Oral and topical exposure to glyphosate in herbicide formulation impact the gut microbiota and survival rates of honey bees

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Abstract
Honey bees are important agricultural pollinators that rely on a specific gut microbiota for regulation of immune system and defense against pathogens. Environmental stressors that affect the bee gut microbial community, such as antibiotics and glyphosate, can indirectly compromise bee health. Most of the experiments demonstrating these effects have been done under laboratory conditions with pure chemicals. Here, we investigated the oral and topical effects of variable concentrations of glyphosate in herbicide formulation on the honey bee gut microbiota and health under laboratory and field conditions. In all of these conditions, the formulation, dissolved in sucrose syrup or water, affected the abundance of beneficial bacteria in the bee gut in a dose-dependent way. Mark-recapture experiments also demonstrated that bees exposed to the formulation were more likely to disappear from the colony, once reintroduced after exposure. Although no visible effects were observed for hives exposed to the formulation in field experiments, challenge trials with the pathogen Serratia, performed under laboratory conditions, revealed that bees from hives exposed to the formulation exhibited increased mortality compared to bees from control hives. In the field experiments, glyphosate was detected in honey collected from exposed hives, showing that worker bees transfer xenobiotics to the hive, thereby extending exposure and increasing the chances of exposure to recently emerged bees. These findings show that different routes of exposure to glyphosate-based herbicide can affect honey bees and their gut microbiota.
Importance

The honey bee gut microbial community plays a vital role in immune response and defense against opportunistic pathogens. Environmental stressors, such as the herbicide glyphosate, may affect the gut microbiota, with negative consequences for bee health. Glyphosate is usually sprayed in the field mixed with adjuvants, which enhance herbicidal activity. These adjuvants may also enhance undesired effects in non-targeted organisms. This seems to be the case for glyphosate-based herbicide on honey bees. As we show in this study, oral exposure to either pure glyphosate or glyphosate in commercial herbicide formulation perturbs the gut microbiota of honey bees, and topical exposure to the formulation also has a direct effect on honey bee health, increasing mortality in a dose-dependent way and leaving surviving bees with a perturbed microbiota. Understanding the effects of herbicide formulations on honey bees may help to protect these important agricultural pollinators.
1. Introduction

Honey bees are important agricultural pollinators whose populations have declined over the past decade. The reasons for colony failures are not fully understood but have been linked to environmental stressors, such as the spread of pathogens and parasites (1–6), reduction of food resources (7), and pesticide exposure (8–11). More recently, the herbicide glyphosate has been found to disrupt the gut microbiota of honey bees, reducing the abundance of beneficial bacterial species (12).

Glyphosate is the main active ingredient of many herbicide formulations used to kill unwanted vegetation not only in crop areas but also in non-agricultural settings, such as industrial sites, parks, railroads, roadides, recreational and residential areas (13). Its use is growing in connection with genetically engineered, herbicide-tolerant crops (14, 15). In such formulations, glyphosate is found in its salt form, which affects its absorption by the targeted organism. Different salt forms of glyphosate are applied in the field (16), along with surfactants, such as polyethylated tallow amine, to enhance herbicide efficacy (17). These glyphosate-based formulations are commercially available at different concentrations which reach up to 48% w/v of glyphosate as the main active ingredient, based on product labelling. These are usually recommended to be diluted in water before spraying on target plants, with concentrations ranging from 0.4% to 7% glyphosate. Once inside the plant, it is the glyphosate acid that binds to the target enzyme in susceptible plants and causes the herbicidal effect.

Glyphosate inhibits the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) in the shikimate pathway. This stops the production of essential aromatic metabolites, such as aromatic amino acids (phenylalanine, tryptophan, and tyrosine), folate cofactors, benzoid and naphtoid coenzymes, phenazines, siderophores, and others (18). A deficit in aromatic amino acids leads to a reduction in protein synthesis and, ultimately, to the organism’s death. All plants and some microorganisms, but not animals, contain a functional shikimate pathway and therefore are potentially susceptible to glyphosate. Thus, glyphosate can only be used in genetically engineered crops carrying a tolerant version of EPSPS, which is commonly derived from Agrobacterium spp. (19).
EPSPS enzymes from different organisms are classified as class I\(\alpha\), I\(\beta\) or II based on their biochemical properties and phylogenetic distinctions. Class I\(\alpha\) enzymes are naturally sensitive to low concentrations of glyphosate and occur naturally in all plants and some Bacteria, whereas class I\(\beta\) enzymes are found in Archaea (20). On the other hand, class II enzymes usually tolerate higher doses of glyphosate than class I enzymes and in nature occur exclusively in Bacteria (21). Moreover, classes I and II EPSPS diverge by more than 30% in amino acid sequence (22). Previous studies have shown that most bee gut bacterial species encode a functional shikimate pathway with a class I\(\alpha\) or a class II EPSPS, whereas others lack this enzyme, suggesting that some bee gut bacteria are selectively inhibited by glyphosate (12).

Honey bees have coevolved with a beneficial, specialized and socially transmitted gut microbiota, comprised of five to eight dominant bacterial members (23). These members belong to different taxa including *Snodgrassella* (24), *Gilliamella* (24), *Bifidobacterium* (25), *Lactobacillus* Firm-4 (26), *Lactobacillus* Firm-5 (26–28), *Bartonella* (29), *Frischella* (30) and *Commensalibacter*. These bacterial taxa are specialized and diverse in terms of metabolic capabilities, i.e., they inhabit specific niches and play specific roles in the bee gut (23, 31, 32). For example, *Snodgrassella* forms a biofilm layer in the ileum (33) and stimulates the host immune system (34). *Snodgrassella* is also involved in cross-feeding interactions with other bacteria, such as *Gilliamella* (28, 35), which in turn detoxifies the gut environment by metabolizing toxic sugars (36), and helps in digestion of recalcitrant components of the bee diet along with *Bifidobacterium* (35). *Lactobacillus* acidifies the bee gut, potentially inhibiting the proliferation of some opportunistic pathogens (37). The microbiome as a whole promotes host weight gain (38) and regulates immune signaling pathways (34, 39). Regarding glyphosate susceptibility, some bee gut bacterial species contain a functional shikimate pathway and encode either a susceptible, class I\(\alpha\) EPSPS (*Snodgrassella*, *Gilliamella*, *Frischella* and *Bifidobacterium*) or a tolerant, class II EPSPS (*Bartonella*), whereas other bacteria contain a truncated shikimate pathway with a class II EPSPS (*Lactobacillus* Firm-4) or lack the gene that codes for EPSPS (*Lactobacillus* Firm-5) (12). In the absence of a functional shikimate pathway, these *Lactobacillus* may rely on the uptake of aromatic...
amino acids from the gut environment, which may come from the bee diet or from other gut bacteria that produce these metabolites.

Honey bees can be directly exposed to high concentrations of glyphosate and other components of the formulation when foraging during herbicide application (up to 2.0 g/L), or when collecting pollen (up to 629 mg/kg) and nectar (up to 31.3 mg/kg) from plants that have been recently treated with the herbicide (40). They can also be exposed to residues of glyphosate when collecting contaminated water (up to 3.1 mg/L) (41–43). Moreover, glyphosate residues have been detected in commercialized and natural honey (up to 0.3 mg/L) (44–47), and even in larval bees (up to 19.50 mg/kg) (40).

Recent studies demonstrating that the honey bee gut microbiota is affected by exposure to glyphosate were mostly performed with the pure chemical and under laboratory conditions (12, 48, 49). Commercial formulations usually contain adjuvants, such as surfactants, to enhance herbicidal efficacy. Since honey bees can be exposed to glyphosate when collecting contaminated nectar, pollen and water sources, or when foraging during herbicide application, we decided to investigate whether the microbial perturbations observed with pure glyphosate would also be observed when bees are orally or topically exposed to glyphosate in herbicide formulations. Moreover, we investigated the impacts of the same formulation on the bee gut microbiota under field conditions, under a worst-case scenario in which bees are directly exposed to the formulation. Our findings suggest that oral or topical exposure to glyphosate, pure or in herbicide formulation, can affect the honey bee gut microbiota under laboratory and field conditions.

2. Results

2.1. Oral exposure of honey bees to glyphosate, pure or in herbicide formulation, under laboratory conditions – effects on gut microbial composition

Age-controlled bees raised under laboratory conditions were divided into three groups which were fed either sucrose syrup, 1.0 mM glyphosate dissolved in sucrose syrup, or Roundup® formulation corresponding to 1.0 mM glyphosate dissolved in sucrose syrup (Figure 1A). After five days of exposure, bees were sampled, and their
microbial communities were evaluated by extracting DNA from their guts and performing 16S rRNA amplicon sequencing and qPCR analyses. From these data, we obtained estimates of absolute abundances for the main bacterial taxa in control and treatment bees.

