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Disinfectant dodecyl dimethyl benzyl ammonium chloride (DDBAC) disrupts gut microbiota, phospholipids, and calcium signaling in honeybees (Apis mellifera) at an environmentally relevant level

Qiangqiang Li\textsuperscript{a,1}, Xiaofeng Xue\textsuperscript{a,1}, Suzhen Qi\textsuperscript{a}, Liuwei Zhao\textsuperscript{a}, Wenwen Zhang\textsuperscript{b}, Man Fan\textsuperscript{a}, Liming Wu\textsuperscript{a,}\textsuperscript{*,} Miao Wang\textsuperscript{a,}\textsuperscript{*}  
\textsuperscript{a} Institute of Apicultural Research, Chinese Academy of Agricultural Sciences (CAAS), Beijing 100093, China  
\textsuperscript{b} College of Life and Health Sciences, Anhui Science and Technology University, Bengbu 233100, China

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One of the impacts of the Coronavirus disease 2019 (COVID-19) pandemic has been a profound increase in the application amounts of disinfectants. Dodecyl dimethyl benzyl ammonium chloride (DDBAC) is a widely used disinfectant, yet its hazards to non-target species remain largely unknown. We are unaware of any studies assessing DDBAC’s impacts on honeybee, a pollinator species that is a useful indicator of environmental pollution essential for many forms of agricultural production. Here, we assessed the potentially negative effects of DDBAC on honeybees. After conducting a formal toxicity evaluation of DDBAC on honeybee mortality, we detected an accumulation of DDBAC in the honeybee midgut. We subsequently studied the midgut tissues of honeybees exposed to sub-lethal concentrations of DDBAC: histopathological examination revealed damage to midgut tissue upon DDBAC exposure, microbiome analysis showed a decreased abundance of beneficial midgut microbiota, lipidomics analysis revealed a significant reduction in cell membrane phospholipids with known functions in signal transduction, and a transcriptome analysis detected altered expression of genes involved in calcium signaling pathways (that variously function in calcium absorption, muscle contraction, and neurotransmission). Thus, our study establishes that DDBAC impacts honeybee midgut functions at multiple levels. Our study represents an early warning about the hazards of DDBAC and appeals for the proper stewardship of DDBAC to ensure the protection of our ecological environment.

1. Introduction

Quaternary ammonium compounds (QACs) have been widely used as disinfectants for decades, and their use has recently undergone a surge owing to the ongoing coronavirus disease 2019 (COVID-19) pandemic (Li et al., 2020; Wang et al., 2020). Benzalkonium chloride compounds (BACs) are one class of QACs used as cationic surfactants (Zhang et al., 2015). Dodecyl dimethyl benzyl ammonium chloride (DDBAC) is a widely used disinfectant, yet its hazards to non-target species remain largely unknown. We are unaware of any studies assessing DDBAC’s impacts on honeybee, a pollinator species that is a useful indicator of environmental pollution essential for many forms of agricultural production. Here, we assessed the potentially negative effects of DDBAC on honeybees. After conducting a formal toxicity evaluation of DDBAC on honeybee mortality, we detected an accumulation of DDBAC in the honeybee midgut. We subsequently studied the midgut tissues of honeybees exposed to sub-lethal concentrations of DDBAC: histopathological examination revealed damage to midgut tissue upon DDBAC exposure, microbiome analysis showed a decreased abundance of beneficial midgut microbiota, lipidomics analysis revealed a significant reduction in cell membrane phospholipids with known functions in signal transduction, and a transcriptome analysis detected altered expression of genes involved in calcium signaling pathways (that variously function in calcium absorption, muscle contraction, and neurotransmission). Thus, our study establishes that DDBAC impacts honeybee midgut functions at multiple levels. Our study represents an early warning about the hazards of DDBAC and appeals for the proper stewardship of DDBAC to ensure the protection of our ecological environment.
DDBAC (C9H18NCl) is one of the most commonly used BACs, serving as an active ingredient in commercial sanitizers, disinfectants, and phase transfer agents (Tezel and Pavlostathis, 2009). However, its widespread use has led to DDBAC residue contamination of wastewater and even surface water worldwide (Ferrer and Furlong, 2001; Martínez-Carballo et al., 2007). Its residual concentration has been detected up to 6.5 mg/L in 2020 China (Wang et al., 2020). DDBAC contamination in the environment is also known to circulate among water, soil, animals, plants, and microorganisms, which can be toxic to organisms and threaten human health (Arrebola-Liébanas et al., 2014).

