

ESTIMATING BROAD-SENSE HERITABILITY OF THE GUT MICROBIOME IN WESTERN  
HONEY BEES (*APIS MELLIFERA*)

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## **Abstract**

The honey bee gut microbiome, crucial for bee health, may be shaped by both environmental and genetic factors. While differences in microbiomes exist between honey bee subspecies, it is unclear if genetic variation within a population influences this variation. This study estimated the broad-sense heritability of gut microbiome traits to explore patriline effects. We analyzed 500 workers from two colonies using 16S rRNA sequencing for microbiome characterization and genotyped them at 11 microsatellite loci. Results showed that patriline genetics marginally influenced the relative abundance of dominant microbiome taxa (18% to 24%) but did not significantly impact presence/absence of bacterial taxa or alpha and beta diversity. These findings suggest that environmental factors play a more substantial role in shaping the honey bee gut microbiome, indicating that microbiome-based interventions could benefit honey bees across populations.

## **Dedication**

I dedicate this thesis to my supportive, loving, and hard-working family, who fostered my career interest in animals since childhood, and without whom I would not be able to follow my passions.

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## **List of Abbreviations**

**PCR** – Polymerase Chain Reaction

**ONT** – Oxford Nanopore Technologies

**UMI** – Unique Molecular Identifier

**QIIME2** – Quantitative Insights Into Microbial Ecology 2

**OTU** – Operational Taxonomic Units

**GLM** – Generalize Linear Model

**FDR** – False Discovery Rate

# **Estimating broad-sense heritability of the gut microbiome in Western honey bees (*Apis mellifera*)**

## **1 Introduction**

### **1.1 Gut microbiome general background**

The gut microbiome is a complex symbiotic community of microorganisms found in the gastrointestinal tract of a host, and is predominantly of bacteria, as well as archaea, viruses, and fungi. The importance of understanding the various factors that affect and can be affected by the gut microbiome is currently an active area of research in biology, due to its increasingly apparent role in influencing overall host health. Generally, the gut microbiome has been found to play a role in the regulation of gastrointestinal function (Jones et al., 2022; Nayak, 2010; Trompette et al., 2014; Zhu et al., 2014), central nervous system function (Mu et al., 2016; Ojeda et al., 2021; Sharon et al., 2016; Suchodolski, 2018), fetal development (Sanidad & Zeng, 2020; Vuong et al., 2020), immune system function (Bonilla-Rosso & Engel, 2018; Ferguson et al., 2018; Geuking et al., 2014; Kwong et al., 2017; Rooks & Garrett, 2016), and psychological health (Chok et al., 2021; Foster et al., 2021; Jiang et al., 2018; Sumich et al., 2022) in a diversity of animals. Functional performance of the microbiome depends on the abundance and diversity of bacteria found in the typical host population (Benson et al., 2010; Dąbrowska & Witkiewicz, 2016; Grieneisen et al., 2021; Kurilshikov et al., 2017; Parks et al., 2013; Ponsuksili et al., 2021). The benefits of the gut microbiome to host health can be interrupted by dysbiosis, which is the imbalance of bacterial diversity and abundance of the typical host gut. Dysbiosis of the gut microbiome can be caused by dietary changes, use of medications, stress, presence of parasites, infections, inflammation of the intestines, environmental changes, or host genetics, which in turn can lead to the onset of chronic gastrointestinal conditions or increase the severity of existing health issues (Cresci & Bawden, 2015; David et al., 2014; Engel et al., 2012; Hacquard et al., 2015; Muegge et al., 2011; Romero et al., 2019; Shanahan et al., 2021; Xu & Knight, 2015; Zheng et al., 2018).

## 1.2 Gut microbiome of Western honey bees

Western honey bees (*Apis mellifera*) are not only known for their important role as pollinators (Canada, 2022; Mockler & Tardif, 2015), but also for their ability to serve as a model organism to study complex gut microbiomes (Engel et al., 2016; Wang et al., 2018; Zheng et al., 2018). Recently, honey bee populations have been declining due to exposure to multiple-stressors (French et al., 2024) and human modified environments (Richardson et al., 2023), making it imperative that we understand the various factors that influence honey bee health. One such factor is the state of their own gut microbiome, which has been linked to both positive and negative effects on individual and colony-level health (Kešnerová et al., 2020; Motta & Moran, 2024; Raymann & Moran, 2018; Ye et al., 2021). A healthy gut microbiome, has been shown to have a plethora of benefits to honey bees such as promoting immunity and detoxification ability against toxins and toxicants (Dong et al., 2022; Wu et al., 2020), protection against polystyrene microplastic exposure (Wang et al., 2021), and protection against bacterial infections (Janashia et al., 2016). Unfortunately, the honey bee gut microbiome is susceptible to dysbiosis caused by environmental and anthropogenic factors. For example, dysbiosis can be caused by an altered diet (Ricigliano et al., 2017; Zheng et al., 2019), infestation of parasites such as *Varroa*, *Nosema*, and *Lotmaria* (Abuldahab et al., 2022; Arismendi et al., 2020; Paris et al., 2020), and consequent diseases like nosemosis and chalkbrood (Khan et al., 2020; Paris et al., 2020; Raymann & Moran, 2018). Exposure to agrochemicals (Al Naggari et al., 2022; Kakumanu et al., 2016; Motta & Moran, 2020; Rouzé et al., 2019; Syromyatnikov et al., 2020; Wu et al., 2022), artificial diets (Powell et al., 2023; Ricigliano et al., 2022), and antibiotics (Duan et al., 2021; Motta & Moran, 2020) can also cause dysbiosis.

### 1.2.1 Acquisition and development of the gut microbiome

The gut microbiome of honey bees changes over the course of their lifetime and routes of acquisition are age-dependent. Prior to emergence, the gut microbiome varies drastically at various stages of

development (Zheng et al., 2018). At the larval stage, the gut microbiome is sparse and primarily in the midgut, and bacteria are acquired from contact with food secreted by their sisters (i.e. nurse bees). Abundance of bacterial cells at this stage is very low and often undetectable. At the pupal stage, the entire gut microbiome is eliminated due to ecdysis, the shedding of the gut lining during pupation (Zheng et al., 2019). When they emerge as adults, workers initially have no gut microbiome, but they start feeding immediately. Their guts get exponentially colonized by bacteria over the course of a few days. This occurs through trophallaxis and social interactions with nurse bees, interacting with fecal matter, and interacting with the general hive environment. The gut microbiome structure stabilizes 4-6 days post-emergence (Powell et al., 2014). Drastic changes in gut microbiome do not tend to occur in adult bees from typical behaviours, but dysbiosis by external factors may cause noticeable changes. Following emergence, 95% of the gut microbiome is found in the hindgut, which is composed of the rectum and ileum (Kwong & Moran, 2016).

### **1.2.2 Composition and functions of the gut microbiome**

The honey bee gut is comprised of a core gut microbe community that are typically found in the guts of almost all adult honey bees. These include: *Bifidobacterium*, *Gilliamella*, *Lactobacillus*, and *Snodgrassella* (Martinson et al., 2012). Additionally, there are also other genera that are present at relatively lower abundance, namely, *Bartonella*, *Commensalibacter*, and *Frischella* (Botero et al., 2023; Dong et al., 2022; Kwong & Moran, 2016).

*Gilliamella* is a core bacterial genus found in the guts of honey bees and plays a crucial role in the bees' ability to metabolize various carbohydrates, including some that can be toxic to them, such as mannose, arabinose, xylose, and rhamnose (Zheng et al., 2016). Alongside *Gilliamella*, *Bifidobacterium* is also vital for helping bees digest tough plant materials like pollen, breaking down complex carbohydrates such as pectin and hemicellulose (Zheng et al., 2019). *Snodgrassella* on the other hand, helps with forming

biofilms in the honey bee gut, which protects against various pressures from the environment (Motta et al., 2024) and protection against pathogens (Horak et al., 2020). *Lactobacillus* has also shown to positively impact neurological processes in honey bees by promoting memory behaviours (Zhang et al., 2022). Additionally, *Lactobacillus* also helps protect the host's immune system via regulating the Toll pathway (Lang et al., 2022).

