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The Herbicide Glyphosate Negatively Affects Midgut Bacterial Communities and Survival of Honey Bee during Larvae Reared in Vitro

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Supporting Information

ABSTRACT: Effects of glyphosate on survival, developmental rate, larval weight, and midgut bacterial diversity of *Apis mellifera* were tested in the laboratory. Larvae were reared *in vitro* and fed diet containing glyphosate 0.8, 4, and 20 mg/L. The dependent variables were compared with negative control and positive control (dimethoate 45 mg/L). Brood survival decreased in 4 or 20 mg/L glyphosate treatments but not in 0.8 mg/L, and larval weight decreased in 0.8 or 4 mg/L glyphosate treatments. Exposure to three concentrations did not affect the developmental rate. Furthermore, the intestinal bacterial communities were determined using high-throughput sequencing targeting the V3−V4 regions of the 16S rDNA. All core honey bee intestinal bacterial phyla such as Proteobacteria (30.86%), Firmicutes (13.82%), and Actinobacteria (11.88%) were detected, and significant changes were found in the species diversity and richness in 20 mg/L glyphosate group. Our results suggest that high concentrations of glyphosate are deleterious to immature bees.

KEYWORDS: *glyphosate, chronic exposure, Apis mellifera, larvae, midgut bacterial community*

INTRODUCTION

Honey bees account for at least 80% of total pollinating insects of major crops,[1] and they are important for the balance of natural ecosystems while supplying many bee products. Glyphosate is a broad-spectrum postemergent herbicide for weed control on large-scale crops in the last few decades[2] to become one of the most commonly used agrochemicals worldwide.[3] Glyphosate becomes potentially bioavailable to the foraging bees from pollen, nectar, water, and dusts[4,5] and brought back to the hive. The fact that honey bees are potentially exposed to glyphosate motivates us to test chronic toxicity.

The impacts of glyphosate on honey bees have been evaluated. In caged bees, AChE activity slightly decreased in response to glyphosate.[6] Field-realistic doses of glyphosate reduced short-term memory and impaired more complex forms of associative learning in foragers.[7] Honey bees that had been fed with solution containing 10 mg/L glyphosate spent more time performing homeward flights than control bees.[8] Caged honey bees that were exposed to realistic doses (1.25, 2.50, and 5.0 ng/bee) of glyphosate for 10 days via contaminated syrup decreased in consumption over time, and β-carotene and at-ROH both decreased with increasing doses of glyphosate.[9] Glyphosate mixed with atrazine, cadmium, and iron may affect different reactions occurring in the metabolic pathway of vitamin A in the honey bee.[10]

Honey bee brood (immature bees: eggs, larvae, and pupae) is crucial to colony fitness. Most studies were focused on the effect on adults, but honey bee brood might in fact be exposed to glyphosate in their natural environment through the consumption of contaminated resources or through a direct exposure. Glyphosate toxicity tests poorly considered effects on honey bee brood.[11] An in vitro methodology has been developed for rearing bee larvae.[12] This technique can be used to determine pesticide toxicity to larvae.[13−16]

Honey bee population declines have been attention to potential agents affecting their health including their microbiota.[17] Guts of adult workers contain a distinctive and specialized microbiota, dominated by nine bacterial species clusters each representing a complex of related strains.[18] Three major bacterial classes that are active in the gut (γ-Proteobacteria, Bacilli, and Actinobacteria), all of which are predicted to participate in the breakdown of complex macromolecules, the fermentation of component parts of these macromolecules, and the generation of various fermentation products.[19] Intestinal microbiota can benefit honey bees by helping to digest food, detoxifying harmful molecules, providing essential nutrients, protecting against invasion by pathogens and parasites, neutralizing dietary toxins, and modulating development and immunity, and its balance is linked to health status of the host.[19−24]

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The gut community also differs between castes and may change with the age of the individual and the colony, likely reflecting the effects of host physiology, diet, and the environment in shaping microbiome composition.[25−28] Insect gut bacterial diversity was determined by environmental habitat, diet, developmental stage, and phylogeny of host.[29] Intestinal microbiota abundance and diversity have been used as parameters on which the impact of transgenic plants on honey bees have been tested.[30−34] The level of cell death in the midgut of 400 ppm glyphosate-treated larvae was highest at 69% mortality.[35] Whether glyphosate affects intestinal microbiota abundance and diversity of honey bees needs further analysis.

