RESEARCH ARTICLE

Figures and figure supplements

Volatile DMNT directly protects plants against *Plutella xylostella* by disrupting the peritrophic matrix barrier in insect midgut

Chen Chen *et al*

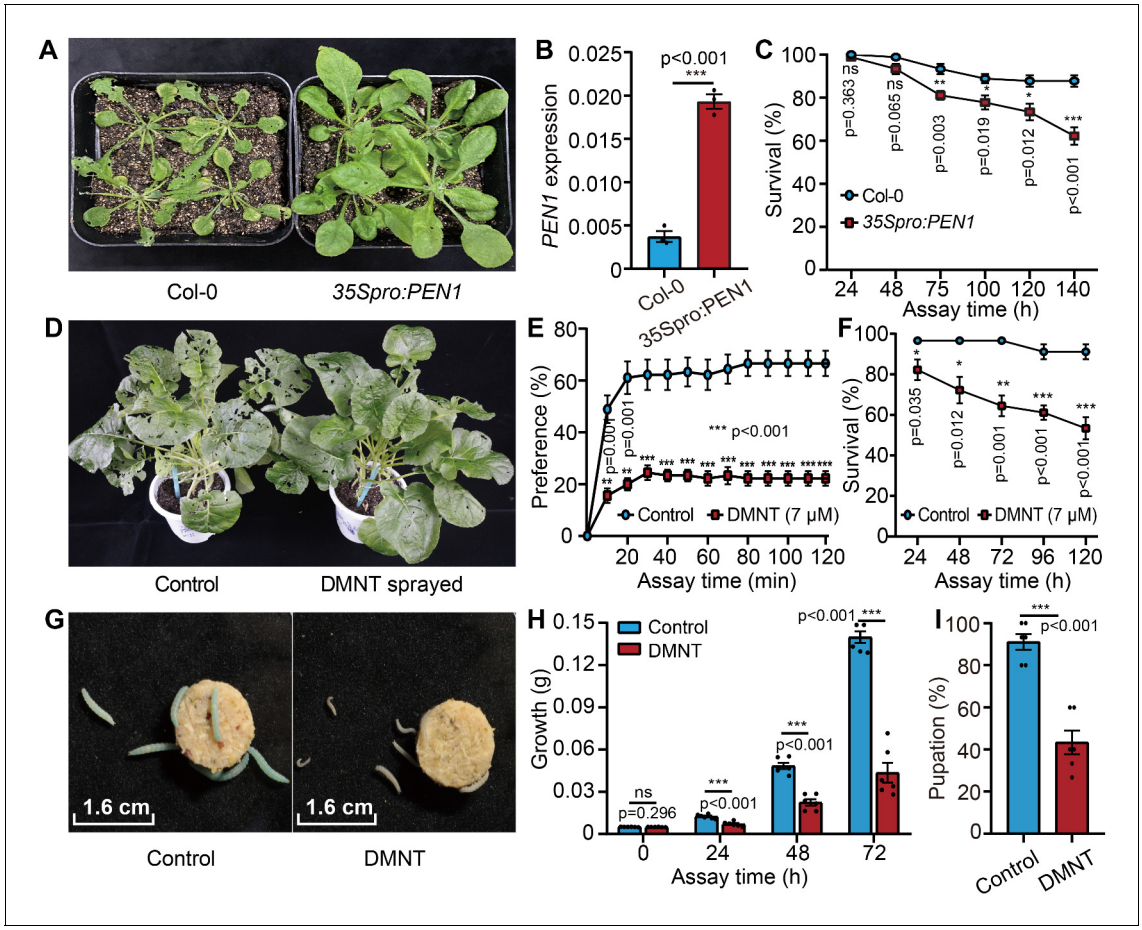

Figure 1. (3E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) repels and kills *P. xylostella* larvae. (**A**) Pentacyclic triterpene synthase 1 (*PEN1*) overexpression in *A. thaliana* results in high resistance to *P. xylostella* infestation. *PEN1* encodes the key enzyme responsible for DMNT biosynthesis in *Arabidopsis* plants. (**B**) *PEN1* is overexpressed in *35Spro:PEN1* transgenic *Arabidopsis* plants. (**C**) *35Spro:PEN1* transgenic *Arabidopsis* plants cause lower survival of *P. xylostella* than wild type control. (**D**) *B. napus* plants sprayed with DMNT show strong resistance to *P. xylostella* larvae. (**E**) *P. xylostella* larvae can sense and are repelled by DMNT. The preference test system is illustrated in ***Figure 1—figure supplement 2***. (**F**) DMNT treatment significantly lowers the survival of *P. xylostella* larvae. (**G**) Phenotypic comparison between DMNT-fed larvae and control larvae (treated with the DMNT solvent paraffin oil). (**H**) Growth inhibition of *P. xylostella* larvae by DMNT. (**I**) Reduced pupation of *P. xylostella* larvae by DMNT treatment. Error bars represent the standard error of six independent biological replicates, with 15 larvae per replicate. Asterisks indicate significant differences (*p<0.05, **p<0.01, ***p<0.001, ns, not significant; two-tailed unpaired *t*-test).

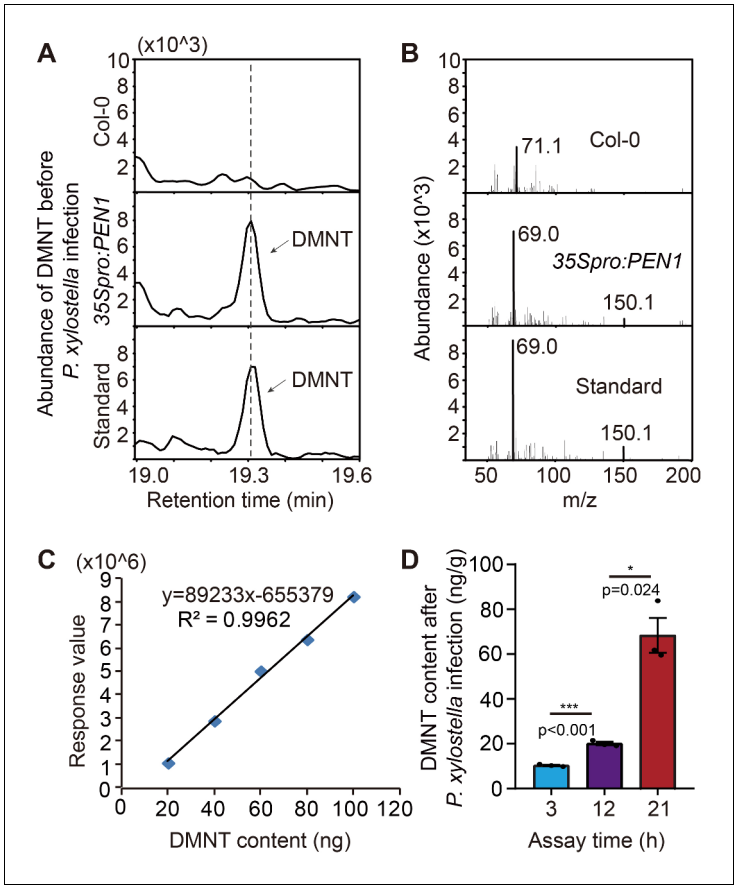

Figure 1—figure supplement 1. (3E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) is enriched in *35Spro:PEN1* transgenic *A. thaliana* plants and can be highly induced by *P. xylostella* infestation. (**A**) Gas chromatography-mass spectrometry analysis of DMNT in Col-0 and *35Spro:PEN1* transgenic *A. thaliana* plants. The DMNT standard is shown at the bottom. (**B**) Ionogram traces of DMNT captured from Col-0 and *35Spro:PEN1* transgenic plants. (**C**) A linear standard curve of DMNT quantification, which was dissolved in methanol. (**D**) DMNT in *35Spro:PEN1* transgenic *A. thaliana* plants can be continuously induced by *P. xylostella* infestation for 3–21 hr. Asterisks in (**D**) indicate significant differences (*p<0.05, ***p<0.001; two-tailed unpaired *t*-test).

