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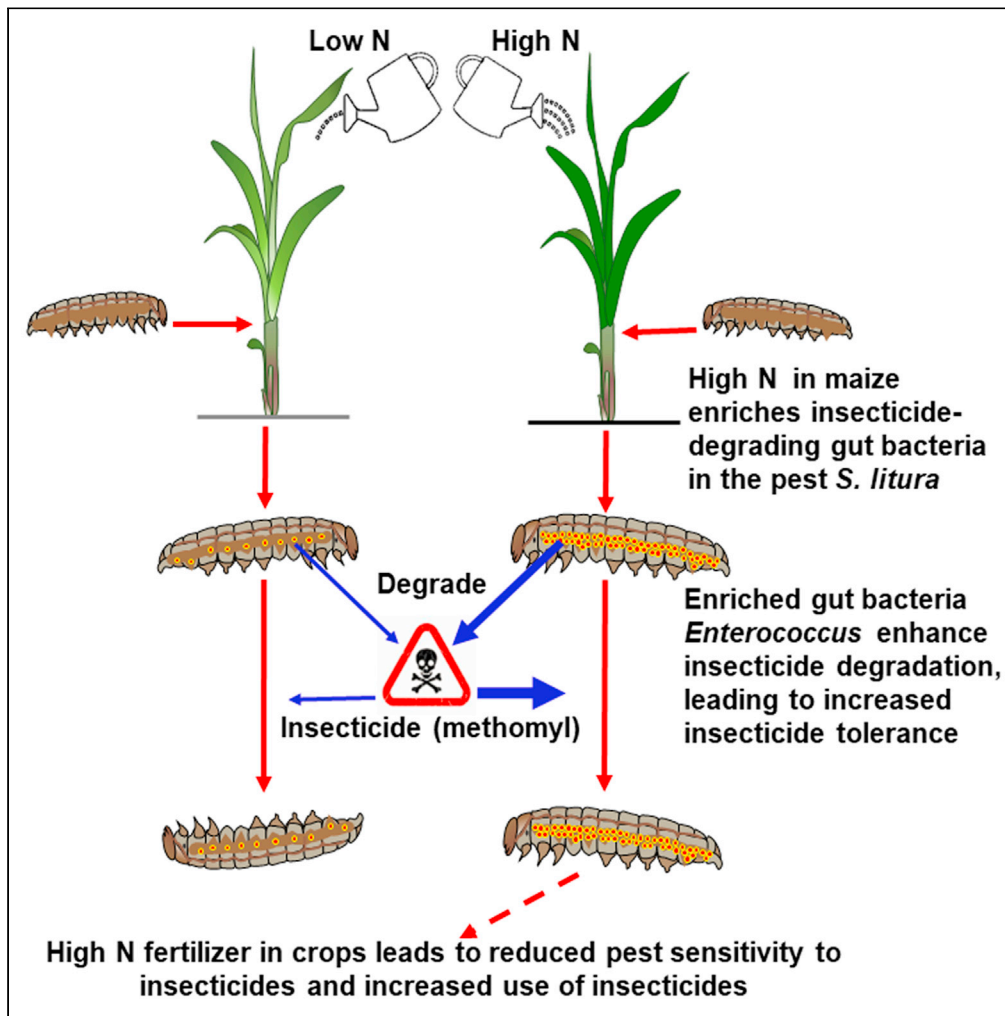
High nitrogen in maize enriches gut microbiota conferring insecticide tolerance in lepidopteran pest *Spodoptera litura*

High N in maize enriches insecticide-degrading gut bacteria in the pest *S. litura*

Degrade

Enriched gut bacteria *Enterococcus* enhance insecticide degradation, leading to increased insecticide tolerance

Insecticide (methomyl)

High N fertilizer in crops leads to reduced pest sensitivity to insecticides and increased use of insecticides

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Highlights

High N applied in maize plants enhances insect tolerance to the insecticide methomyl

High N promotes the gut bacterial proliferation in the genus *Enterococcus*

Two gut bacterial strains (*E. mundtii* and *E. casseliflavus*) degrade methomyl

Depleting the gut microbiota in *S. litura* increased larval sensitivity to methomyl

Article

High nitrogen in maize enriches gut microbiota conferring insecticide tolerance in lepidopteran pest *Spodoptera litura*

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SUMMARY

Abuse of chemical fertilizers and insecticides has created many environmental and human health hazards. We hypothesized that high nitrogen (N) in crops changes insect gut microbiota leading to enhanced insecticide tolerance. We investigated the effect of high N in maize on gut microbiota and insecticide tolerance of the polyphagous pest *Spodoptera litura*. Bioassays showed that high N applied in both maize plants and artificial diets significantly enhanced larval growth but reduced larval sensitivity to the insecticide methomyl. High N promoted the gut bacterial abundance in the genus *Enterococcus*. Inoculation with two strains (*E. mundtii* and *E. casseliflavus*) isolated from the larval guts increased larval tolerance to methomyl. Incorporation of antibiotics in a high-N diet increased the larval sensitivity to methomyl. These findings suggest that excessive application of N fertilizer to crops can increase insecticide tolerance of insect pests via changing gut microbiota, leading to increased use of insecticides worldwide.

INTRODUCTION

Since the early 20th century, industrial scale synthesis of ammonia (NH_4) removed a major constraint of nitrogen (N) in agricultural production. Concomitantly, extensive use of insecticides substantially reduced agricultural losses due to insect pests. Combined application of N fertilizers and insecticides have greatly contributed to increased global food production and human population growth (Galloway et al., 2008; Jarman and Ballschmiter, 2012). However, excessive use of chemical fertilizers has also created an array of environmental hazards, including groundwater contamination, eutrophication, increased emissions of greenhouse gases such as nitrogen oxides, ammonia, and nitrous oxide contributing to global warming (Guo et al., 2010; Nicholson, 1967). Excessive use of insecticides has also generated an array of severe environmental and food safety hazards. Pest populations have rapidly evolved insecticide resistance, which is a global problem that severely compromises efficiency of pest management (Gould et al., 2018).

Nitrogen (N) is one of the most important nutrient elements required for growth and reproduction of both plants and herbivorous insects (Chen et al., 2010). It is the vital nutrient element that mediates plant-insect interactions. The N content in host plants is a key determinant of the fecundity of herbivorous insects at both individual and population levels (Awmack and Leather, 2002; Mattson, 1980). Most studies show that high-N fertilizer application to crops significantly increases herbivore feeding preference, food consumption, population density and the chance of survival, growth, and reproduction (Bentz and Larew, 1992; Jansson and Smilowitz, 1986; Jauset et al., 2000; Minkenberg and Ottenheim, 1990; Moon et al., 2010; Nevo and Coll, 2001). An optimal amount of fertilizer is needed so as to attain an ideal crop yield without financial loss and environmental pollution. Excessive N application that exceeds the optimal amount of fertilizer application makes many crop plants more susceptible to pests and significantly contributes to the increased pest populations. Dramatic increases in populations of major insect pests of rice are closely associated with the long-term excessive application of N fertilizers (Lu et al., 2007). Much higher densities of cotton aphids were found in plants fertilized with high N compared with low-N-fertilized plants (Cisneros and Godfrey, 2001). High N results in decreased concentration of plant secondary metabolites and increased nutritional values as food for phytophagous insects (Bryant et al., 1987; Kainulainen et al., 1996; Stout et al., 1998). Increasing N application to maize significantly increased larval weight and survival

and female fecundity of *Sesamia calamistis* (Lepidoptera: Noctuidae) (Setamou and Schulthess, 1993). Consequently, farmers have to use more insecticides to control insect pests. Conversely, low N induces host plant anti-herbivore resistance. Nitrogen deficiency in barley (*Hordeum vulgare*) induced plant defense responses, leading to failure of development of green peach aphid (*Myzus persicae*) (Comadira et al., 2015). However, direct relation between N fertilizers and insecticide consumption is rarely examined.

