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Imidacloprid Decreases Honey Bee Survival Rates but Does Not Affect the Gut Microbiome

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ABSTRACT Accumulating evidence suggests that pesticides have played a role in the increased rate of honey bee colony loss. One of the most commonly used pesticides in the United States is the neonicotinoid imidacloprid. Although the primary mode of action of imidacloprid is on the insect nervous system, it has also been shown to cause changes in insects' digestive physiology and alter the microbiota of *Drosophila melanogaster* larvae. The honey bee gut microbiome plays a major role in bee health. Although many studies have shown that imidacloprid affects honey bee behavior, its impact on the microbiome has not been fully elucidated. Here, we investigated the impact of imidacloprid on the gut microbiome composition, survivorship, and susceptibility to pathogens of honey bees. Consistent with other studies, we show that imidacloprid exposure results in an elevated mortality of honey bees in the hive and increases the susceptibility to infection by pathogens. However, we did not find evidence that imidacloprid affects the gut bacterial community of honey bees. Our *in vitro* experiments demonstrated that honey bee gut bacteria can grow in the presence of imidacloprid, and we found some evidence that imidacloprid can be metabolized in the bee gut environment. However, none of the individual bee gut bacterial species tested could metabolize imidacloprid, suggesting that the observed metabolism of imidacloprid within *in vitro* bee gut cultures is not caused by the gut bacteria. Overall, our results indicate that imidacloprid causes increased mortality in honey bees, but this mortality does not appear to be linked to the microbiome.

IMPORTANCE Growing evidence suggests that the extensive use of pesticides has played a large role in the increased rate of honey bee colony loss. Despite extensive research on the effects of imidacloprid on honey bees, it is still unknown whether it impacts the community structure of the gut microbiome. Here, we investigated the impact of imidacloprid on the gut microbiome composition, survivorship, and susceptibility to pathogens of honey bees. We found that the exposure to imidacloprid resulted in an elevated mortality of honey bees and increased the susceptibility to infection by opportunistic pathogens. However, we did not find evidence that imidacloprid affects the gut microbiome of honey bees. We found some evidence that imidacloprid can be metabolized in the bee gut environment *in vitro*, but because it is quickly eliminated from the bee, it is unlikely that this metabolism occurs in nature. Thus, imidacloprid causes increased mortality in honey bees, but this does not appear to be linked to the microbiome.

KEYWORDS honey bee, imidacloprid, metabolism, microbiome

The decline of honey bee colonies over the last decade has been attributed to several factors, such as pathogens, parasites, genetics, climate change, and a loss of foraging habitat (1, 2). In addition, growing evidence suggests that the extensive use of

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pesticides has played a role in the increased rate of colony loss (3–5). One of the most commonly used pesticides for the control of insect damage to crops in the United States is the neonicotinoid imidacloprid. Imidacloprid acts on the nicotinic acetylcholine receptor (nAChR) to interfere with the nervous system of insects (6). Imidacloprid is absorbed by plants and spreads to all tissues, including pollen and nectar, through the vascular system (1, 2, 6). Thus, imidacloprid can be transmitted to the hive via the transport of pollen and nectar and subsequently through social contact (3–5, 7). At field-realistic levels, imidacloprid in nectar and pollen is not immediately lethal to honey bees (6, 8), but it alters bee behavior, resulting in impaired homing abilities (9–12), immunocompetence (13), and a susceptibility to infection by the parasitic microsporidian *Nosema* (14–17). Moreover, foraging bees prefer food containing imidacloprid or other neonicotinoids, potentially increasing exposure (18). Two recent, large-scale long-term studies provide additional evidence for the detrimental effects of neonicotinoids on honey bees, reflected in the decreased bee survival in some hives exposed to neonicotinoids (4, 5).

The importance of the gut microbiome in the health of animal hosts has become increasingly evident. In honey bees, the gut microbiome has been shown to play a role in metabolism, growth and development, protection against pathogens, and immunity (19–22), and several results suggest that gut bacterial community imbalance in honey bees leads to an increased susceptibility to pathogen infection (22, 23) and an elevated mortality of workers in hives (22).

Despite the extensive research on the effects of imidacloprid on honey bees, it is still unknown whether it impacts the community structure of the gut microbiome or whether any of the resident gut bacteria of honey bees are capable of metabolizing imidacloprid. Although the primary mode of action of imidacloprid is on the insect nervous system, it also changes the digestive physiology (24). Additionally, imidacloprid impacts the structure, genetic diversity, and catabolic activity of soil microbial communities (25) and has been shown to alter the microbiota of *Drosophila melanogaster* larvae (26). Some bacteria can metabolize imidacloprid, but several of the metabolites are more toxic than imidacloprid itself (27). For example, the most widely reported metabolites of imidacloprid are 4-hydroxy imidacloprid (IMI-IV) and 5-hydroxy imidacloprid (IMI-V); both products spontaneously form olefin, which is 10 times more toxic than imidacloprid to insects (27). However, the metabolism of imidacloprid to IMI-V and the spontaneous conversion to olefin usually happens under acidic conditions (28).

Here, we investigated the impact of imidacloprid on the gut microbiome composition, survivorship, and susceptibility to pathogens of honey bees. We also performed *in vitro* experiments to determine if honey bee gut bacteria can grow in the presence of or can metabolize imidacloprid. Consistent with other studies, we found that the exposure to imidacloprid resulted in an elevated mortality of honey bees in the hive and an increased susceptibility to infection by the opportunistic pathogen *Serratia*. However, we did not find evidence that imidacloprid affects the gut bacterial community of honey bees. We did find some evidence that imidacloprid can be metabolized in the bee gut environment *in vitro*, but because it is very quickly eliminated from the bee, it is unlikely that this metabolism occurs in nature. Furthermore, none of the tested isolates of core bee gut bacteria were capable of metabolizing imidacloprid. Thus, imidacloprid causes increased mortality in honey bees, but this mortality does not appear to be linked to the microbiome.

