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Identification of the Toxic Compounds in *Camellia oleifera* Honey and Pollen to Honey Bees (*Apis mellifera*)

Zhen Li, Qiang Huang, Yu Zheng, Yong Zhang, Xin Li, Shiqing Zhong, and Zhijiang Zeng*

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ABSTRACT: Identifying the components of *Camellia oleifera* honey and pollen and conducting corresponding toxicological tests are essential to revealing the mechanism of *Camellia oleifera* toxicity to honey bees. In this research, we investigated the saccharides and alkaloids in honey, nectar, and pollen from *Camellia oleifera*, which were compared with honey, nectar, and pollen from *Brassica napus*, a widely planted flowering plant. The result showed that melibiose, manninotriose, raffinose, stachyose, and lower amounts of santonin and caffeine were found in *Camellia oleifera* nectar, pollen, and honey but not in *B. napus* nectar, pollen, and honey. Toxicological experiments indicated that manninotriose, raffinose, and stachyose in *Camellia oleifera* honey are toxic to bees, while alkaloids in *Camellia oleifera* pollen are not toxic to honey bees. The toxicity mechanism of oligosaccharides revealed by temporal metabolic profiling is that oligosaccharides cannot be further digested by honey bees and thus get accumulated in honey bees, disturbing the synthesis and metabolism of trehalose, ultimately causing honey bee mortality.

KEYWORDS: *Camellia oleifera, saccharides, alkaloids, toxic mechanism, Apis mellifera*

INTRODUCTION

Nectar is a sweet substance secreted by plant nectar exosomes and mainly consists of saccharides, proteins, amino acids, vitamins, flavonoids, glycosides, alkaloids, and water.[1−5] Honey bees process nectar into honey as food for their colony.[6] Plant flowers secrete sweet nectar that, by attracting more pollinators, increases the number of offspring produced by the plant.[7] For honey bees, the collected plant pollen is a high-quality source of protein for them individually or as a population and can help them reproduce healthy offspring.[8] Thus, plant diversity is a prerequisite for insect pollinator diversity.[9] However, it should be noted that monoculture, which is common for commercial crop cultivation, reduces vegetation and pollinator diversity.

Camellia oleifera is a woody oil crop traditionally cultivated in Asia, and oil can be extracted from its fruit. Camellia oil is an edible oil with liver-protecting effects that have potential benefits for human health.[10] In recent decades, the habitat of *Camellia oleifera* pollinating insects has been reduced by factors such as crop planting homogenization, environmental pollution, and artificial destruction of vegetation.[11] Additionally, the self-incompatibility characteristics of *Camellia oleifera* further limit the fruiting rate and camellia oil yield.[12] Overall, inadequate and inefficient pollination is a key factor limiting the development of the camellia oil industry.[13] When honey bees (*Apis mellifera* and *Apis cerana*) pollinate *Camellia oleifera*, colonies can begin to exhibit the decay of small larvae and pupae and bloating of adults. The likely main chemical components that cause honey bee toxicity have been previously reported to be the oligosaccharides and alkaloids contained in the nectar and honey of *Camellia oleifera*,[14−16] but these inferences have not been confirmed.

Kang and Fan were the first to identify raffinose and stachyose, which are not contained in common honey, in *Camellia oleifera* nectar and honey.[17] As the concentration of oligosaccharides (raffinose and stachyose) fed to honey bees increases, the bees' mortality rate also increases.[18] Further *in vitro* hydrolysis of oligosaccharides in *Camellia oleifera* honey followed by liquid chromatography analysis yielded oligosaccharide-bound galactose content up to 31.4%.[19] However, the honey bees could tolerate no more than 5% galactose content.[19] The toxicity of raffinose and stachyose to honey bees has been evaluated in standard laboratory tests on adult bees.[20] Toxicological tests showed that 10% raffinose was lethal to *A. mellifera* at a rate of 81% over 16 days.[20] Furthermore, a reduction of 99% of *A. mellifera* adult bees owing to in adverse effects over 16 days was observed with 8% stachyose addition.[20] This series of experiments showed that perhaps oligosaccharides and galactose in *Camellia oleifera* nectar and honey are the main cause of honey bee toxicity. However, in practice, most studies on the composition of *Camellia oleifera* nectar and honey have only focused on the kinds of oligosaccharides, and the absolute composition of oligosaccharides and alkaloids in *Camellia oleifera* nectar, pollen, and honey is not yet known. There is also a gap in research on the mechanism of toxicity of *Camellia oleifera* nectar, pollen, and honey to honey bees.

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To improve the pollination efficiency of *Camellia oleifera*, which is of substantial agricultural and economic importance, we examined the contents of saccharides and alkaloids in honey, nectar, and pollen from *Camellia oleifera* and those from a common flowering plant (*Brassica napus*) that showed no toxicity to honey bees by using high-performance liquid chromatography (HPLC) and HPLC quadrupole time-of-flight mass spectrometry (HPLC/Q-TOF-MS) techniques. The aim was to explore the causes of honey bee toxicity induced by *Camellia oleifera* nectar, pollen, and honey and to provide an empirical for resolving the problem of toxicity to honey bees after visits to *Camellia oleifera* and enhancing the pollination efficiency of *Camellia oleifera*.

MATERIALS AND METHODS

Nectar, Pollen, and Honey Collection. *Camellia oleifera* nectar, pollen, and honey (subsequently abbreviated as CON, COP, and COH, respectively) were taken from the *Camellia oleifera* plantation in Shengqiao Town, Changning City, Hunan Province, and the sampling period time was from October to November 2021. The *Camellia oleifera* nectar was collected using a vacuum aspirator (Beijing Dalong Xingchuang Experimental Instrument Co., Beijing, China) and gentle handling was required to avoid pollen contamination of the nectar during the aspiration process. Moreover, the *Camellia oleifera* pollen and honey were obtained from *A. mellifera* colonies, where *Camellia oleifera* pollen was removed from the hind feet of the honey bees by using a pollen stripper at the hive entrance and then dried utilizing a dryer, while *Camellia oleifera* honey was collected as capped mature honey (42.5° Bé) extracted from the combs by centrifugation. All samples were stored at −80 °C for subsequent analysis. *B. napus* nectar, pollen, and honey (hereafter referred to as BNN, BNP, and BNH, respectively) were obtained from winter-sown *B. napus* grown in Anyi County, Jiangxi Province, and samples were collected from March to April 2022. The sampling method was performed using the same techniques to collect *Camellia oleifera* nectar, pollen, and honey.