### **1.3 Relationship between host genetics and the gut microbiome**

Despite being a highly active field of research, we still know relatively little about the honey bee gut microbiome including how genetics contributes to the diversity and relative abundance of gut microbiome within colonies. One study for example, examined the impact of host genetics on the gut microbiome using subspecies of honey bees, and found strain-level differences in gut microbiota (Wu et al., 2021). These differences however could result from differences in genetics, differences in the environment, or both. Furthering our understanding of the role host genetics plays in the community structure of honey bee gut microbiome can assist researchers and beekeepers to develop or improve interventions and management strategies to enhance honey bee gut health.

#### **1.3.1 Family genetics within honey bee colonies**

Quantitative geneticists estimate heritability of traits to examine the degree to which genetics influences phenotypic variance (Falconer, 1960; Lynch & Walsh, 1998). Broad-sense heritability and narrow-sense heritability are two ways to estimate heritability of traits (Messiaen et al., 2012; Snedden et al., 2007; Wang et al., 2013). Broad-sense heritability ( $H^2=Vg/Vp$ ) is defined as the proportion of phenotypic variance ( $Vp$ ) that is influenced by total genetic variance ( $Vg$ ) (Lynch & Walsh, 1998), while narrow-sense heritability ( $h^2=Va/Vp$ ) is the proportion of phenotypic variance that is influenced by additive genetic variance ( $Va$ ) (Lynch & Walsh, 1998).

Honey bees have several properties that allow researchers to estimate the genetic contribution to phenotypic variation. Honey bees are haplodiploid where unfertilized (haploid) eggs result in male offspring, known as drones, while fertilized (diploid) eggs often result in female offspring, which develop as either workers or queens (Beye et al., 2003; Gempe & Beye, 2011). Queen bees are polyandrous and mate with 10 to 20 males on average (Winston, 1991). Workers inherit 100% of their father's genome and 50% of their mother's genome, and so workers sired by the same haploid father are 75% related to each other and are called 'super-sisters'. On the other hand, half-sisters sired by different haploid fathers are 25% related to each other.

The existence of multiple patriline groups within the same colony, i.e. honey bee workers that experience the same maternal and environmental effects but vary with respect to their father's genetics, has allowed honey bee biologists to relatively easily estimate broad-sense heritability for worker phenotypic traits by examining the degree to which different patrilines within a colony differ phenotypically. Here, significant differences in worker phenotype between patrilines is taken as evidence for heritability. Previous honey bee patriline studies have shown that traits such as division of labor among workers (Kolmes et al., 1989), foraging behaviours (Mattila & Seeley, 2011), emergency queen rearing (Châline et al., 2003), olfactory learning ability (Laloi & Pham-Delegue, 2010), and tolerance to some pesticides (Tsvetkov et al., 2023) are influenced to some degree by host genetics.

### **1.3.2 Study aim and hypothesis**

This study aims to investigate broad-sense heritability of the gut microbiome in *A. mellifera*. To do this, we will quantify the gut microbiomes using 16s rRNA gene sequencing of the V4 hypervariable region from extracted gut DNA and simultaneously genotype each worker at 11 microsatellite loci to determine their patriline genetics. We will then estimate broad-sense heritability to test our hypothesis that gut

microbiome properties significantly vary between patriline. The gut microbiome properties we will explore include relative abundance, presence/absence, and alpha diversity (within-community diversity), and beta diversity (between-community diversity) of bacteria taxa at the genus and family level. Our study aims to provide key knowledge on how genetics influence within-colony variability of the gut microbiome, and this knowledge can potentially be leveraged by selective breeding programs and beekeepers to monitor and potentially improve the gut health quality of colonies.

## **2 Methodology**

### **2.1 Sample collection and dissections**

We used two honey bee colonies with naturally mated queens (Colonies A12 and A18) at York University's research apiary (Toronto, ON, Canada). Two frames with ready-to-emerge brood from both colonies were collected and placed in separate incubators at 33°C (Powell et al., 2014; Tsvetkov et al., 2023; Vernier et al., 2020). These frames were inspected daily, and newly emerged workers were marked by gently tapping the thorax with non-toxic enamel paint (Testors) using a soft-bristled paint brush. Bees were marked with a unique color representing their specific colony and their specific emergence day. The marked bees were re-introduced into their respective colonies after marking. Since it takes 4 to 6 days for the gut microbiome to develop post-emergence (Powell et al., 2014), we waited for 6 days before the marked bees were collected, freeze-killed in -20°C, and stored in individual 1.5 mL micro-centrifuge tubes at -80°C until ready for dissections. A total of 752 workers were found and collected out of 909 marked bees from Colony A12, and a total of 579 workers were found and collected out of the 768 marked bees from Colony A18.

Each bee was dissected with a pair of blunt and sharp forceps cleaned with Sparkleen 1 detergent (Fisherbrand), disinfected with ethanol, and flame sterilized. First, a whole bee was transferred from the 1.5  $\mu$ L tube to a sterile Petri dish placed on top of dry ice. Then, the head and abdomen were separated from the thorax, and the abdomen was stored in a 1.5  $\mu$ L tube to thaw on ice. Meanwhile, the wings and legs were then separated from the thorax, and a sterile No. 22 surgical blade was used to cut the thorax into two halves. One half of the thorax was used for DNA extraction. The thawed abdomen was then placed on a new sterile Petri dish. Gripping at the stinger, the gut was gently pulled out from inside the abdomen. Lastly, sharp forceps were used to gently cut away the stinger and midgut, leaving the hindgut, which was used for DNA extraction.

## **2.2 DNA extraction**

We extracted genomic DNA from both the thorax and hindgut of 250 bees from each colony. The thorax DNA was used for patriline assignment, and the hindgut DNA was used for microbial taxonomic assessment. For each bee, we placed half of the dissected thorax into a 2 mL bead mill tube (Fisherbrand) containing five to six 1.4 mm ceramic beads (Fisherbrand) and the dissected hindgut into a 2 mL bead mill tube containing eight to nine 1.4 mm ceramic beads. We added 350  $\mu$ L of TL Buffer to each bead mill tube and homogenized samples in the Bead Mill 24 Homogenizer (Fisherbrand). Each thorax was homogenized at 5 m/s for 15 seconds and each hindgut was homogenized at 5 m/s for 30 seconds. We used the Mag-Bind Blood & Tissue DNA HDQ 96 Kit (Omega Bio-Tek) and the KingFisher Flex system (ThermoFisher Scientific) to conduct automated DNA extraction as published in Tsvetkov et al. (2023) with modifications to the incubation temperature, incubation duration, and elution buffer volumes as follows. We added 20  $\mu$ L of Proteinase K, mixed well by pipetting up and down, and incubated samples at 55°C for 2 hours. During the incubation period, we prepped 6 deep-well plates with the following reagents per well: 2 plates with 600  $\mu$ L of VHB Buffer, 1 plate with 600  $\mu$ L of SPM Buffer, 1 plate with 500  $\mu$ L of Nuclease-free Water, 1 plate for tip-pickup, and 1 plate for the lysate containing 290  $\mu$ L of AL

Buffer, 400  $\mu\text{L}$  of HDQ Binding Buffer, and 20  $\mu\text{L}$  of Mag-Bind particles HDQ. The prepped plates were placed back carefully into their respective packaging to avoid evaporation of reagents until ready for the extraction system. Following the incubation period, we centrifuged the samples at 9.0 rcf for 10 minutes, transferred 300  $\mu\text{L}$  of clear supernatant to an intermediate set of 1.5 mL micro-centrifuge tubes, and centrifuged the samples again at 9.0 rcf for 10 minutes. We then transferred 250  $\mu\text{L}$  of clear supernatant into the lysate plate and added 5  $\mu\text{L}$  of RNase A. We incubated the plate at room temperature for 10 minutes during which we prepped 1 shallow-well plate with Elusion buffer. We used 80  $\mu\text{L}$  of Elusion buffer for thorax DNA extractions and 70  $\mu\text{L}$  of Elusion buffer for hindgut DNA extractions. Following the 10-minute incubation, we placed the plates into the KingFisher Flex system in the required order and ran extraction protocol. Once the extraction was completed, we transferred the final elute into a final set of 1.5 mL micro-centrifuge tubes. We checked DNA quality using gel electrophoresis and then stored samples at  $-80^{\circ}\text{C}$ .