Both glyphosate and Roundup® formulation affected the abundance of beneficial bacteria in the guts of honey bees (Figure 1B) by significantly decreasing the absolute abundance of *Snodgrassella* when compared to control group (Figure 1C, Figure S1). The absolute abundances of *Gilliamella* and *Bifidobacterium* also decreased in exposed bees, but this decrease was only significant in the group exposed to Roundup® formulation (Figure 1D-E).

Moreover, principal coordinate analysis of gut community compositions demonstrated that bees treated with pure glyphosate or Roundup® formulation clustered together and apart from those of controls; this was true for analyses based on both Bray-Curtis dissimilarities, which reflect relative abundances (Figure 1F, Table S1), and weighted UniFrac dissimilarities, which include phylogenetic relatedness (50) (Figure S1, Table S1). These results support and extend previous work showing that glyphosate perturbs the bee gut microbiota by reducing the abundance of beneficial bacterial species (12); glyphosate-based formulations have similar effects.

### 2.2. Oral exposure of honey bees to glyphosate in herbicide formulation – effects on hive recovery rates and gut microbial composition

Considering the effects observed for both glyphosate and Roundup® formulation on the honey bee gut microbiota, we decided to conduct more experiments with the formulation. Based on recommended applications, the concentrations used for weed control vary according to location and type of weed, ranging from 0.4% to 7.0% Roundup® formulation. We tested a concentration of 0.1% Roundup® formulation, which is lower than the minimum concentration recommended to spray in the field and is in the same magnitude of glyphosate concentrations detected in pollen and nectar from recently exposed plants (40).

In Fall 2018, hundreds of worker bees were collected from inside a hive and divided into two groups, control and treatment, which were fed sterile sucrose syrup or
0.1% Roundup® dissolved in sucrose syrup, respectively (Figure 2A). These bees were marked on the thorax with different paint colors, white or pink, respectively, and three days after treatment under laboratory conditions, the bees were reintroduced to their hive. Three days after hive reintroduction, all remaining marked bees were recovered: only 27.6% of Roundup®-treated bees were recaptured, which was significantly lower than the 44.0% of control bees recaptured (Figure 2B).

We also sampled control and treatment bees throughout this experiment: at the end of treatment (day 0 post-treatment), at the time of recovery (day 3 post-treatment), and two days after the recovery time (day 5 post-treatment), and evaluated their gut microbial compositions by 16S rRNA amplicon sequencing and qPCR analyses. Overall, treated bees exhibited a significant reduction in the absolute abundance of gut bacteria when compared to control bees at days 3 and 5 post-treatment (Figure 2C). This decrease in total bacterial abundance was accompanied by a significant reduction in the absolute abundance of the core bacterial members Snodgrassella (Figure 2D) and Gilliamella (Figure 2E) at days 3 and 5 post-treatment, as well as Bifidobacterium (Figure 2F) and Lactobacillus Firm5 (Figure 2G) at day 5 post-treatment. As found for the laboratory experiment described above, principal coordinate analysis of community compositions based on 16S rRNA amplicons demonstrated that treated bees diverged from controls at days 3 and 5 post-treatment, and this result was supported by both Bray-Curtis or weighted UniFrac dissimilarities (50) (Figure S2, Table S2).

This recovery experiment was replicated four more times using bees collected from different hives in Spring or Summer, 2019 (Figure S3, Table S3). Two of these experiments found significant decreases in recovery rates for treatment groups (Figure S3A, Figure S3G), but two others found no significant differences between control and treatment recovery rates (Figure S3D, Figure S3F). We also performed a color-bias validation experiment, which showed no significant difference in recovery between bees marked either pink or white, the colors used in these experiments (Figure S3H).

We also processed bees from control and treatment groups from two more replicate experiments. This time, total bacteria and/or Snodgrassella abundance were measured by qPCR (Figure S3). In the second experiment, which showed significant differences in recovery rates between groups, we observed a similar decrease in
abundance for *Snodgrassella* in treated bees at both days 0 and 3 post-treatment (Figure S3C), but not for total bacteria (Figure S3B). In the third experiment, in which we did not see a significant difference in recovery rates between control and treatment bees, we also did not observe significant changes in *Snodgrassella* abundance after treatment (Figure S3E).

### 2.3. Oral exposure of honey bees to glyphosate in herbicide formulation under field conditions

#### 2.3.1. Effects on the gut microbiota

In parallel with the previously described experiments, we conducted more experiments to evaluate the potential effects of Roundup® exposure to honey bees under field conditions. Honey bee hives were established at two sites on private land in Driftwood, TX, in 2018 and 2019, in both cases approximately two months before starting experiments. The first field experiment was performed at site 1 in August/September 2018, whereas the second field experiment was performed at sites 1 and 2 in August/September 2019.

For the first field experiment, 10 hives were randomly selected at site 1, divided into two groups and exposed to either of the following conditions: five hives were treated weekly for one month with a single dose of 0.1% Roundup® in sucrose syrup (0.5 L), and five other hives were treated with 0.5 L sucrose syrup on the same treatment schedule (Figure 3A, Table S4). Sampling of bees began before initial treatment at Week 0 (August 1st, 2018). At each sampling point, we compared microbial abundance and composition between groups using 16S rRNA gene community profiling and qPCR analyses.

Though selected at random, bees from hives in the control group initially exhibited lower loads of total gut bacteria than did bees from hives in the treatment group (Figure 3B), with fewer *Lactobacillus* Firm-4 and Firm-5 (Week 0 in Figure S4). Hives were treated immediately after sampling at Week 0. One week after first exposure (Week 1, August 8th, 2018), bees were sampled again and none of the differences previously observed remained (Figure 3B, Figure S4). However, significant decreases in *Snodgrassella* and *Commensalibacter* abundances were observed in bees from...
Roundup®-treated hives (Figure 3C and Figure S4, respectively). The same scheme of treatment was followed for the next two weeks, in which bees were first sampled and then treated each week. At Weeks 3 (when the last treatment was provided to the hives, August 22nd, 2018) and 4 (one week after finishing treatment, August 29th, 2018), Snodgrassella abundance remained significantly lower in bees from Roundup®-treated hives (Figure 3C). Bifidobacterium also decreased in abundance in bees from these hives (Figure 3D). One month after finishing treatment (Week 7, September 19th, 2018), the effects on the microbiota of bees from Roundup®-treated hives not only persisted but extended to most of the core bacterial species in the bee gut. We detected a significant decrease in abundance for total bacteria (Figure 3B), with reduced loads of Snodgrassella (Figure 3C), Bifidobacterium (Figure 3D), Lactobacillus Firm-5 (Figure S4), Gilliamella (Figure S4) and Commensalibacter (Figure S4), and a significant increase in absolute abundance for Bartonella (Figure S4) and Frischella (Figure S4).

We also checked abundance for environmental bacteria in the bee gut. During the treatment, there was an increase in abundance for Lactobacillus kunkeei at Week 3 and an increase in abundance for Fructobacillus at Weeks 3 and 4 (Figure S4). These two bacterial groups are associated with floral nectar and are commonly found in honey bee hives (51).

A second field experiment was performed in 2019 at two different sites, as an attempt to evaluate the effects of different concentrations and exposure levels of Roundup® formulation delivered in two different matrices, sucrose syrup or water. At site 1, 14 hives were randomly selected to replicate the experiment performed in 2018 and to include a treatment group in which hives were exposed to a lower dose (0.001%) of Roundup® formulation (Figure 4A, Table S4). Treatment and sampling schemes were the same as applied in the previous season. However, this time guts were pooled to extract DNA and to perform 16S rRNA amplicon sequencing and qPCR analyses. The lower dose of Roundup® formulation tested did not affect the abundance nor the composition of the honey bee gut microbiota. On the other hand, the higher dose (0.1% Roundup®) significantly decreased the abundance of Snodgrassella during the treatment (Weeks 1 and 3) and one week after finishing treatment (Week 4). This was similar to the experiment performed in 2018, but no effects were observed one month...
after finishing treatment (Figure 4B). In 2019, we did not observe significant effects on other members of the gut community (Figure S5). Since we collected random bees from the hives, thus not controlling for age, pooling guts may have masked effects due to the potential presence of outliers, such as recently emerged bees, known to have fewer gut bacteria, or bees that were not exposed to the treatment.

