, pathogens, diet, and other factors on queen microbiomes. Future studies should investigate the long-term effects of fecal induction and collection on queen survival and colony stability.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

EVSM and JR conceived the research. EVSM, JS, MB, and TFS performed the experiments. EVSM conducted the analyses and wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript and approved the final version.

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DATA AVAILABILITY

16S rRNA amplicon sequencing data are available on NCBI BioProject PRJNA1234195. Other data are included in this article and its supplementary information files.

DECLARATIONS

Competing interests The authors declare no competing interests.

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