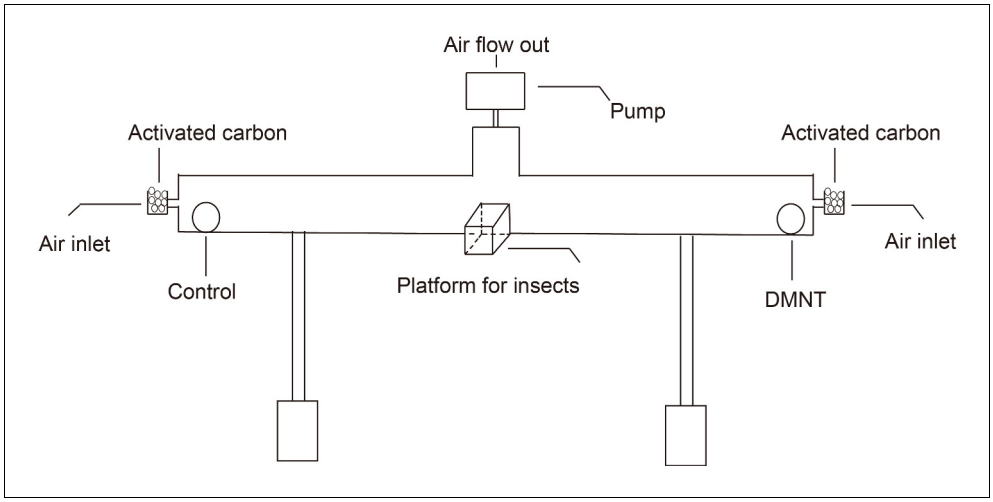

Figure 1—figure supplement 2. A schematic representation of the principle behind the insect choice test system. The horizontal box indicates the glass test tube. Equal amounts of activated carbon are placed at both ends of the test tube to avoid air contamination. The air flow is generated by an air pump. In all experiments, (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and control samples are placed on either end of the glass test tube, while 15 *P. xylostella* larvae are put on the platform located in the center of the test tube for the choice test.

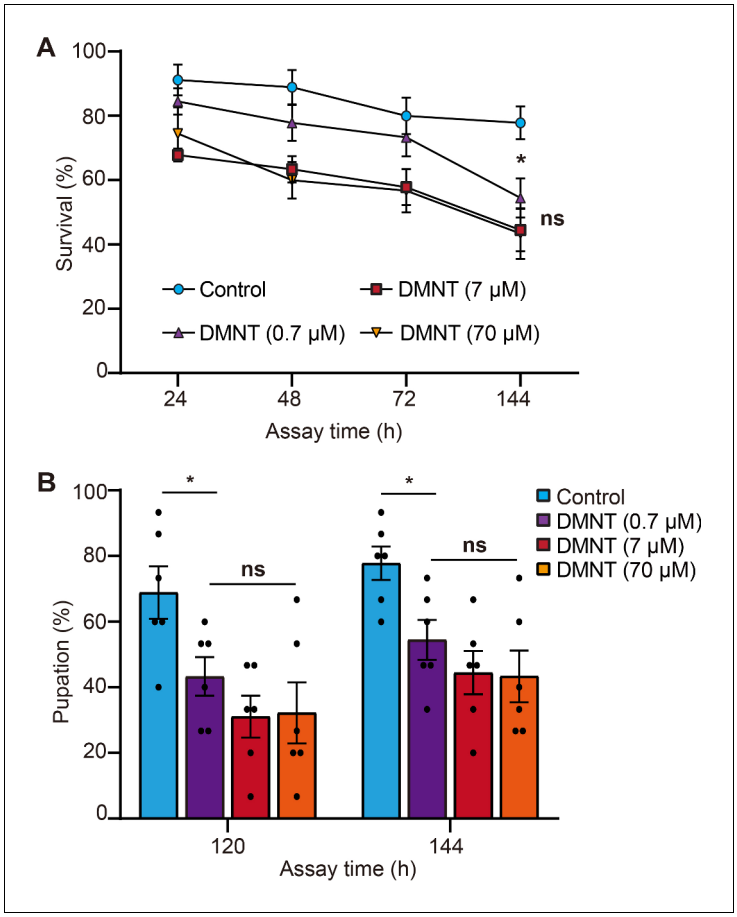

Figure 1—figure supplement 3. (3E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) shows a slight dosage-dependent influence on the survival and pupation rates of *P. xylostella* larvae. (**A**) DMNT shows no dosage-dependent effects on the end of survival or pupation (**B**) of *P. xylostella* larvae. Error bars in (**A, B**) represent the standard error of six biological replicates, with 15 larvae per replicate. Asterisks indicate significant differences (*p<0.05; ns, not significant; two-tailed unpaired *t*-test).