Animal guts harbor trillions of microorganisms that play fundamental roles in many aspects of biology, including metabolism, growth, reproduction, and immune function. Animal diets can have a profound effect on the gut environment and microbial composition (Scott et al., 2013). The gut microbiota of insects has also been reported to be influenced by diets (Yun et al., 2014). For example, diet is an important factor shaping the gut microbial community in adults and larvae of dung beetles (*Euoniticellus intermedius* and *Euoniticellus triangulatus*) (Shukla et al., 2016). Different diets can markedly change both the abundance and composition of gut microbiota of *Adelphocoris suturalis* (Luo et al., 2021).

The microbial community in the gut tract of a host organism is critical to the development, diseases, and immune responses of the host (Engel and Moran, 2013; Douglas, 2015). Characterization of the gut microbiota and their functions in insect pests is crucial for pest management. Insect gut tracts create a unique environment that harbors the most densely populated microbiota, which provide many beneficial services to their hosts (Engel and Moran, 2013). Gut microbes can influence a wide range of processes such as physiology, ecology, and evolution of host insects (Oliver and Martinez, 2014). Increasing evidence shows that the insect herbivore-associated microbes can profoundly shape plant-insect interactions by suppressing plant defenses and detoxifying defensive phytochemicals (Shikano et al., 2017). For example, gut bacteria in coffee berry borer (*Hypothenemus hampei*) can degrade caffeine and use caffeine as a sole source of carbon and nitrogen (Cejanavarro et al., 2015). Gut microbiota of pine weevil (*Hylobius abietis*) are able to degrade conifer diterpenes and increase nutrition of their diet to increase insect fitness (Berasategui et al., 2017). Bacteria from oral secretions of the fall armyworm *Spodoptera frugiperda* inhibit the activity of plant defensive proteins polyphenol oxidase and trypsin proteinase inhibitors (trypsin PIs), and thereby enhance caterpillar growth in tomato (Acevedo et al., 2017). Colorado potato beetle (*Leptinotarsa decemlineata*) larvae exploit bacteria in their oral secretions as a decoy to deceive plants into incorrectly perceiving the threat as microbial infection to activate salicylic acid signaling pathway, and consequently suppress jasmonic acid signaling pathway and antiherbivore defenses in tomato (Chung et al., 2013). Most insect gut bacteria are commensal, but some can become pathogenic and have negative effects on their host (Mason et al., 2021).

Previous studies demonstrated that gut microbes are involved in insecticide degradation in several insect species. For example, bean bug *Riptortus pedestris* can easily acquire *Burkholderia* gut symbiont from surrounding soil, which degrades fenitrothion and confers insecticide resistance to host insects (Kikuchi et al., 2012); honeybee (*Apis mellifera*) gut microbiota promotes host endogenous detoxification capability via regulation of P450 gene expression (Wu et al., 2020); and gut microbes in *Drosophila melanogaster* metabolize the insecticide imidacloprid to nitro-reduced metabolites (Fusetto et al., 2017). In the wild population of oriental fruit fly *Bactrocera dorsalis* a gut bacterium plays a critical role in host resistance to organophosphate insecticide trichlorphon (Cheng et al., 2017). Gut-associated bacteria are subjected to the selection pressure imposed by insecticides applied on their hosts and influence the metabolization of insecticides in *Spodoptera frugiperda* (Almeida et al., 2017). A widespread endosymbiont, *Wolbachia*, can protect the small brown planthoppers (*Laodelphax striatellus*) against the commonly used insecticide, buprofezin (Li et al., 2020).

Since N is a key nutrient element for all organisms and diets have profound influence on insect gut microbiota (Yun et al., 2014; Shukla et al., 2016; Luo et al., 2021), we hypothesized that high rate of N in maize will change gut microbial biota in *S. litura* larvae, leading to enhanced tolerance of herbivore insects to insecticides, because N is a limited but critical element in numerous compounds involved in primary metabolic activities of plants, insects, and microbes. In this study we used maize (*Zea mays* L.), tobacco cutworm (*Spodoptera litura*), and insecticide methomyl to examine the effect of high N application in maize plants on insecticide sensitivity of the larvae fed on the plants. We evaluated the changes in the growth, insecticide sensitivity, and gut bacteria of *S. litura* larvae fed on hydroponic maize seedlings cultivated with different N concentrations. We then isolated and cultivated gut bacteria with the capacity to metabolize the insecticide methomyl from the larvae fed on high-N-cultivated plants. Finally, we evaluated changes in larval sensitivity to insecticide methomyl after inoculation and manipulation of gut bacteria. We found
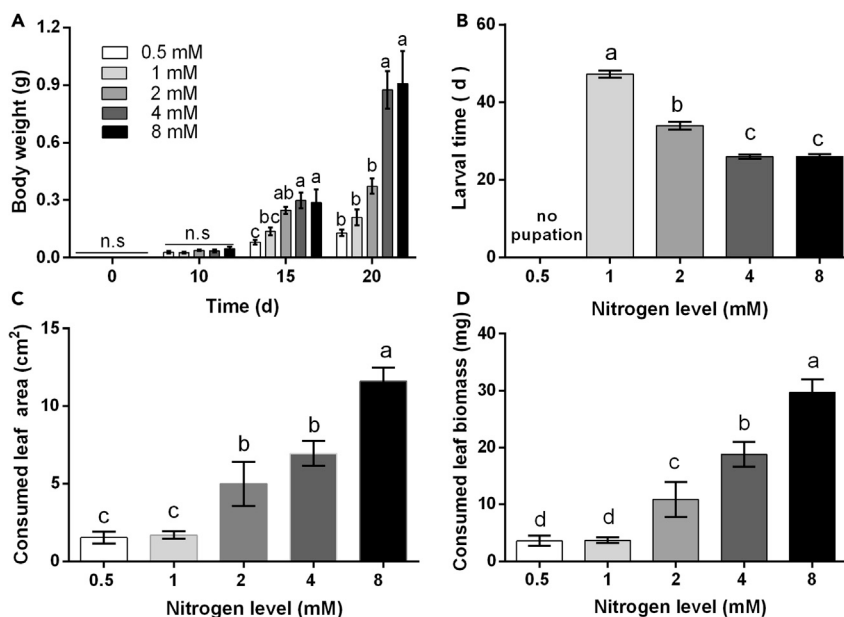
Figure 1. Growth and feeding performance of *Spodoptera litura* larvae fed on maize seedlings hydroponically grown under different nitrogen levels

Body weight (A) and larval duration (B) after feeding on maize plants with different nitrogen levels. Newly hatched first-instar larvae were inoculated on hydroponic 14-days-old maize plants cultured with different nitrogen levels (0.5–8.0 mM). Consumed leaf area (C) and biomass (D) of hydroponic 30-day maize plants cultured with different nitrogen levels (0.5–8.0 mM) infested by third-instar larvae for 4 days. Consumed leaf area was measured by leaf scanning. Consumed leaf biomass was calculated by leaf water content. Values are mean $\pm$ standard error (n = 15). Different letters above bars indicate significant differences among treatments ($p < 0.05$) according to one-way ANOVA with Tukey's multiple comparison test.

that high N application on maize plants increased insecticide tolerance of *S. litura* larvae fed on maize and changed gut microbiota. High N promoted gut bacterial proliferation in the genus *Enterococcus*, and two *Enterococcus* bacterial strains isolated from the larval guts were able to degrade methomyl.

RESULTS

Effects of different N rates on larval growth and performance on maize plants

In the larval developmental trial, 15 and 20 days after inoculation there were significant differences among different N treatments ($p < 0.001$) but no significant difference was found 10 days after the treatment ($p = 0.285$, Figure 1A). Twenty days after inoculation the body weights of larvae on maize plants with 4 and 8 mM N were 6.7- and 7.0-fold, respectively, compared with the 0.5 mM N treatment. No significant difference in larval body weight was observed between 4 and 8 mM N treatments, and no significant differences were found among 0.5, 1.0, and 2.0 mM N treatments at 20 days.

No larva successfully pupated when fed on plants with 0.5 mM N (Figure 1B). Larval duration significantly decreased with increasing N rates ($p < 0.001$). The larval stage lasted for 47 and 34 days when fed on plants with 1.0 and 2.0 mM N, respectively, whereas it lasted for 26 days when fed on plants with 4.0 and 8.0 mM N.

