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Effects of Toxicant Exposure on Honey Bee and Bumble Bee Microbiomes and Impacts on Host Health

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Effects of Toxicant Exposure on Honey Bee and Bumble Bee Microbiomes and Impacts on Host Health

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Microbiology

by

Jason Rothman

June 2019

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ABSTRACT OF THE DISSERTATION

Effects of Toxicant Exposure on Honey Bee and Bumble Bee Microbiomes and Impacts on Host Health

by

Jason Rothman

Doctor of Philosophy, Graduate Program in Microbiology
University of California, Riverside, June 2019
Dr. Quinn McFrederick, Chairperson

Bees are important insect pollinators in both agricultural and natural settings who may encounter toxicants while foraging on plants growing in contaminated soils. How these chemicals affect the bee microbiome, which confers many health benefits to the host, is an important but understudied aspect of pollinator health. Through a combination of 16S rRNA gene sequencing, LC-MS metabolomics, ICP-OES spectroscopy, quantitative PCR, culturing, microbiome manipulation, and whole organism exposure studies, I attempt to establish the effects that toxicants have on social bees and their associated microbes.

The microbiome of animals has been shown to reduce metalloid toxicity, so I exposed microbiome-inoculated or uninoculated bumble bees to 0.75 mg/L selenate and found that inoculated bees survive longer when compared to uninoculated bees. I also showed that selenate exposure altered the composition of the bumble bee microbiome and that the growth of two major gut symbionts – Snodgrassella alvi and Lactobacillus bombicola – was unaffected by this exposure.
Due to the pervasiveness of environmental pollution in bee habitats, I exposed bumble bees to cadmium, copper, selenate, imidacloprid, and hydrogen peroxide and found that each of these compounds can be lethal to bees. I also showed that most of these chemicals can affect the diversity of the bee microbiome and that there is interstrain variation in toxicant tolerance genes in the major bee symbionts *Snodgrassella alvi* and *Gilliamella apicola*.

As exposure to cadmium or selenate has been shown to affect animal-associated microbes, I assayed the effects of these chemicals on honey bees and observed shifts in the bee microbiome at multiple timepoints. I also found that exposure to selenate and cadmium changes the overall bee metabolome and may cause oxidative damage to proteins and lipids. Lastly, I found that bee-associated bacteria can bioaccumulate cadmium but generally not selenate.

In this dissertation I demonstrated that bee-associated bacteria are generally robust to toxicant exposure, but that chemicals can alter the composition of both bumble bee and honey bee microbiomes. I also show that toxicants affect bee metabolism, and that the bee microbiome plays an important role in maintaining host health when challenged with toxicants.
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Chapter One:

Introduction
Introduction

Pollination is a vital service that insects provide for both wild and managed ecosystems. For example, agricultural production of many crops is greatly enhanced with insect pollination [1], and in the United States alone, bee pollination provides over $10 billion annually in crop yields [2]. As the largest group of insect pollinators, bees diversified following flowering plants, indicating a history of millions of years of coevolution [3]. There are approximately 20,000 species comprising the bee clade Anthophila (Order: Hymenoptera) [4], with varied lifestyles ranging from completely solitary to eusocial [4]. Examples of insect pollinators found specifically in the state of California include nearly 1,600 species of native bees [5], and introduced and managed species such as the alfalfa leafcutting bee (Megachile rotundata), the blue orchard bee (Osmia lignaria), the European honey bee (Apis mellifera) and bumble bees (Bombus spp.) [2]. In this dissertation, I will focus on experiments involving two species of commercially-available, social bees: Apis mellifera and the common eastern bumble bee Bombus impatiens.

Recent studies have shown that the populations of several bee species are declining in various parts of the world. For example, bee species richness was shown to decline in parts of Europe by over 50% since 1980 [6], wild bumble bee populations are declining in North America (with some declining over 90%) [7, 8] and Europe [9], and managed honey bee colonies have steadily declined in the United States [10]. While it is unlikely that one single stressor is responsible for bee decline, research has shown that
the combination of lack of floral resources driven by land-use change, parasites, and pesticides is driving major bee population reduction in both natural and agricultural regions [11]. Specifically, much of the current research is focused on pathogen spillover from commercial bee colonies [12], Deformed Wing Virus and its vector, *Varroa destructor* [13], nontarget exposure to neonicotinoid pesticides [14], and habitat loss [15]. While these stressors are likely the major causes of bee decline, bees face other environmental pressures such as metal or metalloid exposure when foraging on plants growing in polluted areas [16, 17]. Because bees may face many simultaneous and diverse stressors [18, 19], more research into neglected stressors such as metals and metalloids should be conducted.

Heavy metals, metalloid, and other xenobiotic contamination can be found in industrialized areas around the world [20, 21]. For example, the non-essential heavy metal cadmium can be found in soils near heavy industries such as mining and battery production [22], copper can be deposited from mining [23], selenium (both elemental and ionic forms) through lubricant production and other industries [24], and neonicotinoid pesticides from agriculture [14]. As mentioned previously, bees can come into contact with compounds such as those listed above through foraging on plants growing in contaminated areas, and bees have been shown to bring those chemicals to their colony [16]. For example, *Stanleya pinnata* can biomagnify selenate and these plants have been found to contain over 2000 mg/kg selenate [25]. As an even more extreme example, *Chamaecrista fasciculata* can accumulate cadmium to over 4000 mg/kg [26]. Bees appear to tolerate levels of metals and metalloids similar to other insects, however larvae
are particularly sensitive, which may cause negative colony-wide effects and ultimately cause the colony to collapse [27–30]. Bees generally cannot detect the presence of metals in flowers, as bees will forage on selenate- [31] and aluminum-containing [32] nectar, although bees seem to avoid nectar containing nickel [32]. Aside from mortality, metals exposure can cause behavioral and metabolic effects: copper alters feeding behaviors [33], manganese increases foraging time [17], multiple heavy metals increase cellular detoxification and redox activity [19, 34, 35], and metal exposure reduces immunocompetence [36].

Eusocial corbiculate bees – which includes the honey bees, bumble bees, and stingless bees – harbor simple and distinct microbiomes that are fairly consistent in colonies worldwide [37, 38]. This close microbial relationship in corbiculate bees is in contrast to the largely environmentally-acquired microbes that solitary and primitively eusocial non-corbiculate bees associate with [39–41]. It is thought that the bee microbiome is transmitted largely through contact with colony mates [42], and is the result of a long history of symbiosis between the host and microbes [37]. Over 95% of the honey bee gut bacterial community is populated by nine species clusters [43]: *Snodgrassella alvi* [44], *Gilliamella apicola* [44], two lactobacilli clades (*Lactobacillus Firm*-4 and Firm-5) [38, 45], *Frischella perrara* [46], *Bartonella apis* [47], *Bifidobacterium asteroides* [48], *Parasaccharibacter apium* [49], an Acetobacteraeaceae clade now thought to be *Commensalibacter intestini* [38], and *Bombella apis* [50]. Bumble bees have a distinct microbiota that differs somewhat from honey bees but shares the same ancestry [37]: *Bombus* species tend to harbor *Snodgrassella alvi, Gilliamella*
*apicola, Lactobacillus* Firm-4 (generally less abundant than in honey bees) and Firm-5, and Acetobacteraceae clade bacteria, while possessing their own unique species of *Bifidobacterium* [51], *Bombiscardovia coagulans* [52], and *Candidatus Schmidhempelia bombi* [53]. The bumble bee microbiome appears more labile than the honey bee microbiome, as there may be other bumble bee gut enterotypes present [54]. Aside from the “core” microbiota, social bees associate with other species of bacteria often present in the colony or environment, such as *Lactobacillus kunkeei* [55] and species within the family *Enterobacteriaceae* [56]. While the microbiomes of social bees are largely consistent, considerable strain variation exists in many of the major symbionts, so deeper analyses of these microbiomes is needed [57–59].

The symbiotic bee microbiome has been shown to provide numerous benefits to the bee host including defense against pathogens such as trypanosomes [60–62] and bacteria [63], immune system stimulation [64], body mass gain [65], and the digestion of pectin and toxic sugars [66, 67]. Similarly, when the microbiome is in a state of dysbiosis or absent, bees are more susceptible to bacterial or fungal infections [68, 69], although strains of core bacteria may positively associate with *Nosema ceranae* infections so the intricacies of host/microbe/pathogen still need further study [70, 71].

Investigating the effects of metals pollution on animal-associated microbes is becoming an important sub-field of microbial ecology and ecotoxicology [72, 73], and bees are well-suited to this type of research due to their simple rearing conditions and consistent microbiome [74]. Research in the field of metal and metalloid exposure on animal microbiomes is gaining popularity and impact: for example, studies have shown
that cadmium exposure affects the microbiomes of earthworms [75] and rats [76], copper on mice [77], and selenium in mice [78, 79] and bumble bees [80]. In addition to shifting the composition of the host-associated microbiome, recent research has begun investigating the potential for the animal microbiome to protect the host against metals and metalloid toxicity. It has been shown that animal microbiomes reduce mortality in insects challenged with chromium and lead [81], and in mice challenged with arsenic [82], copper, [83], cadmium, [84, 85], chromium [86], and aluminum [87], and we extend this growing body of research to bee-associated bacteria through work in this dissertation.

The major goal of this dissertation is to test novel hypotheses involving the exposure of metals, metalloids, and other toxicants on bumble bees and honey bees, their symbiotic microbiomes, and isolated microbes: 1) Does selenate exposure affect the bumble bee microbiome and its major symbionts, and does the microbiome reduce mortality upon selenate challenge? 2) Is cadmium, copper, selenate, imidacloprid, or hydrogen peroxide harmful to bumble bees, do these chemicals affect the bee microbiome, what genes are present in bee symbionts that may affect toxicant tolerance or susceptibility, and is there interstrain variation in these genes in major bee symbionts? 3) Does cadmium or selenate exposure affect the honey bee microbiome and metabolome, and can bee-associated bacteria bioaccumulate these chemicals?

We attempt to test the above-mentioned hypotheses through the experiments spanning the following three dissertation chapters: Chapter two investigates the effects that selenate exposure has on the bumble bee microbiome through 16S rRNA gene sequencing, in vitro exposure to two major gut symbionts – Snodgrassella alvi and
*Lactobacillus bombicola* – and the ability of the microbiome to reduce mortality upon selenate challenge. Chapter three assays the effects of cadmium, copper, selenate, imidacloprid, and hydrogen peroxide on bumble bee mortality and the composition of the microbiome through 16S rRNA gene sequencing. We also survey possible genetic bases in detoxification or tolerance in the genomes of 120 total strains of the major gut symbionts *Snodgrassella alvi* and *Gilliamella apicola* through Rapid Annotation using Subsystem Technology (RAST) genome annotations [88]. Lastly, Chapter four examines the longitudinal effects of selenate and cadmium exposure on the honey bee microbiome through 16S rRNA gene sequencing, the consequences of these exposures on the honey bee metabolome through untargeted metabolomics, and the bioaccumulation potential of nine strains of bee-associated bacteria through *in vitro* culturing and inductively coupled plasma optical emission spectroscopy (ICP-OES).
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Chapter Two:

The bumble bee microbiome increases survival of bees exposed to selenate toxicity
Abstract

Bumble bees are important and widespread insect pollinators who face many environmental challenges. For example, bees are exposed to the metalloid selenate when foraging on pollen and nectar from plants growing in contaminated soils. As it has been shown that the microbiome of animals reduces metalloid toxicity, we assayed the ability of the bee microbiome to increase survivorship against selenate challenge. We exposed uninoculated or microbiota-inoculated Bombus impatiens workers to a field-realistic dose of 0.75 mg/L selenate and found that microbiota-inoculated bees survive slightly, but significantly longer than uninoculated bees. Using 16S rRNA gene sequencing, we found that selenate exposure altered gut microbial community composition and relative abundance of specific core bacteria. We also grew two core bumble bee microbes – Snodgrassella alvi and Lactobacillus bombicola – in selenate-spiked media and found that these bacteria grew in the tested concentrations of 0.001 mg/L to 10 mg/L selenate. Furthermore, the genomes of these microbes harbor genes involved in selenate detoxification. The bumble bee microbiome slightly increases survivorship when the host is exposed to selenate, but the specific mechanisms and colony-level benefits under natural settings require further study.
Introduction

Bumble bees (*Bombus* spp.) are important insect pollinators for a multitude of food crops and native plants [1]. Commercially, over one million bumble bee colonies are used annually to pollinate high-value greenhouse crops such as tomatoes and peppers [2]. Recently, it has been established that many wild bumble bee populations are steadily declining in North America [3] with some species having declined over 90% [4]. European bumble bees are simultaneously facing serious decline [5]. Much of these declines have been attributed to exposure to land use change, pesticides [6], parasites and pathogens [7], and heavy metals [8].

Selenium pollution is a worldwide problem stemming from industries such as mining, coal combustion and lubricant production as well as the leaching of selenium from seleniferous soils through rainfall or agricultural irrigation [9]. Plants growing in selenium-contaminated areas can accumulate high levels of the metalloid in their pollen and nectar which, once foraged upon by bees and other insect pollinators, can be toxic [10]. For example, in 2011, Quinn *et al.* found that bumble bees are not deterred from foraging on the selenium-accumulating plants *Brassica juncea* and *Stanleya pinnata* (flowers were found to contain up to 3200 mg/kg selenium) and that elevated levels of selenium accumulated both in their corbicula pollen and inside of the foraging bees [11]. Likewise, in 2012, Hladun *et al.* found that honey bees (*Apis mellifera*) readily foraged on *Raphanus sativus* plants that were grown in seleniferous soil and collected pollen containing up to 2830 mg/kg selenium [12]. Accumulating selenium in the tissues of
foraging honey bees has been shown to be detrimental to both the individual forager bees plus the health of the whole colony [13–15]. Metals may also have sub-lethal effects on bees, as foraging efficiency is decreased when honey bees are exposed to manganese [16] and bumble bees are exposed to nickel [17, 18].

Selenium-tolerant microbes have been identified in a wide variety of environments, including bacteria isolated from beetle larvae [19], polluted water [20], mangrove soil [21], bioreactors [22], and endophytic bacteria from hyperaccumulator plants [23]. Additionally, some bacteria are known to reduce pernicious selenate and selenite ions to elemental selenium, thus significantly reducing toxicity [24], and facilitating removal from the bacterial cell [25]. Not all bacteria, however, can tolerate metals and metalloids. For example, the microbiota of mice can be altered when exposed to lead, cadmium [26] or selenium [27]; human gut microbes change in response to arsenic and lead [28]; and the gut microbial community of the Mongolian Toad is affected by a combination of copper, cadmium, zinc and lead [29]. In light of these studies, using the microbiome to reduce metalloid toxicity is now starting to be explored, with Coryell et al. establishing that the mouse gut community assists in reducing host mortality upon arsenic exposure [30].

Previous research shows that the microbial associates of insects can detoxify some metals from the environment. In 2013, Senderovich and Halpern found that the bacteria associated with the pollution-tolerant chironomid midges (Diptera: Chironomidae) detoxified lead and hexavalent chromium, which potentially reduces metals’ harmful effects [31]. Likewise, Wang et al. showed that a strain of Alcaligenes
*faecalis* isolated from beetle larvae (*Monochamus alternatus*) reduced selenite to the less toxic form of elemental selenium [19]. Still, there is little published research on the effects of selenium exposure on the microbiome of insects, despite the pervasive nature of selenium contamination in the environment [32] and the importance of considering the microbiome of insects in entomological studies [33]. We seek to address this gap in the literature using bees, as in addition to being an emerging as a model for this type of research [34], bees are frequently exposed to toxicants such as selenium when foraging, while the effects of environmental pollution on bumble bees and other wild bees is still an understudied field [35].