RESULTS AND DISCUSSION

Imidacloprid does not significantly affect the gut microbiome composition of honey bees. Adult worker bees were collected from brood frames from a single hive. The bees were fed filter-sterilized sucrose syrup or imidacloprid suspended in filter-sterilized sucrose syrup for 3 days before being returned to the hive. To determine if imidacloprid exposure affects the composition of the gut microbiome, the bees were marked and sampled at several time points postexposure (days 0 [D0], 3 [D3], and 5

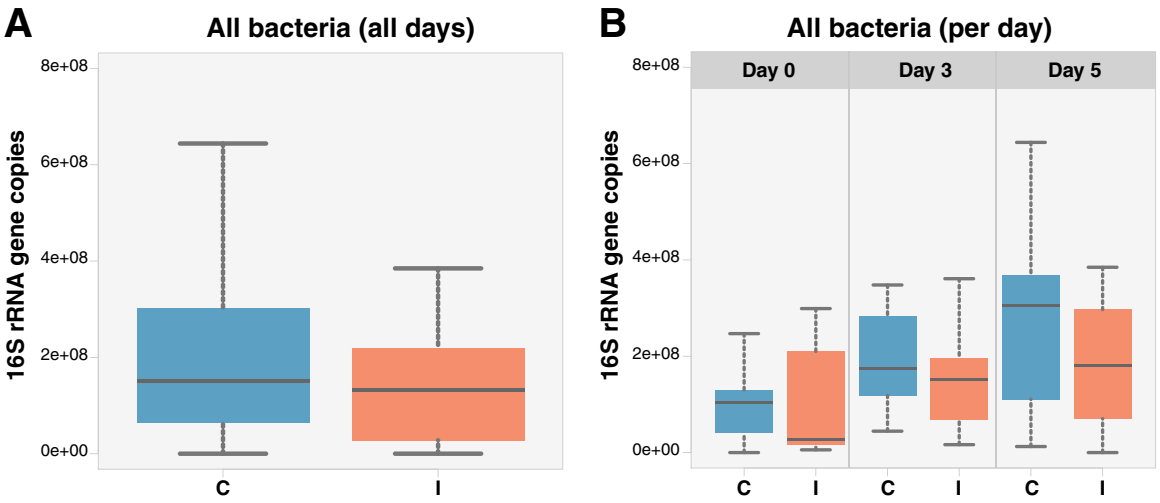

FIG 1 Absolute abundances of gut bacteria after imidacloprid exposure. (A) Boxplot of total bacterial 16S rRNA gene copies estimated by qPCR for all control (C) and imidacloprid-exposed (I) bees. (B) Boxplot of total bacterial 16S rRNA gene copies estimated by qPCR for control (D0, $n = 15$; D3, $n = 12$; and D5, $n = 11$) and imidacloprid-exposed (D0, $n = 14$; D3, $n = 14$; and D5, $n = 15$) bees at each sampling day after imidacloprid exposure. Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. No significant differences were observed in total bacterial abundance between control and imidacloprid-exposed bees ($P > 0.05$ Wilcoxon rank sum tests).

[D5]). The community composition of the gut microbiome was assessed using deep amplicon sequencing of a region (V4) of the bacterial 16S rRNA gene.

Because imidacloprid has been shown to change the physiology of insect guts (24), alter the microbiota of fly larvae (26), and change the microbial community composition of soil (25), we hypothesized that the exposure to imidacloprid would alter the gut microbiota of honey bees. However, we did not find evidence that imidacloprid impacts the honey bee gut microbiome. No significant changes in the gut bacterial community size (Fig. 1) or composition (Fig. 2; see also Fig. S1 in the supplemental material) were found at any time point after imidacloprid exposure ($P > 0.05$, Wilcoxon rank sum tests), and no differences in alpha diversity (diversity within individuals) or beta diversity (diversity between groups) were observed between control and exposed bees ($P > 0.05$, Wilcoxon rank sum tests) (Fig. 3A and B). Furthermore, a principal-coordinate analysis (weighted UniFrac [29]) showed that the gut community compositions of exposed and control bees were similar between the two groups (Fig. 3C).

Our results suggest that imidacloprid does not impact the gut bacterial community of honey bees. Jones et al. recently evaluated the microbiomes of honey bees foraging in two different environmental landscapes, one next to a neonicotinoid (thiamethoxam)-treated oilseed rape field and one not (30). Along with the landscape and diet, the authors proposed that neonicotinoid exposure could impact microbiome composition (30). Our results weigh against this proposal, though strains of core bee gut bacteria at different locations may be impacted differently by particular compounds. For example, individual isolates of *Gilliamella apicola* vary in gene repertoires and corresponding catabolic capabilities (31). Because we only tested the effects of imidacloprid on the microbiome in a single hive, it is possible that other hives with different genetic backgrounds or gut bacterial compositions could be more impacted by imidacloprid.

In *D. melanogaster*, imidacloprid exposure caused an increase in the abundance of two indigenous microbiota members, *Acetobacter* and *Lactobacillus*, in third instar larvae (26). However, no significant changes in bacterial abundance were observed in adult flies (26). These results are consistent with ours, as we only tested the effects of imidacloprid on adult bees with established gut microbiota. Because pesticides can alter the bees' immune responses (13, 32, 33), it is possible that imidacloprid affects the early colonization of the microbiota by interfering with the bees' ability to regulate bacterial populations.