This study aimed to develop an approach to evaluate potential effects of glyphosate on honey bee brood reared in vitro at realistic exposure rates. We evaluated survival, developmental rate, larval weight, and midgut bacterial communities of in vitro-reared honey bees exposed chronically to varying concentrations of glyphosate as larvae under controlled laboratory conditions. The concentrations (0.8, 4, and 20 mg/L) of glyphosate were based on concentrations recommended for spraying and on those measured in natural environments, from 1.4 to 7.6 mg/L.[2,36] Glyphosate 0.8 and 4 mg/L do not exceed those recommended for aquatic and terrestrial weed control or those measured in natural environments, and 20 mg/L glyphosate is unlikely to be encountered in the field and thus represents a worst case scenario. We used the pesticide dimethoate at a concentration of 45 mg/L as positive control using the same experimental procedures. This is the first study on effects of glyphosate on intestinal bacterial diversity of honey bee based on rearing brood in vitro. This study is important for understanding bacterial transmission, the contribution of both pesticides and intestinal microbiota to honey bee health, the risk assessment of pesticides to honey bee brood, and reduction of pesticide threat to honey bees.

MATERIALS AND METHODS

Glyphosate. Glyphosate (product number P109919−250 mg, purity 99.5%) was purchased from Aladdin, Inc. (Qigang Rd. Fengxian, Shanghai, China).

Honey Bee Rearing Conditions. All honey bees were obtained from the Institute of Apicultural Research apiaries (40°00′28″N, 116°12′18″E), Chinese Academy of Agricultural Sciences, in Beijing during June−August 2017. The colonies were of mixed race, *Apis mellifera*, housed in standard Langstroth-style equipment, and managed per common best management pratices for the region. Honey bee larvae were reared in vitro according to reported methods.[12,15,16] Our discussion of the in vitro timeline corresponds to Schmehl et al.'s[12] Table 3, column 3, where all time points from grafting as day D = 0 or D0 are discussed. Honey bee queens were caged on a wax comb (D-4) for 24 h to lay eggs. At D0 (75 h after the queens were released), the larvae were transferred from the comb to sterile, 48-well tissue culture plates (STCPs) with 20 μL of diet A (royal jelly 44.25%, glucose 5.3%, fructose 5.3%, yeast extract 0.9%, and water 44.25%) prepared in each well. On D2 (48 h after grafting), each larva was fed 20 μL of diet B (royal jelly 42.95%, glucose 6.4%, fructose 6.4%, yeast extract 1.3%, and water 42.95%). On D3, 4, and 5, each larva was fed 30 μL, 40 μL, and 50 μL, respectively, of diet C (royal jelly 50%, glucose 9%, fructose 9%, yeast extract 2%, and water 30%). The larval STCPs were placed horizontally in a larval growth chamber maintained at 94% R.H. and 35 °C. Larvae were transferred from the larval STCP to the prepared pupal STCP when all available diet had been consumed (as early as D6). Pupal STCPs were maintained at 75% R.H. and 35 °C. Adult worker bees began to eclose as soon 18 days after grafting. Emerging adults were collected at least twice daily and were maintained in hoarding cages with ad libitum access to pollen and 50% sugar water solution (w/v).[12]

Experimental Design. Glyphosate was dissolved in water to prepare stock solution, and the solvent accounted for 0.5% of the volume of the final diets. The following treatments were conducted: glyphosate 0.8, 4, and 20 mg/L, negative control and positive control (45 mg/L dimethoate). Four replicates were conducted for each treatment. Larvae tested within each replicate were sourced from a single colony and each replicate was sourced from a different colony.

A surplus of larvae was grafted for each replicate. On D2, a minimum of 16 robust larvae per replicate were randomly selected for each treatment group and fed 20 μL of diet B containing the test solution appropriate to the group's assigned treatment. On D3, 4, and 5, the larvae were fed 30, 40, and 50 μL, respectively, of diet C containing the appropriate test solution.

End Points. Larval survival was noted by viewing larvae under a dissecting microscope at which time spiracular movement (opening/closing) was noted. The individual was considered dead if no spiracular movement was detected. Pupal survival was monitored daily by visual inspection of the pupae. Dead prepupae and pupae were recognized by occasional black or white subdermal necrotic stains or visible wilting. Any larvae or pupae determined to be dead were removed from the plates. Survival rates was assessed each day for each treatment group. Additionally, the developmental rate was calculated for each treatment. Furthermore, larval fresh body weight at D6 was calculated for each larva immediately prior to transfer to the pupal plate. Finally, five new emerged bees were randomly selected for each treatment group (negative control, glyphosate 0.8, 4, and 20 mg/L), and their midguts were isolated on ice using sterile forceps. Five brood frames from different colonies were placed in screened enclosures in a climate controlled room, and new emerged bees from five colonies were collected as hive control. There were not enough individuals of positive control for test midgut bacterial diversity because of high mortality. The total midgut bacterial DNA from each sample was extracted referring to protocols described by Jia et al.[34] The V3−V4 region of the bacterial 16S rRNA gene was targeted with the barcoded primer pair 341*f*/806r for the microbial community diversity analysis. The purified products were sequenced on the Illumina HiSeq 2500 platform. The obtained sequences were normalized to make the samples compared at the same sequencing depth for the following analysis.