Bumble bees are known to host a simple and distinct gut microbiome that comprises core bacterial species within the genera *Snodgrassella*, *Gilliamella*, *Lactobacillus*, *Bombiscardovia*, *Schmidhempelia* and *Bifidobacterium* [36–40]. The honey bee and bumble bee microbiota is transmitted throughout the colony by social interactions between nest mates [36] resulting in host specific relationships within each clade of the corbiculate Apids (subfamily: Apinae) [38]. The microbiota has been shown to defend bumble bees against pathogens such as *Crithidia* spp. [36, 41] and microbial dysbiosis of the core microbes has been suggested to encourage *Nosema* spp. establishment in honey bees [42]. Likewise, the honey bee microbiome is known to positively affect host health, by promoting weight gain [43], metabolizing toxic sugars [44], degrading pectin [45] and stimulating immune function [46].

Here we investigate the complex interplay between selenate exposure and the bumble bee microbiome using *in vitro*, *in vivo* and *in silico* methods. First, we ask: Does
the *Bombus impatiens* microbiome increase survivorship against selenate toxicity? Second, are there effects of selenate exposure on the bees’ microbial gut community and individual bacterial strains? Third, is there natural resistance against selenate exposure in the bumble bee core gut bacteria *Snodgrassella alvi* and *Lactobacillus bombicola in vitro*, and is there a possible genomic basis of bacterial selenate tolerance?
Materials and Methods

Bee Husbandry for the Selenate-Challenge Experiment

We conducted two separate experiments to assay the effects of microbiome inoculation on selenate-challenged bees. For a pilot experiment (Experiment 1, see below for experimental design details), we obtained three commercial Bombus impatiens colonies from the Biobest Group (Biobest USA Inc., Romulus, MI), and for the fully factorial experiment (Experiment 2, see below for experimental design details), we obtained four commercial B. impatiens colonies from Koppert Biological Systems, Inc. (Howell, MI). Each colony contained approximately 50 workers, a gravid queen, pollen and a proprietary sugar solution. As the supplied sugar solution typically contains antibiotics and antifungal compounds [47], we immediately replaced it with sterile 60% sucrose and allowed the bees access ad libitum. We also provided the colony with pollen patties ad libitum and kept the colonies in environmentally-controlled rooms at 29°C under constant darkness at the University of California, Riverside. We allowed the colonies to grow undisturbed for two weeks before starting the experiment.

Uninoculated and Microbiome-Inoculated Bumble bees

In order to manipulate the bumble bee gut microbiota, we used a modified version of previously-described protocols [36, 43, 48]. We removed dark-colored cocoons from
each of the three bee colonies and aseptically extracted the pupa from within each cocoon. We then placed pupae in a sterile, 48-well tissue culture plate (Corning, Corning, NY) and incubated the plates at 29°C with 70% humidity. Once the bees had eclosed, we placed them into cohorts of 6 colony mates in 475mL polypropylene containers (WebstaurantStore, Lancaster, PA). We then autoclaved a mixture of 40% sucrose and pollen and provided the bees with this mixture ad libitum for two days.

To determine whether a healthy microbiota increases survivorship in bumble bees under selenate challenge, we fed selenate or a sterile sucrose control to bees that we either inoculated with gut microbes or left uninoculated. In order to inoculate bees with a stable microbial community, we fed cohorts of bees microbes harvested from workers corresponding to their source colony. To do this, we aseptically dissected the whole guts from three mature workers and macerated these in an autoclaved 40% sucrose and pollen solution. We then fed this homogenate ad libitum to the bees for two days, followed by sterile 40% sucrose for a total of five days to allow the microbes to stably colonize the gut [49]. The uninoculated bees did not receive microbes and were given a solution of sterile 40% sucrose and sterile pollen only.

Selenate Exposure Challenge and Statistics

We conducted two separate experiments to determine if the microbiota can increase bumble bee survival when challenged with field-realistic levels of selenate. In a preliminary experiment (Exp. 1), we challenged \( N = 87 \) sham-inoculated bees and \( N = 68 \)
microbiota-inoculated bees with 0.75 mg/L selenate. This concentration was designed to represent exposure to a conservative concentration of selenate compared to what bees may naturally encounter when foraging upon plants in highly contaminated areas (up to 3200 mg/kg) [11], and from past greenhouse experiments (nectar up to 110 mg/kg and pollen 710 mg/kg) [10], as well as previously reported selenate toxicity to honey bees [15]. As this preliminary experiment did not control for the effects of inoculation on bumble bee survival, we ran a second fully factorial experiment that crossed two factors: microbiota or sham inoculation and selenate spiked sucrose feed or sucrose only feed to control for the effects of a microbiome on control bees (Exp. 2). We challenged 80 bees (40 microbe-inoculated and 40 uninoculated) assigned to cohorts of 4-7 colony mates with a solution of either 0.75 mg/L sodium selenate (Alfa Aesar, Ward Hill, MA) or 0 mg/L sodium selenate in 40% sucrose. In addition to the treatments, we also concurrently exposed 80 bees (43 microbe-inoculated and 37 uninoculated) bees to 40% sucrose with no selenate to serve as controls. We allowed bees to feed ad libitum for up to 10 days, censused mortality daily, and removed dead bees immediately upon discovery.

We analyzed the mortality data using the Cox Proportional Hazards function with Mixed Effects [50] on colony of origin, microbe-inoculation, and selenate treatment in R. We also checked to ensure that our data did not violate the proportional hazards assumptions of the Cox Regression with the function “cox.zph” in the R package “Survival,” [51], and graphed the survivorship data with the “survminer” package in R [52]. We used Schwartz’s Bayesian Information Criterion (BIC) to choose the model that best fit our data and compensated for mixed effects through a penalized log likelihood.
Effects of Sub-Lethal Doses of Selenate on the Bumble Bee Microbiome

To explore the effect of selenate on the bumble bee microbiome, we acquired three new bumble bee (*Bombus impatiens*) colonies containing less than 10 workers, a gravid queen, pollen and proprietary sugar solution (Koppert Biological Systems). We maintained the colonies in the same way as described above. We then isolated 20 individual mature workers from each colony (N=60 total) in 60mL polypropylene containers (WebstaurantStore, Lancaster, PA) and provided them either 60% sucrose (control, N = 30) or 60% sucrose spiked with 0.5 mg/L sodium selenate (treatment, N = 30). Bees fed *ad libitum* for four days before we assessed mortality and stored the bees at -80°C. We used these bees for DNA extraction and 16S rRNA gene amplicon sequencing as described below.

DNA Extraction and Next-Generation Sequencing of the Bacterial 16S rRNA gene

We used a modified DNA extraction protocol based on Engel et al. 2013, [53], Pennington et al. 2017 [54] and Pennington et al. 2018 [55]. Using sterile technique, we dissected whole guts out of each bee and placed them into 96-well bead-beating plates (Qiagen, Valencia, CA) containing 50-100 µL of 0.1mm glass beads, one 3.4mm steel-chrome bead (Biospec, Bartlesville, OK), and Qiagen lysis buffer, then homogenized the mixture with a Qiagen Tissuelyser at 30 Hz for 6 minutes. We included four blanks to control for contamination, which were included in all library preparation and sequence
processing steps. We extracted total DNA from each sample with the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) by following the manufacturer’s protocol for tissue samples.

We prepared Illumina MiSeq libraries for paired-end sequencing as in McFrederick and Rehan 2016 [56], Pennington et al. 2017 [57], and Rothman et al. 2018 [58]. We incorporated the genomic DNA primer sequence, an eight-mer barcode sequence, and Illumina adapter sequence as in [59]. We used the primers 799F-mod3 (CMGGATTAGATACCCCKGG) [60] and 1115R (AGGGTTGCGCTCGTTG) [59] to amplify the V5-V6 region of the 16S rRNA gene. We used the following reaction conditions for PCR: 4 µL of DNA, 0.5 µL of 10µM 799F-mod3 primer, 0.5 µL of 10µM 1115R primer, 10 µL sterile water and 10 µL 2x Pfusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), an annealing temperature of 52°C, and 25 cycles in a C1000 Touch thermal cycler (BioRad, Hercules, CA). We then used the PureLink Pro 96 PCR Purification Kit (Invitrogen, Carlsbad, CA) to clean up the resulting amplicons. We subsequently performed a second PCR reaction using 1 µL of the cleaned PCR amplicons as a template with the primers PCR2F (CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC) and PCR2R (AATGATACGGCGACCACCGAGATCTACACTCTTTCTAATGACAGC) to generate the Illumina adapter sequence [59]. We performed PCR with the following reaction conditions: 0.5 µL of 10µM PCR2F primer, 0.5 µL of 10µM PCR2R primer, 1 µL of cleaned PCR amplicon, 13 µL of sterile water and 10 µL of 2x Pfusion High-Fidelity DNA polymerase for 15 cycles at an annealing temperature of 58°C. We used 18
µL of the resulting amplicons for normalization with the SequlPrep Normalization kit and followed the supplied protocol (ThermoFisher Scientific, Waltham, MA). We pooled 5 µL of each of the normalized library and performed a final clean up with a PureLink PCR Purification Kit (Invitrogen, Carlsbad, CA). We then quality checked the amplicons on a 2100 Bioanalyzer (Agilent, Santa Clara, CA) and sequenced the libraries using a V3 Reagent Kit at 2 X 300 cycles on an Illumina MiSeq Sequencer (Illumina, San Diego, CA) in the UC Riverside Genomics Core Facility.

Raw sequencing data are available on the NCBI Sequence Read Archive (SRA) under accession numbers SRR6788889-SRR6788898, SRR6788969-SRR6788978, SRR6788989- SRR6789000 and SRR6789009-SRR6789022.

16S rRNA Gene Quantitative PCR for Bacterial Abundance in Bees

We used quantitative PCR (qPCR) to validate our methods of rearing uninoculated or microbe-inoculated bees. We extracted DNA in the same way as above from individual control-treated bees that survived the full 10 days, as there was likely bacterial proliferation or degradation in bees that died during the experiment. We then ran 16S rRNA gene qPCR on the extracted DNA in triplicate using the following recipe: 2 µL DNA, 5 µL SsoAdvanced master mix (BioRad, Hercules, CA), 0.2 µL 10 µM forward primer (TCCTACGGGAGGCAGCAGT), 0.2 µL 10 µM reverse primer (GGACTACCAGGTATCTAATCCTGTT) [61], and 2.6 µL of sterile ultrapure water. We used a protocol consisting of an initial denaturation step of 95 °C for three minutes,
followed by 95 °C for 10 seconds and an annealing/extension step of 59 °C for 30 seconds repeated 39 times on a BioRad C1000 Touch thermal cycler. We compared our samples to a standard curve of $1 \times 10^2 - 1 \times 10^8$ copies of the 16S rRNA gene cloned into a TOPO-TA plasmid (Invitrogen, Carlsbad, CA), with all qPCR efficiencies between 90% - 100% and $R^2$ above 0.98 and tested our data for statistical significance using Welch’s two-tailed t-test in R. Lastly, we validated the DNA extractions by running PCR targeting a region of the bee 18S rRNA gene on each sample and verifying that there was a positive band on an agarose gel, as in Meeus et al. 2009 [62].

Bioinformatics and Statistics

We used QIME2-2017.12 [63] to process the 16S rRNA gene sequences. We viewed the sequence quality of our sequences and removed the low-quality ends. Then, we used DADA2 [64] to identify exact sequence variants (ESVs; 16S rRNA gene sequences that are identical), remove chimeric sequences and quality filter the data. We assigned taxonomy to the ESVs using the q2-feature-classifier [65] with the SILVA 16S rRNA gene database [66]. We also conducted local BLASTn searches against the NCBI 16S ribosomal RNA sequences database (accessed March 2018). We then removed reads matching mitochondria and contaminants [67] as identified in our blank samples from the feature tables. After filtering out contaminants, we aligned the representative sequences against the SILVA reference alignment with MAFFT [68] and generated a phylogenetic tree using FastTree v2.1.3 [69]. We used this tree and the filtered feature table to analyze
alpha diversity, sampling depth, and to generate a Generalized UniFrac distance matrix [70]. We visualized the UniFrac distance through Principal Coordinates Analysis (PCoA), Non-metric Multidimensional Scaling (NMDS), and used R v3.4.1 [71] to plot the data. We analyzed the alpha diversity of our samples through the Shannon Diversity Index and assessed statistical significance through the Kruskal-Wallis test in QIIME2. We also used the R packages “vegan” [72] to test for statistical significance through Adonis on the distance matrix, “ggplot2” for graphing and “DESeq2” to analyze differentially abundant ESVs representing at least 1% proportional abundance between treatments [73]. To minimize the likelihood of a Type I error due to differential data dispersion in our Adonis testing, we analyzed the distance matrix with PERMDISP (permutational dispersion of beta diversity with 999 permutations).

Bacterial Culture Conditions and Inhibitory Concentration Analyses

To determine inhibitory concentrations of field-realistic doses of selenate on two representative members of the bumble bee core gut microbiota, we grew liquid cultures of *Snodgrassella alvi* wkB12 in Tryptic Soy Broth (TSB) (Becton, Dickinson and Co., Franklin Lakes, NJ) and *Lactobacillus bombicola* DSM-28793 in De Man, Rogosa and Sharpe + 0.05% cysteine (MRSC) broth (Research Products International, Mt. Prospect, IL). We incubated each culture at 37°C under a 5% CO₂ atmosphere inside a Type C Biobag (Becton, Dickinson and Co., Franklin Lakes, NJ) with a CO₂ generation ampule and grew the cultures to an OD₆₀₀ of 1.0. We then transferred 1 µL of the cultures to 199
µL of TSB or MRSC spiked with five concentrations of sodium selenate (0.001 mg/L, 0.01 mg/L, 0.1 mg/L, 1.0 mg/L and 10 mg/L) in triplicate along with 0 mg/L selenate controls and media blanks under the same conditions as above. We allowed the cultures to grow for 48 hours and read the cultures’ OD₆₀₀ with a VarioSkan Lux microplate reader (ThermoFisher Scientific, Waltham, MA). We then tested statistical significance with one-way ANOVA, Tukey’s HSD post-hoc testing, and normality through the Shapiro-Wilk test with the R package “car” [74].

