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Imidacloprid decreases honey bee survival but does not affect the gut microbiome

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Running Head: Effects of imidacloprid on the honey bee microbiome

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Abstract

Accumulating evidence suggests that pesticides have played a role in the increased rate of honeybee colony loss. One of the most commonly used pesticides in the US is the neonicotinoid imidacloprid. Although the primary mode of action of imidacloprid is the insect nervous system, it has also been shown to cause changes 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 of honey bees, and susceptibility to pathogens. Consistent with other studies, we show that imidacloprid exposure results in elevated mortality of honey bees in the hive and increases 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 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 of honey bees, and susceptibility to pathogens. We found that exposure to imidacloprid resulted in elevated mortality of honey bees, and increased 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.

Introduction

The decline of honey bee colonies over the last decade has been attributed to several factors such as pathogens and parasites, genetics, climate change, and loss of foraging habitat (1, 2). In addition, growing evidence suggests that the extensive use of pesticides has played a role in the increased rate of colony loss (3–5). One of the most commonly used pesticides for 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 through the hive via 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 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 detrimental effects of neonicotinoids on honey bees, reflected in decreased bee survival in some hives exposed to neonicotinoids (4, 5).

The importance of the gut microbiome in 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 increased susceptibility to pathogen infection (22, 23) and elevated mortality of workers in hives (22).

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 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 the insect nervous system, it also changes the digestive physiology (24). Additionally, imidacloprid impacts the structure, genetic diversity, and catabolic activity of soil

93 microbial communities (25) and has been shown to alter the microbiota of
94 *Drosophila melanogaster* larvae (26). Some bacteria can metabolize imidacloprid,
95 but several of the metabolites are more toxic than imidacloprid itself (27). For
96 example, the most widely reported metabolites of imidacloprid are 4-hydroxy
97 imidacloprid (IMI-IV) and 5-hydroxy imidacloprid (IMI-V); both products
98 spontaneously form the olefin, which is 10 times more toxic than imidacloprid to
99 insects (27). However, the metabolism of imidacloprid to IMI-V and the spontaneous
100 conversion to olefin usually happens under acidic conditions (28).

101 Here we investigated the impact of imidacloprid on the gut microbiome
102 composition, survivorship of honey bees, and susceptibility to pathogens. We also
103 performed *in vitro* experiments in order to determine if the honey bee gut bacteria
104 can grow in the presence of or can metabolize imidacloprid. Consistent with other
105 studies, we found that exposure to imidacloprid resulted in elevated mortality of
106 honey bees in the hive, and increased susceptibility to infection by the opportunistic
107 pathogen *Serratia*. However, we did not find evidence that imidacloprid affects the
108 gut bacterial community of honey bees. We did find some evidence that
109 imidacloprid can be metabolized in the bee gut environment *in vitro*, but because it
110 is very quickly eliminated from the bee it is unlikely that this metabolism occurs in
111 nature. Furthermore, none of the tested isolates of core bee gut bacteria were
112 capable of metabolizing imidacloprid. Thus, imidacloprid causes increased mortality
113 in honey bees, but this mortality does not appear to be linked to the microbiome.
114

115 **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. Bees were fed filter-sterilized sucrose syrup or imidacloprid suspended in filter-sterilized sucrose syrup for three days before being returned to the hive. In order to determine if imidacloprid exposure affects the composition of the gut microbiome, bees were marked and sampled at several time points post-exposure (Days 0, 3, and 5). The community composition of the gut microbiome was accessed 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 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, Fig S1) were found at any time-point post 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 3 A and B). Furthermore, principal coordinate analysis (weighted UniFrac (29)) showed that the gut community compositions of exposed and control bees are similar between the two groups (Fig 2 C).

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 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 bees' immune responses (13, 32, 33), it is possible that imidacloprid affects early colonization of the microbiota by interfering with the bees' ability to regulate bacterial populations.

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* (wkB2, wkB9, wkB332, wkB339), *Lactobacillus* Firm-4 (26254, 26255), *Lactobacillus* Firm-5 (wkB8, wkB10), *Gilliamella apicola* (wkB1, wkB7, M1-2G, M6-3G), *Bifidobacterium* (wkB338, wkB344), *Bartonella apis* (wkB233A), and *Frischella perrara* (PEB095) (Fig 4 A). While *G. apicola* did exhibit slight, non-significant 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 supernatant 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 three days of incubation (Fig 4 B), or in whole gut samples when compared to controls after six days of incubation (Fig 4 C).