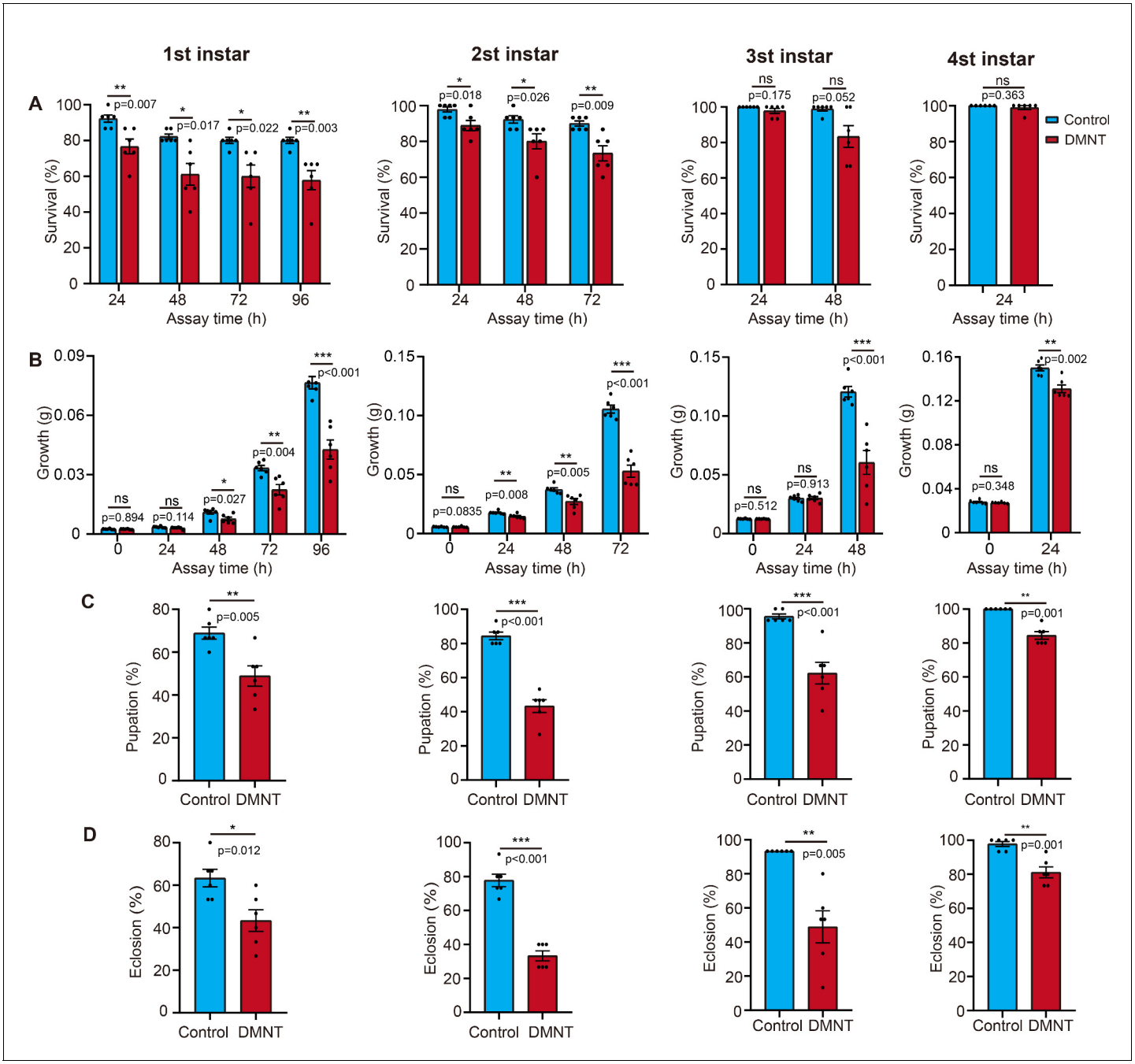

Figure 1—figure supplement 4. (3E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) treatment affects *P. xylostella* larvae of different ages. First- to fourth-instar *P. xylostella* larvae were tested. (**A**) Survival rate. (**B**) Body weight. (**C**) Pupation rate. (**D**) Eclosion rate. Error bars represent the standard error of six biological replicates, with 15 *P. xylostella* larvae per replicate. Asterisks indicate significant differences (*p<0.05, **p<0.01, ***p<0.001; ns, not significant; two-tailed unpaired *t*-test).

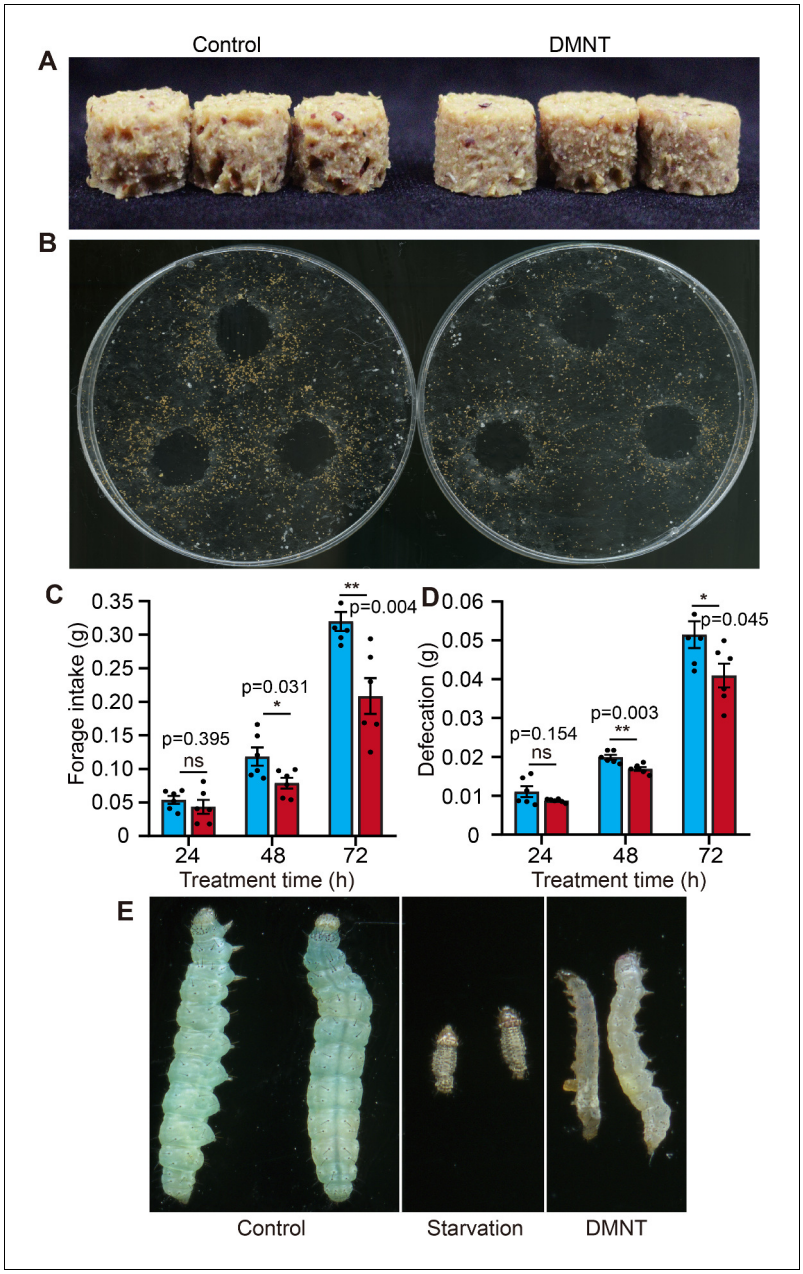

Figure 1—figure supplement 5. (3E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) treatment lowers forage intake and defecation of *P. xylostella* larvae. (**A, C**) Reduced forage intake in *P. xylostella* larvae fed with DMNT. (**B, D**) Reduction in defecation of DMNT-fed *P. xylostella* larvae compared to the control. Error bars in (**C, D**) represent the standard error of six biological replicates, each consisting of 15 *P. xylostella* larvae. The same amount of forage was used in all assays. (**E**) The length of DMNT-treated *P. xylostella* is different from that of starved larvae. Asterisks indicate significant differences (*p<0.05, **p<0.01; ns, not significant; two-tailed unpaired *t*-test).