DDBAC has been assigned as a Category III antiseptic active ingredient by the Food and Drug Administration (FDA, USA), indicating that available data are insufficient to classify it as safe and effective and that further testing is required (Food and Drug Administration, 2016). The antimicrobial effect of DDBAC is predominantly attributable to its destruction of the lipid bilayer of bacterial cell membranes, interference with the activities of membrane-bound enzymes, and dissipation of the proton motive force (Tezel and Pavlostathis, 2011; Gravel et al., 2017). Prolonged use of topical medications containing DDBAC has also been reported to induce the disruption of human corneal cell membranes, cause impairment of epithelial barrier functions, and increase lyso- phospholipids, while also decreasing phospholipids in human epithelial corneal cells (Chang et al., 2000; Georgiev et al., 2011; Magny et al., 2020). Further, long-term DDBAC exposure can cause neurotoxic and genotoxic effects including DNA damage and cell division inhibition in invertebrate, cladoceran, mammalian, and plant cells (Ferk et al., 2007; Lavorgna et al., 2016; Sreevidya et al., 2018). Given these indications that DDBAC exposure can deleteriously affect eukaryotic cells, analysis of the hazards of DDBAC in the environment is warranted.

Honeybee (Apis mellifera) has become a widely examined indicator species for researching environmental pollution, owing to its environmental dependence such as a need of over 7 km² foraging area for nectar, pollen, and water (Badiou-Bénéteau et al., 2013). Increases in environmental pollutants are currently threatening honeybee survival, with the collapse of honeybee colonies causing significant agricultural losses and ecological destruction (Hung et al., 2018). Since the survival of honeybees depends on gathering water, plant nectar, and pollen, DDBAC contamination in soil and water (or its migration to plants), and even the abuse of DDBAC-containing beekeeping disinfectants, bring potential hazards to honeybees. Thus, assessing the potential hazards of DDBAC exposure to honeybees can provide an early warning for the health effects of DDBAC on honeybee colonies, and may support development of guidelines for the proper stewardship of DDBAC to ensure protection of our ecology and agriculture.

In the present study, we performed toxicity tests to explore how lethal and sublethal concentrations of DDBAC affect adult honeybees. The in situ distribution of DDBAC in the honeybee midgut was determined using MALDI-MS imaging analysis, and the impacts of DDBAC on honeybee midgut tissue were assessed by histopathological examination. Furthermore, the impacts of DDBAC exposure on the microbial community, phospholipid composition, and signaling transduction of the honeybee midgut were investigated through microbiome, lipidomics, and transcriptome analyses.

2. Materials and methods

2.1. Chemicals and reagents

DDBAC (≥99 %) was purchased from Sigma-Aldrich LLC. (USA). A series of concentrations of DDBAC including 1 mg/L, 10 mg/L, 100 mg/L, 1 g/L, 5 g/L, 10 g/L, and 50 g/L, were prepared in 50 % sucrose solution. Trifluoroacetic acid (TFA, HPLC grade, ≥99 %) and 2-mercaptobenzothiazole (2-MBT, ≥98 %) were purchased from Sigma-Aldrich LLC. (USA) and Yuanye Bio-Technology Co., Ltd. (Shanghai, China), respectively. 2-MBT was prepared at a concentration of 12 mg/mL in 80:20 (v/v) methanol:water, containing 0.2 % TFA, for matrix coating before DDBAC detection by MALDI-MS imaging analysis. Chloroform, acetonitrile, methanol, isopropanol, ammonium acetate, and ammonium fluoride (HPLC grade, ≥99 %) were purchased from Fisher Scientific Inc. (USA) for lipidomics analysis.

2.2. Obtain newly emerged adult honeybees in colony

Six honeybee (Apis mellifera L.) colonies were maintained at a suburban apiary in Beijing, China. Each colony (containing one sister queen and three frames) was maintained separately to avoid interference. In the beginning, a marked empty frame was added to each colony to support monitoring of egg laying. After 21 days, the newly emerged honeybees were collected from each marked frame to rear in the lab for acute and chronic toxicity tests.

2.3. Acute and chronic toxicity tests

The newly-emerged-honeybees (<24 h old) collected from colony were reared in the lab for acute and chronic toxicity tests. Thirty honeybees were collected in one plastic transparent container (10 cm × 10 cm × 6 cm) and cultured in an incubator (30 ± 2 °C, 50 ± 10 % relative humidity, darkness). A 50 % sucrose solution containing a series of concentrations of DDBAC was provided as food for the toxicity tests on honeybees as described (Zhu et al., 2020; Qi et al., 2020).