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 imidacloprid metabolite concentrations were below our analytical detection limits. To investigate this, we

183 evaluated the production of imidacloprid metabolites using high-resolution LC-MS.
184 Gut homogenates were extracted from 12 bees and pooled (six bees per sample,
185 WG1 and WG2). These two pooled samples were cultured and then incubated in a
186 buffer media with 1 mg/mL imidacloprid. Aliquots of the gut/imidacloprid solution
187 were used for metabolite extraction at Days 2 and 7. In both pooled samples, we
188 detected nitrosoguanidine and guanidine (Fig 5), which were identified as part of a
189 degradation pathway of imidacloprid in *Pseudomonas* sp. (34). Guanidine has also
190 been identified as an imidacloprid degradation product in *Leifsonia* sp. (35). The
191 production of nitrosoguanidine and guanidine may contribute to imidacloprid
192 toxicity in insects, as these transformations alter the 'magic nitro' group of the
193 pesticide (=N-NO_2), which is responsible for the insect selectivity of neonicotinoids
194 (34). We also identified the imidacloprid metabolite IMI-V in both pooled samples
195 after seven days of incubation (Fig 5 A and B), indicating that imidacloprid can be
196 degraded in the bee gut environment. The signal intensity of all metabolites
197 (nitrosoguanidine, guanidine and IMI-V) was lower in the WG2 sample pool than in
198 WG1 (Fig 5 A and B).

199 Because IMI-V can be converted into olefin under acidic conditions (28), we
200 investigated if lowering the pH conditions of our incubated samples could induce
201 production of olefin. WG1 was selected because it had the strongest signal for IMI-V,
202 the olefin precursor (Fig 5 A). The WG1 culture that had been incubated for 7 days
203 in the previous experiment was acidified to mimic the environment of the bee
204 rectum (pH 5.5) (20), where the highest density of bacteria occurs in the bee gut
205 (36). After lowering the pH, olefin was detected in WG1 (Fig 5 C), 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 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. Following incubation 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 product of IMI-V, so its presence following sample acidification would strongly indicate that IMI-V had been produced from imidacloprid, 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 ten of the 12 bees whose guts were exposed to imidacloprid in cultures and acidified after incubation (Fig S2). Olefin was not detected in non-acidified samples.

From these *in vitro* experiments, a majority of bees tested had gut environments capable of producing olefin from imidacloprid. The increased insecticidal activity of olefin suggests (27) this could have consequences on host health. However, our experimental design does not allow us to pin-point 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 oxidation of IMI into IMI-V via *Cyp6g1* in insects (38). Therefore, host cells that may have been extracted along with 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 has been 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/L) given to bees in this study was sub-lethal (Fig S3). Additionally, we checked for the presence of imidacloprid in control and exposed bees using high-resolution LC-MS based on extractions of individual bees (40) and did not detect imidacloprid in control or exposed bees immediately following treatment (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/L). 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 could detect in this study (41–45), which indicates that imidacloprid is quickly eliminated from or metabolized by bees (45). 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

252 metabolism) and the hindgut, which contains most of the bee bacterial biomass.
253 However, the dose administered in Suchail et al. (41) was higher than the dose
254 administered here. Therefore, at field realistic concentrations, imidacloprid is likely
255 mostly metabolized in the bee before it reaches the hindgut. Thus, the metabolism
256 of imidacloprid into IMI-V and olefin observed in our *in vitro* gut cultures, may not
257 occur in the bee gut in nature. Because none of the core bee gut bacterial isolates
258 tested could metabolize imidacloprid and imidacloprid did not affect the
259 microbiome composition, this suggests that even if imidacloprid is present in the
260 hindgut, it is metabolized by bee enzymes and not the resident microbiota.
261
262 *Imidacloprid exposure decreases honey bee survival in the hive and increases*
263 *susceptibility to pathogens*
264 Adult worker bees were collected from brood frames from two hives. Bees
265 were exposed to imidacloprid for three days in the lab as described above. The
266 impact of imidacloprid exposure on honey bee survival in the hive was evaluated by
267 returning marked bees to their original hive following exposure and censusing bees
268 three days after reintroduction. Consistent with other studies, we found that
269 imidacloprid exposure resulted in decreased survival in the hive (Fig 6 A). In both
270 experiments we recovered over 60% of the control bees whereas less than 40% of
271 imidacloprid-exposed bees were recovered (Fig 6 A). This decrease in survival could
272 be due to behavioral defects (9–12), increased susceptibility to pathogens (14–17),
273 or altered immunocompetence (13) induced by exposure to imidacloprid.