### **2.3 Microsatellite genotyping**

To investigate paternal relatedness of honey bee workers, we performed genotyping of 11 hypervariable microsatellite loci for each of the 250 workers per colony (Shaibi et al., 2008; Tsvetkov et al., 2023). Protocols in Tsvetkov et al. (2023) and Shaibi et al. (2008) were followed with PCR conditions optimized for our samples as follows. We amplified samples at 11 hypervariable microsatellite loci (**Table S1**). Each PCR reaction contained 0.5  $\mu\text{L}$  of 10  $\mu\text{M}$  forward primer, 0.5  $\mu\text{L}$  of 10  $\mu\text{M}$  reverse primer, 12.5  $\mu\text{L}$  of Taq 2X Master Mix (New England Biolabs), and 1.5  $\mu\text{L}$  of sample thorax DNA. Volume of nuclease-free water and concentration of  $\text{MgCl}_2$  were optimized for each primer pair (**Table S2**). We performed PCR amplification using the Eppendorf Mastercycler EPGradient Thermal Cycler with the following cycler conditions: DNA denaturation at  $95^{\circ}\text{C}$  for 5 minutes; 35 cycles of  $90^{\circ}\text{C}$  for 30 seconds, primer annealing at  $55^{\circ}\text{C}$  or  $52.8^{\circ}\text{C}$  for 30 seconds (annealing temperature was optimized separately for each primer pair (**Table S2**),  $72^{\circ}\text{C}$  for 1 minute; primer extension at  $72^{\circ}\text{C}$  for 20 minutes; hold at  $4^{\circ}\text{C}$ . We

poolplexed the PCR product into 3 groups (**Table S3**) and sent to The Center for Applied Genomics at The Hospital for Sick Children (Toronto, ON, Canada) for capillary electrophoresis and microsatellite fragment analysis using the following products: SeqStudio Flex 22203-040 genetic analyzer, instrument software version 1.1.1 (ThermoFisher Scientific), and the GeneScan 500(-250) LIZ size standard (ThermoFisher Scientific).

We used the Microsatellite Plugin in Geneious Prime (version 2024.0.2) to call allele peaks (Tsvetkov et al., 2023). Since workers are diploid, we set the expected number of allele peaks to 2. For each bee at each locus, we called the two peaks with the highest relative fluorescence. We called the peak twice using the option to duplicate homozygotes if only one peak was present. We corrected miscalls by adjusting the peak to align with the highest point of its relative fluorescence. Locus HB-SEX-02 was removed from further analysis for both colonies due to large volume of miscalls that could not be corrected and highly variable peaks with no distinct pattern of relative fluorescence within each sample. Post-corrections, we generated allele tables for Colony A12 (**Table S4**) and Colony A18 (**Table S5**). A total of 18 samples from Colony A12 and 10 samples from Colony A18 contained missing allele information at one or more microsatellite loci and were excluded prior to assignments.

## **2.4 Patriline assignment**

Patriline assignment was conducted for 232 workers from Colony A12 and 240 workers from Colony A18 out of the 500 genotyped workers. We calculated allele frequencies at each locus and inferred the queen's genotype at each locus by (1) determining the two alleles with the highest frequency, and (2) checking if each worker possessed at least one of the two alleles with the highest frequency. Every individual in Colony A12 was homozygous for the same allele at Locus AC006, which made this locus uninformative, and was consequently removed prior to assignments. We used COLONY software version 2.0.7.1 for Linux (Jones & Wang, 2010) to assign patrilines by grouping workers into full sibship groups

(Conflitti et al., 2022) using the following program parameters: (1) female polygamy and male monogamy mating system without inbreeding or clone interference, (2) dioecious haplodiploid species, (3) medium run length, (4) full-likelihood analysis method, (5) no male genotypes, (6) queen's inferred genotype for candidate female, (7) one known mother and all maternal sibs, (8) no paternity or maternity exclusions, and (9) 0% allelic dropout rate. Workers from both colonies were assigned patriline based on COLONY's best family configuration results. We verified the results by checking if every individual in the assigned patriline contained an identical haplotype in both Colony A12 (**Table S6**) and Colony A18 (**Table S7**).

A total of 18 patriline (P1-P18) were assigned in Colony A12 using 9 informative microsatellite loci, and a total of 23 patriline (P1-P23) were assigned in Colony A18 using 10 informative microsatellite loci. The number of found patriline falls within the typical range of 10 to 20 mates on average for a honey bee queen (Brodschneider et al., 2012; Estoup et al., 1994; Withrow & Tarpy, 2018). Only patriline with 5 or more offspring were used for analyses, which were patriline P1-P14 in Colony A12 and P1-P14 in Colony A18. The distribution of genotyped workers into patriline and the patriline selected for analyses is visually presented in **Figure S1**.

## **2.5 16s rRNA gene sequencing**

We followed the protocol for Oxford Nanopore Technologies (ONT) sequencing in Hebert et al. (2023) and Floyd et al. (2023) with modifications to the number of samples and controls used as follows. We amplified the samples at the V4 hypervariable region of the 16s rRNA gene using the 515F and 806R primers (**Table S8**). Each PCR reaction contained 0.5  $\mu$ L of 10 mM 515F forward primer, 0.5  $\mu$ L of 10 mM 806R reverse primer, 3  $\mu$ L of Multiplex PCR Master Mix (Qiagen), 3  $\mu$ L of nuclease-free water, and 2  $\mu$ L of sample gut DNA. We used 2  $\mu$ L of nuclease-free water for the negative control and 2  $\mu$ L of 0.52 ng/ $\mu$ L microbial community DNA standard (Zymo Research) for the positive control. For multiplexing,

we added an 24x8 combination of unique molecular identifiers (UMI) to asymmetrically tag each well in 3 96-well plates (**Table S8**), resulting in 250 DNA samples, 18 positive controls, and 18 negative controls per colony all uniquely tagged prior to amplification. PCR amplification was performed using the Eppendorf Mastercycler EPGradient Thermal Cycler with the following optimized cyclers conditions: DNA denaturation at 94°C for 3 minutes; 35 cycles of 94°C for 45 seconds, primer annealing at 52.5°C for 1 minute, 72°C for 10 minutes; primer extension at 72°C for 10 minutes; hold at 4°C. PCR product was stored at -20°C until ready for library preparation.

Two ONT libraries, one per colony, were prepared using the Ligation Sequencing Kit V14 (SQK-LSK114). Pooling, purification, quantification, adapter ligation, and final clean-up were performed as published in Hebert et al. (2023). Two ONT libraries were sequenced using a MinION Mk1B sequencing device (MN46811), MinION flow cells (FLO-MIN114), and MinKNOW software version 24.02.6. The sequencing parameters were as follows: (1) minimum read length of 20 bp, (2) no real-time basecalling, (3) POD5 read output, (4) 72 hours run time. Flow cells used for both colonies consisted of over 800 pores found for sequencing and were considered fit to proceed with sequencing.

## **2.6 Microbial taxonomic assessment**

We used ONT's open-source Dorado software (model dna\_r10.4.1\_e8.2\_sup@v4.3.0) to perform basecalling of sequence reads into FASTQ files. We used the MiniBar (Krehenwinkel et al., 2019) in Python version 3 to demultiplex the ONT sequenced reads into individual sample files using the following parameters: (1) barcode edit distance value 4, (2) primer edit distance value 4, (3) no trimming, and (4) default option for remaining parameters. Primers and adapters were trimmed in the 5' to 3' direction, followed by the 3' to 5' direction using Cutadapt version 4.8 (Martin, 2011). Reads were size filtered down to 240-260 base pair length. Filtered reads were processed through a bioinformatics pipeline for microbial taxonomic assessment using Quantitative Insights Into Microbial Ecology 2 version 2021.11

(QIIME2). Filtered FASTQ reads were converted to qiime2 artifacts using the import tool. Then, sequence reads were dereplicated using VSEARCH. Sequences were clustered into operational taxonomic units (OTU) using VSEARCH closed-reference clustering at 98% identity against the SILVA 16s rRNA gene database. Clustered OTUs were then classified using the VSEARCH feature classifier at 98% identity against the SILVA 16s rRNA gene database. Finally, OTUs were collapsed at two taxa levels: genus and family.

