**Methods**

*DNA extraction and library preparation*

Bee bread microbiome samples were removed from pipette tips and homogenized using a flame-sterilized metal rod and spatula. A total of 0.05 g ± 0.008 g of bee bread was transferred to a ZR BashingBead Lysis Tube (Zymo Research, Irvine, CA, USA). We extracted DNA using the Zymo Quick-DNA Fecal/Soil Microbe Microprep Kit applying the standard non-soil sample protocol. DNA samples were eluted in 20 µL of sterile water and frozen at -20° C until preparation of amplicon libraries.

The V4 region of the bacterial 16S rRNA gene was amplified using the 515-F (GTGCCAGCMGCCGCGGTAA) (1, 2) and 806-R (GGACTACHVGGGTWTCTAAT) (3) primer pair. Primers included both unique sample barcodes and Illumina adapters. DNA was amplified in a 12.5 µL reaction containing 6.25 µL PCR master mix containing Taq polymerase, 0.5 µL 515F primer, 0.5 µL 806R primer, 4.75 µL MilliQ water, and 1.00 µL sample DNA. PCR cycling conditions were 94°C for 5 min followed by 35 cycles of 94°C for 45 seconds, 50°C for 60 seconds, and 72°C for 90 seconds and a final extension step of 72°C at 10 min. PCR products were then checked on a 1% agarose gel, stained with SYBR Green Nucleic Acid Stain. Bands of target DNA were visualized under UV-light. Equimolar libraries were sequenced on Illumina’s MISEQ platform using V3 chemistry (2 x 250 PE) at the University of Colorado Next Generation Sequencing Facility (Boulder, Colorado).

*Sequence data processing*

Amplicon sequence data were processed in R using the DADA2 pipeline (4). Sequences were first demultiplexed using idemp. Next, primers were removed using Cutadapt (v. 3.7) (5). Trimmed reads were then passed to DADA2 for quality filtering, error learning, dereplication, merging, and chimera removal. Briefly, the following quality filtering parameters were used: trunQ = 11, maxEE = c(1,1), and truncLen = c(150,140). All base pairs were used to learn errors (nbases =1 x 109), and sequences were merged with a minimum overlap of 10 base pairs (bp) and zero mismatches. Chimeras were then removed using the “consensus” method of the removeBimeraDenovo() function. Sequences passing through our processing pipeline were then subjected to a final trimming to include only sequences between 245 bp and 258 bp. Taxonomy was assigned using the Silva V138 database (6). ASVs with taxonomic assignments of non-bacteria, unassigned at the kingdom level, chloroplasts, or mitochondria were removed for all downstream analyses. Phylogenetic trees were built using the DECIPHER and PHANGORN packages in R. The ASV table, sample metadata, taxonomic information, and phylogenetic tree were then passed to Phyloseq for visualization and statistical testing (7).Rarefied abundances were used for calculating α-diversity metrics and a core microbiome. All other analyses were performed using within-sample proportional abundances, calculated as sum the of reads assigned to each taxon divided by the total reads for that sample.

*Statistical analyses of sequence data*

After verifying the assumptions, ANOVA was used to assess differences in α-diversity (Y~Sampling location\*Miticide treatment\*Sampling timepoint). For all statistical testing, we report α = 0.05 for statistical significance and α = 0.1 for marginal significance. Patterns in β-diversity were assessed with both weighted and unweighted unifrac distances, using the adonis function of the vegan package (8) (Ydist ~ Sampling location\*Miticide treatment\*Sampling timepoint). β-diversity patterns were then visualized using NMDS.

*Core microbiome assignments*

A core bee bread microbiome was assigned by summarizing the rarefied taxon table at the genus level using the phyloseq package (7). Next, we assigned core membership using the taxa\_core() function, found in the phylosmith package. We defined core membership based upon the following criteria. A taxon must be: 1) present in 100% of sampling locations, 2) present in at least 50% of samples within each sampling location, and 3) have a relative abundance of greater than 0.01% within each sample. Patterns of β-diversity in the core were assessed using the same statistical approaches and visualizations as for the entire dataset.

**Results**

*Summary of sequence data pre-processing*

Following pre-processing, 76.9% of total raw reads were retained, for a mean sample read depth of 8,020. ASV assignment using DADA2 produced 1,337 ASVs. After rarefaction to 1,100 reads, the dataset consisted of 851 ASVs. Rarefaction at 1,100 reads sufficiently captured richness as demonstrated in by plots of rarefaction curves (Figure S1). For subsequent analyses, we report 69 independent samples in the rarefied dataset and 72 for the within-sample proportional abundances dataset.

*Alpha (α) diversity*

ANOVA showed sampling location to be a significant predictor of species richness, Shannon diversity (H`), and Chao1 (p < 0.05, F < 2.702) (Figure S2). The interaction of sampling timepoint \* sampling location was a significant predictor of species richness and Chao1 (p < 0.05, F < 2.871), while sampling location \* miticide treatment was significant for Shannon diversity (H`) (p < 0.05, F < 2.718). No clear trends in directionality were observed for α-diversity metrics across sampling locations, miticide treatments, or sampling timepoints.

