A METAGENOMIC ANALYSIS OF THE HONEY BEE GUT MICROBIOME FOLLOWING ORAL IMIDACLOPRID EXPOSURE

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science in Biology

Middle Tennessee State University
August 2018

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ACKNOWLEDGEMENTS

I would like to thank my wonderful parents and my partner Shaun for their love and support. Thank you to my committee and my advisor Dr. Frank Bailey for their guidance. I would also like to thank Dr. Natàlia Garcia-Reyero and Dr. Chuan-yu Hsu at Mississippi State for performing the sequencing and Brock Arivett for his help with the Qiime 2 analysis.

ABSTRACT

Thought to play a significant role in their health, the intestinal microbiota of honey bees is a growing subject of interest. Imidacloprid, one of the highest selling insecticides worldwide, is transferred to the nectar and pollen of treated plants and therefore is likely to be ingested by foraging workers. Little is known about the effects of imidacloprid (and pesticides in general) on bee microbiota. The purpose of this study was to test the hypothesis that exposure to the pesticide imidacloprid alters the community structure of commensal bacteria in the honey bee (*Apis mellifera*) gut. Workers were kept in captivity and fed a sucrose solution containing imidacloprid at 5 μg/L, a field realistic dose. The relative abundance of bacterial taxa in the gut was determined using MiSeq. Oral imidacloprid exposure did not appear to impact the community structure of the honey bee gut microbiome.

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INTRODUCTION

Economic value of Honey Bees

Honey bees (*Apis mellifera*) are a large agricultural asset. In 2012, pollination services provided by managed honey bee hives in the US generated gross revenue of $655.6 million. The cultivation of 90-130 crops, constituting up to a third of the US diet, is supported by these services (1). In recent years bee keepers have complained of high annual losses of their hives, totaling 42.1% for April 2014 through April 2015 and 34.2% for 2013-2014, which are high above the 18.7% that keepers report as economically acceptable (2). In spite of this, the number of reported honey-producing colonies in the US has remained stable over the last 20 years, likely due to increased vigilance in colony management and renewal by keepers. Globally, the number of hives is increasing (3). However, an analysis by Aizen and Harder (4) pointed to the increased reliance on pollinator-dependent crops, which they concluded is growing faster than the global stock of honey bees. The high reliance on pollinator-dependent crops illustrates the importance of understanding honey bee stressors before a potential population crisis occurs. No species can be considered perpetually safe from decline. The introduction of new stressors to bees is a looming possibility that makes the understanding and mitigation of current stressors an essential precaution.

Gut Microbiota

Characteristics of the honey bee microbiome suggest a long held symbiotic relationship with *Apis mellifera*. Abundant in sister clades, it's taxonomic diversity is relatively low. Most of the bacterial species comprising the honey bee gut microbiome are found only in the guts of *Apis* spp. and do not grow in other environments (5). Several studies characterizing honey bee gut microbiota using non-culture methods have revealed eight core phylotypes (16S sequences with >97% similarity) that are consistently present across colonies from North America, Europe, and Australia (6). These phylotypes constitute approximately 95% of the bacteria in the gut (Table 1). The hindgut, divided into the ileum and rectum, harbors 95% of all bacteria in the gut. However, the ileum is dominated by *Gilliamella apicola*, *Snograssella alvi*, and *Frischella perrara*, while the rectum is dominated by Firm-4 and Firm-5 *Lactobacillus* spp. and *Bifidobacterium* spp. Relative concentrations of specific operational taxonomic units (OTUs) vary not only among colonies, but also significantly between same-age individuals within a colony (7).

TABLE 1 The core bacterial gut community and its distribution (5)

Phylotype	Phylum	Further classification	Primary location
Gamma-1	Gammaproteobacteria	*Gilliamella apicola*	Adult midgut, adult hindgut (ileum)
Gamma-2	Gammaproteobacteria	*Frischella perrara*	Adult hindgut (ileum)
Beta	Betaproteobacteria	*Snodgrassella alvi*	Adult hindgut (ileum)
Firm-4	Firmicutes	*Lactobacillus*	Adult hindgut (rectum)
Firm-5	Firmicutes	*Lactobacillus*	Adult hindgut (ileum, rectum)
Bifido	Actinomycetes	*Bifidobacterium*	Adult hindgut (rectum)
Alpha-1	Alphaproteobacteria	*Bartonella*	Adult gut
Alpha-2	Alphaproteobacteria	Acetobacteraceae	Larval gut, adult crop, honey, nectar, some in adult hindgut

The previously described microbiome is specific to adult honey bees. The larvae have a low quantity and diversity of gut bacteria composed primarily of environmental Acetobacteraceae and *Lactobacillus* spp. Larval gut bacteria disappear during pupation, and upon emergence from the pupal state, the gut is initially free of bacteria. The adult microbial gut community is then established and reaches a fairly stable state in 3-5 days. Transmission occurs in part by a fecal-oral route as well as trophyllaxis (5).

Animal gut microbiota often have an important role in the immunity and nutrition of their hosts, and this may be especially true for honey bees. A metagenomic study found genes in *G. apicola* for enzymes involved in the degradation of pectin, a polysaccharide abundant in pollen that is indigestible by bees (8). These bacterial enzymes may increase the nutritional value of pollen for the bees. *G. apicola* is also able to utilize

monosaccharides that can be naturally present in nectar or released during the breakdown of pollen and are toxic to bees (9). Specific lactic acid bacteria found in the guts of larvae can inhibit the in vitro growth of the highly virulent bacterium, *Paenibacillus larvae,* responsible for American Foulbrood disease (10). Dysbiosis, an imbalance of the gut microbiota, has also been correlated with an increase in susceptibility to the protozoan parasite *Lotmaria passim* (11). Therefore, disruption of the normal concentrations of honey bee gut microbiota may render colonies more susceptible to pathogens and have long term impacts on colony survival and production.