In the short-term feeding trial, the third instars were inoculated on maize plants with different N rates and fed only for 4 days. The larvae fed on high-N plants consumed significantly more leaves than those on low-N plants ($p < 0.001$, Figures 1C and 1D). The larvae in 0.5 and 1 mM treatments consumed the least leaf area (1.59 and 1.63 cm^2) and leaf biomass (3.60 and 3.69 mg). The most consumed leaf area (12.81 cm^2) and biomass (32.83 mg) were found in plants with 8 mM N supply.

Effects of different N rates on larval sensitivity to insecticide

Four days after feeding on plants provided with different N, larval body weight increased with N level ($p < 0.001$, Figure 2A). The nitrogen contents of plant leaves were shown in Figure S1. Feeding on low-N plants (0.5 and 1 mM)
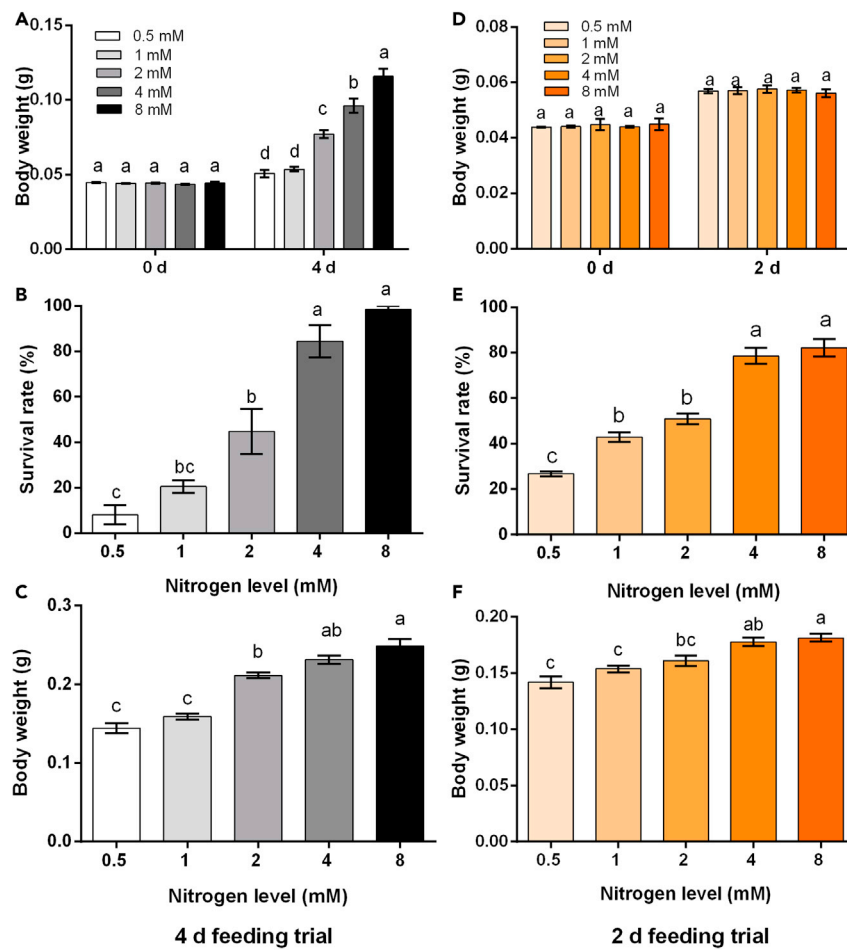
Figure 2. Growth and insecticide (methomyl) sensitivity of *Spodoptera litura* larvae fed on maize seedlings hydroponically grown under different nitrogen levels

Maize seedlings were hydroponically cultivated with different levels of nitrogen for 30 days. The third-instar larvae were transferred to the leaves for 4 (A, B, C) and 2 days (D, E, F) and then used for topical application with methomyl (B, E) and exposure to diets containing methomyl (C, F).

(A, D) Body weight of *S. litura* larvae 4 and 2 days after feeding on maize plants.

(B, E) Survival rate of *S. litura* larvae 24 h after topical application with methomyl (2 μL× 0.015 μg/μL per larva).

(C, F) Body weight of *S. litura* larvae 24 h after diet exposure to methomyl (0.005 mg/g). Values are mean ± standard error from three replicates (20–25 larvae/replicate). Different letters above bars indicate significant differences among treatments ($p < 0.05$) according to one-way ANOVA with Tukey's multiple comparison test (A, C, D, F) and chi-square test with a Bonferroni correction for multiple tests (B, E).

S. litura larvae gained little weight. The larvae fed on the highest-N plants gained the heaviest weight. More intriguingly, feeding on plants provided with different N rates led to significant difference in survival after topical application with methomyl ($p < 0.001$, Figure 2B). The larval survival rate significantly increased with N supply to host plants. Larvae fed on maize plants supplied with the highest rate of N (8.0 mM) showed very high survival (98.4%). However, those fed on maize plants suppled with low rate of N (0.5 mM) showed very low survival (8.1%).

After larvae were reared on plants provided with different N rates and then exposed to diets containing methomyl, significant difference in larval sensitivity to the insecticide was observed ($p < 0.001$, Figure 2C). Larvae fed on plants provided with high N rates (4 and 8 mM) showed significantly heavier body weight relative to low N rates (0.5 and 1 mM) when growing on artificial diets containing methomyl. Body weight of the larvae at 4 and 8 mM increased by 60% and 70%, respectively, relative to low N rate (0.5 mM) 24 h after growing on diets containing methomyl (Figure 2C). No significant difference in larval weight was observed between 4 and 8 mM N rates or between 0.5 and 1.0 mM rates (Figure 2C).

To exclude the possible effect of body weight on larval sensitivity to the insecticide, third-instar larvae were reared on plants treated with different N for only 2 days. In this short time period, no significant difference in body weight was found among larvae fed on plants with different N rates (p = 0.907, Figure 2D). However, two days feeding on plants provided with different N rates led to significant difference in larval sensitivity to the insecticide in both topical application and diet administration experiments with methomyl (p < 0.001, Figures 2E and 2F). The larval survival rate significantly increased with N supply to host plants regardless of similar body weight. The survival rates at 0.5 and 8 mM N were 26.8% and 82.1%, respectively, 24 h after topical application with methomyl (Figure 2E). There was no significant difference in body weight between larvae fed on plants provided with 2 mM and 4 mM N (Figure 2F). No significant differences in survival rate and body weight were found between larvae fed on plants provided with 4 mM and 8 mM N (Figures 2E and 2F).

Gut microbiota affected by different N supply in maize

In the nine representative gut samples from larvae fed on maize plants cultured at three N rates, we obtained a total of 72,910 sequences and grouped them into 79 OTUs at 97% similarity cut-off level. Rarefaction curves of the nine samples almost reached equilibrium, indicating that most bacterial sequences obtained by the MiSeq sequencing system reflected the abundance and diversity of microbiota (Figure S2). Alpha diversity was estimated by five indices including number of OTUs, ACE, Chao1, Shannon, and Simpson indices. Four indices (number of OTUs, Chao1, ACE, and Shannon) were lower in gut microbiota of larvae fed on high-N plants, but no significant differences were found among the other three N treatments (Table S1).

The phylogeny-based weighted UniFrac principal coordinate analyses considering relative abundances of OTUs showed that samples from different N treatments clustered independently. The fact that the same treatment samples can be grouped into a cluster confirms that these gut samples share similar microbial compositions (Figure 3A).

The heatmap for clustering with relative abundance of species at genus level is shown in Figure S3. According to the clustered heatmap, the genera that accounted for different proportion were presented by different colors and locations of clustering in heatmap. Abundance of *Rhodobacter*, *Devosia*, *Bacillus*, *Enterococcus*, and *Staphylococcus* was significantly higher in the guts of larvae fed on high-N-treated plants than that on low-N plants.

The specific species that showed significant difference between high N (8 mM) and low N (0.5 mM) treatments at each taxa level was identified using linear discriminant analysis effect size (LEfSe) (Figure 3B). We found significantly different abundant functional profiles of the gut microbiota between the high N (8 mM) and low N (0.5 mM) treatments. A total of five taxa with discrepancy in relative abundance were presented in high and low N groups, respectively. At the genus level, the relative abundance of *Enterococcus* was remarkably higher in the guts of larvae fed on high-N plants.