## **2.7 Data filtering and preparation**

We filtered and prepped data tables to appropriately estimate heritability for three different gut microbiome properties: relative abundance, presence/absence, and community diversity of bacteria present.

First, we generated observed OTU count tables for both the genus and family level from the qiime2 artifacts using the qiime2R package in R version 4.4.1. We then used the 18 negative controls per colony to determine mistag frequency and filtered the OTU sample data to remove detections likely caused by mistagging using a mistag-filtering program developed for R by Richardson (Richardson, 2022). A few samples from both colonies were removed after mistag filtering as there was no observed data across the sample. We also found that ‘Mitochondria’ and ‘Chloroplast’ are classified by the reference database as bacteria as seen in previously published microbiome research (Bridson et al., 2022). While mitochondria and chloroplasts have a complex relationship with ancestral bacteria (Martin et al., 2015), they are not considered to be part of families or genera within the bacterial kingdom and were therefore removed from the list of OTUs. Unknown bacteria were also removed. We then rarefied our OTU tables down to the rarefaction threshold, determined by calculating Good’s coverage, to control for any differences in sampling effort and sequencing depth across different samples using the vegan package (Schloss, 2024). After rarefaction, we isolated and compared our positive control data to the composition of the original

microbial community standard used to verify presence of expected microbes. We converted our tables to relative abundance and presence/absence tables. Not all bacteria were suitable to be analyzed for all three properties. For heritability of community diversity, all taxa present in a sample were used to calculate Shannon diversity index. A total of 38 families and 66 genera were used for community diversity analyses (**Table S9**). Only taxa that were present in at least 15% of the individuals within each colony were selected for relative abundance or presence/absence analyses to ensure the data for the assumptions of a parametric GLM model. A total of 8 genera and 7 families passed the 15% threshold. Further, the distribution of data for each bacterium was visually checked for residual plots and histograms. Bacteria that (1) were present in all or nearly all individuals did not qualify and (2) had data with gaussian or gamma family distribution, were selected for relative abundance heritability analysis. The other bacteria had zero inflated data and were selected for presence/absence heritability analyses using binomial family distribution.

## 2.8 Statistical Analyses

We performed all statistical analyses using R version 4.4.1. By utilizing nested generalized linear models and the respective model fits, we estimated broad sense heritability ( $H^2$ ) of gut microbiome properties examining the phenotypic variance that is explained by patriline relative to the total phenotypic variance (Laloi & Pham-Delegue, 2010; Tsvetkov et al., 2023). To estimate the heritability of microbiome properties for each bacterium, we first ran two generalized linear models (GLMs). In both GLMs, the microbiome property of the bacterium was used as the response variable. In model 1, the first explanatory variable was colony, and the second added explanatory variable was patriline nested with the colony. In model 2, the explanatory variable was just colony. We then calculated deviance-based R-squared ( $R^2$ ) for both models and subtracted  $R^2$  for model 2 from  $R^2$  for model 1 to determine the deviance explained by just the effect of patriline.  $H^2$  is twice the patriline variance for haplodiploid organisms. We used the p.adjust function from the R stats package to conduct multiple test correction using the Benjamini-

Hochberg method to estimate the false discovery rate (FDR) at a significance level of 0.05, and reported the adjusted p-values. We also assessed gut community structure using Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity to evaluate beta diversity across the 14 patriline in two honey bee colonies. This analysis was performed at both the genus and family levels using the bray method with the vegdist function from the vegan package. PCoAs, boxplots, and barcharts were plotted using ggplot2 package.

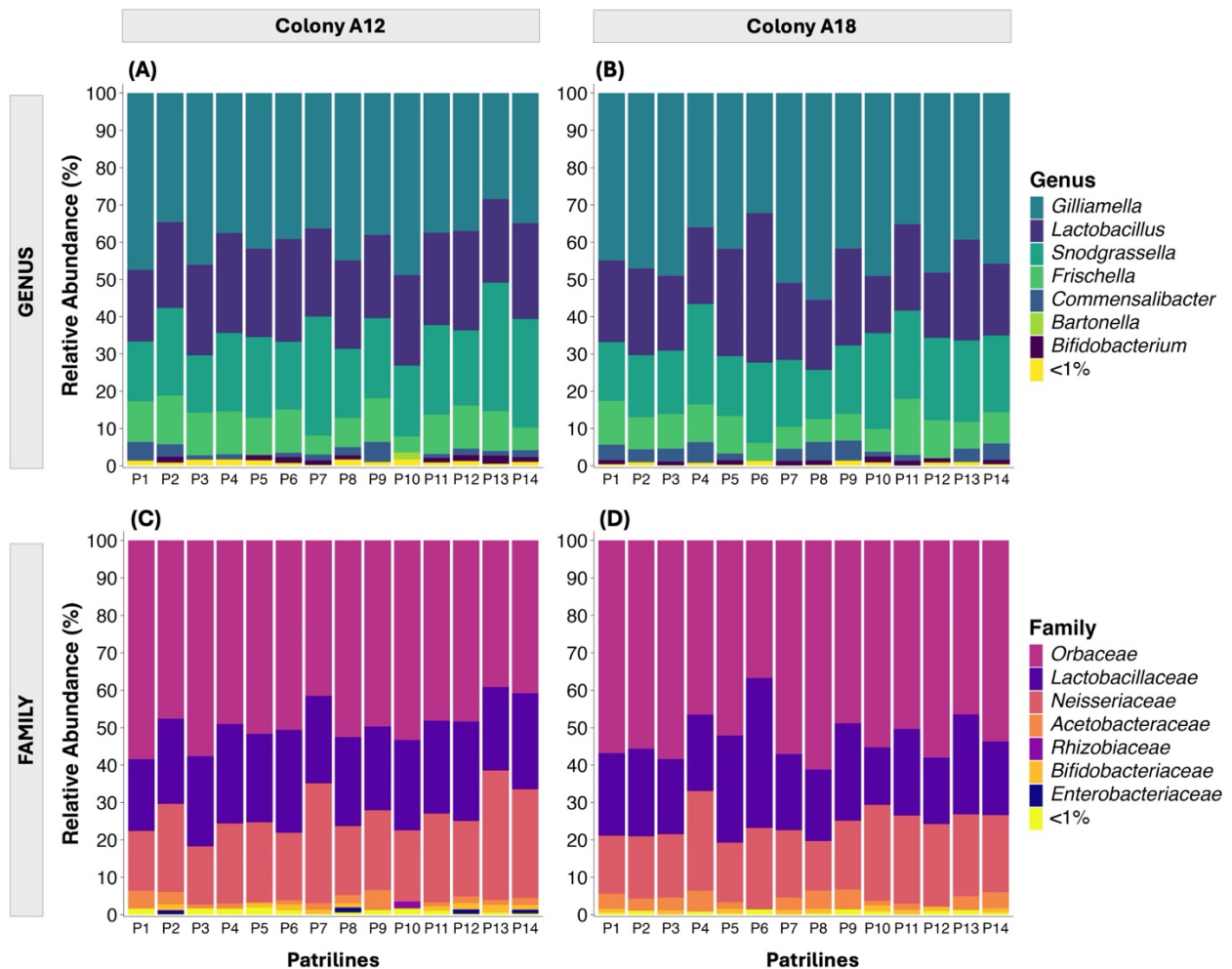
### 3 Results

#### 3.1 Honey bee gut microbiome composition and abundance

The gut microbiome of honey bees was profiled across 14 different patriline in two colonies (A12 and A18). We found that 38 bacterial families and 66 bacterial genera were identified across 500 honey bee hindguts using 16S rRNA gene sequencing (**Table S9**). There were 7 to 8 prominent taxa for both family and genus level classification (**Figure 1**).