Genomic Basis of Resistance to Selenate

We used the National Microbial Pathogen Data Resource’s Rapid Annotations using Subsystem Technology (RAST) server to annotate the publicly available genomes of bacteria usually found within the bumble bee gut and other bacteria that were found to be differentially abundant between selenate exposure and controls [75, 76]. We then searched through the genomes’ subsystems for genes encoding selenium-containing proteins and functional genes corresponding to selenate reductases, selenocysteine acid metabolism, and the genes involved in uptake of selenate/selenite. To verify the accuracy of the RAST annotations, we also searched for protein homology using Swiss-Prot [77] and considered proteins with greater than 50% amino acid sequence identity to be homologous. Accession numbers for each representative strains’ assembled genome are as follows: *Bifidobacterium bohemicum* DSM-22767 (GCA_000741525.1),
*Bifidobacterium bombi* DSM-19703 (GCA_000737845.1), *Bifidobacterium commune* R-
52791 (GCA_900094885.1), Bombiscardovia coagulans DSM-22924
(GCA_002259585.1), Commensalibacter intestini A911 (GCA_000231445.2),
Gilliamella apicola wkB30 (GCA_000695585.1), Gilliamella bombi LMG-29879
(GCA_900103255.1), Gilliamella intestini R-53144 (GCA_900094935.1), Lactobacillus
apis Hma11 (GCA_000970735.1), Lactobacillus bombicola R-53102
(GCA_900112665.1), Lactobacillus mellis Hon2 (GCA_000967245.1), Candidatus
Schmidhempelia bombi Bimp (GCA_000471645.1), Serratia marcescens WW4
(GCA_000336425.1), and Snodgrassella alvi wkB12 (GCA_000695565.1).
Results

The Bumble Bee Microbiome Increases Survival Against Selenate Toxicity

In both the preliminary experiment (Exp. 1) and the fully factorial experiment (Exp. 2), the inoculated bumble bee microbiome significantly increased bee survival when exposed to selenate. In the preliminary experiment (Exp. 1) the inoculated microbiome significantly increased bee survival ($N = 155, Z = -3.27, P = 0.001$); (Cox mixed-effects model fitted with penalized log-likelihood: $\chi^2 = 54.34, \text{d.f.} = 2.7, P < 0.001$, BIC = 40.70), with microbiome-inoculated bees experiencing a 42% increase in mean survival, although no bees lived the full 10 days. This result was replicated in Exp. 2 ($N = 160, Z = -3.12, P = 0.002$); (Cox mixed-effects model fitted with penalized log-likelihood: $\chi^2 = 88.81, \text{d.f.} = 3.6, P < 0.001$, BIC = 73.00) with proper controls. We continued Exp. 2 for 10 days and found that selenate-challenged bees inoculated with a microbiome experienced a 20% increase in mean survival (Fig. 2.1). In the absence of selenate exposure microbe inoculation did not significantly affect mortality when compared to uninoculated bees ($N = 80, Z = -0.57, P = 0.57$). Our selenate exposure data did not violate the assumptions of the Cox Proportional Hazards Model by inoculation treatment ($\rho = -0.09, \chi^2 = 0.63, P = 0.43$), colony of origin ($\rho = -0.05, \chi^2 = 0.26, P = 0.68$), selenate treatment ($\rho = 0.19, \chi^2 = 3.61, P = 0.06$) or globally ($\chi^2 = 4.53, P = 0.48$).

Lastly, we verified that the uninoculated bees had depauperate microbiota compared to inoculated bees in our fully factorial experiment (Exp. 2) through qPCR
targeting the bacterial 16S rRNA gene on our control samples as in Powell et al 2014 and Kesnerova et al 2017 [49, 78]. Inoculated bees had a mean 16S rRNA gene copy number of $6.88 \times 10^8$ versus $8.89 \times 10^4$ for uninoculated bees (Welch’s two-tailed t-test, $t = 3.13$, $P = 0.004$).

Sub-Lethal Selenate Exposure Alters the Microbiome of Bumble Bees

Alpha Diversity and Library Coverage

There was a total of 276,126 quality-filtered reads with an average of 5,210 reads per sample ($N = 53$) that were clustered into 86 filtered Exact Sequence Variants (ESVs). Through rarefaction analysis, we determined that we had representative coverage of bacterial species diversity at a depth of 2,385 reads per sample. We found that there was a significant increase in the alpha diversity (as measured by the Shannon Diversity Index) of the bees’ microbial community when treated with sodium selenate ($H = 7.95$, $P = 0.005$).

Beta Diversity and Differential Abundance of Bacterial Taxa

In order to discern patterns in the beta diversity of the whole microbial gut community of the bumble bees, we plotted the relative proportional abundance of ESVs comprising at least 1% of each sample (Fig. 2.2). Overall, we found our samples were
dominated by the genera *Snodgrassella, Gilliamella, Lactobacillus, Bifidobacterium, Commensalibacter, Bombiscardovia* and *Serratia*. We also performed Principal Coordinates Analysis (PCoA) and Non-Metric Multidimensional Scaling (NMDS) analysis on the Generalized UniFrac distance matrix that compared selenate-treated bees with controls (Fig. 2.3). Overall, there was no obvious clustering by treatment in both the two-dimensional NMDS (stress = 0.18) and PCoA ordinations. As we performed the experiment on individual bees from three separate colonies, we then analyzed the UniFrac distance matrix with Adonis (PERMANOVA with 999 permutations) using both treatment and colony of origin as explanatory variables in the model. We found that there was a significant effect of selenate treatment ($F = 2.9, R^2 = 0.05, P < 0.001$), colony ($F = 3.30, R^2 = 0.12, P < 0.001$) and interaction between treatment and colony ($F = 1.87, R^2 = 0.07, P = 0.005$) after four days of continuous exposure, and that our data was not heterogeneously dispersed ($F = 0.89, P = 0.35$). Our analyses found that while there are significant effects of treatment and colony, the small $R^2$ indicates that the impact of treatment on the overall beta diversity is slight, and the more physiologically important effects of selenate exposure are likely found at the individual ESV level.

We analyzed the 16S amplicon data with “DESeq2” to identify ESV changes within the bumble bee microbiome. Through analyzing ESVs present at greater than 1% proportional abundance, we found nine differentially abundant ESVs (Benjamini and Hochberg corrected for multiple comparisons $P_{adj} < 0.05$) between selenate-treated bees and controls in the following genera: 2 ESVs of *Commensalibacter intestini*, 3 ESVs of *Gilliamella apicola*, 2 ESVs of *Lactobacillus bombicola* and 2 ESVs of *Snodgrassella*
alvi. Each of the ESVs were less proportionally abundant in selenate-treated bees except C. intestini (Fig. 2.4).

Genes Involved in Selenium Ion Uptake and Processing

By using RAST subsystem analyses and UniProt BLAST searches, we identified the presence or absence of genes that encode the production of selenium-containing proteins, selenate reductases, selenocysteine metabolism and genes involved in selenium ion uptake/release. We found that G. apicola wkB30 and S. alvi wkB12 only had one putative selenocysteine-containing enzyme each (both had formate dehydrogenase EC 1.17.1.9 based on sequence homology), while no other bacteria commonly found in bumble bee guts or our samples contained any selenoproteins. Many of the non-bumble bee-specific taxa had at least some active selenoproteins. Each of the other core bumble bee gut bacteria or non-core bacteria that were differentially abundant in our study had putative genes corresponding to the sulfate and thiosulfate import ATP-binding protein CysA [79], the putative [80] selenite-inducible transporter TsgA [80], the selenate/selenite transporter DedA [81], L-seryl-tRNA(Sec) selenium transferase SelA [82], the selenocysteine-specific translation elongation factor SelB [83], the selenide/water dikinase SelD [84] and the selenophosphate-dependent tRNA 2-selenouridine synthase 2-SeU [85]. Only Candidatus Schmidhempelia bombi had no enzymes for the uptake or release of selenium ions or selenoprotein metabolism.
Members of the Bumble Bee Microbiota React Differently to Selenate Exposure

Through ANOVA testing, we did not detect an overall significant difference in bacterial growth after 48 hours for either *Snodgrassella alvi* wkB12 (*F*(5,12) = 2.389, *P* = 0.101) or *Lactobacillus bombicola* (*F*(5,12) = 0.282, *P* = 0.914), at any dose of sodium selenate (Tukey’s HSD *P*~adj~ > 0.05 for each concentration) (Fig. 2.5). Our data did not violate the assumption of normality, based on the Shapiro-Wilk Test (*P* = 0.892 and 0.613, respectively). We note that these bacteria grew somewhat poorly, indicating that our culturing conditions are not optimal for bee symbionts. Similar OD readings, however, have been reported in other studies using different strains of these bacteria [86].
Discussion

The *Bombus impatiens* microbiome plays a role in the reduction of host mortality when bees are exposed to field-realistic doses of selenium. We found that selenate toxicity was slightly higher in uninoculated bees than in those that we inoculated with a microbial community obtained from their colony mates, and that lacking a microbiome did not affect survivorship of bees receiving only control treatments. To the best of our knowledge, this is the first time that the gut microbiome of any insect has been shown to increase the survivorship of its host against selenate poisoning. We show that while ingestion of selenate inflicts mortality on all tested bumblebees, the microbiome-inoculated bees have slightly reduced mortality, which on a wider colony level, may have a positive effect on resource-gathering, colony hygiene, and ultimately overall colony health. Future research into the colony-level effects of selenate poisoning on bumble bees should be investigated in a similar manner to Hladun et al 2015 [14], who showed that selenate had a deleterious effect on honey bee colony health.

Other work has linked the insect microbiota to metal/metalloid detoxification. Senderovich and Halpern [31] showed that bacteria associated with *Chironomus transvaalensis* egg masses and larvae reduced lead- and hexavalent chromium-induced mortality. Our research extends this work, as *Chironomus* spp. typically exhibit a lifestyle whereby adults do not feed [87], do not possess a functional microbiome, and are unlikely to orally ingest pollutants. Likewise, Wang *et al.* recently showed that a strain of bacteria isolated from beetle larvae can reduce selenite [19], although as beetles undergo
complete metamorphosis, this bacteria is unlikely to persist into the adult stage. These are important distinctions, as our study shows that the microbiome of adult bumble bees increases survivorship upon metalloid challenge during their final life stage. Furthermore, socially acquired core bumble bee gut bacteria appear to mainly drive the increase in survivorship. As pupal bees shed their larval gut and lose any alimentary tract bacteria upon eclosion [36], any microbially-mediated effect on larvae may be lost before the adult bees are exposed to the toxicant. Additionally, as we are studying coevolved, socially-transmitted core microbes, these symbiotic bacteria share an intricate relationship with their host that includes defensive functions [36]. Our system serves as a useful model for studying transmittable symbiont-induced phenotypes that increase survival against environmental toxicants in social insects. Lastly, it has been shown that the gut microbiota is involved in reducing arsenic-induced mouse mortality [30], and our research contributes to the growing body of literature that implicates the animal microbiome in increased host survival when challenged with metalloids.

We found ESVs of the gut symbionts *S. alvi*, *G. apicola* and *L. bombicola* in lower proportional abundance in selenate-treated bees versus controls. We also found two *C. intestini* ESVs in higher proportional abundance of in selenate-treated bees. This contrasts with our culture-based results in which *S. alvi* and *L. bombicola* were not affected by field-realistic, low doses of selenate. This conflicting result may be due to the compositional nature of microbiome data [88] in which other changes in proportional abundance may not reflect changes in absolute abundance. Likewise, these bacteria grew poorly in culture, which may mask the true effects of dose-dependent selenate exposure
as an artifact, although strains of *S. alvi* and other Firm-5 lactobacilli have grown to similar OD$_{600}$ readings in previous studies [86]. We also cannot examine genomic differences between cultured strains and taxa identified in our bee samples as we are unable to discern their entire genomes from a 16S rRNA gene sequencing survey. Strain level diversity in the honey bee gut microbiota is high [45], and future experiments are needed to fully understand selenotolerance in the bumble bee microbiota. While there is no published research on the interactions of bee symbionts and selenium, non-bee-associated bacteria are known to accumulate selenium in culture (Calomme, Van den Branden, and Vanden Berghe 1995), or can respire less toxic elemental selenium [19, 24, 25]. This may be a mechanism for increasing host survivorship upon selenate challenge, and future studies should investigate the ability of symbionts to accumulate or respire selenium.

There are interesting patterns that develop when examining the genomes of bacterial genera typically associated with bumble bees: *Bifidobacterium* spp., *Bombiscardovia coagulans* and *Lactobacillus* spp. appear only to uptake selenium ions via the transporter DedA. *Snodgrassella alvi* wkB12 possesses DedA along with CysA, which is involved in selenium ion transport and can incorporate selenocysteine into proteins which may contribute to selenate resistance. Candidatus *S. bombi* and *G. apicola* wkB30 also use selenocysteine but lack any obvious method of selenate uptake, although *G. apicola* genomes may vary between strains [44] and more investigation into their selenium metabolism is needed. While most differentially abundant bacteria were less proportionally abundant in the selenate treatments, one taxon was notably more abundant:
Commensalibacter. The effects of selenium on this genus are unknown, although it does not possess DedA, but appears selenotolerant *in vivo*. Notably, we only obtained ESVs of *Commensalibacter* and the opportunistic bee pathogen *Serratia* (Sánchez-Bayo et al. 2016) from one colony, indicating this colony may be suffering from dysbiosis or disease and the apparent selenotolerance may be due to the compositional nature of our data.

Selenoproteins are common throughout several insects species, and their genomes often contain enzymes for selenium metabolism [89]. Conversely, bumble bees and other hymenopterans are not known to incorporate selenium into proteins [90]. However, the mechanisms of increased bee survival may be host-mediated, as gut microbes could induce changes in host gene expression to generally allow for detoxification. Likewise, selenate-induced stress may synergize with the lack of a microbiome in our uninoculated bees, and we may be observing the combination of multiple insults on bee health. This may decrease host survivorship rather than the microbiome itself increasing survivorship, and more research needs to be conducted to understand the mechanisms of multiple stressors on bees. Microbial inducement of the immune system has been shown in honey bees [46] and stimulation of detoxification gene expression and immune function has been shown to occur in bumble bees [91], so the presence of the bees’ microbiota may be influencing the bees ability to detoxify selenate. Lastly, the bacteria may simply be forming a physical barrier, but more research is needed to test these competing hypotheses.

Our results illustrate that the bumble bee microbiome slightly increases host survival when exposed to selenate and that bacteria within the core microbiome are
tolerant to field-realistic doses of selenate. Selenate causes shifts in the relative abundance of core microbes at the individual ESV level. As the mechanisms of the microbiome-induced increase in host survival upon selenate challenge are unknown, future research should investigate the ability of bacterial symbionts to metabolize and detoxify selenate in the host. Similarly, many bee species commonly encounter metal and metalloid contamination in the environment [8] and more studies are needed to assess the effects of other toxicants on their microbes.
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Figure 2.1: Survival plot of the fully factorial experiment 2. Microbiome-inoculated bees lived significantly longer than uninoculated bees when challenged with 0.75 mg/L sodium selenate ($N = 160, Z = -3.12, P = 0.002$). Microbe inoculation did not affect mortality when compared to uninoculated bees in our controls ($N = 80, Z = -0.57, P = 0.57$). Shaded areas signify 95% confidence intervals, and dashed lines indicate 50% survival probability.
Figure 2.2: Stacked bar plot showing the relative proportion of bacterial genera that were present at greater than 0.1% abundance in each sample. Individual sample treatments are indicated by “C” for control and “Se” for selenate exposure, and colony of origin is denoted by H3, H6 or H9.
Figure 2.3: A) Nonmetric Multidimensional Scaling (stress= 0.18) and B) Principal Coordinates Analysis plot of the Generalized UniFrac distance matrices of individual bumble bee worker guts when exposed to sodium selenate versus controls. Red points indicate control treatments and blue points denote selenate treatments. Colored ellipses designate 95% confidence intervals around the centroid median of the points.
Figure 2.4: Log$_2$fold change of the proportionally differentially abundant Exact Sequence Variants as measured by DESeq2 between selenate-treated bees and controls, colored by genus. We analyzed ESVs that were present in at least 1% proportional abundance, and each of the illustrated ESVs were found to be significantly different ($P_{adj} < 0.05$). Error bars denote the standard error of the Log$_2$fold change.
Figure 2.5: Bar plots of the effects of sodium selenate exposure on *Snodgrassella alvi* and *Lactobacillus bombicola* growth after 48 hours. Growth was not significantly affected for either *S. alvi* ($F_{(5,12)} = 2.389, P = 0.101$) or *L. bombicola* ($F_{(5,12)} = 0.282, P = 0.914$) at any concentration. Error bars denote standard error.
Chapter Three:

The effects of cadmium, copper, selenate, hydrogen peroxide, and imidacloprid on

the bumble bee microbiome and the lethality of these compounds
Abstract

Bumble bees (*Bombus* spp.) are important and widespread insect pollinators that can be exposed to environmental toxicants through foraging upon plants growing in contaminated soils. How these compounds affect the bumble bee microbiome, which confers many health benefits to the host, is an important but unknown aspect of bee health. As these toxicants take many forms, we exposed *Bombus impatiens* workers to varying concentrations of selenate, cadmium, copper, the neonicotinoid pesticide imidacloprid, and hydrogen peroxide spiked into their diet. We first measured each chemicals’ LC$_{50}$. Due to the importance of the microbiome, we also exposed bumble bee workers to sublethal concentrations of each chemical and sequenced the 16S rRNA gene to survey their gut microbial communities. We show that each compound tested except imidacloprid significantly altered the composition of the bee microbiome and may cause gut dysbiosis. Lastly, we annotated bumble bee symbiont genomes to understand the potential genomic basis for toxicant tolerance and show that there is inter-strain variation in the symbionts’ genomic repertoire. Overall, our study shows that environmental toxicants can cause bee mortality and affect the bee microbiome.
Introduction

Bumble bees (*Bombus* spp.) are valuable pollinators for both managed crops and wild flowering plants worldwide [1]. For example, wild bumble bees were found to provide sufficient pollination services at a majority of watermelon farms in the Eastern United States [2]. Over one million managed bumble bee colonies per year are responsible for the pollination of many greenhouse-grown crops [3]. It is therefore worrisome that bumble bee populations are declining in both Europe [4] and North America [5], with the main contributors likely being habitat destruction, pesticides, and disease [6, 7]. While these stressors are likely the major causes of bee decline, other environmental insults such as foraging on metal- and metalloid-contaminated food sources can negatively impact honey bees living in polluted areas [8, 9], and effects of these toxicants on other bee species such as bumble bees warrants further investigation.

