THE PENNSYLVANIA STATE UNIVERSITY

THE SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Is there a Trade-off? Impacts of Antibiotic Treatments on Gut Microbiota and Immune Gene Expression in Honey Bees (*Apis Mellifera*)

NICHOLAS TOMASKO
SPRING 2023

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biochemistry and Molecular Biology
with honors in Biochemistry and Molecular Biology

Reviewed and approved* by the following:

Margarita López-Uribe, Ph.D.
Lorenzo L. Langstroth Early Career Professor
Associate Professor of Entomology
Thesis Supervisor

Joseph Reese, Ph.D.
Professor of Biochemistry and Molecular Biology Honors Advisor

*Signatures are on file in the Schreyer Honors College.

ABSTRACT

Antibiotics serve as fundamental treatments for bacterial infections in all organisms. However, these treatments can also deplete beneficial microbes that maintain immune homeostasis. Considering the links between beneficial microbes and immunity, it is critical to understand whether changes in the microbiome can reduce the individual's ability to fight infection and decrease the long-term effectiveness of antibiotic treatments. Here, we used honey bees (*Apis mellifera*) to study the immunological effects of antibiotics. Honey bees are often treated with antibiotics when infected with devastating bacterial pathogens, such as *Paenibacillus larvae*: the causative agent of American foulbrood. To quantify the consequences of antibiotic treatment for immune function, we fed honey bees oxytetracycline followed by an immune challenge of heat-killed *Escherichia coli* (*E. coli*) to characterize changes in key gut bacteria and test for immunosuppression effects. We collected honey bees from five colonies and treated them in a laboratory incubator over 10 days. We then quantified the relative expression of three immune genes and quantified key groups of gut bacteria through qRT-PCR to assess changes in immune gene expression and gut microbiota abundance after each treatment. Antibiotic treatments caused general depletion in the honey bee microbial communities and a corresponding weak downregulation in AMPs, with the combined oxytetracycline and heat-killed *E. coli* treatment inducing a microbial rebound reflective of a typical immune response. Heat-killed *E. coli* treatments did not induce a significant response in immune gene expression. We also did not find significant survival differences between treatments.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

OTC	Oxytetracycline
AMPs	Antimicrobial Peptides
S. alvi	*Snodgrassella Alvi*
E. coli	*Escherichia coli*
HK	Heat-Killed
HKA	Heat-Killed *E. coli* and Antibiotic Treatment
ATB/AB	Antibiotic
w/v	Weight by volume
DWV	Deformed Wing Virus
Ef-1 alpha	Elongation Factor 1-alpha
RPS5	40S Ribosomal Protein S5
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
S. marcescens	*Serratia marcescens*
Bifido	*Bifidobacterium*
Lacto	*Lactobacillus* Firm-5
Apid	*Apidaecin*
Def-2	*Defensin-2*
Hym	*Hymenoptaecin*

ACKNOWLEDGEMENTS

I want to first thank Dr. Margarita López-Uribe for making this research possible. I was a reserved undergraduate student interested in research at a time of such incredible uncertainty, yet Dr. López-Uribe graciously went out of her way to provide me with an opportunity to become involved in her lab, and I never looked back. Her guidance and leadership assisted in organizing this project and ensuring its completion. The final product is a representation of her input in formulating the study and providing substantial editing efforts to refine my writing. Additionally, the López-Uribe lab is an exceptional group of researchers who were always eager to share tips and help me along the way. I am honored to be part of the lab as I continue to grow my knowledge of pollinators and hopefully hand off my work to another member who can take my explorations to greater heights.

I also want to thank my mentor, Chauncy Hinshaw, who provided me with a foundational knowledge of all lab techniques and strategies utilized in this study. Chauncy was fundamental in generating these experiments and helping organize a project that both adhered to my interests and provided results significant to the pollinator world. Chauncy not only led our bee collection efforts but also constructed all figures illustrated in this study. He also provided longitudinal emotional support during the extensive troubleshooting periods of our experiments. His contributions are what fuel this project and give us the opportunity to share our work with the public. I am forever grateful for Chauncy and his mentorship throughout my time at Penn State – from counting bees in the field at the crack of dawn to reviewing my near-impossible microbiology homework – his guidance was instrumental in helping me realize my passion for research and subsequently traverse my windy career journey.

I want to thank Dr. Joseph Reese for supplying the necessary expertise throughout the thesis writing process, as well as plenty of career advice as I navigated my undergraduate experience. His knowledge – coupled with the support from fellow BMB faculty members and the Schreyer Honors College – was significant in completing this thesis.

Finally, I want to thank my family and friends for their endless love and support. I would not be anywhere close to where I am at today without their constant presence and tremendous advice as I continue embracing new challenges in both healthcare and science.

FUNDING

None of my research would have been possible without the financial support provided by the Apes Valentes Undergraduate Student Award. I want to thank Ms. Mychelle Hunter for providing me with this incredible award, as well as Dr. Heather Hines and Susan Bass for assisting in the funding process. I also want to thank Audrey Harrod and Chauncy Hinshaw for taking care of the logistics required to make all purchases.

Additionally, I had the chance to attend the American Bee Research Conference this year in Jacksonville, Florida to present my ongoing project. Participating in this conference would not have been possible without financial assistance from both the Eberly College of Science and the Schreyer Honors College. I am grateful for their help and the support from Penn State to broadcast my research to beekeepers and fellow researchers who shared countless pieces of advice.

Chapter 1

Introduction

The western honey bee (*Apis mellifera*; hereafter honey bee) plays a significant ecological role as a pollinator of natural plants and managed crops. Our nationwide agricultural sector relies heavily on pollination for assisting in crop production and ensuring high yields for all cultivated plants. Bees function as particularly effective pollinators due to their global presence, many foragers per colony, and relatively simple management. In fact, bee-pollinated crops are responsible for supplying the most nutritious foods to the human diet (Khalifa et al., 2021). Honey bees have especially high value as crop pollinators due to their foraging behavior and ability to pollinate various plant species (Hung et al., 2018). Due to recent downward trends in global bee populations, optimizations in beekeeping and pathogen treatment for honey bees have the potential for large impacts on human well-being and overall sustainable development (Patel et al., 2021). Since honey bees contribute to the sustainability of our cropping systems, prioritizing their health is paramount.

In humans, the gut microbiome functions fundamentally in immune homeostasis by inducing innate and adaptive response mechanisms. Considering this synergism between humans and their gut microbes, alterations in our microbiome unsurprisingly shift typical immune responses and even contribute to the prevalence of some autoimmune disorders (Wu & Wu, 2012). Gut microbes not only protect our bodies from pathogens but also aid in the acquisition of nutrients and metabolites through vital host-microbe interactions. However, incomplete knowledge of gut microbiome function continues to leave research gaps concerning the true implications of large disturbances to gut microbial communities (Lozupone et al., 2012). Thus,

microbiome research becomes more accessible in model systems like honey bees, where eight prevalent microbiota species provide a conserved and relatively simple environment for study (Moran 2015). The honey bee microbiome also presents strong connections to the human microbiome through similarities in their biology, including the acquisition of microbes via social contact and the specialization of bacterial species within hosts (Zheng et al., 2018; Raymann et al., 2017; Tian et al., 2012). Studying microbiome regulation processes in honey bees provides an experimental advantage not possible in humans, leading to insights that can benefit the health of both organisms.

A significant threat to honey bee health exists in the variety of bacterial infections that may attack individuals and quickly spread through the ecology. To treat these infections, beekeepers often use one of the three approved antibiotics: oxytetracycline, tylosin, or lincomycin (Cripps 2021). One of the most lethal infections, American Foulbrood (AFB), is caused by the gram-positive bacterium *Paenibacillus larvae* (Ortiz-Alvarado et al., 2020). This highly contagious infection spreads quickly; killing larvae and inflicting significant losses for beekeepers. Oxytetracycline (OTC) serves as one of the common methods to control the spread of infection and salvage colony health (Genersch 2010). However, antibiotic treatments bring alternative consequences: changes in honey bee lipid production, altered behavioral development, and gut microbiome depletion (Ortiz-Alvarado et al., 2020; Raymann et al., 2017). The implications for reduced microbiome diversity and abundance pose questions concerning the downstream immune effects of honey bees lacking stable microbiomes in their guts. Previous research demonstrates how long-term tetracycline exposure decreases bacterial strain diversity and induces the production of resistance loci (Tian et al., 2012). In a study by Raymann et al. (2017), honey bees treated with tetracycline exhibited increased mortality as a result of

microbiome depletion and further mortality after exposure to a subsequent bacterial pathogen. Considering these immunity-related effects of antibiotics – as well as the prospect of antibiotic-resistant bacteria that exacerbate infection – it is essential to understand possible health trade-offs in using antibiotic treatments.

The honey bee microbiome assists the bees' metabolism of many nutrients and pollen components, as well as immune and hormone signaling pathways that formulate an overall symbiotic relationship with their host (Kešnerová et al., 2017). Exposing honey bees to high doses of antibiotics can not only lower resident bacteria levels within the honey bee microbiome but also lead to subsequent decreases in antimicrobial peptides (AMPs) (Duan et al., 2021; Motta et al., 2022). AMPs are often measurements for immune competency in honey bees, as their upregulation serves as a reference for an inducible innate immune response. Several studies have revealed how lowering gut microbial levels produces a cascade of downregulation in AMPs from the Toll or RNA interference pathway, which ultimately decreases honey bee fitness (Duan et al., 2021; Motta et al., 2022; Barrosso-Arévalo et al., 2019). Therefore, we hypothesize there is a 'trade-off' when the downregulation of immune genes in honey bees after antibiotic treatment makes it more arduous to fend off other immune challenges. In other words, honey bees with depleted microbiomes and fewer AMPs at their disposal lack "immune flexibility" (Evans et al., 2006). Stimulating these AMPs can be done via a variety of infections, including heat-killed (HK) bacteria. In the study by Horak et al., 2020, HK *Snodgrassella alvi* (*S. alvi*) and *E. coli* produced large immune responses by upregulating AMPs while also eliminating any confounding variables associated with a live bacterium. Similarly, honey bee larvae showed increases in AMPs both 24 and 48 hours after a live *E. coli* infection, demonstrating a robust immune response (Gätschenberger et al., 2013). These results, along with the study from

Raymann et al. (2017), support a manipulated bacterial infection as a worthy immune challenge that could test the relative strength of the honey bee immune system.

In this study, we employ a common antibiotic treatment of OTC on honey bees and supplement it with an immune challenge of HK *E. coli* (Ortiz-Alvarado et al., 2020; Horak et al., 2020). With this experimental setup, we test how a depleted microbiome interacts with AMPs to shape the honey bee immune response. These results help our understanding of the complex effects of antibiotics on honey bee immunity and potentially reveal patterns in honey bee microbiota dynamics that are present across many other biological systems, including humans. Additionally, this information may be relevant for beekeepers regarding the efficacy of using large antibiotic doses when treating honey bee infections. The experimental design includes controls for both OTC and *E. coli* treatments and an overall treatment-free control that stay consistent throughout the course of the experiment (Figure 2). Based on previous studies, antibiotic consumption has been shown to reduce relative AMP and microbiota levels after 3-9 days of treatment (Raymann et al., 2017; Soares et al., 2021; Daisley et al., 2020; Jia et al., 2022; Duan et al., 2021; Motta et. al, 2022). However, honey bees exposed to HK *E. coli* showed a contradictory spike in AMPs in response to the immune challenge (Gätschenberger et al., 2013; Horak et al., 2020). Thus, the honey bees exposed to a combined treatment were predicted to exhibit AMP and microbiota levels that fall somewhere in between the isolated OTC or *E. coli* exposures, with the combined response being somewhat comparable to the control bees. In the present study, honey bees treated with antibiotics revealed trends within their relative microbiota abundance that suggest their ability to overcome a large antibiotic dose and lower gut microbial diversity. Despite OTC knocking down many key microbes in the honey bee gut microbiome, the immune gene levels were only slightly downregulated. Thus, honey bee AMP levels showcase

weak correlations to their core gut microbes after OTC exposure and require a more robust and potentially lethal immune challenge to induce higher levels of expression.

Chapter 2

Materials and Methods

Bee Collection and Incubation

Honey bees were collected from a local State College (PA, USA) apiary over two days from April-May 2022. A total of 5 colonies were analyzed over 2 rounds of collection. At the apiary, smoke was applied to the brood frames, and falcon tubes with breathing holes were used to scrape nurse bees off active frames. Bees were then chilled on ice and transferred to their respective cages with an estimation that 25-45 bees ended up in each cage. Cages were designed as plastic cups with breathing holes down the sides and around the removable base of the cup (Williams et al., 2015; Evans et al., 2009). Cages with honey bees were then transferred to a Curtis Matheson Scientific, Inc. Equatherm incubator (Thermo Fisher Scientific, Waltham, MA, United States) kept at approximately 30^{o}C. Hive humidity was reproduced in the incubator by putting pipette boxes full of water at its base (Williams et al., 2015). Water levels and incubator temperature were monitored daily.

Antibiotic and Heat-Killed *E. coli* Treatments

To design an efficient oral feeding mechanism, 15mL falcon tubes were punctured with two small holes near the cap and filled with the respective treatment solution. These tubes were then inverted and inserted through the opening of the cup's lid and positioned so that the holes were accessible for feeding (Figure 1). The control solution was made up of 50% sucrose w/v (Kešnerová et al., 2017). The OTC antibiotic treatment solution was prepared at a final concentration of 450µg/mL suspended in 50% sucrose w/v (Raymann et al., 2017). Both final concentrations were achieved by combining 12.5mL of 1800µg/mL of OTC with 37.5mL of

66.67% sucrose. Treatment cages during the 'antibiotic stage' were kept in place for 5 days and sucrose solutions were filled daily to ensure that they would not deplete (Raymann et al., 2017; Soares et al., 2021). One cage was observed to consume around 1mL of solution per day, so 2mL per day was prepared for each respective treatment. After 5 days, the HK and HKA cages received the immune challenge. The HK *E. coli* treatment was prepared by suspending heat-killed cells (InvivoGen, San Diego, CA, United States) in water to create a 10^{10}cells/mL stock, which was transferred to the feeding tube at a final concentration of $5x10^{7}$cells/mL in 50% w/v sucrose. Treatment during the 'heat-killed *E. coli* stage' was terminated after 2 days (Horak et al., 2020). All honey bees were treated with 50% w/v sucrose for the remaining 3 days of incubation (Figure 2). The cages were then removed from the incubator and solution tubes were taken out before freezing all bees at -80^{o}C. Bees were then counted and placed in separate tubes for nucleic acid extraction.

Figure 1. Experimental setup in incubator.

Inverted 15mL Falcon tubes are shown in the center of the cup to allow for optimal feeding. Cup positions were shuffled after daily mortality check-ins and water was monitored in pipette boxes to ensure

consistent humidity. Thermometer in the lower right-hand corner was used to maintain an incubator temperature around 30ºC.