At the genus level, the core bacterial genera identified were *Gilliamella*, *Lactobacillus*, *Snodgrassella*, and *Bifidobacterium* (**Table S10**). *Gilliamella* showed variation in relative abundance ranging from 28.43% to 48.81%, and 32.18% to 55.49% in Colony A18. *Lactobacillus* showed variation in relative abundance ranging from 19.24% to 27.55% in Colony A12, and 20.10% to 40.16% in Colony A18. *Snodgrassella* had a relative abundance ranging from 15.37% to 34.45% in Colony A12, and 13.15% to 26.97% in Colony A18. Lastly, *Bifidobacterium* was found in smaller abundance ranging from 1.12% to 2.19% in in Colony A12, and 1.04% to 1.60% in Colony A18.

At the family level, the core bacterial families identified were *Orbaceae*, *Lactobacillaceae*, *Neisseriaceae*, *Acetobacteraceae*, and *Bifidobacteriaceae* (Table S11). Relative abundance of *Orbaceae* ranged from 39.14% to 58.44% in Colony A12, and 36.73% to 61.20% In Colony A18. *Lactobacillaceae* showed relative abundance ranging from 19.17% to 27.46% in Colony A12, and 20.07% to 40.08% in Colony A18. *Neisseriaceae* showed relative abundance ranging from 15.45% to 34.58% in Colony A12, and 13.21% to 26.67% in Colony A18. Lastly, *Bifidobacteriaceae* showed smaller relative abundance ranging from 1.12% to 2.07% in Colony A12, and 1.05% to 1.60% in Colony A18.



**Figure 1.** The honey bee gut microbiome profiled from different patriline within two colonies. Stacked bar plots represent the average relative abundance of bacterial taxa found in the hindgut of honey bees at the genus and family level. Relative abundance of bacteria at the genus level is shown for **(A)** Colony A12 and **(B)** Colony A18. Relative abundance of bacteria at the family level is shown for **(C)** Colony A12 and **(D)** Colony A18. Each bar represents a different patriline (P1–P14) found within each colony. Taxa with average abundance of <1% across all patrilines are presented as a sum of averages in a single group at both the genus and family level.

### 3.2 Heritability of the relative abundance of honey bee gut microbiome taxa

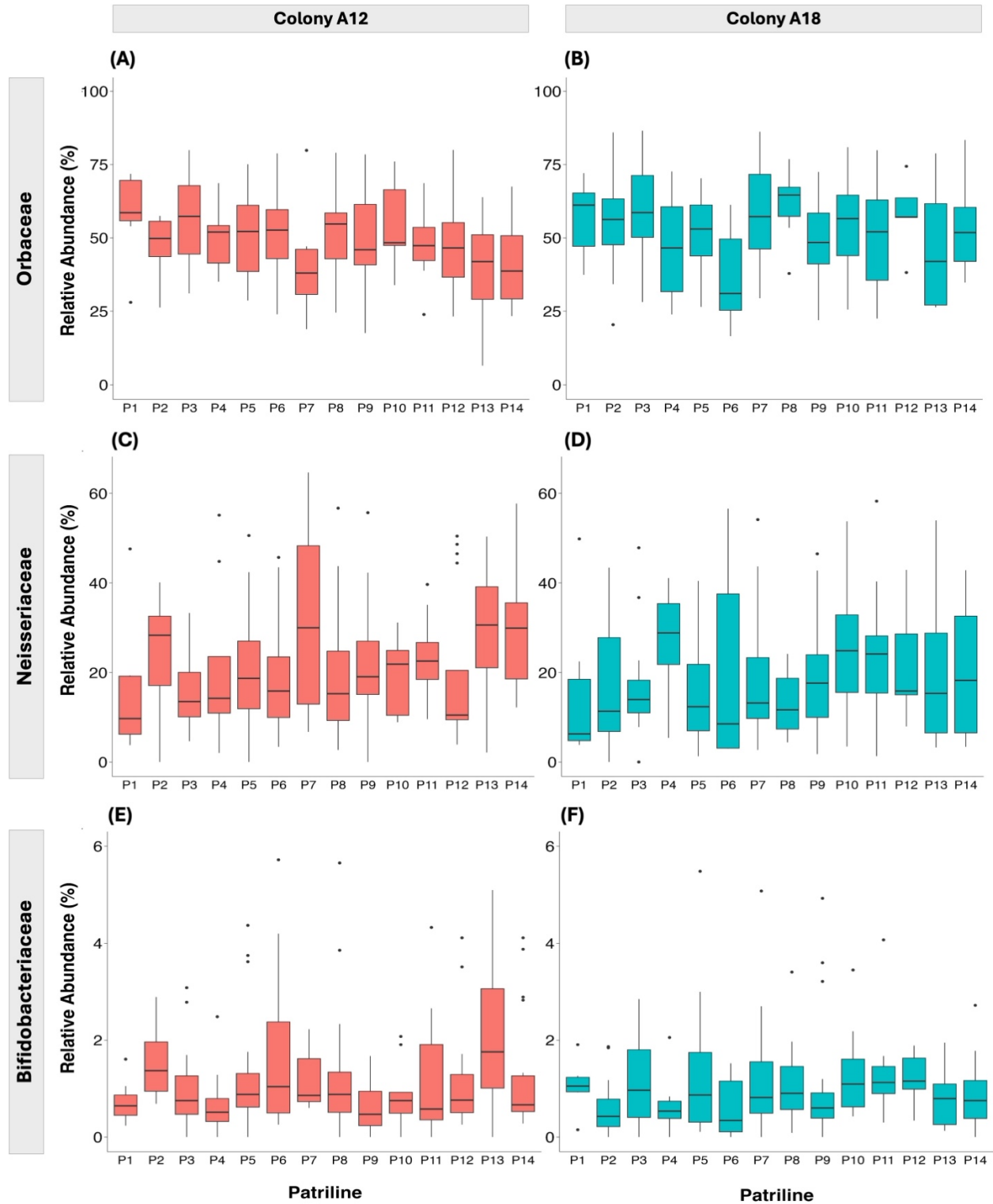
The analysis of relative abundance data revealed some marginally statistically significant effects of patriline on the relative abundance of certain bacterial families and genera. At the genus level, some bacteria had marginally significant heritability estimates for relative abundance at <10% FDR. For example, the broad sense heritability of the relative abundance of *Gilliamella*, *Snodgrassella*, *Bifidobacterium* was 18% ( $H^2 = 0.18$ , adjusted p-value = 0.0507), 20% ( $H^2 = 0.20$ , adjusted p-value = 0.0507), and 24% ( $H^2 = 0.24$ , adjusted p-value = 0.0641), respectively. The relative abundance of *Lactobacillus*, on the other hand, was not significantly heritable ( $H^2 = 14\%$ , adjusted p-value = 0.1528).

At the family level, some bacteria had marginally significant heritability estimates for relative abundance at <10% FDR. For example, the broad sense heritability of the relative abundance of *Orbaceae*, *Neisseriaceae*, and *Bifidobacteriaceae* was 21% ( $H^2 = 0.21$ , adjusted p-value = 0.0148), 20% ( $H^2 = 0.20$ , adjusted p-value = 0.0170), and 22% ( $H^2 = 0.22$ , adjusted p-value = 0.0991), respectively. The relative abundance of *Lactobacillaceae*, on the other hand, was not significantly heritable ( $H^2 = 14\%$ , adjusted p-value = 0.1598).

**Table 1.** Broad sense heritability of relative abundance of bacterial genera and families in 500 honey bees across two colonies. Generalized linear models were performed to test the significance of colony and patriline level effects on the relative abundance of bacteria: Chi-square (Chisq) statistics, degrees of freedom (Df), p-values, false discovery rate (FDR) corrected p-value for patriline effect, model fits for  $H^2$  calculations ( $R^2$ ), and broad-sense heritability ( $H^2$ ) are presented. Refer to section 2.8 in Methodology for model specifications.

