"Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees"
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Introduction

The composition of animal gut microbiomes is important for maintaining host health. Previous studies demonstrated that antibiotic exposure could allow infection by decreasing presence of native gut community members, allowing pathogen propagation. Infection following antibiotic treatment had not been extensively studied in bees, an organism that is often treated with antibiotics by beekeepers to prevent infection. To study the impact of antibiotics on the honeybee gut microbiome, the authors compared the size and composition of the gut community of tetracycline exposed treatment bees to control bees at time intervals of 0, 3, 5, and 7 days after antibiotic treatment. Survivorship was monitored after reintroduction to the hive to see what effect antibiotic exposure had on longevity. Infection experiments using the pathogenic bacterial strain *Serratia* kz11 were performed on control and tetracycline-treated bees to determine whether susceptibility to opportunistic pathogens was increased following antibiotic treatment, and how mortality differed between groups.

Bees are a useful model organism for understanding host-microbe interactions. Bees acquire their microbiota through social contact, like mammalian species. Antibiotic resistance occurs in pathogens that infect bees, a rising issue in human pathogens as well, due to long-term antibiotic use. The bee gut microbiome is relatively simple and is made up of eight core species that make up 95-99% of the gut bacteria. This makes close monitoring of fluctuations in the gut microbiome feasible in a laboratory setting, so antibiotic-induced changes can be identified accurately. Bees are an incredibly important species as global pollinators, and bee populations are declining, so understanding the role of the bee gut microbiome in maintaining host health and the effects of antibiotic use on longevity is important in conservation efforts, in addition to the relevance of this research to human microbiome studies.

The authors used PCR and deep amplicon sequencing of 16s rRNA genes to assess the community size and composition of the gut microbiota. The relative and absolute abundances of core species of the gut microbiomes are reported for control and treatment bees, at each time point sampled post-treatment, in excel file S2 Data (s2.xlsx), with sheet 1 giving relative abundances and sheet 2 giving absolute abundances. Shannon's H index was used to measure alpha diversity within each sample, and Bray-Curtis dissimilarity to measure beta diversity between all samples. The results from these analyses are given in excel file S3 Data (s3.xlsx), with sheet 1 giving the Shannon H values for each bee at each time point sampled, and sheet 2 giving the Bray-Curtis dissimilarity matrix. This file additionally includes unweighted and weighted UniFrac distance matrices (a measure of beta diversity, on sheet 3 and sheet 5, respectively), and results of principal coordinate analysis (PCoA) on the unweighted and weighted UniFrac distances (sheets 4 and 6, respectively), including proportion explained and eigvals. The S3 Data contains all the data used to produce Figure 3. To replicate this figure, I used the S2 Data to independently produce alpha and beta diversity data for Figure 3A and 3B and performed PCoA on the UniFrac distance matrices to generate the data needed for Figure 3C and 3D.

Age-controlled (hatched on the same day) and non-age-controlled bees from control and tetracycline-treated groups were exposed to the pathogenic bacterial strain *Serratia* kz11 over two days. The survival counts for age-controlled bees are reported in S7 Data (s7.xlsx), and survival counts for non-age-controlled bees are reported in S8 Data (s8.xlsx). A follow-up bacterial challenge to confirm that *Serratia* is an opportunistic pathogen was performed. Control and treatment bees were exposed to either *Serratia* kz11, *E. coli* K-12 (a non-pathogenic lab strain), *S. alvi* wkB2, or *Lactobactillus* sp. wkB8 (members of the core bee gut microbiome). The survival counts for these bees are found in the S10 Data (s10.xlsx).

Data Analysis and Python Code

To perform the alpha and beta diversity analyses used in this study, the authors used the QIIME workflow core_diversity_analysis.py, with default parameters (pg. 14). Since it was not possible to replicate this analysis in the same way using sequencing reads, I used the relative abundance data from the S2 Data to compute Shannon's H index values and Bray-Curtis dissimilarity matrix. I imported the S2 Data sheets as csv

files into pandas data frames, and created a class alpha_beta_div to store the data, with a method (split) to split the data into control and treatment groups, and further separate these data into a dictionary with post-treatment day associated with abundance data. I defined a method (shannon_h) to calculate Shannon's H index for each sample by summing the product of the relative abundance and natural log of the relative abundance for each gut species and multiplying this value by -1. I defined a method (bray_curtis_calc) which used the pdist function with argument metric = "braycurtis" from scipy.spatial.distance to create a Bray-Curits dissimilarity matrix. Wilcoxon rank sum tests were performed for control vs. treatment groups on each post-treatment day using the scipy.stats.ranksums function. The results from these two analyses, split by control and treatment groups, were plotted using the matplotlib.pyplot.boxplot function, further separated by post-treatment day. Plot details were customized to match the plots in Figure 3 as closely as possible.