Bumble bees harbor a distinct gut microbiome that is largely consistent worldwide and mainly contains members from the bacterial genera *Bombiscardovia, Bifidobacterium, Gilliamella, Lactobacillus, Candidatus Schmidhempelia*, and *Snodgrassella* [10–14], although a second, possibly pathogen-associated enterotype has also been identified [15]. As with other eusocial bees, this simple microbiota is mainly transmissible through contact between members of the colony, which ultimately maintains a strong symbiotic fidelity between the bees and their microbial associates [13]. While the members of the microbiome remain generally consistent, factors such as land cover and forage availability [16, 17], antibiotic exposure [18] *Nosema* infection, [19], and pesticides [20] have been shown to alter the proportions of individual taxa of
the gut microbial community. The bee microbiome positively affects bee health, including protection against the trypanosome pathogens *Crithidia* [11, 21] and *Lotmaria* [22], metabolism of toxic sugars and weight gain [23], immune system stimulation [24], and defense against the opportunistic bacterial pathogen *Serratia marcescens* [25]. Similarly, in the absence of a healthy microbiome, bees experience increased mortality [26], gut epithelial scabs [27], and possible multi-disease infections [28].

While flowers provide the nutrition that nearly all bees need to thrive, flowers can also harbor compounds that are toxic to bees. Some of the toxicants that occur in pollen and nectar are translocated from contaminated soils, while others are used as plant defenses. For example, selenium and its ions are found throughout many environments, having been deposited by industry and leaching from seleniferous soils [29]. Copper can be deposited from mining [30] and agricultural use as a broad-spectrum biocide [31]. Cadmium is a toxic, non-essential metal found in polluted soils near production of batteries, pigments, and metal alloys [32] and is considered a worldwide health concern. Bees also encounter non-metallic compounds such as imidacloprid, an extremely toxic manmade neonicotinoid insecticide widely used for pest control in agriculture [33] and hydrogen peroxide, a defense compound commonly found in flower nectar [34] that bees can encounter during floral visitation.

Bees can contact selenium ions, copper, or cadmium by foraging on the pollen and nectar of plants growing in contaminated areas, and in turn will bring these pollutants back to their colony [35]. For example, the hyperaccumulator plant *Stanleya pinnata* can biomagnify selenium into its nectar at concentrations over 2000 mg/L [36]. Little
research has been done on the toxicity of these elements on bumble bees, as only the
Median Lethal Concentration (the concentration of a substance that will kill 50% of a
population after a certain amount of time; hereafter LC$_{50}$) for cadmium has been
established in Bombus terrestris [37]. Similarly, the LC$_{50}$ of selenium ions, copper, and
cadmium is known in honey bees as reported by Hladun et al. in 2012 [38] and Di et al.
in 2016 [39] but has not been reported in B. impatiens. The LC$_{50}$ of imidacloprid has been
established for several species of bee including Apis mellifera, Bombus impatiens, Osmia
lignaria, and Megachile rotundata along with deleterious effects at sublethal
concentrations ranging from reduced feeding to inhibiting the proboscis extension
response to death [40], and there is no known literature on the lethality of hydrogen
peroxide on any bee species.

The effects of metals and metalloids on the microbiomes of animals is emerging
as an integral part of the ecotoxicology of harmful compounds [41]. Cadmium [42],
selenium [43], and copper [44] alter mouse microbiomes, while cadmium alters spider
and carp microbiomes [45, 46], and copper and cadmium affect Mongolian Toad
microbiomes [47]. The effects of imidacloprid have been shown to alter the larval
microbial community of Drosophila melanogaster [48], although conversely, did not
affect the microbiome of honey bees [49]. Lastly, reactive oxygen species (ROS; as
measured by hydrogen peroxide) reduced the abundance of alimentary tract bacteria in
Aedes aegypti mosquitos, which indicates a sensitivity of gut microbes to ROS [50]. The
microbiome potentially protects its host from metal(loid) toxicity: Chironomid-associated
bacteria detoxified chromium and lead [51], in mice models, microbes detoxified copper
[52], and arsenic [53], beetle larvae-associated bacteria reduced selenite [54], and the microbiome of bumble bees reduced selenate-induced mortality [55].

Given the importance of environmental toxicants and the microbiome in host health, we investigated the interactions between multiple poisons, *B. impatiens*, and its associated microbes. We addressed three major questions: First: What is the lethality of these toxicants to bumble bees? Second: Does selenate, copper, cadmium, imidacloprid or hydrogen peroxide affect the bumble bee microbial community and if so, how? Third: Are there genes associated with detoxification to those compounds in major bumble bee gut symbionts and do those genes vary between strains of *Snodgrassella alvi* and *Gilliamella apicola*?
Materials and Methods

Bumble bee rearing and determination of the bumble bee LC$_{50}$ for each compound

In order to establish the LC$_{50}$ (concentration of a substance that causes mortality in 50% of a population) we purchased 10 new commercial bumble bee (*Bombus impatiens*) colonies from Koppert Biological Systems, Inc. (Howell, MI) that contained a mated queen, pollen, and proprietary sugar solution. We immediately replaced the proprietary sugar solution with 60% sucrose and provided the colonies with pollen patties ad libitum. To allow the colonies to develop, we kept them under constant darkness at 29°C at the University of California, Riverside. We allowed the colonies to populate undisturbed for two weeks before starting the experiment, then collected 60 bees from each of three colonies (N = 180 bees for each treatment) and sorted them by colony into cohorts of five bees in 475mL polypropylene containers (WebstaurantStore, Lancaster, PA). We exposed the bees to the following treatments: 10 mg/L, 1.0 mg/L, 0.1 mg/L, 0.01 mg/L, 0.001 mg/L, and 0 mg/L spiked into 60% sucrose for sodium selenate, cadmium chloride, and imidacloprid. We used the concentrations of 100 mg/L, 10 mg/L, 1.0 mg/L, 0.1 mg/L, 0.01 mg/L and 0 mg/L copper chloride spiked into 60% sucrose, and 1.25 mM, 1.0 mM, 0.5 mM, 0.25 mM, 0.01 mM, 0.001 mM, 0.0001 mM, and 0 mM hydrogen peroxide spiked into 60% sucrose. We allowed the bees to feed ad libitum for 14 days while we recorded mortality daily and used R to calculate statistical values through ANVOA tests and the R package “drc” [56] to calculate LC$_{50}$ values.
Bumble bee microbiome response to sub-lethal doses of toxicants

We purchased three bumble bee colonies from Koppert Biological Systems, Inc and reared the bees in the same manner as above. To expose bees to toxicants, we isolated 60 mature workers from each colony (N = 180) in 60mL polypropylene containers (WebstaurantStore, Lancaster, PA). We exposed bees to the chemical treatments by chronically feeding 30 bees 60% sucrose spiked with either 0.25 mg/L cadmium chloride (Sigma Aldrich, St. Louis, MO), 0.5 mg/L sodium selenate (Alfa Aesar, Ward Hill, MA), 25 mg/L copper chloride (Sigma Aldrich, St. Louis, MO), 0.001 mg/L imidacloprid (Sigma Aldrich, St. Louis, MO), 0.85 mg/L hydrogen peroxide (Fisher Scientific, Waltham, MA), or 60% sucrose as a control (N = 30 per treatment). We allowed the bees to feed on either toxicant-spiked or control sucrose ad libitum for four days and then stored the bees at -80°C.

DNA extractions and 16S rRNA gene sequencing library preparation

We used a DNA extraction protocol based on Engel et al 2013, [57], Pennington et al 2017 [58], and Rothman et al 2018 [59]. We first surface sterilized the bees using a 0.1% sodium hypochlorite wash followed by three rinses with ultrapure water. We then used sterile forceps to dissect the whole gut out of each bee and transferred the gut into DNeasy Blood and Tissue Kit lysis plates (Qiagen, Valencia, CA) containing approximately 100 µL of 0.1mm glass beads, one 3.4mm steel-chrome bead (Biospec,
Bartlesville, OK) and 180 µL of buffer ATL, followed by homogenization with a Qiagen Tissuelyser at 30 Hz for 6 minutes. We followed the remainder of the Qiagen DNeasy Blood and Tissue Kit protocol after homogenization. We also included four blanks to control for reagent contamination, which we extracted, prepared and sequenced in the same way as samples.

We prepared 16S rRNA gene libraries for paired-end Illumina MiSeq sequencing using the protocol from McFrederick and Rehan 2016 [60], Pennington et al. 2017 [61] and Rothman et al. 2018 [17]. We incorporated the 16S rRNA gene primer sequence, unique barcode sequence, and Illumina adapter sequence as in [62]. We used the primers 799F-mod3 [63] and 1115R [62] to amplify the V5-V6 region of the 16S rRNA gene. We used the following reaction conditions for PCR: 4 µL of template DNA, 0.5 µL of 10µM 799F-mod3, 0.5 µL of 10µM 1115R, 10 µL PCR grade water and 10 µL Pfusion DNA polymerase (New England Biolabs, Ipswich, MA), and an annealing temperature of 52°C for 30 cycles in a C1000 Touch thermal cycler (BioRad, Hercules, CA). We then removed excess primers and dNTPs with a PureLink Pro 96 PCR Purification Kit (Invitrogen, Carlsbad, CA). We used the cleaned PCR products as template for a second PCR reaction using 1 µL of the cleaned PCR amplicons as a template with the primers PCR2F and PCR2R to complete the Illumina adapter sequence [62]. We performed PCR with the following reaction conditions: 0.5 µL of 10µM forward primer, 0.5 µL of 10µM reverse primer, 1 µL of template, 13 µL of ultrapure water and 10 µL of Pfusion DNA polymerase at an annealing temperature of 58°C for 15 cycles. We normalized the resulting libraries with a SequalPrep Normalization kit by following the supplied protocol
(ThermoFisher Scientific, Waltham, MA). We pooled 5 µL of each normalized library and performed a final clean up with a single column PureLink PCR Purification Kit. We checked the normalized amplicons on a 2100 Bioanalyzer (Agilent, Santa Clara, CA) and sequenced the multiplexed libraries using a V3 Reagent Kit at 2 X 300 cycles on an Illumina MiSeq Sequencer (Illumina, San Diego, CA) in the UC Riverside Genomics Core Facility. Raw sequencing data are available on the NCBI Sequence Read Archive (SRA) under accession numbers SRR6788889 – SRR6789022, and microbiome data of selenate versus control treatments have been published in Rothman et al. 2019 [55].

Microbiome bioinformatics and statistics

We used QIME2-2018.6 [64] to process the 16S rRNA gene sequence libraries. First, we trimmed the low-quality ends off the reads with QIIME2 and then used DADA2 [65] to bin our sequences into exact sequence variants (ESVs; 16S rRNA gene sequences that are 100% matches), remove chimeras, and remove reads with more than two expected errors. We assigned taxonomy to the ESVs using the q2-feature-classifier [66] trained to the 799-1115 region of the 16S rRNA gene with the SILVA database [67]. We also conducted local BLASTn searches against the NCBI 16S microbial database (July 2018). We filtered out ESVs from the resulting feature table that corresponded to reagent contaminants as identified in our blanks or were assigned as chloroplast or mitochondria by the feature-classifier. We used the MAFFT aligner [68] and FastTree v2.1.3 to generate a phylogenetic tree of our sequences [69]. We then used this tree and ESV table
to analyze alpha diversity and to tabulate UniFrac distance matrices. We visualized the UniFrac distances through two-dimensional Non-metric Multidimensional Scaling (NMDS) and Principal Coordinates Analysis (PCoA) with the R package “ggplot2” [70]. We analyzed the alpha diversity of our samples through the Shannon Diversity Index and the Kruskal-Wallis test in QIIME2. Lastly, we tested our beta diversity data for statistical significance in R v3.5.1 [71] with the packages “vegan” [72] and “DESeq2” [73].

Genome annotations

To identify the genomic basis for toxicant tolerance/susceptibility we annotated the genomes of bee symbionts with The RAST Server (Rapid Annotations using Subsystems Technology) [74] using whole genome sequencing data obtained from the National Center for Biotechnology Information (NCBI). We annotated genomes from strains of the following species: *Bifidobacterium bombi, Bifidobacterium commune, Bombiscardovia coagulans, Candidatus Schmidhempelia bombi, Commensalibacter intestini, Gilliamella apicola, Lactobacillus bombicola, Serratia marcescens, and Snodgrassella alvi.*
Results

Bumble bee mortality and LC50 results to chemical exposure

We found that each of the compounds tested significantly reduced *B. impatiens* survivorship after seven days of continuous exposure: Selenate (F(5,30) = 30.04, P < 0.001, Tukey’s HSD P_adj < 0.05 for 1 and 10 mg/L), imidacloprid (F(5,30) = 15.18, P < 0.001, Tukey’s HSD P_adj < 0.05 for 0.1, 1, and 10 mg/L), cadmium (F(5,30) = 24.03, P < 0.001, Tukey’s HSD P_adj < 0.05 for 1 and 10 mg/L), copper (F(5,30) = 19.29, P < 0.001, Tukey’s HSD P_adj < 0.05 for 100 mg/L only), and hydrogen peroxide (F(10,40) = 31.54, P < 0.001, Tukey’s HSD P_adj < 0.05 for 0.5, 0.75, 1, and 1.25 mM). We also calculated the LC50 after seven days continuous exposure for each of the toxicants: Selenate: 0.78 mg/L, imidacloprid: 0.28 mg/L, cadmium: 0.86 mg/L, copper: 70.8 mg/L, and hydrogen peroxide 0.12 mM (Fig. 3.1). We exposed bees to treatments for a total of 14 days, but we found high mortality in the control treatments after seven days, so we did not calculate LC50 concentrations for the later time points.

