



# Glyphosate perturbs the gut microbiota of honey bees

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**Glyphosate, the primary herbicide used globally for weed control, targets the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway found in plants and some microorganisms. Thus, glyphosate may affect bacterial symbionts of animals living near agricultural sites, including pollinators such as bees. The honey bee gut microbiota is dominated by eight bacterial species that promote weight gain and reduce pathogen susceptibility. The gene encoding EPSPS is present in almost all sequenced genomes of bee gut bacteria, indicating that they are potentially susceptible to glyphosate. We demonstrated that the relative and absolute abundances of dominant gut microbiota species are decreased in bees exposed to glyphosate at concentrations documented in the environment. Glyphosate exposure of young workers increased mortality of bees subsequently exposed to the opportunistic pathogen *Serratia marcescens*. Members of the bee gut microbiota varied in susceptibility to glyphosate, largely corresponding to whether they possessed an EPSPS of class I (sensitive to glyphosate) or class II (insensitive to glyphosate). This basis for differences in sensitivity was confirmed using in vitro experiments in which the EPSPS gene from bee gut bacteria was cloned into *Escherichia coli*. All strains of the core bee gut species, *Snodgrassella alvi*, encode a sensitive class I EPSPS, and reduction in *S. alvi* levels was a consistent experimental result. However, some *S. alvi* strains appear to possess an alternative mechanism of glyphosate resistance. Thus, exposure of bees to glyphosate can perturb their beneficial gut microbiota, potentially affecting bee health and their effectiveness as pollinators.**

herbicide is known to affect the growth of microorganisms (13–15), and the health of bees is intrinsically related to their distinct gut microbial community (16, 17). The honey bee gut microbiota is dominated by eight bacterial species: *Lactobacillus* spp. Firm-4, *Lactobacillus* spp. Firm-5 (phylum Firmicutes), *Bifidobacterium* spp. (phylum Actinobacteria), *Snodgrassella alvi*, *Gilliamella apicola*, *Frischella perrara*, *Bartonella apis*, and Alpha 2.1 (phylum Proteobacteria) (18). Each of these species exhibits strain diversity corresponding to differences in metabolic capabilities and tolerances to xenobiotics (19, 20). Newly emerged workers (NEWs) are nearly free of gut bacteria and acquire their normal microbial community orally through social interactions with other workers during the first few days after emergence (21). Bees deprived of their normal microbiota show reduced weight gain and altered metabolism (22), increased pathogen susceptibility (17), and increased mortality within hives (23).

In this study, we investigated the effects of glyphosate exposure on the size and composition of the honey bee gut microbiome. We found the microbiome was affected by glyphosate exposure during and after gut colonization, and that glyphosate exposure during early gut colonization increased mortality of bees exposed to an opportunistic pathogen. Additionally, bee gut bacteria differ in glyphosate susceptibility. We explored the molecular mechanisms of this variability in glyphosate tolerance by expressing the EPSPS of bee gut symbionts in *E. coli*. Some bee gut bacteria tolerate glyphosate by virtue of a class II EPSPS, but a few strains with susceptible class I EPSPS depend on other,

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The broad-spectrum herbicide glyphosate [*N*-(phosphonmethyl)glycine] has long been the primary weed management system, and its use is growing in connection with crops genetically engineered to be resistant to glyphosate (1, 2). Its mechanism of action, inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the shikimate pathway, prevents the biosynthesis of aromatic amino acids and other secondary metabolites in plants and some microorganisms (3). EPSPS catalyzes the reaction between phosphoenolpyruvate (PEP) and shikimate 3-phosphate (S3P) (4), and glyphosate is a competitive inhibitor that blocks the PEP-binding site (5). EPSPS enzymes from different organisms vary in molecular weight (46–178 kDa) and sequence homology (6) and form two phylogenetic clusters that differ in tolerance to glyphosate. Class I enzymes are sensitive to glyphosate and are present in all plants and in some bacteria, such as *Escherichia coli* (4); class II enzymes are only found in some bacteria, such as *Staphylococcus aureus*, and can tolerate high concentrations of glyphosate (7, 8).

Animals lack the shikimate pathway, which is why glyphosate is considered one of the least toxic pesticides used in agriculture (9). However, some evidence suggests that glyphosate affects nontarget organisms, for example, changing the behavior of honey bees (10), reducing reproduction of soil-dwelling earthworms (11), and affecting the growth of microalgae and aquatic bacteria (12). Glyphosate is also associated with changes in plant endophytic and rhizosphere microbiomes (2) and with disturbances of gut microbiota of animals living near agricultural sites (13).