Chemicals and Reagents. The following reagents were used: methanol (TEDIA Reagent Co., Fairfield, OH, USA), ultrapure water (Beijing G-Clone Biotechnology Co., Beijing, China), acetonitrile (Fisher Scientific Inc., Waltham, MA, USA), 16 saccharide standards (Sigma, St. Louis, MO, USA; purity ≥98%), chromatography-grade sodium hydroxide (Shanghai Macklin Biochemical Technology Co., Shanghai, China), chromatography-grade ethanol (Shanghai Macklin Biochemical Technology Co.), mass spectrometry-grade methanol (Thermo Fisher Technology Co., Ltd., Waltham, MA, USA), mass spectrometry-grade formic acid (Shanghai Macklin Biochemical Technology Co.), water (Millipore, Billerica, MA, USA), aqueous ammonium acetate solution (Beijing G-Clone Biotechnology Co., Ltd., Beijing, China), and santonin and caffeine (Sigma; purity ≥99%). Yeast extract powder was obtained from Angel Yeast Co. Ltd. (Yichang, China). Acetone and sucrose were obtained from Aladdin Biochemical Co., Ltd. (Shanghai, China).

HPLC Method for the Determination of Saccharides in Nectar, Pollen, and Honey. *Saccharide Standard Preparation and Construction of the Standard Curves.* To prepare mixed standards, 900 μL of 50% methanol/water solution (v/v) was added to a 10 mL volumetric flask, along with 100 mg of the standards in Table S1. Then, 50% methanol/water solution (v/v) was added to fix the volume to 10 mL, and the solution was vortexed, mixed well, and diluted 100-fold with methanol to obtain the standard stock solution (the concentration of all 16 saccharides was 100 μg/mL). Before testing, each standard solution was diluted according to the gradient shown in Table S2 and loaded into a 1.5 mL injection vial. The fitting degrees of the 16 saccharide standards were all ≥0.9901, indicating a strong linear relationship. The results are shown in Table S3.

Nectar, Pollen, and Honey Preparation for the Detection of Saccharides. First, 400 μL of *Camellia oleifera* or *Brassica napu* nectar sample (three biological replicates of each sample) was pipetted accurately, respectively, diluted 500-fold, and then tested on the HPLC instrument. Exactly 50 mg of pollen or honey (three biological replicates of each sample) was weighed in a 2 mL Eppendorf tube and 700 μL of 80% ethanol solution was added to the pollen or honey-containing tube. The tube was shaken at 50 °C for 2 h, after which 700 μL of H_2O was added and centrifuged at 10,000 rpm for 3 min. The supernatant was transferred into a 1.5 mL injection vial, and the final assay was performed on the HPLC instrument.

HPLC Analysis. Thermo ICS 5000 liquid chromatography with an electrochemical detector (Thermo Fisher Technology Co., Ltd) was employed for the analysis of saccharide fractions in nectar samples. A CarboPac PA20 liquid chromatographic column (150 × 3.0 mm, 4 μm) was used. The analytical method was conducted as described in the Supporting Information.

HPLC/Q-TOF-MS Method for Characterization of Alkaloid Species and HPLC Method for Determination of Alkaloid Content in Nectar, Pollen, and Honey. *Nectar, Pollen, and Honey Pre-Treatment for Characterization of Alkaloid Species.* The pre-treatment of nectar, pollen, and honey samples was conducted, with appropriate modifications, using the method of Du et al.[21] First, 1 g (±0.01 g) of the four kinds of the samples (three biological replicates of each sample) were weighed in a 50 mL centrifuge tube, to which was added 1.25 mL of water, 50 μL of ammonia, and 0.5 mL of acetonitrile, and the solutions were mixed well. After adding NaCl to saturation, samples were vortexed for 2 min and centrifuged at 1,000 rpm for 10 min to remove the upper organic phase. Then, 2 mL of acetonitrile was added to the lower aqueous phase, and samples were vortexed for 2 min and then centrifuged, until the upper organic phase merged. The resulting upper organic phase was dried under a gentle stream of nitrogen and fixed in 2 mL of a 0.05 mol/L HCl−methanol mixture (8:2, v/v). Then, 0.2 g of primary−secondary amine powder was added to the fixing solution, and samples were vortexed and mixed for 1 min, followed by centrifugation for 3 min at 15,000 rpm. The supernatant was filtered through a 0.22 μm filter and injected into a 1.5 mL injection vial for measurement.

HPLC/Q-TOF-MS Analysis. Characterization of alkaloid species analysis was performed on the Agilent 1290 Infinity II HPLC system coupled with Agilent 6546B with quadrupole TOF tandem mass spectrometry (Agilent Technology Inc., Santa Clara, CA, USA). The analytical method was performed as described in the Supporting Information.

The identification of alkaloids was carried out from the library of 57 alkaloids that were summarized in an Agilent PCDL software Ver. B.06.00 (Agilent Technologies Inc.,), which includes the name, molecular formula, qualitative ions, and quantitative ions of each published known compound.[21−23] See Table S4 for details.

Quantification of Alkaloid Content by HPLC and Standard Working Curve Plotting for Santonin and Caffeine. First, 0.02 g of the santonin and caffeine standards was weighed using an accurate balance (Shimadzu, Tokyo, Japan). Then, methanol−water (6:4, v/v) solution was used to prepare mixed standard santonin and caffeine solutions of 2, 1, 0.5, 0.25, and 0.125 mg/mL, respectively, which were used to form the standard working curve of both. At the same time, the lowest concentration of the single standard solution was diluted step by step until the signal-to-noise ratio (S/N) = 3, which in turn was used to establish the limits of detection (LOD) of the instrument for the two compounds.

HPLC Analysis. The alkaloids in these samples were separated via liquid chromatography and were determined by two rounds of mass spectrometry. Quantification of alkaloids was subsequently undertaken by HPLC analysis for samples identified as containing alkaloids. The Agilent 1290 Infinity II HPLC system equipment consisted of a vacuum degasser, a quaternary pump, an autosampler, and a UV detector (Agilent Technologies Inc., USA). The analytical method was performed as described in the Supporting Information.

Toxicity of Oligosaccharides in *Camellia Oleifera* Honey to Larvae and Adult Worker Bees. *Insects and Oligosaccharide Diet Preparation.* Three healthy colonies were reared at the Honeybee Research Institute, Jiangxi Agricultural University, China (28.46 N,

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115.49 E) to provide larvae and adult worker bees for this experiment. Each colony represents a biological replicate.