At site 2, another 23 hives were randomly selected to be treated with similar concentrations of Roundup® formulation as in site 1 (lower dose, 0.001%, or higher dose, 0.1%) in sucrose syrup (single dose at Week 0), reducing exposure to one occurrence, or in water (single doses at Weeks 0 and 2), changing the exposure matrix (Figure 5A, Table S4). Single doses of 0.001% Roundup® in sucrose syrup or in water did not significantly affect the abundance of the core bacterial members in the honey bee gut when compared to the control group over a period of two months. On the other hand, Snodgrassella abundance significantly decreased in the guts of bees treated with 0.1% Roundup® at Weeks 1, 3 and 4, regardless of exposure matrix, syrup or water (Figure 5B). Other changes were observed, such as increases in the abundance of Lactobacillus Firm-4 (Week 1) and Bartonella (Week 7) in bees treated with 0.1% Roundup® in water (Figure S6). There was also an increase in abundance of environmental bacteria, such as species of Enterobacteriaceae, in the guts of bees treated with the formulation at Week 3 (i.e., three weeks after treatment was provided) (Figure S6).

2.3.2. Effects on susceptibility to Serratia infection

Bacterial challenge assays were performed under laboratory conditions with bees collected at Week 4 of each field experiment performed in 2018 and 2019. In 2018, bees collected from hives treated with 0.1% Roundup® in syrup and exposed to the opportunistic bacterial pathogen Serratia exhibited increased mortality compared to bees collected from control hives exposed or not to Serratia, or bees from hives treated with 0.1% Roundup® in syrup but not exposed to Serratia (Figure 3E). We did not observe the same pattern in 2019 for bees sampled from site 1: both control and treatment groups exhibited similar mortality rates when exposed to Serratia, but higher than those not exposed to Serratia (Figure 4C).
Effects observed at site 2 in 2019 were more similar to what was observed in 2018. This time, bees collected from hives treated with a single dose of 0.1% Roundup® in syrup or water and exposed to *Serratia* exhibited increased mortality to that observed for bees collected from control hives exposed to *Serratia* (Figure 5C). This effect was not observed for bees collected from hives treated with 0.001% Roundup® in syrup or water and exposed to *Serratia* (Figure 5C). To determine whether this increased mortality was attributable to the effects of the formulation on the gut microbiota or to direct effects on bees, we included control groups not exposed to *Serratia*. In bees collected from hives treated with the formulation, but not exposed to *Serratia*, survival rates were only significantly affected by the formulation when bees were treated with 0.1% Roundup® in water (Figure 5C), suggesting that direct and indirect effects of the formulation on bees may play a role in the increased susceptibility to *Serratia*, depending on the route of exposure.

### 2.3.3. Glyphosate transference to hive compartments

During the first field experiments in 2018, we observed that treatments given to the colonies were being depleted overnight. Because of that, we investigated whether the bees were consuming the entire treatment solution or storing part of it in hive combs. One week after the last treatment, on Week 4, honey from uncapped hive combs were sampled and processed to detect glyphosate by high-resolution LC-MS. Interestingly, glyphosate was detected in samples collected from all treatment hives in the range of 800–1600 µg/mL, but not in control hives (Figure 3F).

Because of this intriguing fact, in 2019, we decided to collect uncapped honey from the colonies throughout the experiments in both sites 1 and 2. As expected, at Week 0, before the beginning of treatments, glyphosate was not detected in uncapped honey collected from the hives (Figure 4D and Figure 5D). However, after Week 1 all the treatment hives in site 1 contained increasing concentrations of glyphosate, which were at the same magnitude of the doses provided in site 1 (0.001% or 0.1% glyphosate in herbicide formulation), and glyphosate remained in the hives even one month after finishing exposure (Figure 4D).
A similar trend was observed at site 2 for the hives treated with sucrose syrup containing Roundup® formulation. However, this time the initial peak in concentration decreased with time, but still persisted even two months after finishing a single treatment (Figure 5D). Hives treated with the formulation in water also contained contaminated uncapped honey, but concentrations were lower than the ones detected for hives treated with 0.1% Roundup® in syrup. Glyphosate concentrations in uncapped honey were similar in hives treated with either 0.001% or 0.1% Roundup® in water, and contamination persisted even several weeks after treatment. This suggests that bees can also be exposed to glyphosate in contaminated water sources near agricultural sites. The low amount of glyphosate detected in hives suggests that most of the water was consumed or used by the bees, although part of it evaporated (Figure S7).

Interestingly, very low concentrations of glyphosate were detected in a few control hives after treatment, suggesting cross-contamination between groups carried by the bees.

2.4 Topical exposure of honey bees to glyphosate in herbicide formulation – effects on survival rates and gut microbial composition

A worst-case scenario would occur when bees are sprayed directly and thus topically exposed to high concentrations of glyphosate-based formulations at the time of application. Therefore, we investigated the topical effects of Roundup® on the health and the gut microbiota of honey bees. For that, we tested different concentrations of the formulation, ranging from 0.05% to 3% Roundup® in water, and monitored the effects on bees and on the gut microbiota under both laboratory and hive conditions. After spray exposure of bees under laboratory conditions, survival was monitored for the next 24 hours (Figure 6A). Significant increases in mortality were observed for bees sprayed with 0.5%, 1.0% or 3.0% Roundup® when compared to bees sprayed with water (Figure 6B). This increased mortality was observed for the 3.0% Roundup®-exposed group in all of the survival monitoring times (6, 9, 12 and 24 hours after topical exposure), whereas the effects on the 0.5% and 1.0% Roundup®-exposed groups were only apparent 12 hours after topical exposure (Figure S8). A dose-response of bee survival to topical exposure was also observed, with an ED$_{50}$ value, i.e., the half maximal effective concentration of formulation at which bee survival is reduced by 50%, of 1.25 ± 0.38%
glyphosate in herbicide formulation (Figure S9). In initial trials, bees sprayed with 1.0% glyphosate in water did not die more than bees sprayed with only water even 24 hours after exposure, suggesting that other components of the formulation are responsible for the increased mortality of these topically exposed bees (Figure S10, Table S5).

After topically exposed bees were released back to the hive, recovery rates were measured at day 3 post-exposure. Compared to control bees, which were sprayed with water, significant reductions in recovery rates were observed for the groups sprayed with 0.5, 1.0 and 3.0% Roundup® in water (Figure 7, Table S6). We repeated this experiment for specific concentrations (0.1, 1.0 and 3.0% Roundup®) and found similar results. For the replicate experiments, each concentration was evaluated individually along with a control group (Figure S11).

Surviving bees (from the laboratory experiment) and recovered bees (from the hive experiment) were sampled to investigate whether the gut microbiota was affected by topical exposure to Roundup® formulation. For this, we checked *Snodgrassella* abundance by qPCR and used it as an indicator of microbiota perturbation. Interestingly, *Snodgrassella* abundance was reduced in the guts of recovered bees sprayed with 1.0 or 3.0% Roundup® (Figure 7C), but no significant effects were observed for bees kept under laboratory conditions (Figure 6C). This suggests that Roundup® formulation in water, besides reducing survivorship, can also affect the gut microbiota of topically exposed honey bees.

### 3. Discussion

A main impact of herbicide use on ecosystems, including bees and other invertebrates, is the loss of wild plants that provide food and shelter (52, 53). But impacts on susceptible organisms other than plants can also occur. Recent studies have demonstrated that glyphosate, an herbicide with bacteriostatic properties, can affect the microbiota of animals (12, 48, 49, 54–59). The consequences of such microbial perturbations for hosts are still not fully understood and likely vary depending on the roles of the microbiota in host health. Honey bees, for example, have coevolved with a specific gut bacterial community (60), with benefits ranging from metabolic contributions (35, 36, 38) to host immune system stimulation (34, 39). Dysbiosis has
negative consequences for bee health, such as increased susceptibility to infection by pathogens (61, 62).

It was previously shown that glyphosate, under laboratory conditions, perturbs the honey bee gut microbiota by reducing the abundance of *Snodgrassella* (12, 48), a primary bacterial colonizer that forms a biofilm on the ileum wall that probably facilitates the establishment of secondary colonizers, such as *Gilliamella*, *Bifidobacterium* and *Lactobacillus*. Experiments that demonstrated these effects were performed with pure glyphosate. However, glyphosate is commonly found in herbicide formulations in the form of a salt mixed with surfactants to increase water solubility and penetration of the targeted organisms. Thus, the effects of glyphosate in herbicide formulations may differ from effects of pure glyphosate (63).