Honey bees and bumble bees are major pollinators of flowering plants, including many crops. When foraging, they can be exposed to a variety of xenobiotics, such as glyphosate. This

## Significance

**Increased mortality of honey bee colonies has been attributed to several factors but is not fully understood. The herbicide glyphosate is expected to be innocuous to animals, including bees, because it targets an enzyme only found in plants and microorganisms. However, bees rely on a specialized gut microbiota that benefits growth and provides defense against pathogens. Most bee gut bacteria contain the enzyme targeted by glyphosate, but vary in whether they possess susceptible versions and, correspondingly, in tolerance to glyphosate. Exposing bees to glyphosate alters the bee gut community and increases susceptibility to infection by opportunistic pathogens. Understanding how glyphosate impacts bee gut symbionts and bee health will help elucidate a possible role of this chemical in colony decline.**

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The authors declare no conflict of interest.

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Data deposition: All new sequence data are available on NCBI BioProject (accession nos. PRJNA432210 and PRJNA480015).

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yet unknown, mechanisms for tolerance. Overall, our results show that glyphosate exposure can perturb the gut microbiota of honey bees, and that compositional shifts typically favor species tolerant to glyphosate and disfavor sensitive species.

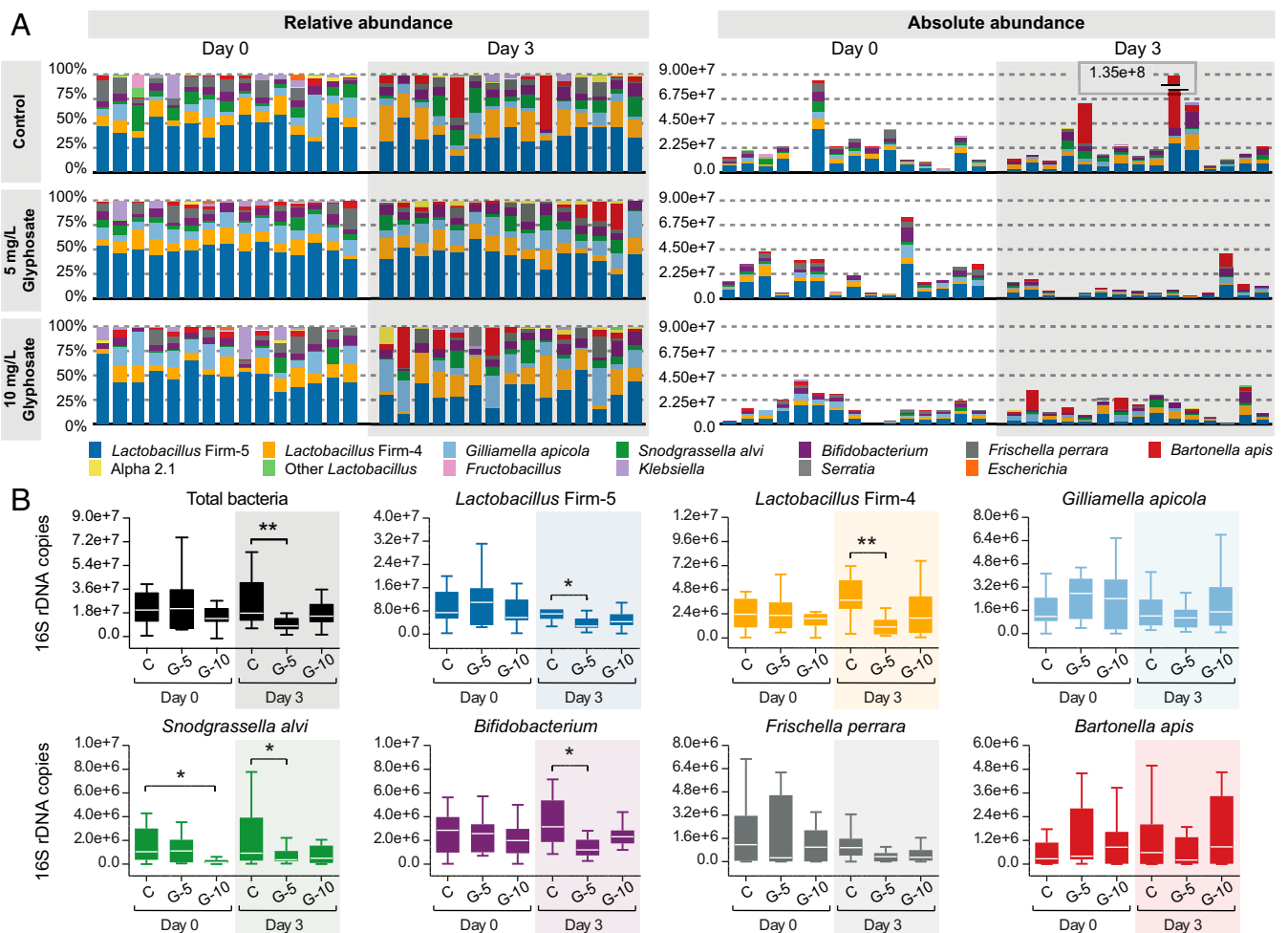
## Results and Discussion

**Glyphosate Perturbs the Honey Bee Gut Bacterial Community.** Hundreds of adult worker bees were collected from a single hive, treated with either 5 mg/L glyphosate (G-5), 10 mg/L glyphosate (G-10) or sterile sucrose syrup (control) for 5 d, and returned to their original hive. Bees were marked on the thorax with paint to make them distinguishable in the hive. Glyphosate concentrations were chosen to mimic environmental levels, which typically range between 1.4 and 7.6 mg/L (24), and may be encountered by bees foraging at flowering weeds. To determine the effects of glyphosate on the size and composition of the gut microbiome, 15 bees were sampled from each group before reintroduction to the hive (day 0) and postreintroduction (day 3), and relative and absolute abundances of gut bacteria were assessed using deep amplicon sequencing of the V4 region of the bacterial 16S rRNA gene and quantitative PCR (qPCR).

At day 0, glyphosate exposure had little effect on the bee gut microbiome size, but the absolute and relative abundances of the core species, *S. alvi*, were significantly lower in the G-10 group (Fig.