Isolation and identification of methomyl-degrading bacteria from *S. litura* gut

Using mineral media with methomyl as sole carbon and N source, nine bacterial strains were isolated from the guts sampled from *S. litura* larvae fed on maize seedlings under high N level (8 mM) (Table S2). The 16S rRNA gene sequences showed the highest similarity of these strains with *Enterococcus mundtii* QU 25 (GenBank:NC_022878.1) and *Enterococcus casseliflavus* EC20 (GenBank:NC_020995.1), with similarity values of 100% and 99%, respectively, indicating that all nine isolated strains belonged to the genus *Enterococcus*.

Methomyl degradation by isolated gut bacteria

Two representative strains, M2A1 (*E. mundtii*) and M2B1 (*E. casseliflavus*) were selected from the nine isolated strains to examine their ability to degrade methomyl (Figure 4). HPLC analysis showed that, after 24 h bacterial inoculation, the methomyl content in culture solution was significantly decreased compared with control (p = 0.004). In 24 h the degradation rates of M2A1 and M2B1 were 18.3% and 11.2%, respectively.

Larval insecticide sensitivity affected by different N rates in artificial diets

To exclude possible effects of other plant traits that are affected by different N rates on larval insecticide sensitivity, three N rates (high, normal, and low N) were set up by incorporating three levels of casein in
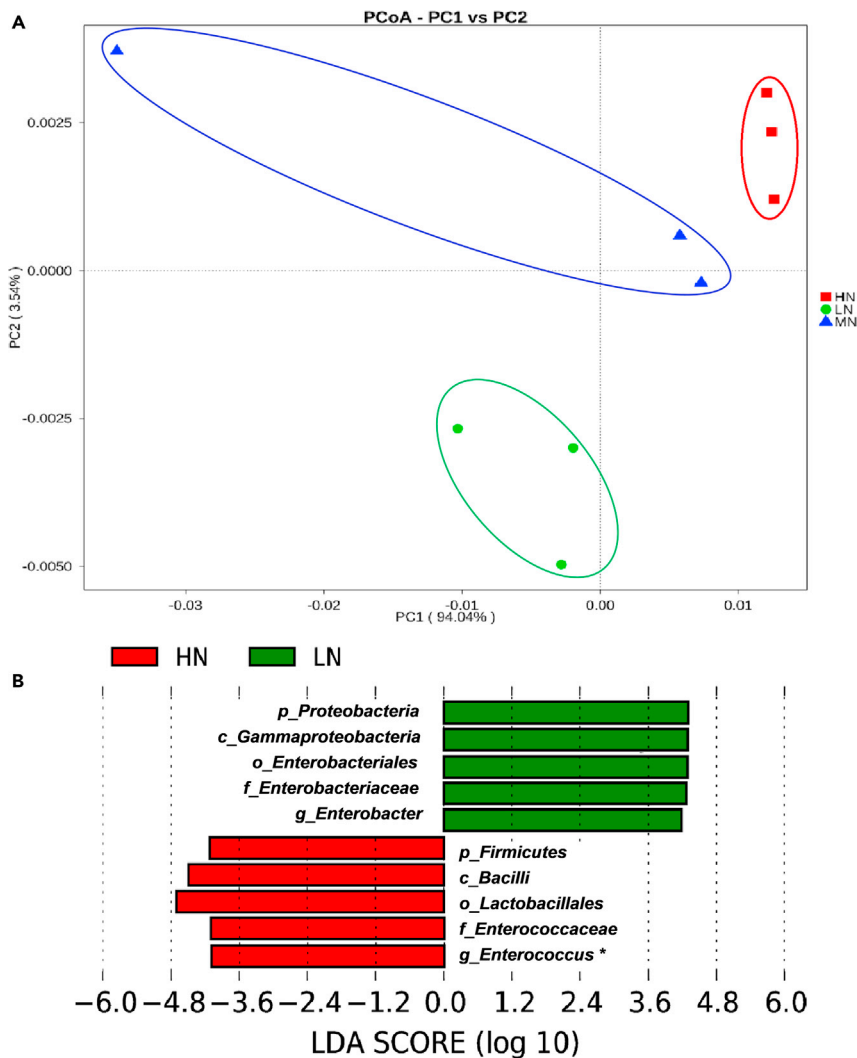
Figure 3. Gut microbiota of *Spodoptera litura* larvae fed on 30-days-old maize seedlings hydroponically grown under different nitrogen levels

Maize seedlings were hydroponically cultivated with different levels of nitrogen (8 mM, HN; 2 mM, MN; 0.5 mM, LN) for 30 days.

The third-instar larvae were reared on the leaves for 4 days. Then the larval midguts were collected for metagenomic analysis.

(A) Principal coordinate analysis (PCoA) plots based on the weighted UniFrac metric for bacterial communities. The red square represents gut samples in high-nitrogen treatments (8 mM, HN), blue triangle represents gut samples in medium-nitrogen treatments (2 mM, MN), and green circle represents gut samples in low-nitrogen treatments (0.5 mM, LN).

(B) A linear discriminant analysis effect size (LEfSe) method identifies the significantly different abundant functional profiles of the gut microbiota between the treatments of high N (8 mM) and low N (0.5 mM) at phylum (p), class (c), order (o), family (f), and genus (g) taxonomic levels. The histogram LDA score showed the biomarkers with statistical difference between groups. The influencing degree of species was expressed by the length of bar in the histogram.

artificial diets. After 2 days larval feeding on different N level artificial diets, no significant difference in the body weight was found among different N levels (p = 0.53, Figure S4A). However, after topical application with methomyl, the survival rate of larvae fed on high-N diet was significantly higher than those on low-N diet (p < 0.001, Figure S4B). The survival rate of larvae on higher-N diet was increased by 87.2%, whereas that fed on normal N diet was intermediate. The body weight of larvae fed on high-N diet with methomyl was significantly higher than those on low-N diet (p < 0.001, Figure S4C). The pattern of difference was similar to the result of topical application.
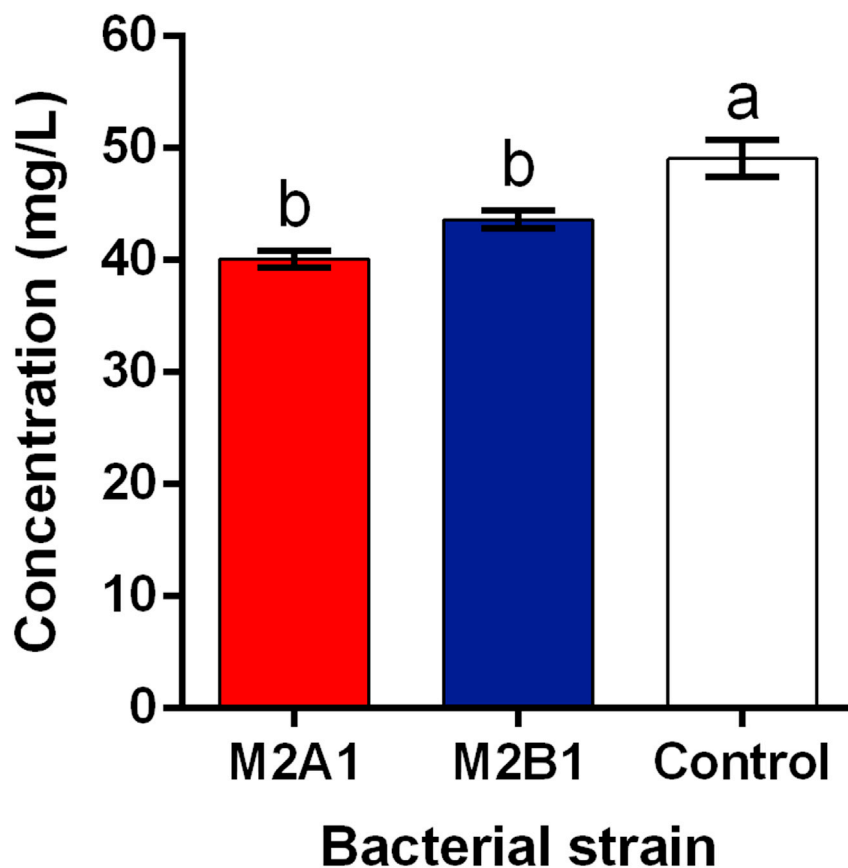
Figure 4. The concentration of methomyl in culture medium inoculated with bacterial strains M2A1 and M2B1 isolated from guts of *Spodoptera litura* larvae fed on maize seedlings hydroponically grown under high nitrogen level (8 mM)

The concentration of methomyl was analyzed by high-performance liquid chromatography (HPLC) after bacterial strain M2A1 and M2B1 inoculation in mineral medium with 50 mg/L methomyl and shaken at 30°C, 180 rmp for 24 h. Values are mean ± standard error from three replicates. Different letters above bars indicate significant differences among treatments ($p < 0.05$) according to one-way ANOVA with Tukey's multiple comparison test.

