

Supplementary Information

Gut bacteria prime and reinforce mature leaf oviposition preference in beetles via plant mediated feedback

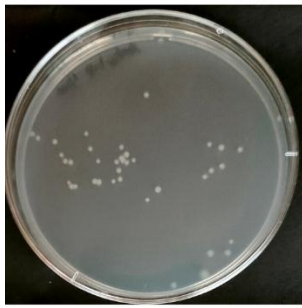
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This PDF file includes:

Supplementary Text
Fig. S1 to S9

A

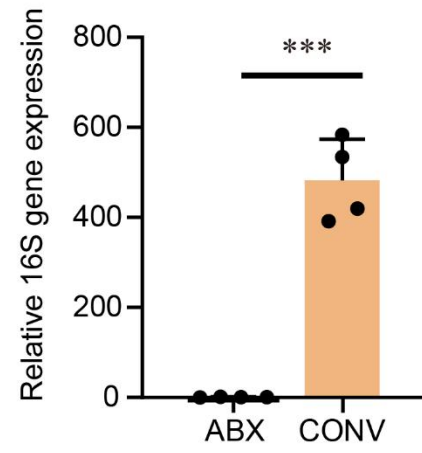


ABX

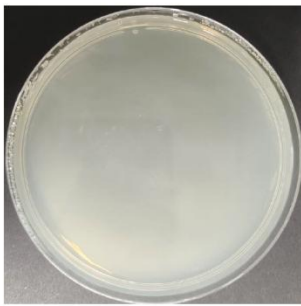


CONV

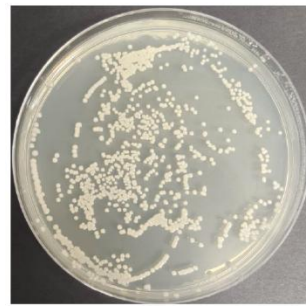
B



C

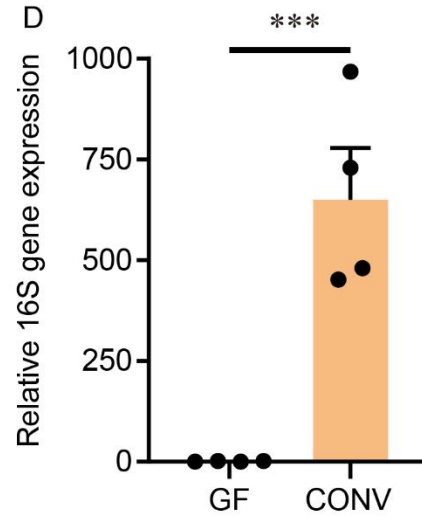


GF



CONV

D



E

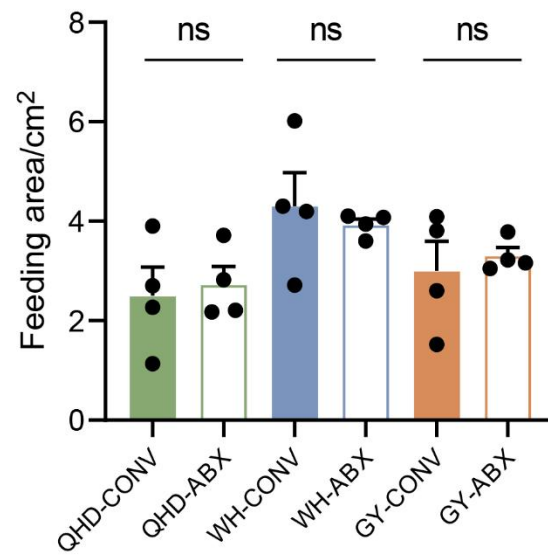


Figure S1. Confirmation of microbial load reduction and assessment of *Plagiodera versicolora* adult feeding performance. The efficacy of antibiotic treatment (ABX) and germ-free rearing (GF) in reducing or eliminating host-associated bacteria, and their potential side effects on adult feeding, were evaluated. (A) Reduction of culturable bacteria in antibiotic-treated (ABX) adult homogenates verified by plate culture. Representative LB agar plates show significantly fewer bacterial colonies from ABX adults compared to numerous colonies from conventional (CONV) adults. (B) qPCR analysis of bacterial load in ABX adult samples. Relative abundance of the bacterial 16S rRNA gene confirms a significant reduction of bacteria in ABX adults ($n = 4$, 3 guts per replicate) compared to CONV adults ($n = 4$, 3 guts per replicate). (C) Absence of culturable bacteria in GF adult homogenates verified by plate culture. Representative LB agar plates show no bacterial colonies from GF adults, while CONV adults yield numerous colonies. (D) qPCR analysis of bacterial load in adult