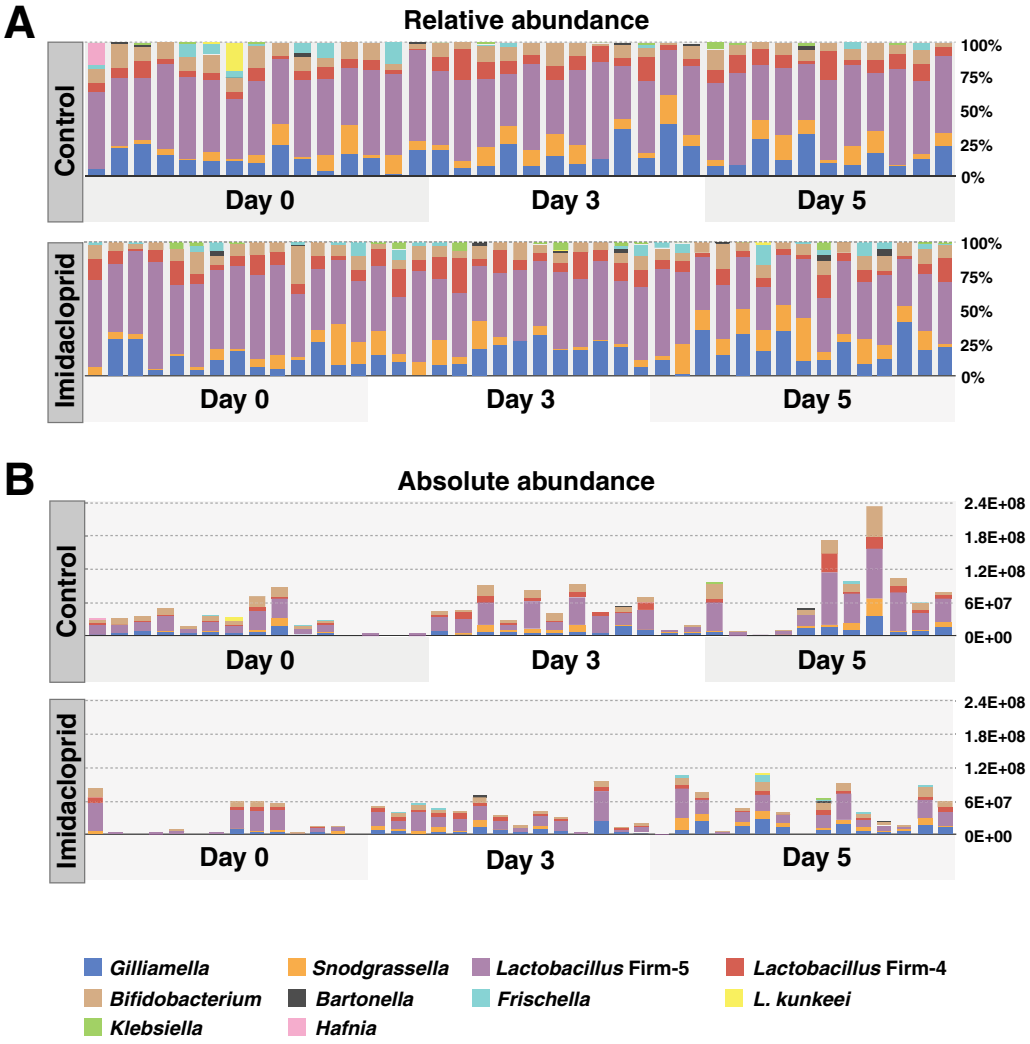

FIG 2 Honey bee gut bacterial compositions at days 0, 3, and 5 after 3 days of imidacloprid exposure. Stacked column graphs showing the relative abundance (A) and absolute abundance (B) of bee gut bacterial species in 15 control day 0, 14 imidacloprid day 0, 12 control day 3, 14 imidacloprid day 3, 11 control day 5, and 15 imidacloprid day 5 bees.

Imidacloprid is not metabolized by the resident honey bee gut bacteria. One possible explanation as to why imidacloprid does not impact the bee gut microbiome could be because it is not toxic to the gut bacteria or because they have the ability to metabolize it. To test these possibilities, we performed *in vitro* experiments in which individual isolates of honey bee gut bacteria or entire gut communities were cultured in the presence of imidacloprid (1 mg/ml). Imidacloprid did not affect the growth of representative strains of the resident bee gut bacterial species, which include *Snodgrassella alvi* (strains wkB2, wkB9, wkB332, and wkB339), *Lactobacillus* Firm-4 (strains 26254 and 26255), *Lactobacillus* Firm-5 (strains wkB8 and wkB10), *Gilliamella apicola* (strains wkB1, wkB7, M1-2G, and M6-3G), *Bifidobacterium* (strains wkB338 and wkB344), *Bartonella apis* (wkB233A), and *Frischella perrara* (strain PEB095) (Fig. 4A). While *G. apicola* did exhibit slight nonsignificant growth inhibition in the presence of imidacloprid, this appears to be due to the solvent used to solubilize imidacloprid. To test if any of these strains can degrade imidacloprid, the supernatants of the bacterial cultures and controls were extracted, and samples were analyzed using liquid chromatography-mass spectrometry (LC-MS). No measurable degradation of imidacloprid was observed in bacterial cultures after 3 days of incubation (Fig. 4B) or in whole-gut samples compared to that in controls after 6 days of incubation (Fig. 4C).

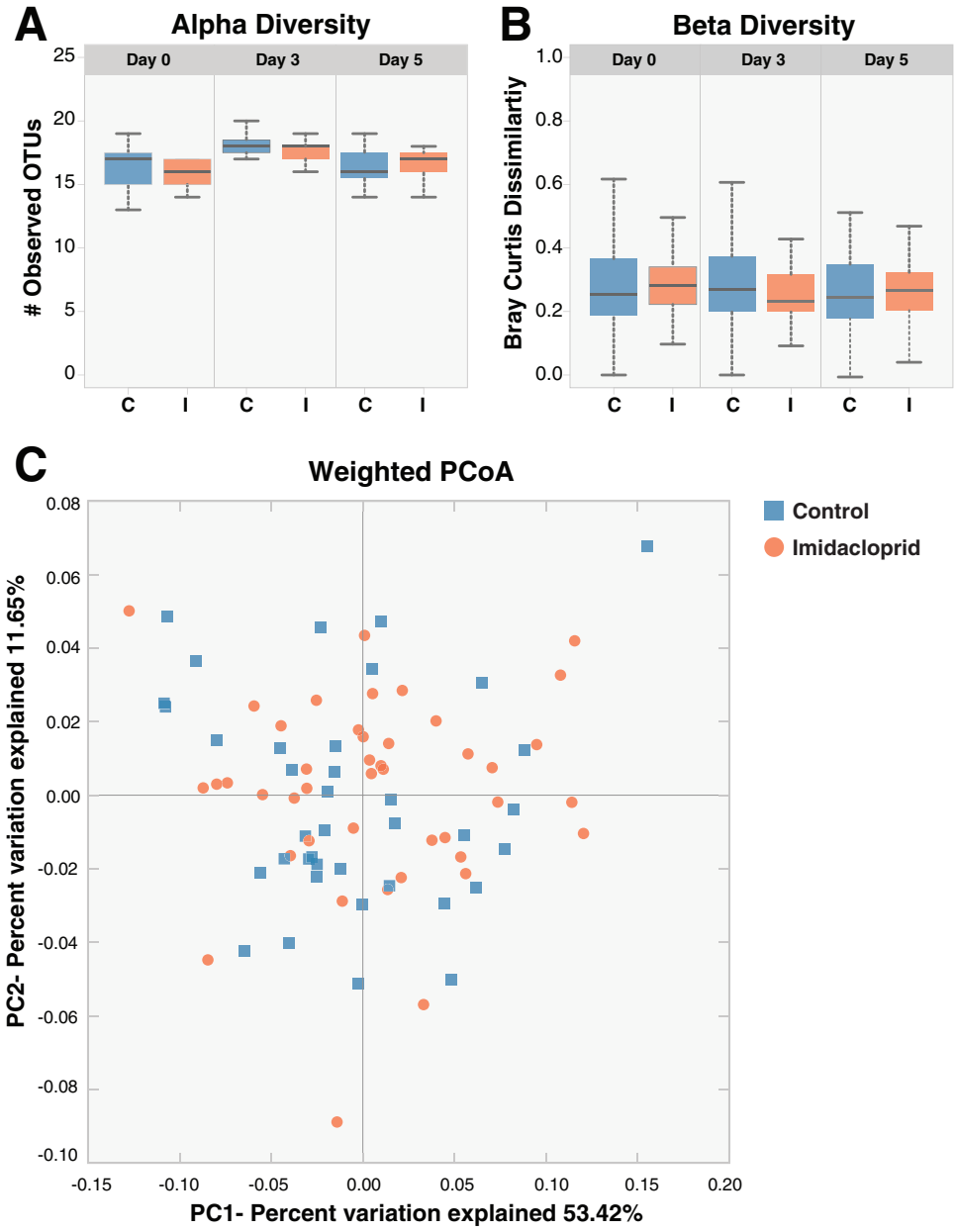

FIG 3 Alpha and beta diversities of imidacloprid-exposed and control bees. (A) Differences in alpha diversity between control (C) and imidacloprid-exposed (I) bees at each time point (measured by observed OTUs). (B) The average Bray-Curtis dissimilarity in gut communities among control bees versus between control bees and exposed bees. Box-and-whiskers plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles. No significant differences were found between control and exposed bees ($P > 0.05$ Wilcoxon rank sum tests). (C) Principal-coordinate analysis using weighted UniFrac.