*Beta (β) diversity*

To account for variation within each sampling location, strata = “Sampling Location” was used to restrict permutations within each site. We report sampling location to be the dominate predictor of β-diversity for both weighted and unweighted unifrac distances (p < 0.001, R2 = 0.31, F5,36 = 8.149, p < 0.001, R2 = 0.15, F5,36 = 2.768, respectively) (Figure 2 A and B, Table S1). Additionally, sampling time was significant for both weighted and unweighted distances (p < 0.05, R2 = 0.03, F1,36 = 4.256, p < 0.001, R2 = 0.03, F1,36 = 2.950, respectively). Miticide treatment was found to be significant for unweighted unifrac distance (p < 0.05, R2 = 0.029, F2,36 = 1.269), but not for weighted unifrac (p = 0.34). We report no statistically significant pairwise differences among miticide treatments when both time points were considered together.

To better understand the effect of miticide treatment on the bee bread microbiome, we subset our dataset to include only those samples collected after application (e.g., sampling time two). Again, “strata = Sampling Location” was used. Besides sampling location, we only report a marginally significant interaction of sampling location x miticide treatment for unweighted unifrac (p = 0.056, R2 = 0.28, F10,18 = 1.139) (Table S1). As per pairwise PERMANOVA testing, miticide treatment was shown to be a marginally significant predictor of community differences in the CON\_and\_ORG hives.

*Core bee bread microbiome*

Core membership assigned at the genus level showed 14 genera that met our thresholds of abundance and prevalence. While representing only 4.3% of the total genera present in our dataset, the assigned core microbiome accounted for 85.7% of the total reads (Figure 3). PERMANOVA testing showed sampling location to remain the dominant predictor of community composition even within the core (unweighted: p < 0.01, R2 = 0.20, F5,34 = 4.733), though this was only marginally significant for weighted unifrac (p =0.07, R2 = 0.35, F5,34 = 8.824) (Table S1). Additionally, the interaction terms of sampling location x miticide treatment (weighted: p = 0.052, R2 = 0.15, F10,34 = 1.905, unweighted: p < 0.05, R2 = 0.17, F10,34 = 1.917) and sampling location x sampling timepoint (weighted: p < 0.001, R2 = 0.13, F5,34 = 3.318, unweighted: p < 0.001, R2 = 0.16, F5,34 3.659) were also statistically significant predictors of beta-diversity in the core microbiome.

Figures:

Figure 1. Descriptive map of sampling locations. CAPTION HERE.

Diagram

Description automatically generated

Figure 2. (Un)Weighted unifrac ordinations of bee bread microbiome composition. Panels A and B represent the entire dataset. Panels C and D represent our assigned core microbiome. Bold faced text indicates statistical significance (p < 0.05) as per PERMANOVA.