Imidacloprid and Dysbiosis

Imidacloprid is a commonly used neonicotinoid that acts as an insect neurotoxin. It binds to nicotinic acetylcholine receptors (nAChRs), ultimately overstimulating the receptors and interfering with the transmission of nerve impulses. It is significantly more toxic to insects than vertebrates due to its high specificity for arthropod nAChRs. Comprising 27% of the worldwide insecticide market in 2010 (12), neonicotinoids have been subject to scrutiny for their effects on non-target organisms, including honey bees and other important pollinators (13). Oral ingestion through pollen and nectar is of primary concern. Imidacloprid is a water soluble, systemic insecticide that, when applied to the soil or seeds, can be translocated to the nectar and pollen. An average field realistic concentration in nectar or pollen is difficult to pin-point, but 1-10 μg/L is within a typical range. Concentrations are usually higher in pollen than nectar and may also vary by plant species depending on the efficiency of translocation (14).

Many studies have been published on the direct effects of imidacloprid on honey bees with varying results. Several have implicated imidacloprid in impairing memory formation and immunity, but use dose concentrations that bees are unlikely to be exposed to naturally. Evidence of field-realistic doses impacting colony survival and fitness is sparse (15, 16). However, indirect effects, such as the hypothetical impairment of immunity as a result of pesticide induced dysbiosis, are often more difficult to measure. Little is known about how pesticides may affect the gut microbiota, which are a probable factor in honey bee health. The aim of this study is to investigate the effects of consuming a field-realistic concentration of imidacloprid on the relative abundance of bacterial OTUs in the *A. mellifera* gut.

METHODS

Materials

Hives

A total of 10 hives were used for this experiment. None of the hives showed signs of microbial infection or parasitic infestation and had not received chemical treatments or preventatives within a minimum of 10 months prior. Hives 1 and 2 were located in Woodbury, TN (35°51'7.97"N, 86° 3'59.13"W). Hives 3 and 4 were located at the MTSU farm (35°53'11.61"N, 86°16'43.11"W). Hives 5-10 were located at a second MTSU farm location (35°53'7.14"N, 86°16'29.55"W). Hives 5, 6, 8, and 10 were Varroa mitesensitive. Hives 5, 6, and 10 were obtained from the same breeder and have shared ancestry, but the exact relatedness between the hives is unknown. Hive 8 is a split from one these hives. Hive 7 is most likely a split from hive 9, and hive 2 is a split from hive 1.

Hoarding Cages

Hoarding cages (Fig 1) were crafted from Diamond Daily™ 12 oz disposable polypropylene containers purchased from Walmart. The plastic lid of each container served as the base of the hoarding cage, with the bottom of the container serving as the top of the hoarding cage. In the top of each cage, 70 ventilation holes approximately 2 mm in diameter were created by pressing the tip of a hot soldering iron through the plastic. With a razor knife, a 1.5 x 1.5 cm square was cut from each of the container lids

and secured to the lid with a piece of tape. This “door” served as a means of removing dead bees from the containers.

Feeding devices were created from 1.5 mL conical centrifuge tubes by making two small holes approximately 1 cm from the bottom of each tube and a single small hole in the center of the cap. Two feeding tubes were inserted through two holes drilled into the top of each cage.

FIG 1 Hoarding cages

Feeding and Treatment Solutions

Crystallized sucrose (MP Biomedicals, cat no. 194018, lot no. QR13496) was used to prepare a 50% sucrose solution by measuring 125 g of sucrose, adding tap water to the sucrose to reach a total volume of 250 mL, heating the mixture to dissolve the crystals, and autoclaving the final solution.

Imidacloprid Pestanal™ (Sigma-Aldrich, CAS # 138261-41-3) with 100.0% purity (HPLC area %) was used to prepare a 128 mg/L stock solution by dissolving 32 mg of imidacloprid in 250 mL of ultrapure water. The stock solution was sterilized by vacuum filtration through a 0.45 µm cellulose acetate membrane.

To prepare the treatment feeding solutions, a 5 mg/L imidacloprid solution was prepared by adding 39 µL of 128 mg/L imidacloprid stock solution to 961 µL of sterile ultrapure water. Next, 30 µL of the 5 mg/L imidacloprid solution was added to 29.97 mL of 50% sucrose solution in each of four sterile 50 mL conical tubes, resulting in the 5 µg/L treatment solution. All solutions were mixed well before use.

For the control group feeding solutions, 30 mL of the 50% sucrose solution was dispensed into each of four sterile 50 mL conical tubes. A 30 µL volume was removed from each tube and replaced with ultrapure water.

Experiment

Collection and conditions

Workers for the control and treatment groups were collected in the late morning on 8/11/2017 by placing open hoarding cages over the hive entrances, waiting until enough workers had filled the cages, and then quickly closing the cages. In a few cases, mild agitation of the hive was required to stimulate flight. Each of the 10 hives provided a replicate for the treatment group and a replicate for the control group, with each cage containing a single replicate, equaling 20 cages in total. The cages were transported back to the lab. Several workers died after transportation for unknown reasons. Workers were

carefully removed from cages in an attempt to equalize the number of bees in each cage. This was only marginally successful, resulting in various numbers of workers in each cage. At the start of the experiment the control and treatment cages contained a total of 168 (12 to 26 per cage) and 189 (12 to 34 per cage) bees, respectively. The cages were kept in a single, dark, 35°C incubator for 4 days and removed once daily for feeding.

Replicates for a baseline group were collected from hives 3 through 10 on 8/29/2017 and hives 1 and 2 on 9/11/2017. Bees from the baseline group were transported to the lab after collection and chilled until immobile. They were placed in tubes (one per replicate) filled with 95% ethanol, as in Moran et al. (7), and stored at 5°C until dissection.