I used the weighted and unweighted UniFrac distance matrices provided in the S3 Data file to perform PCoA analyses for subplots C and D. UniFrac is a distance metric used to compare similarity of communities based on phylogenetic relatedness. Since the UniFrac distance matrix was generated using the 16s rRNA reads, I couldn't reproduce this part of the analysis, but I used the pcoa function from skbio.stats.ordination to perform PCoA on the weighted and unweighted UniFrac matrices from the S3 Data sheets, which I imported as pandas DataFrames from csv files. I defined a function map_index which returns a list mapping the subgroups of the plot, and another function plot_pcoa_groups which plotted the points based on index map groups, so the markers would match the colors and shapes of the plots in Figure 3C and 3D, and sub-groups of data could be differentially labeled. I plotted PC1 on the x-axis and PC2 on the y-axis using matplotlib.pyplot.scatter, and used the proportion_explained attribute of my PCoA results to add the proportion explained by each principal coordinate to my axis labels. Plot details were customized to match the plots in Figure 3 as closely as possible.

To plot Figure 5, I used the S7 and S8 Data. This figure displays survivorship counts (A, C) and percentages (B, D) for age-controlled (A, B) and non-age-controlled (C, D) control treatment bees over 10 days. Treatments for this figure include tetracycline and *Serratia*. Figure 6 uses the S10 Data and displays survivorship counts (A) and percentages (B) over 10 days for control and tetracycline-treated bees exposed to four different bacterial strains, including *Serratia*. For each excel file, I imported the data as a pandas DataFrame. I defined a class called workers_group to separate the DataFrame by the treatment groups listed in the column "Treatment" and create an object of class treatment_group for each of these groups. The class treatment_group further split the groups by column "Cup" and computed survivorship counts and percentages of bees remaining in each cup for 10 days following treatment. The counts were calculated by summing the "Start" column and subtracting bees from the preceding day's count based on the number of "Stop" column values for that day. I stored this data in a numpy array. The array values were divided by the number of bees on day 1 to compute the survival percentage for each day. For each treatment group, the mean of survival counts and percentages of all cups was calculated and plotted as a line, with counts and percentages from each cup plotted as points in the same color. These plots were made using methods plot_count and plot_survival in my workers_group class, which called the functions matplotlib.pyplot.plot and matplotlib.pyplot.scatter.

Results, Figures, and Conclusions

Figure 3 compares the differences in gut microbiome diversity in control bees versus tetracycline-treated bees at four time points during a week post-treatment. 3A presents alpha diversity (within individuals), measured using Shannon's H index. Alpha diversity is not significantly different between groups on day 0 but is lower in tetracycline-treated bees compared to control bees for all other days post-treatment ($p < 0.0001$ for days 3 and 5), and after a week is not recovered to the original level of diversity ($p < 0.05$ for day 7). H values are more varied in the treatment group than the control group following tetracycline exposure, with a greater range between minimum and maximum values. 3B displays beta diversity (diversity between individuals), measured using Bray-Curtis dissimilarity. Beta diversity values are not significantly different between groups on day 0 but is significantly lower in control bees compared to treatment bees for all other days following antibiotic treatment ($p < 0.0001$). PCoA reveals that gut microbiota community composition for control bees

on all days and treatment bees on day 0 is tightly clustered, and comparatively dispersed for treatment bees on all other post-treatment days (3C and 3D). Since honeybees have simple gut microbiomes, the dispersed community compositions seen in 3C and 3D indicate a great extent of disruption caused by antibiotics on the microbiome, and this disruption remains a week after exposure. The results of my analysis for Figure 3A and 3B differed from the values in the S3 Data for Shannon's H values and Bray-Curtis dissimilarity because my analysis was based on relative abundances, while the S3 analysis was run directly on the sequencing reads. However, the p-values from my Wilcoxon rank sum tests were significant at the same levels as the p-values in 3A and 3B between treatment and control groups on all days. So, although my plots have different values than the plots in 3A and 3B, the trends in alpha and beta diversity of control and treatment groups over time remained consistent and between-group differences were significant at the same levels in my analysis as the original analysis. This analysis demonstrates the substantial impact of antibiotic treatment on gut microbiome diversity and highlights the long-lasting changes in gut community composition following antibiotic exposure.