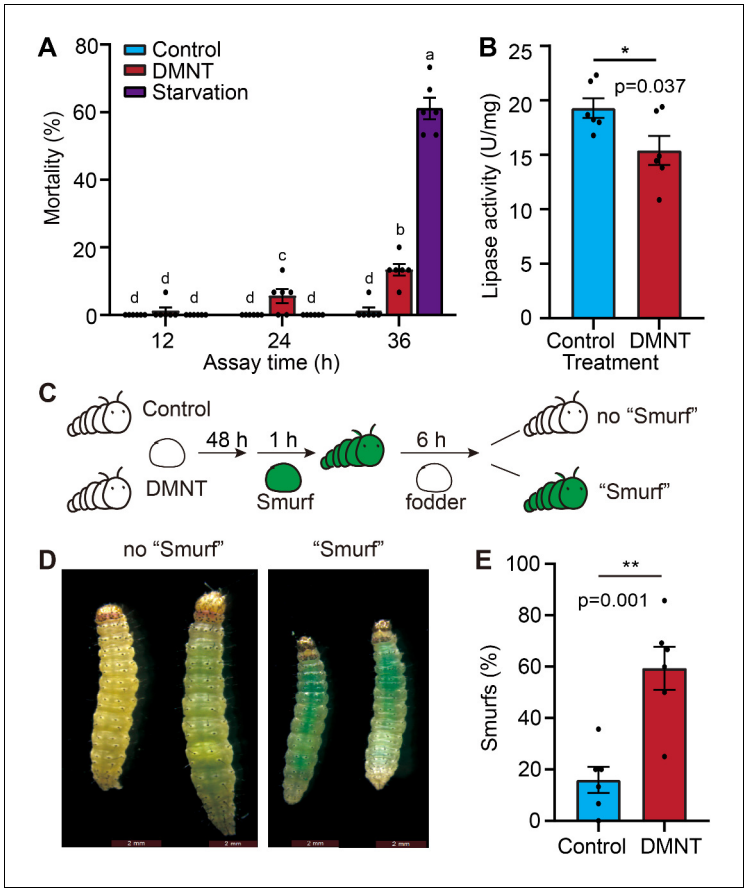

Figure 2. The midgut barrier of *P. xylostella* larvae is damaged by (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) treatment. (**A**) Larvae die earlier from treatment with DMNT than from starvation. (**B**) Decreased lipase activity in the midgut of *P. xylostella* larvae upon DMNT treatment. (**C**) The principle of the Smurf test. Larvae are fed with forage loaded with the dye erioglaucine disodium salt for 1 hr before returning to normal forage without dye. After 6 hr, the extent of dye retention is monitored. (**D**) Representative images of DMNT-treated larvae showing dye retention, as evidenced by their blue appearance, like the Smurf cartoon character. (**E**) Quantification of results shown in (**D**). The different letters in (**A**) indicate a significant difference (one-way ANOVA). Error bars in (**B, E**) represent the standard error of six independent biological replicates, with 15 larvae per replicate. Asterisks in (**B, E**) indicate significant differences (*p<0.05, **p<0.01, ns, not significant; two-tailed unpaired *t*-test).

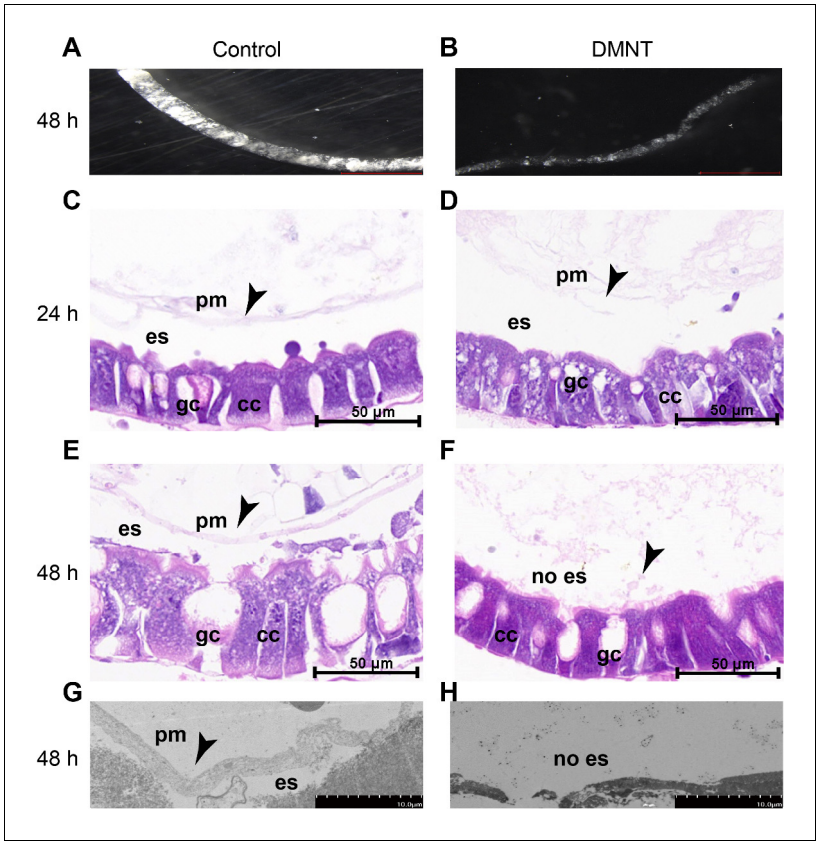

Figure 3. The peritrophic matrix (PM) structure is damaged by (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) treatment. (**A, B**) PM ultrastructure of control (**A**) and DMNT-fed (**B**) *P. xylostella* larvae for 48 hr. Note the thin and delicate PM in DMNT-fed larvae. (**C–F**) Transverse section and hematoxylin-eosin staining show damage of the PM by DMNT after exposure for 24 hr (**C, D**) and 48 hr (**E, F**). (**G, H**) The PM is damaged by DMNT feeding, as shown by transmission electron microscopy. Arrowheads indicate the PM. es: delimits the ectoperitrophic space; gc: goblet cells; cc: columnar cells.

