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RESEARCH ARTICLE

Effect of genetically modified maize expressing the Cry1Ab and EPSPS proteins on growth, development, and gut bacterial diversity of the non-target arthropod *Locusta migratoria*

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Abstract

The widespread cultivation of genetically modified (GM) crops has raised concerns for their safety. Here, we evaluated the effects of a GM maize variety expressing the Cry1Ab (14.76 ± 0.87 μg/g FW) and EPSPS proteins (191.55 ± 15.69 μg/g FW) on the life-history traits and gut bacterial community of a non-target arthropod, *Locusta migratoria*, in the laboratory. We found that GM maize had no significant effect on the survival or body weight of different development stages of *L. migratoria*. The midgut and hindgut bacterial diversities and compositions were determined using high-throughput sequencing targeting the V3–V4 regions of the 16S rRNA. No significant changes were found in the species diversity or abundance between insects in the GM-fed treatment and the non-GM control. Furthermore, the concentration of Cry1Ab and EPSPS in the gut was determined after digestion of GM maize. Results showed that the contents of Cry1Ab/EPSPS rapidly decreased and were hard to detect after 72 h. Based on the parameters assessed, we can conclude that the GM maize variety examined has no significant adverse effect on *L. migratoria*.

Keywords Genetically modified maize · Non-target species · Gut bacteria · Environmental risk assessment

Introduction

Genetically modified (GM) crops have been commercially available worldwide since 1996, and by 2019, there were more than 190.4 million hectares of GM crops planted in over 29 countries (ISAAA 2019). The global adoption of GM crops shows that the technology has proved itself environmental-friendly as well as able to produce significant socio-economic benefits (Smyth et al. 2015; Brookes and Barfoot 2018). China has devoted great effort to developing GM crops, which now include dozens of insect-resistant crops that produce Cry proteins derived from the bacterium *Bacillus thuringiensis* (Bt) (Liu et al. 2016a). One of the new GM maize varieties, DBN9936, is genetically engineered to express the Cry1Ab toxin and the CP4-EPSPS protein, and it confers resistance to both the Asian corn borer (*Ostrinia furnacalis*) and the oriental armyworm (*Mythimna separata*), as well as tolerance to

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the herbicide glyphosate herbicide (Zhang et al. 2021). No studies have been conducted on the potential effects of Cry1Ab/EPSPS-expressing Bt maize on the herbivore *Locusta migratoria*. The maize cultivar DBN9936 assessed in our study obtained its Chinese Production Application Security Certificate in 2019 and will likely be commercialized (Liang et al. 2021). Since its use has not yet been promoted in China, this risk assessment before their commercial release is relevant.

While Bt crops can contribute to increased productivity, their possible environmental impacts on non-target organisms have been a potential concern (Clark et al. 2005). The effects of GM crops on non-target arthropods (NTAs) have been well-studied through bioassays in many insects, especially in predator insects (Yaqoob et al. 2016). However, studies assessing risk for non-target herbivores are still inadequate (Prasifka et al. 2007; Ramirez-Romero et al. 2008; Li et al. 2010). In recent studies, no evident negative impacts of GM crops were found on non-target arthropods, but the bioassays used often concentrated on typical life table parameters, such as survival (Lu et al. 2014a, b; Wang et al. 2012). The indirect effects of GM crop proteins on the bacterial community, disease resistance, and immune responses of NTAs are also worthy of attention. Gut microbiota play a vital role in insect digestion, nutrient provisioning, detoxification of dietary toxins, and maintenance of gut homeostasis (Engel et al. 2012; Lee et al. 2015; Dai et al. 2019). The gut bacterial community shows a highly sensitive response to environmental factors; thus, this community's composition can be used as a parameter to evaluate the impact of GM plants on insects (Zhang et al. 2019).

During the assessment of risk to NTAs, the test species chosen such have, or potentially have, high exposure to Bt proteins (Garcia-Alonso et al. 2006). *Locusta migratoria* is a voracious insect that preferably feeds on maize, making it likely to be exposed to Bt toxins. Moreover, this locust is a significant link in regional food chains where it occurs, being a food resource for both vertebrate and invertebrate animals (Ochiai et al. 2020). When non-target herbivores feed on a GM plant, they ingest Bt proteins and potentially transfer them along to their predators (Meissle and Romeis 2018). Thus, evaluating the effects of GM maize on locusts can provide useful information on potential risks of GM crops.