Statistical Analysis. Statistical analyses were performed using the SAS 9.2 software program (USA).[37] The survival rate data were tested with a Kaplan−Meier analysis. Furthermore, ANOVAs and Tukey's HSD tests were used to compare developmental rate or larval weight at D6 among the experimental groups. The normalized sequences were classified into operational taxonomic units (OTUs) at 97% similarity using UCLUST (Version 1.2.22). The taxonomy of the OTUs was assigned by blasting against SILVA database 128 with default parameters. Alpha diversity included Chao1 richness estimator, and Shannon-wiener diversity index was performed with Mothur (Version v.1.30). Beta diversity including unweighted unifrac distances between samples was performed with QIIME (Version 1.8.0).

RESULTS

Survival. Negative control average total survival was 92.2%, and positive control (45 mg/L dimethoate) average total survival was 5.0%. There were no statistical differences between survival of larvae fed 0.8 mg/L glyphosate and that of larvae fed the negative control diet ($\chi^2 = 0.5146$, $p = 0.4732$, Figure 1). However, survival of larvae fed 4 mg/L ($\chi^2 = 9.5226$, $p = 0.0020$) or 20 mg/L ($\chi^2 = 6.3739$, $p = 0.0116$) of glyphosate was a statistically significant decrease compared to that of larvae fed the negative control diet. Survival of larvae fed 0.8 mg/L ($\chi^2 = 125.8273$, $p < 0.0001$), 4 mg/L ($\chi^2 = 98.8458$, $p < 0.0001$), or 20 mg/L ($\chi^2 = 90.9743$, $p < 0.0001$)

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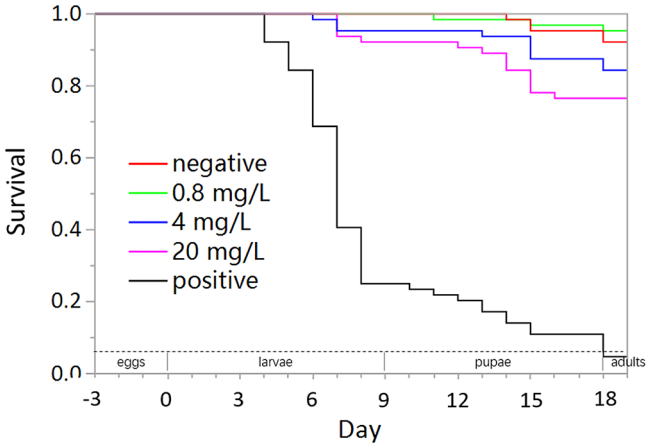

Figure 1. Total survival of honey bees exposed to glyphosate during larval development on D2 thru D5 after grafting (N = 4 replicates of 16 larvae/replicate, or 64 larvae, per test substance). Larvae were fed a dimethoate-contaminated diet (45 mg/L) as a positive control and no contaminated diet as a negative control. D18 on the figures corresponds to D21 from egg laying to adult emergence for the honey bee.

of glyphosate was significantly higher than those fed positive control diet.

Developmental Rate. A one-way ANOVA was conducted to compare the effect of glyphosate on the developmental rate of larvae (F_{239} = 1.89, p = 0.1134), pupae (F_{225} = 1.96, p = 0.1019), and both combined (total) (F_{226} = 0.89, p = 0.4715). Exposure to glyphosate did not affect the larval, pupal, and total developmental rate (Figure S1).

Larval Weight. A one-way ANOVA was conducted to compare the effect of glyphosate on larval weight on D6. Exposure to 0.08 or 4 mg/L glyphosate significantly decreased the larval weight (F_{260} = 14.62, p < 0.0001, Figure 2).