For acute toxicity tests, 2 mL of 50 % sucrose solution containing different concentrations (1, 5, 10, and 50 g/L) of DDBAC were provided as food in each cage continuously for 48 h. A sucrose solution with no DDBAC served as a control. For each treatment group, three replicates with thirty honeybees in each replicate were assessed. The test solutions were prepared fresh and renewed daily. The mortality of honeybees was calculated every 24 h.

For chronic toxicity tests, 2 mL of 50 % sucrose solution containing different concentrations (1, 10, and 100 mg/L) of DDBAC were provided as food in each cage continuously for 14 days. A sucrose solution with no DDBAC served as a control. For each treatment group, three replicates of thirty honeybees each were assessed. The test solutions were prepared fresh and renewed daily. The mortality of honeybees was calculated every 24 h.

After chronic toxicity tests, the surviving honeybees exposed to different concentrations of DDBAC (1, 10, and 100 mg/L) for 14 days were sampled for further investigation, including MALDI-MS analysis, histopathology examination, and gut microbiota characterization.

2.4. Dissection of honeybee midgut

We dissected the honeybee midgut following previously established methods (Wang et al., 2021). Briefly, the honeybees were anesthetized at ~80 °C for 5 min before dissection. Then a pair of forceps was used to pick up the thorax, while another pair of forceps was used to pick up the stinger positioned at the bottom of the abdomen. By carefully pulling forwards from the stinger, the complete gut was extracted. The midgut was cut away from the whole gut, then the midgut contents were squeezed out into a 1.5 mL sterile centrifuge tube, immediately frozen in liquid nitrogen, and stored at ~80 °C for gut microbiota characterization. The midgut tissue was washed with PBS buffer solution (pH 7.4, 0.1 mol/L), quickly frozen on ice, and then transferred to ~20 °C for storage. For the lipidomics and transcriptome analysis, the dissected midguts were immediately frozen in liquid nitrogen and stored at ~80 °C.

2.5. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) imaging analysis for DDBAC in situ detection in honeybee midgut

MALDI-MS imaging analysis was used to investigate the in situ distribution of DDBAC in the honeybee midgut. After chronic toxicity assays, the surviving honeybees exposed to three sublethal concentrations
of DDBAC (1, 10, and 100 mg/L) for 14 days were sampled, and three biological replicates per group were set for MALDI-MS imaging analysis. The dissected honeybee midgut tissues were cryo-sectioned vertically at biological replicates per group were set for MALDI-MS imaging analysis.

The dissected honeybee midgut tissues were cryo-sectioned vertically at −20 °C into 12 μm thick slices using a Leica CM1860 cryostat (Leica Microsystems Inc., Wetzlar, Germany). The tissue slices were then immediately thaw-mounted on conductive sides of indium tin oxide (ITO)-coated microscope glass slides (Bruker Daltonics, Wetzlar, Germany) and then coated with 2-mercapto-benzothiazole (2-MBT) using a Bruker Daltonics ImagePrep electronic matrix sprayer (Bremen, Germany) for DDBAC detection (Norrish and Caprioli, 2013). All MS data were acquired and recorded by an Autoflex Speed MALDI/TOF mass spectrometer (Bruker Daltonics, Billerica, MA). The instrument was equipped with a MALDI ion source using a 355 nm, 2000 Hz solid-state Smartbeam Nd: YAG UV laser (Azura Laser AG, Berlin, Germany). Mass spectra were acquired over the mass range from 200 to 500 Da for DDBAC detection. The MALDI-MS profiling and imaging data were viewed and processed by Bruker FlexAnalysis 3.4 and FlexImaging 4.1 software.

2.6. Histopathology examination of honeybee midgut tissue

Samples were collected from the surviving honeybees exposed to different concentrations (1, 10, and 100 mg/L) of DDBAC for 14 days after the chronic toxicity test. The dissected honeybee midgut was washed with a PBS buffer solution (pH 7.4, 0.1 mol/L) and then fixed with 4 % paraformaldehyde in the PBS buffer solution overnight for a histopathology examination. The fixed tissues were dehydrated in an ethanol series and cleared in xylene before being embedded in liquid paraffin. Tissues were then sliced transversely and stained with hematoxylin-eosin (HE). The stained slides were imaged with a light microscope (Nikon Eclipse Ci, Japan).