In this study, we describe the effects of a new GM maize variety, expressing the Cry1Ab and EPSPS proteins, on the non-target insect *L. migratoria*. Our goals were (1) to determine the effect of GM maize on the growth and development of *L. migratoria*, (2) to quantify the spatiotemporal dynamic of the Cry1Ab/EPSPS proteins in the gut of *L. migratoria*, and (3) to determine if this GM maize affects the gut bacterial diversity of *L. migratoria*. These findings will provide information about a new aspect of risk assessment of GM crops on NTAs.

Materials and methods

Plant materials

The GM maize varieties DBN9936 (producing Cry1Ab/EPSPS) and the near-isogenic cultivar DBN318 (non-GM maize) were obtained from Beijing Dabeinong Biotechnology Co. Ltd. (DBNBC) (Beijing, China). All plants were grown in the same greenhouse in plots near each other, at China Agricultural University. Maize plants were grown following local agricultural practices, but without pesticide applications. The maize leaves of each variety were collected for ELISA tests at (1) the V3 leaf stage, (2) the V6-V8 leaf stage, (3) the VT tasseling stage, and (4) the R1 silking stage. The harvested leaf section was cut approximately 20 cm from the leaf tip. The samples were then placed immediately in liquid nitrogen and stored at − 80 °C until analyzed to determine the levels of Cry1Ab and CP4 EPSPS.

Insect rearing

The migratory locusts used in this study were obtained from the Key Bio-control Laboratory for locusts at the China Agricultural University, Beijing. Newly emerged first instar nymphal locusts were transferred into a wire cage (50 cm high by 15 cm in diameter) and fed fresh leaves at V6–V8 stage of either DBN9936 or DBN318. The leaves were replaced every 12 h. The locusts were reared in an insectary at 30 ± 1 °C, 60 ± 10% RH, and a 16:8 h L: D photoperiod.

Levels of Cry1AB and EPSPS in maize

The levels of the Cry1Ab and EPSPS proteins in maize leaves were quantified using the proprietary ELISA Kit (EnviroLogix Quantiplate Kit, Portland ME, USA) (Niu et al. 2013), performed according to the manufacturer's instructions. The quantitative detection limits of the Cry1Ab and CP4 EPSPS proteins for this kit were 0.1 ng/g and 0.1%, respectively. About 500 mg in total of fresh leaves of GM maize at the V3 leaf, V6–V8 leaf stage, VT tasseling stage, or R1 silking stage were collected from a pool of three randomly selected plants. Before grinding leaf tissues, the samples were washed five times with PBST buffer (made of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.05% Tween-20, at pH 7.4). Washed leaves were then mixed with 1 mL of extracting buffer (provided with the kits) and ground on ice using an electric grinding rod. The mixture was centrifuged (7000 × g, 15 min) and the resulting liquid was used to determine the concentration of Cry1Ab and EPSP protein by assessing the sample's optical densities (ODs), which were measured using the SpectraMax

i3x Multi-Mode Detection Platform (Molecular Devices, Sunnyvale, USA). Three experimental replicates were run for each sample type.

Insect bioassays

One hundred locusts, be newly emerged from their eggs, were individually fed with fresh leaves at V6–V8 stage of DBN9936, while another 100 were fed with DBN318, both for 35 days. Maize leaves fed to test insects were washed with sterile water before use. Survival of locusts was recorded on a daily base, and the body weights of individual locusts were measured immediately after molting.

Quantification of Cry1Ab/EPSPS proteins in the gut tract of L. migratoria

Newly emerged nymphs were fed with fresh non-GM maize leaves (DBN318) until the 4th instar. One day after molting, the nymphs in the treatment group were fed with 0.2 g of leaves of DBN9936 at V6–V8 stage, the GM maize. After feeding, the locusts were again fed with the non-GM maize, DBN318, until they molted to the 5th instar. The control locusts were only fed with leaves of DBN318 maize throughout the experiment. Each treatment (maize variety) had three replicates, with 50 insects per replication. At 6 h, 12 h, 24 h, 48 h, and 72 h after the consumption of GM maize, the foreguts, midguts, and hindguts of ten locusts in each of the treatment or control were collected to determine the concentration of Cry1Ab and EPSPS. The feces present at each sampling time were also collected for the protein assay. Each sample was homogenized in 1 mL of extraction EnviroLogix buffer, then centrifuged at 12, 000 × g for 10 min; then, the Cry1Ab and EPSPS proteins of the resultant supernatant were detected in ELISA plates.