Here, we demonstrate that glyphosate in herbicide formulation also affects the gut microbiota of honey bees, regardless of route (oral or topical) or source (sucrose syrup or water) of exposure. Under field conditions, a single oral exposure to the herbicide formulation was enough to reduce the abundance of *Snodgrassella* in the bee gut. This effect persisted in subsequent weeks during the experiments, regardless of whether further exposures occurred. In some trials, the effects of the formulation extended to other beneficial bacteria, such as *Bifidobacterium*, and persisted even one month after treatments ended. This is probably due to glyphosate, and possibly other components of the formulation, accumulating in parts of the hive environment, such as in honey combs, which would prolong the time of exposure. The decrease in abundance of gut-restricted bacteria led to an increase in environmental bacteria, such as *Fructobacillus* and *Lactobacillus kunkeei*, two prolific bacterial groups found in nectar and in honey bee hives (51).

These microbial perturbations were not linked to any apparent negative effect on colony fitness. Indeed, microbiota perturbation due to glyphosate exposure is not expected to produce an immediate, obvious increase in bee death (40), but more subtle effects of nutritional stress or increased susceptibility to bacterial infection (12).

Because sick honey bee workers are excluded from hives to prevent spread of disease to nestmates (64), dead bees are unlikely to accumulate in the hive. Therefore, we investigated the impacts of glyphosate-based formulation on bees by performing
infection and mark-recapture experiments. Infection experiments were done with bees collected from hives used in the field experiments after herbicide exposure. We challenged these bees with *Serratia marcescens*, an opportunistic bacterium that can cause disease in adult bees (3, 4). This was done in the laboratory to avoid the spread of pathogens through the apiary. Under these conditions, we observed reduced survival rates for bees collected from hives exposed to the formulation and challenged with *S. marcescens* in two of three trials. Moreover, we observed reduced recovery rates for bees orally exposed to the formulation in three of five mark-recapture experiments using bees from a different apiary. Together, these experiments suggest that honey bees are affected by glyphosate-based formulation, but effects vary based on colony status.

Bees can also be topically exposed to glyphosate-based herbicides during application, and concentrations under these circumstances are very high. Here we show that honey bees sprayed with different concentrations of glyphosate-based formulation exhibit increased mortality compared to bees sprayed with pure glyphosate or water. This suggests that surfactants or other unknown adjuvants in the formulation are responsible for the increased mortality, as observed in other studies (65, 66). We performed experiments under both laboratory and hive conditions, and survival rates were dose-dependent and similar among these experiments. Surprisingly, topical exposure to the formulation also affected the gut microbiota of honey bees, showing that topically exposed, surviving bees will have a defective gut microbiota and may become more susceptible to other environmental stressors, such as opportunistic bacterial pathogens.

The effects of glyphosate and its formulations have been tested for other microbial communities, both *in vitro* and *in vivo*. Common beneficial gut bacteria of animals, including *Bifidobacterium, Enterococcus* and *Lactobacillus*, were more susceptible to a glyphosate-based herbicide *in vitro* than were some pathogens, such as *Clostridium* and *Salmonella* (58), but this was not observed in another study (67). Studies of rats have shown that glyphosate-based herbicides can affect behavior (54), as well as the gut microbiome (54, 57, 68). Other studies have suggested that sufficient dietary supplementation of aromatic amino acids counters the negative effects of glyphosate or glyphosate-based formulations *in vitro* in representative bacteria of the
human gut microbiome, and also in vivo in rat gut microbiota (56). Another study (57) tested effects of chronic exposure at environmental concentrations of glyphosate (0.1 ppb) and found negative effects on the gut microbiota. The impacts on microbiota may be stronger in cases of poor nutrition, especially low availability of aromatic amino acids. The EPSPS enzyme varies among bacterial species in presence and in tolerance to glyphosate. Among core members of the bee gut, Snodgrassella, Bifidobacterium, and Gilliamella contain a functional shikimate pathway and can produce their own aromatic amino acids. Exposure of bees to glyphosate directly inhibits the growth of Snodgrassella, Bifidobacterium, and sometimes Gilliamella. This inhibition may in turn decrease the abundance of aromatic amino acids, and depress growth of bacteria lacking EPSPS, such as Lactobacillus, which rely on the uptake of aromatic amino acids produced by other community members. We observed this correlation not only in this study, but also in previous experiments (12). Most of the nutrients that bees acquire from pollen and nectar are absorbed in the proximal region of the gut (midgut), and only hard-to-digest components of the pollen cell wall pass to the distal region of the bee gut (ileum and rectum), where the microbial community resides (69–71). A mutagenesis study of Snodgrassella (72) showed that genes for production of aromatic amino acids (including the gene encoding EPSPS) were required for survival in the bee gut. Metabolomic analyses of different bee gut compartments demonstrate higher levels of the aromatic amino acids tryptophan and tyrosine in the ileum and rectum of bees with a normal microbiota as compared to microbiota-free bees, whereas midgut concentrations of aromatic amino acids are similar in both groups (38).

These experimental observations and predictions assume that the primary effect of glyphosate is inhibition of the EPSPS enzyme. Indeed, glyphosate resistance in many genetically modified organisms is based on the expression of a class II EPSPS from Agrobacterium tumefaciens (73). However, glyphosate does not only inhibit class I EPSPS enzymes but can also act as a potent chelator for micronutrients that are essential cofactors of enzymes and help in the stabilization of proteins in most organisms (74). Glyphosate binds divalent cations, such as Mg$^{2+}$, Ca$^{2+}$, Fe$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$, and forms stable complexes. Moreover, some gut-associated Lactobacillus require high amounts of Mn for protection from oxidative damage (75, 76),
and thus could be inhibited if Mn is chelated by glyphosate. A reduction in the 
bioavailability of cations could compromise organism growth in a way similar to the 
depletion of aromatic amino acids.

Glyphosate exposure does not immediately kill plants, which can persist as a 
source of contaminated food for bees for at least 5 days after spraying (40); glyphosate-
resistant weeds and crops may provide contaminated nectar and pollen for even longer.
Bees can also be exposed to the herbicide when collecting water, from ponds and 
ditches, or plant resin to make propolis. Since foraging bees bring nectar, pollen and 
propolis materials back to the colony, exposure can be extended to young worker bees.
For example, glyphosate was quantified in pollen (100-600 mg/kg) and nectar (10-30 
mg/kg) collected by foraging bees in a semi-field experiment (40). Researchers also fed 
hives with glyphosate, but not the formulation, dissolved in sucrose syrup but did not 
find effects on mortality of bees (40).

In our field experiments, we also detected transfer of glyphosate from bees to 
hive compartments. Uncapped honey exhibited glyphosate concentrations in the same 
magnitude of concentration provided in the sucrose syrup treatment. This probably 
happened because treatments were provided inside the hive, instead of spraying 
nearby plants, to avoid cross-contamination. Unfortunately, this precludes any 
comparisons between the glyphosate concentrations detected in our experiments with 
concentrations detected in honey in other studies (44–47), but it does show that bees 
can directly transfer contaminated resources to hive compartments. A recent study 
using bees under laboratory conditions showed that glyphosate has persistent impact 
on the gut microbiota of young adult workers whether exposure occurs during or after 
microbiota establishment, and that this impact is dose-dependent (E. V. S. Motta and N. 
A. Moran, submitted for publication).

Therefore, several variables could dictate the magnitude of effects of glyphosate 
or herbicide formulations on honey bees and their gut microbiota. From the herbicide 
side, level and route of exposure are the main factors. From the bee side, variation in 
colony health and population densities, exposure to different pathogens and parasites, 
as well as differences in nutritional status due to food resource availability and seasonal 
variations are likely key.
4. Materials and Methods

4.1 Chemicals and solutions

Glyphosate standard, in the acid form, was purchased from Research Products International, USA (Lot: 32612-38399). Roundup PROMAX® formulation was purchased from an agricultural retailer; this formulation contains 48.7% (w/v) or 660 g/L of potassium salt of glyphosate, equivalent to 540 g/L of the acid, glyphosate.

For the oral exposure experiments, glyphosate standard was initially dissolved in distilled water, then diluted to the final concentration with filter-sterilized 0.5 M sucrose syrup, whereas the formulation was directly diluted in filter-sterilized 0.5 M sucrose syrup. For the topical exposure experiments, both standard and the formulation were directly diluted in tap water. The final concentrations used in the experiments were achieved by taking into consideration the initial concentration of glyphosate acid in the formulation.