Previous studies have shown that imidacloprid causes 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* str. kz11 (22), and survival was monitored for ten days. Bees exposed to imidacloprid and subsequently exposed to *Serratia* str. kz11 exhibited increased mortality when compared to control bees, bees exposed to imidacloprid only, or bees exposed to *Serratia* only (Fig 6 B; $P<0.0001$). This result is consistent with observations for *D. melanogaster*; flies exposed to imidacloprid were more susceptible to *S. 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 following imidacloprid exposure (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 establishment of the microbiota by interfering with the bees' ability to regulate bacterial populations. This possibility warrants further investigation. We found no

297 evidence that the core species of the bee gut microbiota can metabolize
298 imidacloprid. Olefin can be produced from *in vitro* whole gut incubations, possibly
299 reflecting the metabolic activities of rare gut community members; however,
300 residual host or abiotic processes during incubations may be responsible for this
301 metabolism. Since imidacloprid is quickly eliminated from bees, we predict that
302 little or none reaches the bee hindgut, where the most of the bacterial community
303 resides, and if it does, it is metabolized by bee enzymes rather than the resident
304 microbiota. Even so, the lack of effect of imidacloprid on the bee gut microbiota is
305 not surprising since the primary targets of this insecticide are insect neural
306 transporters. In this specificity, imidacloprid differs from antibiotics and glyphosate,
307 which are agrochemicals expected to impact organisms other than animals. As
308 expected, imidacloprid exposure decreases bee survival in hives, even though it is
309 quickly eliminated from the bee, potentially through activity of bee-produced
310 enzymes. Our results suggest that imidacloprid increases susceptibility to
311 opportunistic pathogens, possibly by altering immune function.
312
313 **Materials and Methods**
314 *Chemicals and solutions*
315 Imidacloprid was obtained from Toronto Research Chemicals, Canada
316 (Catalog number: I274990). For experiments with bacterial isolates and bee gut
317 cultures, a 1 mg/mL imidacloprid solution was prepared by dissolving 100 mg
318 imidacloprid in 1 mL acetone-methanol 1:1, and then adding 99 mL of culture media.

For experiments with honey bees, a 1mg/mL imidacloprid stock solution was prepared by dissolving 10 mg imidacloprid in 10 mL methanol. To test for sub-lethal doses of imidacloprid, the stock solution was diluted to 500 µg/L, 200 µg/L and 50 µg/L imidacloprid using filter-sterilized 0.5 M sucrose syrup (Fig S3). The 500 µg/L 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 testers paint. One group was fed 500 µg/L imidacloprid suspended in sterilized sucrose syrup (imidacloprid), and the other group was fed only sterile sucrose syrup (control). After three days of exposure, 15 bees from each group were sampled, and the remaining bees were returned to the hive. Bees were also sampled from the hive at Days 3 and 5 post exposure (15 bees per group per day). DNA extractions were performed on all sampled bees using the protocol from (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'-GTTTGATCMTGGCTCAG-3' and 5'-TGCCTCCCGTAGGAGT-3'. The community profiling, including amplification, library preparation, and sequencing (Illumina MiSeq 2X250) 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. 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 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 dataset. 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 honeybee gut microbiota, 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 3500 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 D0;n=15, D3;n=12, and D5;n=11 and for treatment samples of D0;n=14, D3;n=14, and D5;n=15. Abundance of the bee gut bacterial species was estimated by correcting for absolute abundance (estimated by qPCR) and taking into account 16S rRNA gene copy number, as in (22). All alpha and beta

365 diversity estimates were performed in QIIME.