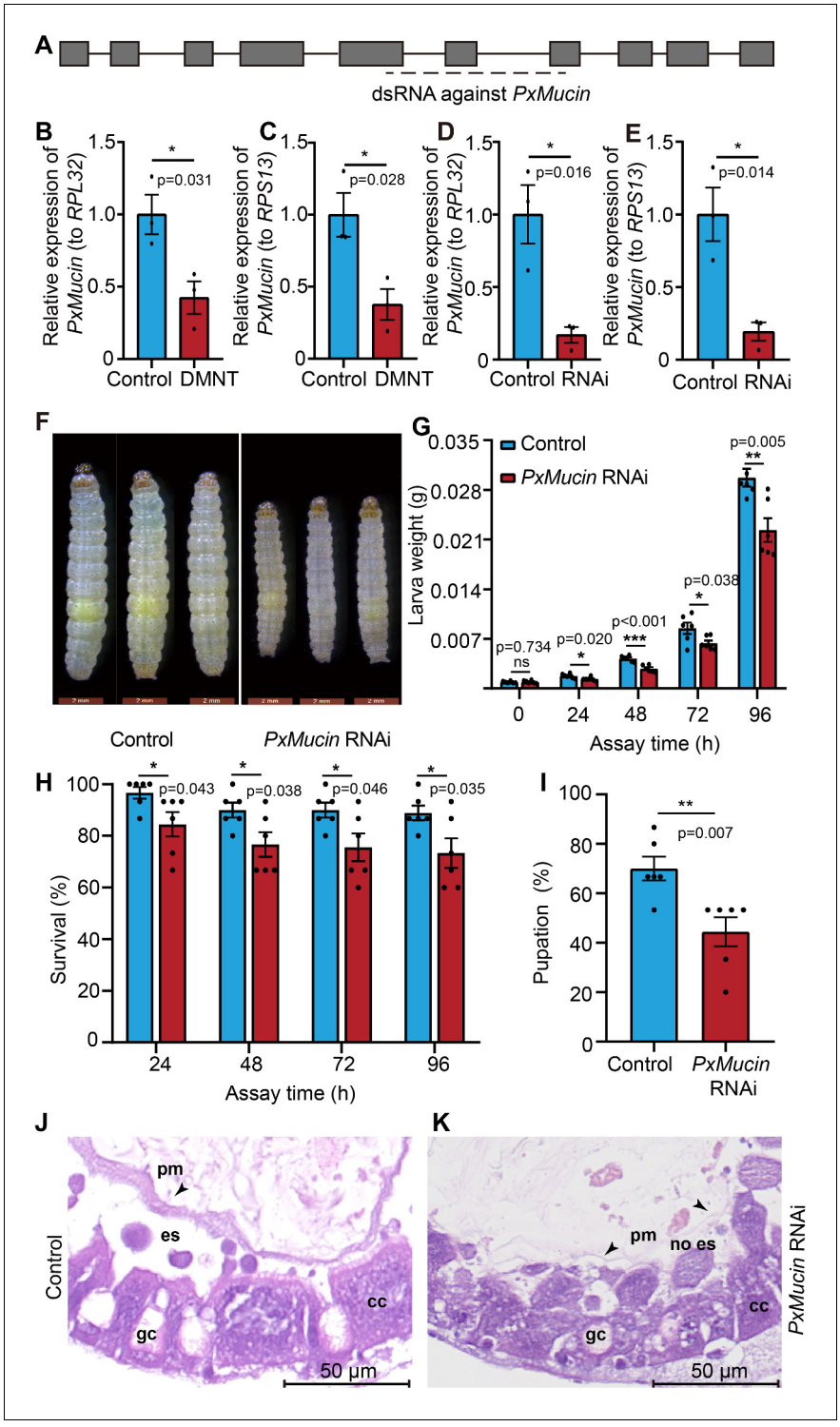

Figure 4. (3E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) disrupts the peritrophic matrix (PM) structure of *P. xylostella* larvae via repression of the mucin-like gene *PxMucin*. (**A**) Gene structure of *PxMucin*. Boxes represent exons, and horizontal lines represent introns. The horizontal dashed line indicates the targeted regions by double-stranded RNA (dsRNA). (**B, C**) DMNT treatment downregulates the expression of *PxMucin*. (**D, E**) Successful RNA interference (RNAi) of *PxMucin* by dsRNA feeding. (**F–I**) Larvae fed with dsRNA against *PxMucin* show impaired body development (**F**), weight (**G**), survival (**H**), and pupation (**I**) rates. (**J, K**) PM structure from control larvae (**J**) and larvae-fed *PxMucin* dsRNA (**K**). Note how *PxMucin* dsRNA feeding phenocopies DMNT treatment. Error bars in (**B–E**) represent the standard error of three independent biological replicates, with 30 *P. xylostella* larvae per

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replicate. Error bars in (**G–I**) represent the standard error of six independent biological replicates, with 15 *P. xylostella* larvae per replicate. Asterisks indicate significant differences (*p<0.05, **p<0.01, ***p<0.001, ns, not significant; two-tailed unpaired *t*-test).

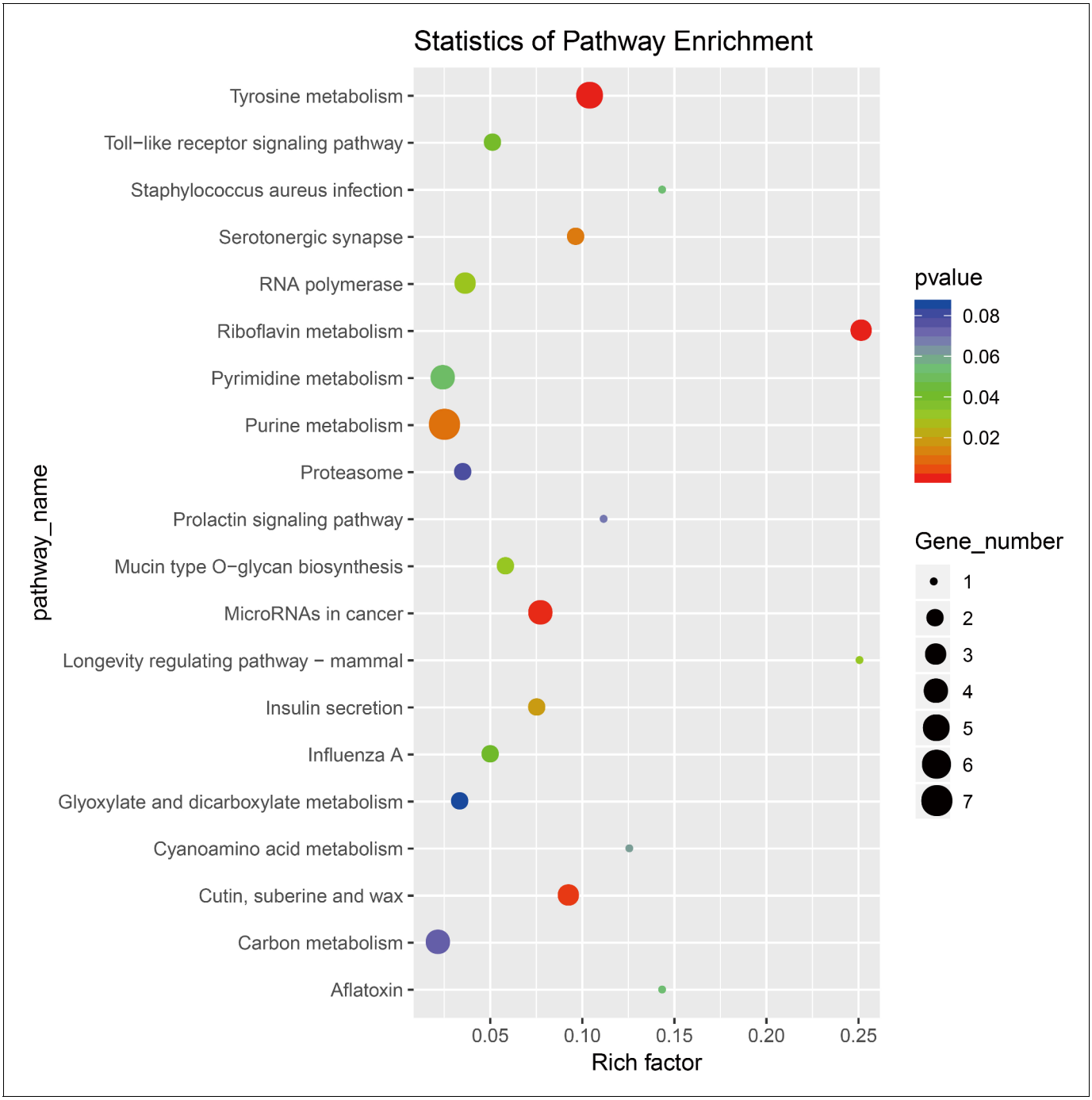

Figure 4—figure supplement 1. Transcriptomic analysis of the differentially expressed genes in the *P. xylostella* larva after (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) treatment at 12 hr. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the pathway enrichment from the differently expressed genes after DMNT treatment is shown.