4.2 Honey bee rearing

For the laboratory and hive recovery experiments, honey bees (the European Apis mellifera) were obtained from outside hives kept on the rooftop of J. T. Patterson Laboratories Building at UT-Austin (latitude: 30.287913, longitude: -97.736183). Hives are assigned with a unique number and name, which usually change when requeening is required. For the field experiments, honey bees were obtained from hives kept on two different sites at Driftwood, TX (Site 1: latitude: 30.114998, longitude: -98.0212251; Site 2: latitude: 30.115057, longitude: -98.0249667). These hives did not exhibit signs of brood or adult bee diseases during the experiment. The surrounding environment is rural and is not actively farmed, so we expect no glyphosate exposure from other sources.

In the field experiments, we provided weekly single doses of 0.5 L of a 1:1 (w/v) sucrose solution to the hives in site 1 (August 2018 and 2019), and a single dose of the same sucrose solution to the hives in site 2 (August 2019). Based on (40, 77), a small hive would require 135 g/day of nectar and 4.5 g/day of pollen for normal development.

We provided 500 g sugar (assuming 50% sugar content in the sucrose solution), which
should be consumed in 3-4 days, and would probably reduce foraging activity during that time.

4.3 Oral exposure experiments

4.3.1 Laboratory conditions

A brood frame was obtained from a honey bee hive kept at UT-Austin, transferred to a frame cage and placed in an incubator at 35°C and ~60% relative humidity to simulate hive conditions until adults emerged. Newly emerged bees were transferred to cup cages and divided into three groups, which were fed (1) sterile sucrose syrup, (2) 1.0 mM glyphosate dissolved in sterile sucrose syrup or (3) 1.0 mM glyphosate in herbicide formulation (or Roundup®) dissolved in sterile sucrose syrup. All groups were allowed to acquire their normal microbiota during treatment by adding a suspension of freshly-prepared gut homogenate from hive bees to the bee bread, as described in previous studies (12, 33). After five days of treatment, 8 bees from each group were sampled and stored at -80°C.

4.3.2 Hive recovery

Hundreds of worker bees were collected from inside of hive #8 (Imperial) in Fall 2018, immobilized at 4°C, and split into two groups, control and treatment, which were marked with white and pink paint on the thorax, respectively. Then, bees were transferred to cup cages in groups of 40 bees, with 10 replicates per condition. The control group was fed sterile sucrose syrup, while the treatment group was fed 0.1% Roundup® in sterile sucrose syrup. During treatment, bees were maintained in an incubator (at 35°C and ~60% relative humidity) and mortality was censused on a daily basis. After three days of treatment, bees from each group were quickly immobilized with CO₂ and pooled together in plastic containers. 15 bees were randomly sampled from each group and stored at -80°C. This was considered as Day 0 post-treatment. 348 bees from each group were returned to the original hive by placing them in the top hive box (containing no frames). Three days after reintroduction to the hive, or Day 3 post-treatment, marked bees were temporarily recaptured to be counted. For that, every frame of the hive was repeatedly inspected until no more marked bees could be found.
15 bees were sampled from each group, and those remaining were released back to the hive to be sampled again at Day 5 post-treatment. All sampled bees were placed in Falcon tubes and stored at -80°C. This experiment was repeated 4 more times in different seasons, using bees from different hives, and swapping group colors as described in Table S3. Treatment, release and recovery steps followed the same protocol described for the first experiment, but bees were only sampled at Days 0 and 3 post-treatment. Moreover, a color marking validation experiment was conducted with worker bees from hive #9 (Alsatian); in this case both groups were fed sterile sucrose syrup (Table S3).

4.4 Topical exposure experiments

4.4.1 Laboratory conditions

Experiments 1 and 2: Hundreds of worker bees were taken from inside of hive #3 (Firefly) in Spring 2019, immobilized at 4°C, then transferred to cup cages in groups of 40 bees, with a total of 24 cup cages. Bees were maintained in an incubator at 35°C and ~60% relative humidity, and provided with sterile sucrose syrup in tubes attached to the top of the cup cage. The following day, filter paper lining, sterile sucrose syrup tubes and dead bees were removed from cup cages, and the remaining bees were sprayed from the top of the cup cage with ~1.2 mL of either tap water or 1.0% Roundup® in tap water (12 cup cages or 436 bees per group). Mortality rates were censused six hours after topical exposure. This experiment was replicated in Summer 2019, but with a total of 10 cup cages or 385 bees per group.

Experiments 3 and 4: Hundreds of worker bees were taken from inside of hive #3 (Firefly) in Summer 2019, immobilized at 4°C, then transferred to cup cages in groups of 40 bees, with a total of 20 cup cages. Bees were maintained in an incubator at 35°C and ~60% relative humidity, and provided with sterile sucrose syrup in tubes attached to the top of the cup cage. The following day, sucrose syrup tubes and dead bees were removed from cup cages, and the remaining bees were split into 4 groups which were sprayed from the top of the cup cage with ~1.2 mL of either tap water, 0.1% Roundup®, in tap water, 1.0% Roundup® in tap water or 1.0% glyphosate in tap water (10 cup cages or 386-388 bees per group). Mortality rates were censused at 6h and 24h after
This experiment was replicated in the same season using bees from hive #0 (Avocado), with 7 cup cages or 266 bees per group, and mortality rates were censused at 6h, 9h, 12h and 24h after topical exposure.

Experiments 5 and 6: Hundreds of worker bees were taken from inside hive #1 (Leviathan) in Fall 2019, placed in a cold room until immobilized, then transferred to cup cages in groups of 40 bees, with a total of 48 cup cages. Bees were maintained in an incubator at 35°C and ~60% relative humidity, and provided with sterile sucrose syrup in tubes attached to the base of the cup cage. The following day, dead bees were removed, and the cup cages were split into 6 groups which were sprayed with ~1.2 mL of either tap water, 0.05%, 0.1%, 0.5%, 1.0% or 3.0% Roundup® in tap water (8 cup cages or 310 bees per group). Mortality rates were censused at 6h, 9h, 12h and 24h after topical exposure. This experiment was replicated in the same season using bees from hive #6 (Pyrenees), with 10 cup cages or 397 bees per group.

These topical exposure experiments performed under laboratory conditions are summarized in Table S5.

4.4.2 Hive recovery

Experiments 1, 2, 3 and 4: Hundreds of worker bees were collected at four different times from inside hive #2 (Newfoundland) in Fall 2019 and placed at 4°C. After approximately 3 hours, immobilized bees were marked on their thorax with their respective group colors, either green or blue, for control or treatment. These bees were placed into cup cages in groups of 40 bees with 10 replicates per group, provided sucrose syrup in tubes attached to the base of the cup cage, and put into an incubator at 35°C and ~60% humidity. The next day, dead bees were removed, and the remaining ones were briefly immobilized with CO₂ and sprayed from the top of the cup cage with ~1.2 mL of either tap water or Roundup® formulation dissolved in tap water (0.1%, 1.0%, 1.0% and 3.0% formulation for experiments 1, 2, 3 and 4, respectively). After 1 hour of topical exposure, bees were released in front of hive #2. After 3 days, marked bees were recovered.

Experiment 5: Hundreds of worker bees were collected one single time from inside of hive #6 (Pyrenees) in Fall 2019 and placed at 4°C. After approximately 3
hours, immobilized bees were marked on their thorax with their respective group colors (white, green, orange, pink, yellow or blue) for control and different treatments. These bees were placed into cup cages in groups of 40 bees with 7 replicates per group, provided sucrose syrup in tubes attached to the base of the cup cage, and put into an incubator at 35°C and ~60% humidity. The next day, dead bees were removed, and the remaining ones were briefly immobilized with CO₂ and sprayed from the top of the cup cage with ~1.2 mL of either tap water or Roundup® formulation dissolved in tap water (0.05%, 0.1%, 0.5%, 1.0% and 3.0% formulation, respectively). After 1 hour of topical exposure, bees were released in front of hive #6. After 3 days, marked bees were recovered.

These topical exposure experiments followed by hive recovery are summarized in Table S6.

4.5 Field experiments

Several honey bee hives were transferred to two different sites on privately owned land in Driftwood, TX, approximately 2 months before the beginning of experiments in 2018 and 2019. Hives were comprised of 8 frames and approximately 8,000 to 10,000 worker bees, which were allowed to forage freely. To the best of our knowledge, there were no nearby flowering crops and few flowering wild plants, probably due to the dry season that preceded the experiments in 2018 (Figure S12). However, flowering plants were more abundant in 2019, probably due to the rainy season that preceded the experiments in 2019 (Figure S12). Moreover, colonies were provided with a limited number of food resources to encourage subsequent feeding. All colonies were generally assessed during the period of the experiment, and no behavioral or physical abnormalities were observed.