Sample collection and DNA extraction of gut microbes

The locusts used for gut microbial assay have been grown under the conditions described in the paragraph: "Insect rearing." Fourth instar locusts, on day 3 after molting, were collected and washed with 75% ethanol. Then, the bodies were rinsed three times with sterile water before dissection. The midguts and hindguts of locusts from the GM-fed group and the control were collected. Each sample contained the midgut and hindgut tissues from 10 locusts, respectively. Three replicates of such samples were obtained and examined for both the treatment and the control. According to protocols described by Jia et al. (38), bacterial DNA was extracted from the pooled mid- and hindguts of ten locusts, for each of three samples. The quantity and quality of the DNA were measured with a NanoDrop 2000C spectrophotometer (Thermo Scientific, USA).

Amplification and sequencing of the V3–V4 region of the 16S rRNA gene

The V3–V4 region of the 16S rRNA gene was targeted with the barcoded primer pair 338F/806R primers for the bacterial community diversity analysis. The DNA samples were sequenced on the Illumina Miseq PE300 platform. The sequencing data were filtered and spliced to remove chimeras using GIIME (v1.8.0) software. Normalized reads were classified into Operational Taxonomic Units (OTUs) at 97% similarity. The taxonomy of the OTUs was assigned by blasting against SILVA database. Alpha diversity included Chao1 richness estimator, and the Shannon–Wiener index was calculated with Mothur (version 1.31.2). Beta diversity included binary Jaccard distances between samples was calculated with the UniFrac algorithm. The 16S rRNA gene sequences are available at the National Center for Biotechnology Information' s Sequence Read Archive (accession no. PRJNA80018).

Data analysis

Survival rates of locust nymphs were tested with a Kaplan–Meier analysis. Differences between groups were tested for statistical significance using the log-rank test. Data from the validation bioassay were found to be normally distributed and were analyzed using Student's *t*-test. The alpha diversity analysis was compared between the treatment and the control with Student's *t*-test. The taxonomic distribution analysis was based on the OTUs of the dominant bacterial genera, and differences were assessed with Student's *t*-test. All statistical analyses were conducted using the software package SPSS ver. 20 for Windows 2007 (SPSS, Inc. Chicago, IL).

Results

Effect of GM maize on growth and development of L. migratoria

The survival rate for *L. migratoria* nymphs fed either GM maize (DBN9936) or the non-GM control (DBN318) maize for 35 days was > 75% in both cases. There was no significant difference between the treatment and the control for any time period ($\chi^2 = 0.30$, $P = 0.58$) (Fig. 1). The GM maize produced no significant change of body weight of *L. migratoria* in any instar ($P = 0.8210$) (Fig. 2).

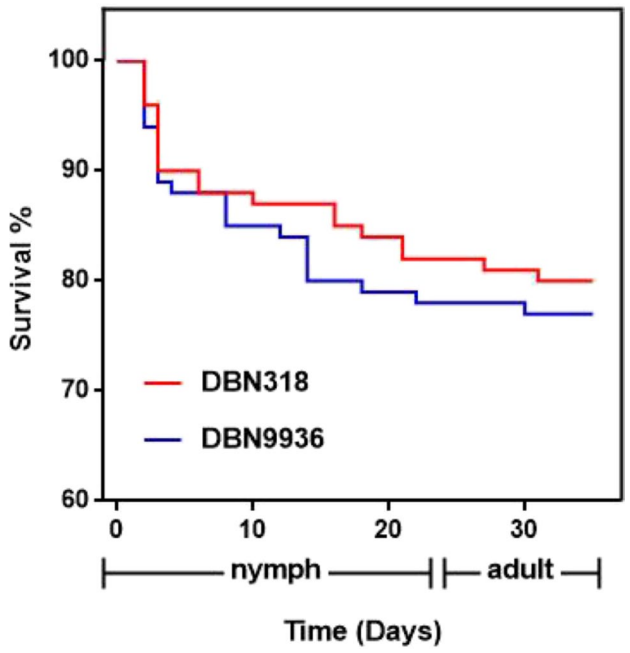

Fig. 1 Total survival of *Locusta migratoria* fed with DBN9936 (GM maize) or DBN318 (isogenic non-GM maize) for 35 days ($n = 100$). Kaplan–Meier method was used to analyze locust survival data