Amplicon sequencing alpha diversity and library statistics

We obtained 743,529 quality-filtered 16S rRNA gene sequences with a mean frequency of 5,467 reads per sample (N = 136) that were clustered into 113 Exact Sequence Variants (ESVs; sequences that are 100% identical). We determined that our samples had a representative coverage of bacterial diversity at a sequencing depth of
2,182 reads through rarefaction analysis, as the curves reached saturation at approximately 1,110 reads. Overall, alpha diversity was significantly different due to treatment (Shannon’s H = 24.21, \( P < 0.001 \)), although pairwise Kruskal-Wallis testing indicated that only selenate treatments had significantly higher diversity as compared to controls (Benjamini-Hochberg corrected \( P_{adj} < 0.05 \)).

Beta diversity and differential abundance of bacterial taxa

Regardless of treatment, we found that the gut communities of our samples were composed of bacteria of the genera *Gilliamella, Snodgrassella, Lactobacillus, Bifidobacterium, Bombiscardovia, Commensalibacter, and Serratia*, while other genera accounted for less than 1% of the relative abundance. To clearly visualize the bumble bee gut microbiota, we graphed the taxonomy of bacteria present in greater than 1% proportional relative abundance in each sample as a stacked bar plot (Fig. 3.2) and beta-diversity through Principal Components Analysis (PCA, Fig. 3.3), with only copper clearly clustering separately from control. We analyzed the Generalized UniFrac distance matrix of our samples with Adonis (999 permutations PERMANOVA) using both colony and treatment as covariates and found that overall, there was a significant effect of treatment (\( F = 4.57, R^2 = 0.14, P < 0.001 \)), colony (\( F = 6.71, R^2 = 0.08, P < 0.001 \)) and interaction of these factors (\( F = 1.63, R^2 = 0.10, P < 0.001 \)). As we had multiple separate treatments in our experiment, we analyzed the pairwise interactions between each unique treatment versus control and found that each treatment except imidacloprid caused a
significant change to the beta diversity of the bees’ microbiomes (Benjamini-Hochberg corrected for each treatment $P_{adj} < 0.02$; imidacloprid: $P_{adj} = 0.96$).

We used the R package “DESeq2” to observe changes in the relative proportional abundances of ESVs in our treatments versus controls. Several ESVs significantly differed in abundance ($P_{adj} = < 0.05$, Fig. 3.4): In cadmium treatments: one *Commensalibacter* ESV; copper treatments: two *Serratia*, four *Gilliamella*, two *Bombiscardovia*, one *Commensalibacter*, and two *Lactobacillus* ESVs; hydrogen peroxide: one *Commensalibacter* ESV; selenate treatments: two *Commensalibacter*, two *Lactobacillus*, two *Snodgrassella*, and two *Gilliamella* ESVs; lastly, we did not find any differentially abundant ESVs in our imidacloprid-treated bees.

Genomic basis of chemical resistance

Through our RAST annotations, we identified several genes that are involved in resistance to the compounds assayed in our experiments. We used the following RAST subsystems to narrow our searches: “Cobalt-zinc-cadmium resistance”, “copper homeostasis”, “copper homeostasis copper tolerance”, “copper transport system”, “oxidative stress tolerance”, “selenate/selenite uptake”, and “selenocysteine metabolism”. Several bee symbionts and other bacteria identified by our next-generation sequencing study had some or all of the following genes annotated in their genomes. For selenium ion resistance, we found genes corresponding to the selenium ion transporters DedA [75], TsgA [76], and putative selenium ion and sulfate importer CysA [77]. For cadmium ion
resistance, we found the genes CzcABC, which encode the components of a cation transporter [78], along with its response regulator CzcD [79], and a cadmium-responsive transcriptional regulator, CadR [80]. We identified the following genes involved in copper resistance: A copper-translocating ATPase [81], two copper-binding multicopper oxidases [82, 83] (SufI and CueO, respectively), genes encoding the likely copper-binding proteins ScsABCD and CutEF [84], components of a copper-sequestering protein complex CopCD [85], and a copper-responsive transcriptional regulator, CueR [86]. Lastly, we searched for genes involved in responding to oxidative stress and found genes encoding the antioxidant DNA binding protein Dps [87], paraquat-inducible superoxide dismutase (SOD) PqiAB, Mn- and Fe-SODs [88], the SOD response regulon SoxS [89], a LysR-family peroxide-inducible transcriptional regulator [90], ferroxidase, a ferric uptake regulation protein (FUR) [91], the zinc/copper uptake regulation protein Zur, which may protect against oxidative stress [92], the antioxidant gene NnrS [93], an Fnr-like transcriptional regulator [94], catalase/peroxidase [95], and alkyl hyperoxide reductase C (AhpC) [96]. As S. alvi and G. apicola genomes are known to vary widely between strains [97, 98] and there are several genomes for each taxon publicly available, we specifically compared the above-mentioned detoxification/tolerance genes across strains within these species (53 strains of S. alvi and 67 strains of G. apicola). We found that G. apicola had notable variation across genes involved in responding to oxidative stress (specifically NnrS, SoxS, Fnr, and catalase), copper tolerance (the copper-translocating ATPase and SufI), cadmium tolerance (CadR), and overall selenate tolerance. There was less overall variation in detoxification/tolerance genes across S. alvi strains: We found
strain variation in copper (CueR, CueO, and the copper-translocating ATPase) and cadmium tolerance (CzcA and CadR), while there was no genetic variation in oxidative stress response or selenate tolerance (Fig. 3.5).
Discussion

The bumble bee microbiome is affected by exposure to selenium, cadmium, copper, and hydrogen peroxide, but not the insecticide imidacloprid. Furthermore, there are individual ESVs of symbiotic or pathogenic bacteria that are tolerant or susceptible to these chemicals. When bumble bees forage for pollen and nectar, they are likely often also obtaining compounds that alter the composition of their gut microbiomes. Antibiotic perturbation to the honey bee gut microbiome increases mortality and alters the bees’ susceptibility to the opportunistic pathogen *Serratia* [18], and it is likely that environmental toxicants have similar effects. As we were not able to assess mortality in germ-free versus bees with conventional microbiomes in our current work, however, future work elucidating the fitness consequences of the toxicant-induced microbiome perturbations that we show here is needed. Previous studies have examined whether the microbiome is affected by poisons in honey bees or *Drosophila melanogaster* exposed to imidacloprid [48, 49], mice to cadmium or copper [44], spiders and carp to cadmium [45, 46], and bumble bees to selenate [55]. We extend this work by screening a broad panel of toxicants and further show that members of the bee microbiota vary in their tolerance to the chemicals. While other studies have isolated metal-tolerant [51, 99, 100], and imidacloprid-tolerant microbes [49] from animal hosts, ours is the first to study the effects of metal(loid)s and peroxide on the entire microbial community. Lastly, we establish the LC$_{50}$ of each of the tested chemicals in the common eastern bumble bee, *Bombus impatiens*, showing that field-realistic doses of most of these chemicals can cause mortality.
By exposing bees to either selenate, cadmium, copper, imidacloprid or hydrogen peroxide, we show that each toxicant is lethal to bumble bees in varying concentrations. For example, the seven-day LC$_{50}$ for selenate is under that which bees can commonly encounter on flowers in contaminated areas [101]. *Bombus impatiens* is sensitive to cadmium, with a seven-day LC$_{50}$ of 0.86 mg/L, which is within the range of levels found in plants growing in polluted soil or in greenhouse experiments [102]. The LC$_{50}$ of bumble bee foragers exposed to copper is similar to honey bee foragers (70.8 mg/L versus 72 mg/L) [39]. While these concentrations are above the levels likely encountered when foraging on plants in contaminated areas [102], studies have shown that larvae are much more sensitive to copper than adult foragers and could be killed by field-relevant doses of copper being brought back to the colony [39]. The LC$_{50}$ of imidacloprid for *B. impatiens* is 0.28 mg/L which is within the scope of previously-reported results [103]. Although sub-LC$_{50}$ doses did not cause significant mortality, it is likely that bees exposed to neonicotinoids at sublethal doses suffer other effects, including reduced brood production [104], reduced foraging [105], and susceptibility to pathogens [49]. Lastly, we show that H$_2$O$_2$ is toxic to bees at an LC$_{50}$ of 0.12 mM and that bees appeared to avoid the highest doses. This represents a fairly low dose, considering that H$_2$O$_2$ can be found in the nectar of some plants in concentrations of over 4 mM [34]. While the acute effects of consuming peroxide are unknown, our data suggests that bumble bees are sensitive to peroxide exposure, possibly due to possessing fewer detoxification genes than most insects [106], although we note that honey bees are known to produce H$_2$O$_2$ as an immune response [107] and both honey bees and bumble bees are capable of detoxifying
physiological H$_2$O$_2$ levels [108, 109]. Our data suggest that exposure to these chemicals should be studied further, and studies should focus on the complex interactions between bees, gut microbes, parasites, and their environment, so that we may understand more about the subtle effects of stressors on pollinator health.

The bumble bee gut microbiome exhibited a variety of responses to the various toxicants that we challenged them with. Copper led to an increase of the opportunistic pathogen *Serratia*, which suggests that this chemical leads to gut dysbiosis, or a departure from the normal and presumably healthy gut community (Rayman, Shaffer, Moran 2017, Raymann, Coon et al. 2018). Similarly, alpha diversity of the bumble bee microbiome increased with selenate exposure. Selenate exposure allowed non-core bacteria to proliferate, while core symbiont ESVs were less proportionally abundant, further supporting our hypothesis of dysbiosis [110]. Copper exposure had the most dramatic effect on the bees’ microbial communities as the samples showed the clearest separation in our PCoA ordination and copper exposure changed the proportional abundance of 13 individual ESVs. When challenged with copper, most ESVs increased in proportional abundance, including a striking proportional increase in *Serratia*. Two *G. apicola* ESVs and two *S. alvi* ESVs, however, decreased in abundance. The decrease in proportional abundance of two *G. apicola* ESVs is noteworthy, as there were also two *G. apicola* ESVs that significantly increased in abundance, suggesting that there is variation between copper tolerance within this taxon, similar to other genomic differences within bee symbionts [97]. Lastly, due to the compositional nature of whole-microbiome sequencing
data [111], we may simply be seeing an overgrowth of *S. marcescens* in copper treatments and may not truly be reducing the absolute abundance of symbiotic bacteria.

Genomic analyses suggest putative mechanisms by which the bumble bee gut microbiome is affected by copper and selenate. Each core symbiont varies in its complement of selenium ion resistance genes, with *Bifidobacterium bombi*, *Bombiscardovia coagulans*, *L. bombicola*, and *S. alvi* [98, 112, 113] all possessing the selenate transporter DedA [75], while *G. apicola* does not. All annotated strains of *S. alvi* possess the sulfate/selenium ion transporter CysA, while some strains of *G. apicola* possess this gene, along with the selenium ion transporter TsgA [76]. There is variation in the selenium ion transport genes between strains of *G. apicola*, with strains having zero or one copy of CysA, and zero to three copies of TsgA. Copper resistance genes include: the suppressor of copper sensitivity genes ScsABCD and copper tolerance lipoprotein genes CutEF [84], multicopper oxidases CueO [83] and SufI [82], cytochrome c heme-copper lyase [114], Copper Resistance Protein D [114], a copper-responsive transcriptional regulator (CueR) [86], and a copper-translocating ATPase. Similar to selenium ion tolerance genes, between-strain variation exists in copper tolerance within the bacterial species *S. alvi* and *G. apicola*: Strains of *S. alvi* contain varying numbers of genes of CueO, CueR, and a copper-translocating ATPase, while *G. apicola* exhibited strain variation in SufI and copper-translocating ATPase genes. The strain variation in *G. apicola* copper translocating genes may underlie the differential abundance of *G. apicola* strains under selenate challenge, although our 16S rRNA gene data do not allow us to
determine if the ESVs that became proportionally more abundant have these copper-translocating genes.

Cadmium, imidacloprid, and hydrogen peroxide all had moderate (cadmium and hydrogen peroxide) to no (imidacloprid) effects on the microbiome. Cadmium significantly changed the bumble bees’ bacterial community but resulted in the decreased proportional abundance of only one ESV of Commensalibacter. Imidacloprid had no significant effects on the diversity of the gut microbiome in B. impatiens. These results agree with a previous experiment that found imidacloprid did not affect the microbial community of honey bees, and that the bee gut community does not appear to metabolize the neurotoxin [49]. As imidacloprid targets acetylcholine receptors in insects [115] it is perhaps not surprising that the bumble bee gut microbiome is not affected by this insecticide. Hydrogen peroxide modestly changed the microbial community of B. impatiens at our field-realistic treatment dose and increased the proportional abundance of one ESV of Commensalibacter. Hydrogen peroxide can be found at high levels in flower nectar and is thought to be an antimicrobial defense [116], so the bumble bee gut microbiome may therefore be routinely and persistently exposed to hydrogen peroxide.

This history of hydrogen peroxide exposure may explain why each of the members of the core bee gut microbiome have various combinations of genes to cope with oxidative stress, including: the antioxidant protein Dps [87], H$_2$O$_2$-inducible regulator [90], a superoxide response regulator [89], catalase [95], paraquat-inducible-and other superoxide dismutases [117], the antioxidant gene NnrS [93], and others. While S. alvi did not exhibit any genetic strain variation in oxidative stress response, G. apicola
did: There was a variable presence of SoxS and an Fnr regulator, while few strains possessed NnrS. Cadmium resistance is not as clear, as *Commensalibacter intestini* possesses a full complement of the genes CzcABC that code for a cadmium efflux pump [78], along with two regulators for cadmium resistance [79, 80], but is still susceptible to the treatment *in vivo*. Core bumble bee symbionts’ cadmium resistance pathways are more depauperate with *Bifidobacterium bombi, Bombiscardovia coagulans, G. apicola, L. bombicola*, possessing two or fewer cadmium resistance transcriptional regulators (CadR), and no functional CzcABC genes [98, 112, 113]. The symbiont *G. apicola* had some variation in CadR, while *S. alvi* exhibited substantial strain variation, with few strains possessing CzcA, and strains containing zero to three copies of CadR. These results suggest that individual core members of the bee microbiome are largely resilient to cadmium on a community-level scale, and we hypothesize that the bacteria may be partitioning the detoxification of cadmium between each other, as has been shown in other metabolic processes [98].
Conclusion