Feeding

On the first 2 days after collection, bees in all cages were fed un-dosed sucrose solution. On days 3 and 4, bees in the treatment cages were fed a sucrose solution containing 5 µg/L imidacloprid. On day 1, before placing the cages in the incubator, 1 mL of sucrose solution was dispensed into each of the feeding tubes and the tube caps were closed. On days 2-4, fresh feeding solutions were provided by removing each feeding tube, replacing it with an unused tube, and dispensing 1 mL of the appropriate feeding solution into each tube. An additional tube containing 1 mL of sucrose solution was placed in the incubator to control for evaporation of the feeding solutions. Any dead bees were removed from the cages before each feeding session. After each 24 hr feeding period, the

evaporation control tube and the used feeding tubes were weighed and recorded in order to determine the amount of food consumed.

At the end of the experiment, each cage was placed in a freezer just until the bees were immobile. For each replicate, all of the bees within the cage were collectively placed in tubes filled with 95% ethanol and stored at 5°C until dissection.

Dissection and Homogenization

The bees were dissected with gloved fingers and flame-sterilized forceps until 10 complete intestinal tracts were obtained from each replicate (Fig 2), with the exception of replicate 7T, from which only 4 intestinal tracts were obtained. Each dissected bee was rinsed by submersion and agitation in fresh 95% ethanol and then placed in a sterile petri dish. The head was pulled off of the thorax to detach the esophagus, and the entire intestinal tract was removed through the anterior end of the bee by grasping the last abdominal segment with forceps and gently pulling. Due to enlargement of the rectum in most of the control and treatment bees, the careful removal of additional segments from the abdominal exoskeleton was necessary in order to remove the rectum intact.

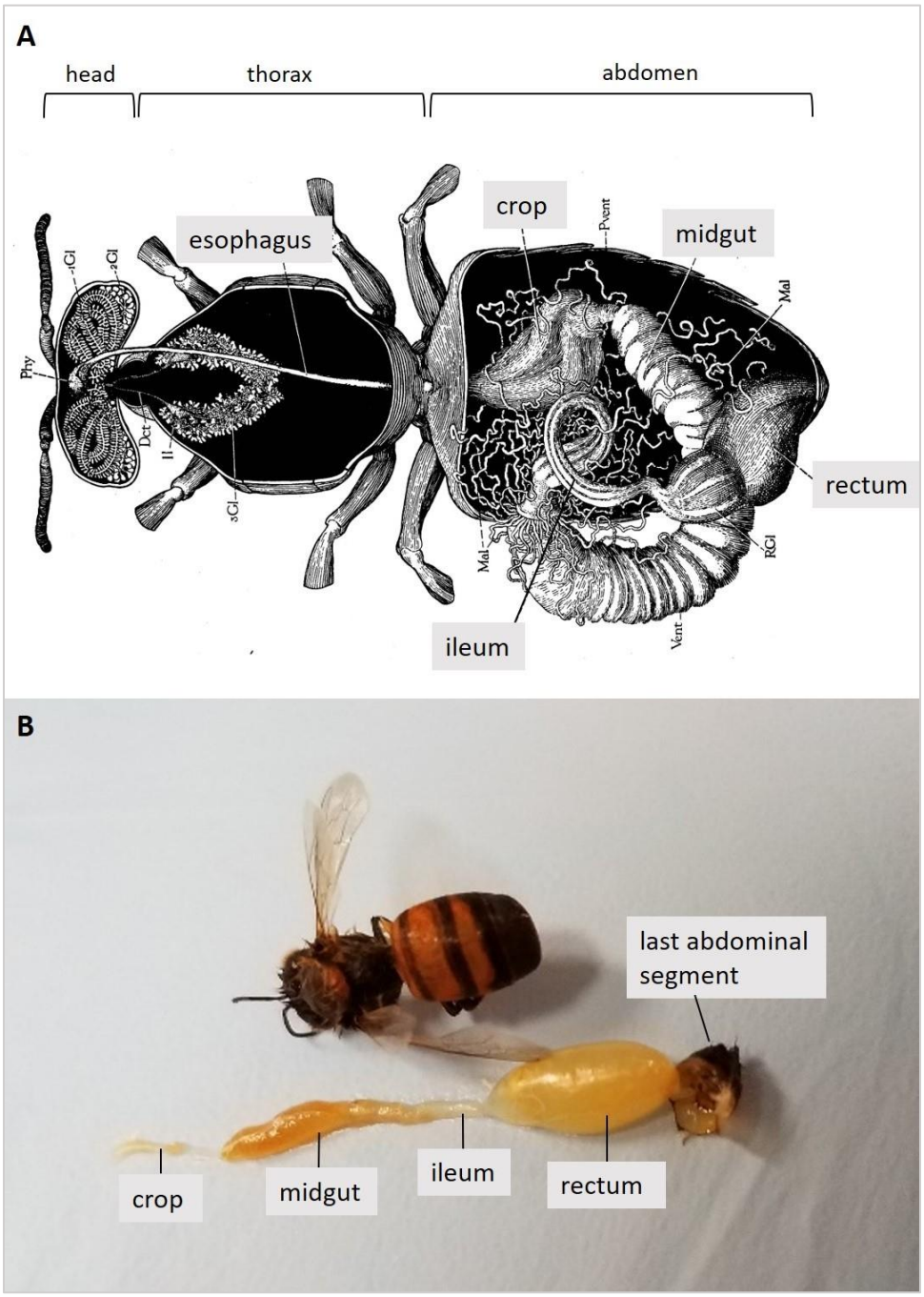

FIG 2 (a) Gastrointestinal anatomy of the honey bee modified from Snodgrass (17). (b) The intestinal tract after dissection

For each group, the intestinal tracts were placed directly into an ethanol and flame sterilized glass tissue grinder. A 0.5 mL volume of sterile ultrapure water was added to the grinder and the guts were pulverized. The resulting liquid was divided into two microcentrifuge tubes and stored at -20°C until the DNA was extracted. After thawing to extract the DNA, additional homogenization with a Tissue-Tearor (7mm probe, medium speed, 6-8 seconds) was needed to liquify the guts enough such that a sample could be drawn into a pipette. The Tissue-Tearor was cleaned between samples by submersion of the tip in bleach, followed by rinsing with 70% ethanol and a final rinse with sterile ultrapure water.