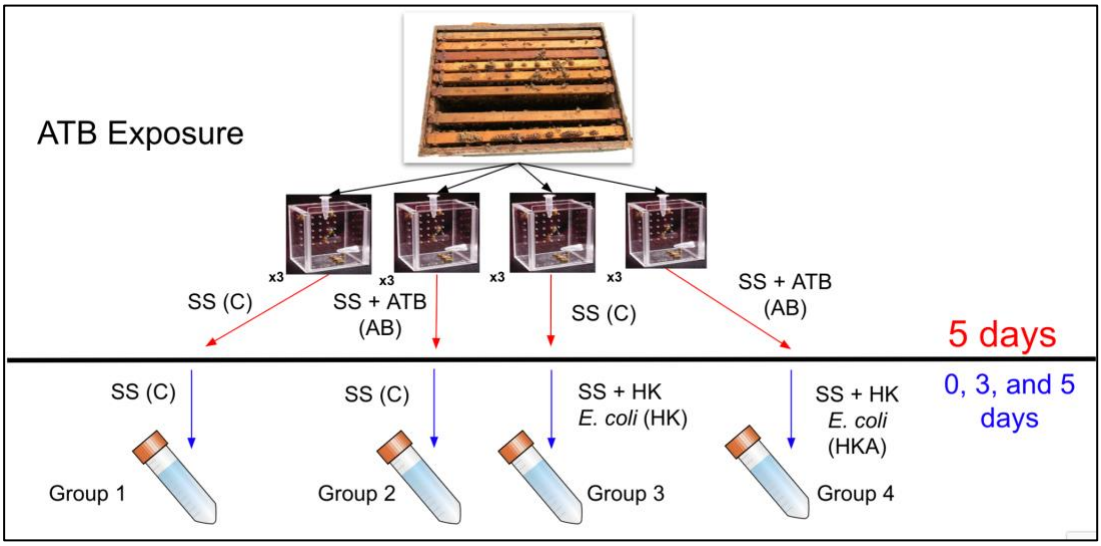

Figure 2. Experimental design of antibiotic and heat-killed *E. coli* treatments.

Treatments were separated into four different groups. Group 1 was a control group fed with only sugar syrup (SS) for 10 days (C). Group 2 was fed sugar syrup with OTC for 5 days followed by sugar syrup for 5 days (AB). Group 3 was fed sugar syrup for 5 days, sugar syrup with HK *E. coli* for 2 days, and then sugar syrup for the final 3 days (HK). Group 4 was fed sugar syrup with OTC for 5 days, sugar syrup with HK *E. coli* for 2 days, and then sugar syrup for the final 3 days (HKA). Honey bees were collected from their cages at 0, 3, and 5 days post-treatment.

Immune Gene and Microbe Selection

We first selected three immune genes to characterize changes in expression: *apidaecin*, *hymenoptaecin*, and *defensin-2*. *Apidaecin* is a proline-rich AMP that presents a unique genomic structure; splicing variation possibly provides its mechanism for pathogen defense (Evans et al., 2006). *Apidaecin* levels also increase in honey bees fed oral treatments of honey bee guts or *Snodgrassella alvi* (Kwong et al., 2017). *Hymenoptaecin* is a unique AMP with a rather high

glycine content and a lack of any proline-rich region. Similar to *apidaecin*, its expression is heavily induced by a honey bee infection, including a live *E. coli* treatment. This response was previously observed to be the result of possible ionic interactions between the basic (+5) *hymenoptaecin* peptide and the negatively charged *E. coli* outer membrane (Casteels et al., 1992). The expression of both *apidaecin* and *hymenoptaecin* has also been shown to upregulate after 5 days of heat-killed *E. coli* treatment (Horak et al., 2020). *Defensin-2*, a cysteine-rich AMP displaying 55.8% similarity to its isoform *Defensin-1*, functions in individual honey bee immunity from its synthesis in fat body cells (Danihlík et al., 2015; Klaudiny et al., 2005; Ilyasov et al., 2013). The peptide has previously displayed upregulation in response to lipopolysaccharide treatment, which is a major component of Gram-negative bacterial cell walls (Richard et al., 2008). We also quantified levels of Deformed Wing Virus (DWV) to obtain a larger picture of the immune interactions taking place in the treated honey bees. DWV is a prominent and widespread pathogen in Western honey bees, and DWV loads modulate immune gene expression (Mookhploy et al., 2021). Thus, quantifying the virus controls the possibility of viral infection superseding the experimental treatments with its effect on honey bee immune competence.

In addition, we selected three gut microbes for microbiome analysis: *Lactobacillus* Firm-5, *Bifidobacterium,* and *Snodgrassella alvi*. All three microbes have been found to be among the eight species that predominate in the guts of worker honey bees (Moran 2015) and observed to deplete significantly within a week of tetracycline treatment (Raymann et al., 2017; Jia et al., 2022). *Lactobacillus* Firm-5 and *Bifidobacterium* are both Gram-positive bacteria that reside in the rectum of adult bee hindguts and function in carbohydrate metabolism (Moran 2015; Lee et al., 2014). On the other hand, *S. alvi* is a Gram-negative bacterial species located in the ileum

wall of the adult hindgut. Extensive studies on *S. alvi* suggest direct correlations with both an upregulation in AMPs and protection against external pathogens, including *Serratia marcescens* (*S. marcescens*). Finally, a Universal 16S rRNA primer set was used to quantify the overall bacterial load, which encompasses all species in the extracted guts (Kešnerová et al., 2017). Considering these prospective functions, all three microbes were specifically chosen due to their roles in assisting honey bee nutrition and possible pathogen defense.

Nucleic Acid Extraction and Preparation

Zymo Research DNA and RNA extraction kits were used for both DNA and RNA extraction (Zymo Research, Irvine, CA, United States). We used forceps to remove the midgut and hindgut of each honeybee for DNA extraction, with the remainder of the abdomen being extracted for RNA. Ten bees from each unique treatment were pooled together in one sample to obtain each nucleic acid (Figure 3). Lysis for all samples was conducted with 18-22 beads and samples were put in a bead-beating sequence at 6.00m/s for 3 cycles at 30 seconds intervals in a BeadBlaster^{TM}24 (Benchmark Scientific, Edison, NJ, United States). The RNA extraction protocol was modified to include 800µL of lysis buffer in the original tube and a longer 3-minute lysate spin-down step. Half of the supernatant was then transferred to the initial filtration column. All centrifugation steps for RNA extraction were completed at 16,000xg and the RNA was eluted using nuclease-free water. The DNA extraction was similarly modified to include 800µL of DNA genomic buffer in the original lysis tube. All centrifugation steps for DNA extraction were completed at 10,000xg and DNA was eluted with manufacturer-provided DNA elution buffer from Zymo Research. RNA and DNA concentrations were quantified using the SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, United States). All eluted nucleic acid was stored at -80^{o}C. RNA extractions from all 55 samples were

then converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, United States). Final reaction volumes include a 10µL final volume of the following manufacturer reagents: 2.0µL of 10X RT buffer, 0.8µL of 25X dNTP mix, 2.0µL of 10X RT random primers, 1.0 of MultiScribe™ Reverse Transcriptase, and 4.2µL of nuclease-free water. These reagents were combined with 10 µL of RNA in each RT reaction to obtain an eventual cDNA concentration of 50ng/µL (Applied Biosystems).

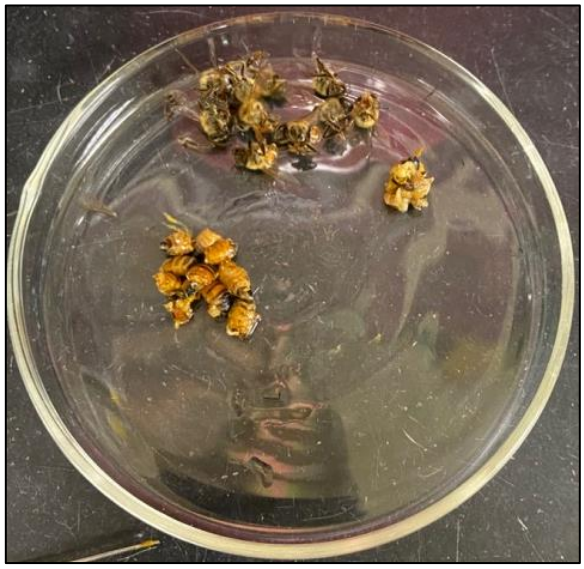

Figure 3. Simultaneous RNA and DNA extraction example.

Honey bee hindguts and midguts were first extracted via forceps from the posterior abdomen and compiled for DNA extraction (upper right). The remaining fat bodies and abdominal tissue were compiled for RNA extraction (middle left).

qRT-PCR Relative Quantification

The relative expression of *apidaecin, hymenoptaecin, defensin-2,* and DWV was quantified for each cDNA sample. The relative abundance of *Lactobacillus* Firm-5*, Bifidobacterium, S. alvi,* and total bacteria was then quantified for each gDNA sample. All bacterial and immune gene primers were obtained from previous experiments (Kešnerová et al., 2017; Vannette et al., 2015;

Cornman et al., 2013; Zaobidna et al., 2017; Ryabov et al., 2014; Hinshaw et al., 2021). After testing primary efficiency, ef-1 alpha and RPS5 were selected as appropriate reference genes through consistent expression among all samples (Lourenço et al., 2008; Jeon et al., 2020). All qRT-PCR primers are included in Table A1.

Concentrations of gDNA and cDNA were first diluted to 10ng/µL working solutions. All plates were prepared with PowerUp™ SYBR™ Green master mix (Applied Biosystems). Every qRT-PCR reaction consisted of 2µL (20ng) of nucleic acid, 5µL of master mix, 0.5µL of forward primer, 0.5µL of reverse primer, and 2µL of nuclease-free water. Reactions were organized in 384-well plates using the QuantStudio 5 Real-Time PCR System (Applied Biosystems) and cycling conditions were as follows: 1 cycle of 2 minutes at 50ºC and 2 minutes at 95ºC (initial activation and denaturation) followed by 40 cycles of 15 seconds at 95ºC (denaturation) and 1 minute at 60ºC (annealing and extension). All reactions were run in triplicate and each primer set was organized with a negative control of nuclease-free water to ensure no on-plate contamination.

After running qRT-PCR, Ct values for each sample were calculated by taking the mean of the three technical replicates. The geometric mean of the Ct values from the two reference genes (√(RPS-5)(ef-1 alpha)) were then taken and subtracted from the Ct of each target to obtain a ΔCq (Vandesompele et al., 2002). A mean ΔCq of all control samples was then calculated and subtracted from the ΔCq of each experimental sample to find ΔΔCq values. The relative amount of microbe and immune gene transcripts was ultimately obtained using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The mean $2^{-\Delta\Delta CT}$ values calculated at each time point for a specific treatment were then used for subsequent relative expression analyses.

Statistical Analysis

All analyses were conducted in R version 4.2.2 using the packages lme4, emmeans, and corrplot (Bates et al., 2015; Lenth 2023; Wei and Simko 2021). To test for any differences in microbe abundance or immune gene expression between treatments, we ran generalized linear models with the expression of each target as the response variable and experimental treatment as a fixed effect. In order to fit normality assumptions, we log-transformed some response variables before running analyses. When testing for differences in immune gene expression, we also included the log-transformed relative abundance of DWV as an offset in the models. We used a Tukey post-hoc test to calculate the estimated marginal means between treatment groups and identified significantly different groups while controlling for multiple comparisons. Additionally, we ran a correlation analysis between all targets, calculating the Pearson's correlation coefficient and plotting those which were significant at alpha < 0.05.

Chapter 3

Results

Honey Bee Mortality

We did not find a significant difference in bee survival from any given treatment (OTC, HK *E. coli*, or the combined HKA treatment) (Figures 4-9). In the first round of bee collection, Colony 3 revealed particularly high levels of mortality, and qRT-PCR results from this colony were later excluded from analysis after discovering that it lacked a queen. However, Colony 3 mortality results were still included for interest and to further identify any potential trends (Figures 4, 6, and 8).

Prior to the start of HK *E. coli* treatment, healthy colonies did not show significant losses. In Round 1, no healthy colony lost more than two bees in any of the treatments. However, without a queen, Colony 3 lost 5 control bees and 25 bees fed OTC (Figure 4). Round 2 demonstrated even healthier trends, with neither colony losing more than a single bee and Colony 2 maintaining 100% survival for all treatments (Figure 5).

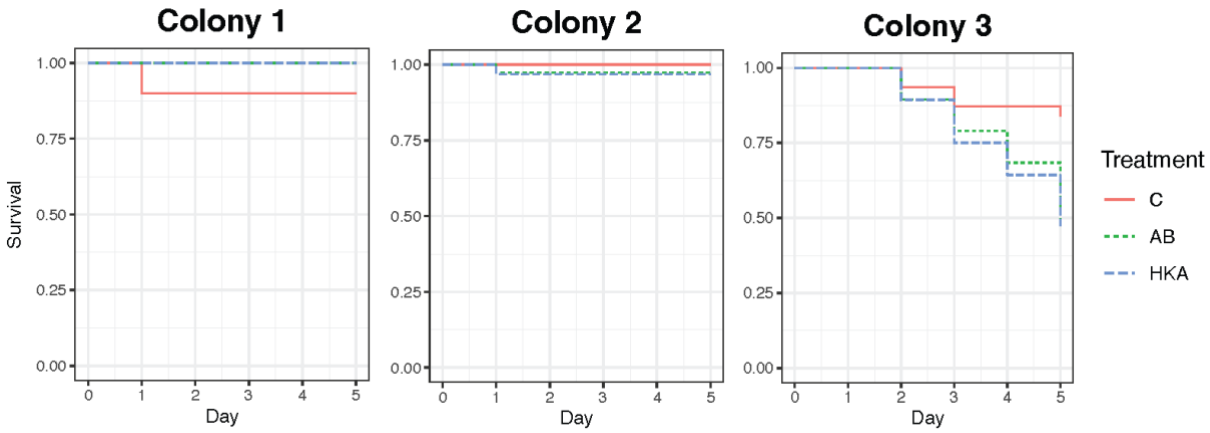

Figure 4. Round 1 mortality curves 0 days post-treatment with heat-killed *E. coli* exposure. Mortality curves are shown in plots testing survival vs. the number of days in the incubator. Survival was measured as a proportion with 1.00 corresponding to a cage with no mortality (100% survival). Plots were

separated by colony. Over 5 days, healthy colonies did not show differences in mortality. AB and HKA treatment groups in Colony 3 exhibited higher mortality than the control group.