2.7. Characterizing the gut microbiota

Following the chronic toxicity testing, survival honeybees exposed by different concentrations (1, 10, and 100 mg/L) of DDBAC for 14 days were sampled and dissected to obtain midgut contents. Midgut contents from six adult honeybees of each treatment group (described in 2.4) were pooled together as a biological sample, and five replicates per treatment group were assessed. Genomic DNA was extracted using an E.Z.N.A.® DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer’s instructions. The extracted DNA was amplified for the V3-V4 high variant region of the 16S rRNA gene using primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGAC-TACHVGGWTCAAT-3′). Sequencing was performed using the Illumina MiSeq PE300 platform (Illumina, San Diego, USA). The effective tags were then clustered using the UPARSE algorithm into Operational Taxonomic Units (OTUs), based on a 97 % similarity cutoff. The taxonomic information was annotated against the Silva 16S rRNA database by the RDP classifier algorithm with a 70 % confidence threshold. The numbers of sequences for each classification level were used to compare the abundance and diversity of gut microbiota among different groups to get the specific bacteria with significant changes.

2.8. Exposure of honeybee colonies to DDBAC

Six honeybee (Apis mellifera L.) colonies were maintained at a suburban apiary in Beijing, China. Each colony (containing one sister queen and three frames) was maintained separately to avoid interference. Six colonies were randomly divided into two groups (control and DDBAC) with each group containing three colonies (biological replicates). Initially, a marked empty frame was added to each colony to support monitoring of egg laying. After 21 days, the newly emerged honeybees were collected from each marked frame and tagged on their thorax with different colors (each colony was marked with one color). All the marked honeybees were then re-introduced to their colony.

Considering that 10 mg/L DDBAC is the environmentally relevant dose in China (Wang et al., 2020), and the results of our chronic toxicity test showing the toxic effects of 10 mg/L DDBAC on the honeybee, we chose 10 mg/L as the concentration at which to evaluate DDBAC exposure on honeybee colonies to mimic field conditions. As DDBAC is widely used and ubiquitous in the environment and even abused as a beekeeping disinfectant to spray on honeybee colonies, DDBAC exposure to honeybee colonies is diverse and complicated. Thus, we exposed the honeybee colonies to DDBAC by spraying so as to mimic actual conditions to comprehensively evaluate the exposure effects. We began treatment the next day by evenly spraying the DDBAC group colonies with a 50 % sucrose solution containing 10 mg/L DDBAC, while the control group was sprayed with the same amount of 50 % sucrose solution without DDBAC. The treatment was performed every other day with an equal amount of spray applied to each colony (5 mL for each side of each frame).

The labeled honeybees in each colony were collected on day 6 and day 18. Within a colony, there is an age-related division of labor among adult worker honeybees: a honeybee works as a nurse at 6 days after eclosion and switches to working as a forager at around 18 days after eclosion. The adult honeybees in these two stages (nurse and forager) are significantly different in physiology and behavior (Graham, 2008). We chose these two time points to characterize the potentially distinct responses of relatively young (nurse) and relatively old (forager) adult honeybees to DDBAC exposure. Samples without DDBAC exposure were collected at 6 and 18 days after eclosion and labeled as CK6 and CK18, respectively. Samples under 10 mg/L DDBAC exposure for 6 and 18 days were labeled as D6 and D18, respectively. These methods were used to collect samples for lipidomics and transcriptome analyses.

2.9. UPLC-Q-TOF-MS/MS-based lipid detection

2.9.1. Lipid extraction

The honeybees sampled from DDBAC-treated colonies (described in 2.8) were dissected to collect midguts. Six adult honeybee midguts from one colony were pooled together as a biological sample. Three honeybee colonies from each group were prepared into three biological samples. A 50 μg sample was added to 100 μL water, vortexed for 5 min, and then mixed with 400 μL chloroform and methanol solution (2:1, vol/vol), and vortexed for 5 min. After centrifugation at 3000 rpm for 15 min at 4 °C, the lipid-containing subnatant was transferred and dried using nitrogen. Finally, dried lipid extracts were redissolved in a chloroform and methanol solution (2:1, vol/vol) and then analyzed by LC-MS/MS.

2.9.2. UPLC-Q-TOF-MS/MS analysis

An Agilent UPLC-Q-TOF-MS/MS system equipped with an Agilent InfinityLab Poroshell 120 EC-C18 column (3.0 × 100 mm, 2.7 μm) was employed. A gradient elution program was used for sample separation. Mobile phase A was 10 mM ammonium acetate, 0.2 mM ammonium fluoride in 9:1 water/methanol; mobile phase B was 10 mM ammonium acetate, 0.2 mM ammonium fluoride in 2:3:5 acetonitrile/methanol/isopropanol. The flow rate was set to 0.4 mL/min. The MS parameters were set as follows: drying gas temperature, 250 °C; drying gas flow rate, 11 L/min; sheath gas temperature, 300 °C; sheath gas flow rate, 12 L/min; nebulizer pressure, 35 psi; VCap, 3000 V; fragmentor voltage, 160 V; collision energy, 20 V; mass ranges, 100–1700 m/z, acquired in negative ionization mode. Mass accuracy was calibrated using reference ions 112.985597 and 1033.988109. MassHunter Lipid Annotator 1.0 software was used for lipid identification with a combination of Bayesian scoring, a probability density algorithm, and non-negative least squares fit to search a theoretical lipid library (modified Lipid-Blot) developed for LC/MS by Kind et al. (2013).