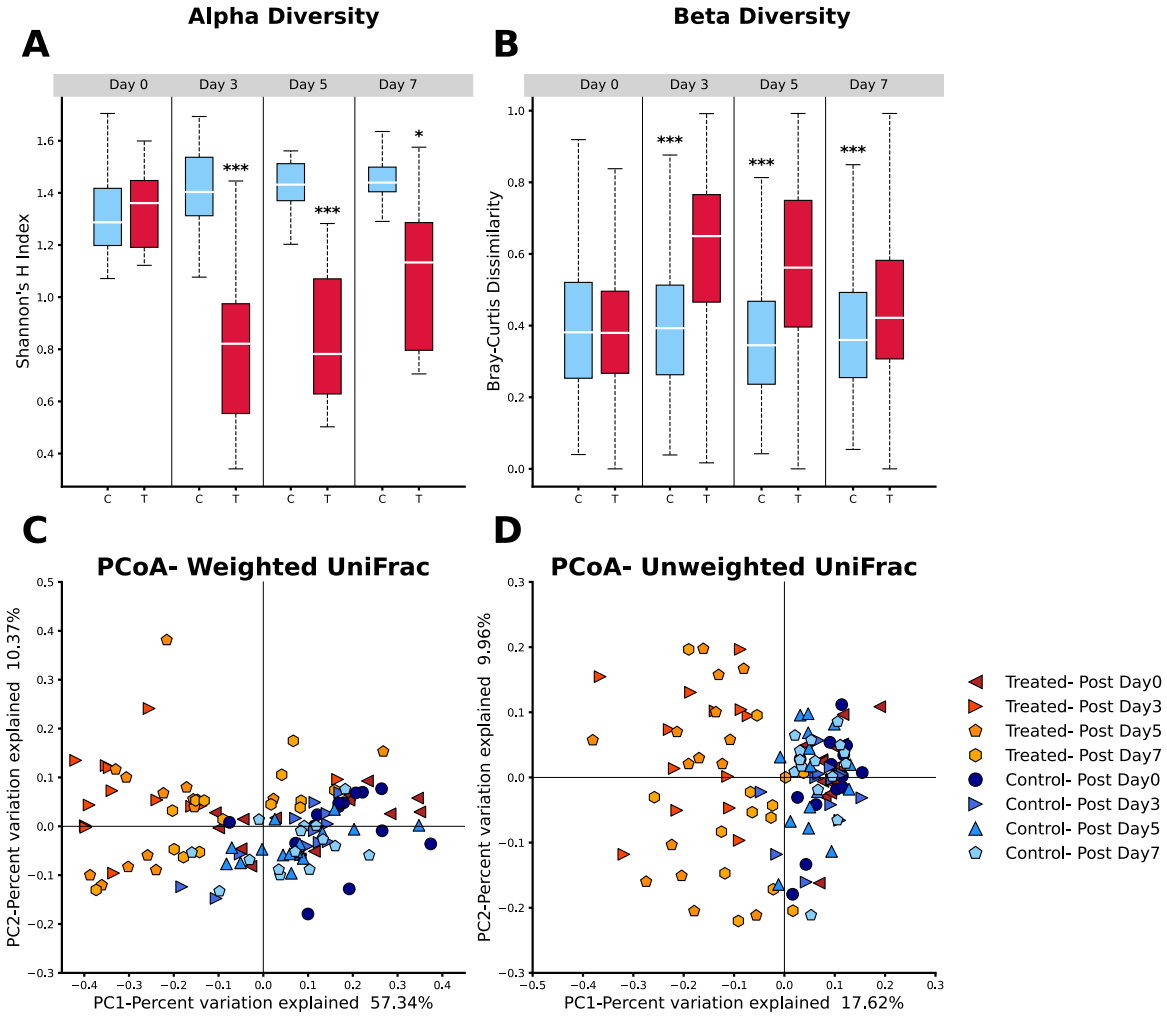

Figure 3.

Figure 5 compared the survivorship between control and tetracycline-treated bees following treatment, as well as for control and treatment bees exposed to *Serratia* kz11. The survivorship of control bees not exposed to tetracycline or *Serratia* was highest of all groups over the 10 days following treatment, and the bees exposed to both antibiotic and *Serratia* had the lowest survival of the groups on each day after day 0. This experiment demonstrates that the reduction in gut microbiome size and diversity resulting from antibiotic exposure can allow infection by opportunistic pathogens, causing increased mortality compared to individuals

exposed to the pathogen alone, the antibiotic alone, or neither. Figure 6 verifies that *Serratia* kz11 is an opportunistic pathogen, following the same method as the experiment in Figure 5 but with the addition of exposure to two core gut microbiome species (*S. alvi* wkB2 and *Lactobactillus* sp. wkB8) and a non-pathogenic *E. coli* strain. The survivorship of all control and treatment bees not exposed to *Serratia* is similar, with control bees not exposed to any bacteria having the highest survival of all groups by Day 10, with bees treated with tetracycline and exposed to *Serratia* having lower survival than all other groups, and control bees exposed to *Serratia* having the second lowest survival. This experiment supports the results from Figure 5, demonstrating that a combination of antibiotic treatment and pathogen exposure results in considerably higher mortality than pathogen exposure or antibiotic treatment alone. This is an especially important finding given that beekeepers often use antibiotic treatment in an attempt to prevent infection by pathogens, but this practice may just cause bees to become more susceptible to opportunistic pathogens, resulting in higher overall mortality in a hive.