366

367 *Quantitative PCR to estimate bacterial abundance*

368 The bacterial abundances within control bees (D0;n=15, D3;n=12, and
369 D5;n=11) and treatment bees (D0;n=14, D3;n=14, and D5;n=15) were estimated
370 using quantitative PCR. Universal 16S rRNA gene primers 27F (5'-
371 AGAGTTTGATCCTGGCTCAG-3') and 355R (5'-CTGCTGCCTCCCGTAGGAGT-3') were
372 used to amplify total copies of the 16S rRNA gene of each sample on an Eppendorf
373 Mastercycler ep realplex instrument (Eppendorf, Hauppauge, NY, USA). Triplicate
374 10-µL reactions were carried out with 5 µL iTaq universal SYBR Green (Bio-Rad,
375 Inc.), 1 µL (each) 3µM primer, 2 µL H_2O, and 1 µL 100X dilutions of template DNA.
376 The cycling conditions consisted of 95 °C for 3 min and 40 cycles of two-step PCR at
377 95 °C for 3 s and at 60 °C for 20 s. Quantification was based on standard curves from
378 amplification of the cloned target sequence in a pGEM-T vector (Promega, Madison,
379 WI, USA). Values were adjusted to account for dilution.

380

381 *Exposure of bee gut bacteria and whole bee guts to imidacloprid*

382 Previously isolated honey bee gut bacterial strains of *Snodgrassella alvi*
383 (wkB2 (47), wkB9, wkB332, wkB339 (48), *Gilliamella apicola* (M1-2G, M6-3G, wkB7
384 (31), wkB1 (47)), *Frischella perrara* (PEB095 (49)), *Bifidobacterium asteroides*
385 (wkB338, wkB344) and *Bartonella apis* (wkB233A) were grown in InsectaGro.
386 Strains of *Lactobacillus* Firm-5 (wkB8 and wkB10 (50)) and Firm-4 (26254 and
387 26255) were grown in MRS. *Lactobacillus mellis* strain DSM 26254 and *Lactobacillus*

388 *mellifer* strain DSM 26255 were purchased from DSMZ – Deutsche Sammlung von
389 Mikroorganismen und Zellkulturen GmbH. All strains were cultured at 35 °C in 5%
390 CO_2 overnight. The 600 nm optical density (OD) of each bacterial culture was
391 measured, and cells were washed with 1x PBS and diluted to a concentration of 1
392 OD in the respective culture media. A 10 µL aliquot of each bacterial suspension was
393 transferred in triplicate to a 96-well plate containing 190 µL media, 190 µL media
394 with solvent, or 190 µL media with 1 mg/mL imidacloprid. Controls consisted of
395 media, media with solvent, or media with 1 mg/mL imidacloprid. The plates were
396 incubated in a plate reader (Tecan) at 35 °C and 5% CO_2 for 72 h. OD was measured
397 at 600 nm every 6 h.
398 The guts from 12 healthy worker bees were extracted, pooled into two
399 samples (6 bees in each sample) to increase screening potential (WG1 & WG2), and
400 cultured in tryptic soy broth (TSB) for 24 hours at 35 °C and 5% CO_2. Bacterial cells
401 were isolated by centrifugation (7,000 rcf, 10 min), washed and concentrated in 0.2
402 M Na_2HPO_4/KH_2PO_4 (pH 8) buffer. The pooled gut community (WG1 & WG2) and a
403 bacteria-free control were incubated for 6 days at 35 °C and 5% CO_2 with 1 mg/mL
404 imidacloprid and 50 mg/mL of sucrose in Na_2HPO_4/KH_2PO_4 buffer.
405
406 *Detection of imidacloprid in bacterial and whole bee gut cultures*
407 Supernatant of bacterial cultures (200 µL, *G. apicola* strains wkB1 and M6-3G,
408 *S. alvi* strains wkB2 and wkB9, *Bifidobacterium* strains wkB338 and wkB334, and
409 *Lactobacillus* Firm-5 strains wkB8 and wkB10) and aliquots of the pooled gut
410 community cultures (200 µL, collected at days 0-4 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 C18 column (2.1 × 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 2900 V, drying gas temperature 300 °C, gas flow 9 L/min, and nebulizer pressure 40 PSI. Imidacloprid identification was performed through comparison of elution time and mass spectra to standard samples. Targets in standards and in extracts were observed as $[M+H^+]$ (protonated species) and $[M+Na^+]$ (sodiated species). Data were analyzed with MassHunter Qualitative Analysis (Agilent). For bacterial cultures, the relative concentration of imidacloprid was compared to controls after 3 days of exposure based on imidacloprid peak area.