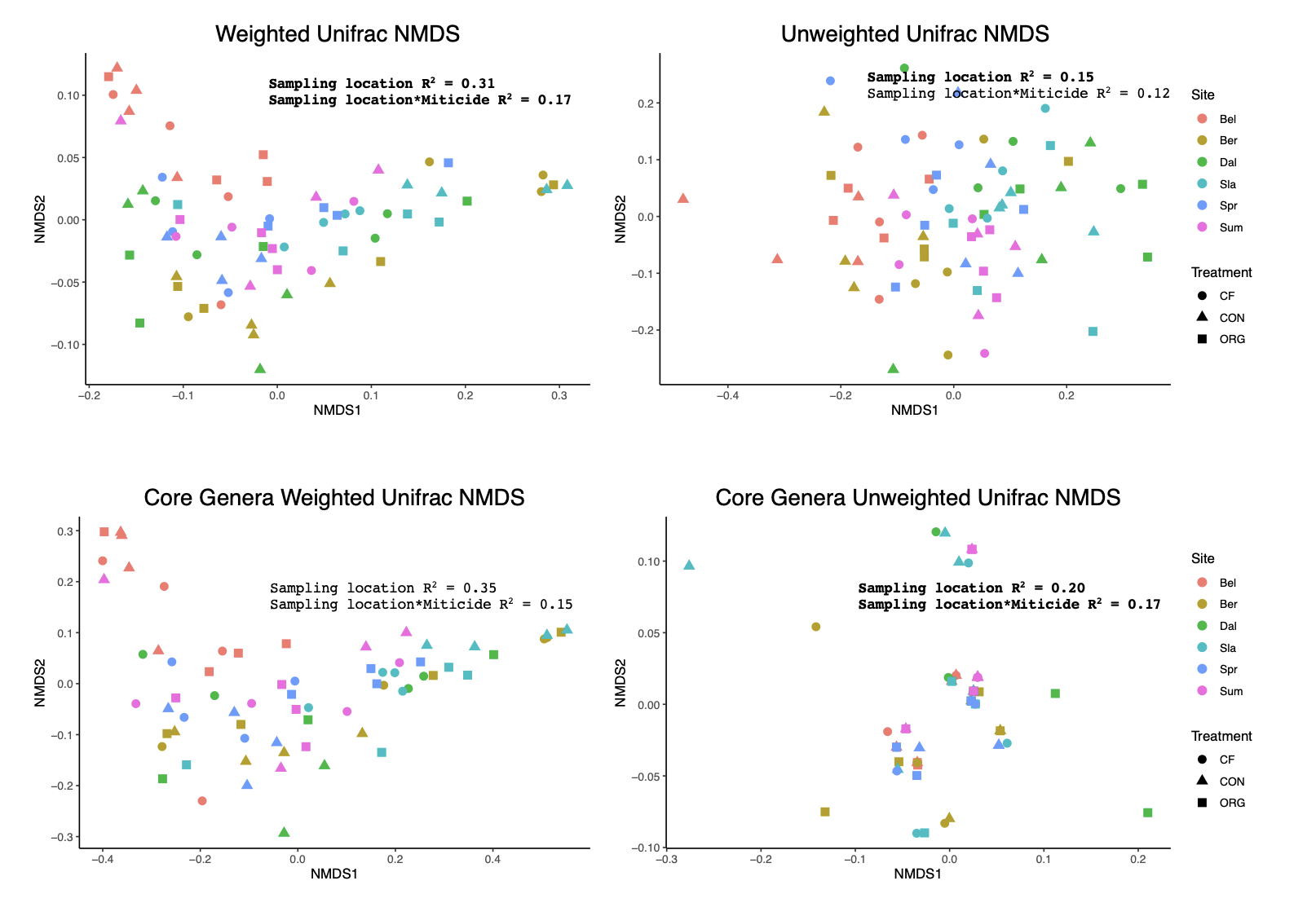


Figure 3. Double bar plot showing abundance and prevalence distribution of core and non-core taxa. Bars above the black line at 0 indicate the taxon prevalence across sites. Bars below the black 0 line indicate the proportional abundance of total reads. Bars are colored by core inclusion status, with salmon bars denoting core (14 taxa) and teal bars denoting non-core (311 taxa).

Chart

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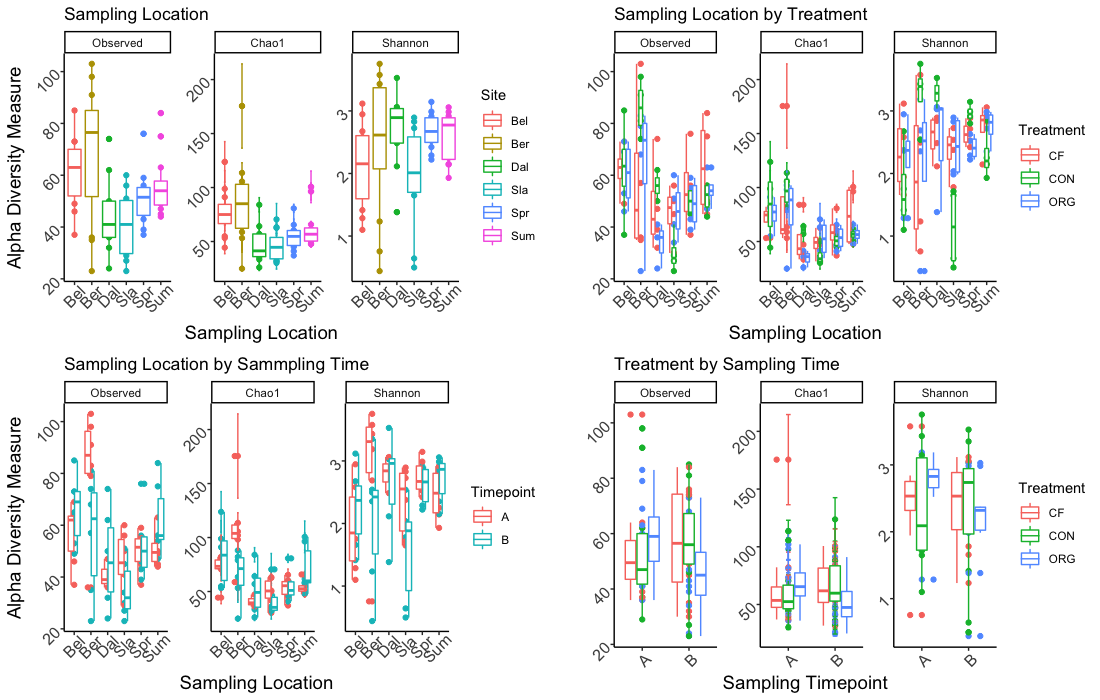
Supplementary Figures:

SFigure1. Rarefaction curves depicting leveling off at approximately 1,000 reads. Samples were rarified at 1,100 reads.

A picture containing diagram

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SFigure2. Boxplots of alpha diversity metrics (Observed number of taxa, Chao1, and Shannon diversity (H`)). Horizontal line inside box represents median. Top of box indicates 75th quartile. Bottom of box indicates 25th quartile. Whiskers represent 1.5 x IQR.

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