Bees have been recognized for their use as bioindicators to monitor environmental pollution [118], and the bee microbiome is rapidly becoming a useful model to study host-microbe interactions [119]. Our interdisciplinary study reports the LC$_{50}$ for selenate, cadmium, copper, imidacloprid, and hydrogen peroxide, and shows that the composition of the bumble bee microbiome can be altered through exposure to metals, a metalloid, and oxidative stress, but not a neonicotinoid pesticide. Through RAST subsystem analyses, we identified several potential genomic bases for tolerance or susceptibility to each toxicant, and that in some cases, there is substantial between-strain variation in these genes in the core gut bacteria *S. alvi* and *G. apicola*. This variation suggests that the bee gut microbiome harbors diverse strains that may be resilient to various environmental challenges. As we have indicated, there is a wide diversity in stress response genes between bee symbiont strains, and culture-based toxicology assays should be conducted to characterize their individual susceptibility to toxicants *in vitro*. We suggest that future studies investigate the multipartite interactions between host, symbiont and their habitats, and the potential for microbiomes and hosts to reciprocally protect each other from environmental insults.
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Figure 3.1: Bar plots indicating the survivorship after seven days when exposed to varying doses of cadmium, copper, imidacloprid, hydrogen peroxide, and selenate. Each chemical significantly increased mortality after seven days of constant exposure ($P < 0.001$). Error bars denote the standard error of the mean, and asterisks indicate which concentration of each chemical significantly increased mortality compared to control (Tukey’s post-hoc HSD $P_{adj} < 0.05$).
Figure 3.2: Stacked bar plot showing bacterial genera that were present at greater than 1% abundance in each sample. Individual sample treatments are indicated by “C” for control, “Cd” for cadmium, “Cu” for copper, “I” for imidaclopid, “P” for hydrogen peroxide, and “Se” for selenate exposure. Colony of origin is denoted by H3, H6, or H9.
Figure 3.3: PCA plot of the Generalized UniFrac distance matrix of our samples. Overall, treatment (F = 4.57, R² = 0.14, P < 0.001), colony (F = 6.71, R² = 0.08, P < 0.001), and an interaction of these factors (F = 1.63, R² = 0.10, P < 0.001) significantly affected the microbiomes of our samples. Post-hoc testing showed that each treatment except imidacloprid significantly altered the beta diversity of the bees’ microbiomes (BH corrected P_adj < 0.02; imidacloprid: P_adj = 0.96).
Figure 3.4: Log$_2$fold change of proportionally differentially abundant Exact Sequence Variants (ESV) between treatments and controls, colored by genus. Each treatment had at least one significantly different ESV except imidacloprid (BH corrected $P_{adj} < 0.05$). Error bars denote the standard error of the Log$_2$fold change.
Figure 3.5: Illustration of the toxicant-tolerance genes found in strains of *Snodgrassella alvi* and *Gilliamella apicola* as annotated by RAST. Colored cells represent the copy number of each gene, row names indicate the bacterial strain, and column names denote the gene abbreviation or name. Nonstandard gene abbreviations are as follows: “H$_2$O$_2$.activator” is a peroxide-inducible genes activator, “FUR” is a ferric uptake regulation protein, “Fnr” is a fumarate and nitrate reduction regulatory protein, “Zn.Cu.UR” is a zinc/copper uptake regulation protein, and “Cu.ATPase” is a copper-translocating ATPase.
Chapter Four:

Cadmium and selenate exposure affects the honey bee microbiome and metabolome while bee-associated bacteria show potential for bioaccumulation
Abstract

Honey bees are important insect pollinators used heavily in agriculture and can be found in diverse environments. Bees may encounter toxicants such as cadmium and selenate by foraging on plants growing in contaminated areas, which can result in negative health effects. Honey bees are known to have a simple and consistent microbiome that conveys many benefits to the host, and toxicant exposure may impact this symbiotic microbial community. We used 16s rRNA gene sequencing to assay the effects that sublethal cadmium and selenate treatments had over seven days and found that both treatments significantly altered the composition of the bee microbiome. Next, we exposed bees to cadmium and selenate then used untargeted LC-MS metabolomics to show that chemical exposure changed the bees’ metabolite profiles and that compounds which may be involved in proteolysis and lipolysis were more abundant in treatments. Lastly, we exposed several strains of bee-associated bacteria in liquid culture and found that each strain removed cadmium from their media, but only Lactobacillus Firm-5 microbes assimilated selenate, indicating a possible probiotic use for these microbes to reduce metals burden on their host. Overall, our study shows that metal and metalloid exposure can affect the honey bee microbiome and metabolome, and that strains of bee-associated bacteria can bioaccumulate these toxicants.
Introduction

Pollination services provided by bees are critical to agricultural crop production and native plant fitness. These insects are responsible for increasing the yield of over half of food crops, and benefiting a wide variety of natural flora [1, 2]. Of the pollinating insects, European honey bees (*Apis mellifera*) are the most intensely-used species of bee in agriculture and contribute billions of dollars in food production in the United States alone [3]. Along with other species, bee populations are declining around the world, most likely due to a combination of disease, pesticides, and land use change resulting in a lack of floral forage [4]. Relatively unstudied stressors include metals and metalloids that are harmful to bees and can affect their overall health when bees forage on plants grown in contaminated areas [5, 6]. As bees may encounter various pollutants across environments [7], the capacity for diverse environmental stressors to affect bee health needs to be understood and mitigated.

Heavy metal and metalloid contamination can be found in industrialized areas around the world [5]. Here, we chose to study cadmium and selenium due to their importance in agricultural and industrialized areas. Cadmium is a non-essential toxic heavy metal that is deposited near industries such as mining and battery production [8] and has been found in croplands [9]. Selenate is an ionic form of selenium that is found in soils near such industries as glass making and ink production or is deposited in naturally seleniferous agricultural soils [10]. As mentioned above, bees may contact metals and metalloids when foraging on plants growing in polluted areas [11, 12]. Plants can translocate toxicants from the soil into their pollen and nectar, which bees then forage
upon and bring back to their colonies [5]. The biomagnification potential of plants with different metals and metalloids varies widely. For example, flowers of the hyperaccumulator plant *Stanleya pinnata* have been found to contain over 2000 mg/kg selenate [13], and partridge pea pollen was shown to accumulate over 4000 mg/kg cadmium in greenhouse experiments [14]. In contrast, radishes grown in high concentrations of lead did not accumulate the metal in their flowers [11]. As the concentrations of cadmium or selenate that have been measured in flowers are well above the levels shown to elicit effects in honey bee foragers and larvae [5, 15], bees living in contaminated areas are likely stressed and less healthy than those living in pristine areas. Bees are often unaware of the metallic content of nectar and pollen, as bees will freely forage on plants grown in selenate-contaminated soil [12] and aluminum-containing nectar [16]. Conversely, bumble bees tend to avoid nectar spiked with nickel [16], indicating that bees are able to detect some metals. The ability of diverse bees to detect diverse metals and metalloids warrants further study.

Honey bees harbor a simple and distinct microbiome that is largely consistent in all colonies worldwide [17, 18]. This symbiotic relationship between microbe and host is thought to be the result of a long-lasting coevolutionary history [19] and is largely maintained through contact between colonymates [20]. The bee microbiome is involved in many aspects of host health including metabolizing toxic sugars [21], resistance to trypanosomes [22–24], bacterial pathogen defense [25], stimulating the immune system [26], and increasing weight gain in adult bees [27]. Due to the importance of the bee microbiome, one would expect reduced vitality when this symbiotic microbiome is absent.
or in a state of dysbiosis [28]. Indeed, when the microbiome of social bees is perturbed or absent, bees are more susceptible to *Nosema* and *Serratia* infection [29–31], gut scab formation by *Frischella perrara* [32], and selenate toxicity [33].

The interaction between environmental metal pollution and animal microbiomes is an emerging field of study [34, 35], and bees are rapidly becoming excellent model organisms for this type of research [36]. There has been a fair amount of investigation into the interactions between cadmium and animal microbiomes [37]. Previous work has shown that cadmium exposure significantly alters the microbiome of rats [38], mice [39–41], earthworms [42], and spiders [43]. To date, no research has investigated these interactions in any insect species. Much less is known about the effects of selenium on gut microbial communities, but it has been shown that there are subtle alterations in these microbe populations when exposed to selenium ions in mice [44, 45] and through our work with bumble bees [33]. In light of the effects of toxicant exposure on the microbiome, research is now being conducted on the ability of this microbial community to protect its host against environmental insults. For example, it was recently shown that the gut microbiome protects against arsenic challenge in mice [46], selenate toxicity in bumble bees [33], and lead or chromium exposure in chironomids [47]. Likewise, various *Lactobacillus* spp. are known to accumulate copper [48], cadmium [49–51], aluminum [52], and chromium [53], which suggests that members of this genus have the potential to be administered as probiotics to reduce host metal burden.

While mortality in bees is relatively straightforward to assess, sublethal doses of toxicants can affect alter bee physiology in more subtle ways. For example, at the
organismal level, exposure to manganese increases bee foraging time [6], copper affects feeding behavior [54], and imidacloprid alters nest behaviors [55]. Metabolically, exposing bees to heavy metals increases detoxification enzyme activity [56, 57] and metallothionein-like protein levels [58], while affecting their overall redox system [59] which may indicate a general response to toxic metal stress. Similarly, metals have been shown to hamper immunocompetence in bees [60], ants [61], and moths [62]. While studies that investigate individual pathways or enzymes are useful, they may be missing subtle and important changes in the overall metabolism of an organism. By using untargeted metabolomics, we can now examine many metabolic compounds and pathways simultaneously [63] and attempt to broadly cover the metabolism of toxicants in bees. Metabolomics have been used to characterize bees’ metabolism of the insecticidal compounds thiacloprid [64] and nicotine [65], but neither metals nor metalloids. Here we use untargeted metabolomics to investigate the metabolites bees produce in response to selenate and cadmium exposure.

Here we investigate the interactions between the honey bee, its symbiotic microbiome, and exposure to selenate and cadmium. We ask three questions: First, is the bee microbiome affected by exposure to selenate or cadmium, and does the microbial response vary over time? Second, what is the bioaccumulation potential of bee-associated bacteria grown in media spiked with selenate or cadmium? Third, what are the metabolic effects of selenate and cadmium exposure as measured through untargeted LC-MS metabolomics?
Materials and Methods

Bee Care and Cage Rearing

We moved one frame of brood each from five healthy honey bee colonies with marked Italian queens and housed them in a hive body at 35°C and 50% humidity under constant darkness. We then allowed the bees to emerge, mixed the newly emerged workers (NEWs) to randomize their colony of origin and placed NEWs into 13 cm x 10.5 cm x 6.5 cm wire cages equipped with feeders containing 35mL of deionized water and 35mL 50% sucrose. We also provided a pollen patty to each cage of bees consisting of 269g corn syrup, 113g sucrose and 113g of Bee Pro (Mann Lake, Hackensack, MN). To inoculate the newly emerged workers with their “core” microbiome, we collected 50 mL of foragers from the source hives of the NEWs, immobilized the bees at 4°C, aseptically dissected out the abdomens and macerated the whole abdomens in 50% sucrose. We added 1 mL of the resulting slurry to 34 mL of 50% sucrose solution and fed it to the NEWs. We allowed the bees to feed *ad libitum* on the mixture for two days before replacing the feeders with 50% sucrose. We allowed the bees to feed for three more days to fully establish a microbiome [20].

Once the bees had an established microbiome, we prepared treatment feeding solutions of 50% sucrose (as a no metal/metalloid control), 50% sucrose spiked with 0.6 mg/L sodium selenate or 50% sucrose with 0.24 mg/L cadmium chloride (Alfa Aesar, Ward Hill, MA) and pollen patties spiked with either 6.0 mg/L selenium or 0.46 mg/L cadmium as in Hladun et al 2015 [5]. We again allowed the bees to feed *ad libitum*. We
sampled three bees from each cage after two, four, and seven days of exposure and immediately placed the samples on dry ice, followed by long-term storage at -80 °C.

DNA Extractions and 16S rRNA Gene Sequencing Library Preparation

We used a DNA extraction protocol based on Engel et al 2013 [66], Pennington et al 2017 [67] and Rothman et al 2018 [68]. We gently vortexed whole bee samples in 0.1% sodium hypochlorite followed by three rinses with ultrapure water for surface-sterilization. We then used sterile forceps to dissect the whole gut out of each bee and transferred the gut into DNeasy Blood and Tissue Kit lysis plates (Qiagen, Valencia, CA) containing approximately 100 µL of 0.1mm glass beads, one 3.4mm steel-chrome bead (Biospec, Bartlesville, OK) and 180 µL of buffer ATL. We then homogenized the guts with a Qiagen Tissuelyser at 30 Hz for 6 minutes. We followed the remainder of the Qiagen blood and Tissue protocol after homogenization. We also included blanks to control for reagent contamination, which we extracted, prepared and sequenced in the same fashion as samples.

We prepared 16S rRNA gene libraries for paired-end Illumina MiSeq sequencing using the protocol from McFrederick and Rehan 2016 [69], Pennington et al. 2017 [70] and Rothman et al. 2018 [71]. We incorporated the 16S rRNA gene primer sequence, unique barcode sequence, and Illumina adapter sequence as in [72]. We used the primers 799F-mod3 [73] and 1115R [72] to amplify the V5-V6 region of the 16S rRNA gene with the following PCR reaction conditions: 4 µL of template DNA, 0.5 µL of 10µM
799F-mod3, 0.5 µL of 10µM 1115R, 10 µL PCR grade water and 10 µL Pfu DNA polymerase (New England Biolabs, Ipswich, MA), with an annealing temperature of 52°C for 30 cycles in a C1000 Touch thermal cycler (BioRad, Hercules, CA). We then removed excess primers and dNTPs with a PureLink Pro 96 PCR Purification Kit (Invitrogen, Carlsbad, CA). We used the cleaned PCR products as template for a second PCR reaction using 1 µL of the cleaned PCR amplicons as a template with the primers PCR2F and PCR2R to complete the Illumina adapter sequence [72]. We performed PCR with the following reaction conditions: 0.5 µL of 10µM forward primer, 0.5 µL of 10µM reverse primer, 1 µL of template, 13 µL of ultrapure water and 10 µL of Pfu DNA polymerase at an annealing temperature of 58 °C for 15 cycles. We normalized the resulting libraries with a SequaPrep Normalization kit following the supplied protocol (ThermoFisher Scientific, Waltham, MA). We pooled 5 µL of each normalized library and performed a final clean up with a single column PureLink PCR Purification Kit. Lastly, we checked the normalized amplicons on a 2100 Bioanalyzer (Agilent, Santa Clara, CA) and sequenced the multiplexed libraries using a V3 Reagent Kit at 2 X 300 cycles on an Illumina MiSeq Sequencer (Illumina, San Diego, CA) in the UC Riverside Genomics Core Facility.
Bioinformatics and Statistics

We used QIME2-2019.1 [74] to process the 16S rRNA gene sequence libraries. First, we visualized and trimmed the low-quality ends of the reads with QIIME2, then used DADA2 [75] to assign our sequences into exact sequence variants (ESVs; 16S rRNA gene sequences that are 100% matches), remove chimeras, and remove reads with more than two expected errors. We assigned taxonomy to the ESVs using a the q2-feature-classifier [76] trained to the 799-1115 region of the 16S rRNA gene with the SILVA database [77]. We also conducted local BLASTn searches against the NCBI 16S microbial database and nt/nr (accessed April 2019). We filtered out features from the resulting ESV table that corresponded to contaminants as identified in our blanks [78] or were present at only one read (singletons). We used the MAFFT aligner [79] and FastTree v2.1.3 to generate a phylogenetic tree of our sequences [80]. We used the resulting tree and ESV table to analyze alpha diversity and to tabulate a generalized UniFrac distance matrix [81] for beta diversity comparisons. We visualized the UniFrac distances through two-dimensional Principal Components Analysis (PCA) with the R package “ggplot2” [82]. We analyzed the alpha diversity of our samples through Shannon’s diversity index and Faith’s phylogenetic diversity using the Kruskal-Wallis test in QIIME2. We tested our beta diversity data for statistical significance in R v3.5.1 [83] with the packages “vegan” [84] and “DESeq2” [85].
Bacterial Accumulation of Cadmium or Selenate

In order to assay the ability of bee-associated bacterial species to remove cadmium or selenate from their environment, we streaked out individual colonies of the strains wkB2 and wkB12 of *Snodgrassella alvi* on plates containing tryptic soy agar (Neogen, Lansing, MI) + 5% defibrinated sheep blood (Hemostat Labs, Dixon, CA) (TSAB) in a 5% CO2 environment, *Lactobacillus bombicola*, and the *Lactobacillus* Firm-5 strain wkB8 on De Man Rogosa and Sharpe (MRS) + 0.05% cysteine agar (MRSC, Research Products Inc., Mt. Prospect, IL), and *L. micheneri, L. timberlakei, L. quenuiae, L. kunkeei* strain 3L, and *L. apinorum* in MRS + 2% fructose agar (MRSF, Research Products Inc., Mt. Prospect, IL). We then transferred individual colonies of the *S. alvi* strains into 15 mL of Insectagro media (Corning Inc., Corning, NY), *L. bombicola*, and *Lactobacillus* sp. wkB8 into 15mL of MRSC media, and *L. micheneri, L. timberlakei, L. quenuiae, L. kunkeei, and L. apinorum* into 15mL of MRSF media spiked with either 1 mg/L sodium selenate or 1 mg/L cadmium chloride (Alfa Aesar, Ward Hill, MA). We incubated the *S. alvi* cultures at 37°C in a 5% CO2 atmosphere without shaking and the *Lactobacillus* spp. at 32°C shaking at 150 rpm for two days. All assays were conducted in triplicate and we also included sterile media samples spiked with or without 1 mg/L of each treatment as controls.