samples. Relative abundance of the bacterial 16S rRNA gene confirms near-complete removal of bacteria in GF adults ($n = 4$, 3 guts per replicate) compared to CONV adults ($n = 4$, 3 guts per replicate). For all 16S rRNA qPCR analyses, CT values greater than 38 were considered below the detection limit and were assigned a value of 0. (E) Comparison of the average 24-h feeding area (cm^2) between single CONV and ABX adults from three geographic populations: Qinghuangdao (QHD), Wuhan (WH), and Guiyang (GY) ($n = 4$). No significant differences were observed between treatments across all sites, indicating that antibiotic treatment did not impair adult feeding capacity. Bars represent means $\pm$ SEM. Statistical comparisons were made using a two-sided independent samples t-test on log2-transformed values. *** $p < 0.001$. All p values are listed in Supplementary Dataset 3.

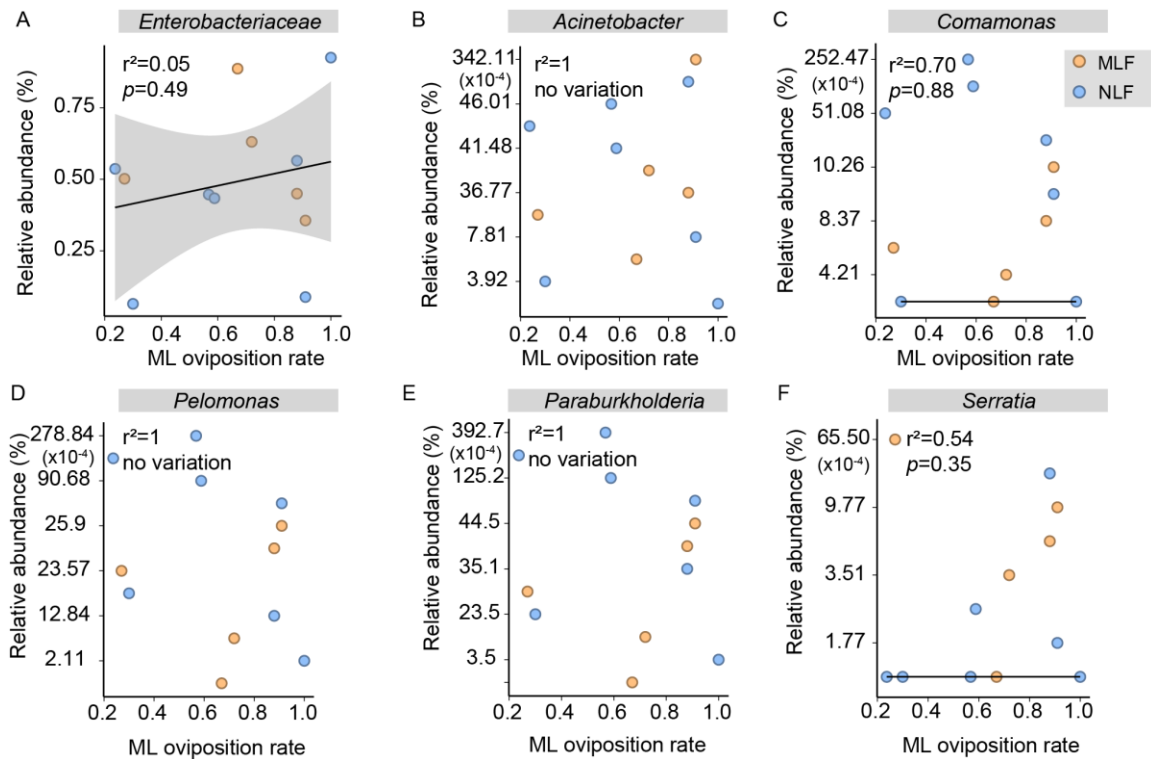


Figure S2. Correlations between maternal oviposition preference for mature leaves and gut bacterial relative abundance in *Plagioderia versicolora*. Correlations between the proportion of eggs laid on mature leaves (ML oviposition rate; x-axis) and the relative abundance (%) of gut bacterial taxa (y-axis). Data points represent beetles from the Mature Leaf Fed (MLF, orange circles, $n = 5$) and New Leaf Fed (NLF, blue circles, $n = 7$) groups. Panels show correlations for: (A) *Enterobacteriaceae*, (B) *Acinetobacter*, (C) *Comamonas*, (D) *Pelomonas*, (E) *Paraburkholderia*, and (F) *Serratia*. Linear regression lines, R-squared values (r^2), and p values are provided for each correlation.