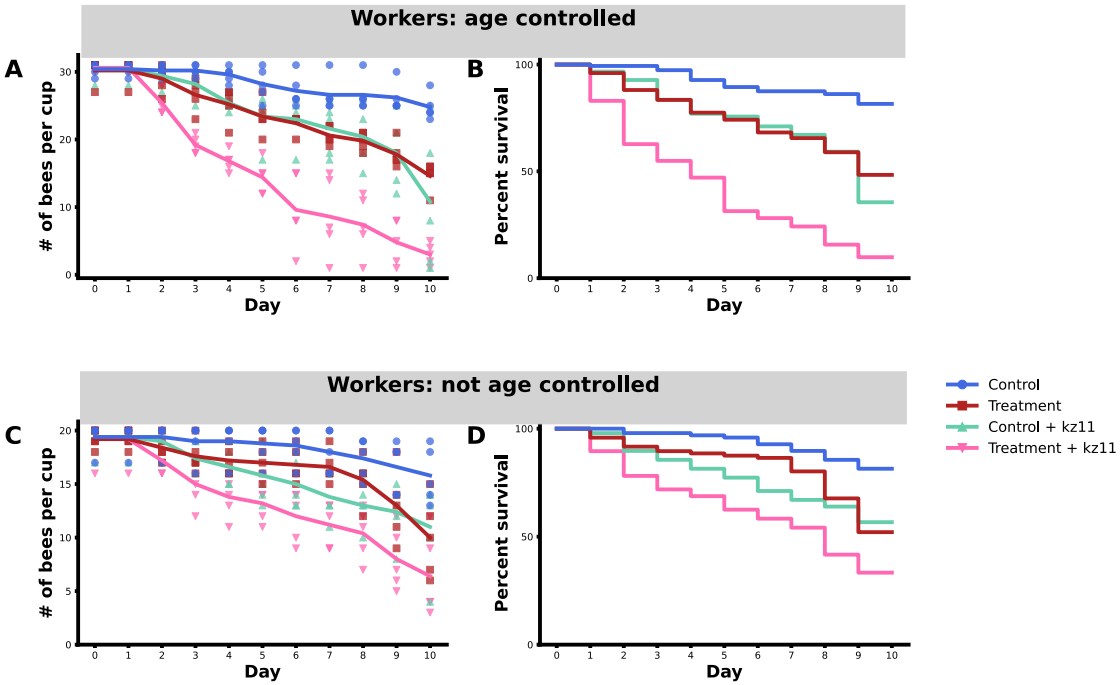

Figure 5.

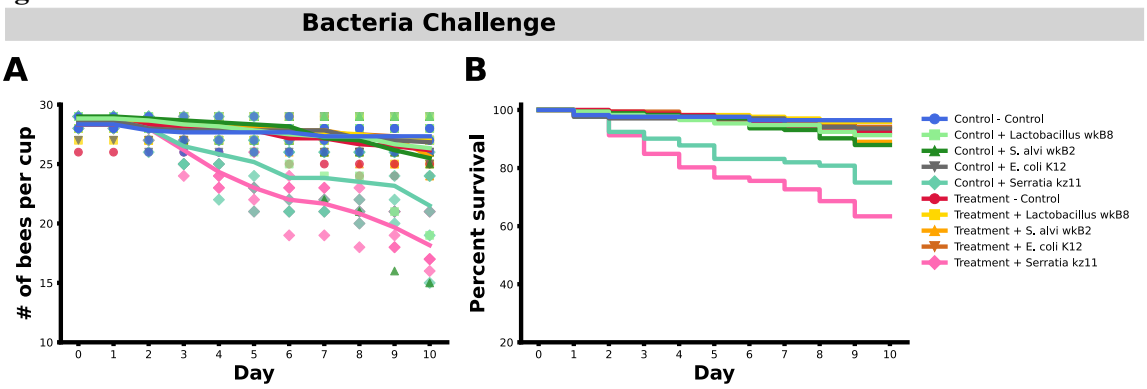

Figure 6.

Overall, this study demonstrates the lasting consequences of antibiotic treatment, and validates the risk of opportunistic pathogen infection during dysbiosis of gut microbiome communities. The results of this study provide important considerations for beekeeping practices and indicate the importance of studying the impacts of frequent antibiotic use on more complex gut communities, such as the human gut microbiome.