Bacteria	Colony effect			Patriline effect			FDR correction for patriline effect	Model 1	Model 2	Broad-sense Heritability
	Chisq	Df	p value	Chisq	Df	p value	adjusted p value	$R^2$	$R^2$	$H^2$
<b>Genera</b>										
<i>Gilliamella</i>	6.42	1	0.0113	41.87	26	0.0253	0.0507*	0.1042	0.0139	0.18
<i>Snodgrassella</i>	2.54	1	0.1107	46.11	26	0.0089	0.0507*	0.1049	0.0055	0.2
<i>Lactobacillus</i>	1.54	1	0.2143	34.31	26	0.1274	0.1528	0.0732	0.0031	0.14
<i>Bifidobacterium</i>	4.18	1	0.0408	39.6	26	0.0427	0.0641*	0.1299	0.0124	0.24
<b>Families</b>										
<i>Orbaceae</i>	4.88	1	0.0272	49.4	26	0.0037	0.0148*	0.1157	0.0104	0.21
<i>Neisseriaceae</i>	2.5	1	0.1137	46.27	26	0.0085	0.0170*	0.1052	0.0054	0.2
<i>Lactobacillaceae</i>	1.39	1	0.2378	33.08	26	0.1598	0.1598	0.0704	0.0028	0.14
<i>Bifidobacteriaceae</i>	3.06	1	0.0802	37.03	26	0.0743	0.0991*	0.1176	0.009	0.22

\* <10% FDR



**Figure 2.** Relative abundance of bacterial families in the honey bee hindgut across patriline within two honey bee colonies. Only bacterial families that showed significant patriline effects at an FDR threshold of 10% are presented. Box plots represent the relative abundance for 14 patriline in each colony. Each panel represents the distribution of relative abundance for *Orbaceae* (**A and B**), *Neisseriaceae* (**C and D**), and *Bifidobacteriaceae* (**E and F**) across patriline in Colony A12 (red) and Colony A18 (blue).

### 3.3 Heritability of the presence/absence of honey bee gut microbiome taxa

The analysis of presence/absence data revealed no significant effect of patriline on the presence/absence of certain bacterial families and genera (**Table 2**). At the genus level, there was no significant heritability estimates for presence/absence at <10% FDR for any of the bacteria. For example, the broad sense heritability of the presence/absence of *Commensalibacter*, *Brachybacterium*, *Frischella*, and *Bartonella* was 10% ( $H^2 = 0.10$ , adjusted p-value = 0.7642), 8% ( $H^2 = 0.08$ , adjusted p-value = 0.7642), 11% ( $H^2 = 0.11$ , adjusted p-value = 0.7642), and 10% ( $H^2 = 0.10$ , adjusted p-value = 0.7642), respectively.

At the family level, there was also no significant heritability estimates for presence/absence at <10% FDR for any of the bacteria. For example, the broad sense heritability of the presence/absence of *Acetobacteraceae*, *Enterobacteriaceae*, and *Rhizobiaceae* was 10% ( $H^2 = 0.10$ , adjusted p-value = 0.3591), 18% ( $H^2 = 0.18$ , adjusted p-value = 0.1913), and 11% ( $H^2 = 0.11$ , adjusted p-value = 0.3591), respectively.

**Table 2.** Broad sense heritability of presence/absence of bacterial genera and families in 500 honey bees across two colonies. Generalized linear models were performed to test the significance of colony and patriline level effects on the relative abundance of bacteria: Chi-square (Chisq) statistics, degrees of freedom (Df), p-values, false discovery rate (FDR) corrected p-value for patriline effect, model fits for  $H^2$  calculations ( $R^2$ ), and broad-sense heritability ( $H^2$ ) are presented. Refer to section 2.8 in Methodology for model specifications.

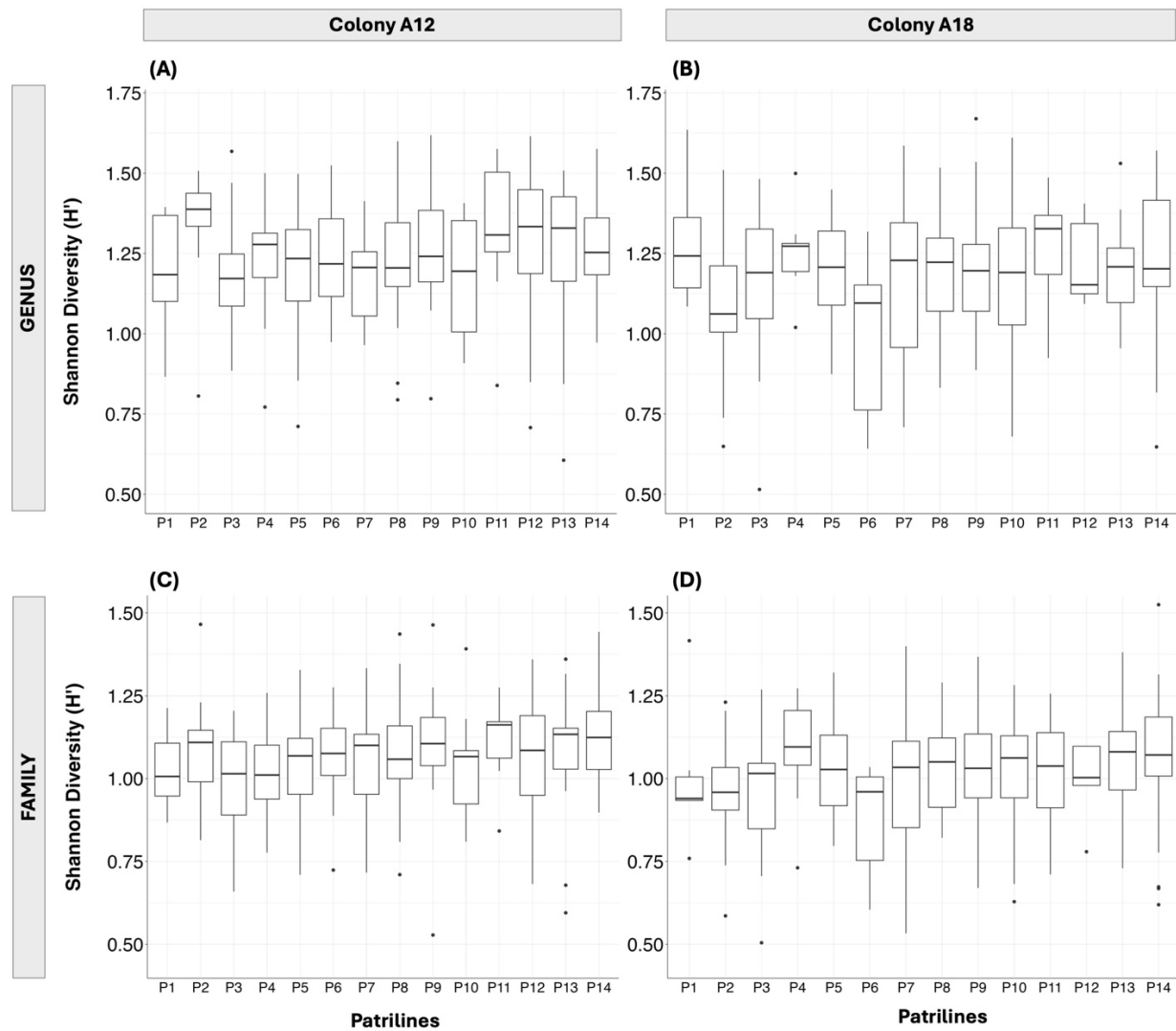
	Colony effect			Patriline effect			FDR correction for	Model	Model	Broad-
	Chisq	Df	p value	Chisq	Df	p value	patriline effect	1	2	sense
<b>Bacteria</b>							adjusted p-value	$R^2$	$R^2$	Heritability
<b>Genera</b>										
<i>Commensalibacter</i>	14.67	1	0.0001	30.68	26	0.2403	0.7642	0.0766	0.0248	0.1
<i>Brachybacterium</i>	30.88	1	$2.7 \times 10^{-8}$	20.56	26	0.7642	0.7642	0.101	0.0606	0.08
<i>Frischella</i>	0.71	1	0.3989	21.66	26	0.7071	0.7642	0.0579	0.0018	0.11
<i>Bartonella</i>	0.26	1	0.6069	26.16	26	0.4542	0.7642	0.0511	0.0005	0.1
<b>Families</b>										
<i>Acetobacteraceae</i>	13.25	1	0.0003	28.94	26	0.314	0.3591	0.0715	0.0225	0.1
<i>Enterobacteriaceae</i>	10.9	1	0.001	37.76	26	0.0638	0.1913	0.1163	0.0261	0.18
<i>Rhizobiaceae</i>	0.09	1	0.7637	27.99	26	0.3591	0.3591	0.0538	0.0002	0.11