1 and *SI Appendix, Fig. S1*). The effects of glyphosate exposure on the bee gut microbiome were more prominent at day 3, after treated bees were returned to the hive. The total number of gut bacteria decreased for both treatment groups, relative to control, but this drop was significant only for the G-5 group, which also exhibited more severe compositional shifts (Fig. 1). The absolute abundances of four dominant gut bacteria, *S. alvi*, *Bifidobacterium*, *Lactobacillus* Firm-4 and Firm-5 were decreased (Fig. 1), and the relative abundance of *G. apicola* increased in the G-5 group (*SI Appendix, Fig. S1*). Surprisingly, only *Lactobacillus* Firm-5 decreased in absolute abundance in the G-10 group (Fig. 1). This experiment was repeated using bees from a different hive and season, and similar trends were observed (*SI Appendix, Fig. S2*). As in the first experiment, significant reductions in abundance were observed for *S. alvi* in bees treated with glyphosate (*SI Appendix, Fig. S2*).

The relative lack of effects of the G-10 treatment on the microbiota composition at day 3 posttreatment is unexplained, but may reflect other effects of glyphosate on bees. Our recapture method fails to sample bees that died or abandoned the hive. Since bees exposed to glyphosate may exhibit impaired spatial processing, compromising their return to hives (10, 24), bees in the G-10 group that consumed more glyphosate-laced sugar syrup before reintroduction to the hive may have been less likely to return to the hive after foraging. Since fewer than 20% of bees



**Fig. 1.** Changes in gut microbiota composition following glyphosate exposure of honey bees with established gut communities. (A) Stacked column graph showing the relative and absolute abundances of gut bacterial species in control bees and bees treated with 5 mg/L or 10 mg/L glyphosate at posttreatment days 0 and 3. Each column represents one bee. (B) Boxplots of bacterial 16S rDNA copies for control (C) and glyphosate-treated (G-5 and G-10) bees at posttreatment days 0 and 3 ( $n = 15$  for each group and time point). Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. \* $P < 0.05$  and \*\* $P < 0.01$ , Wilcoxon rank sum test followed by Bonferroni correction.

reintroduced to the hive were recovered, recovered bees may not represent the total effect of glyphosate on treatment groups.