After incubation, we pelleted the bacterial samples via centrifugation at 5,000 rpm for 10 minutes, followed by three washes with 18 MΩ ultrapure water and subsequent centrifugations. We then transferred the supernatant and washes to 110 mL Teflon-lined
vessels and added 5 mL of TraceMetal Grade concentrated HNO₃ (ThermoFisher Scientific, Waltham, MA) followed by digestion in a 570 W microwave oven (CEM Corp., Matthews, NC) for 20 minutes. Lastly, we diluted the samples with TraceMetal grade 6 M HCl (ThermoFisher Scientific, Waltham, MA), heated them for 20 minutes at 90 °C, and filtered the samples through a 0.45-micron syringe filter as in Hladun et al 2015 [5]. We then analyzed the selenium and cadmium concentrations in the samples via inductively coupled plasma spectroscopy (ICP) with a Perkin-Elmer Optima 7300DV ICP-OES in the Environmental Sciences Research Laboratory at UC Riverside and analyzed our bacterial accumulation data by one-way ANOVA with Tukey’s HSD for post-hoc comparisons.

Sample preparation for untargeted metabolomics

We sampled three bees from 13 cages after four days of continuous exposure to the above-mentioned treatments and immediately placed the samples on dry ice, followed by long-term storage at -80 °C. We then pooled three bee abdomens from each cage, freeze-dried the samples, and homogenized the abdomens to a fine powder at 4°C using a bead mill homogenizer. Next, we extracted 10-12 mg of the powder in a 1.5 mL tube with 100 µL of ice-cold extraction solvent (30:30:20:20 acetonitrile:methanol:water:isopropanol) per 1 mg of tissue. We sonicated the samples for 5 minutes in an ice bath, then vortexed them for 30 min at 4°C. Lastly, we
centrifuged the samples at 16,000 x g for 15 min at 4° C and analyzed the supernatant with Liquid Chromatography - Mass Spectrometry (LC-MS).

Untargeted LC-MS metabolomics

We used a Synapt G2-Si quadrupole time-of-flight mass spectrometer (Waters, Milford, MA) coupled to an I-class UPLC system (Waters) for LC-MS analyses in the UC Riverside Metabolomics Core Facility. We carried out separations on a CSH phenyl-hexyl column (2.1 x 100 mm, 1.7 µM) (Waters, Milford, MA), with the following mobile phases: A. Water with 0.1% formic acid and B. Acetonitrile with 0.1% formic acid at a flow rate of 250 µL/min at 40° C. We injected 2 µL of sample extract, and the gradient was as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 40% B; 24 min, 100% B; 26.5 min, 100% B; 27 min, 1% B. We operated the MS in positive ion mode (50 to 1200 m/z) with a 100 ms scan time and acquired MS/MS data at 1 MS/MS scan per MS scan. We set source and desolvation temperatures to 150° C and 600° C, respectively. We set the desolvation gas flow to 1100 L/hr and cone gas flow to 150 L/h, with all gases being nitrogen except the collision gas, which was argon, and set capillary voltage to 1 kV. We generated a quality control sample by pooling equal aliquots of each sample and analyzed this pool every 3-4 injections to monitor system stability and performance. We analyzed samples in random order and used a leucine enkephalin infusion for mass correction.
Metabolomics data processing

We processed the metabolite data (peak picking, alignment, deconvolution, integration, normalization, and spectral matching) with Progenesis Qi software (Nonlinear Dynamics, Durham, NC). We normalized the resulting data to total ion abundance and removed features with a coefficient of variation greater than 20% or an average abundance less than 200 in the quality control injections as in Barupal et al. 2018 and Dunn et al. 2011 [86, 87]. To aid in the identification of features belonging to the same metabolite, we assigned features a cluster ID using RAMClust [88]. We searched against several mass spectral metabolite databases including Metlin, Massbank of North America [91, 92], and an in-house database in the UC Riverside Metabolomics Core Facility. After metabolites were identified and quantified, we used MetaboAnalyst v4.0 [93] for data handling, log2-normalization, statistical testing through Welch’s t-test (identified metabolites only), ordination generation, quantitative metabolite pathway enrichment analysis (MSEA), and heatmap plotting. Additionally, we built Jaccard distance matrices and tested our treatments for statistical significance through Adonis testing (PERMANOVA with 999 permutations) in the “vegan” R package [84].

Genomic annotations and metal/metalloid detoxification genes

We downloaded publicly-accessible genome sequences from the National Center for Biotechnology Information (NCBI) for the following bacterial species: Bartonella apis, Bifidobacterium asteroides, Bombella intestini, Commensalibacter intestini,
Frischella perrara, Gilliamella apicola, Lactobacillus apinorum, L. apis, L. bombicola, L. helsingborgensis, L. kullabergensis, L. kunkeei, L. mellifer, L. melliventris, L. micheneri, L. quenuiae, Lactobacillus sp. wkB8 (Firm-5), L. timberlakei, and Snodgrassella alvi (see supplemental file SF1 for accession numbers). We then used the RAST Server (Rapid Annotations using Subsystems Technology) [94] to annotate the genomes and identify genes from the subsystem categories “Cobalt-zinc-cadmium resistance,” “Uptake of selenate and selenite,” and “Selenocysteine metabolism” to find a genomic basis for toxicant tolerance and uptake.
Results

Alpha diversity of the honey bee gut microbiome when exposed to selenate or cadmium

We obtained 6,879,949 quality-filtered 16S rRNA gene reads that clustered into 126 Exact Sequence Variants (ESVs) across 263 samples, with an average of 26,160 reads per sample. Through rarefaction analyses, we determined that we had acceptable diversity and coverage at a sequencing depth of 10,340 reads per sample, which left us with 249 samples which we used for diversity analyses. We did not find that treatment exposure or sampling timepoint significantly affected alpha diversity as measured by Shannon’s diversity index (P = 0.22 and P = 0.06, respectively) or Faith’s phylogenetic diversity (P = 0.82 and P = 0.15, respectively).

Beta diversity of the honey bee gut microbiome when exposed to selenate or cadmium

Across all of our samples, we found the following genera of bacteria present at greater than 1% proportional abundance of 16S rRNA gene reads: *Lactobacillus, Snodgrassella, Bartonella, Gilliamella, Bifidobacterium, Commensalibacter, Frischella,* and *Bombella* (Fig. 4.1). We analyzed the beta diversity of our samples through Adonis testing (PERMANOVA with 999 permutations) of the generalized UniFrac distances and found that overall, treatment (F = 2.96, R² = 0.02, P = 0.004), sampling timepoint (F = 2.11, R² = 0.02, P = 0.017), and the interaction between treatment and timepoint (F = 1.68, R² = 0.02, P = 0.023) significantly affected the microbiome of our samples,
although our PCA ordinations did not show any obvious clustering by these terms (Fig. 4.2). As we had two toxicant treatments, we also analyzed the generalized UniFrac distances of each treatment versus controls. We found that within cadmium exposure, treatment ($F = 2.39, R^2 = 0.02, P = 0.036$), timepoint ($F = 2.19, R^2 = 0.03, P = 0.016$), and the interaction between treatment and timepoint ($F = 2.33, R^2 = 0.03, P = 0.01$) all significantly affected the bee microbiome. Similarly, we analyzed the beta diversity of our selenate-exposed samples, and found that treatment ($F = 3.13, R^2 = 0.02, P = 0.005$), and the interaction between treatment and timepoint ($F = 1.78, R^2 = 0.02, P = 0.044$) significantly altered the bee gut community, while timepoint alone did not ($F = 1.55, R^2 = 0.02, P = 0.105$). As the effects of treatment and timepoint on the bees’ gut microbial communities were subtle, we then proceeded to analyze the differential abundance of individual ESVs.

Differential abundance of individual ESVs by treatment and sampling timepoint

To establish more biologically meaningful effects, we analyzed the differential abundance of individual ESVs present in at least 1% proportional abundance across all samples using DESeq2. As we had multiple treatments and multiple sampling timepoints, we compared ESVs in treatments versus control at each timepoint and found the following ESVs to be significantly differentially proportional across our analyses (Benjamini-Hochberg corrected $P_{adj} < 0.05$, Fig. 4.3): After two days continuous exposure, we observed an increase of one *Commensalibacter* ESV in both treatments, a
decrease of one ESV each of *Frischella* and *Lactobacillus* and increase in another ESV of
*Lactobacillus* and *Bartonella* in selenate treatments, and a decrease of an ESV of
*Lactobacillus, Snodgrassella,* and *Bartonella* in cadmium treatments. After four days
continuous exposure, we saw a decrease in an ESV of *Bifidobacterium* and *Lactobacillus*
but an increase in another *Lactobacillus* ESV in both treatments, an increase in an ESV of
*Lactobacillus* and *Snodgrassella* in selenate treatments, and a decrease in an ESV of
*Bartonella* and *Commensalibacter,* three ESVs of *Lactobacillus* and an increase in an
ESV of *Gilliamella.* Our last sampling timepoint was seven days of continuous exposure,
where we saw a decrease of two *Lactobacillus* and one *Snodgrassella* ESV, and an
increase in another *Lactobacillus* ESV in both treatments, while we observed a decrease
in a *Lactobacillus* ESV in cadmium treatments only.

The response of individual ESVs to treatments varied throughout the experiment.
For example, several ESVs of *Lactobacillus* Firm-5 were affected by the treatments:
“*Lactobacillus25*” was negatively impacted by both toxicants, while “*Lactobacillus29*”
grew to a much higher proportional abundance after seven days of exposure. Other ESVs
showed interesting trends: After two days of continuous exposure to cadmium, an ESV of
*Bartonella apis* showed a slight decrease in proportional abundance, while selenate
caused a large upshift in abundance, then these proportions generally leveled off for the
remainder of the experiment. Similarly, an ESV of *Commensalibacter* showed a pattern
of increase proportional abundance after two days of exposure to both treatments, then
again leveled off. Lastly, an ESV of *S. alvi (“Snodgrassella9”)* was generally found in
less proportional abundance in treatments compared to controls
As our bacteria grew in three separate types of media, we separately analyzed the ability of our bacterial strains to remove selenium and cadmium from their respective media. We found that overall, *S. alvi* strains significantly removed cadmium from their media ($F\left(2,4\right) = 18.16, P = 0.01$), and post-hoc testing indicated that both strains did so significantly ($\text{wkB12 } P_{\text{adj}} = 0.010$, $\text{wkB2 } P_{\text{adj}} = 0.022$). Neither *S. alvi* strain accumulated selenium ($F\left(2,5\right) = 1.35, P = 0.34$, Tukey’s HSD $P_{\text{adj}} > 0.05$). Next, we found that both *L. bombicola* and wkB8 removed cadmium ($F\left(2,5\right) = 9.58, P < 0.001$, Tukey’s HSD $P_{\text{adj}} < 0.001$ for each), and selenium ($F\left(2,5\right) = 8.25, P = 0.026$, Tukey’s HSD $P_{\text{adj}} = 0.05$ and 0.02 respectively). We also found that each strain grown in MRSF significantly removed cadmium from their media ($F\left(5,11\right) = 17.15, P < 0.001$) with Tukey’s HSD testing indicating that each strain accumulated cadmium ($P_{\text{adj}} < 0.009$ for all strains). Lastly, while the overall model showed significance, none of the MRSF strains significantly removed selenium from their media ($F\left(4,8\right) = 5.57, P = 0.02$, Tukey’s HSD $P_{\text{adj}} > 0.05$ for all post-hoc analyses) (Fig. 4.4). We also analyzed each media without bacterial inoculation or toxicant addition in duplicate and found that no media contained measurable cadmium, while each medium contained selenium (Insectagro: 0.142 mg/L, MRSC: 0.260 mg/L, and MRSF: 0.308 mg/L), albeit at levels lower than our treatment. Overall metabolite diversity and effect of treatment
We compared the metabolomic profiles of our samples through univariate and multivariate statistics. We generated Jaccard distance matrices of the metabolites identified from our LC-MS to assess the overall differences in composition. We then used Adonis to analyze our results for statistical significance and generated Partial Least Squares - Discriminant Analysis (PLS-DA) plots to visualize the effects of treatments on our samples. Overall, exposure to cadmium and selenate significantly altered the honey bee metabolome (Cd: F = 2.14, R² = 0.26, P = 0.047; Se: F = 5.23, R² = 0.43, P = 0.013). Likewise, we saw obvious clustering by treatment and calculated good model fitting for our PLS-DA ordinations for both cadmium (R² = 0.83, Q² = 0.69, Fig. 4.5) and selenate treatment (R² = 0.96, Q² = 0.86, Fig. 4.5) on the honey bee metabolome.

Differential abundance of individual metabolites and biochemical pathways

We performed two-tailed Welch’s t-tests on log2-transformed individual metabolites identified in our samples between treatments to assess statistical significance and corrected for multiple comparisons with a Benjamini-Hochberg adjusted p-value (P_{adj}). We then generated performed MSEA analyses to assay the effects that treatment may have had on the bees’ metabolic pathways. We obtained a profile of 391 metabolites, of which were able to positively identify 58. When examining honey bees treated with cadmium versus controls, we found two metabolites significantly differed between treatment and control (P_{adj} < 0.05): A coumaric acid-like molecule and a tetrasaccharide. MSEA analysis indicated that the phospholipid biosynthesis pathway was significantly
different (\(P_{\text{adj}} = 0.004\); Fig. 4.6). Next, we examined the effects of selenate exposure on honey bees, and found that seven metabolites were significantly different between selenate and control treatments (\(P_{\text{adj}} < 0.05\)): a coumaric acid-like molecule, a tetrasaccharide, two short-chain peptides, a LysoPC(16:0), a LysoPC(16:1), and phosphocholine. Again, MSEA analyses showed that phospholipid biosynthesis was impacted by treatment (\(P_{\text{adj}} = 0.01\); Fig. 4.6).

Genes involved in cadmium or selenate resistance/transport

We annotated the genomes of bee-associated bacteria present in our amplicon sequencing study or selenium/cadmium accumulation experiment with RAST and found some or all of the following genes present in each genome: The selenium ion transporters DedA [95], TsgA [96], and putative selenium ion and sulfate importer CysA [97], components of selenocysteine metabolism, SelA, SelB, and SelD [98], the genes CzcABC, which encode the components of a cation transporter involved in cadmium resistance [99], the cadmium response regulator CzcD [100], and a cadmium-responsive transcriptional regulator, CadR [101].