Our combined *in vitro* and *in vivo* results suggest that the bacterial members of the bee gut community are not affected by imidacloprid and cannot degrade it. However, it is possible that degradation occurs at very low rates, especially in the complex whole bee gut samples, and that variations in the imidacloprid metabolite concentrations were below our analytical detection limits. To investigate this, we evaluated the production of imidacloprid metabolites using high-resolution LC-MS. Gut homogenates were extracted from 12 bees and pooled (six bees per sample, WG1 and WG2). These two pooled samples were cultured and then incubated in a buffer medium with 1 mg/ml imidacloprid. Aliquots of the gut-imidacloprid solution were used for metabolite extraction at days 2 and 7. In both pooled samples, we detected nitrosoguanidine and

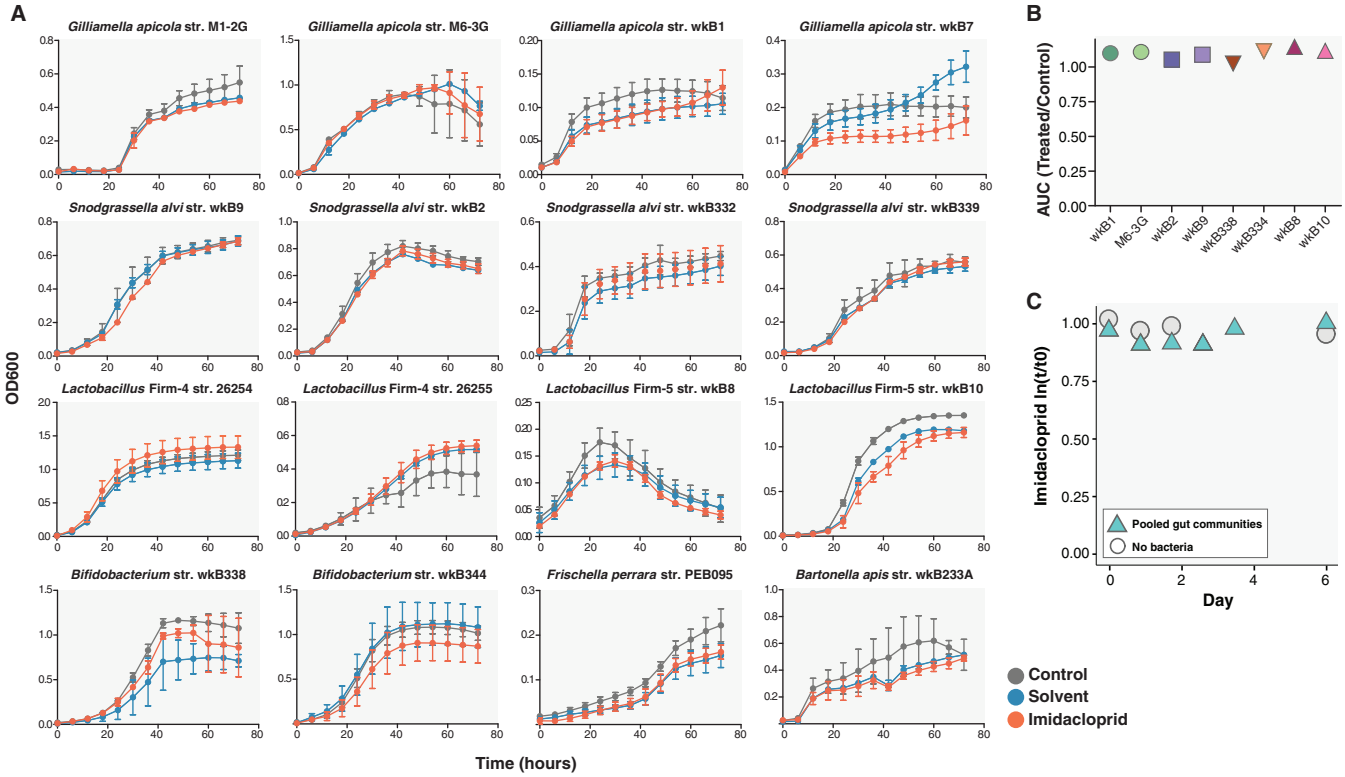

FIG 4 *In vitro* exposure of bee gut bacteria to imidacloprid. (A) Bacterial growth curves of *Gilliamella apicola*, *Snodgrassella alvi*, *Lactobacillus* Firm-4 and Firm-5, *Bifidobacterium asteroides*, *Frischella perrara*, and *Bartonella apis* strains cultured in medium (control), medium with solvent, or medium with 1 mg/ml imidacloprid. Experiments were performed in triplicates, and each data point represents the average optical density (at 600 nm, with standard deviation bars) measured every 6 h. (B) Relative concentrations of imidacloprid in bacterial cultures compared to controls after 3 days of exposure based on imidacloprid peak area (area under the curve [AUC]) obtained from LC-MS analyses. (C) Relative concentrations of imidacloprid in pooled gut community cultures compared to controls at days 0 to 4 and 6 displayed as the neutral logarithm of the ratio over the initial levels.

guanidine (Fig. 5), which were identified as part of a degradation pathway of imidacloprid in *Pseudomonas* sp. (34). Guanidine has also been identified as an imidacloprid degradation product in *Leifsonia* sp. (35). The production of nitrosoguanidine and guanidine may contribute to imidacloprid toxicity in insects, as these transformations alter the "magic nitro" group of the pesticide (═N—NO_2), which is responsible for the insect selectivity of neonicotinoids (34). We also identified the imidacloprid metabolite IMI-V in both pooled samples after 7 days of incubation (Fig. 5A and B), indicating that imidacloprid can be degraded in the bee gut environment. The signal intensities of all metabolites (nitrosoguanidine, guanidine, and IMI-V) were lower in the WG2 sample pool than in WG1 (Fig. 5A and B).

Because IMI-V can be converted into olefin under acidic conditions (28), we investigated if lowering the pH conditions of our incubated samples could induce the production of olefin. WG1 was selected because it had the strongest signal for IMI-V, the olefin precursor (Fig. 5A). The WG1 culture that had been incubated for 7 days in the previous experiment was acidified to mimic the environment of the bee rectum (pH 5.5) (20), where the highest density of bacteria occurs in the bee gut (36). After lowering the pH, olefin was detected in WG1 (Fig. 5C), suggesting that if the bee gut can produce IMI-V, this metabolite has the potential to be converted into olefin in the rectum, due to the low pH of this compartment.