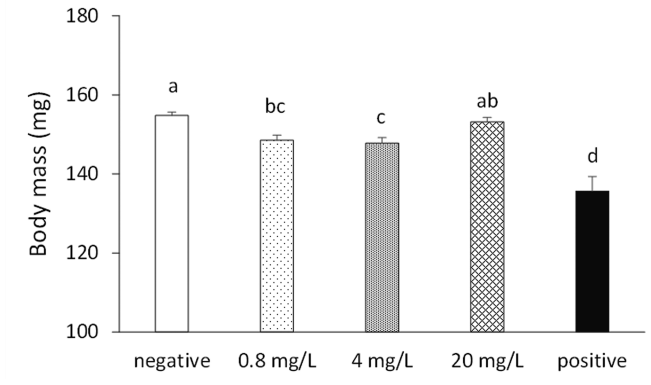

Figure 2. Body weight on D6 of honey bee larvae reared in vitro and exposed to glyphosate in the diet on D2 thru D5 after grafting. Larvae were fed a dimethoate-contaminated diet (45 mg/L) as a positive control or no contaminated diet as a negative control. Bars with the same letter are not different at $\alpha \leq 0.05$.

Analysis of Bacterial 16S rRNA Gene Sequences. Paired-end sequencing of 16S rRNA V3−V4 gene produced a total of 1 207 609 raw reads from 20 samples. Following initial quality trimming, we retained 806 568 reads. Removing suspect or low abundance quality reads yielded 796 378 effective reads in the final analysis. A total of 408 699 high-quality sequences were identified, and the average length of effective sequences reads was 417 bp. The total numbers of OTUs were 8149 (Table S1).

Intestinal Bacterial Community Composition and Diversity. To identify the phylogenetic diversity of midgut bacterial communities in honey bees, all effective reads were classified into different taxonomies according to the QIIME using default settings, and the taxonomic distributions at phylum and class levels were summarized in Figure 3. On the

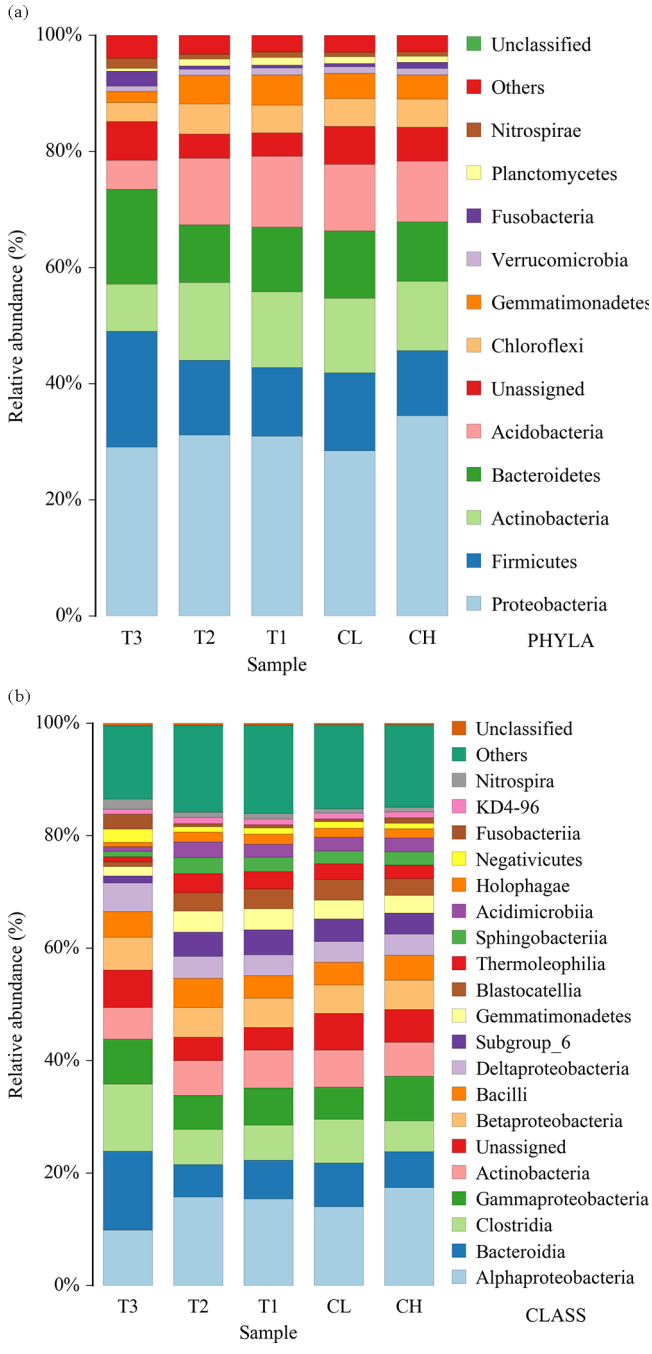

Figure 3. Relative abundance of the dominant midgut bacterial communities in honey bees (*Apis mellifera*) at phylum (A) and class (B) levels. Each bar represents the average relative abundance of each bacterial taxon within a group. CH, hive controls (newly emerged bees from colonies); CL, laboratory controls (negative controls, newly emerged bees reared in vitro); T1, 0.8 mg/L glyphosate; T2, 4 mg/L glyphosate; T3, 20 mg/L glyphosate.