DNA Extraction

DNA was extracted from each sample using the Qiagen DNeasy® PowerSoil® kit as per manufacturer's instructions. In summary, 250 µL of the sample was added to the PowerBead tube and vortexed. Solution C1 was added to the tube, which was then vortexed at maximum speed for 10 min. The tube was centrifuged (all centrifugation steps were at 10,000 x g) for 30 s and the supernatant was transferred to a clean tube. After the addition of solution C2 to the supernatant, the tube was incubated at 4°C for 5 minutes, centrifuged (1 min), and the supernatant transferred to a clean tube. After the addition of solution C3 to the supernatant, the tube was incubated at 4°C for 5 minutes, centrifuged (1 min), and the supernatant transferred to a clean tube. Solution C4 was added to the supernatant, and the tube was vortexed. The sample was loaded onto an MB Spin Column and centrifuged (1 min), discarding the flowthrough. Solution C5 was

added to the spin column which was centrifuged (30 s). The flowthrough was discarded and DNA was eluted from the spin column by adding 100 µL of Solution C6 (10 mM Trist-HCl, pH 8.5) to the column and a final centrifugation (30 s). The resulting flowthrough was divided into two 50 µL aliquots and stored at -20°C. The quality of each sample was analyzed using a Nanodrop blanked with Solution C6. QC values are shown in Table 2.

TABLE 2 QC values of the DNA samples

Replicate	DNA conc. (ng/µL)	A260	A280	260/280	260/230
1C	21.3	0.426	0.233	1.83	1.66
2C	19.8	0.395	0.208	1.9	1.94
3C	16.7	0.334	0.186	1.8	1.55
4C	23.4	0.468	0.257	1.82	1.51
5C	20.3	0.406	0.23	1.76	1.87
6C	11.5	0.231	0.132	1.74	1.29
7C	24.1	0.481	0.261	1.84	2.09
8C	25.3	0.505	0.278	1.82	2.64
9C	16.8	0.335	0.194	1.73	1.56
10C	20.3	0.405	0.225	1.8	2.94
1T	22.6	0.452	0.245	1.84	1.67
2T	21.9	0.439	0.24	1.83	1.51
3T	22.4	0.447	0.241	1.86	1.79
4T	24.8	0.497	0.276	1.8	1.84
5T	17.4	0.347	0.19	1.82	1.28
6T	18.4	0.368	0.202	1.83	2.25
7T	8.1	0.161	0.091	1.78	3.12
8T	23.3	0.466	0.253	1.84	2.56
9T	16.2	0.324	0.183	1.77	2.8
10T	19	0.38	0.212	1.79	1.24
1B	23.1	0.461	0.261	1.77	1.81
2B	29.9	0.498	0.335	1.78	1.52
3B	27	0.54	0.298	1.81	1.9
4B	31.1	0.622	0.348	1.79	1.92
5B	24.5	0.49	0.274	1.79	1.7
6B	26.3	0.527	0.285	1.85	1.94
7B	26.7	0.535	0.296	1.81	2.01
8B	25.8	0.517	0.282	1.83	1.97
9B	21.9	0.438	0.244	1.79	1.69
10B	23.1	0.462	0.249	1.86	1.51

Sequencing

Multiplexed amplicon libraries were prepared using the NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 from Bioo Scientific (catalog #4203-01), as per the manufacturer's protocol (V16.08). An initial PCR amplification produced approximate 450 bp fragments spanning the 254 bp fourth hypervariable domain (V4) of microbial 16S rRNA genes. A second PCR amplification integrated flow cell binding domains and 12 base pair indices unique to each sample. The kit primers used for both PCR steps are shown in Table 3. Agencourt AMPure XP Magnetic Beads were used for cleanup after both PCR amplification steps.

TABLE 3 NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 primers used in the library preparation

Primer	5'→ 3' Sequence
16S V4 Forward	GACGCTCTTCCGATCTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
16S V4 Reverse	TGTGCTCTTCCGATCTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
PCR II Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCT
PCR II Reverse	CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXXXGTGACTGGAGTTCAGA CGTGTGCTCTTCCGATCT

Sequencing was performed with the Illumina MiSeq with MiSeq Control Software v2.6 using the Illumina MiSeq 600-cycle Reagent Kit v3. The MiSeq reads were

demultiplexed with MiSeq Reporter Software and Qiime 2 was used for the remainder of the analysis.

RESULTS AND ANALYSIS

Mortality and Consumption

The number of live bees per cage each day is presented in Table 4. From this data the mean percent mortality (the number of deaths / the starting number of bees x 100) over the 4-day experimental period and the 95% confidence limits were calculated. There was no significant difference in percent mortality between the control (8.6 ± 6.5) and treatment (11.9 ± 12.7) groups.

The daily amounts of feeding solution consumed per bee for each replicate are shown in Table 5. FIG ***3*** 3 shows the mean consumption per bee and 95% confidence interval for the control and treatment groups each day. No significant difference in consumption between the control and treatment groups was observed. For both the control and treatment groups, consumption on day 3 was significantly lower than on days 1, 2, and 4.