\* <10% FDR

### 3.4 Alpha diversity of honey bee gut microbiome

Alpha diversity of the gut microbiome was assessed using the Shannon diversity index across the 14 patrilines in both colonies (**Figure 3**). The heritability of Shannon diversity was estimated at both the genus and family levels (**Table S12**). At the genus level, the broad-sense heritability ( $H^2$ ) of the Shannon diversity index was estimated to be 12% ( $H^2 = 0.12$ , adjusted p-value = 0.3665) for Colony A12, but neither of these were significant, indicating no genetic influence on alpha diversity across patrilines. Similarly, in Colony A18, the heritability of genus-level alpha diversity was comparable, though not

statistically significant, suggesting that genetic differences among patrilines do not influence the overall genus diversity. At the family level, the Shannon diversity index showed a broad-sense heritability estimate of 11% ( $H^2 = 0.11$ , adjusted p-value = 0.5549) for Colony A12. This indicates no genetic effect on the alpha diversity of bacterial families within the gut microbiome. Colony A18 reflected a similar pattern, with no significant patriline effect observed, reinforcing our previous observations that the genetic influence on microbiome diversity at both the genus and family levels is limited.

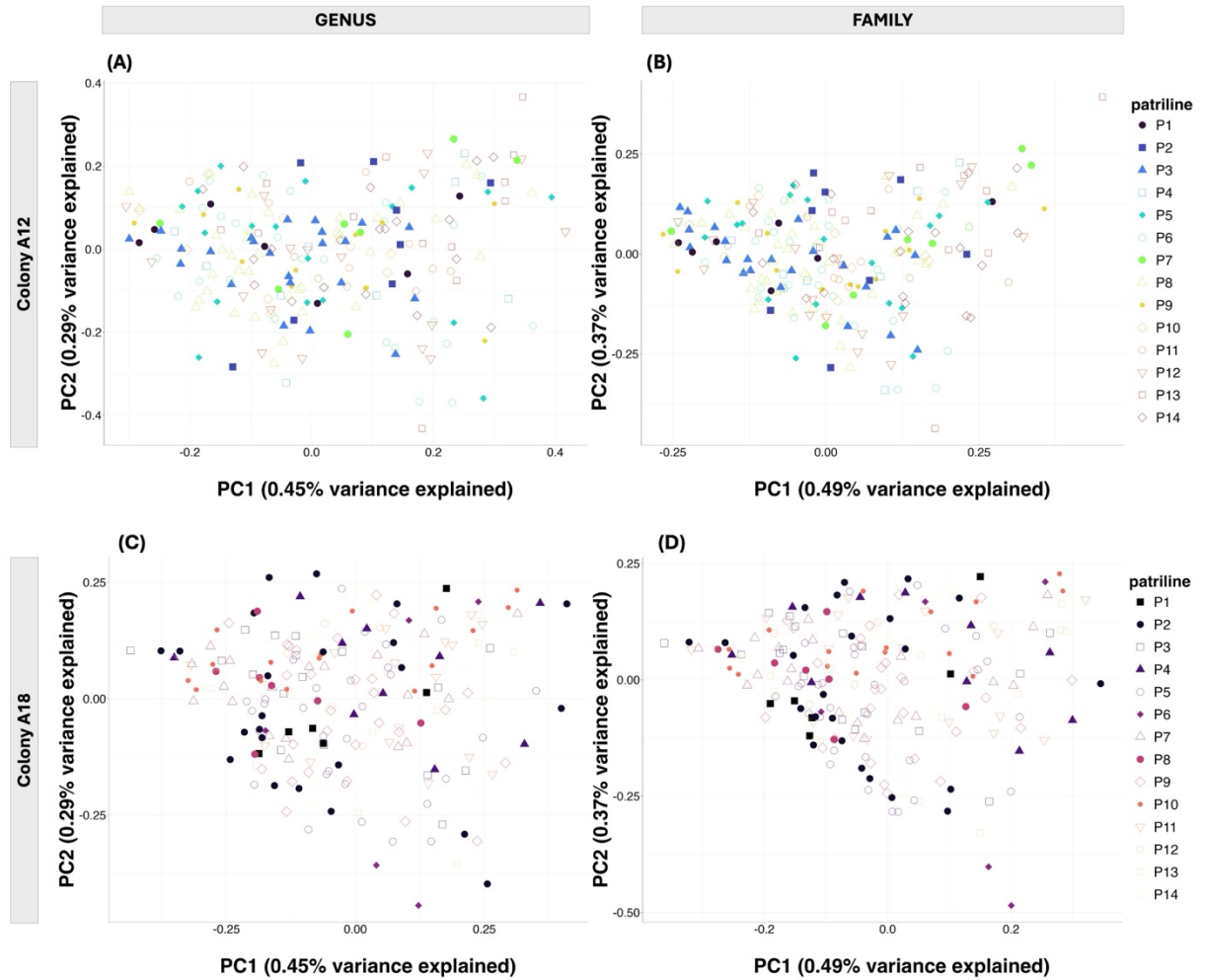


**Figure 3.** Alpha diversity of the honey bee gut microbiome across patriline in two colonies. Boxplots representing the Shannon Diversity Index ( $H'$ ) of gut microbiome diversity across 14 patriline in each honey bee colony at the **(A)** genus level and **(C)** family level for Colony A12; and at the **(B)** genus level and **(D)** family level for Colony A18. The heritability ( $H^2$ ) estimates for Shannon diversity are shown in **Table S12**.

### **3.5 Beta diversity of the honey bee gut microbiome**

The gut microbiome community structure was assessed using Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity to evaluate beta diversity across the 14 patriline in two honey bee colonies at both the genus and family levels (**Figure 4**).

In Colony A12, PCoA plots did not reveal distinct clustering of gut microbiome profiles by patriline at either the genus (**Figure 4A**) or family level (**Figure 4B**). The first principal coordinate (PC1) explained 0.45% of the variance at the genus level and 0.49% at the family level, while the second principal coordinate (PC2) explained 0.38% and 0.37% of the variance, respectively. Similarly, in Colony A18, no clear clustering by patriline was observed at the genus (**Figure 4C**) or family level (**Figure 4D**). The variance explained by the first and second principal coordinates was consistent with Colony A12, with PC1 explaining 0.45% of the variance at the genus level and 0.49% at the family level, and PC2 explaining 0.39% and 0.35% of the variance, respectively.



**Figure 4.** Principal Coordinate Analysis (PCoA) of the gut microbiome community in two honey bee colonies based on Bray-Curtis dissimilarity. Panels (A) and (C) represent Colony A12, while panels (B) and (D) represent Colony A18. The gut microbiome data are analyzed at the genus and family levels. Patriline are color-coded as indicated in the legend. The percentage of variance explained by the first and second principal coordinates is shown on the axes.

## 4 Discussion

We studied the genetics of the honey bee's gut microbiome by profiling it in 500 workers from 2 colonies and asking if gut microbiome properties were influenced by patriline identity. We used 16s rRNA gene sequencing to profile the gut microbiome and our analysis uncovered a common suite of bacterial lineages previously discovered in other studies (Benson et al., 2010; Moritz & Heisler, 1992; Motta & Moran, 2024; Powell et al., 2014; Raymann & Moran, 2018).