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basis of the average relative abundance, Proteobacteria (30.86%), Firmicutes (13.82%), Actinobacteria (11.88%), Bacteroidetes (11.84%), and Acidobacteria (10.12%) were the most abundant phyla. The classes with the high abundance (>5%) of bacteria were α-Proteobacteria (14.50%), Bacteroidia (8.17%), Clostridia (7.53%), γ-Proteobacteria (6.88%), Actinobacteria (6.22%), and β-Proteobacteria (5.34%).

The difference of the midgut microbial community composition in different treatments was evaluated further using Hierarchical cluster analysis. Hierarchically clustered heat maps of all the abundant genera (relative abundance >1%) are shown in Figure 4. This figure shows that mostly all of the presented bacterial taxa here were clustered together corresponding to different treatments. This figure demonstrates that the midgut bacterial composition of bees fed 20 mg/L glyphosate diet was different with other groups.

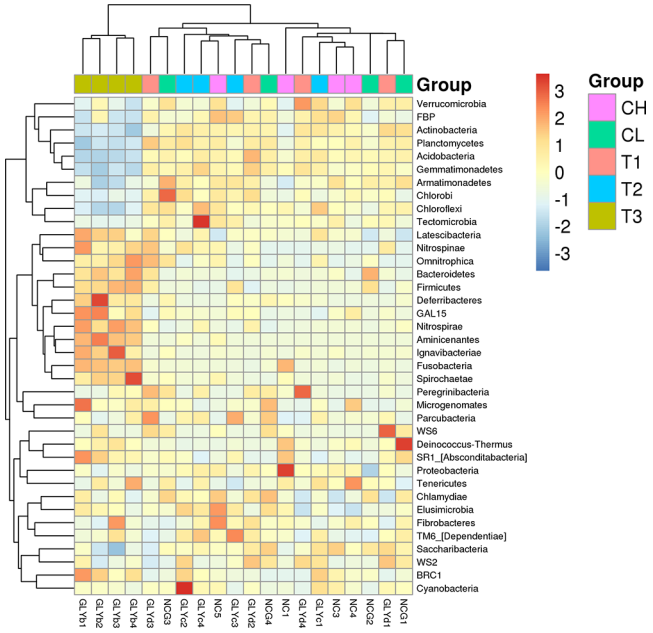

Figure 4. Hierarchically clustered heat map analysis of the highly represented bacterial taxa (at the phylum level) found in the midgut of *Apis mellifera* (relative abundance >1%) across the five treatments. The relative percentages (%) of the bacterial phyla are indicated by varying color intensities according to the legend at the top of the figure. The color key for the *Z* score indicates correspondence between blue−red coloring and standard deviations from the mean abundance of each bacteria. CH, hive controls (newly emerged bees from colonies); CL, laboratory controls (negative controls, newly emerged bees reared in vitro); T1, 0.8 mg/L glyphosate; T2, 4 mg/L glyphosate; T3, 20 mg/L glyphosate.

To determine if honey bee midgut microbiota community structures were altered by the glyphosate, the bacterial diversity was analyzed across different treatments. The bacterial diversity was characterized by calculating the alpha diversity parameters. Two representatively alpha diversity parameters including the Chao index and the Shannon index were selected for community richness comparison. Comparison diversity indexes among groups were compared using a one-way ANOVA with no significant differences in Chao index (F_{19} = 1.37, p = 0.2922) and Shannon index (F_{19} = 0.27, p = 0.8930) noticed across the five treatment groups (Figure 5).

For a better analysis of the relationships between gut microbiota community structures of the honey bees across five treatments, the beta diversity was performed via principal component analysis (PCA) and principal coordinate analysis (PCoA) of unweighted unifrac distances (Figure 6). The midgut microbiota of bees fed 20 mg/L glyphosate diet were distinguishable with other groups (Figure 6). There was a statistically significant decrease in the beta diversity fed 20 mg/L glyphosate diet compared to those fed hive control or laboratory control diet (Figure 7).