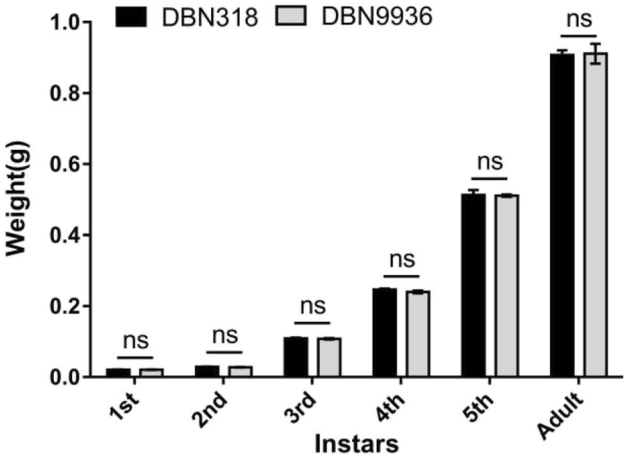

Fig. 2 The body weight of *Locusta migratoria* of different instars fed with different maize varieties (DBN9936 or DBN318 maize). Error bars represent standard errors. The statistical significance was assessed with Student's *t*-tests

Cry1Ab/EPSPS protein levels in gut tracts of L. migratoria fed with GM maize

The Cry1Ab and EPSPS levels in leaves of GM maize at the V6–V8 leaf stage was 14.76 ± 0.87 μg/g and 191.55 ± 15.69 μg/g fresh weight (FW), respectively. The Cry1Ab/EPSPS contents of GM maize at other stages are shown in Table S1. No Cry1Ab or EPSPS proteins were detected in the non-GM maize variety.

Concentrations of Cry1Ab and EPSPS in the gut declined at 6, 12, 24, 48, and 72 h after locusts were fed 0.2 g GM maize, showing that Cry1Ab and EPSPS proteins were gradually degraded in the gut. At 6 h, the concentrations of Cry1Ab in foregut, midgut, hindgut, and feces of locusts were 1044.33 ± 39.75 ng/g, 906.44 ± 90.83 ng/g, 499.08 ± 78.31 ng/g, and 183.85 ± 28.76 ng/g, respectively. The concentration of Cry1Ab in midgut of locust was higher than that of other regions of the gut 24 or 48 h after being fed with GM maize, and the concentrations of Cry1Ab in the gut were very low after 72 h (Fig. 3).

The concentration of the EPSPS protein in the foregut of locust was highest (2394.78 ± 200.87 ng/g) 6 h after being fed with GM maize. The concentration of the EPSPS protein in the midgut of locusts was 2147.16 ± 147.44 ng/g at 12 h after being fed with GM maize, and the concentration of EPSPS significantly decreased and hard to detect at 72 h after locusts were fed with GM maize. These proteins were not detected in the gut or feces of locusts fed with the non-GM control DBN318 variety.

Gut bacterial diversity in midgut of L. migratoria fed with GM maize

Paired-end sequencing of the 16S rRNA V3-V4 gene produced a total of 789,738 raw reads from 12 samples. Removing suspect or low abundance quality reads yielded 768,756 effective reads in the final analysis. The lengths of sequences were mainly distributed between 420 to 440 bp.