TABLE 4 The number of live bees per cage each day

Replicate	Day 1	Day 2	Day 3	Day 4
1T	13	13	13	11
2T	17	17	17	17
3T	17	17	16	16
4T	19	19	19	19
5T	25	25	23	21
6T	20	20	20	19
7T	12	12	9	4
8T	15	14	14	13
9T	15	15	15	15
10T	34	34	34	33
Mean	18.7	18.6	18.0	16.8
1C	12	12	11	11
2C	17	17	17	17
3C	15	15	15	13
4C	15	15	15	15
5C	15	15	13	12
6C	18	18	18	18
7C	14	14	14	13
8C	26	26	20	18
9C	16	16	15	15
10C	18	18	18	18
Mean	16.6	16.6	15.6	15.0

TABLE 5 The amount of feeding solution consumed per bee each day in micrograms

Replicate	Day 1	Day 2	Day 3	Day 4
1T	78	71	56	106
2T	101	54	17	79
3T	67	41	43	64
4T	81	37	58	92
5T	80	39	30	49
6T	73	52	27	37
7T	105	50	70	98
8T	60	56	49	75
9T	101	38	8	33
10T	58	47	36	55
Mean	80.40	48.50	39.40	68.80
SD	17.04	10.53	19.45	25.42
SE	5.39	3.33	6.15	8.04
CI	10.78	6.66	12.30	16.08
1C	154	84	71	84
2C	85	48	34	78
3C	71	35	47	66
4C	75	39	29	83
5C	80	53	56	73
6C	90	47	31	45
7C	71	60	68	86
8C	47	36	66	71
9C	128	106	69	100
10C	55	68	25	54
Mean	85.60	57.60	49.60	74.00
SD	32.48	22.86	18.58	16.10
SE	10.27	7.23	5.88	5.09
CI	20.54	14.46	11.75	10.18

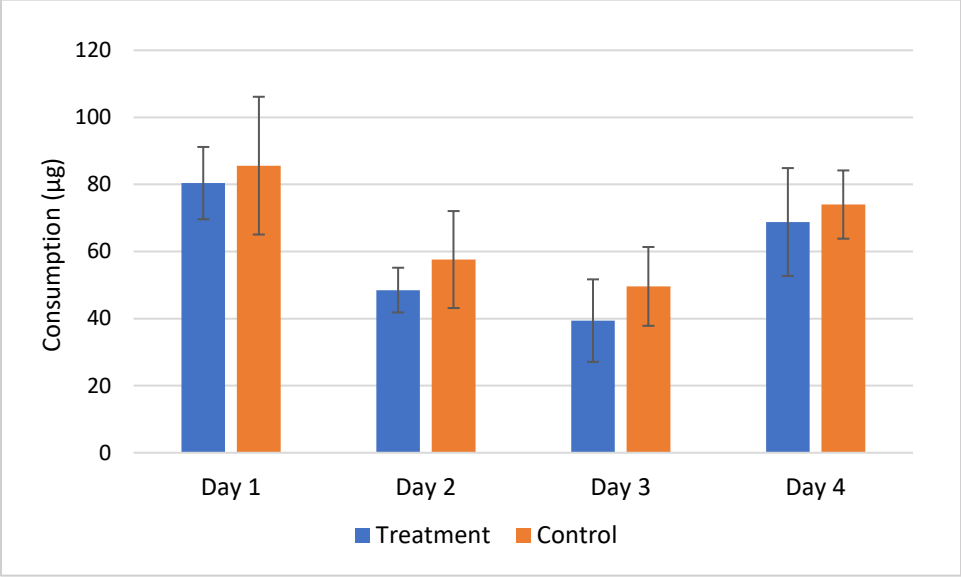

FIG 3 The mean and 95% confidence limits of the amount of sucrose solution consumed per bee each day in micrograms

Metagenomics

Only the forward reads were used in the analysis due to poor quality scores of the reverse reads. For undetermined reasons, the MiSeq data for replicate 8T from the treatment group could not be successfully analyzed, so it has been excluded from the results of the metagenomic analysis. The following steps were performed using multiple plugins within Qiime 2. Reads were quality filtered and trimmed to 190 bp with the Deblur plugin. Chloroplast and mitochondrial reads were filtered from the reads with filter-seq. Multiple sequence alignment was performed on the filtered reads with the Qiime 2 mafft plugin and highly variable positions were filtered from the alignment with mask. A phylogenetic tree was generated with FastTree and the tree was midpoint rooted.

Alpha and Beta Diversity

Alpha and beta diversity analyses were performed in Qiime 2 with a sampling depth of 99,346 reads. Pielou's Evenness and Faith's Phylogenetic Diversity metrics were calculated to look for changes in alpha diversity between the baseline, control, and treatment groups (Fig 4).

Pairwise Kruskal-Wallis tests showed less evenness in the baseline group compared to both the treatment (p = 0.008981) and control (p = 0.023342) groups (Table 6). Phylogenetic diversity was greater for the baseline group compared to the treatment (p = 0.022243) and control (p = 0.049366) groups (Table 7). There were no significant differences in evenness or phylogenetic diversity between the treatment and control groups.

Weighted UniFrac, unweighted UniFrac, Jaccard, and Bray-Curtis distance matrices were calculated and PERMANOVA (999 permutations) was used pairwise for each metric to look for differences in composition (β-diversity) between groups (Tables 8 – 11). For all metrics, there were significant differences between the baseline and control groups and between the baseline and treatment groups (α = 0.05). No differences between the control and treatment groups were observed.