We individually analyzed the remaining gut homogenates from each of the 12 bees from our pooled samples (WG1 and WG2) for the presence of olefin to assess how widespread the ability to produce this toxic metabolite is across individuals. After incubations with imidacloprid and acidification, individual bee samples were scanned for the presence of olefin. We did not analyze the samples for IMI-V; olefin is the

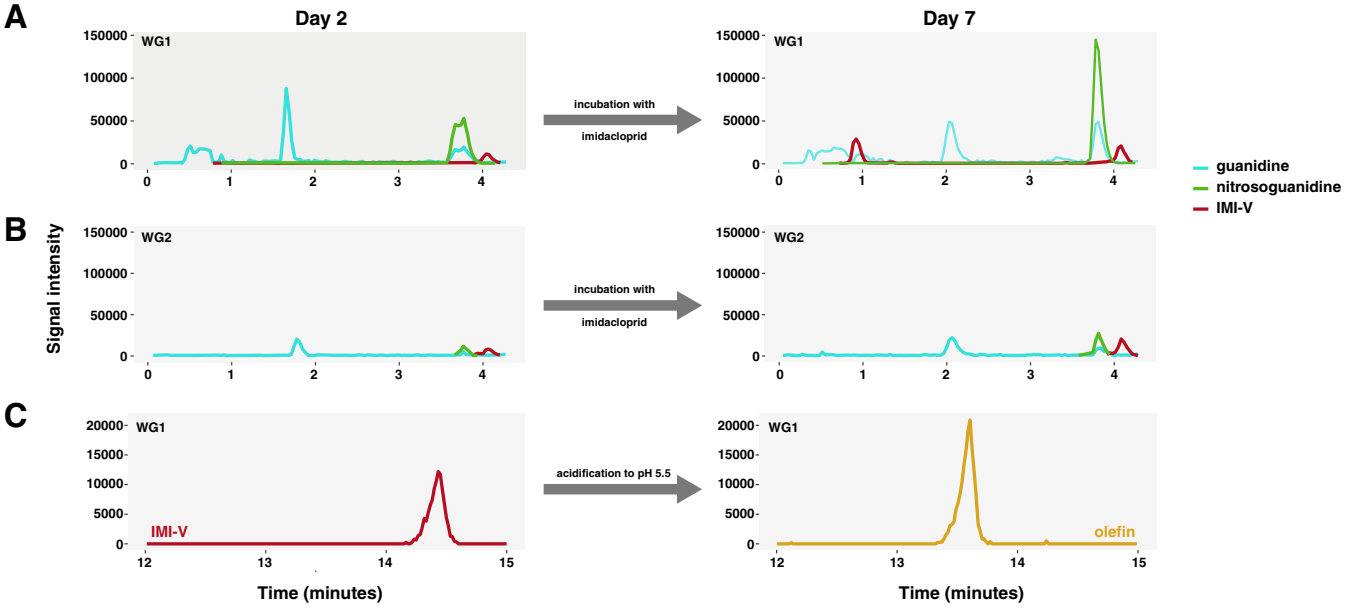

FIG 5 Imidacloprid metabolite production by the honey bee gut. (A and B) Imidacloprid metabolites nitrosoguanidine, guanidine, and IMI-V were detected in WG1 and WG2 and in increasing amounts from day 2 to day 7. Signal intensity was greater in the WG1 pool of samples. (C) Olefin, which was until then undetected in all other samples, was detected following acidification to pH 5.5 (at 13.6 min).

product of IMI-V, and so its presence following sample acidification would strongly indicate that IMI-V had been produced from imidacloprid and then converted to olefin under the low pH conditions of the bee gut (20). The biomass for individual bees was very low, making the results difficult to interpret; but, we confidently detected olefin in 10 of the 12 bees whose guts were exposed to imidacloprid in cultures and acidified after incubation (see Fig. S2). Olefin was not detected in nonacidified samples.

From these *in vitro* experiments, a majority of the bees tested had gut environments capable of producing olefin from imidacloprid. The increased insecticidal activity of olefin (27) suggests this could have consequences for host health. However, our experimental design does not enable us to pinpoint the bee microbiome as the cause. Microsomal cytochromes (CYP450) involved in phase I of cellular detoxification are involved in IMI metabolism (37) and can mediate the oxidation of IMI into IMI-V via *Cyp6g1* in insects (38). Therefore, host cells that may have been extracted along with the gut contents could be responsible for the observed metabolism. Furthermore, abiotic oxidation of IMI into IMI-V may have occurred during our *in vitro* experiments independently from the gut microbiota. In fact, the IMI-V metabolite was previously detected in autoclaved soil samples treated with IMI (39).

Imidacloprid is quickly cleared from the honey bee. It is difficult to estimate the concentrations of imidacloprid that honey bees encounter in the field. However, the dose (500 μg/liter) given to bees in this study was sublethal (see Fig. S3). Additionally, we checked for the presence of imidacloprid in control and exposed bees using high-resolution LC-MS on extractions of individual bees (40) and did not detect imidacloprid in control or exposed bees immediately following treatment (see Fig. S4). Since the bee gut is a complex matrix, we investigated whether components in the bee gut were inhibiting/masking the detection of imidacloprid in treatment samples by adding imidacloprid to control samples before extraction. In imidacloprid-added control samples, we were able to detect imidacloprid, indicating that this method is suitable for detecting amounts of imidacloprid greater than the limit of detection of our mass spectrometer (50 μg/liter). These results are consistent with those of several other studies that usually detect imidacloprid in bees or in their body compartments at very low concentrations, smaller than what we could detect in this study (41–45), which indicates that imidacloprid is quickly eliminated from or metabolized by bees (45).

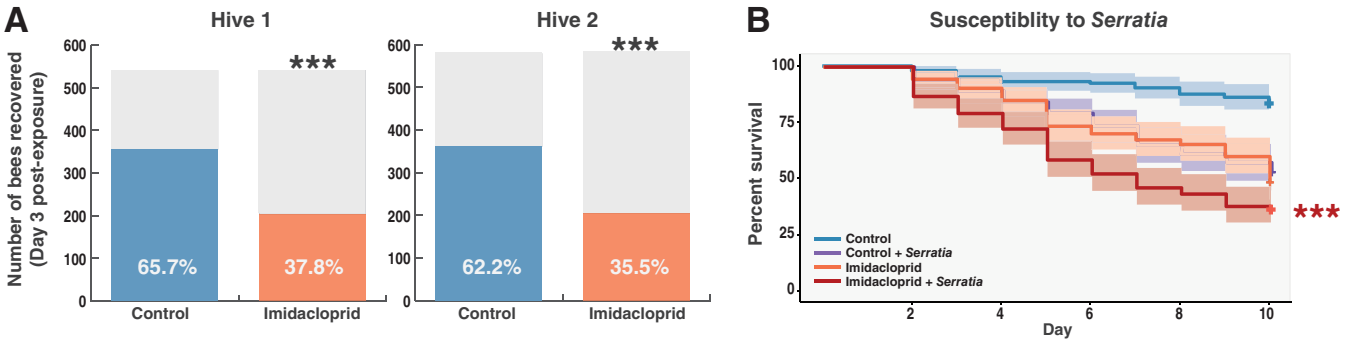

FIG 6 Effects of imidacloprid on honey bee fitness. (A) Survival rates of honey bees returned to the hive after 3 days of exposure to imidacloprid. Numbers of workers recovered from the hive on day 3 posttreatment ($P < 0.0001$, chi-square test). (B) The percent survival of age-controlled bees after *Serratia* exposure, shown as Kaplan-Meier survival curves. Statistical analyses were performed using the coxph model (***, $P < 0.0001$) implemented in the "survival" package (51) in R.