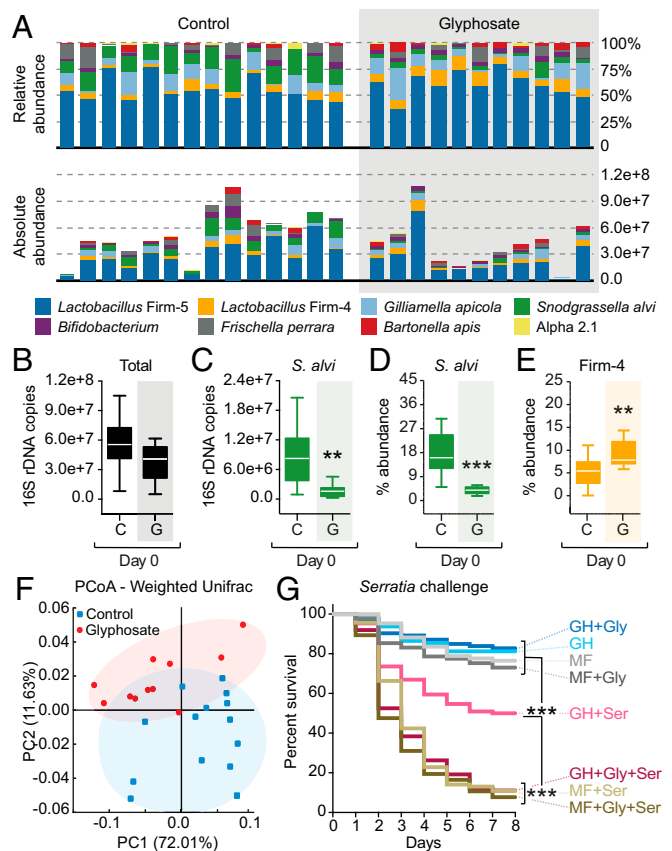
**Glyphosate Affects Early Gut Bacterial Colonization.** Glyphosate arrests bacterial growth without directly killing the cells, so we hypothesized that it would have a greater effect on actively dividing cells present during early gut colonization. To test this, NEWs, which are nearly free of gut bacteria (21), were simultaneously exposed to an inoculum consisting of their normal microbial community and to glyphosate. This simultaneous exposure is relevant to field situations, since glyphosate has been detected in hives and honey samples (25, 26), indicating that honey bee foragers can transport residues of this herbicide to the colony and contaminate other bees, including NEWs, and food resources. Also, glyphosate is a stable, water-soluble chemical that can persist in the environment for long periods (10).

Assessment of gut microbiomes, as described in the previous section, identified all eight core gut taxa in both control and treatment groups (Fig. 2A), showing that glyphosate does not eliminate colonization by any core member. Average total bacterial abundance was slightly lower in glyphosate-treated bees, but this was not statistically significant (Fig. 2B). *S. alvi* was the most strongly affected member of the gut microbiota and decreased in both absolute and relative abundance, while *Lactobacillus* Firm-4 increased in relative abundance (Fig. 2C–E and *SI Appendix*, Fig. S3). Based on relative abundance, gut community compositions of glyphosate-treated bees differed from those of controls (principal coordinate analysis of weighted UniFrac) (27), perMANOVA test with 9,999 permutations;  $P = 0.0078$ , pseudo-F statistic = 6.66) (Fig. 2F). Thus, glyphosate exposure during early development of the gut community can interfere with normal colonization by altering the abundance of beneficial bacterial species.

Typically, captive honey bees do not defecate, and dead bacterial cells and the released DNA accumulate in the gut (23). Thus, we also analyzed changes in bacterial abundance after glyphosate exposure by extracting both DNA and RNA from the guts of treatment and control bees in a second colonization experiment. We included a positive control group, in which bees were exposed to tylosin, an antibiotic used in beekeeping. This antibiotic treatment was expected to perturb the microbiota, but the decrease was significant only for RNA samples (*SI Appendix*, Fig. S4). Glyphosate exposure resulted in nonsignificant decreases in total bacteria for both DNA and RNA assays. We also checked changes in absolute abundance for three core bacterial species, *S. alvi*, *Lactobacillus* Firm-4, and *Lactobacillus* Firm-5. Tylosin treatment resulted in reductions for 16S rRNA copies (*SI Appendix*, Fig. S4). Effects of glyphosate treatment were specific to *S. alvi*, which was the only assayed species showing significant reductions in absolute abundance, observed for both DNA and RNA assays (*SI Appendix*, Fig. S4). This experiment suggests that measures based on DNA are partly obscured by DNA from dead bacterial cells, although this effect does not entirely mask shifts in bacterial abundance.