Larval insecticide sensitivity affected by reducing gut bacteria by antibiotics

Antibiotic addition in high-N diets inhibited 96.1% bacteria ($p < 0.001$, Figure 5A). Two days feeding on antibiotics-containing diets did not show any significant difference in larval growth ($p = 0.085$, Figure 5B). However, reduction of gut bacteria by antibiotics led to 39.4% reduction in survival rate ($p = 0.006$, Figure 5C) and 13.5% reduction in body weight of *S. litura* ($p = 0.025$) following methomyl treatment (Figure 5D).

Larval insecticide sensitivity affected by inoculation with isolated gut bacteria

After 2 days feeding on low-N diet inoculated with bacterial strain M2A1 and M2B1, the numbers of bacterial cells in the gut of each larva were 1.5×10^8 and 1.8×10^8, which was significantly higher than those in the gut of larvae without bacterial inoculation (control), which was 9.5×10^7 CFU ($p < 0.001$, Figure 6A). The bacterial inoculation did not show obvious effect on larval growth ($p = 0.96$, Figure 6B). However, inoculation with either M2A1 or M2B1 significantly increased survival rate ($p < 0.001$, 13.5% and 19.0% increase, respectively, Figure 6C) and enhanced body weight ($p = 0.009$, 35.1% and 23.5% increase, respectively) after diet incorporation with the insecticide (Figure 6D).

DISCUSSION

An increasing supply of N and other nutrients essential for plant growth is critical for enhancing crop production. Demands for N fertilizers are likely to grow substantially to nourish a growing global population.
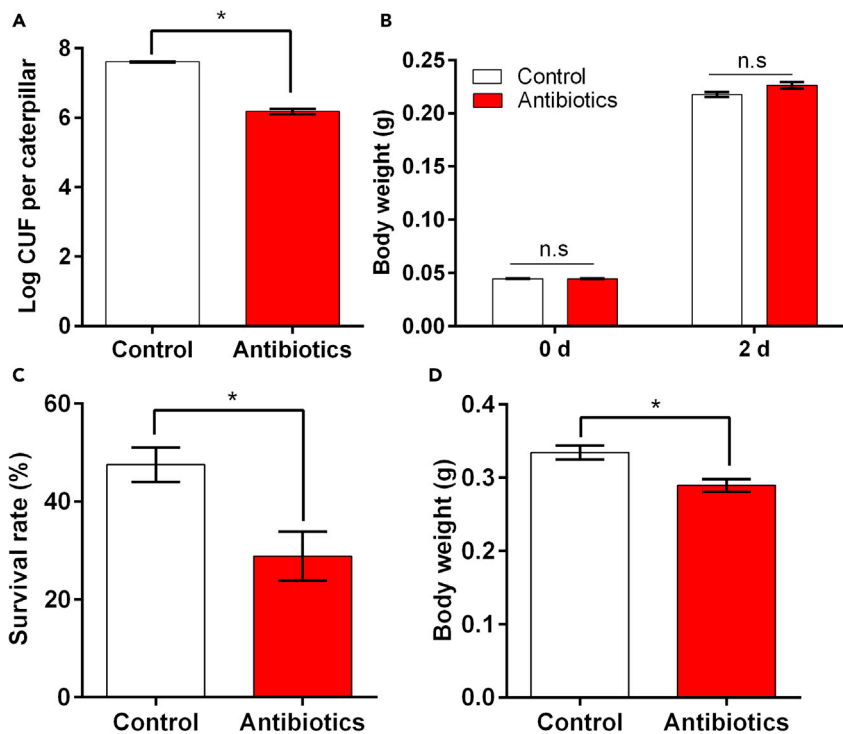
Figure 5. Effects of antibiotics on gut bacterial abundance, larval growth, and insecticide (methomyl) sensitivity of *Spodoptera litura*

Antibiotics-incorporated diets contained 0.02% neomycin, 0.1% chlortetracycline, and 0.006% streptomycin. Third-instar larvae were reared on antibiotics-incorporated and un-incorporated artificial diets containing 16 g casein/L for 48 h and then topically applied with 2 μL× 0.04 μg/μL methomyl or transferred to a diet containing 0.01 mg/g methomyl for 24 h.

(A) Number of bacterial cells in the gut of a caterpillar. CFU, colony-forming unit.

(B) Body weight of *S. litura* larvae fed on antibiotics-containing and control diets.

(C) Survival rate of *S. litura* 24 h after topical application with methomyl.

(D) Body weight of *S. litura* larvae 24 h after diet incorporation with methomyl. Values are mean ± standard error from three replicates (25 larvae/replicate in B, C, D). Significant differences ($p < 0.05$) between antibiotic treatments and controls are indicated by asterisk above bars ((A, B, D) Student's t test, (C) Chi-square test).

However, increased application of nitrogen fertilizer in crops normally results in remarkable increases in feeding preference, food consumption, growth, reproduction, and population density of insect pests, which may further lead to increased use of insecticides (Gordon, 1961). Based on the data from the International Fertilizer Association and Food & Agriculture Organization of the United Nations, a large-scale analysis was conducted to assess the relationship between N fertilizer and insecticides consumption in past decades in seven countries (Figure S5). Increases in N fertilizer consumption were linearly associated with increases in insecticides consumption in China, Japan, Canada, United Kingdom, and Australia, although no significant relationship was found in the United States and Netherlands due to new legislation and technology such as introduction of transgenic insect-resistant plants (Cattaneo et al., 2006; Oskam et al., 1992; Viray, 2009). This result suggests the positive correlation between N fertilizers and insecticides.

The increasing pest resistance to insecticides is closely associated with the long-term excessive application of nitrogen fertilizers. Samuel et al. (2020) showed that exposure to inorganic fertilizers has a greater effect on insecticide-susceptible *Anopheles arabiensis* as compared with resistant strains, where the primary advantage is increased insecticide tolerance. Lu et al. showed that female adults of brown planthopper *Nilaparvata lugens* Stål fed on rice plants with a high-nitrogen fertilizer were more tolerant to adverse environmental stresses including the insecticide buprofezin than those fed on rice plants with a low-N fertilizer (Lu et al., 2005). However, the underlying mechanism is completely unknown.