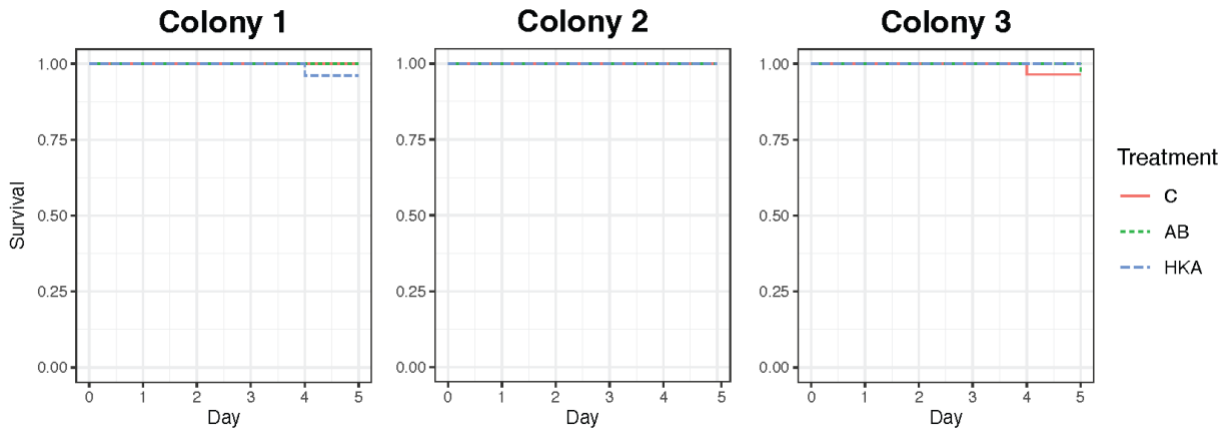

Figure 5. Round 2 mortality curves 0 days post-treatment with heat-killed *E. coli* exposure. Mortality percentages hovered around 100% for all colonies. Over 5 days, no colony exhibited significant mortality.

At 3 days post HK *E. coli* exposure, a few more bees were lost with the introduction of an immune challenge, yet most sample sizes were maintained. In Round 1, Colony 1 stayed healthy while Colony 2 showed a slight spike (>12%) in losses for both control and HKA treatments. Colony 3 continued to struggle; losses in all treatments increased significantly, with notable mortality percentages of 55% and 85% for HKA and AB treatments, respectively (Figure 6). In Round 2, bees generally withstood the immune challenge with no colony losing more than three bees over eight days (Figure 7).

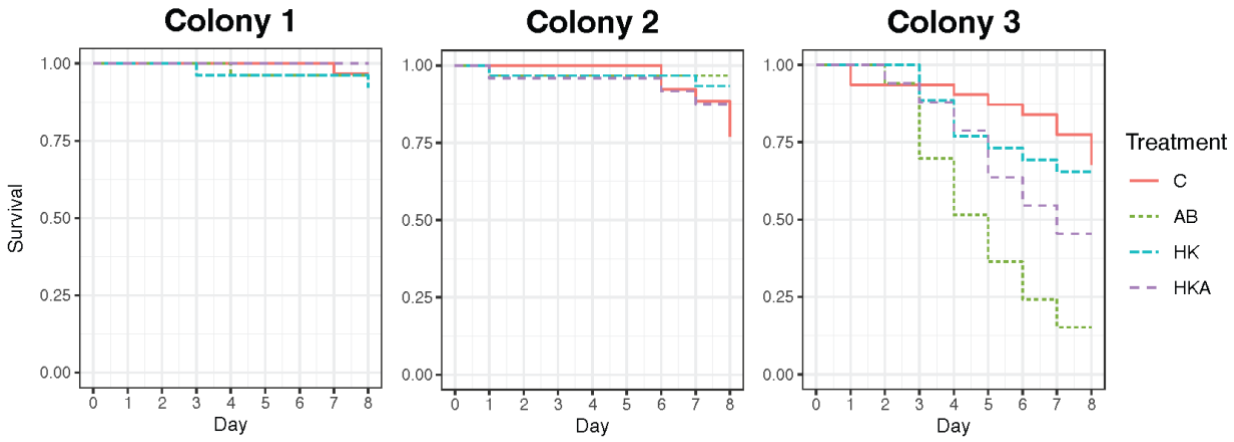

Figure 6. Round 1 mortality curves 3 days post-treatment with heat-killed *E. coli* exposure. Colony 2 started exhibiting losses in control and HKA treatments. Colony 3 showed even more losses from AB treatment and over 50% losses from HKA. This mortality curve resembles a heightened version of the survival data patterns from previous experiments involving multiple tetracycline and bacterial treatments (Raymann et al. 2017; Horak et al. 2020).

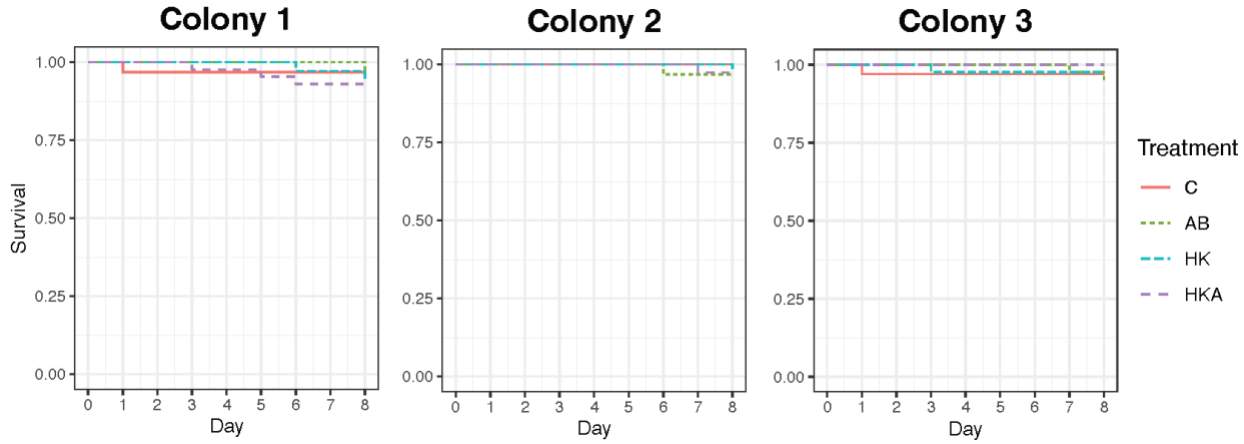

Figure 7. Round 2 mortality curves 3 days post-treatment with heat-killed *E. coli* exposure. Mortality percentages continued to hold steady at 100% for all colonies. Over 8 days, no colony exhibited significant mortality.

At 5 days Post HK *E. coli* exposure, mortality generally increased. In Round 1, Colony 1 lost a significant number of bees in only the HKA treatment, which accounted for 15% mortality. In addition, Colony 2 had 25% losses in all treatments, with HK inflicting the highest losses at

34%. Colony 3 struggled once again, with all treatments hovering around losses of 50% and higher (Figure 8). In Round 2, bees continued performing better and Colony 1 struggled the most with greater than 25% mortality in both AB and control treatments. Colony 2 had moderate mortality of 20% with both HK and control treatments, and Colony 3 revealed minimal losses with the HKA treatment inducing the highest mortality of 7% (Figure 9).

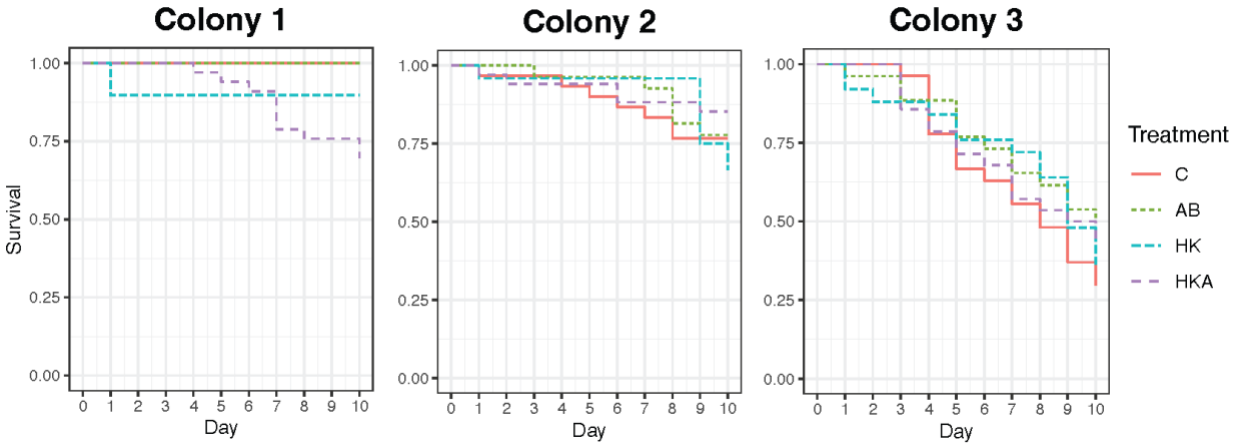

Figure 8. Round 1 mortality curves 5 days post-treatment with heat-killed *E. coli* exposure.

More losses accumulated in bee cages for varying treatments. In Colony 1, the HKA treatment lowered survival most noticeably, while all treatments show slightly increased mortality in Colony 2. Mortality levels of around 70% were also observed in every Colony 3 treatment group.

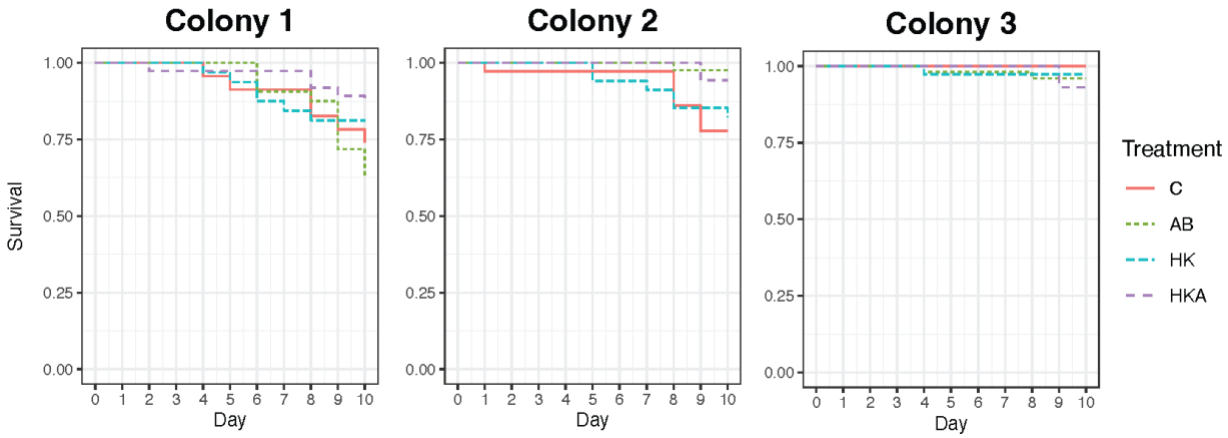

Figure 9. Round 2 mortality curves 5 days post-treatment with heat-killed *E. coli* exposure.

At 5 days, Round 2 bees started to lose strength. Low survival rates persisted in Colony 1 among all treatments and in Colony 2 mainly due to HK *E. coli* and control treatments. Colony 3 maintained its strength with limited mortality throughout.

Overall, while OTC and HK *E. coli* treatments appeared to induce higher levels of mortality at specific time points, similar correlations in control groups nullified any significant differences. Sporadic spikes in mortality were exhibited in each treatment and did not stay consistent across multiple colonies or time points. Thus, our experiments do not support high doses of OTC or HK *E. coli* significantly lowering honey bee survival.

Microbe Relative Quantification

Our results showed moderate depletion trends across all three core gut microbes after 5 days of antibiotic (OTC) treatment. Some differences were not statistically significant (*p-value < 0.05), yet noticeable decreases in relative abundance were observed in all cases. Using the Universal 16S rRNA primer set, we also observed a general decrease in bacterial abundance. After HK *E. coli* treatment, microbial abundances revealed a subsequent replenishing effect, favoring abundance levels consistent with the control and HK treatment groups. Thus, it appears that honey bees regained colonization by gut microbes in response to the immune challenge that counteracted earlier OTC-induced depletion.

After 5 days of OTC treatment and prior to the introduction of HK *E. coli*, honey bee microbes started to exhibit lower levels of abundance. At this time point, OTC induced significant depletion in *S. alvi* and a more noticeable depletion in all measured microbes (Figure 10). While *Bifidobacterium* did not show any depletion trends with OTC at this time point, its abundance

was slightly lower than the control. On the other hand, *Lactobacillus* Firm-5 did not respond to OTC at Day 0.

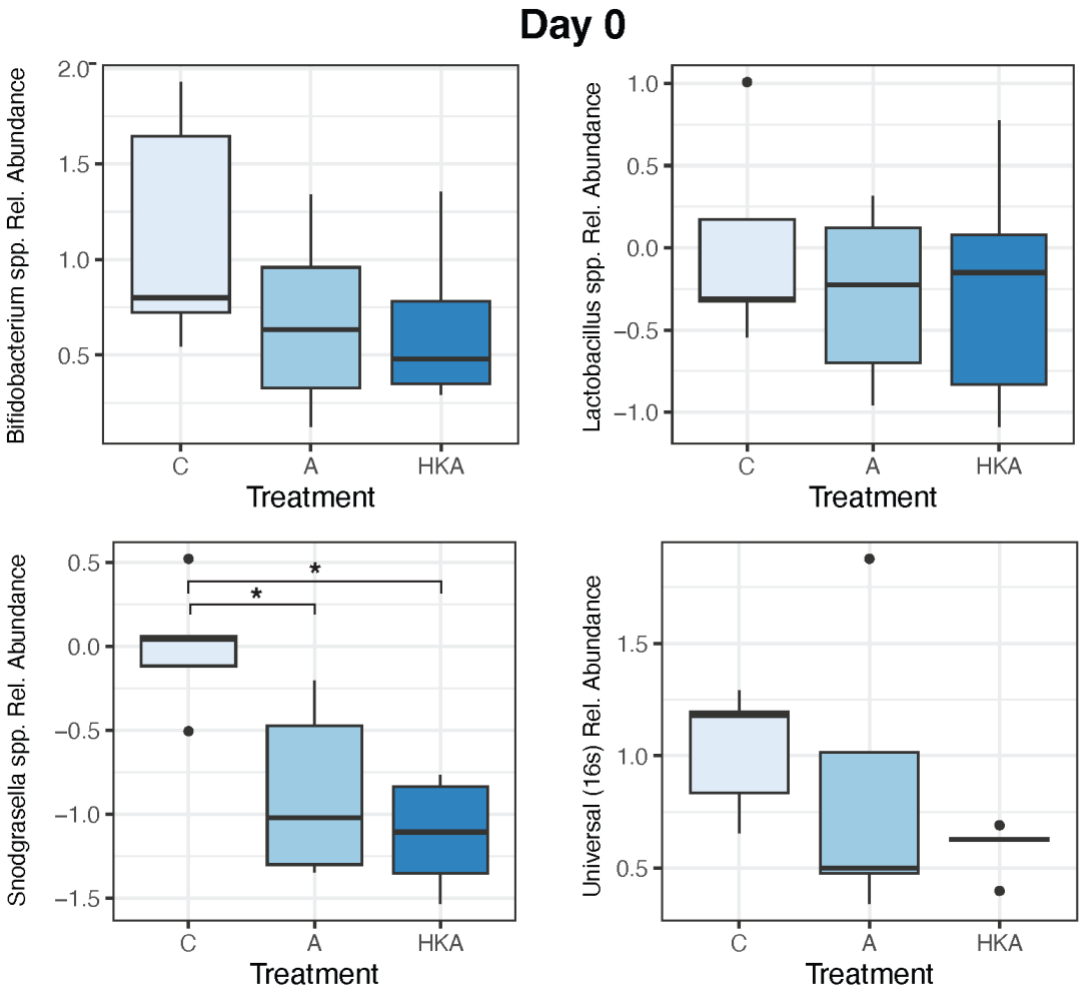

Figure 10. Relative microbial abundances 0 days post-treatment with heat-killed *E. coli* exposure. OTC significantly depleted *S. alvi* after five days of treatment (**p-value* < 0.05; GLM). Both Universal and *Bifidobacterium* revealed abundance levels trending toward depletion, but *Lactobacillus* Firm-5 did not deviate from the control.