The diversity of bacterial communities in the midguts of locusts was determined using the alpha diversity method. The observed number of species (Obs) and Chao index were used to estimate community richness, while the Shannon index was applied for estimating community diversity. The

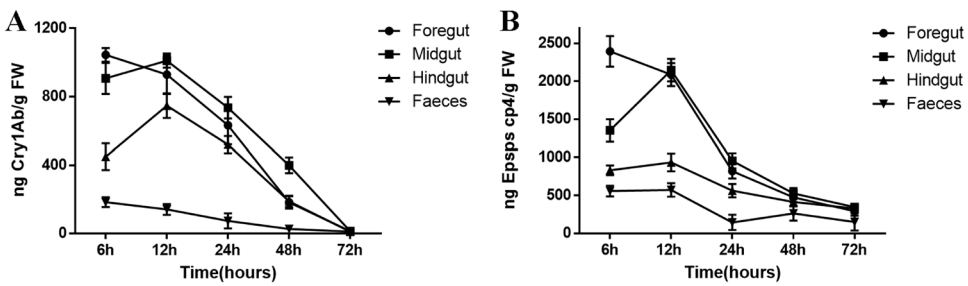

Fig. 3 Concentrations of Cry1Ab (**A**) and EPSPS (**B**) of the foregut, midgut, hindgut, and feces of 4th instar *Locusta migratoria* fed with DBN9936 leaves. Error bars indicate SE ($n = 3$)

gut bacterial diversity indices (Shannon index and Chao 1 index) of midguts of *L. migratoria* fed with GM maize showed no significant difference from that of locusts fed non-GM maize ($P = 0.653$, $P = 0.993$, respectively). The observed number of species of gut bacteria was relatively low in locusts fed with GM, and showed no significant difference from the control locust gut community ($P = 0.682$) (Fig. 4A).

The taxonomic distributions of midgut bacteria at class and order levels are summarized in Fig. 5. Based on the average relative abundance, the most abundant orders were Enterobacteriales (30.6%), Pseudomonadales (19.3%), Rhizobiales (4.1%), and Flavobacteriales (4.5%) in the midgut of locust. The relative abundance of Enterobacteriales was 30.0% after being fed with GM maize (Fig. 5A). The compositions of the dominant bacterial genera showed no significant difference between treatment and control ($P > 0.05$ for all).

Bacterial diversity in hindguts of L. migratoria fed with GM maize

The alpha diversity indices Obs, Chao, and the Shannon index of hindgut microbes of locusts fed with GM maize

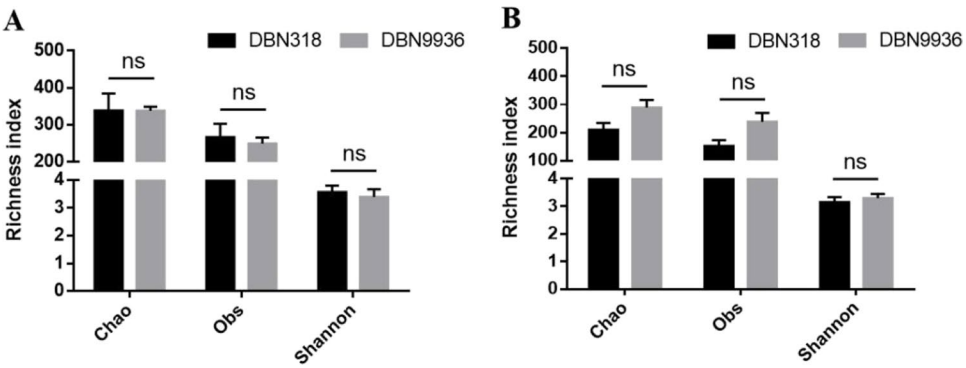

Fig. 4 Effects of maize varieties on diversity metrics of bacteria in the midgut (**A**) and hindgut (**B**) of locusts. Richness measured as Chao, observed species (Obs), and Shannon indices of gut bacterial communities from different treatment. Statistical comparison was based on Student's *t*-test (*$P < 0.05$)