We set out to test the hypothesis that a high rate of N fertilizer in crop plants (maize) will increase the N content in plant tissues (Figure S1) and supply as insect (*S. litura*) diets, which may nourish more insect
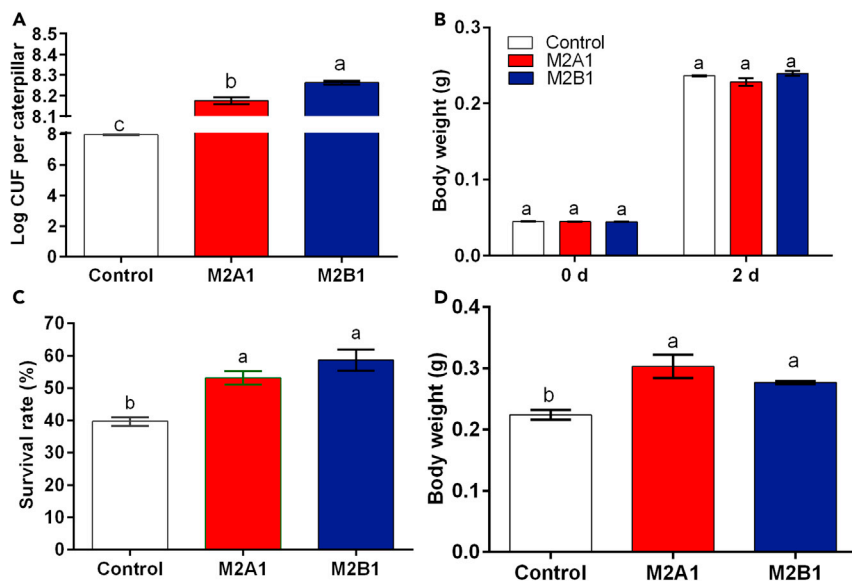
Figure 6. Effects of gut bacterial inoculation with M2A1 and M2B1 on growth and insecticide (methomyl) sensitivity of *Spodoptera litura* larvae fed on artificial diet without casein addition

Third-instar larvae were reared on low-N artificial diet (without casein addition) inoculated with bacterial strains M2A1 and M2B1 for 48 h, and then topically treated with 2 μL × 0.02 μg/μL methomyl or transferred to a diet containing 0.01 mg/g methomyl for 24 h.

(A) Number of gut bacterial cells per larva. CFU, colony-forming unit.

(B) Body weight 48 h after feeding on diet inoculated gut bacteria and control.

(C) Survival rate of *S. litura* larvae 24 h after topical application with methomyl.

(D) Body weight of *S. litura* larvae 24 h after diet incorporation with methomyl. Values are mean ± standard error from three replicates (25 larvae/replicate). Different letters above bars indicate significant differences among treatments (p < 0.05) according to one-way ANOVA with Tukey's multiple comparison test (A, B, D) and chi-square test with a Bonferroni correction for multiple tests (C).

gut bacteria, leading to increased insecticide tolerance of insect pests and increased use of insecticides during crop production. Our results supported the hypothesis showing that high N applied to maize plants significantly increased the pest larval growth and reduced larval sensitivity to methomyl.

The results of the present work show that high N applied on maize, one of three most important food crops, significantly improved larval growth and development of *S. litura*. However, when the N rate was 4 mM the larval growth reached a climax (Figures 1A and 1B), suggesting that this N level is sufficient for larval growth. Similar results have been reported in other insects, for example, *Liriomyza trifolii* females responded to increased tomato leaf N with significantly increased feeding and fecundity (Minkenberg and Ottenheim, 1990). Larvae of two *Pieris* butterflies that fed on highly nutritious foliage showed increased growth and a shorter development period (Hwang et al., 2008). Most evidence to date suggests that more N application may have a positive effect on insect growth and populations as in our study. Increased body weight may decrease insect sensitivity to insecticides due to dilution effect, leading to increased insecticides use. Also, increased body weight means enlarged body size or improved insect nutrition, which can significantly influence the host insect susceptibility to insecticides (Kliot and Ghanim, 2012; Robertson and Preisler, 1992). A shorter development period promotes population growth, which may also result in increased use of insecticides. In this study, we found that larvae of the polyphagous pest *S. litura* feeding on high-N plant had markedly reduced sensitivity to methomyl even when there was no difference in body weight of larvae grown in different levels of N (Figures 2D–2F). It is well known that body size is closely associated with insect tolerance to insecticides. Within 2 days body weight showed no obvious difference among the different N treatments, which excludes the possible effects of body size on larval tolerance to the insecticide, suggesting other mechanisms involved in the enhanced insecticide tolerance in N-rich larvae.

Many studies have demonstrated that gut microbes can enhance insecticide resistance (Almeida et al., 2017; Cheng et al., 2017; Fusetto et al., 2017; Kikuchi et al., 2012; Li et al., 2020; Wu et al., 2020). We

observed variation in microbiome composition in the guts of insects feeding on plants cultivated at different N levels (Figure 3). Bioinformatics analyses showed that larval guts of *S. litura* in high N treatment harbored more *Enterococcus* bacteria. We isolated nine bacterial strains from the guts of *S. litura* on media containing insecticide methomyl as the sole source of C and N. Interestingly, all these insecticide-degraded strains belong to the genus *Enterococcus*. Two representative strains, *E. mundtii* M2A1 and *E. casseliflavus* M2B1, were verified to be able to degrade the insecticide (Figure 4). The *Enterococcus* spp. are commonly found in nature and gut colonization (Lebreton et al., 2014). Their existence has been detected in numerous lepidopteran species (Paniagua et al., 2018). *Enterococcus* sp. was found to dominate in the gut of two specialized Lepidoptera, *Hyles euphorbiae* and *Brithys crini*, that specifically feed on latex-rich *Euphorbia* sp. and alkaloid-rich *Pancratium maritimum*, respectively (Vilanova et al., 2016). *Enterococci* spp. in the guts of the diamondback moth *Plutella xylostella* and silkworm *Bombyx mori* enhanced insecticide resistance of the host (Xia et al., 2018; Chen et al., 2020).

In this study antibiotic incorporation to artificial diet removed most of the gut bacteria and increased the sensitivity of *S. litura* to methomyl (Figure 5). Our results demonstrate that the insect with an intact gut microbiome increased its tolerance to the insecticide, evidenced from the data showing that both laboratory and field populations of *S. litura* were more resistant to the insecticide in the presence of gut bacteria than those in the absence of gut bacteria reduced by antibiotic administration (Gadad and Vastrad, 2016).

To confirm the role of strains M2A1 (*E. mundtii*) and M2B1 (*E. casseliflavus*) in methomyl resistance, we reared the insect larvae on low-N diet and inoculated with the strain M2A1 and M2B1. Inoculation with the two gut bacterial strains led to significantly decreased sensitivity of *S. litura* to methomyl (Figure 6), which further highlighted the importance of these gut microbiota in larval tolerance to insecticides. These two strains also play an important role in other insects. *E. casseliflavus and E. mundtii* in the gut of *Heliconius erato* Phyllis (Lepidoptera: Nymphalidae) show high rates of resistance to rifampicin and erythromycin (Huff et al., 2020). *E. casseliflavus* can support house fly larval development and colonize the gut of teneral adults to various degrees (Anuradha et al., 2014). *E. mundtii* with adherence properties can form a biofilm layer on the gut wall to help insect adapt to altered environments (Mazumdar et al., 2021b). Probiotic characterization of *E. mundtii* was observed from larval gut of *Plutella xylostella* (Mazumdar et al., 2021a). Members of other gut microbiomes can also degrade insecticides *in vitro*. For example, degradation test revealed that the gut-colonizing Burkholderia retains a high degrading activity of the organophosphate compound in the gut of rice bug *Cletus punctiger* (Ishigami et al., 2021). A gut bacteria *Lactobacillus plantarum* was shown to metabolize organophosphate insecticide chlorpyrifos and may be beneficial to reducing chlorpyrifos toxicity in *Drosophila melanogaster* (Daisley et al., 2018).

Since N plays a central role in all metabolic processes as well as in cellular structure and genetic coding, N is a key nutrient element for plants and herbivorous insects, as well as microorganisms. Both herbivorous insects and microorganisms obtain most N directly and indirectly from their food source plants. Therefore, N level in plants is an important determinant of herbivorous insects and associated microorganisms (Awmack and Leather, 2002; Mattson, 1980). Nitrogen fertilizer application can mediate gut microbiomes in insects and other animals (Ding et al., 2019; Bi et al., 2021). Increasing evidence shows that gut bacteria mediate insecticide resistance in a diverse range of insects (Almeida et al., 2017; Cheng et al., 2017; Xia et al., 2018). It has been showed that the gut microbiota is involved in insecticide resistance in *S. litura* (Gadad and Vastrad, 2016). So it is reasonable that gut microbiomes can serve as an intermediary between these N fertilizer and insecticide application.