The first field experiment was performed at site 1 in August/September 2018. Five hives were selected to be part of the control group and were supplemented with 0.5 L of sucrose syrup, whereas other 5 hives were selected to be part of the treatment group and were exposed to 0.5 L of 0.1% Roundup® dissolved in sucrose syrup. Honey bees were sampled from each hive in the beginning of the experiment, Week 0, after which treatments were added in containers allocated inside of each hive to avoid cross-
contamination. Hives were inspected in a weekly basis during which bees were sampled before adding fresh treatments on Weeks 1, 2 and 3. Honey bees were also sampled on Weeks 4 (one week after final treatment) and 7 (one month after final treatment). Fifteen bees from each hive per sampling time were used to extract DNA, totaling 750 samples.

The second field experiment was performed at sites 1 and 2 in August/September 2019. In site 1, hives were supplemented with 0.5 L of sucrose syrup (control, n = 5), 0.001% Roundup® in sucrose syrup (0.001R-S, n = 5) or 0.1% Roundup® in sucrose syrup (0.1R-S, n = 4), following the same treatment and sampling schemes as in the first field experiment. One hive from the 0.1R-S treatment group died on Week 2. 15 bees from each hive per sampling time were dissected and guts were pooled according to hive for DNA extraction, totaling 67 samples.

In site 2, hives were split into 5 groups. Hives from control group (n = 6) were supplemented with 0.5 L of sucrose syrup and 0.45 L of tap water, whereas hives from treatment groups were supplemented with 0.001% or 0.1% Roundup® dissolved in sucrose syrup (0.001R-S: n = 4, 0.1R-S: n = 4) or in water (0.001R-W: n = 4, 0.1R-W: n = 5). Hives were supplemented with a single dose of sucrose syrup at Week 0, as well as with water at Weeks 0 and 2. Sucrose syrup was added in containers allocated inside of each hive, whereas water was added to a glass bottle with punched cap connected to a plastic boardman and attached to the hive entry. Honey bees and uncapped honey were sampled right before adding treatments to the hives (Week 0), and 1, 2, 3, 4 and 7 weeks after providing treatments to the hives. Fifteen bees from each hive per sampling time (except for Week 2) were dissected and guts were pooled respective to hive for DNA extraction, totaling 115 samples.

4.6 *Serratia* infection experiments

*Serratia* infection experiments were performed with worker bees collected from hives used in the field experiments (site 1 in 2018, and sites 1 and 2 in 2019). For each experiment, approximately 200 worker bees were collected from each selected hive at Week 4 (at least three hives per group) and brought back to the laboratory. Then, they were briefly immobilized at 4°C, transferred to cup cages in groups of at least 25 bees, with 6 replicates per group. Cup cages were transferred to growth chambers simulating...
The next day, each group was divided into two subgroups: one subgroup was used as control and provided only sterile sucrose syrup, whereas the other group was challenged with the opportunistic pathogen *Serratia marcescens* strain kz19. For that, a 0.5 OD *S. marcescens* kz19 suspension in sucrose syrup was provided to the cup cages in feeding tubes. Briefly, *S. marcescens* kz19 were grown in LB broth at 37°C the night before the experiment. The 600 nm optical density was measured, cells were washed with PBS and diluted to a concentration of 0.5 OD in proportions of 1 to 4 of PBS and sucrose syrup, respectively. The bacterial suspension was administered in feeding tubes. In each experiment, survivorship was monitored and recorded each day for 10 days. Kaplan–Meier survival curves were generated in GraphPad Prism®.

4.7 DNA extraction, qPCR analysis and 16S rRNA library preparation

For the laboratory and hive experiments, sampled honey bees were placed in sterile Falcon tubes and, while still alive, transferred to a freezer at -80°C. For the field experiments, sampled honey bees were placed in clean tubes and immediately flash frozen on site in a dry ice and ethanol mixture until transferred to a freezer at -80°C.

For the laboratory, hive recovery and 2018 field experiments, DNA was extracted from individual guts, following the protocol previously described (60). For the 2019 field experiments, DNA was extracted from pooled guts (15 dissected guts per hive were pooled) following the same protocol with some modifications: dual extraction with 0.75 mL phenol:chloroform:isoamyl (25:25:1), dual cleaning with 1.0 mL of cold 75% ethanol, and resuspension of DNA pellet in 200 μL water.

All DNA samples were 10-fold diluted to be used as template for qPCR analyses, as described in (12), and for 16S rRNA library preparation.

Library preparation consisted of two PCR steps. PCR 1 was designed to amplify the V4 region of the 16S SSU rRNA gene and was performed in 20 μL triplicate reactions using 515F (5’-tcgtcgcgaagtcggtagttaataagagacagggtacmcgccggtta-3’) and 806R (5’-gtctcgctggagatctgtaaagacagggtactacmvgggtwctaat-3’) primers (both at 200 nM final concentration) and 5 PRIME HotMasterMix (2.5X, Quantabio, MA, USA). Cycling conditions consisted of: 94°C for 3 min; 30 cycles of 94°C for 45 s, 50°C
for 60 s, 72°C for 90 s; then 72°C for 10 min. PCR 1 products were combined, purified
with 0.8x HighPrep™ PCR magnetic beads (MAGBIO®, MD, USA) and diluted to a final
volume of 52.5 μL. PCR 2 was designed to attach dual indices and illumina sequencing
adapter to PCR 1 product and was performed in 25 μL single reactions using a unique
combination of N7XX (5’-caagcagaagagcatacagatrnknnntctcgtgggctgg-3’) and
S5XX (5’-aatgatacgccacggagatatcactacnnnntgctcggcagctc-3’) index primers
(both at 400 nM final concentration) and 5 PRIME HotMasterMix (2.5X, Quantabio, MA,
USA). Cycling conditions consisted of: 94°C for 3 min; 10 cycles of 94°C for 20 s, 55°C
for 15 s, 72°C for 60 s; then 72°C for 10 min. PCR 2 products were purified with 0.8x
HighPrep™ PCR magnetic beads (MAGBIO®, MD, USA), diluted to a final volume of
27.5 μL and quantified fluorometrically (Qubit, Thermo Fisher Scientific Inc.). Samples
(50 ng each) were split into four pooled libraries. The first pooled library consisted of
samples from weeks 1 and 3 of the field experiment performed in 2018 (total of 300
samples). The second pooled library consisted of samples from weeks 0 and 7 of the
field experiment performed in 2018 (total of 300 samples). The third pooled library
consisted of 150 samples from week 4 of the field experiment performed in 2018, 90
samples from the first oral exposure, hive recovery experiment and 24 samples from the
oral exposure, laboratory experiment. The fourth pooled library consisted of 182
samples from the field experiments performed in 2019. Each library was loaded onto an
Illumina iSeq cartridge according to the manufacturer instructions and subjected to
Illumina sequencing on the iSeq platform (2x150 sequencing run, instrument model
number: FS10000184). 5% PhiX was used to check the quality of the runs.

4.8 Processing of 16S rRNA amplicon data
Illumina sequence reads were demultiplexed on the basis of the barcode
sequences by the iSeq software, then processed according to experiment in QIIME 2
version 2019.10 (78). Due to the lack of sufficient overlap between forward and reverse
reads, downstream analyses were performed with forward reads only. Primer
sequences were removed using the cutadapt plugin (79). Then, reads were truncated to
120 base pairs, filtered and denoised, and chimeric reads were removed using the
DADA2 plugin (80). Taxonomy was assigned to amplicon sequence variants (ASVs)
using the SILVA database in the feature-classifier plugin (81). Reads with lower than 0.1% abundance were removed from the dataset using the feature-table plugin, as well as unassigned, mitochondrial and chloroplast reads using the taxa filter-table plugin. The absolute abundance for each bacterial species was estimated by multiplying the total number of 16S rRNA gene copies obtained by qPCR by the percent relative abundance of each species, adjusting based on genomic 16S rRNA gene copy number, as in (62).