**Glyphosate Exposure Makes Young Worker Bees More Susceptible to *Serratia*.** To determine whether glyphosate-induced perturbation of microbiota colonization affects host health, we measured the susceptibility of glyphosate-treated bees to an opportunistic bacterial pathogen. NEWs were exposed to glyphosate in the stage of acquiring their normal microbial community. After 5 d of treatment, bees were challenged with *Serratia marcescens* kzl19, an opportunistic pathogen commonly detected at very low frequencies in the bee gut (28, 29).

For bees lacking gut microbiota, *Serratia* challenge resulted in increased mortality relative to that observed for bees with a conventional gut microbiota, regardless of glyphosate exposure (Fig. 2G and *SI Appendix*, Fig. S5). For bees with a conventional gut microbiota, glyphosate treatment resulted in increased mortality after *Serratia* challenge. To determine whether this increased mortality was attributable to the effects of glyphosate on the gut microbiota or to direct effects of glyphosate on bees, we included

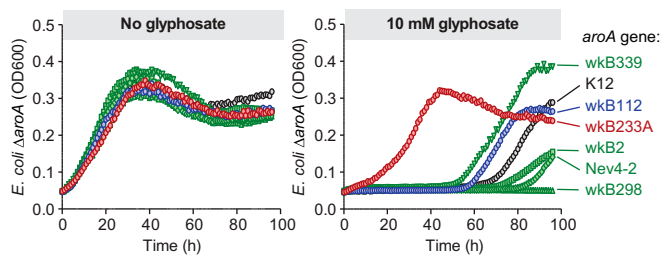


**Fig. 2.** Changes in gut microbiota composition following glyphosate exposure of young honey bees and susceptibility to *Serratia* infection. (A) Stacked column graph showing the relative and absolute abundances of gut bacterial species in control and glyphosate-treated bees. Each column represents one bee. (B–E) Boxplots of total bacterial 16S rDNA copies and of absolute and relative abundances of two gut bacterial species for control ( $n = 14$ ) and glyphosate-treated ( $n = 11$ ) bees.  $**P < 0.01$ , and  $***P < 0.001$ , Wilcoxon rank sum test followed by Bonferroni correction. (F) Principal coordinate analysis of gut community composition using weighted UniFrac (perMANOVA test with 9,999 permutations;  $P = 0.0078$ , pseudo-F statistic = 6.66). (G) The percent survival of age-controlled bees after *Serratia* kzl19 exposure, shown as a Kaplan–Meier survival curve.  $***P < 0.001$ , coxph model implemented in the “survival” package in R. GH, gut homogenate-exposed bees; Gly, glyphosate treatment; MF, microbiota-free bees; Ser, *Serratia* challenge.

control groups not challenged with *Serratia*. In bees exposed to glyphosate, but not challenged with *Serratia*, survival rates were not significantly affected by glyphosate and were much higher than in the *Serratia*-challenged groups (Fig. 2G), demonstrating that a direct effect of glyphosate on bees is not the basis of the high mortality of glyphosate-exposed, pathogen-challenged bees.

Our results show that glyphosate reduces the protective effect of the gut microbiota against opportunistic pathogens and that *S. alvi* is the bacterial species most negatively affected by glyphosate exposure. By itself, *S. alvi* appears to give some protection, but not as much as the whole gut microbiota (*SI Appendix*, Fig. S6). *S. alvi* forms a biofilm on the wall of the gut ileum (18, 21, 30), which may function as a mechanical barrier against pathogen invasion. Some *S. alvi* strains encode type VI secretion systems (31), which could contribute to colonization resistance through contact-dependent inhibition of *Serratia*. Furthermore, host expression of antimicrobial peptides is upregulated after *S. alvi* colonization (32), which could increase resistance to infection by pathogens. Besides a direct protective effect, *S. alvi* may be critical in enabling the full microbiota to assemble, thus enabling greater protection.





**Fig. 4.** Growth curves of *E. coli*  $\Delta$ aroA BW25113 expressing the *aroA* gene from different bee-associated bacterial strains (*B. apis* in red, *E. coli* in black, *G. apicola* in blue, and *S. alvi* in green) cultured in minimal media in the presence or absence of 10 mM glyphosate.

glyphosate resistance: *yhhS*, which encodes a membrane transporter conferring glyphosate tolerance when overexpressed in *E. coli* (39), and *tetC*, which encodes an efflux pump that provides tetracycline resistance to *S. alvi* wkB2 (38). However, these transporters were not able to reverse the delay in *E. coli* growth caused by glyphosate (SI Appendix, Fig. S9). As the glyphosate tolerance exhibited by some *S. alvi* strains does not appear to be due to a resistant EPSPS or to transport by YhhS or TetC, these strains are likely to employ a novel mechanism of glyphosate resistance. Future studies might identify this mechanism and determine the evolutionary origin of resistance.