2.10. RNA-seq analysis

The honeybee midgut samples from colony (described in 2.8) were
dissected and collected for RNA-seq analysis. Six honeybee midguts from one colony were pooled together as a single biological sample. Three biological samples were set up using samples from different colonies in one treatment group. Total RNA from each sample was extracted using TRIzol (15596018, Invitrogen). RNA quality was determined by 2100 Bioanalyzer (Agilent) and quantified using an ND-2000 instrument (NanoDrop Technologies). Sequencing libraries were constructed with a TruSeqTM RNA sample preparation Kit from Illumina (San Diego, CA). The paired-end RNA-seq sequencing libraries were sequenced with an Illumina HiSeq xten/NovaSeq 6000 sequencer (2 × 150 bp read length).

Fig. 1. The toxic effects of DDBAC on adult honeybees. (A) Acute toxicity test showing mortality of adult honeybees after DDBAC exposure at the indicated concentrations at 24 h and 48 h. (B) Survival of adult honeybees exposed to the indicated sublethal concentrations of DDBAC during a 2-week exposure window. (C) In situ distribution of DDBAC molecule in honeybee midgut after DDBAC exposure at the indicated concentrations for 14 days, assessed by MALDI-MS imaging analysis. (D) Profile signals of MALDI-MS imaging analysis showing the accumulation of DDBAC in honeybee midgut after DDBAC exposure at the indicated concentrations for 14 days. (E) Midgut pathology results for honeybees after DDBAC exposure at the indicated concentrations for 14 days; midgut cells were stained by hematoxylin & eosin.
Differential expression analysis was performed using the DESeq2 R package (1.16.1). Genes with an adjusted p-value < 0.05 found by DESeq2 were deemed as differentially expressed. Gene Ontology (GO) functional enrichment and KEGG pathway analyses were carried out using Goatools (https://github.com/tanghaibao/Goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do), respectively. The GO term and KEGG pathways were considered to be significantly enriched when the adjusted P value (P adjust) was < 0.05 using Fisher exact test.

2.11. Statistical analysis

T-test and ANOVA analyses were conducted at a 95 % confidence level using SPSS 21.0 software. Heat-map analysis was conducted using GraphPad Prism 8 software. Alpha-diversity analysis (including Ace, Chao, Shannon, and Simpson index analysis), partial least squares discriminant analysis (PLS-DA), a Kruskal-Wallis H-test, and linear discriminant analysis (LDA) effect size (LEfSe) analysis were performed using the bioinformatics tools on the free online Majorbio i-Sanger cloud platform (https://en.majorbio.com).

3. Results

3.1. The acute and chronic toxicity of DDBAC exposure to adult honeybees

We conducted acute and chronic toxicity tests to assess the hazards of DDBAC to the survival of honeybee. In the acute toxicity test, the mortality of adult honeybees was monitored after DDBAC exposure for 24 h and 48 h. Mortality increased along with the increases in DDBAC exposure time and concentration (Fig. 1A). A 1 g/L DDBAC exposure was considered to be a lethal concentration 50 (LC50) at 48 h, with a 95 % confidence interval (CI, 0.4–2.3 g/L, Y = 1.08 + 1.20 * Log (X), R2 = 0.901).

We subsequently used sublethal DDBAC concentrations (100 mg/L, 10 mg/L, and 1 mg/L) based on 1/10, 1/100, and 1/1000 of the LC50 value for 14-day chronic toxicity tests. The survival rate of honeybees in the vehicle control group was above 95 % (Fig. 1B). There were no significant differences in the survival rates between the vehicle control group and the groups exposed to 1 mg/L or 10 mg/L DDBAC (Fig. 1B). For the 100 mg/L DDBAC-exposure group, the survival rate decreased significantly compared to the control group from day 9 to day 14 (p-value < 0.0001, Fig. 1B). Thus, honeybee mortality caused by DDBAC depends on the concentration and duration of exposure.