4.9 Quantification of glyphosate in honey samples

Approximately 2 mL samples of uncapped honey were collected from hives at site 1 in 2018 (Week 4), and at sites 1 and 2 in 2019 (Weeks 0, 1, 2, 3, 4 and 7). These samples were preserved at -20°C until submitted to an extraction protocol to detect and quantify glyphosate. Briefly, 1.00 ± 0.01 g of honey was weighed in a 50 mL Falcon tube and homogenized with 4.3 mL of a solution of 50 mM acetic acid and 10 mM Na₂EDTA in a vortex for 5 min, as described in (82). Samples were centrifuged at 5,000 rpm for 5 min, and 1 mL was transferred to a HPLC vial and submitted for high-resolution liquid chromatography, mass spectrometry (LC-MS) analysis. LC was performed with an Agilent 1260 Infinity HPLC system using an Acclaim Trinity Q1 column (2.1 mm by 100 mm, 3-μm particle size). The injection volume and the flow rate were 10 μL and 0.25 mL/min, respectively, during an isocratic elution using a mobile phase of 50 mM ammonium formate (pH 2.9, formic acid) for 5 min. Eluting species were detected by an Agilent 6530 Accurate-Mass Q-TOF mass spectrometer equipped with a Jet Stream electrospray ion source in negative mode. The ion source settings were capillary voltage, 3,000 V; nozzle voltage, 2,000 V; fragmentor voltage, 180 V; drying gas and sheath gas temperature, 350 °C; drying gas flow, 10 L/min; sheath gas flow, 11 L/min; nebulizer pressure, 45 lb/in². Glyphosate (C₃H₈NO₅P) was observed in the samples with this LC-MS method as [M–H]⁻ at 168.0067 Da, with a retention time of 2.2 minutes. Glyphosate quantification was performed by preparing analytical curves using the area under the glyphosate extracted ion chromatogram peak of different standard solutions prepared from a 1.0 mg/mL glyphosate stock solution in water: 1.25, 2.5, 5.0, 10 and 25 μg/mL glyphosate for samples collected from hives treated with 0.001% Roundup® in...
sucrose syrup or water; 50, 100, 200, 300, 400 and 500 μg/mL glyphosate for samples collected from hives treated with 0.1% Roundup® in sucrose syrup or water.

Quantification limits (QLs) were obtained by calculating the ratio between the standard deviation of the lower concentration used in the analytical curve and the slope of the analytical curve, then multiplying by 10. One sample from the 0.001% Roundup® group collected at Week 0, site 2 was excluded from the analyses due to contamination. The linear equations obtained from the analytical curves were used to calculate the concentration of glyphosate in the samples. The exact mass weighed for each sample was converted to volume, considering the density of sucrose syrup 1:1 (w/v) equal to 1.22 g/mL. Then, the concentrations obtained from the linear equation were corrected for the dilution factor.

4.10 Statistical analyses

For some oral and topical exposure experiments, comparisons of changes in bacterial abundance between control and treatment groups were performed using the non-parametric Kruskal-Wallis test followed by Dunn’s multiple comparisons test, if significant, in R version 3.5.2 (83). Principal Coordinate Analyses based on Bray-Curtis or weighted Unifrac dissimilarities were plotted using the R package “phyloseq” (84) and statistical tests were performed using pairwise permutational MANOVA (PERMANOVA) tests with 999 permutations in QIIME 2 version 2019.10 (78). Comparisons of changes in recovered or surviving bees between control and treatment groups were performed using the Chi-squared test followed by Bonferroni correction.

For some oral exposure experiments and all the field experiments, generalized linear mixed-effects models assuming Poisson regression were used to compare changes in bacterial abundances between control and treatment bees or hives, respectively, per sampling time. Treatment and sampling time were considered as fixed effects, and bees or bees nested within hives as random effects. Mixed models were fitted using the R package “lme4” (85) and followed by post hoc tests using the R package “emmeans” (86).

For some topical exposure experiments and all the Serratia challenge experiments, comparisons of survival rates between control and treatment groups were
performed using Kaplan–Meier survival curves and the Cox Proportional Hazards Model implemented in the R package “survival” (52). For some topical exposure experiments, dose-response models were fitted using the drm and LL.4 functions to fit and define the structure of the regression model, and the modelFit function to obtain a lack-of-fit test, all in the R package “drc” (87).

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**Data availability**

All sequence data are available on NCBI BioProject PRJNA630698. Final tables and R scripts are available in GitHub (https://github.com/erickmotta/aem-2020). The other data generated during this study are included in this published article and its supplementary information files.

**Competing interests**

The authors declare that they have no competing interests.

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**Figure legends**

Figure 1. The effects of glyphosate and Roundup® formulation on the honey bee gut microbiota.
(A) Newly emerged workers were exposed to 1.0 mM glyphosate in sucrose syrup, 1.0 mM Roundup® in sucrose syrup, or only sucrose syrup for 5 days. (B) Boxplots of absolute abundance of total bacteria, (C) Snodgrassella, (D) Gilliamella and (E) Bifidobacterium in the guts of bees sampled from Control, Glyphosate and Roundup® groups (n = 8 for each group). Groups with distinct letters are statistically different (P < 0.05, Kruskal-Wallis test followed by Dunn’s multiple comparisons test). (F) Principal coordinate analysis of Bray-Curtis dissimilarity of gut community compositions of Control, Glyphosate and Roundup® groups.

Figure 2. Recovery rates and gut microbial changes for honey bees returned to the hive after oral exposure to 0.1% Roundup® formulation. (A) Experimental design. (B) Number of worker bees recovered from the hive at day 3 post-treatment (***P < 0.001, Chi-squared test). (C-G) Scatter plots of bacterial abundances in the guts of control and treatment bees sampled at days 0, 3 and 5 post-treatment (n = 15 for each group and time point), with error bars of 95% confidence interval. Total 16S rRNA gene copies were estimated by qPCR and corrected for rRNA operon number per genome. Generalized linear mixed-effects models assuming Poisson regression were used to compare changes in bacterial abundances between control and treatment bees per sampling time. Mixed models were fitted using the package lme4 and followed by post hoc tests using package emmeans (86). *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 3. Gut microbial changes and susceptibility to bacterial infections in honey bees from hives exposed to Roundup® formulation in site 1, 2018. (A) Field experiment performed in site 1, 2018. 10 hives were split into 2 groups to be exposed to 0.5 L of sucrose syrup or 0.1% Roundup® formulation dissolved in sucrose syrup at weeks 0, 1, 2 and 3. Treatment was placed in a reservoir inside of the hives to avoid cross-contamination. Bees were sampled at weeks 0, 1, 3, 4 and 7. Uncapped honey samples were collected at week 4. (B) Scatter plots of total bacteria, (C) Snodgrassella and (D) Bifidobacterium abundance in the guts of bees sampled from control (sucrose syrup) and treatment (0.1% Roundup® in syrup) groups on weeks 0, 1,
3, 4 and 7, with error bars of 95% confidence interval (n = 5 hives per group, 15 bees per hive) per sampling time. Generalized linear mixed-effects models assuming Poisson regression were used to compare changes in bacterial abundances between control and treatment groups. Mixed models were fitted using the package lme4 (85) and followed by post hoc tests using the package emmeans (86). *P < 0.05, **P < 0.01 and ***P < 0.001. (E) Survival rates of worker bees after *Serratia marcescens* kz19 exposure, shown as a Kaplan–Meier survival curve. Worker bees were sampled from all hives at week 4 and exposed or not to *S. marcescens* kz19 under laboratory conditions for 10 days (n = 5 hives per condition, 3 cup cages per hive, at least 25 bees per cup cage). *P < 0.05, ***P < 0.001, Cox Proportional Hazards Model implemented in the package "survival". (F) Glyphosate concentration detected in uncapped honey samples from control and treatment groups (n = 5 hives per group) at week 4.

**Figure 4. Gut microbial changes and susceptibility to bacterial infections in honey bees from hives exposed to Roundup® formulation in site 1, 2019.**

(A) Field experiment performed in site 1, 2019. 14 hives were split into 3 groups to be exposed to 0.5 L of sucrose syrup, 0.001% or 0.1% Roundup® formulation dissolved in sucrose syrup at weeks 0, 1, 2 and 3. Treatment was placed in a reservoir inside of the hives to avoid cross-contamination. Bees were sampled at weeks 0, 1, 3, 4 and 7. Uncapped honey samples were collected at weeks 0, 1, 2, 3, 4 and 7. (B) Scatter plot of *Snodgrassella* abundance in the guts of bees sampled from control (sucrose syrup) and treatment (0.001% and 0.1% Roundup® in syrup) groups on weeks 0, 1, 3, 4 and 7, with error bars of 95% confidence interval. For group 1: n = 5 for weeks 0, 1, 3 and 4; n = 3 for week 7. For group 2: n = 5. For group 3: n = 4 for weeks 0 and 1; n = 3 for weeks 3, 4 and 7. Each hive is represented by 15 pooled bee guts. Generalized linear mixed-effects models assuming Poisson regression were used to compare changes in bacterial abundances between control and treatment hives per sampling time. Mixed models were fitted using the package lme4 (85) and followed by post hoc tests using the package emmeans (86). *P < 0.05, **P < 0.01 and ***P < 0.001. (C) Survival rates of worker bees after *Serratia marcescens* kz19 exposure, shown as a Kaplan–Meier survival curve. Worker bees were sampled from representative hives from each
group at week 4 and exposed or not to *S. marcescens* kz19 under laboratory conditions for 10 days (*n* = 3 hives per condition, 3 cup cages per hive, at least 25 bees per cup cage). *P* < 0.05, ***P* < 0.001, Cox Proportional Hazards Model implemented in the package “survival”. (D) Glyphosate concentration detected in uncapped honey samples collected from control (*n* = 5, all weeks), 0.001% Roundup®-treated (*n* = 5, all weeks) and 0.1% Roundup®-treated (*n* = 4, weeks 0, 1 and 2; *n* = 3, weeks 3, 4 and 7) hives.