***S. alvi* Strains May Vary in Sensitivity to Glyphosate in Vivo.** Our phylogenetic analysis and cloning experiments demonstrated that, despite displaying variable susceptibility to glyphosate in vitro, all *S. alvi* strains possess a glyphosate-sensitive class I EPSPS. Therefore, we investigated whether this variation in susceptibility occurs in vivo. NEWs were mono-inoculated with two different *S. alvi* strains: wkB2, which grows in the presence of high concentrations of glyphosate, or wkB339, which exhibits a delay in growth in the presence of high concentrations of glyphosate (Fig. 5A). Bees were hand fed bacterial suspensions to inoculate with a control number of *S. alvi* cells, exposed to glyphosate for 3 d, and sampled at days 1 and 3 during exposure. Both *S. alvi* wkB2 and *S. alvi* wkB339 increased in abundance between days 1 and 3 in unexposed bees, consistent with previous findings that *S. alvi* can colonize guts of mono-inoculated bees (30, 40). Based on qPCR estimates of *S. alvi* abundance on day 3, glyphosate exposure had a negative effect on growth of both strains [two-way analysis of variance (ANOVA), treatment effect,  $P < 0.0001$ ]. *S. alvi* wkB339 was more affected by glyphosate exposure, based on significant interaction between strain and treatment (two-way ANOVA,  $P < 0.0204$ ). Correspondingly, the absolute abundance of the glyphosate-sensitive strain, wkB339, was significantly lower in glyphosate-treated bees compared with controls (Tukey's test,  $P < 0.001$ ) or wkB2-treated bees (Tukey's test,  $P < 0.001$ ) (Fig. 5B and C). Potentially, strain differences in glyphosate sensitivity may contribute to the observed variation in the overall decrease in *S. alvi* abundance when bees with their native gut microbiota are exposed to glyphosate.

## Conclusion

As in many animals, honey bees rely on their gut microbial community for a variety of functions, including food processing (25, 26), regulation of immune system (33, 34), and defense against pathogens (17, 27). Perturbations of this system have the potential to lead to negative consequences for host fitness. We found that glyphosate affects the bee gut microbiota composition and that bacterial species and strains within this community vary in susceptibility to glyphosate. Recent experimental and observational studies have provided evidence that dysbiosis affecting the bee gut can increase susceptibility to pathogen invasion (23, 41, 42). Our results also suggest that establishment of a normal microbial community is crucial for protection against opportunistic

pathogens of honey bees. Furthermore, our results highlight one potential mechanism by which glyphosate affects bee health.

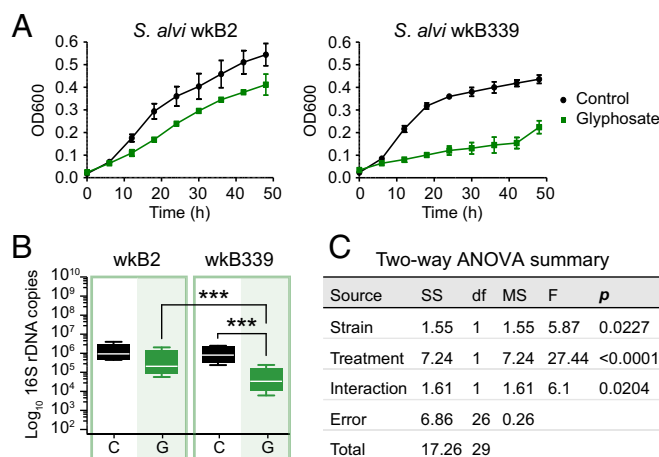
While some species in the bee gut can tolerate high concentrations of glyphosate due to the presence of a class II EPSPS enzyme, others are sensitive due to the presence of a class I EPSPS. A consistent effect of glyphosate on the bee gut microbiota was a negative impact on growth of *S. alvi*, which possesses a sensitive EPSPS. However, some strains of *S. alvi* may tolerate glyphosate through an as yet unknown mechanism. Since bee gut symbionts affect bee development, nutrition, and defense against natural enemies, perturbations of these gut communities may be a factor making bees more susceptible to environmental stressors including poor nutrition and pathogens.