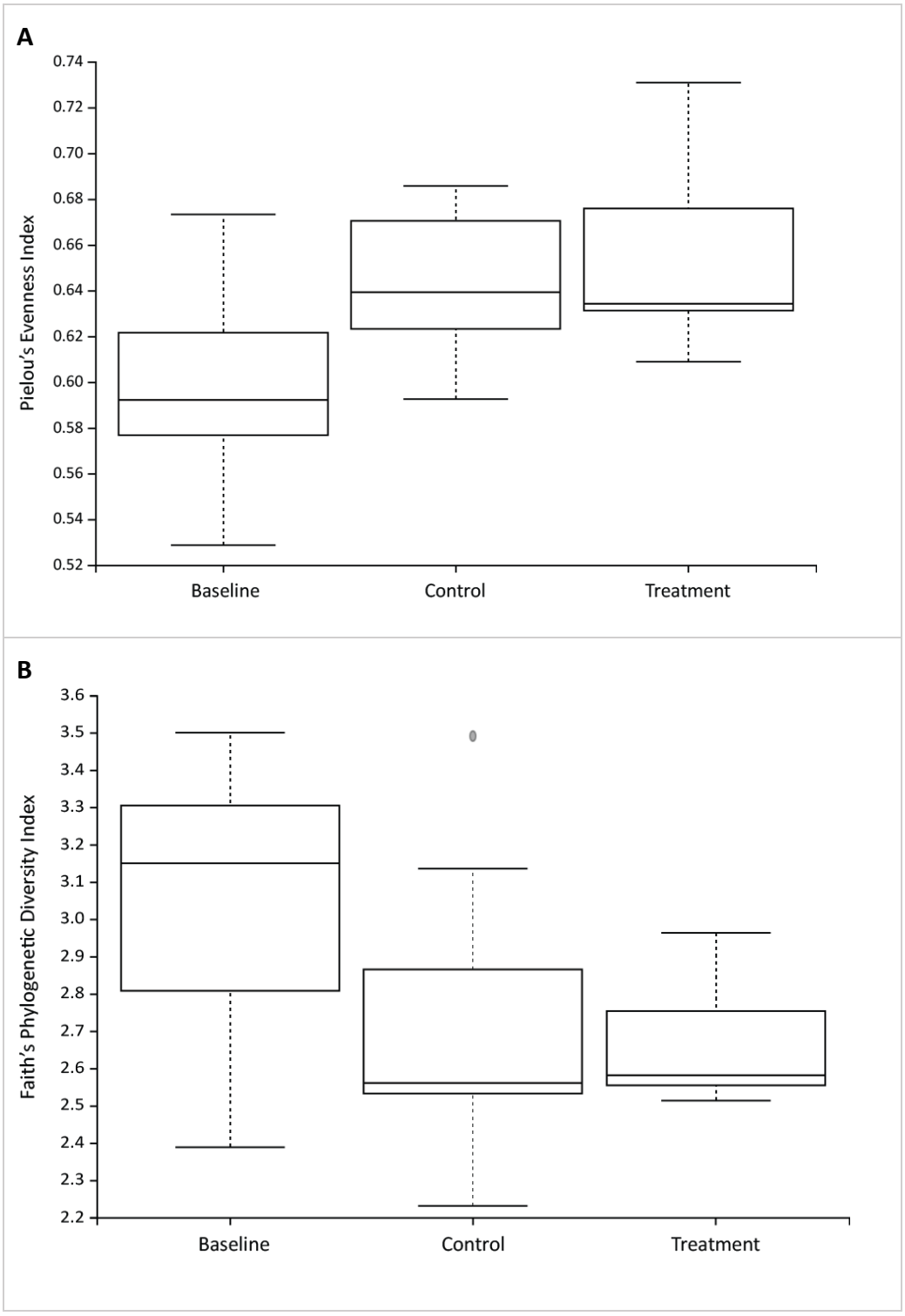

FIG 4 (a) Pielou's Evenness (b) Faith's Phylogenetic Diversity

TABLE 6 Pairwise Kruskal-Wallis Test of Evenness

Group 1	Group 2	H	P-value	Q-value
Treatment (n=9)	Baseline (n=10)	6.826667	0.008981	0.026942
Treatment (n=9)	Control (n=10)	0.24	0.624206	0.624206
Baseline (n=10)	Control (n=10)	5.142857	0.023342	0.035013

TABLE 7 Pairwise Kruskal-Wallis Test of Faith's Phylogenetic Diversity

Group 1	Group 2	H	P-value	Q-value
Treatment (n=9)	Baseline (n=10)	5.226667	0.022243	0.066729
Treatment (n=9)	Control (n=10)	0.326667	0.567628	0.567628
Baseline (n=10)	Control (n=10)	3.862857	0.049366	0.074049

TABLE 8 Weighted UniFrac pairwise PERMANOVA

Group 1	Group 2	Pseudo-F	P-value	Q-value
Treatment	Baseline	8.811648	0.001	0.003
Treatment	Control	1.363237	0.268	0.268
Baseline	Control	8.580722	0.002	0.003

TABLE 9 Unweighted UniFrac pairwise PERMANOVA

Group 1	Group 2	Pseudo-F	P-value	Q-value
Treatment	Baseline	6.955703	0.001	0.0015
Treatment	Control	1.029896	0.43	0.43
Baseline	Control	7.098477	0.001	0.0015

TABLE 10 Bray-Curtis pairwise PERMANOVA

Group 1	Group 2	Pseudo-F	P-value	Q-value
Treatment	Baseline	3.76759	0.003	0.0045
Treatment	Control	1.006112	0.418	0.418
Baseline	Control	5.77019	0.001	0.003

TABLE 11 Jaccard pairwise PERMANOVA

Group 1	Group 2	Pseudo-F	P-value	Q-value
Treatment	Baseline	3.242313	0.001	0.0015
Treatment	Control	0.705343	0.922	0.922
Baseline	Control	3.536829	0.001	0.0015

Relative Abundance

Taxonomy was assigned to reads with a BLAST+ consensus taxonomy classifier coupled the with Silva 128 reference dataset in Qiime 2. Results were exported to Microsoft Excel for plotting and relative abundance analyses. The relative abundances of major bacterial OTUs are presented in Fig 5 and Fig 6. Statistical significance ($\alpha = 0.05$) was determined by Mann-Whitney Tests with Addinsoft XLSTAT (Table 12).