Taken together, our results demonstrate that high N application in maize plants changes gut microbiota of *S. litura* and triggers the shifts to *Enterococcus* spp., leading to increased insecticide tolerance of the pest. This study sheds new light on the pivotal role of gut microbiota in insecticide tolerance in insect pests. Our findings suggest that excessive use of nitrogen fertilizers in crop production may be an important reason for enhanced insecticide tolerance in agroecosystems, leading to globally increasing use of insecticides. Appropriate use of nitrogen fertilizers can minimize the use of insecticides in agriculture.

Limitations of the study

Our study could be limited by the number of sequenced gut sample (n = 3), the optimal sample size at each N concentration should be greater than or equal to five. Our study could also be limited by degradation time inoculated with target bacteria strains. If more gut samples were collected and degradation time was extended beyond 24 h, more detail may have been elucidated.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103726.

AUTHOR CONTRIBUTIONS

L.H., R.Z., and Y.S. designed the experiments. Z.S., C.X., J.W., C.G., and D.C. carried out the experiments. L.H., L.L., A.U.M., and Y.S. analyzed data and prepared the manuscript, and all authors contributed to revisions.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Enterococcus mundtii	This paper	M2A1
Enterococcus casseliflavus	This paper	M2B1
Chemicals, peptides, and recombinant proteins		
soybean powder	JD	NA
wheat bran	JD	NA
yeast powder	Solarbo	Cat#Y8020
Casein	Solarbo	Cat#C9290
agar powder	Solarbo	Cat#A8190
choline chloride	Solarbo	Cat#G8330
sorbic acid	Solarbo	Cat#S5451
Cholesterol	Solarbo	Cat#C8280
Inositol	Solarbo	Cat#I8050
ascorbic acid	Solarbo	Cat#A8100
Neomycin	Solarbo	Cat#N8090
chlortetracycline	Solarbo	Cat#C9100
streptomycin	Solarbo	Cat#S8290
Acetone	Sigma-Aldrich	Cat#270725
Methomyl	Sigma-Aldrich	Cat#74088
NaClO	Sinopharm	Cat#80010462
K_2SO_4	Sinopharm	Cat#10017918
KCl	Sinopharm	Cat#10016308
KH_2PO_4	Sinopharm	Cat#10017690
$MgSO_4$	Sinopharm	Cat#20025118
EDTA-Fe	Sinopharm	Cat#30084726
H_3BO_3	Sinopharm	Cat#10004808
$MnSO_4 \cdot H_2O$	Sinopharm	Cat#10013418
$ZnSO_4 \cdot 7H_2O$	Sinopharm	Cat#10024018
$CuSO_4 \cdot 5H_2O$	Sinopharm	Cat#10008218
$Na_2 MoO_4 \cdot 2H_2O$	Sinopharm	Cat#10019818
$Ca(NO_3)_2 \cdot 4H_2O$	Sinopharm	Cat#80029062
$CaCl_2 \cdot 2H_2O$	Sinopharm	Cat#20011160
NaOH	Sinopharm	Cat#10019718
H_2SO_4	Sinopharm	Cat#10021608
N_2HPO_4	Sinopharm	Cat#20040618
$MgCl_2$	Sinopharm	Cat#10012828
Critical commercial assays		
Illumina MiSeq PE250	Beijing Novogene	NA
Bacterial 16S rRNA sequencing	Shanghai Biosune	NA
Deposited data		

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
insect gut microbiome data	This Paper	GenBank: PRJNA630363.
Experimental models: Organisms/strains		
Spodoptera litura	This paper	NA
Zea mays	Guangdong Academy of Agricultural Sciences,	Yuebai
Oligonucleotides		
799R	Sangon Biotech	AACMGGATTAGATACCCKG
1391F	Sangon Biotech	GACGGGCGGTGWGTRCA
27F	Sangon Biotech	GTTTGATCCTGGCTCAG
1492R	Sangon Biotech	GGTTACCTTGTTACGACTT'
Software and algorithms		
GraphPad Prism 6	GraphPad Prism	NA
ImageJ	ImageJ	NA
QIIME	Knights and Caporaso labs	NA
Excel	Microsoft Corporation	NA
SPSS	SPSS Inc.	NA

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yuanyuan Song (yyuansong@fafu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The raw sequence data of gut community were deposited in the Sequence Read Archive (SRA) service of the GenBank database under the accession number GenBank:PRJNA630363.

This manuscript did not generate new code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Insect materials

The laboratory strain of the tobacco cutworm (*Spodoptera litura*), originally collected from the field in Henan Province (China), were reared on artificial diets that are composed of soybean powder (100 g), wheat bran (40 g), yeast powder (26 g), casein (8 g), agar powder (23 g), choline chloride (1 g), sorbic acid (2 g), cholesterol (0.2 g), inositol (0.2 g), ascorbic acid (8 g), and water (to final volume 1 L) as previously described with minor modification (Zhang et al., 2010). The larvae were maintained at $25 \pm 2°C$ with $70 \pm 5\%$ relative humidity and a photoperiod of 14:10 h (L: D) in a climatic chamber. The adults were provided with 10% honey liquid under the same conditions.

Plant materials

Seeds of maize (*Zea mays* L. cv. Yuebai) were supplied by Crops Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China. Maize seeds were surface-sterilized with 2% NaClO for 15 min, rinsed six times with sterile water, and pre-imbibed in distilled water for 24 h. After pre-germination for 2 d at 28°C, the single seedling was transplanted to a 1-L hydroponic plastic container. The nutrient solution consisted of 0.75 mM K_2SO_4, 0.1 mM KCl, 0.25 mM KH_2PO_4, 0.65 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM EDTA-Fe, 10 μM H_3BO_3, 1 μM $MnSO_4 \cdot H_2O$, 1 μM $ZnSO_4 \cdot 7H_2O$, 0.1 μM $CuSO_4 \cdot 5H_2O$, and 0.035 μM $Na_2 MoO_4 \cdot 2H_2O$. NO_3^- was supplied as $Ca(NO_3)_2 \cdot 4H_2O$ (Wang et al., 2004). The maize seedlings were cultured with five

different N concentrations (0.5, 1, 2, 4 and 8 mM). CaCl$_2$·2H$_2$O was added to maintain a final Ca concentration of 4 mM to exclude possible effect of Ca. The pH was adjusted to 6.3 by addition of NaOH or H$_2$SO$_4$. The nutrient solution was changed twice a week. All plants were grown in a greenhouse at temperatures of 28 $\pm$ 2°C with 75% relative humidity in natural light.

METHODS DETAILS

Larval growth and performance trials

Two types of bioassays, long-term developmental trial and the third instar performance trial, were conducted to determine the effect of different N rates on maize seedlings on larval growth and the performance of *S. litura*.

Long-term developmental trial was conducted to assess the effects of different N nutrients on the development and growth of *S. litura* during the entire larval stage. Fifty newly hatched first-instar larvae were transferred on the top three leaves of 14-days-old hydroponic maize seedlings cultured at N rates of 0.5, 1, 2, 4 and 8 mM (5 larvae/plant, 10 plants/concentration). The leaves were covered with nylon mesh to prevent insect escaping. The larvae were weighed 0, 10, 15 and 20 d after transfer to the plants. The pupation time was also recorded.

Third instar performance trial was conducted to evaluate the effects of different N rates on food consumption by the third instar larvae on maize plants only for 4 d. Each third-instar larva of *S. litura* was transferred on the top third leaf of a 30-days-old hydroponic maize seedling cultured at N rates of 0.5, 1, 2, 4 and 8 mM and reared for 4 d (1 larva/plant, 15 plants/rate). Each consumed leaf was scanned with 600 pixel scanning mode, and the consumed area was calculated with ImageJ software (Igathinathane et al., 2008). According to water content, biomass of the damaged leaves was calculated.

Bioassays for sensitivity of larvae to insecticide

Both topical and oral feeding bioassays were used to evaluate sensitivity of larvae to methomyl due to contact and ingested toxicity of the insecticide. Third-instar larvae (40–50 mg) from artificial diet were transferred on 30-days-old hydroponic maize seedlings cultured at N rates of 0.5, 1, 2, 4 and 8 mM for either 2 d or 4 d (10 larvae/plant, 15 plants/rate). Then one group of the larvae were transferred from plants to standard artificial diet, and were topically applied on the pronotum with methomyl dissolved in acetone at doses of 0.02 μg/larva for 2 d treatment and 0.03 μg/larva for 4 d treatment (1 μL for each larva, the volume 1 μL acetone was used for each larva due to no larval death during the 96-h observation period in the solvent). The survival rate was recorded 24 h after treatment with methomyl. Another group of the larvae were transferred from plants to artificial diet containing 0.005 mg/g methomyl for 24 h and then weighed. Twenty-five larvae were used for each treatment at three independent replicates.