For bee gut degradation rates, changes in imidacloprid concentration were shown as the natural logarithm of the ratio over 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 C18 (50 × 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 APCI (Atmospheric Pressure Chemical Ionization). The flow rate was 0.7 mL/min with mobile phase A (water + 0.1% formic acid) and mobile phase B (acetonitrile + 0.1% formic acid).

Detection of imidacloprid metabolites in whole bee gut cultures

Bee gut homogenates were combined into two pooled samples, containing six individual bee guts each (WG1 & WG2) to maximize screening potential. Pooled homogenates were cultured in tryptic-soy broth (TSB) for 24 hours. Cells were isolated by centrifugation and washed with 0.2 M Na_2HPO_4/KH_2PO_4 (pH 8). 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 (+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 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 C18 column (50 × 2.1 mm, 5 µm particle size). The flow rate was 0.7 mL/min with mobile phase A (water + 0.1% formic acid) and mobile phase B (acetonitrile + methanol + 0.1% formic acid). The gradient mode was 0 min, 5% B; 12-17 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. Ion source settings were: drying gas temperature 350 °C, gas flow 13 L/min, nebulizer pressure 55 PSI. Metabolites identification was performed through comparison of elution time and mass spectra to standard samples. Targets in standards and in extracts were observed as $[M+H^+]$ (protonated species) and $[M+Na^+]$ (sodiated species). Data were analysed with MassHunter Qualitative Analysis (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. 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 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 XDB C-8 column (150 × 4.6 mm, 3.5 µm particle size). The flow rate was 0.6 mL/min with mobile phase A (water + 0.1% formic acid) and mobile phase B (acetonitrile + methanol + 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); 17-23 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. Ion source settings were: drying gas temperature 350 °C, gas flow 10 L/min, nebulizer pressure 45 PSI. Metabolite identification was

480 performed using target m/z (254.0439). Data were analyzed with MassHunter
481 Qualitative Analysis (Agilent).
482
483 *Detection of imidacloprid residues in whole bee bodies*
484 Imidacloprid was extracted from whole bodies of exposed bees following a
485 described protocol (40), with some adaptations. Five bees from control and
486 imidacloprid-treated groups were sampled after 3 days. They were individually
487 transferred to microtubes with steel beads and 25% methanol (1 mL) and
488 homogenized in TissueLyser equipment for 3 min at 250 rpm. The homogenates
489 were centrifuged for 5 min at 14000 rpm at 4 °C, and the supernatants were
490 collected and combined according to their respective group. Combined samples
491 were then diluted with an equal volume of 25% acetic acid and were subjected to a
492 solid-phase extraction using a Discovery DSC-18 column (bed weight 50 mg; volume
493 1 mL). The column was first washed with 1 mL water, and then the samples were
494 eluted 3 times with methanol (200 µL). The samples were dried, resuspended in
495 25% methanol (400 µL), filtered, and submitted for high-resolution LC-MS analysis.
496 Before processing the control sample, a 0.5 µL aliquot of 1 mg/mL imidacloprid was
497 transferred to the microtube to check sample matrix interference in imidacloprid
498 detection. LC was performed with an Agilent 1200 Infinity liquid chromatograph in
499 gradient elution mode using a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 5 µm
500 particle size). The flow rate was 0.6 mL/min with mobile phase A (0.1% formic acid
501 in water) and mobile phase B (acetonitrile). The gradient mode was 0-5 min, 10% B;
502 16 min, 43% B; 17-23 min, 100% B (flow rate changed to 1.2 mL/min); 24 min, 10%