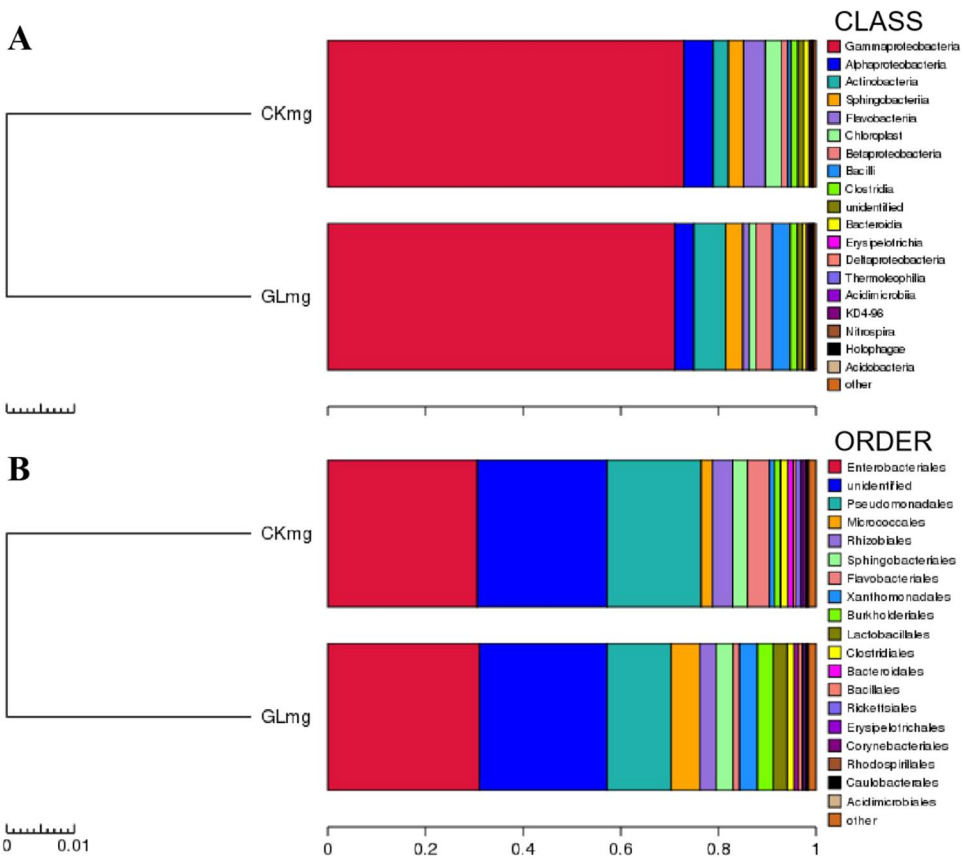

Fig. 5 Relative abundance of the highly represented bacterial taxa communities in the midguts of *Locusta migratoria* at the class (**A**) and order (**B**) levels. Each bar represents the average relative abundance of each bacterial taxon within a group. CKmg midgut in controls, GLmg midgut in GM-fed locusts

showed no significant difference from that of the control ($P = 0.573$, $P = 0.097$, and $P = 0.08$, respectively) (Fig. 4B). Based on average relative abundance, the most abundant bacterial orders in the hindguts of locusts fed GM maize were Enterobacteriales (41.0%), Pseudomonadales (30.9%), Rhizobiales (4.6%), and Sphingobacteriales (4.0%). The abundance of the dominant bacterial genera of the hindguts of locusts fed with GM maize showed no significant difference from that of the control ($P > 0.05$ for all), but the average proportion of main groups of the gut microbes showed some difference. The average relative abundance of Enterobacteriales was slightly lower in GM-fed locusts than control (41.0% vs. 29.0%), while that of Pseudomonadales was higher than in the control (30.9% vs. 28.1%) (Fig. 6A). Clustered heat maps of the top 20 abundant bacterial communities are shown in Fig. S1. This figure shows that most of the bacterial taxa found were clustered together in the hindgut and midgut of locust.

Discussion

Safety evaluations are essential to determine the effect of GM crops on NTAs before the crops become commercially available, and laboratory assessments are important parts of such safety assessments (Romeis et al. 2008). In the current study, the results of the feeding bioassay and the diversity of gut microbes were analyzed to determine the effects of GM maize (expressing Cry1Ab and EPSPS proteins simultaneously) on the non-target organism *L. migratoria.*

Such assays require prior information as to the expression levels of the toxins in the GM crop's tissues before performing risk assessments since the conclusions can be strongly affected in the bioassays with NTAs (Romeis et al. 2008). The levels of toxins in GM crops vary markedly among tissues and growing seasons (Li et al. 2011). This variation makes it essential to quantify the levels of toxins in specific tissues of GM crops at a specific point in time before conducting risk assessment. We quantified Cry1Ab and EPSPS expression in GM maize leaves at different stages and used the GM maize leaves at the jointing stage as the test plant stage (14.76 ± 0.87 μg/g FW for Cry1Ab, 191.55 ± 15.69 μg/g FW for EPSPS). It is worth mentioning that the current safety evaluation on NTAs mainly uses varieties expressing single or multiple insect resistance genes as research objects. Stacked traits in GM plants (especially insect resistance and herbicide tolerance) have been widely employed in recent years, while their joint risk assessment is lacking. Our results showed that DBN9936 maize had no effect on the development traits of locusts, which was consistent with previous work on non-target herbivores (Romeis et al. 2019). For risk assessment of GM crops with dual