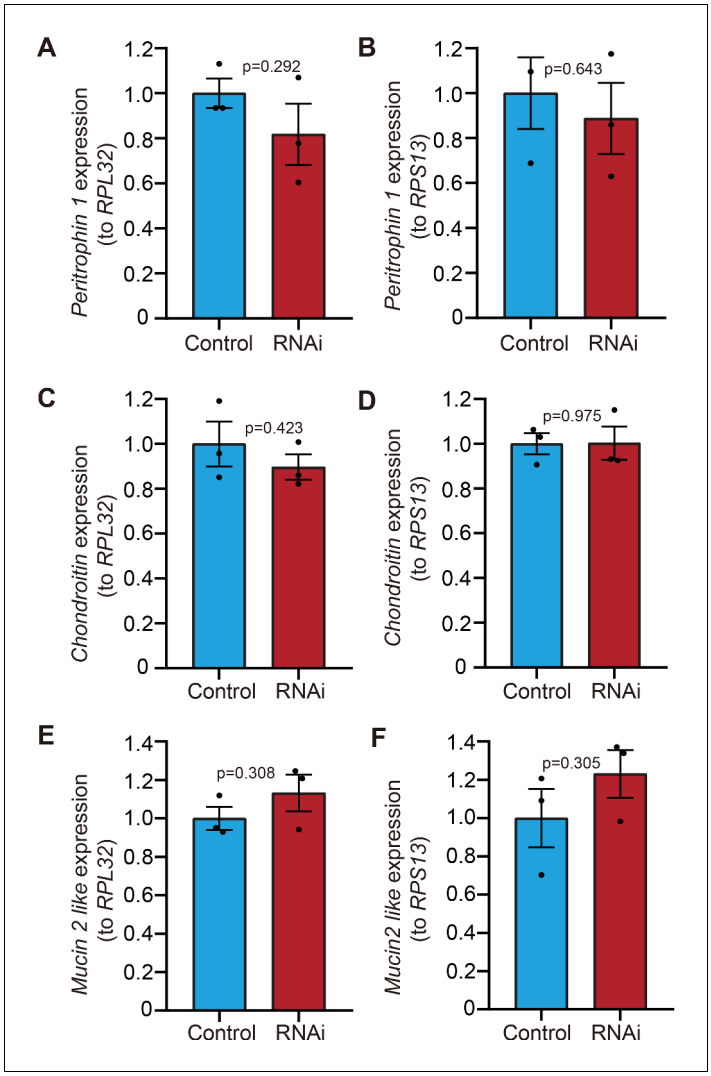

Figure 4—figure supplement 2. The expression of three *PxMucin* homologs is not significantly influenced by the double-stranded RNA (dsRNA) treatment. (**A, B**) *Peritrophin 1 like*. (**C, D**) *Chondroitin*. (**E, F**) *Mucin 2 like*. *RPL32* and *RPS13* were used as internal control ***Fu et al., 2013*** for qRT-PCR. Error bars represent the standard error of three biological replicates. p values of statistical tests are shown in figures (two-tailed unpaired *t*-test).

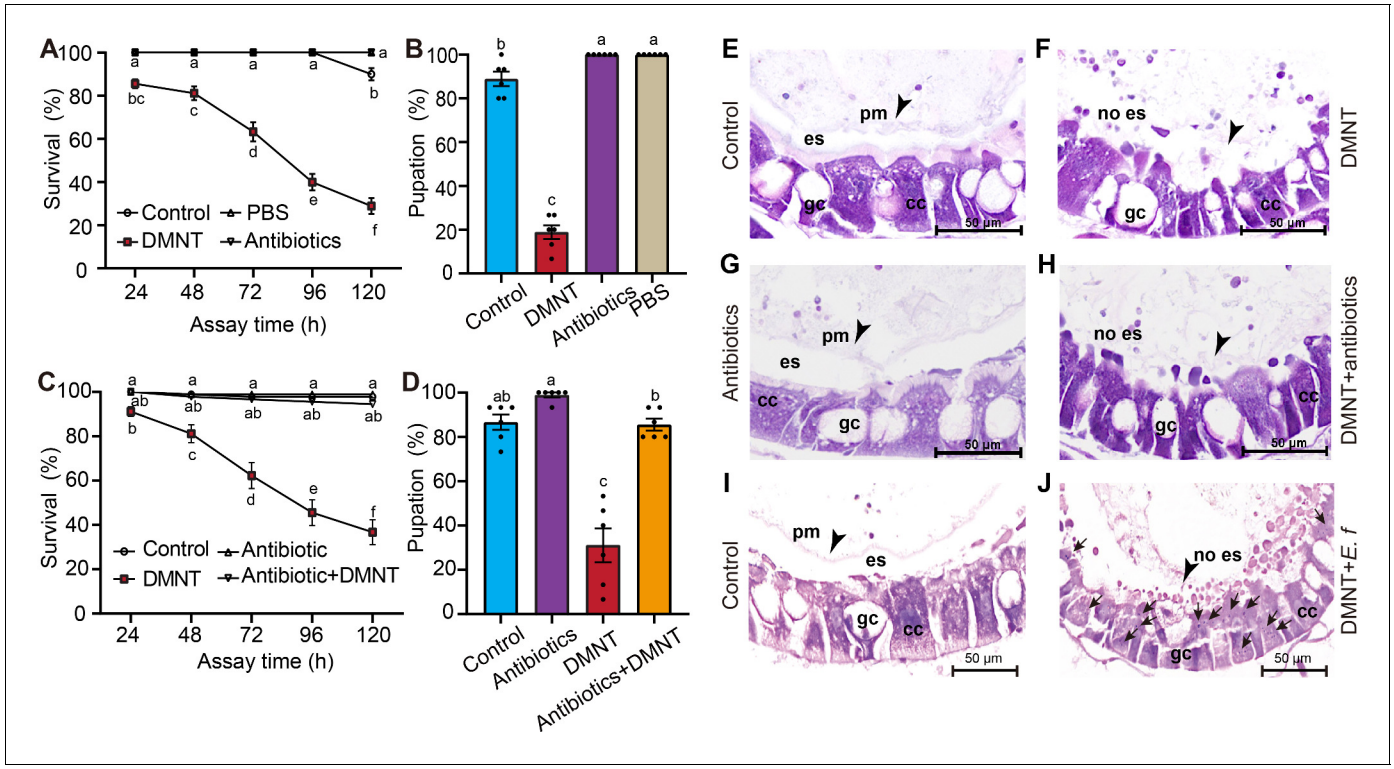

Figure 5. Microbes in the midgut are indispensable for (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT)-mediated killing of *P. xylostella* larvae. (**A, B**) Removal of the microbiota by antibiotics alone has no influence on larval survival (**A**) or pupation rates (**B**). DMNT treatment was used as a control. (**C, D**) Removal of the microbiota by treatment with antibiotics eliminates the adverse DMNT-mediated effects on the survival (**C**) and pupation (**D**) rates of larvae. (**E–H**) Comparison of peritrophic matrix (PM) structure in control larvae (**E**), larvae treated with DMNT alone, (**F**) or in combination with antibiotics (**H**). Note the disruption of the PM, whereas treatment with the antibiotic cocktail alone has no effects (**G**). (**I, J**) *Enterococcus faecalis* (*E. f*) can invade midgut cells when the PM is disrupted by DMNT treatment (**J**), but is restricted within the PM in controls (**I**). Arrows indicate *E. f* cells stained with dye. Arrowheads indicate the PM. es: delimits the ectoperitrophic space; gc: goblet cells; cc: columnar cells. The different letters in (**A–D**) indicate a significant difference (one-way ANOVA).