To identify specific taxa in the midgut microbiota that were affected by the glyphosate, we performed a linear discriminant analysis effect size (LEFSe) analysis that revealed changes in the abundance of bacterial taxa accounted for the observed differences in the midgut microbiota (Figure 8). Statistical analysis using LEFSe analysis showed the relative abundances of Acidobacteria (LDA = 4.6695 and P = 0.0442) and Gemmatimonadaceae (LDA = 4.0403 and P = 0.0118) in glyphosate 0.8 mg/L samples were all significantly higher than those of other honey bee groups. The relative abundances of Sphingobacteriales (LDA = 4.0238 and P = 0.0168), Thermoleophilia (LDA = 4.0994 and P = 0.0115), and Acidimicrobiales (LDA = 4.0220 and P = 0.0258) in glyphosate 4 mg/L samples were all significantly higher than those of other honey bee groups. The relative abundances of Gammaproteobacteria (LDA = 4.0554 and P = 0.0156), Lachnospiraceae (LDA = 4.2309 and P = 0.0363), Prevotellaceae (LDA = 4.2261 and P = 0.0133), and Ruminococcaceae (LDA = 4.1251 and P = 0.0261) in glyphosate 20 mg/L samples were all significantly higher than those of other honey bee groups.

DISCUSSION

Honey bee larvae may be exposed to glyphosate orally given their diets contain pollen and honey. On the basis of the concentrations recommended for spraying and on those

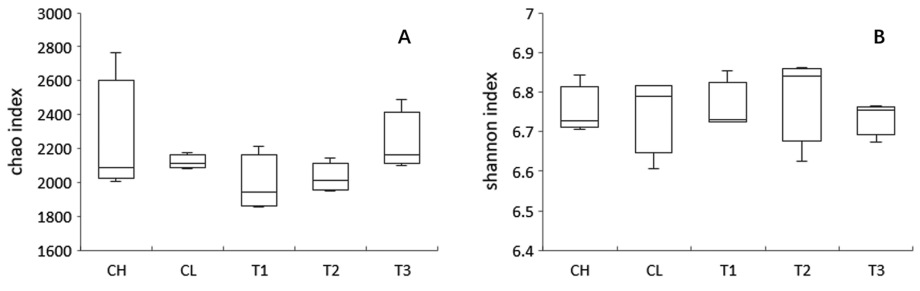

Figure 5. Alpha diversity in honey bee midgut bacteria with respect to glyphosate. The amount of bacterial diversity was determined by comparing Chao index (A) and Shannon index (B).

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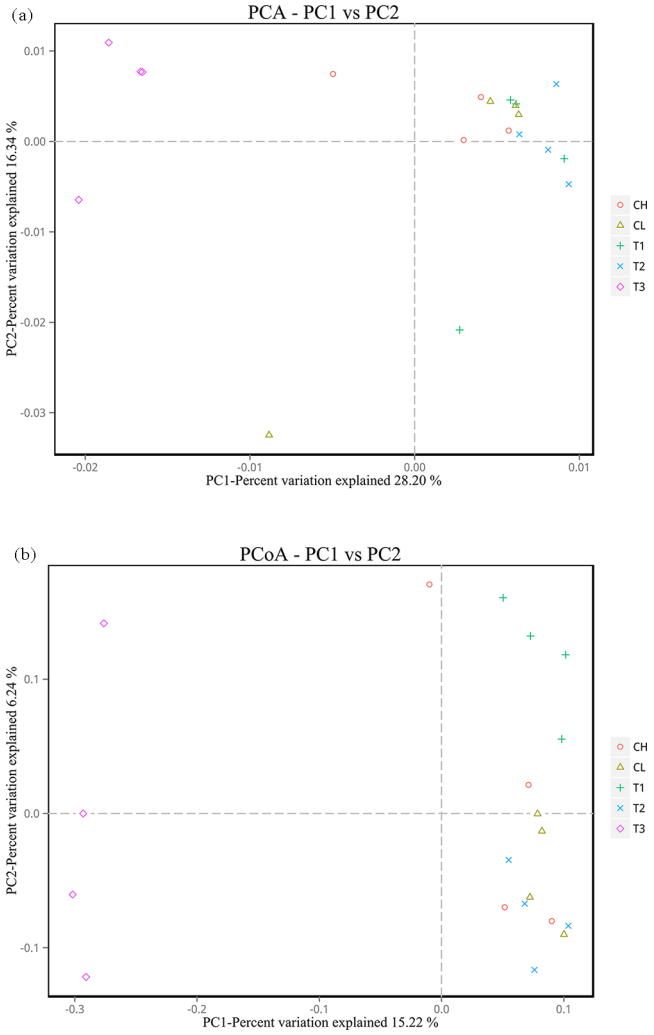