After 3 days of HK *E. coli* treatment, microbial abundances fluctuated. OTC treatment decreased both *Bifidobacterium* and *Lactobacillus* Firm-5 abundance, while *Bifidobacterium* levels significantly dropped off from the control (Figure 11). Additionally, *S. alvi* abundance rebounded from its significantly low level from OTC treatment three days earlier. HKA

treatments induced rebounds in abundance for all three bacteria as well, with *Lactobacillus* Firm-5 responding stronger to the *E. coli* despite prior OTC exposure. Both *Lactobacillus* Firm-5 and *S. alvi* abundance appeared higher from the HKA treatment than the HK *E. coli* alone. All conserved gut microbes also continued to deplete, and the HKA treatment interestingly induced stronger microbial wipeout than the isolated OTC treatment. At this time point, HK *E.coli* induced no observable change in bacterial abundances when compared to the respective control groups. Finally, *Bifidobacterium* was the only microbe with a varying abundance between OTC and non-OTC treatments.

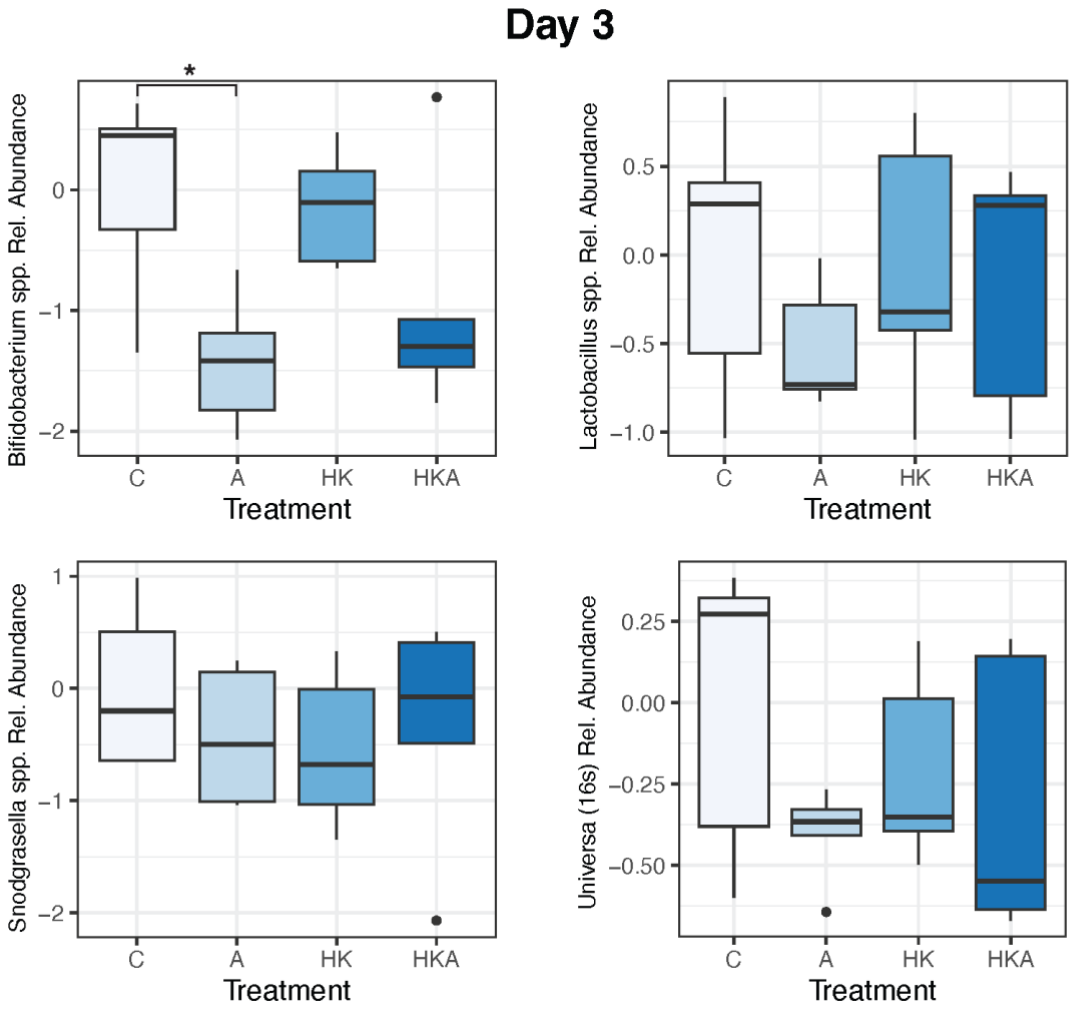

Figure 11. Relative microbial abundances 3 days post-treatment with heat-killed *E. coli* exposure.

OTC significantly depleted *Bifidobacterium* after an additional 3 days of 50% sucrose treatment (**p-value* < 0.05; GLM). *Lactobacillus* Firm-5 and Universal bacterial abundances showed depletion trends while *S. alvi* abundance reflected a level now comparable to the control. After 3 days, HK *E. coli* treatments did not influence *Lactobacillus* Firm-5, Universal bacteria, or *S. alvi* levels. However, the combined HKA treatment led to depletion trends in *Bifidobacterium* and Universal bacteria, but it reacted oppositely for the abundances of both *Lactobacillus* Firm-5 and *S. alvi*.

After 5 days of HK *E. coli* treatment, most of the trends from the previous time point were maintained. Both *Bifidobacterium* and *Lactobacillus* Firm-5 remained depleted from prior OTC treatment, and *Bifidobacterium* significantly decreased from its respective control (Figure 11). *S. alvi* abundance also remained steady at previously observed levels for both OTC and HKA treatments. Abundances of all conserved gut microbes now exhibited a rebounding trend where the effect of both OTC and HKA treatments did not deviate from the control. Generally, HKA treatments induced microbial abundance recovery in response to the immune challenge when compared to the isolated OTC treatment, except for *Bifidobacterium*. For this bacterium, while HKA treatment led to slightly stronger depletion, the two OTC treatments were mainly non-differentiable. Similar to previous observations, isolated HK *E. coli* treatments did not induce significant effects on microbial abundance, as levels of abundance remained consistent with each respective sucrose control. However, abundances do appear slightly higher on Day 5 than on Day 3, which provided stronger indications of the anticipated immune response.

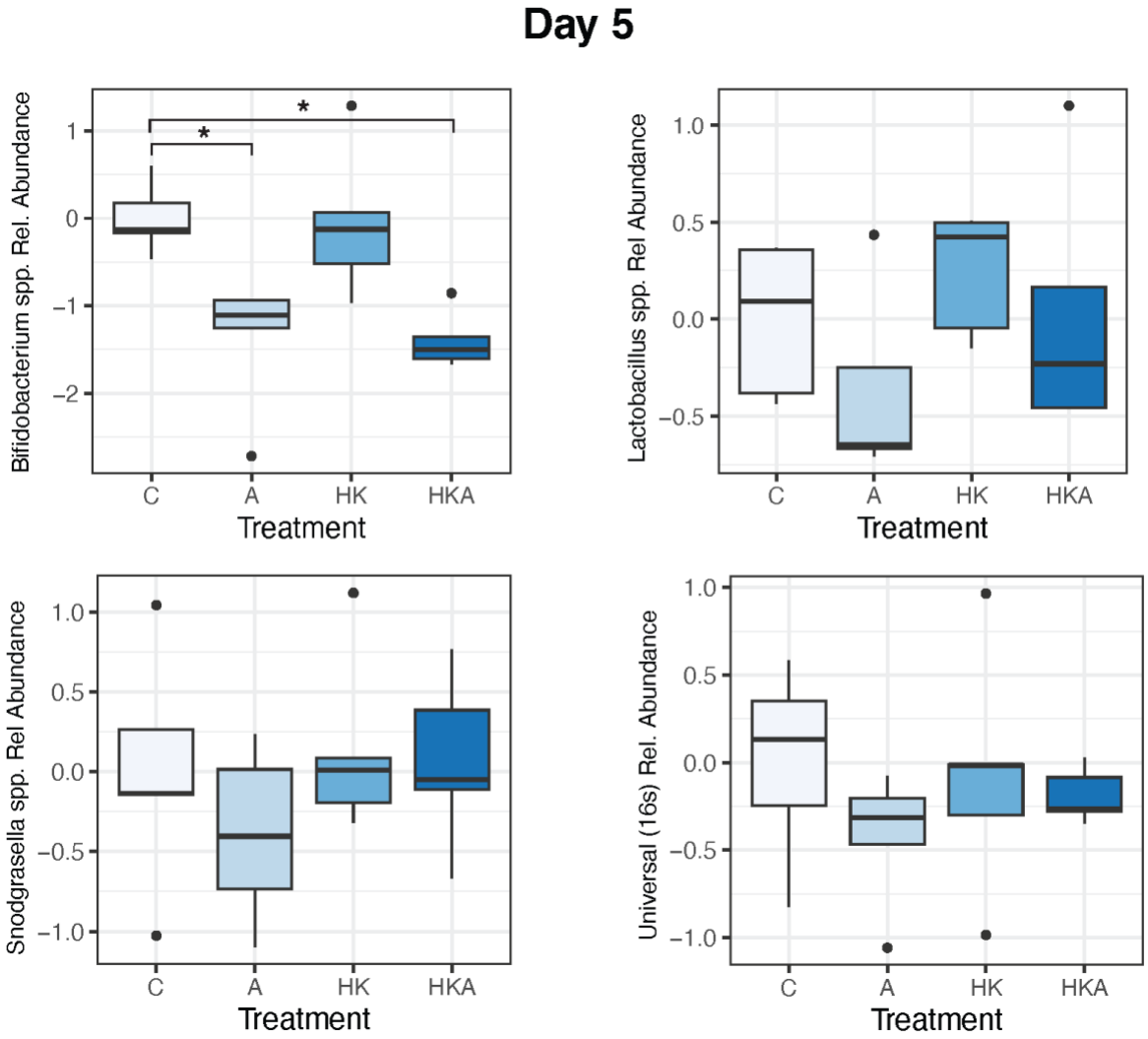

Figure 12. Relative microbial abundances 5 days post-treatment with heat-killed *E. coli* exposure. OTC significantly depleted *Bifidobacterium* after an additional 5 days of 50% sucrose treatment, and the HKA treatment further depleted abundance (**p-value* < 0.05; GLM). HK *E. coli* treatments caused microbe levels comparable to 50% sucrose. HKA treatments only led to microbial depletion in *Bifidobacterium*; other bacterial abundances appeared to recover from the earlier OTC treatment and replenish from HK *E. coli*.

Immune Gene Relative Quantification

Overall, our results displayed inconsistent trends from the HK *E. coli* treatment and a slight level of general immune gene downregulation with OTC. The HKA treatment also varied in its effect on immune gene expression, revealing a level of immune rebound dissimilar to our previous microbial abundance analysis. Thus, levels of all three immune genes appeared to react sporadically to the intended immune challenge, with more variation observed in the combined treatment. Collected honey bees used for the present experiments also possessed low levels of DWV across the board.

At 0 days, both *apidaecin* and *defensin-2* decreased in expression with OTC when compared to their respective controls, but neither decrease is significant. *Hymenoptaecin* levels remained the same after 5 days of OTC. Additionally, aside from one outlier in every treatment where a cage showed high DWV expression, all bees removed from this time point possessed low levels of the virus (Figure 13).

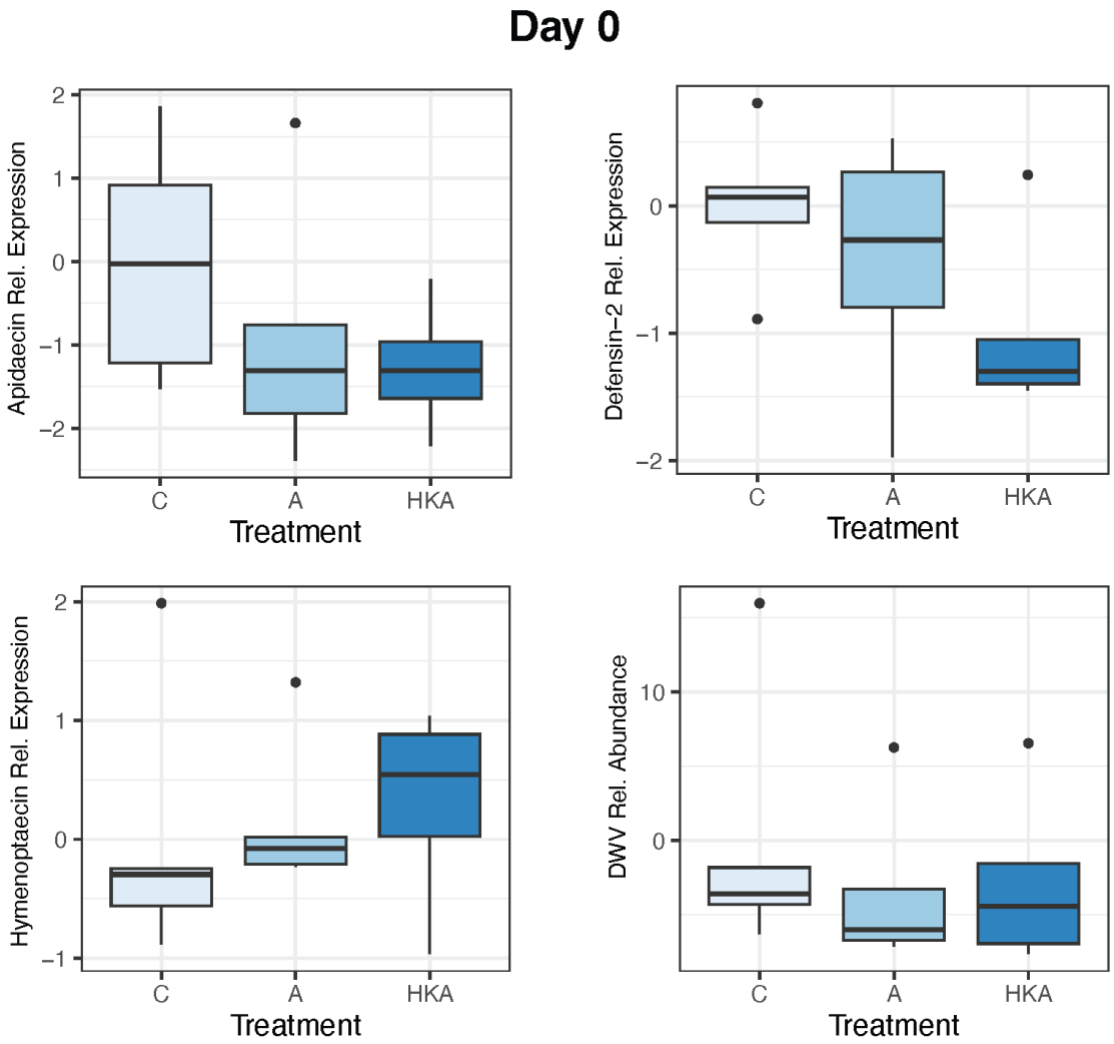

Figure 13. Immune gene and deformed wing virus expression 0 days post heat-killed *E. coli* exposure. OTC induced low expression of *apidaecin* and *defensin-2* after 5 days. However, *hymenoptaecin* levels remained consistent with the sucrose control. DWV did not factor into immune gene expression variation at Day 0 due to low viral loads.