The larvae oligosaccharide diet formulation was based on the method of Roth et al. but without additional fructose and glucose to facilitate the total dissolution of the oligosaccharide (melibiose, manninotriose, raffinose, and stachyose).[24] Individual or combined oligosaccharides were added to the larval diet at doses comparable to those found in *Camellia oleifera* honey. For detailed information on larvae diet formulations, please refer to Table S5. In accordance with the kinds of oligosaccharides added to the larvae diet, the larvae were divided into the melibiose group, manninotriose group, raffinose group, stachyose group, and mixed oligosaccharide group (referred to as Mel, Mann, Raf, Sta, and mix, respectively). Larvae in the mixed group were fed a diet with all four oligosaccharides. In contrast, no oligosaccharides were added to the diet of the control group larvae (CG). The larval diet was allocated into 10 mL sterile centrifuge tubes and stored at −80 °C.

For adult worker bees, the diet was made by dissolving single or combined oligosaccharides in a 50% (w/w) sucrose solution, and four oligosaccharides were added in the same dosage as the corresponding oligosaccharide concentration of *Camellia oleifera* honey. The experimental and control groups of adult worker bees were the same as those of the larvae—also determining the type of oligosaccharides in the diet they were fed. Worker bee diets were formulated daily and ready to use.

Lethal Effects of Oligosaccharides on Larvae and Adult Worker Bees. To harvest same-aged honey bee larvae, queens were caged on a fresh wax comb and allowed to lay eggs for 6 h inside the colony. Then, the comb with eggs was incubated in the top layer of the Langstroth hive. After 96 h, the newly hatched honey bee larvae (2 day-old larvae) were transferred to a 48-well cell culture plate (BOSTER, Wuhan, China) with the sterile queen cell (with 30 μL of larvae diet added in advance). Three biological replicates were set up for each of the five oligosaccharide-fed groups and the control group, and each group was fed one of the six larvae diets. There were three biological replicates from three colonies, respectively, with 48 larvae in each replicate. During 2−6 days of age, six groups of larvae were fed equivalent amounts of diet per day (2 day-old, 30 μL; 3 day-old, 40 μL; 4 day-old, 40 μL; 5 day-old, 50 μL; 6 day-old, 60 μL). All 48-well plates were then incubated at 34.5 °C in a humidity-controlled (85% relative humidity) incubator. From 2 to 8 day-old stages, the numbers of dead larvae were recorded at the same time each day for each group, and the Kaplan−Meier survival curves were plotted.

After collecting the 2 day-old larvae, the comb was returned with the remaining larvae to the hive for further cultivation. The comb was collected from the colony and placed in the incubator at 34.5 °C and 75% relative humidity the day before the worker bees emerge, and the newly emerged worker bees were collected the next day. Of these, 40 worker bees were gently captured from each comb and reared in cup cages, with a total of 120 worker bees in three cup cages (the worker bees in the three cup cages were from three colonies, respectively) per group. Each of the six groups of worker bees was fed one of the six worker bee diets, and adequate diets were supplied daily. These cup cages containing worker bees were kept in an incubator at 34.5 °C with 75% relative humidity; and the number of dead bees was regularly counted, and dead bees were discarded daily until all bees were dead. Notably, it was essential to replace cup cages contaminated with sugar solution and bee feces promptly. The survival time for each group was plotted as a Kaplan−Meier survival curve.

Toxicity of Alkaloids in *Camellia Oleifera* Pollen to Larvae and Adult Worker Bees. The larvae alkaloid diet was prepared according to the method reported by Roth et al., but the amount of fructose and glucose added was modified to make the diets more suitable for larvae growth.[24] The additional amount of caffeine in the larvae's diet was the same as the content in *Camellia oleifera* pollen; instead, for santonin, 10 times the LOD was added. Since caffeine and santonin are insoluble in water, they were dissolved in 500 μL of acetone before the larval diets were prepared. Please refer to Table S6 for larvae diet formulation details. According to the type of alkaloids added to the larvae diets, the larvae were divided into caffeine, santonin, and mixed alkaloids groups and the control group (no alkaloids were added to the diet, but 500 μL of acetone was added). Mixed alkaloid group larvae were fed the diet supplemented with two alkaloids, caffeine and santonin. The four treatment groups were referred to as CG, santonin, caffeine, and mix. The larvae diets were allocated into 10 mL sterile centrifuge tubes and stored at −80 °C.

In adult worker bees, the diet was made by dissolving single or composite alkaloids in a 50% (w/w) sucrose solution (dissolved in advance in 500 μL of acetone and then mixed with 50% (w/w) sucrose solution), and worker bees were grouped as well by the alkaloids in their diets. The extra amount of caffeine added to worker bee diets matches the amount of caffeine in *Camellia oleifera* pollen, and the amount of santonin added was 10 times its LOD. In the control group, no alkaloids were added and only 500 μL of acetone was provided. The two alkaloids were added to the diets of worker bees in the mixed group. The four treatment groups were referred to as CG, santonin, caffeine, and mix. Diets for worker bees are prepared daily and ready to use.

Lethal Effect of Alkaloids on Larvae and Adult Worker Bees. To obtain same-aged honey bee larvae, queens were caged on a fresh wax comb and allowed to lay eggs for 6 h inside the colony. Then, the comb with eggs was incubated in the top layer of the Langstroth hive. After 96 h, the newly hatched honey bee larvae (2 day-old larvae) were transferred to a 48-well cell culture plate with the sterile queen cell (30 μL of larvae diet was added in advance). Three biological replicates were set up for each of the three alkaloid-fed groups and the control group (CG), and each group was fed one of the four larvae diets, respectively. Additionally, there were three biological replicates from three colonies, respectively, with 48 larvae in each replicate. During 2−6 days of age, six groups of larvae were fed equivalent amounts of diet per day (2 day-old, 30 μL; 3 day-old, 40 μL; 4 day-old, 40 μL; 5 day-old, 50 μL; 6 day-old, 60 μL). All 48-well plates were then incubated at 34.5 °C in a humidity-controlled (85% relative humidity) incubator. From 2 to 8 day-old stages, the numbers of dead larvae were recorded at the same time each day for each group, and Kaplan−Meier survival curves were plotted.

After collecting the 2 day-old larvae, the comb was returned with the remaining larvae to the hive for further cultivation. The comb was removed from the colony and placed in the incubator at 34.5 °C and 75% relative humidity the day before the worker bees emerged, and the newly emerged worker bees were collected. Of these, 40 worker bees were gently captured and reared in cup cages from each comb, with a total of 120 worker bees in three cup cages (the worker bees in the three cup cages were from three colonies, respectively) per group. Each of the four groups of worker bees was fed one of the four worker bee diets, and adequate diets were supplied daily. These cup cages containing worker bees were kept in an incubator at 34.5 °C with 75% relative humidity; and the number of dead bees was regularly counted, and dead bees were discarded daily until all bees were dead. For each group, the survival time was graphed as Kaplan−Meier survival curves.