Figure 6. Distances among communities of *Apis mellifera* with different exposure treatments, represented as PCA (A) and PCoA (B) plots of jackknifed unweighted UniFrac distances. Positions of the bacterial communities for each sampled niche along the first three principal coordinate axes are illustrated, along with the percentage of variation explained by each axis. CH, hive controls (newly emerged bees from colonies); CL, laboratory controls (negative controls, newly emerged bees reared in vitro); T1, 0.8 mg/L glyphosate; T2, 4 mg/L glyphosate; T3, 20 mg/L glyphosate.

measured in natural environments,[2,36] we investigated the effects of glyphosate to honey bee larvae reared in vitro.

Our results showed that brood survival was significantly lower for individuals fed diet with 4 mg/L and 20 mg/L glyphosate than for ones fed the negative control diet, and exposure to 0.08 or 4 mg/L glyphosate significantly decreased the larval weight. In contrast, a previous work found no effects of glyphosate on brood survival or mean pupal mass in a realistic exposure scenario.[11] The residues of Roundup (glyphosate at 35 mg/L) reported no effects lethal to honey bees.[13] No adverse effects on brood developmental rate were observed in any of the glyphosate treated larvae. This confirms the previous work on glyphosate that found no effects on brood development in a realistic exposure scenario.[11] Peak glyphosate residues after application at 2.88 kg/ha were 31.3 mg/kg nectar and 574 mg/kg pollen at the first 3 days of exposure, and glyphosate demonstrated rapid decline to 2.78 mg/kg in nectar and 87.2 mg/kg in pollen at day 7.[11] Pollen

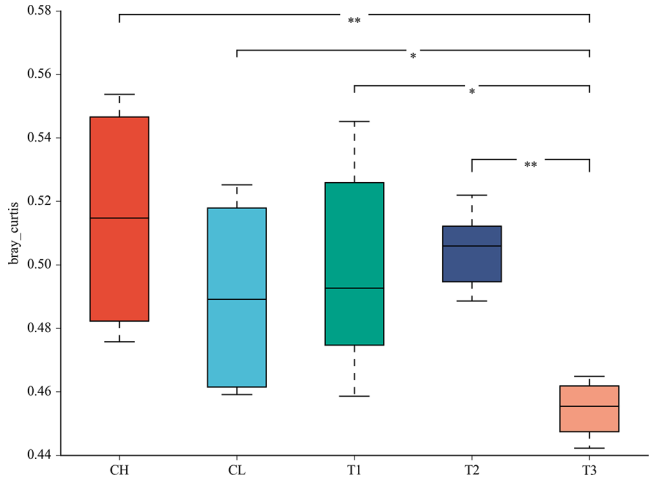

Figure 7. Box plot of bray curtis distances of the midgut microbiota among five treatments. CH, hive controls (newly emerged bees from colonies); CL, laboratory controls (negative controls, newly emerged bees reared in vitro); T1, 0.8 mg/L glyphosate; T2, 4 mg/L glyphosate; T3, 20 mg/L glyphosate. ∗ indicates significance at the 0.05 level, and ∗∗ indicates significance at the 0.01 level.