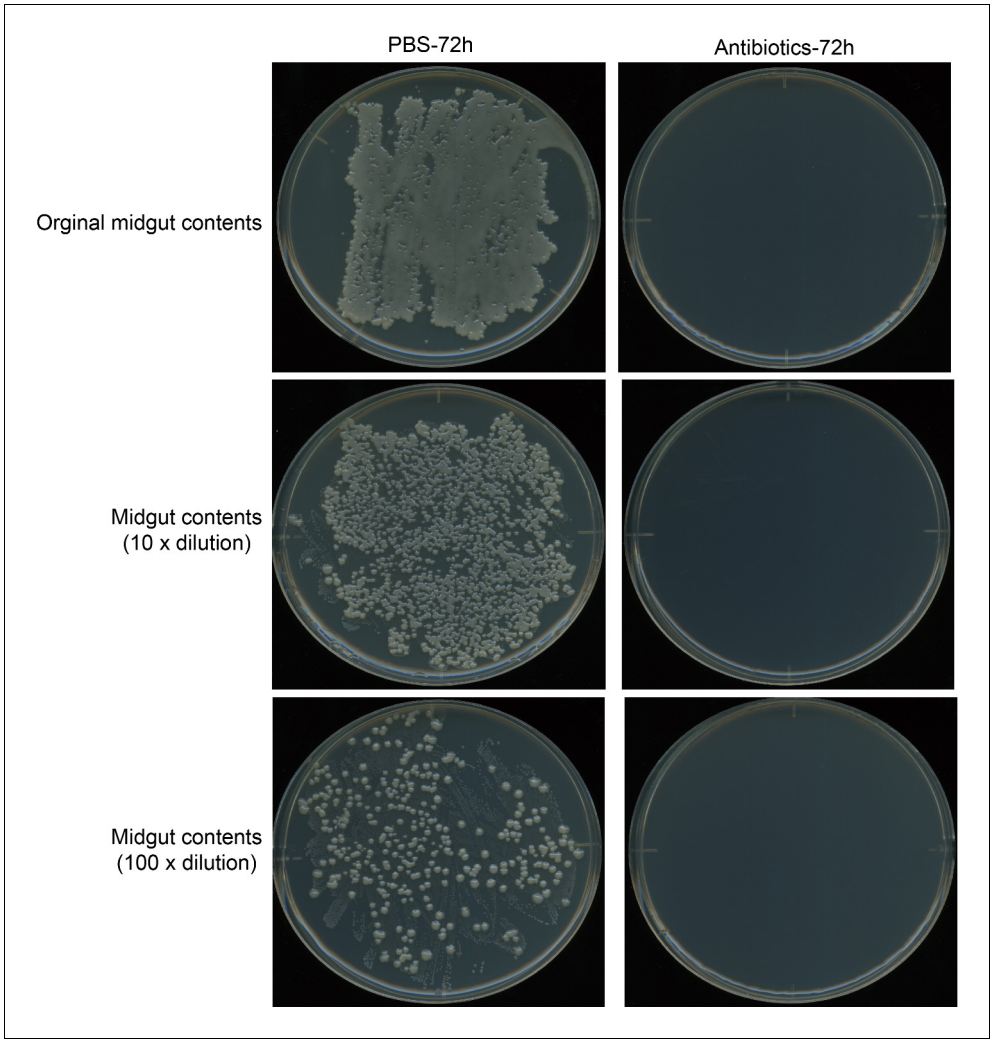

Figure 5—figure supplement 1. The midgut microbiota is completely killed by the antibiotic cocktail. Multiple replicates were carried out with the same concentrations of antibiotics added to forage to feed *P. xylostella* larvae, with similar results. The antibiotic cocktail contains 100 kU/mL penicillin, 100 mg/mL streptomycin, and 50 mg/mL gentamycin.

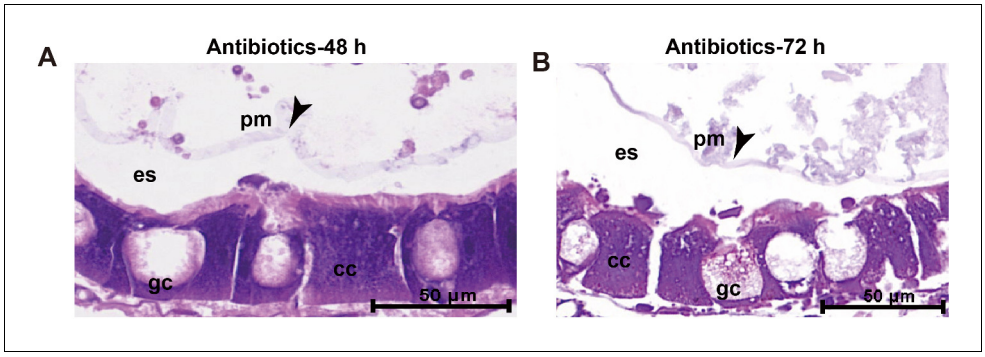

Figure 5—figure supplement 2. Treatment with antibiotics does not influence the integrity of peritrophic matrix (PM) structures. (**A**) 48-hr treatment with antibiotics. (**B**) 72-hr treatment with antibiotics. Arrowheads indicate the PM. es: delimits the ectoperitrophic space; gc: goblet cells; cc: columnar cells. The antibiotic cocktail contains 100 kU/mL penicillin, 100 mg/mL streptomycin, and 50 mg/mL gentamycin.

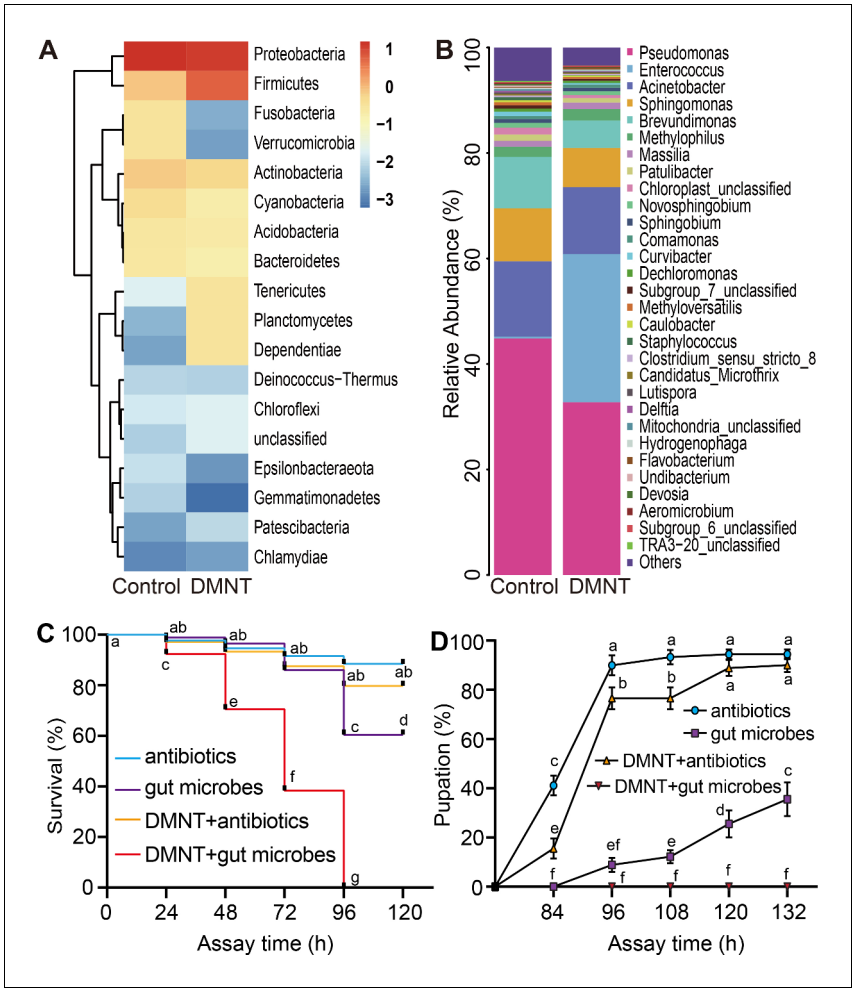

Figure 6. The microbiota composition of *P. xylostella* larvae midgut is affected by (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) treatment. (**A**) Relative abundance of 18 phyla in response to DMNT treatment. (**B**) Effect of DMNT treatment on *Enterococcus* microbial populations. (**C, D**) Feeding with gut microbes decreases the survival (**C**) and pupation (**D**) rates of larvae treated with DMNT. Error bars represent the standard error of six independent biological replicates, with 15 *P. xylostella* larvae each. The different letters in (**C, D**) indicate a significant difference (one-way ANOVA).