Temporal Metabolic Profile of Oligosaccharides in Larvae and Adult Worker Bees. *Larvae and Adult Worker Bees Rearing and Sampling Time Point Conditions.* Oligosaccharide diets (including manninotriose, raffinose, and stachyose individually, respectively) for larvae and worker bees were prepared and supplied to larvae and worker bees. No oligosaccharides were added to the diets of larvae and worker bees in the CG.

4, 5, and 6 day-old larvae were collected at the same time of each day, respectively. Also, before sampling, the larvae were rinsed with sterile deionized water and dried in 2 mL frozen tubes in liquid nitrogen for 1 h before the final storage at −80 °C until subsequent analysis. Each of the three 4 day-old larvae was a single replicate for a total of three biological replicates. Each 5 or 6 day-old larvae was one replicate for a total of three biological replicates. In addition, larvae samples were named based on the type of oligosaccharides in the diet and the age of the larvae. For example, manninotriose group 4 day-old larvae samples were referred to as ML4d.

After the worker bees emerged from the three combs, 200 healthy worker bees were harvested from each of the combs and kept in four groups of cup cages in the incubator at 34.5 °C and 75% relative

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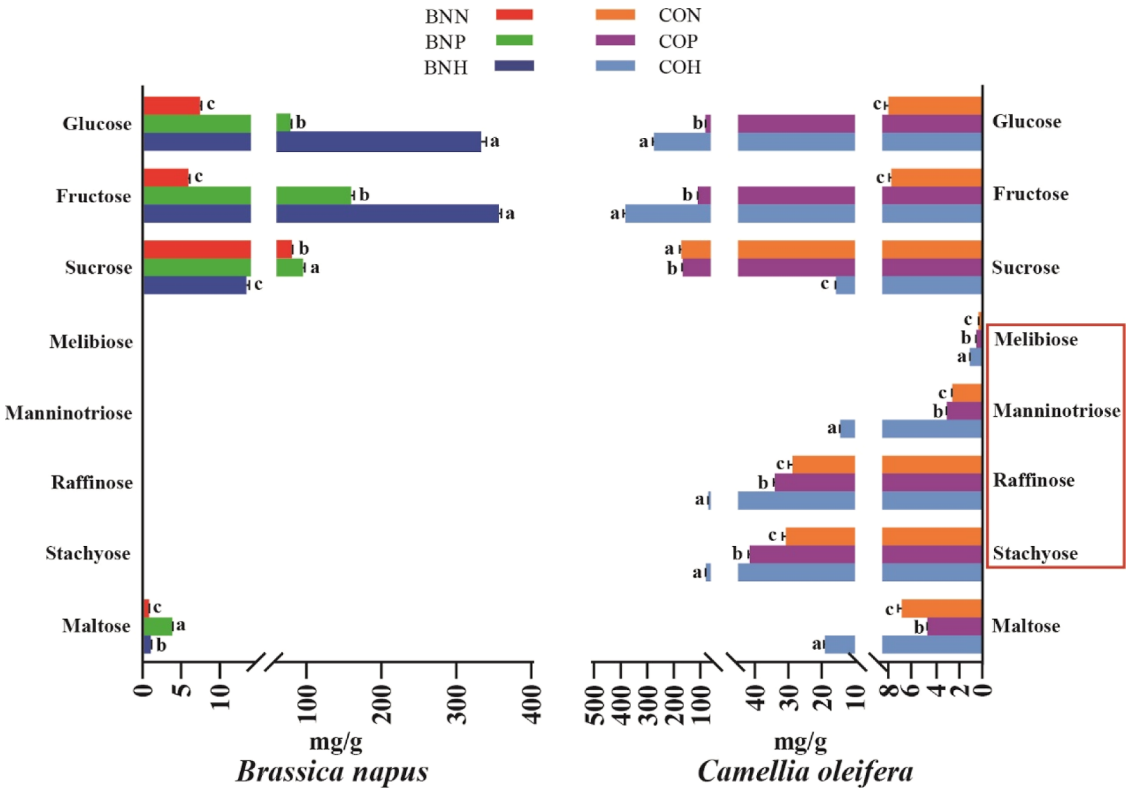

Figure 1. Composition of saccharides in *Brassica napus* and *Camellia oleifera* nectar, pollen, and honey. The same lowercase letter in the three columns of the same saccharide indicates no significant difference ($P > 0.05$), and different lowercase letters indicate significant differences ($P < 0.05$).

humidity. Each group had three cups of worker bees from three different combs, with 50 worker bees per cup. Each of the four groups of worker bees was then fed one of the three oligosaccharide diets or the control diet, respectively. The foregut, midgut, and hindgut of each group of worker bees were completely dissected at 24, 48, and 72 h after feeding, and the guts of 15 worker bees were mixed to form one replicate (five worker bee guts were dissected from each of the three cups and worker bees in each group comprised of one replicate each day), for a total of three biological replicates per day for one group. Fifteen worker bees for each replicate were weighed and recorded with subsequent gut/weight conversions prior to dissection. We referred to worker bee gut samples according to the type of oligosaccharide in their diet and the time point at which they were sampled. For instance, the raffinose group 24 h worker gut sample was abbreviated as RWG 24 h. Samples were stored in 2 mL frozen tubes in liquid nitrogen for 1 h and then kept at −80 °C for subsequent testing.

Saccharide Standards Preparation and Construction of the Standard Curve. Standard curves were constructed for each of the 16 saccharides. All standard curve correlation coefficients were ≥0.9916, indicating that the derived standard curves for the 16 saccharides were reliable (Table S7).

Pre-Treatment of Larvae and Adult Worker Bee Guts for Saccharide Metabolic Profile Analysis. Larvae or worker bee gut samples were lyophilized and ground into powder, and then, 50 mg of sample powder was accurately weighed into a 2.0 mL centrifuge tube. After 700 μL of 80% ethanol was added, samples were shaken at 50 °C for 2 h, then diluted with 700 μL of H_2O, and centrifuged at 10,000 rpm for 3 min. Finally, 800 μL of the supernatant was transferred to a new 1.5 mL injection vial for analysis.

HPLC Analysis. The saccharide compositions of larvae or worker bee gut samples were analyzed utilizing the same equipment and methods used to assay the saccharide compositions of *Camellia oleifera* nectar, pollen, and honey. The analytical method was carried out as described in the Supporting Information.