3.2. DDBAC accumulation detection and histopathological examination of honeybee midgut

Given that honeybees would be exposed to DDBAC by ingesting food and water, we focused on the midgut as the priority tissue for examining the potentially deleterious effects of DDBAC on honeybees. We used MALDI-MS imaging analysis to evaluate the in situ distribution of DDBAC in the midgut samples of the adult honeybee with 14-day exposure to DDBAC at sublethal concentrations (1, 10, and 100 mg/L DDBAC). DDBAC was not detected in the control samples but was observed present in the midguts of DDBAC-exposed honeybees (Fig. 1C and D). The accumulation level of DDBAC in honeybee midgut increased with the elevation of exposure concentration of DDBAC (Fig. 1C and D). DDBAC molecules notably accumulated in the middle of the longitudinal section, where the inner epithelial layer of the honeybee midgut is positioned; the DDBAC abundance decreased towards the outer muscle layer of the midgut (along the edge of the longitudinal section) (Fig. 1C). These findings indicate that DDBAC can be retained and absorbed in the midgut upon DDBAC exposure.

We then performed a histopathology examination of hematoxylin-eosin-stained honeybee midgut tissue sections to assess whether DDBAC exposure affects gut integrity. The epithelium of midgut was arranged closely and orderly in the control samples. The effects of DDBAC were not obvious in the 1 mg/L-DDBAC-exposure samples. However, the epithelium of honeybee midgut became thinner and distorted under 10 and 100 mg/L DDBAC exposure. These results indicated that DDBAC exposure disrupts the honeybee midgut epithelium.

3.3. The impacts of DDBAC exposure on microbial composition of honeybee midgut

To investigate the impacts of DDBAC exposure on honeybee gut microbiota, we used 16s rRNA sequencing (Illumina MiSeq PE300 platform) on 20 samples harvested from adult honeybees exposed to 100 mg/L, 10 mg/L, or 1 mg/L of DDBAC for 14 days. A total of 880,925 high-quality sequences were acquired from the midgut samples, with an average length of 422 bp. A total of 114 unique operational taxonomic units (OTUs) were clustered based on a 97 % similarity cutoff. There are four indexes to evaluate the alpha-diversity of microbial community. The community richness examined by Chao and Ace index indicated no significant differences among the control, 1 mg/L DDBAC, 10 mg/L DDBAC, and 100 mg/L DDBAC groups (Fig. S1). For examining community diversity, Simpson index indicated no significant differences among the different treatment groups, but the Shannon index showed that both 10 mg/L DDBAC and 100 mg/L DDBAC exposure groups had a significant decrease in alpha-diversity compared to the control (Fig. 2A).

A partial least squares discriminant analysis (PLS-DA) can indicate the relative similarity of microbial community composition among different groups (Rabhi et al., 2016). Our PLS-DA plot clustered the control group and DDBAC exposure groups into different quadrants, indicating an obvious change in gut microbiota structure due to DDBAC exposure (Fig. 2B). A linear discriminant analysis (LDA) effect size (LEfSe) analysis indicated that the gut microbiota of the control group had a higher abundance of Lactobacillus and Bifidobacterium compared to the DDBAC-exposed groups (Fig. 2C). A Kruskal-Wallis H test showed that DDBAC exposure significantly reduced the abundance of Lactobacillus (p-value < 0.05) (Fig. 2D), with a concentration-dependent manner. Bifidobacterium abundance was also significantly reduced by DDBAC exposure (p-value < 0.01) (Fig. 2D). Lactobacillus and Bifidobacterium are core residents of honeybee gut microbiota that are widely regarded as beneficial (Alberoni et al., 2016). Their abundance reductions in honeybee gut suggest that DDBAC exposure may threaten honeybee gut health.

3.4. Lipidomic analysis of DDBAC-exposed honeybee midgut

Honeybee colonies were exposed to DDBAC to mimic its effects on honeybees in the field. A sublethal concentration of 10 mg/L DDBAC was chosen because it is the environmentally relevant dose in China (Wang et al., 2020), and the chronic toxicity test results show its toxic effects on honeybees. Midgut samples collected from these 10 mg/L-DDBAC-exposed colonies were analyzed using an LC-MS/MS-based lipidomic analysis to assess the potential hazards of DDBAC exposure on gut phospholipid composition. The control and 10 mg/L DDBAC groups (with triplicate biological samples) were examined at two sampling time points (6 days or 18 days after eclosion). In a colony, adult worker honeybee undergoes an age-related division of labor. Honeybees work as nurses at 6 days after eclosion and switch to working as foragers at 18 days after eclosion. The adult honeybees in these two stages (nurse and forager) are significantly different in physiology and behavior (Graham, 2008). Therefore, we chose these two time points for the lipidomics analysis to characterize the potentially distinct responses of relatively young (nurse) and relatively old (forager) adult honeybees to DDBAC exposure.