Our analysis identified typical bacterial genera such as *Gilliamella*, *Lactobacillus*, *Bifidobacterium*, and *Snodgrassella* within the honeybee hindguts across all different patriline and colonies. Similarly, the significant presence of *Orbaceae*, *Neisseriaceae*, *Bifidobacteriaceae*, and *Lactobacillaceae* at the family level further validate the taxonomic classification results from this study. Additionally, the relative abundance of the bacteria we discovered also align with the typical host gut microbiome, with *Gilliamella* and *Snodgrassella* being amongst the most commonly occurring (Kešnerová et al., 2020), and composing almost half the honey bee hindgut. *Lactobacillus* has also been shown to be highly abundant at 23 – 45% (Kwong & Moran, 2016; Moran et al., 2012) but this was not the case for our study, possibly due to difference in genetics or the environment.

We hypothesized that if some aspects of the honey bee gut microbiome were heritable, then we would observe statistically significant differences in gut microbiome traits between patrilines. We discovered a marginal effect ( $H^2$  approximately 18% to 24%) of genetics on the relative abundance of several dominant bacterial genera and families. For example, *Gilliamella*, *Snodgrassella*, and *Bifidobacterium* all showed marginally significant heritability estimates. Similar genetic effects on lineage relative abundance have been discovered in mice, humans, and pigs (Doms et al., 2022). For example, the genus *Enterococcus* in humans was shown to have a heritable component of 28%, the family *Clostridiaceae* in

humans was shown to have heritability estimate of 30% and 57% in mice, and the family *Fibrobacteriaceae* in pigs has shown to have heritability estimate of 26% in pigs (Doms et al., 2022).

The results above could only be carried out on common gut microbiome lineages whose relative abundance could be objectively quantified across the different patriline and colonies studied (**Figure 1; Table 1; Table 2**). However, we found that a large number of bacterial taxa that inhabit the honey bee gut varied greatly in abundance between individuals, making it difficult to perform any quantitative analysis on them individually. For some these taxa, we decided to study whether the presence or absence of a lineage was influenced by patriline genetics and found no evidence of this, which is consistent with the environment playing a major role in influencing the presence of these rare (or uncommon) taxa. Our results suggest that the genetic influence on the relative abundance of *Lactobacillus* is limited compared to the relatively more heritable genera like *Gilliamella* and *Bifidobacterium*. Genetic influence on the relative abundance of *Lactobacillaceae* is limited compared to the more heritable families like *Orbaceae* and *Bifidobacteriaceae*. These findings indicate that while some bacterial taxa exhibit a weak genetic influence in their relative abundance, the overall influence of patriline on the presence/absence of these taxa is limited. We also found that the overall community diversity of the gut microbiome, as measured by the Shannon diversity index, was not influenced by patriline (**Figure 3**). Overall gut microbiome composition was similar across all patrilines within each colony. Our PCoA analysis of the gut microbiome data from both colonies clearly illustrates this (**Figure 4**); we observed no obvious clustering of gut microbiome samples by patriline, which also indicates a lack of genetic influence at the population level.

Overall, our results suggest that the honey bee gut microbiome is largely influenced by the environment as our measured gut microbiome traits either had low and marginally significant estimates of  $H^2$  (~18 – 24%) or estimates of  $H^2$  that were not significantly different from zero. The honey bee gut microbiome of adult workers is established via trophallaxis with other mature bees within the colony and from

interacting with hive environment (Powell et al., 2014). Our results are thus consistent with the knowledge that workers from specific patrilineages do not preferentially feed or interact with their super-sisters (Moritz & Heisler, 1992), and so the acquisition of the gut microbiome should be fairly uniform across patrilineages.

Several possible mechanisms have been proposed to explain how genetics can influence the structure of bacterial lineages in the host. The host's immune system plays a crucial role in shaping the gut microbiome. Specific genes related to immune response, such as those involved in Toll-like receptor (TLR) and interleukin (IL) signaling pathways, are associated with the presence and abundance of certain microbial taxa (Tanoue et al., 2010; Zheng et al., 2020); Genome-wide association studies (GWAS) have identified various genetic loci associated with the abundance of specific microbial taxa, suggesting a polygenic influence on the microbiome (Stankevic et al., 2024; Xu et al., 2020); Genes involved in growth and development can affect gut morphology, which in turn influences the composition of the gut microbiome (Cohen et al., 2019; Ryu & Davenport, 2022). Variations in gut structure driven by host genetics following these mechanisms can create different environments that favor the growth of specific microbial communities.

While our sampling sizes were robust (250 workers per colony), as with most studies, we typically lack power to detect small genetic effects, and so we cannot fully rule out that genetics may play a relatively small but significant role in influencing the gut microbiome of workers. Further, while we studied two honey bee colonies, heritability is a property of a population and not a species, and so it may be possible that gut microbiome traits may be heritable in other honey bee populations. For example, if the honey bee gut microbiome plays a significant role in colony fitness and host health, then it may be possible that beekeepers have been unknowingly selecting colonies with specific microbiome properties that promote colony fitness, and this artificial selection may lead to very low and insignificant estimates of  $H^2$  because

of the reduced additive genetic diversity which is a component of overall genetic variation (Falconer, 1960; Lynch & Walsh, 1998).

To promote the presence of a typical gut microbiome in honey bees, several targeted interventions can be implemented focusing on ensuring stability of the typical honey bee gut microbiome. These include optimizing diet by ensuring that bees have access to a diverse range of floral resources, which supports a balanced microbial community. Probiotic supplementation with beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium* strains that are natural components of the honey bee gut, can help maintain microbial diversity and resilience. Additionally, reducing exposure to environmental stressors like pesticides and antibiotics, which can disrupt the gut microbiome, is crucial. Finally, managing hive conditions to reduce stress and disease transmission, including controlling Varroa mites and other pathogens, can further support the health and stability of the gut microbiome in honey bee colonies. These interventions collectively contribute to maintaining a healthy and functional gut microbiome, essential for the overall health and productivity of honey bee populations.

Looking at the big picture, while genetics undoubtedly plays a role in shaping the structure of the gut microbiome, its influence on the gut microbiome seems minor compared to that of environmental factors. This limited genetic impact, coupled with the challenges in detecting small genetic effects, suggests that strategies focusing on enhancing the gut microbiome—regardless of the bee's genetic background—could be more promising for improving honey bee health. Such interventions may provide a robust approach to bolster bee resilience, transcending the limitations posed by genetic variability.

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## Appendix A: List of Supplementary Tables

All supplementary tables are available in the additional Supplementary Materials file.

**Table S1.** List of primers and sequences used for microsatellite genotyping at 11 loci for patriline assignment.

**Table S2.** Optimized PCR reaction conditions for amplification of target microsatellite loci.

**Table S3.** Poolplex volumes for microsatellite PCR product.

**Table S4.** Allele table for 250 diploid honey bee workers in Colony A12 determined by fragment analysis at 10 microsatellite loci.

**Table S5.** Allele table for 250 diploid honey bee workers in Colony A18 determined by fragment analysis at 10 microsatellite loci.

**Table S6.** Haplotype distribution across offspring for 18 paternal lineages in Colony A12.

**Table S7.** Haplotype distribution across offspring for 23 paternal lineages in Colony A18.

**Table S8.** List of primers and unique molecular identifiers (UMIs) used of V4 amplification and multiplexing of 250 honey bee workers per colony for 16s rRNA gene sequencing.

**Table S9.** List of 38 bacterial families and 66 bacterial genera identified across 500 honey bee hindguts from 16s rRNA gene sequencing.

**Table S10.** Average relative abundance of bacterial genera in 28 patriline across 2 colonies.

**Table S11.** Average relative abundance of bacterial families in 28 patriline across 2 colonies.

**Table S12.** Broad sense heritability of Shannon diversity of bacterial genera and families in honey bees from two colonies.

## **Appendix B: List of Supplementary Figures**

All supplementary figures are available in the additional Supplementary Materials file.

**Figure S1.** Offspring distribution of 500 genotyped worker bees across patriline in two honey bee colonies.