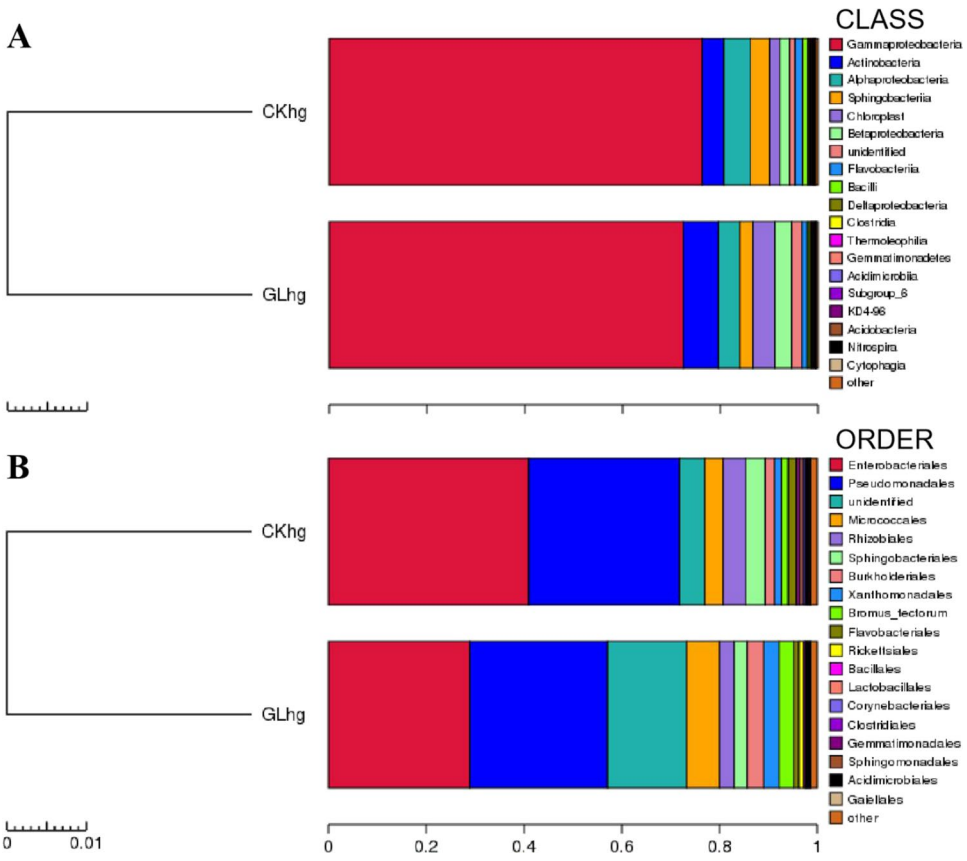

Fig. 6 Relative abundance of the highly represented bacterial taxa communities in hindgut of *Locusta migratoria* at the class (**A**) and order (**B**) levels. Each bar represents the average relative abundance of each bacterial taxon within a group. CKhg hindgut in controls, GLhg hindgut in GM-fed locusts

genes, previous studies on NTAs have mainly focused on predators and pollinators as target NTAs (Han et al. 2010; Liu et al. 2016b). Zhao et al (2013) reported that the transgenic Bt cotton expressing Cry1Ac/Cry2Ab or Cry1Ac/EPSPS did not affect the lady beetle *Propylaea japonica* via consumption of its prey *Aphis gossypii*. Similar results were observed in the plant bug *Adelphocoris suturalis* or the pollinating beetle *Haptoncus luteolus* (Niu et al. 2018). Cotton pollen containing Cry1Ac/EPSPS or Cry1Ac/2Ab as a test substance did not affect the expression of detoxification genes in honeybees (Niu et al. 2017). To the best of our knowledge, this is the first study to determine the effect of GM crops expressing dual proteins on orthopteran insects.