A principal component analysis (PCA) score plot of the LC-MS/MS-based lipidomics data indicated obvious clustering of triplicate samples for each of the four examined groups (Fig. 3A). A volcano plot
highlighted multiple significantly altered compounds among the honeybee midgut under DDBAC exposure of 6 days or 18 days duration comparing to their corresponding controls (CK6 vs D6 and CK18 vs D18). Specifically, we found that there were 100 down-regulated and 107 up-regulated significantly altered lipids in the 6-day comparison, and 331 down-regulated and 118 up-regulated significantly altered lipids in the 18-day comparison (Fig. 3B). Among these compounds, the abundance of phospholipids was dramatically changed during DDBAC exposure (Fig. 3B and Table S1). At both 6 and 18 days, the DDBAC-exposed samples had significantly reduced levels of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) as compared to their time-matched control samples. The level of phosphatidylinositol (PI) was significantly decreased on day 6 of DDBAC exposure and significantly elevated on day 18 in the DDBAC-exposed samples (Fig. 3C and Table S1). Given that PC, PE, PS, and PI are the essential components of cell membrane and can also execute biological functions in signal transduction (Dowhan, 2017), their changes caused by DDBAC exposure indicate potentially destructive effects of DDBAC on honeybee midgut membranes and cellular signaling.

3.5. Transcriptome analysis of honeybee midgut under DDBAC exposure

To examine the effects of DDBAC on the honeybee midgut at transcriptome level, we used RNA-seq to analyze midgut tissues from the 6-day and 18-day sublethal DDBAC exposure and corresponding controls (with triplicate replication), which were sampled from the DDBAC-exposed colonies. The raw data for each sample contained 44–52 million reads (Table S2). In total, 43%–82% of the reads were uniquely mapped to a reference honeybee genome (Weinstock et al., 2006) (Table S2). A PCA of all 12 samples indicated that the RNA-seq data from

Fig. 2. The hazards of DDBAC exposure on the microbial composition of honeybee midgut. (A) The changes in microbial alpha diversity of honeybee midgut under the indicated sublethal concentrations of DDBAC exposure for 14 days based on Shannon and Simpson index analysis. (B) The changes in microbial beta diversity of honeybee midgut under the indicated sublethal concentrations of DDBAC exposure for 14 days based on partial least squares discriminant analysis (PLS-DA). (C) Taxonomic cladogram showing the bacterial taxa meeting an LDA-significant threshold > 4.0. The taxa shaded in different colors are with significant abundance in those corresponding groups. The abundance of those highlighted taxa in the control, 1 mg/L, and 100 mg/L DDBAC groups is significantly higher than that in the 10 mg/L DDBAC group. (D) Differences in the abundance of Lactobacillus and Bifidobacterium among different sublethal concentrations of DDBAC exposures by Kruskal-Wallis H test.
three biology replicates was of sufficient repeatability and quality to enable further analysis (Fig. S2). We used the DESeq2 R package to identify the significant differentially expressed genes (DEGs) after DDBAC exposure (p-value < 0.05). There were 286 up-regulated and 138 down-regulated DEGs in the 6-day comparison (CK6 vs D6), and 203 up-regulated and 122 down-regulated DEGs in the 18-day comparison (CK18 vs D18) (Fig. 4A and B).

GO enrichment analysis revealed enrichment among DEGs for the 6- and 18-day DDBAC exposure samples for functional annotations related to the terms “calcium: sodium antiporter activity”, “voltage-gated channel activity”, “ion binding”, and “integral and intrinsic component of membrane” (Fig. 4C). In a KEGG analysis, the DEGs of the 6-day DDBAC exposure samples were enriched in the “calcium signaling pathway” and calcium channel related pathways, including “hypertrophic cardiomyopathy (HCM)”, “arrhythmogenic right ventricular cardiomyopathy (ARVC)”, “apelin signaling pathway”, “dilated cardiomyopathy (DCM)”, and “endocrine and other factor-regulated calcium reabsorption”. The DEGs for the 18-day DDBAC exposure samples were enriched in the calcium-binding-protein-related pathways, including “HCM”, “phototransduction”, “dopaminergic synapse”, and “pancreatic secretion”.

Shifting from the pathway to the individual transcript level, we examined the expression of multiple components involved in calcium signaling. In the transcriptome data, we detected that the 6-day DDBAC-exposed samples had significantly reduced expression of genes encoding proteins that actively pump Ca\(^{2+}\) ions out of the cell (e.g., NCX and PMCA) (Brini and Carafoli, 2011); and the 18-day DDBAC-exposed samples had significantly elevated expression of genes encoding proteins that transport calcium into the cytoplasm (e.g., RYR, CACN, and CaV3) (Catterall, 2011) (Fig. 5B and Table S3). Additionally, we found significantly elevated expression of genes encoding second messengers of calcium-sensing receptors (e.g., PLC\(\gamma\), PLC\(\beta\)) (Tan et al., 2021) and calcium-binding proteins (e.g., CALM, PKC) (Jin et al., 2019) in the 6- and 18-day DDBAC-exposed samples, respectively (Fig. 5B and Table S3).