At 3 days, *apidaecin* and *defensin-2* levels rebounded from any initial indications of downregulation. *Apidaecin* expression was now standard across all treatments and *defensin-2* expression only differed with a slight downregulation from HK *E. coli*. *Hymenoptaecin* levels responded to the initial OTC treatment and the AMP was significantly downregulated at this time point. The combined HKA treatment for *hymenoptaecin* showed early indications of

upregulation in immune gene expression brought on by HK *E. coli*, yet the isolated HK *E. coli* treatment did not reveal an elevated response. Similar to Day 0, DWV levels remained low in the tested honey bees aside from two outliers (Figure 14).

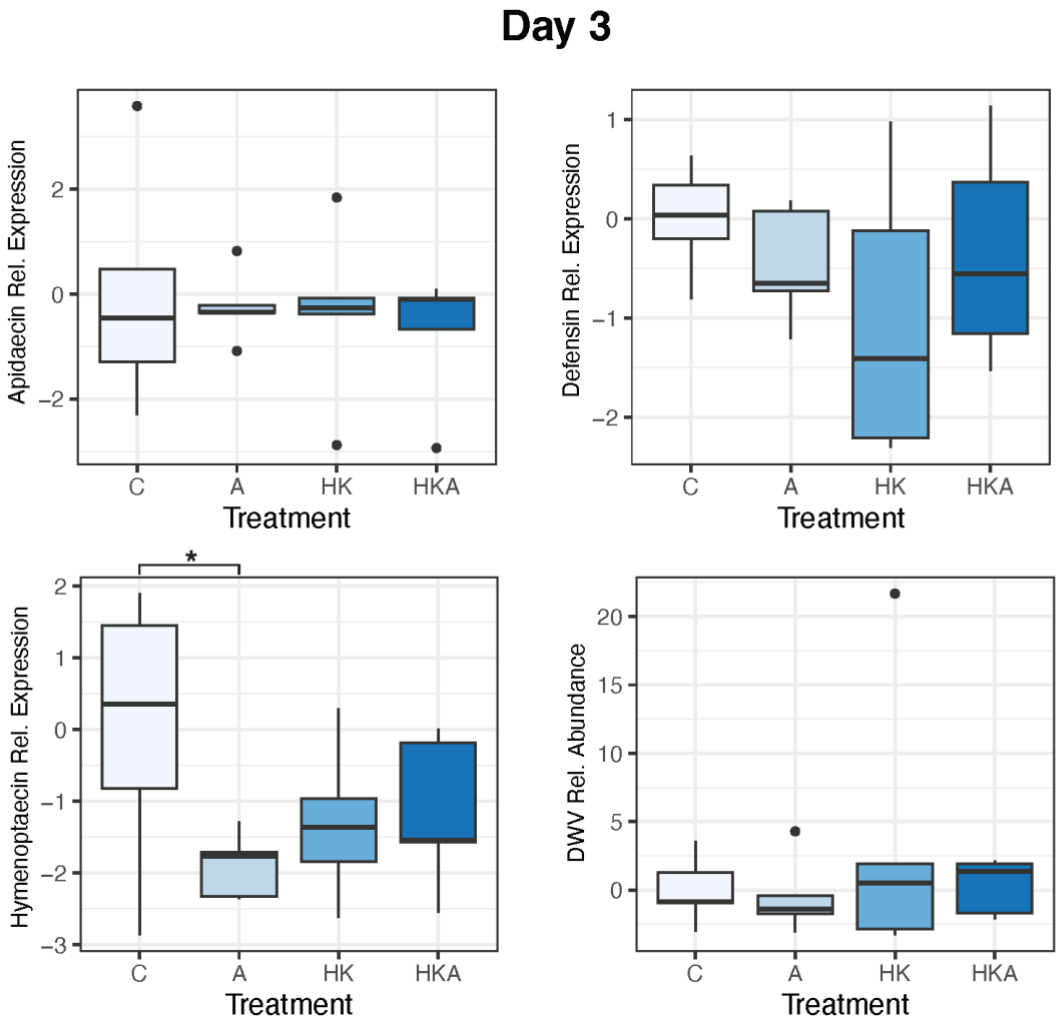

Figure 14. Immune gene and deformed wing virus expression 3 days post heat-killed *E. coli* exposure. OTC induced significantly low levels of *hymenoptaecin* after 8 days (**p-value* < 0.05; GLM). *Apidaecin* and *defensin-2* revealed expression levels consistent with 50% sucrose w/v. Levels of DWV were once again low across all treatments.

At 5 days, most Day 3 trends in immune gene expression were maintained. Aside from a few outliers, all four treatments now induced almost identical levels of *apidaecin* and *defensin-2* expression. The initial downregulation at Day 0 from OTC was recouped for both AMPs and the combined HKA treatment only showed slight upregulation compared to the isolated OTC. Once again, the HK *E. coli* treatment on its own did not elicit any changes in expression for all AMPs. For *hymenoptaecin*, the OTC treatment group rebounded in expression after the significant downregulation on Day 3. Similar to *apidaecin* and *defensin-2*, *hymenoptaecin* expression slightly increased as a result of HKA treatment when compared to isolated OTC, yet the lack of response from HK *E. coli* made it difficult to establish any trends. Moderate DWV abundances were more common at this time point, yet no cage showed any significant levels of expression (Figure 15).

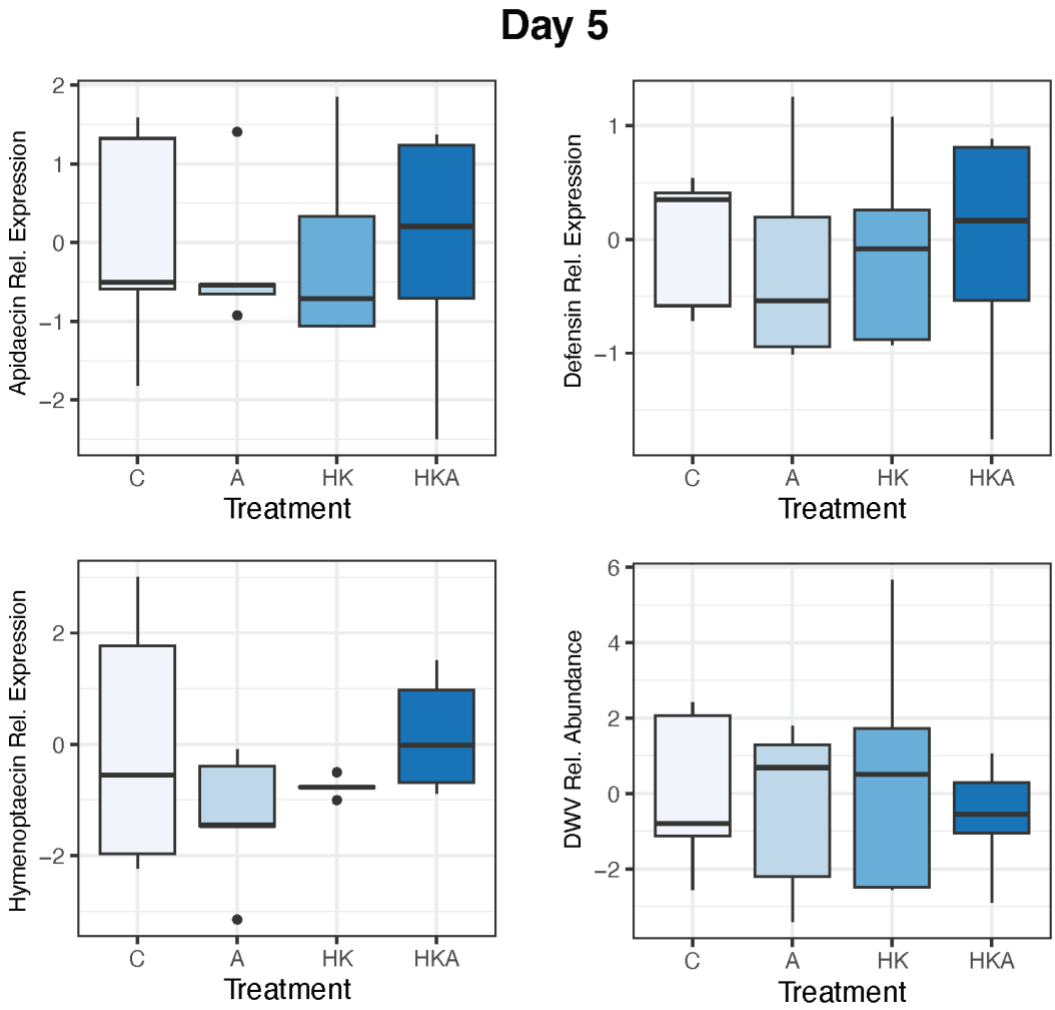

Figure 15. Immune gene and deformed wing virus expression 5 days Post heat-killed *E. coli* exposure. Expression levels for all three tested AMPs were consistent across all four treatments. HKA treatment groups had minimally higher levels of AMP expression compared to the isolated OTC treatment. DWV levels were also slightly higher at this time point, yet viral loads were still very low for all treatment groups.

Associations Between Microbiome and Immune Gene Expression

Due to previous studies connecting the honey bee microbiome to host immune symbiosis, we constructed a correlation chart to identify any patterns between immune gene expression and relative microbial abundances (Kešnerová et al., 2017). Any correlations significant at alpha =

0.05 were labeled with a correlation coefficient where values closer to 1 suggest a stronger correlation. We first discovered similarities in abundance between the quantified key microbes, including correlated levels of *Bifidobacterium* and *S. alvi* with universal bacterial abundance (r = 0.77 and 0.69, respectively). *Bifidobacterium* and *S. alvi* were regulated similarly as well (r = 0.57). Weaker correlations also existed between *Lactobacillus* Firm-5 and *Bifidobacterium* (r = 0.35), as well as between *Lactobacillus* and universal bacterial abundance (r = 0.39)

In addition, we discovered weak correlations among certain immune genes. *Hymenoptaecin* and *apidaecin* expression were positively correlated (r = 0.37). Between immune genes and microbes, both *hymenoptaecin* and *defensin-2* showed weak positive correlations with *S. alvi* (r = 0.42 and 0.37, respectively), while *defensin-2* displayed a slight correlation with universal bacteria abundance (r = 0.35). Finally, *apidaecin* expression weakly corresponded with *Lactobacillus* Firm-5 (r = 0.32) (Figure 16).

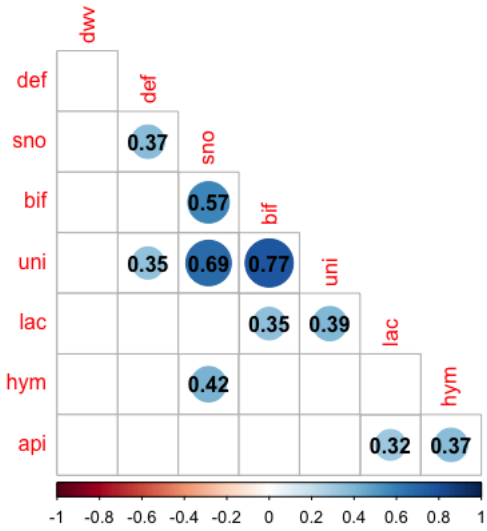

Figure 16. Correlations in expression patterns between tested immune genes and microbes

Patterns identified between core microbes and immune genes. Stronger patterns existed within microbial abundances, including between universal bacteria and all three microbes, as well as between

Bifidobacterium and *S. alvi.* We also found weak correlations between both *hymenoptaecin* and *defensin-2* with *S. alvi*, and between *apidaecin* and *Lactobacillus* Firm-5. Correlation coefficients were labeled within blue dots that vary in color according to correlation strength.

Chapter 4

Discussion

Non-Treatment Induced Honey Bee Losses

In the previous study by Raymann et al. 2017, honey bees treated with live bacteria after tetracycline only showed significant mortality when exposed to *Serratia* kz11. Treatment with other live species, including *E. coli* K-12, did not increase mortality. In general, honey bee workers and their respective larvae fared well when encountering individual treatments of either *E. coli* or tetracycline (Gätschenberger et al., 2013; Jia et al., 2022; Aljedani et al. 2022).

Consistent with these studies, none of our treatments caused high levels of honey bee mortality. All observed mortality patterns occurred randomly and persisted independently of treatment. From Round 1, Colony 3 displayed the most losses stemming from previous maternal stresses in a hive without a queen (Delaplane and Harbo 1987). Colony 2 also exhibited slightly more mortality on average than Colony 1, suggesting higher initial pathogen exposure within the colony or stressors that could have inhibited survival in laboratory conditions (Figures 4, 6, and 8). Colonies 1 and 2 from Round 2 also fared moderately worse than Colony 3, which was likely the result of similar variations within pathogen exposure and rearing conditions (Figures 5, 7, and 9). Therefore, our finding of low honey bee death as a result of OTC, HK *E. coli*, or a combination of the two treatments agrees with previous work linking honey bee tolerance to both individual antibiotic and live bacterial treatments (Gätschenberger et al., 2013; Jia et al., 2022; Aljedani et al. 2022; Bulson et al. 2021; Raymann et al. 2017).

After cross-checking the most unsuccessful cages with levels of DWV expression, cages with higher DWV loads still performed better than some cages with lower viral titers (Table A2).

Thus, moderate DWV titers did not singularly increase mortality within our honey bees. Rather, any influence of pathogens on mortality would have likely been multifactorial in nature (Alburaki et al., 2018). This finding corresponds to previous studies suggesting DWV exposure accounted for only 30% higher mortality (Koziy et al., 2019). In the future, running our experiments with larger sample sizes could reveal more noticeable trends between DWV and honey bee losses. After ruling out DWV, it seems plausible that some level of mortality may be attributed to prior colony exposure from a combination of stressors, such as varroa mite *Varroa destructor*, *Nosema* sp., and bacterial/viral infections (Noël et al., 2020; Alburaki et al., 2018). *Varroa destructor* – an efficient vector of DWV – is viewed by many as one of the main contributors to colony mortality worldwide. In addition to mites, poor nutrition and pesticide consumption within certain colonies might have factored into our small number of bee losses (Alburaki et al., 2018). However, observed mortality was most likely induced by variable rearing conditions, which was primarily shown through improved survival during later rounds of experimentation. While treatments and pathogens may have contributed to honey bee vulnerability, other stressors during incubation seemed to account for most survival differences between colonies.