Statistical Methods. SPSS (version 25.0, IBM Corp., Armonk, NY, USA) software was used for all data analyses. Data are expressed as mean ± standard error (SE). We used one-way analysis of variance (ANOVA) to test differences between treatment groups, and PLSD was used to analyze the significance of differences between the test groups. The probability level at which the differences between groups were considered significant was $P < 0.05$. Kaplan−Meier curves were used to assess observations of the survival of each group of larvae or worker bees and were compared, with log-rank test P values of less than 0.05 considered statistically significant.

RESULTS AND DISCUSSION

In this experiment, we determined the differences in the oligosaccharides and alkaloids between nectar, pollen, and honey from *Camellia oleifera* and *B. napus*. We also identified the components of *Camellia oleifera* nectar, pollen, and honey that are toxic to honey bee larvae and adult worker bees through toxicological tests of oligosaccharides and alkaloids. Moreover, the temporal metabolic profile of three toxic oligosaccharides (manninotriose, raffinose, and stachyose) in larvae and adult worker bees revealed the detailed mechanism of toxicity of *Camellia oleifera* nectar, pollen, and honey to honey bees.

Analysis of the Saccharide Composition of *Camellia oleifera* Nectar, Pollen, and Honey. Sucrose, fructose, and glucose are common saccharides in most nectars.[25−28] A few nectars also contain lower levels of disaccharides, such as maltose and melibiose as well as trace levels of oligosaccharides such as raffinose, melezitose, and stachyose.[29] *Camellia oleifera* nectar, pollen, and honey contained a high level of raffinose and stachyose compared with *B. napus* nectar, pollen, and honey (Figure 1). Meanwhile, two saccharides newly

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Table 1. Characterization and Composition Differences of Alkaloids in *Brassica napus* and *Camellia oleifera* Nectar, Pollen, Honey (n = 3)[a]

	characterization parameters		absolute composition, mg/g					
	precursor ion (m/z)	product ion (m/z)	*Brassica napus*			*Camellia oleifera*		
compound			nectar	pollen	honey	nectar	pollen	honey
santonin	247.13	173.1*	ND	ND	ND	trace	trace	trace
caffeine	194.19	138.05*	ND	ND	ND	trace	0.140 ± 0.001	trace

[a]"*" means quantitative ions.

discovered in *Camellia oleifera* nectar, pollen, and honey were melibiose and manninotriose (Figure 1). The concentrations of the four oligosaccharides (melibiose, manninotriose, raffinose, and stachyose) in *Camellia oleifera* nectar, pollen, and honey were highest in honey, followed by pollen and nectar. *Camellia oleifera* honey was enriched with raffinose and stachyose up to 69.24 and 78.54 mg/g (Table S8), respectively, a result that supports the earlier findings (*Camellia oleifera* honey contains raffinose and stachyose).[17] However, the levels of melibiose and manninotriose were relatively low in *Camellia oleifera* honey (Table S8). It is likely that the melibiose and manninotriose in *Camellia oleifera* honey are partly derived from nectar concentration and hydrolysis of raffinose and stachyose.

Analysis of Alkaloid Composition in *Camellia oleifera* Nectar, Pollen, and Honey. After the nectar, pollen, and honey of *Camellia oleifera* and *B. napus* were characterized by HPLC/Q-TOF-MS, the results were compared to a library of the existing alkaloid compounds.[21−23] Thus, *Camellia oleifera* nectar, pollen, and honey were determined to contain two alkaloids, santonin and caffeine, while *B. napus* nectar, pollen, and honey contained no alkaloids (Table 1; Figure 2a,b). When the two alkaloids in *Camellia oleifera* nectar, pollen, and honey were further quantified by HPLC, their contents in the *Camellia oleifera* nectar and honey were determined to be below the LOD (12.9 μg/kg for santonin and 1.6 μg/kg for caffeine) (Table 1). The santonin level in *Camellia oleifera* pollen was also below the LOD of the equipment, with 1.4×10^5 μg/kg of caffeine in *Camellia oleifera* pollen (Table 1). This indicated that the alkaloid content in *Camellia oleifera* nectar, pollen, and honey was relatively low.

Oligosaccharide Toxicity Tests on Honey Bee Larvae and Adult Worker Bees. After the forager bees have collected nectar and returned to their hive, the worker bees responsible for making honey remove the high level of water and convert the sucrose into simple sugar as it is eventually stored in the capped cells. Honey is the most stored food most consumed by honeybee larvae and adult bees. Therefore, toxicological tests were performed on honey bee larvae and worker bees using four specific oligosaccharides from *Camellia oleifera* honey.

The larvae were fed the individual oligosaccharides melibiose, manninotriose, raffinose, and stachyose and a mixture of these four oligosaccharides (the oligosaccharide content in the diet was comparable to *Camellia oleifera* honey), respectively. The predominant symptoms of toxicity in larvae were abnormal development, blackening, rotting, and shrinkage of the body during the excretion phase (7 day-old) (Figure S1), resembling the symptoms observed in the larvae of the colonies that visited *Camellia oleifera*. All larvae in the Raf, Sta, and mix groups died before reaching 8 days of age, except for the CG, Mel, and Mann groups (Figure 3A). The survival rate of larvae in the Mel group was not significantly different from that in the CG group (Table S9, comparisons no. 1), while the survival rate of larvae in the Mann, Raf, Sta, and mix groups was significantly lower than that in the CG and Mel groups (Table S9, comparisons no. 2, 3, 4, 5, 6, 7, 8, 9). There were no significant differences between the survival rates of larvae in the Raf and Sta groups (Table S9, comparisons no. 13). The survival rate of larvae in the mix group decreased the fastest with increasing age, followed by the Raf and Sta groups, whereas the survival rate of larvae in the Man group decreased a bit more slowly and the survival curve of the Mel group almost coincided with that of the CG group (Figure 3A).

No statistically significant difference was found in the survival of adult worker bees in the Mel group compared to the CG group (Table S11, comparisons no. 1) (Table S10; Figure 3B), whereby the average lifespan of adult worker bees in the Mann, Raf, Sta, and mix groups was significantly lower than that of the CG and Mel groups (Table S11, comparisons no. 2, 3, 4, 5, 6, 7, 8, 9) (Table S10; Figure 3B). Worker bees in the mixed group had the shortest average lifespan of 7.77 days; furthermore, the individual addition of stachyose and raffinose severely shortened worker bee lifespan, while manninotriose also shortened worker bee lifespan but was less lethal (Table S10). Toxic symptoms in worker bees include a swollen abdomen and slow crawling until death.