Each strain of bacteria analyzed had the genes corresponding to cadmium resistance CadR and CzcD, although only \(C.\) intestini had a complete CzcABC protein complex, and presumably higher cadmium tolerance. Likewise, each bacterial strain also had one or more of the putative sulfate/selenium ion transporters TsgA, DedA, and CysA,
which may confer selenate resistance, while only *B. apis* and *L. mellifer* had genes (SelA, SelB, and SelD) corresponding to selenocysteine metabolism.
Discussion

Exposure to cadmium or selenate impacted both the microbiomes and metabolomes of honey bees. Cadmium or selenate treatments subtly changed the composition of the honey bee microbiome and changed the proportional abundance of several ESVs of core symbiotic taxa. While the overall community effects of treatment were slight, we found that core bacteria vary in their response to toxicant exposure. Variation in toxicant tolerance is therefore the likely mechanism driving differential abundance of core taxa in the control and toxicant treatments. We also show that species of bee-associated bacteria can uptake cadmium but generally not selenate, which may provide protection against heavy metals but not metalloid exposure. Lastly, through untargeted metabolomics, we show that there are metabolic responses in bees to sublethal toxicant exposure including likely protein and lipid degradation and increased carbohydrate use.

Previous studies have examined the effects of cadmium on the microbes associated with mice [102], spiders [43], and selenate on bumble bee microbes [33]. Similarly, studies have suggested that microbes can detoxify or sequester toxic metals including arsenic [46], selenate [33], lead and chromium [47], copper [48], and cadmium [49–51]. Our research extends this work by showing that bee-associated microbes can be affected by metalloid exposure, are able to bioaccumulate some of these compounds, and that the metabolome is changed in response to selenate and cadmium poisoning.

Overall, treatment with either selenate or cadmium slightly altered the honey bee microbiome over the course of our experiment. While the community-wide effects of
treatments were subtle, there were remarkable shifts in the proportional abundance of individual ESVs. Changes in the relative abundance of specific ESVs suggest that exposure to cadmium and selenate can negatively affect symbiont growth \textit{in vivo}, which may cause gut dysbiosis and allow disease-causing bacteria or parasites to proliferate in stressed bees [28, 30]. While our results suggest that individual ESVs can be harmed by metal or metalloid exposure, our data are proportional [103] and we may be observing growth in the overall microbiome instead of reduction in individual ESVs or vice versa.

Similar research has been conducted in earthworms showing that cadmium exposure alters the proportional abundance of several ESVs, and that those taxa may be used as bioindicators of cadmium pollution [42], and in mice, where cadmium moderately affects the microbiome [38]. Our results also agree with a previous experiment exposing bumble bees to selenate, where the microbiome was slightly altered, and treatment had an overall apparent negative affect on several gut symbionts [33]. Mouse-associated bacterial taxa also vary in their response to selenite [45], suggesting that toxicants may cause similar changes in the microbiomes of diverse hosts.

As mentioned above, the proportional abundance of several ESVs were reduced or increased by cadmium or selenate treatment while others were apparently unaffected. We used RAST annotations to search for putative mechanisms for toxicant tolerance or sensitivity in bacteria commonly associated with bees. While the RAST annotations show that cadmium resistance and selenium transporter genes are common, the bacterial species that have unique toxicant resistance genes showed ESV-level variation in response to toxicant challenge. As honey bee symbionts have functionally diverse
genomes [104–107], strain variation may therefore explain the discrepancies in treatment response between individual ESVs of bacteria corresponding to the same species. Furthermore, putative toxicant-resistance genes may not predict symbiont response to toxicants in the dynamic environment of the bee gut, within which diverse host/microbe interactions occur [108]. In the case of the bees’ responses to toxicant exposure, immune function may be hampered which could allow for suboptimal population control of gut-associated bacteria [26] leading to the microbiome being in a state of flux. Lastly, as we are sampling at distinct timepoints, we are likely only seeing a snapshot of the bee microbiome and deeper sampling or metagenomic data may be needed to truly understand community-wide responses to toxicant exposure.

The potential for bacteria to protect their host from toxic metal exposure through bioaccumulation is an emerging topic of investigation in several systems including humans [50, 109], mice [48, 51], and insects [47]. We exposed strains of bee-associated bacteria to cadmium in vitro, we and found that all assayed taxa removed a significant amount of cadmium from their growth media after two days of incubation. Notably, strains that removed the most cadmium are enteric bacteria isolated from social bees (Lactobacillus sp. wkB8, L. bombicola, and both strains of S. alvi) [110], which indicates that these symbionts may remove metal from the gut and protect their host when foraging on plants grown in polluted environments. We also found that bacteria associated with solitary bees (L. micheneri, L. quenuiae, L. timberlakei) [111] and bee honey stomachs (L. apinorum and L. kunkeei) [112, 113] removed less cadmium than enteric symbionts, but still may provide some protection to host bees. We exposed the same bacteria to
selenate in vitro and found that only Firm-5 lactobacilli (*Lactobacillus* sp. wkB8 and *L. bombicola*) removed a small but significant amount of the metalloid, while no other strain removed a significant quantity from their media. Although our tested strains of bacteria do not appear to accumulate selenate inside their cells, other bacteria associated with insects has been shown to reduce selenite to elemental selenium [114] and reduce mortality upon selenate challenge [33], so bee-associated taxa may be able to reduce selenate as well. As our sample processing methods involved acidifying the media, we may have overlooked the ability of bee-associated taxa to reduce selenate because any elemental selenium produced would have been redissolved, so future research should continue examining the possibility of bee- and other insect-associated bacteria to produce elemental selenium nanoparticles from selenium ions. Lastly, we note that there are several other bee symbionts that have not been tested for their bioaccumulation potential, so more experiments into the interactions between these bacteria and metals should be conducted.

We used untargeted LC-MS analyses to assay the metabolites present in cadmium- or selenate-treated bees as compared to controls and found that both treatments altered the overall metabolome of the bees along with several individual metabolites. Two metabolites were differentially abundant in both treatments: A tetrasaccharide (lower in treatments) and a coumaric acid-like molecule (higher in treatments). The reduction of tetrasaccharide in toxicant-exposed samples may be due to the increased metabolic demands of coping with added stress, as organisms exposed to metals may expend more energy for repair and detoxification [115], which should deplete
carbohydrates in the bees. The role of the coumaric acid-like molecule is less clear, as bees are not known to produce coumaric acid, and must obtain it from their diet [116]. Coumaric acid has been implicated in upregulating detoxification genes in honey bees [117], but its breakdown products (if any) are unknown, so the “coumaric acid-like” compound that we detected may be part of the bees’ metabolism in response to toxic stress. Further metabolic analyses of coumaric acid under toxicant stress should be investigated. We also observed a fairly large increase in short-chain peptides in selenate-exposed bees, which probably indicates protein degradation as a response to treatment [118] as selenium ions have been shown to increase protein degradation in cell models [119, 120]. Likewise, we saw an insignificant but suggestive increase in short-chain peptide metabolites in cadmium-treated bees which indicates that protein degradation may be a general consequence of metal(loid) exposure [121]. Lastly, we saw a higher proportional abundance of the phospholipid precursor molecule phosphocholine [122] and two lysophosphatidylcholines – products of oxidized phospholipids [123] – in selenate treatments, as well as MSEA indicating an over expression of phospholipid biosynthesis metabolites in both treatments. We posit that the higher proportional abundance of these phospholipid metabolites may be due to the oxidative stress that toxic doses of metals and metalloids can produce [121]. Previous work has shown that the bumble bee microbiome reduces mortality when exposed to selenate [33] and that the bee microbiome is involved in suppressing oxidative damage [27, 29]. The increase in oxidative damage under selenate stress coupled with a hampering of antioxidant activity due to a lack of functional microbiome may explain why mortality is increased in
microbiome-free bees upon selenate challenge. We therefore suggest that future research investigate the ability of antioxidants to remedy the effects of toxicant exposure on insects.
Conclusion

Bees are important insect pollinators that have become an excellent model for studying host/microbe interactions [36] and the toxicology of chemical compounds [124]. Our interdisciplinary study indicates that the honey bee microbiome can be affected by cadmium and selenate exposure, and that there are potentially tolerant or susceptible strains of core symbionts. We also show that several bee-associated strains of bacteria can bioaccumulate cadmium – and to a lesser degree selenate – which may provide a protective mechanism for bees against metal and metalloid pollution and provide putative genes involved in detoxification to these chemicals. Lastly, we report metabolic responses by honey bees upon toxicant exposure and posit that these compounds may cause oxidative damage to proteins and lipids, although much more investigation into the bee metabolome is needed. We suggest that future research investigate the interactions of toxicants and subcellular through organismal responses of both symbionts and honey bees to understand the complex interplay within this system.
References cited:


111. McFrederick QS, Vuong HQ, Rothman JA (2018) *Lactobacillus micheneri* sp. nov., *Lactobacillus timberlakei* sp. nov. and *Lactobacillus quenuiae* sp. nov., lactic acid bacteria isolated from wild bees and flowers. Int J Syst Evol Microbiol. doi: 10.1099/ijsem.0.002758


Figure 4.1: Stacked bar plot showing bacterial genera present at greater than 1% proportional abundance in each sample. Treatments and sampling timepoints are separated by vertical lines.
<table>
<thead>
<tr>
<th>Day</th>
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<td>Day 7</td>
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Figure 4.2: PCA plot of the generalized UniFrac distances of all samples. Overall, treatment ($F = 2.96$, $R^2 = 0.02$, $P = 0.004$), sampling timepoint ($F = 2.11$, $R^2 = 0.02$, $P = 0.017$), and the interaction between treatment and timepoint ($F = 1.68$, $R^2 = 0.02$, $P = 0.023$) significantly affected the bee microbiome. Post-hoc testing showed that both selenate and cadmium treatments significantly affected the beta diversity of our samples ($P < 0.04$ for each).
Figure 4.3: Proportional abundance of individual exact sequence variants (ESVs) that were significantly different between at least one treatment and control as analyzed by “DESeq2,” separated by timepoint. “*” denotes significant differences between treatment and control and error bars denote standard error.
Figure 4.4: Bar plot showing the amount of cadmium or selenium (mg/L) present in either bacteria-inoculated (gray bars) or uninoculated media (black bars) after two days of incubation separated by bacterial strain. “*” denotes significantly different as analyzed by one-way ANOVA and Tukey’s HSD post-hoc tests (BH corrected $P_{adj} < 0.05$), and error bars denote standard error.
Figure 4.5: Partial Least Squares – Discriminant Analysis (PLS-DA) plots of the individual bee metabolomes in treatments versus control. Exposure to cadmium and selenate significantly altered the honey bee metabolome (Cd: \( F = 2.14, R^2 = 0.26, P = 0.047 \); Se: \( F = 5.23, R^2 = 0.43, P = 0.013 \)).
Figure 4.6: Metabolite Set Enrichment Analysis (MSEA) bar plots indicating the fold enrichment of metabolites from biochemical pathways with P value indicated by heat color. The phospholipid biosynthesis pathway was enriched in both cadmium and selenate treatments versus control samples (BH corrected $P_{adj} < 0.05$).
Chapter Five:

Conclusion
Conclusion

Metal, metalloid, and xenobiotic pollution is widespread and can impact pollinators who forage on plants growing in contaminated areas. Likewise, studies involving animal-associate microbiomes and environmental stress continue to be vital to our understanding of how complex symbioses impact the health of both host and microbiome. We have determined the broad effects of two metals, a metalloid, a neonicotinoid pesticide, and a floral oxidative compound on the health and microbial community of the honey bee (*Apis mellifera*) and a species of bumble bee (*Bombus impatiens*). Through research conducted in this dissertation, we have also established selenate tolerance in two major gut symbionts of bees, the potential for the bumble bee microbiome to reduce mortality upon selenate challenge, and possible genomic bases for toxicant tolerance in bee symbionts. Likewise, we investigated within-strain variation in these toxicant tolerance genes in two major bee symbionts, the lethality of these compounds, the metabolomic consequences of metal and metalloid exposure on honey bees, and the potential probiotic use of bee-associated bacteria to reduce metal and metalloid burden on the host.

We showed that the gut microbial community of bumble bees plays an important role in reducing host mortality when challenged with the metalloid ion selenate. Even so, the microbiome can be altered through selenate exposure, with several exact sequence variants (ESVs) of symbiotic bacteria appearing less abundant. We also show that two strains of bee gut-associated bacteria – *Snodgrassella alvi* and *Lactobacillus bombicola* –
tolerate selenate exposure in vitro, and that many of the bumble bee microbiome taxa contain genes putatively involved in selenium ion tolerance. We hypothesized that bacteria are accumulating selenate or reducing it to the less toxic form of elemental selenium, or the presence of a microbiome is somehow stimulating the bees’ own inherent detoxification mechanisms, but these hypotheses need further study. Likewise, we also posited that the microbiome may be forming a physical barrier to selenate, or that we are simply observing the synergistic effects of two stressors: A lack of stable microbiome and selenate toxicity, which may cause higher mortality to microbiome-free bees versus microbiome-inoculated bees.

We characterized the median lethal dose (LC$_{50}$) of cadmium, copper, selenate, the neonicotinoid pesticide imidacloprid, and hydrogen peroxide exposure on bumble bees and showed that each of these compounds except imidacloprid affected the composition of the bees’ symbiotic microbiome. Within this study, we examined the effects that each compound had on individual bacterial ESVs within the bumble bee gut community and found that there are ESVs that appear tolerant to chemical exposure in vivo, while some ESVs appeared susceptible. These contrasting results suggest that there is strain variation within the bee gut microbial community. Lastly, we annotated genomes from 120 total strains of the major symbionts Snodgrassella alvi and Gilliamella apicola and found that substantial strain variation exists in the genetic subsystems corresponding to toxicant tolerance and resistance. These results suggest that strain variation is involved in the microbial response to toxicants and the susceptibility of bees to chemical exposure, and
more genome sequences from diverse strains are needed to understand the dynamic within the bees’ microbiome.

We investigated the effects of selenate and cadmium exposure on the honey bee microbiome through 16s rRNA gene sequencing and found that exposure to these chemicals slightly alters the composition of the gut community. Furthermore, we also showed that individual ESVs of gut symbionts are likely tolerant or susceptible to toxicant exposure and that these effects vary over time. We then used untargeted LC-MS metabolomics to characterize the metabolome of honey bees exposed to selenate and cadmium and observed an overall change in metabolic composition, as well as increases in metabolites that correspond to protein and lipid degradation. Finally, we cultured strains of bee-associated bacteria in cadmium- or selenate-spiked media and show that these bacteria can bioaccumulate cadmium but generally not selenate. Collectively, these results suggest that toxicant exposure can shift the composition of the honey bee microbiome, cause metabolic changes that may be associated with oxidative stress and cellular damage, and that bee-associated bacteria can accumulate metals from their environment which may ultimately reduce exposure to toxic metal stress.

The experimental results obtained from this dissertation have shed light into the interactions between social bees, their associated microbes, and environmental toxicants. We were able to show that the symbiotic microbiome of bees can reduce mortality upon metalloid exposure, bioaccumulate metals, and is generally robust against exposure to two metals, a metalloid, a neonicotinoid pesticide, and a floral oxidative compound, although these microbiomes and some individual microbial taxa can be affected by these
toxicants. Likewise, we showed that major gut symbionts – *Snodgrassella alvi* and *Gilliamella apicola* – exhibit considerable interstrain variation in their toxicant tolerance genes, and that *S. alvi* and *Lactobacillus bombicola* are tolerant of field-realistic metalloid exposure. These collective results suggest that the microbiomes of social bees play a vital role in bee health by reducing the hazards associated with toxicant exposure in agricultural and natural ecosystems. While this dissertation contributes to the literature and collective knowledge in the interdisciplinary fields of microbial ecology, ecotoxicology, and entomology, more research is needed to continue investigating the multipartite interactions between symbionts, their hosts, and the environment. Future research should further investigate the ability of the microbes associated with both social and solitary bees to positively affect pollinator health and cope with environmental stress.