503 B (flow rate changed back to 0.6 mL/min). Eluting species were detected by an
504 Agilent Technologies 6530 Accurate-Mass Q-TOF equipped with an electrospray ion
505 source in positive mode. Ion source settings were: capillary voltage 2500 V, drying
506 gas temperature 350 °C, gas flow 12 L/min, nebulizer pressure 60 PSI. Imidacloprid
507 identification was performed through comparison of elution time and mass spectra
508 to standard samples. Targets in standards and in extracts were observed as $[M+H^+]$
509 (protonated species) and $[M+Na^+]$ (sodiated species). Data were analysed with
510 MassHunter Qualitative Analysis (Agilent).
511
512 *Effects of imidacloprid on honey bee fitness in the hive*
513 Hundreds of honey bee workers were taken from two separate hives located
514 on the UT campus (one was used for the microbiome analysis above). Bees were
515 treated and marked as explained above. Briefly, control bees were fed sterile
516 sucrose syrup for three days, and exposed bees were administered 500 µg/L
517 imidacloprid suspended in sterilized sucrose syrup for three days. Marked bees
518 were returned to their original hive after three days of imidacloprid exposure. Three
519 days after reintroduction to the hive, marked bees were captured and counted.
520
521 *Susceptibility to Serratia infection following imidacloprid exposure*
522 Approximately 700 honey bee workers were taken from a single hive located
523 on the UT campus. Control bees were fed sterile sucrose syrup for three days, and
524 exposed bees were administered 500 µg/L imidacloprid suspended in sterilized
525 sucrose syrup for three days followed by oral exposure to i) *Serratia* kz11 (0.5 OD

600 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 on NCBI BioProject PRJNA432211. All other data generated or analyzed during this study are included in this published article and its supplementary information.

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706
707
708
709 **Figure legends**

710 **Fig 1. Absolute abundance of gut bacteria following imidacloprid exposure. A)**
711 Boxplot of total bacterial 16S rRNA gene copies estimated by qPCR for all control
712 (D0;n=15, D3;n=12, and D5;n=11) and imidacloprid-exposed bees (D0;n=14,
713 D3;n=14, and D5;n=15). **B)** Boxplot of total bacterial 16S rRNA gene copies
714 estimated by qPCR for control (C) and imidacloprid-exposed (I) bees at each
715 sampling day post-imidacloprid exposure. Box-and-whisker plots show high, low,
716 and median values, with lower and upper edges of each box denoting first and third
717 quartiles, respectively. No significant differences were observed in total bacterial
718 abundance between control and imidacloprid exposed bees ($p>0.05$ Wilcoxon rank
719 sum tests).
720
721 **Fig 2. Honey bee gut bacterial composition at Days 0, 3, and 5 after three days**
722 **of imidacloprid exposure.** Stacked column graph showing the **A)** relative
723 abundance and **B)** absolute abundance of bee gut bacterial species in 15 control Day
724 0, 14 imidacloprid Day 0, 12 control Day 3, 14 imidacloprid Day 3, 11 control Day 5,
725 15 imidacloprid Day 5 bees.
726
727 **Fig 3. Alpha and beta diversity of imidacloprid-exposed and control bees. A)**
728 Difference in alpha diversity between control (C) and imidacloprid-exposed (I) bees
729 at each time point (measured by observed OTUs). **B)** The average Bray-Curtis
730 dissimilarity in gut communities among control bees versus between control bees
731 and exposed bees. Box-and-whiskers plots show high, low and median values, with
732 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.

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 media (control), media with solvent, or media with 1 mg/mL imidacloprid. Experiment was performed in triplicate, and each data point represents the average optical density (600 nm, with standard deviation bars) measured every 6 h. **B)** Relative concentration 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 concentration of imidacloprid in pooled gut community cultures compared to controls at days 0-4 and 6 displayed as the neutral logarithm of the ratio over initial levels.

Fig 5. Imidacloprid metabolite production by the honey bee gut. A & 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).

Fig 6. Effects of imidacloprid on honey bee fitness. A) Survival rate for honey bees returned to the hive after three days of exposure to imidacloprid. Number of

workers recovered from the hive on Day 3 post-treatment (P<0.0001, Chi-squared test). **B)** The percent survival of age-controlled bees after *Serratia* exposure, shown as a Kaplan–Meier survival curve. Statistical analyses were performed using the coxph model (***= P<0.0001) implemented in the "survival" package (51) in R.