The results of oligosaccharide toxicology studies have indicated that manninotriose, raffinose, and stachyose in *Camellia oleifera* honey are toxic to honey bee larvae and worker bees, but a low level of melibiose was not harmful to larvae and worker bees. According to the coevolutionary hypothesis, plants and pollinators have evolved in concert with each other to achieve an mutualistic relationships.[30] However, such a mutualism has not been reached between honey bees and *Camellia oleifera*, as the latter produces nectar pollen that shortens the lifespan of pollinators during flowering. This paradoxical situation is likely explained by the fact that *Camellia oleifera* flowers in the cold winter months, and the raffinose and stachyose series of oligosaccharides synthesized within its flowers are energy-storing and low-temperature protectants.[31,32]

Alkaloid Toxicity Tests on Honey Bee Larvae and Adult Worker Bees. Santonin is a lactone compound derived from plants in the genus *Artemisia*. Santonin has been used as an anthelmintic and has obvious central nervous system toxicity.[33] Yet, the toxicity threshold of santonin to honey bees has been unclear. When honey bee larvae and adult worker bees were fed at 10 times the LOD level of santonin, there was no significant effect of santonin on larvae (from 2 to 8 day-old larvae) and worker bee survival rates were comparable to those of the CG group (Table S12, comparisons no. 1; Table S14, comparisons no. 1) (Table S13; Figure 4A,B). Caffeine can be found in the nectar of coffee and citrus plants, and naturally

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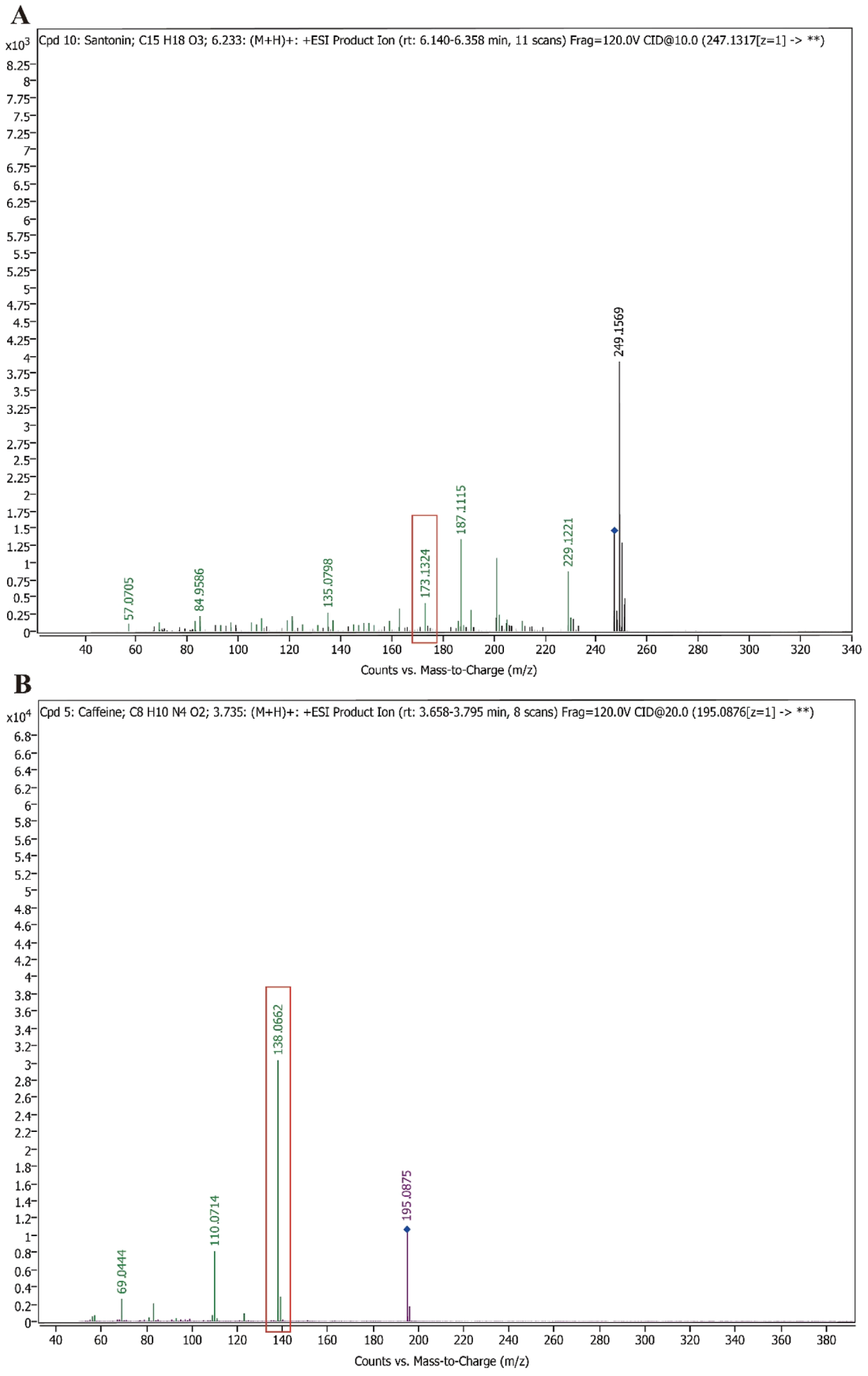

Figure 2. Mass spectrum of the alkaloids from the positive mode analyzed by HPLC/Q-TOF-MS. (A) Mass spectrum of santonin. (B) Mass spectrum of caffeine.

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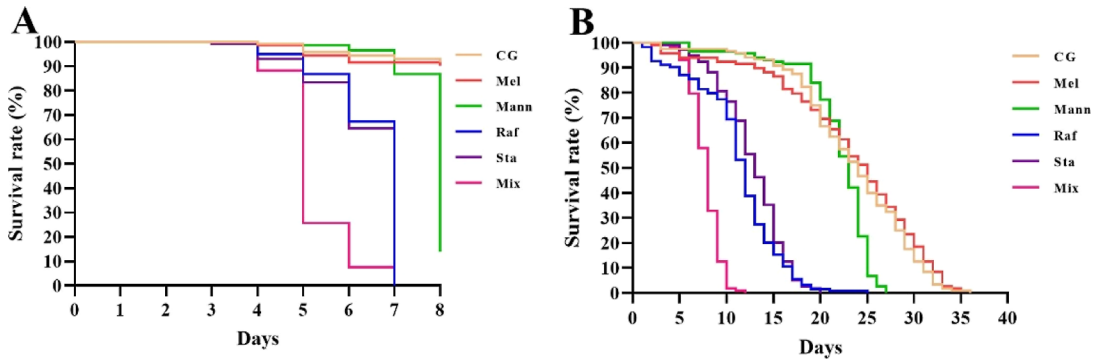

Figure 3. (A) Effect of oligosaccharides on the survival rate of honey bee larvae. (B) Effect of oligosaccharides on the survival rate of honey bee worker bees.