Results from previous studies have also uncovered the impact of long-term OTC exposure in decreasing honey bee survivorship and inducing immune system vulnerability (Raymann et al., 2017; Bulson et al., 2020; Aljedani et al., 2022). Our study did not account for mortality levels of honey bee larvae and the long-term effects of overall hive health with continuous OTC exposure. In addition, studies on *P. larvae* reveal established resistance mechanisms to OTC, further diminishing its effectiveness as an antimicrobial treatment in modern beekeeping (Miyagi et al., 2000). Despite our findings of limited mortality in worker bees with OTC treatment, the

antibiotic may induce more lethal effects earlier in colony development. Continued research on stronger and safer OTC alternatives can further our understanding of how antimicrobials can be utilized to sustain colony health (Kochansky et al., 2001). Our results provide surface-level support for the non-lethal effects of concentrated and short-term antibiotic treatments on honey bee workers. Currently, beekeepers around the world still use OTC and similar antimicrobials to treat bacterial infections in their hives (Genersch et al., 2010; Daisley et al., 2020). Until more viable treatment methods become commonplace, infected honey bees are more likely to endure short spurts of OTC exposure.

OTC Induces General Microbial and Immune Gene Depletion

Previous studies have indicated significant depletion in *S. alvi* 6, 7, and 9 days after 450ng/µL tetracycline treatment (Jia et al., 2022; Soares et al., 2021). *Lactobacillus* Firm-5 and *Bifidobacterium* were also effectively knocked down 3-7 days after the same treatment (Raymann et al., 2017; Daisley et al., 2020; Jia et al., 2022).

In our study, the abundance of quantified microbes decreased in response to OTC treatment. As a broad-spectrum antibiotic, OTC was expected to influence both Gram-positive and Gram-negative members of the honey bee microbiome. Results supported this broad antimicrobial impact with a widespread knockdown of key microbes residing in honey bee guts. Both *S. alvi* and *Bifidobacterium* abundances were significantly depleted after OTC treatment while levels of *Lactobacillus* Firm-5 and universal bacteria only showed moderate decreases (Figures 11-12). Although slightly weaker in comparison, these results still reflected those from previous experiments testing the effect of tetracycline on honey bee gut microbes (Raymann et al., 2017; Jia et al., 2022; Daisley et al., 2020; Soares et al., 2021). This weaker response might also stem

from stronger microbial resistance mechanisms to tetracycline (Tian et al., 2012). In either case, our OTC treatment was successful at reducing the gut homeostasis of honey bees and depleting key microbes that participate in numerous metabolic and immune processes (Moran 2015; Lee et al., 2014; Horak et al., 2020).

Additionally, previous penicillin-streptomycin treatments reduced honey bee fitness through the downregulation of AMPs in 5 and 7-day-old larvae, including *apidaecin*, *hymenoptaecin*, and *defensin-1* (Duan et al., 2021). Glyphosate, a broad-spectrum herbicide, and Tylosin, a commonly used antibiotic in beekeeping, were also previously reported to lower the expression of our three tested AMPs (Motta et al., 2022). In contrast, immune challenge experiments from Horak et al. (2020) showed strong upregulations in *apidaecin* and *hymenoptaecin* AMPs 1-, 2-, and 5-days post-inoculation from both HK *E. coli* and *S. alvi* treatments. Considering these conflicting trends, we anticipated immune gene expression to decrease with the treatment of broad-spectrum OTC and then increase with HK *E. coli*. Expression levels from the combined HKA treatment were then expected to fall somewhere in between these two trends.

Based on our results, honey bee AMPs were weakly downregulated as a result of OTC. Only *hymenoptaecin* expression reduced significantly at any point, and *apidaecin* and *defensin-2* expression moderately decreased at selective time points (Figures 13-15). While previous experiments have shown AMP expression within honey bee larvae to significantly decrease after a broad-spectrum antibiotic treatment, our worker bees retained their ability to mount an immune response (Duan et al., 2021). OTC treatment also seemed to have a weaker effect on AMP expression than previous studies on tylosin and glyphosate (Motta et al., 2022). Similar to the trends observed in microbial abundances, AMP depletion may also require a more robust OTC treatment spanning longer than 5 days. Additionally, RNA obtained from whole-body samples or

entire larvae may reveal AMP expression differences when compared to our samples extracted from dissected fat bodies and abdominal tissue (Duan et al., 202; Motta et al., 2022). In the future, extracting DNA and RNA from 10 distinct bees to include guts for RNA analysis might reveal stronger levels of depletion among the tested immune genes. In either case, our AMP quantification data suggests OTC treatment induces a weaker – but not completely suppressed – honey bee immune response.

The timing of OTC treatment and subsequent HK *E. coli* exposure can be modified in future experiments to more effectively test the honey bee immune system. OTC-induced depletion varied between microbes, as *S. alvi* was significantly depleted directly after 5 days of treatment, whereas the other two microbes and universal bacterial abundances were depleted 3-5 days later (Figures 10-12). Immune genes functioned similarly; *apidaecin* and *defensin-2* expression appeared to deplete at Day 0 despite *hymenoptaecin* requiring three additional days to deplete (Figure 13). Therefore, extending the OTC treatment for another 2-3 days would likely create the widespread knockdown required to induce a more immunocompromised state. While our results still provide insights into the immune effects of a weakened honey bee microbiome, the quick rebound from certain microbes like *S. alvi* may assist in quickly recuperating certain levels of AMP expression.

Honey Bees Overcome OTC to Mount Microbial Immune Response

Based on visual trends, our tested honey bee microbiomes revealed a broad rebounding effect as a result of HK *E. coli* treatment. *S. alvi, Lactobacillus* Firm-5, and universal bacteria all responded quickly to HK *E. coli*, as shown by moderate abundance increases from the HKA combined treatment. None of these increases were significant, yet broad replenishments in

microbial activity were observed for most microbes. However, *Bifidobacterium* responded more significantly to OTC and did not display any rebounding effect from HK *E. coli* (Figures 11-12). Thus, our results indicate fluctuating impacts of OTC on different microbes and the difficulty in assessing microbiome strength after antibiotic treatment. Continuing to study the role of each microbe in honey bee immunity can further enhance our understanding of how detrimental broad-spectrum antibiotics can be for honey bee health. Overall, our short-term OTC treatment was handled well by honey bees through general microbiome replenishment. By studying *Bifidobacterium* levels and more diverse gut microbes longer than 5 days after an immune challenge, we can further support the efficacy of short-term antibiotic treatments.

Heat-Killed *E. coli* Induces Weak Immune Gene Response

Despite noticeable differences in microbiome composition, HK *E. coli* did not induce the anticipated immune response. Expression levels of *hymenoptaecin*, *apidaecin*, and *defensin-2* were all comparable to the control after an isolated HK *E. coli* treatment (Figures 14-15). These results do not correspond to the significant increases in immune AMPs reported by Horak et al. (2020). Differences in methodology, particularly the use of microbiota-free bees, may have caused the result discrepancies. We also opted for a 10% less concentrated $5x10^7$ cells/mL treatment over just 2 days and did not extract RNA from the honey bee guts. In spite of these differences, our study still supports the overwhelming immune responsibilities associated with honey bee microbiota and their ability to suppress many immune challenges (Horak et al., 2020; Steele et al., 2021). These results also reflect previous studies on live *E. coli* infections in honey bee larvae that showed high levels of AMP expression only after initial bacterial clearance (Gätschenberger et al., 2013). In the future, maintaining a heavily concentrated HK *E. coli* treatment for an entire 5 days may reveal the intended AMP response. In either case, while

observing minor alterations in key microbe abundances, a short-term HK *E. coli* treatment will not provoke a strong honey bee immune response. More long-term and potentially lethal pathogens should be used to fully test the vulnerability of the honey bee immune system caused by ATB treatment, including the various live bacteria explored in Raymann et al. (2017). Finally, the difficulty in establishing statistically significant differences between the control groups and experimental treatments stems from the wide range of expression values obtained from a small set of bees, which can be improved by extracting from a larger sample size. Expanding this study across more colonies and including multiple rounds of extractions may increase the precision of our expression data and allow us to identify stronger patterns across different treatments.

Due to its minor effect on AMP expression, an HK *E. coli* treatment may serve as an effective immune primer to protect honey bees from lethal pathogens or future ATB treatment. Prior research reported how insects respond well to immune priming and confer stronger protection mechanisms when faced with a subsequent or secondary pathogen (Cooper & Eleftherianos, 2017). Immune priming in honey bees has already been explored; live *S. alvi* helped control future pathogen treatment of live *S. marcescens* while HK *S. alvi* limited honey bee mortality (Horak et al., 2020). Honey bees inoculated with *S. alvi* wkB2 or *Gilliamella apicola* wkB7 also led to higher survival rates when compared to bees lacking microbiota that faced a live *E. coli* immune challenge (Kwong et al., 2017). Due to its similar Gram-negative and sublethal characteristics that still cause noticeable increases in key microbial abundance, HK *E. coli* offers an alternative immune priming option over live honey bee microbes. Additionally, an isolated HK *E. coli* treatment may be more accessible for beekeepers to obtain and use when treating their hives. The effects of HK *E. coli* and other HK bacteria not only have the possibility to protect honey bees from disease but also upregulate their key microbes prior to a necessary ATB

treatment. Our study offers support for this idea based on noticeable trends in higher AMP expression from the combined HKA treatment over the isolated OTC treatment. These trends exist in all three immune genes tested (Figure 15). Considering all possibilities, future experiments exploring the benefits of initial HK *E. coli* priming prior to ATB hive treatment may uncover optimal ways to utilize antimicrobials in modern beekeeping. Furthermore, continuing to study how certain immune challenges or probiotic treatments can prime the honey bee immune system will provide many answers that support honey bee health moving forward.

Correlation between Microbiome and AMP Expression

It is widely known that gut microbial communities impact the host immune system. Previous research has studied the effect of microbiota treatments in honey bees without initial microbial communities, showing significant increases in AMP expression (Kwong et al., 2017; Horak et al., 2020). Our study supports the results of previous experiments by correlating *hymenoptaecin* and *defensin*-2 expression to *S. alvi*. We also observed *apidaecin* to be associated with *Lactobacillus* Firm-5 abundance, whereas *defensin-2* correlated with general microbe abundance (Figure 16). While none of these correlations were exceptionally strong, they carry more weight since all levels of expression were obtained from a small sample size across many treatments. Like our previous results, extracting RNA from the honey bee guts might show more convincing levels of correlation due to a more localized impact of microbial abundance on gut AMPs (Kwong et al., 2017; Horak et al., 2020).

Nonetheless, our results support the inherent link between the microbiome and innate immunity within honey bees. Researchers proposed that a possible basis behind this complex connection stems from gut AMPs maintaining microbiome homeostasis (Kwong et al., 2017). Our study further reinforces previous indications that bacterial disruption can limit AMP expression and

weaken honey bee immune defenses (Daisley et al., 2020; Li et al., 2017). Our observations of more widespread microbiota depletions ahead of AMP downregulation suggest the importance of microbiome health in downstream AMP expression. Due to some delayed responses in the depletion of key microbes from OTC, it is possible that keeping bees alive past 10 days would have shown stronger AMP downregulation. Taking a more detailed look into the timing involved with microbiome depletion and subsequent shifts in AMP expression will further enhance our understanding of the overall involvement of key microbial communities. Shifting our focus to immune genes both localized in the guts and throughout the honey bee anatomy will also showcase the widespread effects of the gut microbiome and the extent of its influence within an organism.

With everything considered, there is certainly a trade-off to consider when opting for ATB treatments to treat AFB or other bacterial infections in a hive. Clear microbial disruptions within the honey bee microbiome can affect downstream immunity and hinder immune flexibility when responding to future pathogens. However, the strength of diverse gut microbes within honey bees also allows them to rebound from ATB and still provide a layer of immune protection, yet the capacity of that shield likely reduces after long-term ATB exposure (Bulson et al., 2021). Ideally, finding the sweet spot in the timing of ATB treatment will reduce infection levels without compromising future honey bee immunity. Continuing to optimize modern ATB treatments and test the honey bee microbiome will provide even stronger insights into how beekeepers around the world can save their honey bee colonies from infection. Finally, dissecting how the honey bee microbiome interacts and operates on a symbiotic level to sustain immune function will further our knowledge of foundational microbial patterns and allow us to discover strong parallels to human health. Drawing these parallels will only revolutionize how we view our gut

microbes and apply them in clinical settings to reinforce the human immune response and ultimately improve patient outcomes.

BIBLIOGRAPHY

Alburaki, M., Chen, D., Skinner, J. A., Meikle, W. G., Tarpy, D. R., Adamczyk, J., & Stewart, S. D. (2018). Honey Bee Survival and Pathogen Prevalence: From the Perspective of Landscape and Exposure to Pesticides. *Insects*, *9*(2), 65. https://doi.org/10.3390/insects9020065

Aljedani D. M. (2022). Antibiotic treatment (Tetracycline) effect on bio-efficiency of the larvae honey bee (*Apis mellifera jemenatica*). *Saudi journal of biological sciences*, *29*(3), 1477–1486. https://doi.org/10.1016/j.sjbs.2021.11.024

Barroso-Arévalo, S., Vicente-Rubiano, M., Puerta, F. et al. Immune related genes as markers for monitoring health status of honey bee colonies. BMC Vet Res 15, 72 (2019). https://doi.org/10.1186/s12917-019-1823-y

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Bulson, L., Becher, M. A., McKinley, T. J., & Wilfert, L. (2021). Long-term effects of antibiotic treatments on honeybee colony fitness: A modelling approach. *The Journal of applied ecology*, *58*(1), 70–79. https://doi.org/10.1111/1365-2664.13786

Casteels, P., Ampe, C., Jacobs, F., & Tempst, P. (1993). Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (Apis mellifera). *Journal of Biological Chemistry*, *268*(10), 7044–7054. https://doi.org/10.1016/s0021-9258(18)53143-4

Cooper, D., & Eleftherianos, I. (2017). Memory and Specificity in the Insect Immune System: Current Perspectives and Future Challenges. *Frontiers in Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.00539

Cornman, R. S., Lopez, D., & Evans, J. D. (2013). Transcriptional Response of Honey Bee Larvae Infected with the Bacterial Pathogen Paenibacillus larvae. PLOS ONE, 8(6), e65424. https://doi.org/10.1371/journal.pone.0065424

Cripps, C.J. (2021). Veterinary Regulations. In Honey Bee Medicine for the Veterinary Practitioner (eds T.R. Kane and C.M. Faux). https://doi.org/10.1002/9781119583417.ch15

Daisley, B.A., Pitek, A.P., Chmiel, J.A. *et al. Lactobacillus* spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. *Commun Biol* **3**, 534 (2020). https://doi.org/10.1038/s42003-020-01259-8

Danihlík, J., Aronstein, K., & Petřivalský, M. (2015). Antimicrobial peptides: a key component of honey bee innate immunity. *Journal of Apicultural Research*, *54*(2), 123–136. https://doi.org/10.1080/00218839.2015.1109919

Delaplane, K.S., & Harbo, J.R. (1987). Effect of Queenlessness on Worker Survival, Honey Gain and Defence Behaviour in Honeybees. *Journal of Apicultural Research, 26*, 37-42.