DNA extraction, PCR, sequencing, and analysis

Third-instar larvae (40–50 mg) were fed on the top third leaf of 30-days-old hydroponic maize seedlings cultured at N rates of 0.5, 2 and 8 mM for 4 d (10 larvae/plant, 6 plants/concentration). Then the larvae were surfaced-sterilized and dissected to collect gut samples (20 larvae/sample, 3 samples/concentration). Genomic DNA of gut sample was extracted by using a MoBio Powersoil bacterial DNA isolation kit (Carlsbad, CA, USA) as per manufacturer's instructions.

The PCR reactions were carried out in a 20 μL solution containing 10 ng of DNA using 16S rRNA primers 799F (5'-AACMGGATTAGATACCCKG-3') and 1391R (5'-GACGGGCGGTGWGTRCA-3') (Beckers et al., 2016). The PCR conditions were 94°C for 2 min and 30 cycles of 94°C for 30 s followed by 55°C for 30 s, 72°C for 30 s and a final extension step of 72°C for 5 min. The final PCR products were analyzed by electrophoresis in 2% agarose gel followed by staining with ethidium bromide and visualization under ultraviolet light. Sequencing of these barcoded amplicons was performed using Illumina MiSeq PE250 platform (Novogene, Beijing, China). Raw pair end-reads were assembled after filtering adaptor, low-quality reads, and barcodes to generate clean joined reads capturing the complete V5-V7 region of the 16S rRNA gene by Novogene. The generated high quality sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). The sequences were clustered into OTUs at the 97% similarity level using UCLUST clustering and taxonomy of each OTU was classified against the Ribosomal Database Project (Edgar, 2013).

For MiSeq data analysis, rarefaction curves were estimated using the "alpha_rarefaction.py" script in QIIME to test whether the sequencing efforts adequately represented the bacterial diversity within each sample. Two richness estimators (the abundance-based coverage estimator (ACE) and a nonparametric richness estimator based on distribution of singletons and doubletons (Chao1)) and two diversity indices (Shannon and Simpson index) were calculated for the samples using the "alpha_diversity.py" script in QIIME (Xu et al., 2016). The diversity indices of four groups and the relative abundances of different genera were compared using One-way ANOVA test.

The representative sequences of all OTUs were used to construct neighbor-joining trees. The phylogenetic tree together with sample sequence abundance data were used for weighted Unifrac PCoA (principal coordinate analysis) which considers both relative abundance and different branch lengths in a tree, through the online Fast Unifrac program (Hamady et al., 2010). Linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/lefse/) was applied to identify the specific bacterial taxa associated with insect gut microbes fed on high N maize seedling (Segata et al., 2011).

Isolation and identification of methomyl-degrading bacteria

Newly hatched first-instar larvae of *S. litura* were reared on the maize leaves in greenhouse. When they reached the fourth instar stage, larval guts were collected to isolate bacteria that are able to degrade methomyl. Ten larvae were dissected, and gut samples were macerated in 5 mL sterile distilled water, sonicated for 1 min, shaken at 180 rpm for 15 min at 28°C. The mixture was allowed to stand for 30 min at room temperature. The suspension was diluted and spread on the surface of agar plates containing the mineral media (9.5 mM KH_2PO_4, 4.8 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.8 mM Na_2HPO_4 and 20 g/L agar) (Ceja-navarro et al., 2015) with 100 mg/L methomyl. The Petri-dishes were placed in an incubator at 28°C for 5 d. Bacterial growth was monitored every day. Total genomic DNA in all pure colonies was extracted using the DNA extraction Kit (Qiagen, USA) according to the manufacture's protocol. The16S rRNA sequence was amplified using universal primers 27F (5'-GTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTA CGACTT-3'). The PCR conditions were 94°C for 5 min and 30 cycles of 94°C for 30 s followed by 55°C for 30 s, 72°C for 60 s and a final extension step of 72°C for 8 min. The PCR products were sequenced by Shanghai Biosune Biological Technology Co., Ltd. The sequencing results were analyzed using NCBI database for homologous sequences of 16S rRNA.

HPLC analysis of the degradation of methomyl

The fresh bacteria cells were centrifuged at 6000 *g* for 5 min and washed twice with sterilized water, the cell pellets were then resuspended and adjusted to OD 1.0 at 600 nm. They were then inoculated into 1% (v/v) mineral medium with 50 mg/L methomyl and shaken at 30°C and 180 rpm. After 24 h, the culture medium was centrifuged at 10 000 *g* for 30 min, and the supernatant was filtered with 0.22 μm Nylon filter membranes. The 0.5 mL filtrate was added to 0.5 mL of acetonitrile (99.9%, HPLC grade) and injected into a Waters HPLC device to analyze the concentration of methomyl. The HPLC column was an Agilent C18 (4.6 × 250 mm, 5 μm). The mobile phase was 50:50 acetonitrile and pure water with a flow rate of 0.8 mL/min. A UV detector was used with the detection wavelength of 254 nm (Zhang et al., 2017).

Effect of different N rates in diets

To test whether N levels in artificial diet also affect larval sensitivity to insecticide, three levels of casein (0, low N; 8 g/L, normal N; 16 g/L, High N) were set up in artificial diets. Third-instar larvae (40–50 mg) were reared on diets with different N levels for 2 d. Then one group of the larvae were topically applied on the pronotum with methomyl dissolved in acetone at doses of 0.07 μg/larva. The survival rate was recorded 24 h after the insecticide treatment. Another group of larvae were transferred to a diet containing 0.01 mg/g methomyl, and reared for 24 h and then weighed. Twenty-five larvae were used for each treatment with three replications.

To determine whether the gut microbiota are involved in reduced larval sensitivity to insecticide at high N rate, three antibiotics were added to the artificial diet to reduce the microbes present in gut as previously described (Chung et al., 2013; Wang et al., 2016, 2020). Two hundred microliters of sterile water containing 0.02% neomycin, 0.1% chlortetracycline and 0.006% streptomycin were added to an artificial diet (0.5 g) with 16 g/L casein (high N). Third-instar larvae (40–50 mg) were reared on antibiotic-containing and antibiotic-free diets for 2 d. Then one group of larvae were topically applied with methomyl dissolved in acetone at a dose of 0.08 μg/larva. Another group of larvae were fed on diet containing 0.01 mg/g methomyl for 24 h and then weighed as described above.

To confirm that the gut microbiota are involved in insecticide degradation, the larvae were inoculated with bacterial strains M2A1 and M2B1 isolated from guts of *S. litura* larvae fed on maize seedlings hydroponically grown under high nitrogen (8 mM). Fresh bacterial cells were diluted to OD 0.1 at a 600 nm. The bacterial cells were centrifuged at 5000 *g* for 10 min and re-suspended in sterile 10 mM MgCl$_2$ solution, with the volume equal to half volume of medium. Each bacterial suspension (200 μL) or MgCl$_2$ solution (control) was added to an artificial diet without casein addition. Third-instar larvae were allowed to feed on the gut bacterium-inoculated diet for 2 d, receiving freshly prepared diet daily as described previously (Wang et al., 2017). Then two insecticide bioassays, topical application (0.04 μg methomyl/larva) and diet incorporation (0.01 mg/g methomyl), were used to estimate larval sensitivity to the insecticide.

QUANTIFICATION AND STATISTICAL ANALYSIS

The differences of body weight, diversity indices, chemical concentration and bacterial numbers were analyzed using t-test or one-way analysis of variance followed by Tukey's difference (HSD) ($p < 0.05$). The difference of survival rate was investigated using the Chi-square test with a Bonferroni correction for multiple tests. Statistical analysis was performed in IBM SPSS Statistics 22. Graphs were created in GraphPad Prism 6.