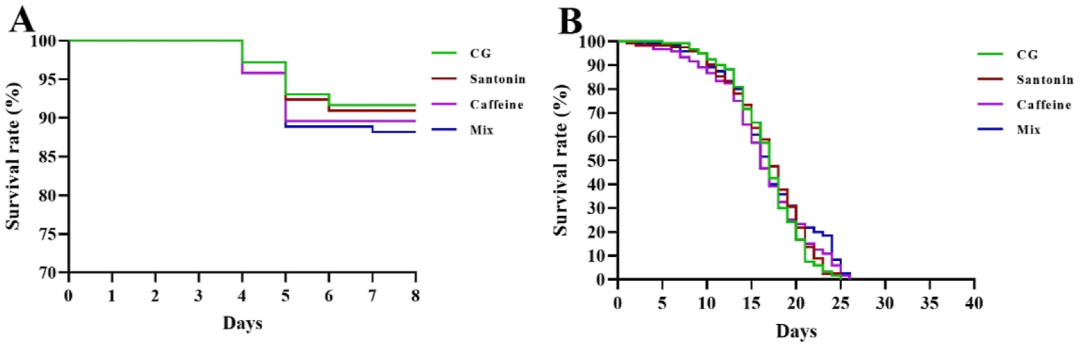

Figure 4. (A) Effect of alkaloids on the survival rate of honey bee larvae. (B) Effect of alkaloids on the survival rate of honey bee worker bees.

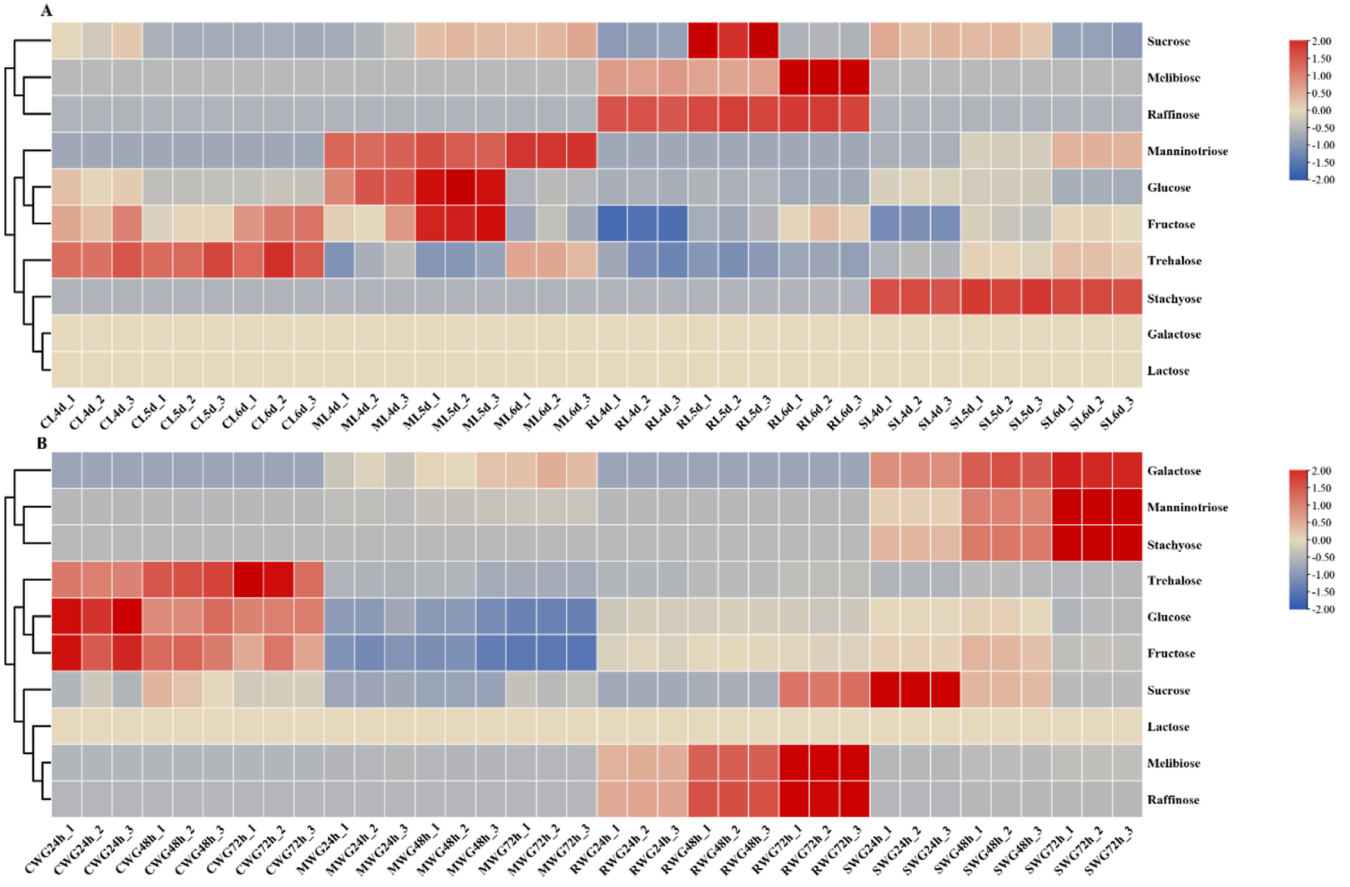

Figure 5. (A) Temporal metabolic heatmap of saccharides in larvae. (B) Temporal metabolic heatmap of saccharides in worker bee gut.

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low levels of caffeine do not harm bees.[34] Honey bees prefer solutions containing low concentrations of caffeine and have even been found to increase visitation rates and learn flower characteristics faster accordingly.[35,36] In the case of larvae and worker bees fed caffeine at levels comparable to those in *Camellia oleifera* pollen, caffeine showed no significant effect on larvae (from 2 to 8 day-old larvae) and worker bee survival rates compared to the CG group (Table S12, comparisons no. 2; Table S14, comparisons no. 2) (Table S13; Figure 4A,B). Of particular concern were larvae and worker bee survival rates in the mix group, which were non-significantly different from those in the CG, santonin, and caffeine groups (Table S12, comparisons no. 1, 2, 3, 4, 5, 6; Table S14, comparisons no. 1, 2, 3, 4, 5, 6) (Table S13; Figure 4A,B). Also, four groups of larvae were normally creamy white in color, with no signs of blackening or decay (Figure S2) and without abdominal bloating in any of the groups of adult worker bees. This means that traces of santonin and caffeine in *Camellia oleifera* nectar, pollen, and honey are not responsible for honey bee toxicity.