Calcium signaling is essential for excitation–secretion coupling in neurons, and for excitation-contraction coupling in muscles. We detected that 18-day DDBAC-exposed samples had the significantly increased expression of genes encoding CREB and BDNF, two regulatory factors essential in neuronal development and survival (Alberini, 2009; Nieto et al., 2013) (Fig. 5B and Table S3). Moreover, we found that the 6-day DDBAC-exposed samples had dramatically altered expression of genes involved in Ca\(^{2+}\)-mediated control of muscle contraction. For example, the significantly elevated expression was detected for genes encoding membrane receptors (e.g., ITGB and DG) (Kopp, 2009) and a regulator of myosin (MLCP) (Yamashiro et al., 2008); and we also detected significantly reduced expression of a gene encoding sarcomere component (actin; ACTG1) (Tanaka et al., 2018) (Fig. 5B and Table S3). These results collectively reveal a DDBAC-induced disturbance of calcium signaling, suggesting that DDBAC may pose a risk for muscle and...
neuron function in honeybee.

4. Discussion

DDBAC is widely used in hospitals, industry, and cosmetics, and is known to reduce the number of microbial infections. However, the effective function of DDBAC is a double-edged sword, as DDBAC residues can be harmful to the environment and non-target organisms. Our study has revealed the negative effects of DDBAC on honeybee health, including disrupted gut tissue, reduced gut beneficial microbiota, altered gut phospholipid composition, and disturbed cellular signaling transduction.

DDBAC is a chlorinated compound containing a benzene ring, which is more toxic than brominated or non-benzene ring QACs compounds (Zhu et al., 2010; Jing et al., 2012; Burton et al., 2001). The reported LC₅₀ of DDBAC for fish species (O. latipes) was about 0.2 mg/L (Kim et al., 2020), and for C. elegans LC₅₀ was up to 3 mg/L (Sreevidya et al., 2018). According to the mortality results of our acute toxicity test, the LC₅₀ of DDBAC for honeybees is 1 mg/L. Therefore, it appears that DDBAC is less toxic to honeybees than to fish and C. elegans. Though current assessments considered DDBAC to be of low toxicity, all the non-target animals continuously undergo chronic toxicity exposure in this...
contaminated environment. The chronic toxicity of DDBAC to honeybee midgut at sublethal concentration has been revealed in our study, thus the risk of DDBAC exposure to pollinators is worthy of our attention.

The health status of honeybees is closely linked with the composition and function of gut microbiota: *Lactobacillus* and *Bifidobacterium* are core residents of honeybee gut microbiota that are regarded as probiotics (Alberoni et al., 2016). Notably, *Lactobacillus* and *Bifidobacterium* have also been associated with immunomodulatory functions in the human intestine (Perdigón et al., 2004). Both *Lactobacillus* and *Bifidobacterium* can exert antimicrobial activity and inhibit the growth of honeybee pathogens such as *Paenibacillus larvae*, *Melissococcus plutonius*, and *Ascosphaera apis* (Vásquez et al., 2012; Killer et al., 2014). Therefore, significant decreases in *Lactobacillus* and *Bifidobacterium* upon DDBAC exposure may leave honeybees vulnerable to pathogenic...
microorganisms. Interestingly, Lactobacillus is known as the dominant flora at the early development stage of adult honeybees and has been proposed to affect their early behaviors including hive cell cleaning (Anderson et al., 2016). As such, it appears that DDBAC may be a hazard proposed to affect their early behaviors including hive cell cleaning flora at the early development stage of adult honeybees and has been linked to beneficial midgut microbiota, as well as significant changes in midgut phospholipid composition, and the altered expression of genes involved in calcium signaling pathways of honeybees upon exposure to sub-lethal concentrations of DDBAC. Therefore, our study establishes that DDBAC exposure deleteriously impacts honeybee midgut functions at multiple levels. Honeybees provide highly valued pollination services for a large variety of agricultural crops. Thus, agents that threaten the health of honeybees are in danger of increasing the risk of global food security. Our study found hazards of DDBAC to honeybee health, highlighting the necessity of proper stewardship and removal of QACs residues to protect honeybee health, ecological environment, and global agricultural production.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107639.

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