Duan, X., Zhao, B., Jin, X., Cheng, X., Huang, S., & Li, J. (2021). Antibiotic Treatment Decrease the Fitness of Honeybee (Apis mellifera) Larvae. *Insects*, *12*(4), 301. https://doi.org/10.3390/insects12040301

Evans, J.D., Armstrong, TN. Antagonistic interactions between honey bee bacterial symbionts and implications for disease. BMC Ecol 6, 4 (2006). https://doi.org/10.1186/1472-6785-6-4

Evans, J. D., Chen, Y., Di Prisco, G., Pettis, J. S., & Williams, V. V. (2009). Bee cups: single-use cages for honey bee experiments. Journal of Apicultural Research, 48(4), 300–302. https://doi.org/10.1080/00218839.2009.11101548

Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J. L., Jiang, H., Kanost, M., Thompson, G. J., Zou, Z., & Hultmark, D. (2006). Immune pathways and defence

mechanisms in honey bees Apis mellifera. Insect molecular biology, 15(5), 645–656. https://doi.org/10.1111/j.1365-2583.2006.00682.x

Gätschenberger, H., Azzami, K., Tautz, J., & Beier, H. (2013). Antibacterial Immune Competence of Honey Bees (Apis mellifera) Is Adapted to Different Life Stages and Environmental Risks. PLOS ONE, 8(6), e66415. https://doi.org/10.1371/journal.pone.0066415

Genersch, E. (2010). American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. Journal of Invertebrate Pathology, 103, S10–S19. https://doi.org/10.1016/j.jip.2009.06.015

Hinshaw, C., Evans, K., Rosa, C., & López-Uribe, M. (2021). The Role of Pathogen Dynamics and Immune Gene Expression in the Survival of Feral Honey Bees. *Frontiers in Ecology and Evolution*, *8*. https://doi.org/10.3389/fevo.2020.594263

Horak, R. D., Leonard, S. P., & Moran, N. A. (2020). Symbionts shape host innate immunity in honeybees. Proceedings. Biological sciences, 287(1933), 20201184. https://doi.org/10.1098/rspb.2020.1184

Hung, K. J., Kingston, J. M., Albrecht, M., Holway, D. A., & Kohn, J. R. (2018). The worldwide importance of honey bees as pollinators in natural habitats. Proceedings. Biological sciences, 285(1870), 20172140. https://doi.org/10.1098/rspb.2017.2140

Ilyasov, R. A., Gaifullina, L. R., Saltykova, E. S., Poskryakov, A. V., & Nikolenko, A. G. (2012). Review of the Expression of Antimicrobial Peptide Defensin in Honey Bees Apis Mellifera L. Journal of Apicultural Science, 56(1). https://doi.org/10.2478/v10289-012-0013-y

Jeon, J. H., Moon, K., Kim, Y., & Kim, Y. H. (2020). Reference gene selection for qRT-PCR analysis of season- and tissue-specific gene expression profiles in the honey bee Apis mellifera. Scientific reports, 10(1), 13935. https://doi.org/10.1038/s41598-020-70965-4

Jia, S., Wu, Y., Chen, G., Wang, S., Hu, F., & Zheng, H. (2022). The Pass-on Effect of Tetracycline-Induced Honey Bee (Apis mellifera) Gut Community Dysbiosis. Frontiers in Microbiology, 12. https://doi.org/10.3389/fmicb.2021.781746

Kešnerová, L., Mars, R. A. T., Ellegaard, K. M., Troilo, M., Sauer, U., & Engel, P. (2017). Disentangling metabolic functions of bacteria in the honey bee gut. *PLOS Biology*, *15*(12), e2003467. https://doi.org/10.1371/journal.pbio.2003467

Khalifa, S. A. M., Elshafiey, E. H., Shetaia, A. A., El-Wahed, A. A. A., Algethami, A. F., Musharraf, S. G., AlAjmi, M. F., Zhao, C., Masry, S. H. D., Abdel-Daim, M. M., Halabi, M. F., Kai, G., Al Naggar, Y., Bishr, M., Diab, M. A. M., & El-Seedi, H. R. (2021). Overview of Bee Pollination and Its Economic Value for Crop Production. Insects, 12(8), 688. https://doi.org/10.3390/insects12080688

Klaudiny J, Albert S, Bachanová K, Kopernický J, Simúth J. Two structurally different defensin genes, one of them encoding a novel defensin isoform, are expressed in honeybee Apis mellifera. Insect Biochem Mol Biol. 2005 Jan;35(1):11-22. doi: 10.1016/j.ibmb.2004.09.007. PMID: 15607651.

Kochansky, J., Knox, D., Feldlaufer, M. F., & Pettis, J. S. (2001b). Screening alternative antibiotics against oxytetracycline-susceptible and -resistant *Paenibacillus larvae*. *Apidologie*, *32*(3), 215–222. https://doi.org/10.1051/apido:2001123

Koziy, R. V., Wood, S. C., Kozii, I. V., van Rensburg, C. J., Moshynskyy, I., Dvylyuk, I., & Simko, E. (2019). Deformed Wing Virus Infection in Honey Bees (*Apis mellifera* L.). *Veterinary pathology*, *56*(4), 636–641. https://doi.org/10.1177/0300985819834617

Kwong, W. K., Mancenido, A. L., & Moran, N. A. (2017). Immune system stimulation by the native gut microbiota of honey bees. Royal Society Open Science, 4(2), 170003. https://doi.org/10.1098/rsos.170003

Lee FJ, Rusch DB, Stewart FJ, Mattila HR, Newton IL. Saccharide breakdown and fermentation by the honey bee gut microbiome. Environ Microbiol. 2015 Mar;17(3):796-815. doi: 10.1111/1462-2920.12526. Epub 2014 Jul 8. PMID: 24905222.

Lenth, R. V. (2023). *Estimated Marginal Means, aka Least-Squares Means [R package emmeans version 1.8.5]*. https://cran.r-project.org/web/packages/emmeans/index.html

Li, J. H., Evans, J. D., Li, W. F., Zhao, Y. Z., DeGrandi-Hoffman, G., Huang, S. K., Li, Z. G., Hamilton, M., & Chen, Y. P. (2017). New evidence showing that the destruction of gut bacteria by antibiotic treatment could increase the honey bee's vulnerability to Nosema infection. *PloS one*, *12*(11), e0187505. https://doi.org/10.1371/journal.pone.0187505

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods, 25(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Lourenço, A.P., Mackert, A., Cristino, A.d.S. *et al.* Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. *Apidologie* **39**, 372–385 (2008). https://doi.org/10.1051/apido:2008015

Lozupone, C., Stombaugh, J., Gordon, J. et al. Diversity, stability and resilience of the human gut microbiota. Nature 489, 220–230 (2012). https://doi.org/10.1038/nature11550

Miyagi, T., Peng, C. Y., Chuang, R. Y., Mussen, E. C., Spivak, M., & Doi, R. H. (2000). Verification of Oxytetracycline-Resistant American Foulbrood Pathogen Paenibacillus larvae in the United States. *Journal of Invertebrate Pathology*, *75*(1), 95–96. https://doi.org/10.1006/jipa.1999.4888

Mookhploy, W., Chantawannakul, P., & Chantawannakul, P. (2021). Effects of Deformed Wing Virus Infection on Expressions of Immune- and Apoptosis-Related Genes in Western Honeybees (Apis mellifera). Insects, 12(1), 82. https://doi.org/10.3390/insects12010082

Moran N. A. (2015). Genomics of the honey bee microbiome. Current opinion in insect science, 10, 22–28. https://doi.org/10.1016/j.cois.2015.04.003

Motta, E. V. S., Powell, J. E., & Moran, N. A. (2022). Glyphosate induces immune dysregulation in honey bees. *Animal microbiome*, *4*(1), 16. https://doi.org/10.1186/s42523-022-00165-0

Noël, A., Le Conte, Y., & Mondet, F. (2020). Varroa destructor: how does it harm Apis mellifera honey bees and what can be done about it?. *Emerging topics in life sciences*, *4*(1), 45–57. https://doi.org/10.1042/ETLS20190125

Ortiz-Alvarado, Y., Clark, D. R., Vega-Melendez, C. J., Flores-Cruz, Z., Domingez-Bello, M. G., & Giray, T. (2020). Antibiotics in hives and their effects on honey bee physiology and behavioral development. Biology open, 9(11), bio053884. https://doi.org/10.1242/bio.053884

Patel, V., Pauli, N., Biggs, E., Barbour, L., & Boruff, B. (2021). Why bees are critical for achieving sustainable development. Ambio, 50(1), 49–59. https://doi.org/10.1007/s13280-020-01333-9

Raymann, K., Shaffer, Z., & Moran, N. A. (2017). Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. *PLOS Biology*, *15*(3), e2001861. https://doi.org/10.1371/journal.pbio.2001861

Richard, F. J., Aubert, A., & Grozinger, C. M. (2008). Modulation of social interactions by immune stimulation in honey bee, Apis mellifera, workers. BMC biology, 6, 50. https://doi.org/10.1186/1741-7007-6-50

Ryabov, E. V., Wood, G. R., Fannon, J. M., Moore, J. W., Bull, J. J., Chandler, D., Mead, A., Burroughs, N. J., & Evans, D. M. (2014). A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (Apis mellifera) Prevails after Varroa destructor-Mediated, or In Vitro, Transmission. PLOS Pathogens, 10(6), e1004230. https://doi.org/10.1371/journal.ppat.1004230

Soares, K. O., Oliveira, C. J. B. D., Rodrigues, A. E., Vasconcelos, P. C., Silva, N. M. V. D., Cunha Filho, O. G. D., Madden, C., & Hale, V. L. (2021). Tetracycline Exposure Alters Key Gut Microbiota in Africanized Honey Bees (Apis mellifera scutellata x spp.). Frontiers in Ecology and Evolution, 9. https://doi.org/10.3389/fevo.2021.716660

Steele, M. I., Motta, E. V. S., Gattu, T., Martinez, D., & Moran, N. A. (2021). The Gut Microbiota Protects Bees from Invasion by a Bacterial Pathogen. *Microbiology spectrum*, *9*(2), e0039421. https://doi.org/10.1128/Spectrum.00394-21

Tian, B., Fadhil, N. H., Powell, J. E., Kwong, W. K., & Moran, N. A. (2012). Long-Term Exposure to Antibiotics Has Caused Accumulation of Resistance Determinants in the Gut Microbiota of Honeybees. MBio, 3(6). https://doi.org/10.1128/mbio.00377-12

Vandesompele, J., De Preter, K., Pattyn, F. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3, research0034.1 (2002). https://doi.org/10.1186/gb-2002-3-7-research0034

Vannette, R., Mohamed, A. & Johnson, B. Forager bees (*Apis mellifera*) highly express immune and detoxification genes in tissues associated with nectar processing. *Sci Rep* **5**, 16224 (2015). https://doi.org/10.1038/srep16224

Wei, T., & Simko, V. (2021). *GitHub - taiyun/corrplot: A visual exploratory tool on correlation matrix*. GitHub. https://github.com/taiyun/corrplot

Williams, G. C., Alaux, C., Costa, C., Csáki, T., Doublet, V., Eisenhardt, D., Fries, I., Kuhn, R., McMahon, D. P., Medrzycki, P., Murray, T. E., Natsopoulou, M. E., Neumann, P. J., Oliver, R., Paxton, R. J., Pernal, S. F., Shutler, D., Tanner, G., Formato, G., & Brodschneider, R. (2013). Standard methods for maintaining adultApis melliferain cages underin vitrolaboratory conditions. Journal of Apicultural Research, 52(1), 1–36. https://doi.org/10.3896/ibra.1.52.1.04

Wu, H. J., & Wu, E. (2012). The role of gut microbiota in immune homeostasis and autoimmunity. *Gut microbes*, *3*(1), 4–14. https://doi.org/10.4161/gmic.19320

Zaobidna,E.,Żółtowska,K. & Łopieńska-Biernat,E.(2017).Expression and Activity of Lysozyme in Apis Mellifera Carnica Brood Infested with Varroa Destructor. Journal of Apicultural Science,61(2) 253-256. https://doi.org/10.1515/jas-2017-0014

Zheng, H., Steele, M. I., Leonard, S. P., Motta, E. V. S., & Moran, N. A. (2018). Honey bees as models for gut microbiota research. Lab animal, 47(11), 317–325. https://doi.org/10.1038/s41684-018-0173-x

APPENDIX

We conducted extensive troubleshooting throughout experimentation to minimize mortality during bee collection and extract quality nucleic acid. All findings are detailed below to share optimizations to help guide future experiments. Modifications are organized in chronological order by when they were introduced into our protocols.

4/2022-05/2022: Bees were collected and fed their respective treatments in cup cages via upside-down transfer pipette bulbs. Air bubbles were visualized in the transfer pipettes and bees were unable to feed despite cutting off the pipettes at various lengths. Ultimately, small 15mL falcon tubes were inverted and poked through the bottom of the cup cages with tiny holes near their base to feed the bees. An immediate reduction in mortality was observed and this was the method used for feeding the bees moving forward.

5/23/22: When beginning extractions, both RNA and DNA ratios appeared a lot lower than desired for confident qRT-PCR testing. Complications likely stemmed from a large pool of 10 bees being extracted, causing inadequate lysis. Many modifications were made to the protocol to increase 260:280 and 260:230 ratios. First, more buffer was added to include 800µL of lysis buffer for the RNA extractions and 700µL of DNA genomic buffer for the DNA extractions. More beads were added to the tubes to total about 18-22 tubes per tube prior to lysis.

8/26/22: More modifications to the extraction protocol were made. Lysate is now spun down at 16,000xg for RNA for 3 minutes after lysis and 10,000xg for 5 minutes for DNA. 400µL of supernatant is removed for RNA and 300µL for DNA. DNA lysate was added to a purple Qia-shredder column before transferring it to the binding column.

9/2/2022: 800µL of DNA genomic buffer is now added to the tubes prior to lysis. 400µL of supernatant can then be transferred to the Qia-shredder column for extraction.

10/25/22: DNA quality assessment. 4 samples were used in qRT-PCR to assess whether poor quality ratios lead to subsequent amplification inefficiency. Amplification appears consistent between samples of varying quality. DNA cleanup was determined not to be required prior to qRT-PCR analysis.

Table A1. Sequences of forward and reverse primers used for relative quantification of gut microbes and immune genes in qRT-PCR.