Analysis of the Temporal Metabolic Profile of Oligosaccharides in Larvae and Adult Worker Bees. To further explore the mechanisms of toxicity of manninotriose, raffinose, and stachyose to honey bees, we investigated the metabolic profiles of these three individual oligosaccharides in honey bee larvae and adult worker bees.

The molecule melibiose (Figure S3A) consists of one moiety of galactose connected to one moiety of glucose via α-1,6 glycosidic bond linkage, and melibiose is an isomer of lactose. Manninotriose (Figure S3B) has a "galactose-galactose-glucose" structure, and raffinose (Figure S3C) is a trisaccharide composed of galactose, glucose, and fructose. Indeed, manninotriose can be considered to have a galactose-melibiose structure, and raffinose can be described as having a melibiose-fructose structure. Two compositions of stachyose (Figure S3D), owing to the fact that it has a "galactose-galactose-glucose-fructose" structure, were considered: (1) raffinose-bound galactose; (2) manninotriose connected to fructose. Honey bees generally break down disaccharides into monosaccharides for energy.[37] However, the metabolic heat map shown in Figure 5A showed that manninotriose, raffinose, and stachyose were not metabolized into individual monosaccharides in the larvae, with the most representative hydrolysis products, melibiose and galactose, not being detected at all. Furthermore, manninotriose, raffinose, and stachyose were enriched in the larvae as age increased, but lower levels of raffinose and stachyose were hydrolyzed by β-fructofuranosidase into melibiose and manninotriose, and fructose is the only individual monosaccharide product of these two oligosaccharides (Table S15). We also found fructose, glucose, and sucrose present in all groups of larvae, and these non-toxic sugars for bees were derived from royal jelly (Table S15; Figure 5A). In contrast to the temporal metabolic profile of saccharides in larvae, raffinose and stachyose in the gut of worker bees can be partially hydrolyzed into melibiose and manninotriose, and the level of melibiose and manninotriose increased with time (Table S16; Figure 5B). This may be owing to the fact that worker bees have a superior ability to secrete β-fructofuranosidase relative to larvae.[38] Raffinose and stachyose are hydrolyzed because both include a sucrose molecule in their structure. Fructose, glucose, and sucrose in the gut of all groups of worker bees originated from their intake of sucrose. Notably, low levels of galactose and melibiose were detected in the gut of worker bees in the manninotriose group, while little galactose and raffinose were measured in the gut of worker bees in the stachyose group worker bees' gut (Table S16; Figure 5B). We speculate that the symbiotic bacteria in the gut of worker bees help the host hydrolyze a relatively small number of α-glycosidic bonds to release galactose.[39] Trehalose is a non-reducing disaccharide consisting of two glucose molecules bound by hemiacetal hydroxyl groups, and it is widely found in bacteria, fungi, caecilians, and some lower plants.[40] In the animal kingdom, trehalose was first reported in insects, where it is found not only in adult insects but also in larvae or pupae, where it is synthesized to store large amounts of glucose for energy.[41] It is worth noting that the trehalose level in larvae and worker bees of the manninotriose, raffinose, and stachyose groups was significantly lower than that of the control larvae and worker bees ($F_{12.85,0.17}$ = 77.83, P < 0.001; $F_{109.97,2.11}$ = 52.14, P < 0.001) (Tables S15, S16). This implies that the addition of manninotriose, raffinose, and stachyose (comparable to their levels found in *Camellia oleifera* honey) to the diet interferes with trehalose synthesis and energy storage in honey bee larvae and worker bees.

A comprehensive saccharide metabolic heatmap for larvae and worker bees indicated that honey bee larvae and worker bees have difficulty digesting manninotriose, raffinose, and stachyose (Figure 5A,B), likely because honey bees do not have enzymes to break down melibiose, manninotriose, raffinose, and stachyose.[42] Furthermore, the accumulation of manninotriose, raffinose, and stachyose in larvae and worker bees interferes with larvae development and causes abdominal bloating in worker bees, which eventually leads to larvae and worker bee mortality. In the future, the solution to the challenge of honey bee poisoning after pollinating *Camellia oleifera* is likely to be characterized by one of two aspects: (1) the use of drugs or probiotics to help honey bees break down oligosaccharides; (2) artificial selection and breeding of honey bee colonies that are resistant to these oligosaccharides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c04950.

Information on saccharide and alkaloid standards, fitted curves, saccharide and alkaloid content in honey, pollen, and nectar; analysis of survival curves of honey bee larvae and worker bees; and absolute content of saccharides in larvae and worker bees (PDF)

HPLC analysis for saccharides in nectar, pollen, honey, larvae, and gut of work bees; HPLC/Q-TOF-MS analysis for alkaloid species in nectar, pollen, and honey; HPLC analysis for alkaloid content in nectar, pollen, and honey (PDF)

CG, melibiose, manninotriose, raffinose, stachyose, and mix group larvae in 7 day-old; CG, santonin, caffeine, and mix group larvae in 7 day-old; chemical structure formula of melibiose, manninotriose, raffinose, and stachyose (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Zhijiang Zeng − *Jiangxi Province Key Laboratory of Honeybee Biology and Beekeeping, Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, P. R.*

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China; orcid.org/0000-0001-5778-4115; Phone: +86-791-83813998; Email: bees1965@sina.com

Authors

Zhen Li − *Jiangxi Province Key Laboratory of Honeybee Biology and Beekeeping, Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, P. R. China*

Qiang Huang − *Jiangxi Province Key Laboratory of Honeybee Biology and Beekeeping, Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, P. R. China*

Yu Zheng − *Jiangxi Province Key Laboratory of Honeybee Biology and Beekeeping, Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, P. R. China*

Yong Zhang − *Jiangxi Province Key Laboratory of Honeybee Biology and Beekeeping, Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, P. R. China*

Xin Li − *Jiangxi Province Key Laboratory of Honeybee Biology and Beekeeping, Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, P. R. China*

Shiqing Zhong − *Jiangxi Province Key Laboratory of Honeybee Biology and Beekeeping, Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, P. R. China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.2c04950

Author Contributions

Z.L.: collecting nectar, pollen, and honey samples, detection of samples, and writing original manuscripts. Q.H.: improving manuscripts. Y.Z.: collecting nectar, pollen, and honey samples and detection of samples. Y.Z.: collecting nectar, pollen, and honey samples. X.L.: collecting nectar, pollen, and honey samples. S.Z.: collecting nectar, pollen, and honey samples. Z.Z.: resources, funding acquisition, and improving manuscripts.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATION

ND, no detected

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