Suchail et al. (41) demonstrated that radiolabeled imidacloprid is readily distributed and metabolized into a few metabolites, including IMI-V and olefin, in different compartments of the bee body, including the midgut (the primary place of metabolism) and the hindgut, which contains most of the bee bacterial biomass. However, the dose administered by Suchail et al. (41) was higher than the dose administered here. Therefore, at field-realistic concentrations, imidacloprid is likely mostly metabolized in the bee before it reaches the hindgut. Thus, the metabolism of imidacloprid into IMI-V and olefin observed in our *in vitro* gut cultures may not occur in the bee gut in nature. None of the core bee gut bacterial isolates tested could metabolize imidacloprid, and imidacloprid did not affect the microbiome composition, which suggests that even if imidacloprid is present in the hindgut, it is metabolized by bee enzymes and not the resident microbiota.

Imidacloprid exposure decreases honey bee survival in the hive and increases susceptibility to pathogens. Adult worker bees were collected from brood frames from two hives. The bees were exposed to imidacloprid for 3 days in the lab as described above. The impact of imidacloprid exposure on honey bee survival in the hive was evaluated by returning marked bees to their original hive after exposure and censusing bees 3 days after reintroduction. Consistent with other studies, we found that imidacloprid exposure resulted in a decreased survival in the hive (Fig. 6A). In both experiments, we recovered more than 60% of the control bees, whereas less than 40% of imidacloprid-exposed bees were recovered (Fig. 6A). This decrease in survival could be due to behavioral defects (9–12), an increased susceptibility to pathogens (14–17), or altered immunocompetence (13) induced by the exposure to imidacloprid.

Previous studies have shown that imidacloprid causes an increased susceptibility to *Nosema* infection (14–17). Here, we tested whether imidacloprid exposure results in increased infection by honey bee-associated strains of the opportunistic bacterial pathogen *Serratia*. Control and imidacloprid-exposed bees were orally exposed to *Serratia* sp. strain kz11 (22), and survival was monitored for 10 days. The bees exposed to imidacloprid and subsequently exposed to *Serratia* sp. strain kz11 exhibited an increased mortality compared to that of control bees, bees exposed to imidacloprid only, or bees exposed to *Serratia* only ($P < 0.0001$) (Fig. 6B). This result is consistent with observations for *D. melanogaster*; flies exposed to imidacloprid were more susceptible to *Serratia marcescens* infection (26). Because imidacloprid has little or no effect on the gut microbiome, this increased susceptibility is likely due to impaired immune function induced by imidacloprid (13).

Conclusions. Our results indicate that imidacloprid has little or no impact on the size or composition of the gut microbiome of adult worker bees with established gut communities. Our results resemble findings for *D. melanogaster*, in which no significant changes in bacterial abundance were observed in adult flies after exposure to imida-

cloprid (26). However, imidacloprid-induced changes in microbiome composition were observed in *D. melanogaster* larvae. Because pesticides can alter bees' immune responses (13, 32, 33), it is possible that imidacloprid could affect the establishment of the microbiota by interfering with the bees' ability to regulate bacterial populations. This possibility warrants further investigation. We found no evidence that the core species of the bee gut microbiota can metabolize imidacloprid. Olefin can be produced from *in vitro* whole-gut incubations, possibly reflecting the metabolic activities of rare gut community members; however, residual host or abiotic processes during incubations may be responsible for this metabolism. Since imidacloprid is quickly eliminated from bees, we predict that little or none reaches the bee hindgut, where most of the bacterial community resides, and if any does, it is metabolized by bee enzymes rather than the resident microbiota. Even so, the lack of effect of imidacloprid on the bee gut microbiota is not surprising, since the primary targets of this insecticide are insect neural transporters. In this specificity, imidacloprid differs from antibiotics and glyphosate, which are agrochemicals expected to impact organisms other than animals. As expected, imidacloprid exposure decreases bee survival in hives, even though it is quickly eliminated from the bee, potentially through the activity of bee-produced enzymes. Our results suggest that imidacloprid increases the susceptibility to opportunistic pathogens, possibly by altering immune function.

MATERIALS AND METHODS

Chemicals and solutions. Imidacloprid was obtained from Toronto Research Chemicals, Canada (catalog number I274990). For experiments with bacterial isolates and bee gut cultures, a 1-mg/ml imidacloprid solution was prepared by dissolving 100 mg imidacloprid in 1 ml acetone-methanol 1:1 and then adding 99 ml of culture medium.

For experiments with honey bees, a 1-mg/ml imidacloprid stock solution was prepared by dissolving 10 mg imidacloprid in 10 ml methanol. To test for sublethal doses of imidacloprid, the stock solution was diluted to 500 μg/liter, 200 μg/liter, and 50 μg/liter imidacloprid using filter-sterilized 0.5 M sucrose syrup (see Fig. S3 in the supplemental material). The 500-μg/liter imidacloprid solution was used for all further experiments.

Effects of imidacloprid on the honey bee gut microbiome. Hundreds of adult honey bee workers were collected from brood frames from a single hive. The bees were brought into the lab and immobilized at 4°C, separated into two groups, and labeled using Testors paint. One group was fed 500 μg/liter imidacloprid suspended in sterilized sucrose syrup (imidacloprid), and the other group was fed only sterile sucrose syrup (control). After 3 days of exposure, 15 bees from each group were sampled, and the remaining bees were returned to the hive. The bees were also sampled from the hive at days 3 and 5 postexposure (15 bees per group per day). DNA extractions were performed on all sampled bees using the protocol from reference 22. Extracted DNA was used for bacterial community profiling based on deep sequencing of the V4 region of the 16S rRNA gene, as amplified by PCR primers 5′-GTTTGATCMTGGCT CAG-3′ and 5′-TGCCTCCCGTAGGAGT-3′. The community profiling, including amplification, library preparation, and sequencing (Illumina MiSeq 2×250) was performed by the Genomic Sequencing and Analysis Facility at the University of Texas at Austin. Sequence reads were processed in QIIME (46). FASTQ files were filtered for quality with split_libraries_fastq.py, allowing a minimum Phred quality score of Q20. Forward and reverse Illumina reads were joined using join_paired_ends.py with default settings. Chimeric sequences were removed using the usearch6.1 detection method implemented in the identify_chimeric_seqs.py script in QIIME. Operational taxonomic units (OTUs) were clustered at 97% identity using the UCLUST algorithm as implemented in pick_open_reference_otus.py. Briefly, sequence reads were initially clustered against the QIIME default reference database (Greengenes v 13.8). Sequences that did not match the QIIME reference data set were subsequently clustered into *de novo* OTUs with UCLUST. Unassigned, mitochondrial, and chloroplast reads were removed from the data set. To eliminate pyrosequencing errors, all OTUs present in less than 0.1% total abundance across all samples were removed. Because the currently available curated 16S rRNA sequence databases do not contain reference sequences for the core species of the honey bee gut microbiota, an additional taxonomic assignment was performed using a local BLAST database of 16S rRNA gene sequences from a reference set of honey bee bacteria. This process yielded a total of 22 OTUs, which were then clustered to the genus level, resulting in 10 genus-level clusters. Downstream analyses, including alpha and beta diversity estimations, were conducted using the QIIME workflow core_diversity_analysis.py, with a sampling depth of 3,500 reads per sample and default parameters. The rarefaction depth was chosen manually to exclude samples represented by exceptionally low numbers of sequences and resulted in final sample sizes for control samples of $n = 15$ for D0, $n = 12$ for D3, and $n = 11$ for D5 and for treatment samples of $n = 14$ for D0, $n = 14$ for D3, and $n = 15$ for D5. The abundance of the bee gut bacterial species was estimated by correcting for absolute abundance (estimated by quantitative PCR [qPCR]) and taking into account 16S rRNA gene copy number, as in reference 22. All alpha and beta diversity estimates were performed in QIIME.