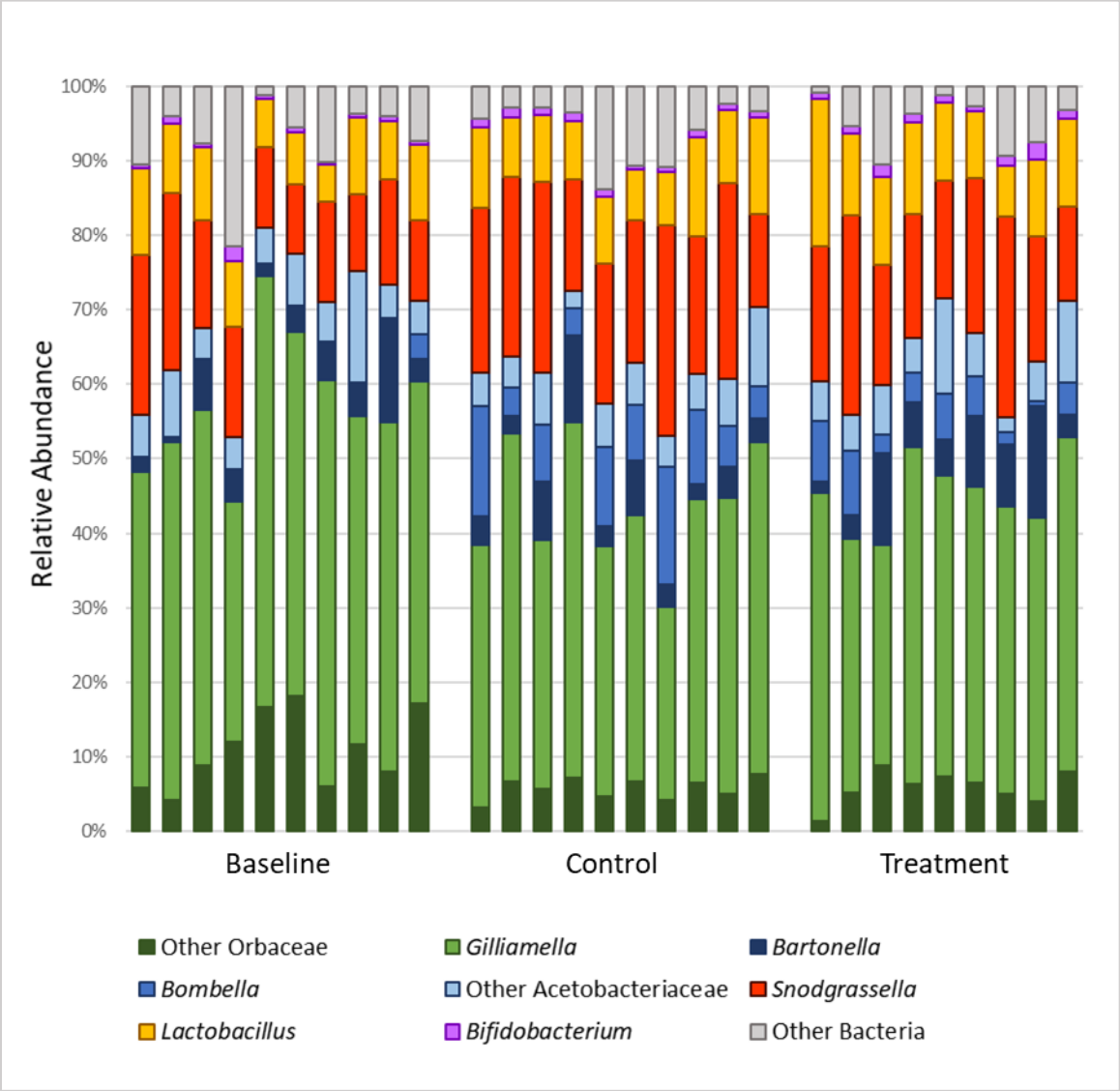

FIG 5 Relative abundance of major bacterial OTUs

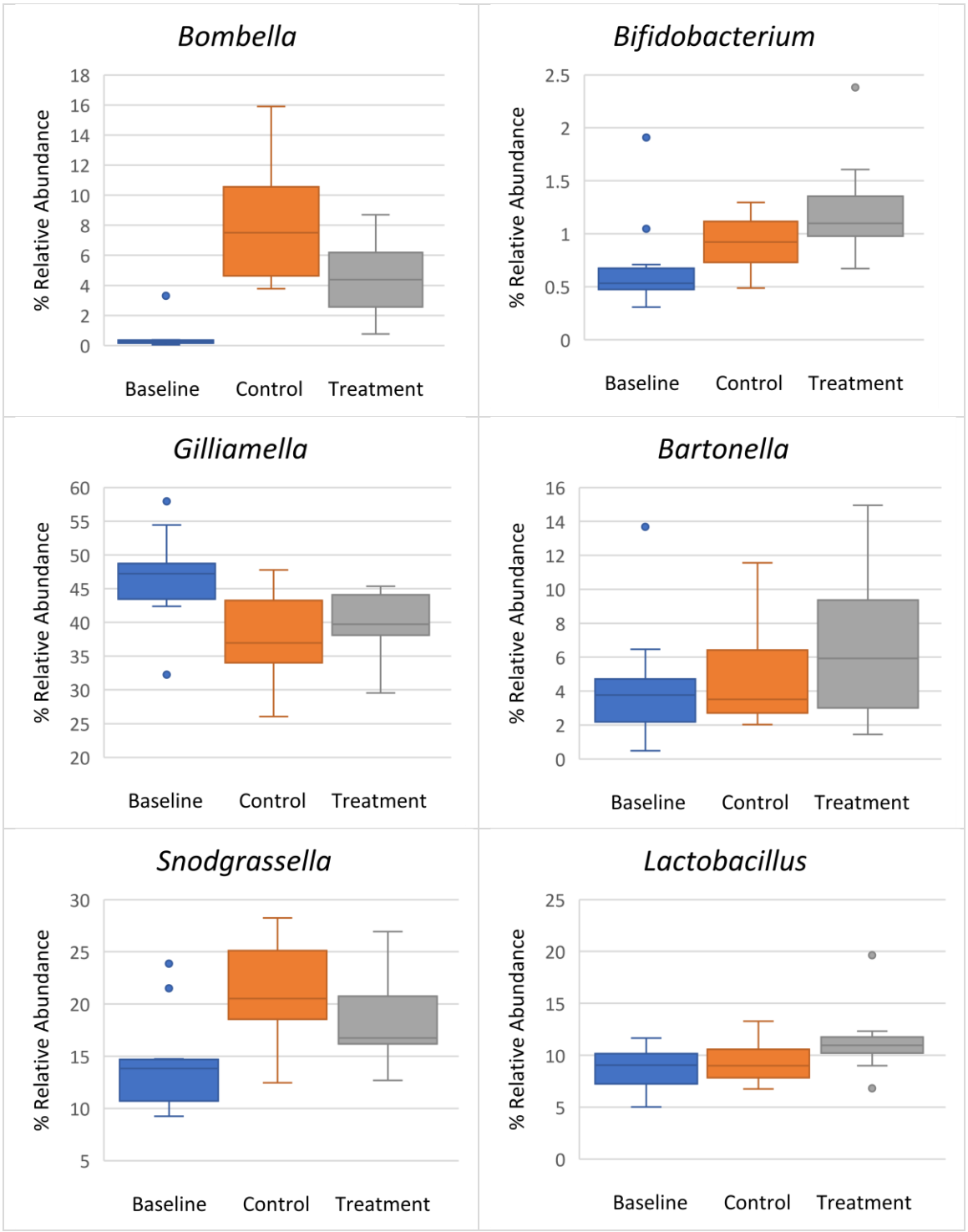

FIG 6 Box and whisker plots of % relative abundance for *Bombella, Bifidobacterium, Gilliamella, Bartonella, Snodgrassella,* and *Lactobacillus.* Dots indicate outliers.

TABLE 12 Mann-Whitney Test p-values for major bacterial OTUs. P-values lower than the significance level (α = 0.05) are shown in bold.

OTU	Baseline Control	Baseline Treatment	Treatment Control
Bombella	**< 0.0001**	**0.000152**	0.09472
Gilliamella	**0.023231**	**0.017212**	0.60378
Lactobacillus	0.630529	**0.027929**	0.182316
Bifidobacterium	**0.035463**	**0.005672**	0.156401
Bartonella	0.853428	0.211024	0.242807
Snodgrassella	**0.006841**	**0.027929**	0.400182

Significant differences in relative abundance of multiple taxa between the baseline and captive (treatment and control) groups were observed. *Bombella, Snodgrassella, and Bifidobacterium* had greater relative abundance in the captive groups than in the baseline group. *Lactobacillus* was also greater in relative abundance in the treatment group, but not in the control group, compared to the baseline group. Only *Gilliamella* showed a significant decrease in the captive groups. These differences were fairly minimal with the exception of *Bombella*, which was 27x and 16x more relatively abundant in the control and treatment groups, respectively.

Analysis of Composition of Microbes (ANCOM) was also applied pairwise in Qiime2 to detect differentially abundant genera. Of the aforementioned genera, only *Bombella* was identified by ANCOM as differentially abundant between the baseline and captive groups. ANCOM also detected a significant difference in *Pantoea*, which was present only in the baseline group with a mean 0.3% relative abundance.

DISCUSSION

Short term oral exposure to imidacloprid under laboratory conditions does not appear to impact the community structure of the honey bee gut microbiome at the tested dose of 5 μg/L. Between the control and treatment groups, no significant differences in evenness, phylogenetic diversity, or relative abundance of bacterial OTUs were observed. There was no increased mortality in the treatment group, nor did the treatment appear to impact consumption. A curious drop and subsequent rise in consumption during the 4-day experimental period was observed in both the control and treatment groups, but the cause of this is undetermined.

There were differences in diversity and relative abundance of certain taxa between the baseline group and control and treatment groups. *Bombella* was markedly less abundant in the baseline group. This could be due to different collection dates for the baseline group. The phylogenetic diversity of the baseline group may be artificially inflated by the presence of environmentally acquired DNA from organisms which have not been reported as normal gut microbiota. *Pantoea* spp., for example, are ubiquitous in the honey bee's environment (18). However, caution must be used in asserting that specific taxa should not be considered as gut microbiota and removing them from analyses.