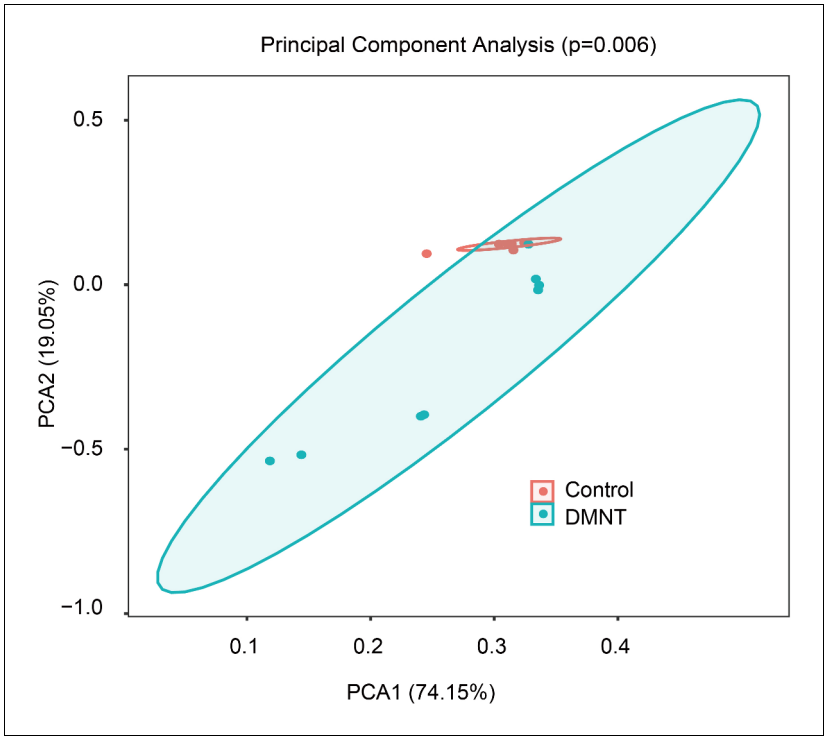

Figure 6—figure supplement 1. (3E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) affects the abundance of microbiota populations in the midgut of *P. xylostella* larvae. Principal component analysis of the 16S rDNA sequencing results. DMNT-treated samples cluster separately from control samples (p=0.006).

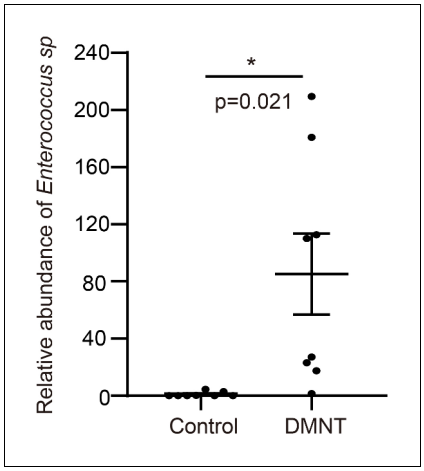

Figure 6—figure supplement 2. *Enterococcus* sp. was enriched around 85-fold in (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT)-treated larvae than the control larvae. Asterisks indicate significant differences (*p<0.05; two-tailed unpaired *t*-test).

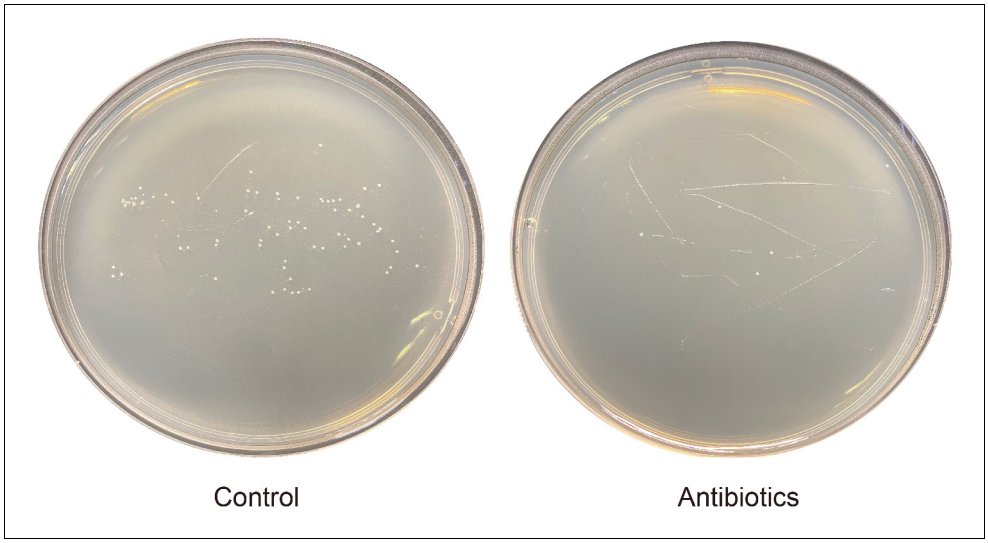

Figure 6—figure supplement 3. The gut microbiota from second-instar *P. xylostella* larvae were nearly killed by the antibiotic cocktail. The antibiotic cocktail contains 100 kU/mL penicillin, 100 mg/mL streptomycin, and 50 mg/mL gentamycin.

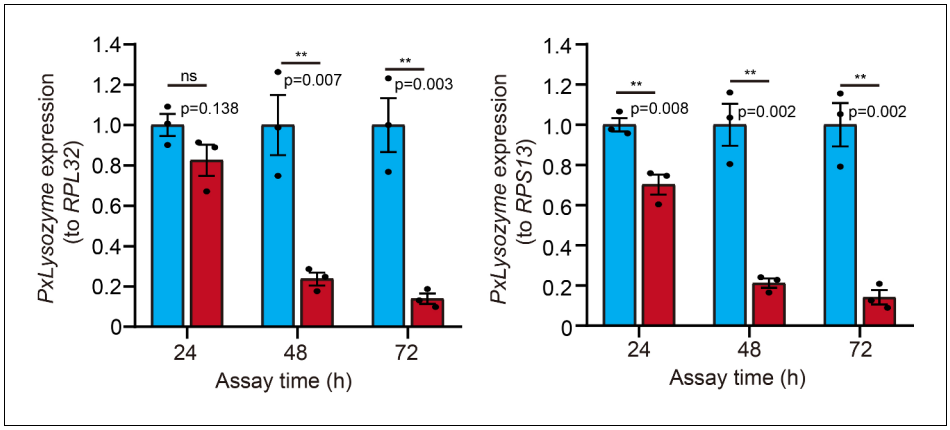

Figure 6—figure supplement 4. *PxLysozyme* expression was downregulated from 48 to 72 hr. Error bars represent the standard error of three independent biological replicates, with 30 *P. xylostella* larvae per replicate. Asterisks indicate significant differences (**p<0.01, ns, not significant; two-tailed unpaired *t*-test).