Figure 5. Gut microbial changes and susceptibility to bacterial infections in honey bees from hives exposed to Roundup® formulation in site 2, 2019.

(A) Field experiment performed in site 2, 2019. 23 hives were split into 5 groups to be exposed to 0.001% or 0.1% Roundup® formulation dissolved in sucrose syrup or water. Treatment in syrup (0.5 L) was provided at week 0 in a reservoir inside of each hive. Treatment in water (0.45 L) was provided at weeks 0 and 2 in a glass bottle with punched cap connected to a plastic boardman and attached to the hive entry. Bees were sampled at weeks 0, 1, 3, 4 and 7. Uncapped honey samples were collected at weeks 0, 1, 2, 3, 4 and 7. (B) Scatter plot of *Snodgrassella* abundance in the guts of bees sampled from control and treatment groups on weeks 0, 1, 3, 4 and 7, with error bars of 95% confidence interval. For group 1: *n* = 6 for weeks 0, 3, 4 and 7; *n* = 5 for week 1. For group 2: *n* = 4 for weeks 0, 3, 4 and 7; *n* = 3 for week 1. For groups 3 and 4: *n* = 4. For group 5: *n* = 5 for weeks 0, 1, 3 and 4; *n* = 4 for week 7 Each hive is represented by 15 pooled bee guts. Generalized linear mixed-effects models assuming Poisson regression were used to compare changes in bacterial abundances between control and treatment groups per sampling time. Mixed models were fitted using the package lme4 (85) and followed by post hoc tests using the package emmeans (86). *P* < 0.05, **P* < 0.01 and ***P* < 0.001. (C) Survival rates of worker bees after *Serratia marcescens* kz19 exposure, shown as a Kaplan–Meier survival curve. Worker bees were sampled from representative hives from each group at week 4 and exposed or not to *S. marcescens* kz19 under laboratory conditions for 10 days (*n* = 3 hives per condition, 3 cup cages per hive, at least 25 bees per cup cage). *P* < 0.05, ***P* < 0.001, Cox Proportional Hazards Model implemented in the “survival” package in R. (D) Glyphosate concentration detected in uncapped honey samples collected from control.
(n = 6) and treatment (0.001% Roundup® in syrup, n = 4; 0.001% Roundup® in water, n = 4; 0.1% Roundup® in syrup, n = 4; 0.1% Roundup® in water, n = 5) groups at weeks 0, 1, 2, 3, 4 and 7.

Figure 6. Survival rates and gut microbial changes of honey bees topically exposed to Roundup® formulation under laboratory conditions.  
(A) Worker bees were split into 6 groups to be sprayed with different concentrations of glyphosate in herbicide formulation in water. Survivorship was monitored for 24 hours under laboratory conditions. (B) Survival rates of worker bees after topical exposure to a glyphosate-based formulation over a period of 24 hours, shown as a Kaplan–Meier survival curve (n = 10 cup cages per group, 38-40 bees per cup cage). ***P < 0.001, Cox Proportional Hazards Model implemented in the package "survival". (C) Boxplots of Snodgrassella abundance in the guts of survived bees 24 hours after spray, measured by qPCR (n = 15 bees per group). Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles. No significant changes were observed, Kruskal-Wallis test.

Figure 7. Recovery rates and gut microbial changes of honey bees topically exposed to Roundup® formulation under hive conditions.  
(A) Worker bees were split into 6 groups and marked on the thorax with different colors of paint. Groups were sprayed with different concentrations of a glyphosate-based formulation in water and released back to their hive. All marked bees were recovered on day 3 post-spray. (B) Recovery rates of worker bees topically exposed to a glyphosate-based formulation on day 3 post-spray, (n = 7 replicates per group, 35-40 bees per replicate). ***P < 0.001, Chi-squared test followed by Bonferroni correction. (C) Boxplots of Snodgrassella abundance in the guts of recovered bees on day 3 post-spray, measured by qPCR (n = 15 bees per group). Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles. Groups with distinct letters are statistically different (P < 0.001, Kruskal-Wallis test followed by Dunn’s multiple comparisons test).
References


A Bee bread with gut homogenate
Bee age (days) 0 1 2 3 4 5 6
Sampling
Exposure
A Bee bread with gut homogenate
B Sucrose syrup
Group 1
1.0 mM Glyphosate
Group 2
1.0 mM Roundup®
Group 3

16S rRNA gene copies
All bacteria
Snodgrassella
Gilliamella
Bifidobacterium

PCoA of Bray–Curtis dissimilarity
PCo1 − 24.7%
PCo2 − 18.4%
16S rRNA gene copies

A: Capture, Release, Sampling

Days

-3 0 3 5

Exposure

Recovery

Control 0.1% Roundup®

Bees recovered on Day 3

***

B: Experiment 1, Oct 2018

Bees recovered on Day 3

***

***

44.0%

27.6%

Non-recovered

Recovered

Sucrose syrup

Group 1

0.1% Roundup® in syrup

Group 2

-3 0 3 5

Days

Sampling

Capture Release

Exposure

Recovery

C: All bacteria

16S rRNA gene copies

5e+7

3e+7

2e+7

0 3 5

Day

D: Snodgrassella

16S rRNA gene copies

1e+7

3e+6

2e+6

0 3 5

Day

E: Gilliamella

16S rRNA gene copies

2e+7

1e+7

0 3 5

Day

F: Bifidobacterium

16S rRNA gene copies

1e+7

5e+6

3e+6

0 3 5

Day

G: Lactobacillus Firm-5

16S rRNA gene copies

2e+7

7e+6

0 3 5

Day
A. Site 1 – 2019

B. Snodgrassella

C. Serratia challenge at week 4

D. Honey samples

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16S rRNA gene copies

Week

Sucrose syrup
0.001% Roundup®
0.1% Roundup®

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Percent survival

Days

Control groups
Serratia-challenged groups

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Glyphosate concentration (μg/mL)

Week

0 300 600 900 1200 1500 1800 2100
**A** Site 2 − 2019

- **Site 2 − 2019**
- **Bee sampling**
- **Honey sampling**
- **Treatments in water**
- **Treatment in sucrose syrup**

**B** Snodgrassella

- **16S rRNA gene copies**
- **Weeks 0, 1, 2, 3, 4, 7**
- **Group 1: sucrose syrup + water (6 hives)**
- **Group 2: 0.001% Roundup® in syrup (4 hives)**
- **Group 3: 0.001% Roundup® in water (4 hives)**
- **Group 4: 0.1% Roundup® in syrup (4 hives)**
- **Group 5: 0.1% Roundup® in water (5 hives)**

**C** Serratia challenge at week 4

- **Percent survival**
- **Control groups**
- **Serratia-challenged groups**
- **Weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, 10**

**D** Honey samples

- **Glyphosate concentration (μg/mL)**
- **Weeks 0, 1, 2, 3, 4, 5, 6, 7**
A. Topical exposure and survival monitoring in the lab

B. Survival after spray

C. Snodgrassella abundance in surviving bees

16S rRNA gene copies

Hours
0 3 6 9 12 15 18 21 24

0 20 40 60 80 100 Percent survival

Water
0.05% Roundup®
0.1% Roundup®
0.5% Roundup®
1.0% Roundup®
3.0% Roundup®

Group 1
Tap water
Group 2
0.05% Roundup®
Group 3
0.1% Roundup®
Group 4
0.5% Roundup®
Group 5
1.0% Roundup®
Group 6
3.0% Roundup®

Survival monitored for 24h

Topical exposure

Days
-1 0 1
0 6h 9h 12h
A. Topical exposure and release to the hive

B. Recovery after 3 days in relation to Roundup® concentration (%)

C. Snodgrassella abundance in recovered bees

Mark recovery on February 21, 2021 by guest

http://aem.asm.org/