Alternatively, the laboratory conditions used in this study may induce changes in gut microbiota. Nectar contains a combination of fructose, glucose, and sucrose. The feeding solutions in this experiment were prepared with sucrose, which is standard for

maintaining honey bees in laboratory settings (19). It is expected that some of the sucrose was hydrolyzed during autoclaving, producing glucose and fructose, but the proportions of these sugars in the final solution may still differ significantly from those in nectar. This diet, compared to the nectar and pollen diet of the baseline group, may have selected for a different population of bacteria. Honey bees also do not defecate in confined spaces (20). The retention of waste in the rectum, which harbors most of the bee gut microbiota, may favor the growth of some bacteria. If laboratory conditions impact the honey bee microbiome, the results of experimentation on captive honey bees may have limited relevance. Ideally experiments such as this one would occur in a natural outdoor environment with entire hives. This would require far more time and resources however, as researchers would most likely need to establish their own hives for use in experimentation with pesticides. Without an enclosure, the results of experiments in natural settings may be confounded by exposure to unknown contaminants within a hive's large foraging range.

Although the major OTUs observed were in agreement with previous characterizations of the gut microbiome, the results of this study showed a notably lower abundance of Firmicutes and higher abundance of Gammaproteobacteria in the guts of honey bees than has been described in other studies (7, 21, 22). These studies used different DNA extraction methods, which may be more effective at lysing the cell walls of gram-positive bacteria, including Firmicutes. A study which also used the MO BIO (Now Qiagen) PowerSoil kit for extraction observed relative abundances that closely resemble

those from this study (23). The Qiagen PowerSoil kit is the standard method for the Earth Microbiome Project and has been used to extract microbiome DNA from other insects in several microbiome studies (24–27). Although a comparison of extraction methods by Rubin et al. (27) found no difference in community composition between PowerSoil and phenol chloroform extraction, additional research comparing DNA extraction methods for honey bee microbiota may be needed.

In summary, findings from this study do not support the hypothesis that oral imidacloprid exposure induces dysbiosis of the honey bee gut microbiome, but do not provide strong evidence to the contrary. A single conservative dose based on estimates of typical field concentrations was tested. Higher doses as well as chronic lower doses may be encountered in the field and warrant testing as well.

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