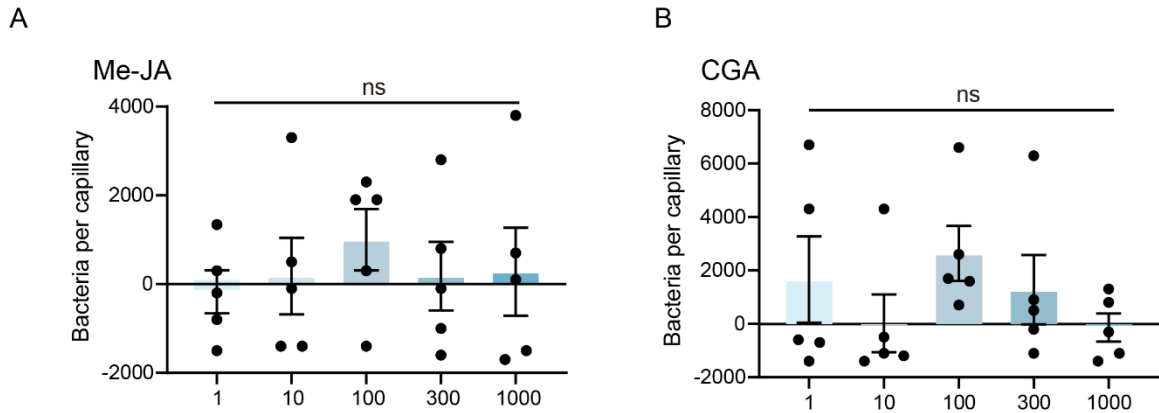


Figure S3. Chemotactic response of *Pseudomonas putida* toward methyl jasmonate (MeJA) and chlorogenic acid (CGA). *In vitro* capillary chemotaxis assays showing the response of *P. putida* toward varying concentrations (1, 10, 100, 300, and 1000 μM) of (A) methyl jasmonate (MeJA) and (B) chlorogenic acid (CGA) ($n = 5$). No significant chemotactic response was observed for either compound across the tested concentration range. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test. "ns" indicates not significant. All p values are listed in Supplementary Dataset 3. Data are presented as mean $\pm$ SEM.