Quantitative PCR to estimate bacterial abundance. The bacterial abundances within control bees (D0, $n = 15$; D3, $n = 12$; and D5, $n = 11$) and treatment bees (D0, $n = 14$; D3, $n = 14$; and D5, $n = 15$) were estimated using quantitative PCR. Universal 16S rRNA gene primers 27F (5′-AGAGTTTGATCCTGG CTCAG-3′) and 355R (5′-CTGCTGCCTCCCGTAGGAGT-3′) were used to amplify total copies of the 16S rRNA gene from each sample on an Eppendorf Mastercycler ep realplex instrument (Eppendorf, Hauppauge, NY, USA). Triplicate 10-μl reactions were carried out with 5 μl iTaq universal SYBR green (Bio-Rad, Inc.), 1 μl (each) 3 μM primer, 2 μl H_2O, and 1 μl 100× dilutions of the template DNA. The cycling conditions consisted of 95°C for 3 min and 40 cycles of two-step PCR at 95°C for 3 s and at 60°C for 20 s. The quantification was based on standard curves from amplification of the cloned target sequence in a pGEM-T vector (Promega, Madison, WI, USA). The values were adjusted to account for dilution.

Exposure of bee gut bacteria and whole bee guts to imidacloprid. Previously isolated honey bee gut bacterial strains of *Snodgrassella alvi* (strains wkB2 [47], wkB9, wkB332, and wkB339 [48]), *Gilliamella apicola* (strains M1-2G, M6-3G, wkB7 [31], and wkB1 [47]), *Frischella perrara* (strain PEB095 [49]), *Bifidobacterium asteroides* (strains wkB338 and wkB344), and *Bartonella apis* (strain wkB233A) were grown in Insectagro. Strains of *Lactobacillus* Firm-5 (wkB8 and wkB10 [50]) and Firm-4 (26254 and 26255) were grown in MRS. *Lactobacillus mellis* strain DSM 26254 and *Lactobacillus mellifer* strain DSM 26255 were purchased from DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. All strains were cultured at 35°C in 5% CO_2 overnight. The optical density at 600 nm (OD_{600}) of each bacterial culture was measured, and the cells were washed with 1× phosphate-buffered saline (PBS) and diluted to an OD_{600} of 1 in the respective culture media. Ten-microliter aliquots of each bacterial suspension were transferred in triplicates to a 96-well plate containing 190 μl medium, 190 μl medium with solvent, or 190 μl medium with 1 mg/ml imidacloprid. The controls consisted of medium, medium with solvent, or medium with 1 mg/ml imidacloprid. The plates were incubated in a plate reader (Tecan) at 35°C and 5% CO_2 for 72 h. The OD_{600} was measured every 6 h.

The guts from 12 healthy worker bees were extracted, pooled into two samples (6 bees in each sample) to increase screening potential (WG1 and WG2), and cultured in tryptic soy broth (TSB) for 24 h at 35°C and 5% CO_2. Bacterial cells were isolated by centrifugation (7,000 relative centrifugal force [rcf], 10 min), washed, and concentrated in 0.2 M Na_2HPO_4-KH_2PO_4 (pH 8) buffer. The pooled gut community (WG1 and WG2) and a bacteria-free control were incubated for 6 days at 35°C and 5% CO_2 with 1 mg/ml imidacloprid and 50 mg/ml of sucrose in Na_2HPO_4-KH_2PO_4 buffer.

Detection of imidacloprid in bacterial and whole bee gut cultures. Supernatants of bacterial cultures (200 μl, *G. apicola* strains wkB1 and M6-3G, *S. alvi* strains wkB2 and wkB9, *Bifidobacterium* strains wkB338 and wkB334, and *Lactobacillus* Firm-5 strains wkB8 and wkB10) and aliquots of the pooled gut community cultures (200 μl, collected at days 0 to 4 and 6) were partitioned with ethyl acetate (100 μl, 2 times), dried, resuspended in methanol (100 μl), and analyzed by LC-MS for imidacloprid detection. LC was performed with an Agilent 1200 Infinity liquid chromatograph in gradient elution mode using a Zorbax Eclipse Plus C_{18} column (2.1 mm by 50 mm, 5-μm particle size). The flow rate was 0.7 ml/min with mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile). The gradient mode was 0 min, 5% B; 5 min, 42.5% B; 6 min, 95% B; 10 min, 95% B; 10.1 min, 5% B. Eluting species were detected by an Agilent Technologies 6130 single quadrupole spectrometer equipped with an electrospray ion source in positive mode. Ion source settings were capillary voltage, 2,900 V; drying gas temperature, 300°C; gas flow, 9 liters/min; and nebulizer pressure, 40 lb/in^2. Imidacloprid identification was performed by comparing elution times and mass spectra to those of standard samples. Targets in standards and in extracts were observed as $[M+H^+]$ (protonated species) and $[M+Na^+]$ (sodiated species). The data were analyzed with MassHunter qualitative analysis software (Agilent). For bacterial cultures, the relative concentration of imidacloprid was compared to that of controls after 3 days of exposure on the basis of the imidacloprid peak area.

For bee gut degradation rates, changes in imidacloprid concentrations were shown as the natural logarithm of the ratio over the initial levels. Aliquots from incubations were extracted using ethyl acetate and resuspended in methanol, as described above. LC was performed with an Agilent 1260 Infinity liquid chromatograph in gradient elution mode using a Zorbax Eclipse Plus 95Å C_{18} column (50 mm by 2.1 mm, 5-μm particle size). Targets in standards and in extracts were observed as $[M+H^+]$ (protonated species) and $[M+Na^+]$ (sodiated species), and ionization was by positive mode atmospheric pressure chemical ionization (APCI). The flow rate was 0.7 ml/min with mobile phase A (water plus 0.1% formic acid) and mobile phase B (acetonitrile plus 0.1% formic acid).