Target	Sequences (5'-3')	Reference
Ef-1 alpha (Endogenous Reference)	F: GGAGATGCTGCCATCGTTAT R: CAGCAGCGTCCTTGAAAGTT	Lourenço et al., 2008
RPS5 (Endogenous Reference)	F: GATGTTTCTCCGTTACGACGAGT R: GAGTTCATCGGCTAAACATTCGG	Jeon et al., 2020
Lactobacillus Firm-5 (Gut Microbe)	F: GCAACCTGCCCTWTAGCTTG R: GCCCATCCTKTAGTGACAGC	Kešnerová et al., 2017
S. alvi (Gut Microbe)	F: CTTAGAGATAGGAGAGTGCCTT R: AACTTAATGATGGCAACTAATGACAA	Kešnerová et al., 2017
Bifidobacterium (Gut Microbe)	F: ATGCAAGTCGAACGGGATCC R: CATCCCATRCCGGTAAACCC	Kešnerová et al., 2017
Universal 16s rRNA Bacteria (Total Gut Microbes)	F: AGGATTAGATACCCTGGTAGTCC R: YCGTACTCCCCAGGCGG	Kešnerová et al., 2017
Hymenoptaecin (Immune Gene AMP)	F: ACAATGGATTATATCCCGACTCGT R: CAATGTCCAAGGATGGACGAC	Vannette et al., 2015

Apidaecin (Immune Gene AMP)	F: TAGTCGCGGTATTTGGGAAT R: TTTCACGTGCTTCATATTCTTCA	Cornman et al., 2013
Defensin-2 (Immune Gene AMP)	F: ATGGATTCCAACGAACCGCT R: ACACACGCCATTTCTGCAAC	Zaobidna et al., 2017
DWV (Viral Pathogen)	F: GTTTGTATGAGGTTATACTTCAAGGAG R: GCCATGCAATCCTTCAGTACCAGC	Ryabov et al., 2014

Table A2. Raw Cq expression values from gut microbe qRT-PCR analysis

Sample	Treatment	Time Point	Ef-1 α	RPS5	*Bifido*	*Lacto*	*S. alvi*	Universal
A 3/1/0	A	0	19.445	19.579	22.093	16.198	17.696	14.83
A 3/2/0	A	0	18.913	19.173	20.077	15.452	16.434	12.79
A 3/3/0	A	0	18.944	19.198	20.705	16.258	17.721	13.908
A 2/1/0	A	0	19.777	20.133	20.507	17.827	16.958	12.815
A 2/2/0	A	0	18.545	18.369	22.456	16.702	17.044	13.225
C 2/2/0	C	0	19.012	19.437	20.52	14.632	16.663	12.755
C 3/1/0	C	0	18.565	18.837	20.145	16.012	15.349	13.074
C 3/2/0	C	0	19.239	19.701	19.495	16.084	15.427	12.868
C 2/1/0	C	0	18.738	19.324	20.875	16.678	15.908	12.54
C 3/3/0	C	0	18.646	19.28	19.217	16.29	15.585	12.989
HKA 3/1/0	HKA	0	18.776	19.028	20.234	15.652	16.719	13.342
HKA 3/2/0	HKA	0	19.451	19.578	20.052	15.263	17.43	13.816
HKA 2/1/0	HKA	0	18.738	18.438	21.079	16.649	17.511	13.036
HKA 3/3/0	HKA	0	18.674	18.849	20.795	17.197	17.425	13.855
HKA 2/2/0	HKA	0	18.984	19.491	21.99	16.316	17.543	13.668
A 3/2/3	A	3	18.6	19.236	21.194	15.791	16.967	12.785
A 2/1/3	A	3	18.886	18.716	22.355	16.056	16.113	13.011
A 3/3/3	A	3	19.176	19.199	20.718	17.221	15.427	12.856

A 2/2/3	A	3	19.355	20.055	22.904	17.603	16.088	13.46
A 3/1/3	A	3	19.005	19.569	21.899	17.223	17.371	13.095
C 2/1/3	C	3	18.806	19.228	18.556	15.446	14.883	11.907
C 2/2/3	C	3	18.768	19.383	18.993	14.637	15.955	11.89
C 3/2/3	C	3	18.995	19.473	19.066	15.492	14.408	11.963
C 3/1/3	C	3	18.501	18.498	19.541	16.832	16.021	12.33
C 3/3/3	C	3	19.086	19.691	21.893	17.03	16.91	13.533
HK 3/1/3	HK	3	19.196	19.683	19.325	15.479	15.553	12.449
HK 3/3/3	HK	3	18.776	19.208	20.413	14.687	17.525	12.781
HK 2/1/3	HK	3	19.218	19.921	19.908	16.875	16.169	12.821
HK 2/2/3	HK	3	18.983	19.357	20.673	16.63	16.745	12.433
HK 3/2/3	HK	3	18.418	19.123	19.488	17.114	16.857	12.766
HKA 2/1/3	HKA	3	18.949	19.446	22.309	15.562	15.896	13.814
HKA 2/2/3	HKA	3	18.98	19.538	21.947	15.434	15.13	12.259
HKA 3/2/3	HKA	3	18.864	19.491	21.618	17.512	16.471	13.248
HKA 3/3/3	HKA	3	18.948	18.948	21.069	16.941	18.527	13.147
HKA 3/1/3	HKA	3	19.027	19.54	18.742	15.726	15.282	12.359
A 3/1/5	A	5	18.638	18.985	21.257	15.525	14.967	12.473
A 3/2/5	A	5	18.854	19.699	21.244	17.55	15.748	12.748
A 2/1/5	A	5	19.113	20.028	21.559	17.927	16.645	13.607
A 2/2/5	A	5	18.743	19.352	23.606	17.352	17.127	13.939
A 3/3/5	A	5	19.096	19.633	21.602	17.065	16.919	13.181
C 2/1/5	C	5	18.739	18.982	19.742	16.83	16.838	13.414
C 3/1/5	C	5	18.91	18.833	18.648	15.695	14.987	11.395
C 3/2/5	C	5	18.835	18.773	19.194	16.696	13.796	11.66
C 2/2/5	C	5	18.624	19.623	19.948	15.928	15.82	12.292
C 3/3/5	C	5	18.644	18.685	19.974	15.871	15.36	12.386

HK 3/2/5	HK	5	18.905	19.339	19.66	15.746	16.077	12.51
HK 2/1/5	HK	5	18.851	19.618	21.262	16.79	15.603	14.019
HK 2/2/5	HK	5	18.742	18.837	20.18	16.198	15.27	12.587
HK 3/1/5	HK	5	19.343	19.987	18.444	16.271	14.545	11.635
HK 3/3/5	HK	5	18.937	18.515	19.543	15.457	15.504	12.11
HKA 3/2/5	HKA	5	18.892	19.416	21.025	14.905	15.812	12.481
HKA 3/3/5	HKA	5	18.788	19.503	21.947	16.244	15.083	13.012
HKA 2/1/5	HKA	5	18.935	19.587	22.301	17.259	14.65	12.747
HKA 2/2/5	HKA	5	19.485	19.42	22.404	17.462	16.02	13.219
HKA 3/1/5	HKA	5	18.586	19.184	21.472	16.557	16.341	12.634

Table A3. Raw Cq expression values from immune gene qRT-PCR analysis

Sample	Treatment	Time Point	Ef-1 α	RPS5	*Apid*	*Def-2*	*Hym*	DWV
A 3/1/0	A	0	18.368	20.938	28.647	27.101	17.712	33.454
A 3/2/0	A	0	16.961	20.24	28.376	25.253	18.863	34.035
A 3/3/0	A	0	16.512	20.535	22.421	23.988	18.531	28.334
A 2/1/0	A	0	17.059	19.954	25.952	24.407	18.43	33.352
A 2/2/0	A	0	16.227	19.721	26.181	27.072	18.196	14.059
C 2/2/0	C	0	17.383	20.842	24.117	26.656	20.318	26.865
C 3/1/0	C	0	18.232	21.181	23.375	25.892	16.792	33.95
C 3/2/0	C	0	16.417	19.383	24.287	24.366	18.27	0
C 2/1/0	C	0	17.058	19.978	27.071	23.633	19.277	29.867
C 3/3/0	C	0	18.337	19.624	27.127	25.095	19.327	29.36
HKA 3/1/0	HKA	0	17.464	20.091	25.445	26.947	17.463	33.952
HKA 3/2/0	HKA	0	16.947	20.25	26.821	26.958	17.742	34.776

HKA 2/1/0	HKA	0	16.946	19.422	27.736	25.993	18.114	13.896
HKA 3/3/0	HKA	0	17.524	19.501	26.276	26.842	16.999	30.073
HKA 2/2/0	HKA	0	15.839	19.454	26.329	23.541	18.954	25.01
A 3/2/3	A	3	17.419	20.77	24.397	25.592	18.396	35.744
A 2/1/3	A	3	17.024	20.014	25.516	25.729	19.392	32.68
A 3/3/3	A	3	17.926	20.237	25.917	24.459	19.035	25.104
A 2/2/3	A	3	17.145	20.529	26.881	24.019	19.642	31.594
A 3/1/3	A	3	18.067	20.868	26.47	25.872	19.482	34.176
C 2/1/3	C	3	19.942	21.793	28.072	25.884	19.556	34.307
C 2/2/3	C	3	17.312	21.235	20.557	25.86	13.96	26.26
C 3/2/3	C	3	19.031	21.145	25.926	25.525	15.492	33.657
C 3/1/3	C	3	18.009	20.142	27.464	24.86	16.057	35.642
C 3/2/3 (Calib 2)	C	3	18.736	21.164	26.128	25.238	15.108	35.57
C 3/2/3 (Calib 3)	C	3	18.998	21.211	26.342	24.482	15.042	34.832
C 3/3/3	C	3	17.989	20.984	29.305	24.034	21.081	29.844
HK 3/1/3	HK	3	17.53	20.375	25.577	24.594	19.076	35.884
HK 3/3/3	HK	3	18.29	20.212	29.938	27.929	18.135	35.544
HK 2/1/3	HK	3	18.608	20.732	26.74	27.193	20.943	29.104
HK 2/2/3	HK	3	17.875	21.014	23.286	23.491	16.474	30.863
HK 3/2/3	HK	3	18.142	19.993	25.989	27.898	18.526	0
HKA 2/1/3	HKA	3	17.066	20.621	25.189	25.083	18.549	33.456
HKA 2/2/3	HKA	3	17.023	21.313	25.722	24.039	16.547	28.52
HKA 3/2/3	HKA	3	16.94	19.865	25.874	26.072	19.553	33.69
HKA 3/3/3	HKA	3	16.776	19.845	24.94	25.435	18	27.354
HKA 3/1/3	HKA	3	19.487	20.077	30.57	23.662	17.57	30.02

A 3/1/5	A	5	17.865	21.054	23.32	25.961	18.057	34.587
A 3/2/5	A	5	17.509	20.067	25.635	25.994	18.968	35.682
A 2/1/5	A	5	17.446	21.367	26.043	23.29	17.542	30.335
A 2/2/5	A	5	17.104	20.341	25.941	24.157	21.299	28.8
A 3/3/5	A	5	17.317	20.109	25.387	25.81	18.864	28.085
C 2/1/5	C	5	17.414	19.738	25.216	25.361	19.487	32.187
C 3/1/5	C	5	16.97	19.835	22.394	23.628	13.904	34.043
C 3/2/5	C	5	17.674	19.894	22.414	25.327	12.472	31.856
C 2/2/5	C	5	17.674	20.69	25.925	24.325	20.45	28.165
C 3/3/5	C	5	17.11	19.831	26.998	23.436	17.326	26.958
HK 3/2/5	HK	5	17.553	20.73	24.552	26.203	18.294	34.784
HK 2/1/5	HK	5	17.034	19.968	25.426	24.353	17.649	22.293
HK 2/2/5	HK	5	17.578	20.242	22.153	24.274	17.69	30.138
HK 3/1/5	HK	5	17.537	20.016	26.214	22.973	18.286	34.342
HK 3/3/5	HK	5	19.113	21.295	27.671	27.24	19.395	29.713
HKA 3/2/5	HKA	5	16.905	20.137	22.616	26.777	16.57	34.651
HKA 3/3/5	HKA	5	16.642	19.718	27.671	23.665	17.495	29.713
HKA 2/1/5	HKA	5	17.926	21.332	25.211	24.172	16.254	33.098
HKA 2/2/5	HKA	5	17.638	21.294	23.365	23.892	15.3	29.886
HKA 3/1/5	HKA	5	17.87	20.29	26.016	25.609	18.136	31.86

Academic Vita

Nick Tomasko

EDUCATION

The Pennsylvania State University **Class of 2023**
B.S. in Biochemistry and Molecular Biology Degree, Minor in Spanish *University Park, PA*
Schreyer Honors College
Mechanicsburg Area Senior High School **Salutatorian, Class of 2019**
High School Diploma *Mechanicsburg, PA*

WORK EXPERIENCE

ScribeAmerica **Jan. 2023 – Present**
Telescribe *Virtual*
- Coordinate with physicians during patient consultations in University of California San Francisco medical system to transcribe information and improve organizational responsibility
- Develop foundational knowledge of medicine through rigorous training and healthcare exposure

Regeneron Pharmaceuticals **June 2022 – Aug. 2022**
QC Virology Intern *Albany NY*
- Optimized molecular biology assays under Dr. Patrick Blatt in 10-week program using Next-Generation Sequencing to identify lower viral and bacterial contaminants within drug production cell lines
- Collaborated extensively with bioinformatics team to advance testing paradigms

The Pennsylvania State University, Department of Entomology **Feb. 2022 – Present**
Undergraduate Research Assistant in the López-Uribe Lab *University Park, PA*
- Broadcasted work during beekeeping conferences to discuss future efficacy of antibiotic treatments in honey bees
- Awarded best student poster at American Bee Research Conference in Jacksonville, FL

LEADERSHIP EXPERIENCE

American Society of Microbiology Student Organization **Aug. 2022 – Present**
President *University Park, PA*
- Revitalize club to welcome faculty talks and connect undergraduate students with Penn State researchers
- Engage in community service activities to increase microbiology exposure to grade-school students

The Pennsylvania State University, Department of Chemistry **Aug. 2021 – Dec. 2022**
Teaching Assistant *University Park, PA*
- Served under Dr. Aaron Garner to facilitate student learning in daily lectures and out-of-class help sessions
- Instructed two weekly recitations of 15-30 students and focused on arduous concepts to improve student outcomes

VOLUNTEER EXPERIENCE

Crisis Text Line **Sept. 2021 – Present**
Crisis Counselor *Virtual*
- Monitor text line by messaging over 250 people in vulnerable situations to ensure their mental and physical safety

UPMC Harrisburg **Mar. 2022 – May 2022**
Patient Liaison *Harrisburg, PA*
- Played authoritative role assisting nurses by checking in on patients and cleaning hospital rooms in neurological unit

The Pennsylvania State University, Department of Chemistry **Aug. 2021 – Present**
Learning Assistant *University Park, PA*
- Actively participated in four separate classes consisting of both general chemistry and organic chemistry
- Tailored teaching methods to specific learners and created supportive environment welcoming to all students

Central Pennsylvania Food Bank **June. 2020 – Nov. 2021**
Volunteer *Harrisburg, PA*
- Promoted food accessibility in downtown Harrisburg by communicating with patrons and loading grocery bags

SKILLS & INTERESTS

- **Skills:** Basic statistical analysis; Microsoft office, Adobe, and PyMOL proficiency; medical documentation; qPCR; nucleic acid extraction; computational drug development; scientific writing; research inquiry
- **Other languages:** Spanish – advanced skills in reading, writing, speaking, and listening
- **Interests:** Intramural sports; sustainability; running; traveling; investment; pollinator health; classic rock & roll