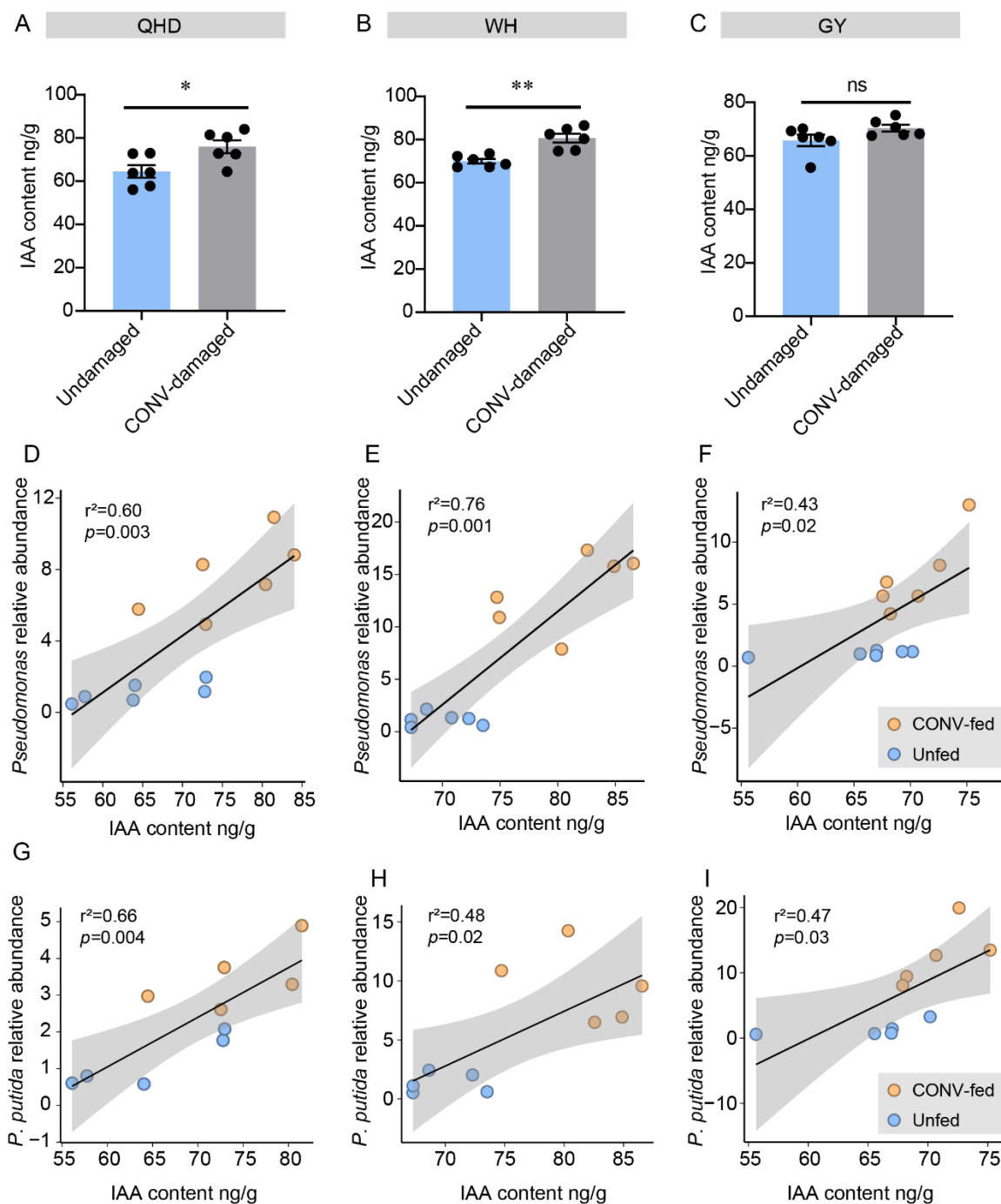


Figure S4. Indole-3-acetic acid (IAA) content in plant leaves following *Plagioderia versicolora* feeding and its correlation with *Pseudomonas* and *P. putida* abundance across different geographical locations. (A-C) IAA content in plant leaves following feeding by *Plagioderia versicolora* adults. Leaves collected from the apical end of shoots recently fed upon by *P. versicolora* adults (CONV-damaged) were compared to undamaged control leaves. Samples were obtained from six individual plants ($n = 6$) at three distinct geographical locations: Qinhuangdao (QHD, A), Wuhan (WH, B), and Guiyang (GY, C). Bars represent means $\pm$ SEM. Statistical significance was determined

using two-sided independent samples t-tests. * $p < 0.05$, ** $p < 0.01$, ns, not significant. (D-F) Correlations between IAA content and the relative abundance of the genus *Pseudomonas* in QHD (D), WH (E), and GY (F) ($n = 6$ for each group). (G-I) Correlations between IAA content and the relative abundance of *P. putida* in QHD (G), WH (H), and GY (I) ($n = 5$ for each group). For all correlation plots (D-I), orange and blue circles represent CONV-damaged and undamaged leaf samples, respectively. The solid line is the linear regression fit, shaded area is the 95% confidence interval. Reported r^2 and p values are from Pearson's correlation. All p values are listed in Supplementary Dataset 3.