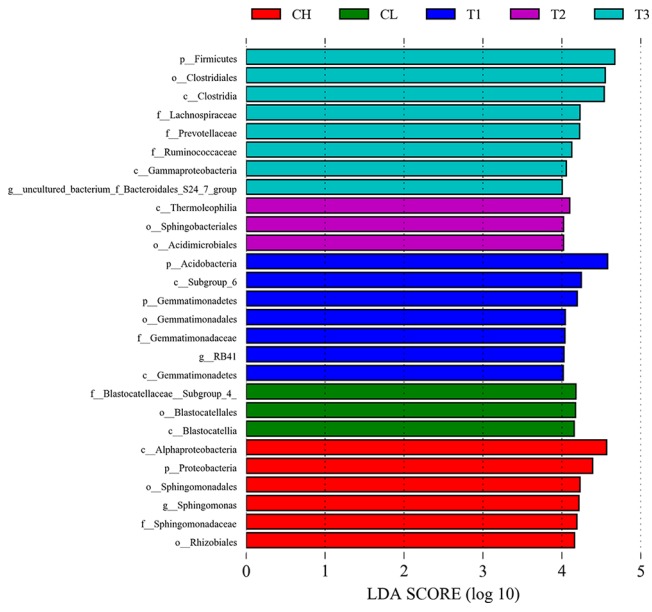

Figure 8. LEFSe analysis illustrating differentially abundant bacteria among samples with different haze levels. LDA scores (bacteria that obtain a log LDA score of >4 are ultimately considered) can be interpreted as the degree of consistent difference in relative abundance among five treatments. CH, hive controls (newly emerged bees from colonies); CL , laboratory controls (negative controls, newly emerged bees reared in vitro); T1, 0.8 mg/L glyphosate; T2, 4 mg/L glyphosate; T3, 20 mg/L glyphosate.

and honey/nectar represent only a small part of larval diet. It seems unlikely that the glyphosate levels found in brood food will approach the maximum residues found in pollen or nectar/honey under normal environmental conditions.

The social lifestyle of honey bees provides opportunities for vertical transmission and horizontal transmission.[38,39] This could enhance bacterial transfer between nest mates and offer opportunities for direct transfer of symbionts from one generation to the next,[39] though opportunities are rather limited.[21] For successful transmission between generations, gut

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microbes can take advantage of host behavior or possess genes that permit transient survival between generations.[40,41] Transmission may be multimodal as there are many fringe environments and interaction networks within the hive and colony that may support bacteria.[25,41,42] Many bacteria associated with the honey bee may be transmitted through nurses feeding beebread,[25] pollen,[43,44] or royal jelly[44−46] to the larvae by oral trophallaxis, through a fecal route,[25] or via the pollination environment.[47] Newly emerged honey bees are colonized by bacteria following adult emergence, attaining their gut bacteria from older workers, the hive environment, or a combination of the two.[25,26,41,48]

Newly emerged adult bees have few or no gut bacteria and are colonized by the normal gut community within the first few days of adult life before leaving the hive.[25,48,49] However, Hroncova et al.[49] reported a significant decrease of bacterial counts in pupae of *A. mellifera* but not a complete sterile gut. Combination of culture-dependent and culture-independent studies indicates that larvae contain taxonomically similar bacterial communities found in adults.[44,45] All of the characteristic bacteria found at low levels in larval guts may survive within brood cells following larval defecation. In this study, honey bee brood are reared in sterile plates containing artificial diet with fructose, glucose, royal jelly, yeast, and water in laboratory. Rearing larvae in vitro exclude many potential routes such as fresh pollen and nectar, bee bread, hive environment, interaction with older worker cohorts, and pollination environment. In rearing honey bee brood in vitro, bacterial transmission routes may be from queen to eggs, nurse feeding day 1 larvae by oral trophallaxis before grafting, comb containing fecal, or royal jelly in artificial diet.

The artificial diet environment does not affect bacterial establishment in newly emerged bees. Honey bee gut communities are dominated by phylum Proteobacteria, Firmicutes, and Actinobacteria.[18,23,50,51] Though the abundances of bacteria were inconsistent with the previous work,[34] our results found all these dominated classes including α-Proteobacteria (14.50%), Bacteroidia (8.17%), Clostridia (7.53%), γ-Proteobacteria (6.88%), Actinobacteria (6.22%), β-Proteobacteria (5.34%), and Bacilli (4.46%). Glyphosate 20 mg/L significantly decreased the intestinal bacterial diversity of honey bees compared with glyphosate 0.8 mg/L, 4 mg/L, hive control, or laboratory control. p_Firmicutes, c_Clostridia, c_γ-proteobacteria, o_Clostridiales, f_Lachnospiraceae, f_Prevotellaceae, and f_Ruminococcaceae increased in 20 mg/L glyphosate samples.

In conclusion, under our experimental conditions, our work showed that glyphosate 4 mg/L and 20 mg/L chronic exposure decreased brood survival, and glyphosate 20 mg/L affected gut bacterial communities of newly emerged bees. There is little information in the literature about the residue levels of glyphosate in brood food in general. However, high concentrations of glyphosate are in fact deleterious to developing honey bees, and our data do not preclude any possible synergisms when they co-occur in bee colonies. Our results are important for understanding bacterial transmission, the contribution of both pesticides and intestinal microbiota to honey bee health, and the risk assessment of pesticides to honey bee brood.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b02212.

OTUs and sequence read number; larval development rate, pupal development rate, and both combined (total) rate of honey bees reared in vitro and exposed to exposed to glyphosate in diet on D2 thru D5 after grafting (PDF)

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Notes

The authors declare no competing financial interest.

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