## Methods

More details are provided in SI Appendix.

**Effects of Glyphosate on the Honey Bee Gut Microbiome.** Adult workers with established gut communities were collected from a hive at University of Texas, Austin (UT Austin), marked on the thorax with paint, fed glyphosate (5 or 10 mg/L) or sterile sucrose syrup for 5 d, and returned to the same hive. Fifteen bees from each group were sampled before and 3 d after reintroduction to the hive. This experiment was repeated using bees from a different hive and different year. DNA was extracted from dissected guts and used as template for qPCR analyses. DNA samples from the first experiment were submitted for Illumina sequencing at the Genomic Sequencing and Analysis Facility (GSAF) at UT Austin. Illumina sequence reads were processed using QIIME 1.9.1 (43).

**Effects of Glyphosate on Early Gut Colonization and Susceptibility to *Serratia* Infection.** Hundreds of late-stage pupae were removed from brood frames and allowed to emerge under sterile conditions in laboratory. (Experiment A) NEWs were exposed to bee gut homogenate for 5 d, then hand fed 1 mM glyphosate or sterile sugar syrup on 2 alternate days. Fifteen bees from each group were sampled 2 d after the last hand feeding. DNA was extracted from dissected guts, used as template for qPCR analyses, and submitted for Illumina sequencing at the GSAF, UT Austin. (Experiment B) NEWs were exposed to a bee gut homogenate or sterile sucrose syrup. Each group was divided into two subgroups and treated with 0.1 mM glyphosate or sterile sucrose syrup for 5 d. After that, half of the subgroups was exposed to the opportunistic pathogen *S. marcescens* kz19, whereas the other half was used as controls.



**Fig. 5.** Variation in *S. alvi* strain sensitivity to glyphosate. (A) Growth curves of *S. alvi* wkB2 and wkB339 cultured in InsectaGro media in the presence or absence of 10 mM glyphosate. Experiment was performed in triplicate, and each data point represents the average optical density (600 nm, with SD bars). (B) Boxplots of *S. alvi* wkB2 and wkB339 abundances in bees exposed or not to 0.1 mM glyphosate for 3 d estimated by qPCR. Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. \*\*\* $P < 0.001$ , two-way ANOVA with Tukey's correction for multiple comparisons. (C) Two-way ANOVA for effects of *S. alvi* strain and glyphosate treatment.

Bees were exposed to similar amounts of glyphosate (~1.7 µg) in experiments A and B.

**S. alvi Colonization During Glyphosate Exposure.** NEWs were hand fed 5 µL sucrose syrup containing ~10<sup>5</sup> cells of *S. alvi* wkB2 or wkB339 or sterile sucrose syrup as control. Each group was divided into two subgroups and treated with 0.1 mM glyphosate or sterile sucrose syrup for 3 d immediately following bacterial exposure. Eight bees were sampled from each subgroup at days 1 and 3, and DNA was extracted from dissected guts. *S. alvi*-specific primers (44) were used to amplify total copies of 16S rDNA of each sample by qPCR.

**In Vitro Experiments with Bee Gut Bacterial Strains.** Honey bee and bumble bee gut bacterial strains (SI Appendix, Table S1) were cultured in InsectaGro or MRS broth in the presence or absence of 10 mM glyphosate in a 96-well plate and incubated in a plate reader at 35 °C and 5% CO<sub>2</sub> for 48 h. Optical density was measured at 600 nm every 6 h. Experiments were performed in triplicate.

**Plasmid Construction and Transformation.** The *aroA*, *yhhS*, and *tetC* genes from various bacterial strains were PCR amplified and cloned into the

arabinose-inducible pBAD30 vector (45) by Gibson assembly (46) and then used to transform *E. coli* strain BW25113 or a derivative lacking the *aroA* gene by electroporation. Primer sequences are listed in SI Appendix, Table S2.

**Growth Rate Analysis of Transformed *E. coli*.** Transformed *E. coli* cells were cultured in duplicate in 24-well plates containing M9 minimal medium (47) with appropriate antibiotics, varying concentrations of glyphosate, and varying concentrations of arabinose. The plates were incubated in a plate reader at 37 °C for 24–96 h. Optical density was measured at 600 nm every hour.

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