Detection of imidacloprid metabolites in whole bee gut cultures. Bee gut homogenates were combined into two pooled samples (WG1 and WG2), each containing six individual bee guts, to maximize the screening potential. Pooled homogenates were cultured in tryptic soy broth (TSB) for 24 h. The cells were isolated by centrifugation and washed with 0.2 M Na_2HPO_4-KH_2PO_4 (pH 8). The cells were concentrated and resuspended in Na_2HPO_4-KH_2PO_4 and incubated with imidacloprid and sucrose (final concentrations 1 mg/ml and 50 mg/ml, respectively, for 7 days). Aliquots were collected for metabolite extraction at day 2 and day 7 and were analyzed by high-resolution LC-MS (plus electrospray ionization [ESI]). Because IMI-V can be converted into highly toxic olefin under acidic concentrations, the WG1 sample that had been incubated for 7 days in imidacloprid and sucrose and showed the highest levels of metabolites was acidified to pH 5.5 with HCl to represent the conditions in the bee hindgut (20).

Aliquots from incubations were extracted using ethyl acetate and resuspended in methanol, as described above. LC was performed with an Agilent 1200 Infinity liquid chromatograph in gradient elution mode using a Zorbax Eclipse Plus C_{18} column (50 mm by 2.1 mm, 5-μm particle size). The flow rate was 0.7 ml/min with mobile phase A (water plus 0.1% formic acid) and mobile phase B (acetonitrile plus methanol plus 0.1% formic acid). The gradient mode was 0 min, 5% B; 12 to 17 min, 95% B; 18 min,

5% B. Eluting species were detected by an Agilent Technologies 6530 Accurate-Mass Q-TOF equipped with an electrospray ion source in positive mode. The ion source settings were drying gas temperature, 350°C; gas flow, 13 liters/min; nebulizer pressure, 55 lb/in^2. Metabolite identification was performed by comparing elution times and mass spectra to those of standard samples. Targets in standards and in extracts were observed as [M+H$^+$] (protonated species) and [M+Na$^+$] (sodiated species). The data were analyzed with MassHunter qualitative analysis software (Agilent).

Individual gut homogenates from the 12 bees that had been pooled into WG1 and WG2 were cultured individually to determine how widespread the ability to produce olefin is across individual bees. The cells were isolated and cultured as described for WG1 and WG2 and acidified to pH 5.5 with HCl. Olefin was measured in these 12 samples. Aliquots from the incubations were extracted using ethyl acetate and were resuspended in methanol, as described above. LC was performed with an Agilent 1200 Infinity liquid chromatograph in gradient elution mode using a Zorbax Eclipse XDB C_8 column (150 mm by 4.6 mm, 3.5-μm particle size). The flow rate was 0.6 ml/min with mobile phase A (water plus 0.1% formic acid) and mobile phase B (acetonitrile plus methanol plus 0.1% formic acid). The gradient mode was 0 min, 10% B; 5 min, 10% B; 16 min, 43% B (flow change to 1.2 ml/min); 17 to 23 min, 100% B (flow change to 0.6 ml/min); 24 min, 10% B. Eluting species were detected by an Agilent Technologies 6530 Accurate-Mass Q-TOF equipped with an electrospray ion source in positive mode. The ion source settings were drying gas temperature, 350°C; gas flow, 10 liters/min; nebulizer pressure, 45 lb/in^2. Metabolite identification was performed using target *m/z* (254.0439). The data were analyzed with MassHunter qualitative analysis software (Agilent).

Detection of imidacloprid residues in whole bee bodies. Imidacloprid was extracted from whole bodies of exposed bees according to a described protocol (40), with some adaptations. Five bees from control and imidacloprid-treated groups were sampled after 3 days. They were individually transferred to microtubes with steel beads and 25% methanol (1 ml) and homogenized in TissueLyser equipment for 3 min at 250 rpm. The homogenates were centrifuged for 5 min at 14,000 rpm at 4°C, and the supernatants were collected and combined according to their respective groups. Combined samples were then diluted with equal volumes of 25% acetic acid and were subjected to a solid-phase extraction using a Discovery DSC_{18} column (bed weight, 50 mg; volume, 1 ml). The column was first washed with 1 ml water, and then the samples were eluted 3 times with methanol (200 μl). The samples were dried, resuspended in 25% methanol (400 μl), filtered, and submitted for high-resolution LC-MS analysis. Before processing the control sample, a 0.5 -μl aliquot of 1 mg/ml imidacloprid was transferred to the microtube to check sample matrix interference in imidacloprid detection. LC was performed with an Agilent 1200 Infinity liquid chromatograph in gradient elution mode using a Zorbax Eclipse Plus C_{18} column (2.1 mm by 50 mm, 5-μm particle size). The flow rate was 0.6 ml/min with mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile). The gradient mode was 0 to 5 min, 10% B; 16 min, 43% B; 17 to 23 min, 100% B (flow rate changed to 1.2 ml/min); 24 min, 10% B (flow rate changed back to 0.6 ml/min). Eluting species were detected by an Agilent Technologies 6530 Accurate-Mass Q-TOF equipped with an electrospray ion source in positive mode. The ion source settings were capillary voltage, 2,500 V; drying gas temperature, 350°C; gas flow, 12 liters/min; nebulizer pressure, 60 lb/in^2. Imidacloprid identification was performed by comparing elution times and mass spectra to those of standard samples. Targets in standards and in extracts were observed as [M+H$^+$] (protonated species) and [M+Na$^+$] (sodiated species). The data were analyzed with MassHunter qualitative analysis software (Agilent).

Effects of imidacloprid on honey bee fitness in the hive. Hundreds of honey bee workers were taken from two separate hives located on the UT campus (one was used for the microbiome analysis above). The bees were treated and marked as explained above. Briefly, control bees were fed sterile sucrose syrup for 3 days, and exposed bees were administered 500 μg/liter imidacloprid suspended in sterilized sucrose syrup for 3 days. The marked bees were returned to their original hive after 3 days of imidacloprid exposure. Three days after reintroduction to the hive, the marked bees were captured and counted.

Susceptibility to *Serratia* infection following imidacloprid exposure. Approximately 700 honey bee workers were taken from a single hive located on the UT campus. The control bees were fed sterile sucrose syrup for 3 days, and the exposed bees were administered 500 μg/liter imidacloprid suspended in sterilized sucrose syrup for 3 days followed by oral exposures to (i) *Serratia* sp. strain kz11 (OD_{600} of 0.5 suspended in sterile sugar syrup) or (ii) sterile sucrose syrup only. Each group contained five replicates with approximately 30 bees per replicate. Survivorship was monitored and recorded each day for 10 days.

Accession number(s). All sequence data are available in the NCBI BioProject database under project number PRJNA432211. All other data generated or analyzed during this study are included in this published article and its supplementary information.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00545-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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