Data on Bt toxin levels s in herbivores are scarce (Meissle and Romeis 2018). Previous studies have focused on levels of toxins in individual whole insect or in the hemolymph, while how toxins are metabolized in the gut is understudied, particularly for dual gene crops (Meissle and Romeis 2018; Wang et al. 2020). Previous reports have indicated that Bt toxins interact with the gut epithelium and damage the cellular structure and permeability after reaching the gut of susceptible insects (Bravo et al. 2004; Hendriksma et al. 2013). Determinations of GM protein concentrations after the proteins or GM plant materials have entered the insect gut are valuable for the study of protein toxicity and metabolic mechanisms. In the current study, we established the spatiotemporal dynamic of proteins in the gut of *L. migratoria* by repeatedly measuring the concentrations of Cry1Ab and EPSPS proteins in the gut of locusts. The concentrations of proteins in the gut and feces of locusts declined with time after locusts were fed with GM maize leaves; the levels of these proteins in guts are various time post ingestion were lower than those in GM maize leaves. Meissle and Romeis (2018) measured Cry1Ab and Cry2Ab levels in several herbivores fed on Bt cotton leaves and found that the concentrations of Bt proteins in individual herbivores ranged from 5 to 50% of the concentrations measured in leaves. Low levels of Bt toxins have also been found in some non-target arthropods (*Nilaparvata lugens* and honey bees) that fed on Bt crops (Hendriksma et al. 2013; Han et al. 2014). The EPSPS protein was hard to detect in the gut of locusts fed on GM maize after 48 h, but a low concentration of Cry1Ab was found in the gut (Fig. 3). Higher metabolic rates of EPSPS were observed in the gut compared with Bt toxin Cry1Ab, which may be associated with the binding of the Bt toxin and the brush border membrane vesicle (BBMV) (Luo et al. 2006). The binding between Bt toxins and BBMV caused a reduction in the metabolic rate (Bravo et al. 2004; Zhao et al. 2016). To figure out the mechanism of slow metabolism, the interaction between Cry1Ab proteins and BBMV in locusts needs further study.

Since the composition of the gut microbiota of herbivorous insects is susceptible to the external environment and the herbivore's consumption, it has been included in risk assessments of GM crops for honeybees (Jiang et al. 2013; Dai et al. 2019). Reports indicated that Bt infections could increase the susceptibility of the host to other pathogens and alter the fitness of insects through disruption of the gut homeostasis (Patil et al. 2013; Motta et al. 2018). We evaluated the effects of GM maize on the midgut and hindgut bacterial communities of locusts by 16S rRNA sequencing. The alpha diversity indices of gut bacteria of locusts fed with GM maize leaves did not show significant changes, which was consistent with former studies on the impact of Cry toxins on gut microbes of honeybees and the microbial community of *Propylea japonica* (Jia et al. 2016, 2017; Martinson et al. 2012; Zhang et al. 2019). The dominant groups of gut bacteria in insects are stable, and only the relative proportions of these groups show differences, depending on host diet, age, and habitat (Dillon et al. 2005; Tan et al. 2015, 2021; Ng et al. 2018). We characterized the dominant bacteria of the locust's midgut, which were in the Enterobacteriales, Pseudomonadales, Rhizobiales, and Flavobacteriales, and we found that there were no significant differences in the gut bacterial composition between locusts fed GM maize versus control maize. The combined assessments of growth, development, and gut microbiota of locusts can increase our ability to accurately estimate both possible direct and indirect effects of GM crops.

In conclusion, GM maize expressing the Cry1Ab and EPSPS proteins had no significant impact on the survival, larval weight, or gut bacterial diversity of *L. migratoria.* The levels of Cry1Ab and EPSPS in the gut of locusts were also determined. These results allow the risk associated with the cultivation of GM maize to be estimated, and the methods developed can be used to assess risk of GM crops in general.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-022-20147-8.

Author contribution Yue Yin: investigation, writing — original draft. Yudi Xu: formal analysis. Kaili Cao: investigation. Xinxin Zhao: validation. Chuan Cao: methodology. Xuehui Dong: funding acquisition. Jingang Liang: review and editing. Wangpeng Shi: methodology, writing — review and editing.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interest The authors declare no competing interests.

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