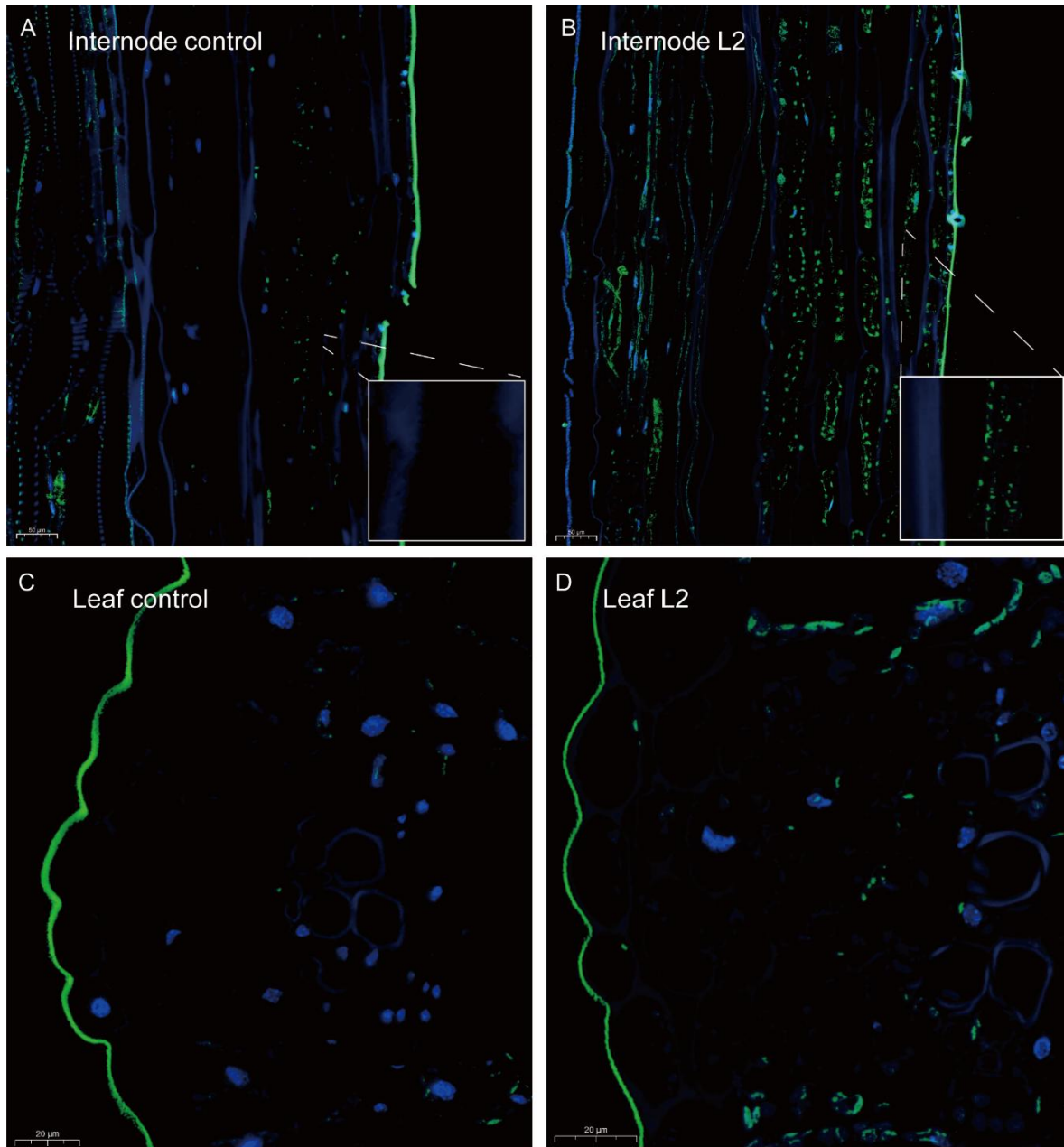


Figure S5. Immunofluorescence localization of eGFP-tagged *P. putida* within willow tissues following beetle feeding. Representative fluorescence microscopy images showing the translocation of eGFP-tagged *P. putida* in longitudinal sections of stem internodes and leaf tissues (3 µm thickness). Samples were collected 12 h after feeding by female beetles. Tissues were fixed in FAA, embedded in paraffin, and stained with an anti-eGFP primary antibody followed by an Alexa Fluor 488-conjugated secondary antibody (green). (A) Internode control section from an undamaged plant. (B) Internode L2 section from the stem adjacent to the damaged leaf, showing bacterial clusters (green) distributed within the vascular bundles. The inset provides a magnified view of the bacterial colonization. (C) Leaf control section from an undamaged plant. (D) Leaf L2 section showing the presence

of *P. putida* within the internal vascular tissues. In all panels, green fluorescence indicates eGFP-tagged *P. putida*, while blue signals represent host plant structures (β -actin stained with TRITC-Phalloidin or autofluorescence). Images were captured using a fluorescence microscope with excitation/emission wavelengths of 488/511 nm for eGFP. Scale bars: 50 μ m (top); 20 μ m (bottom).

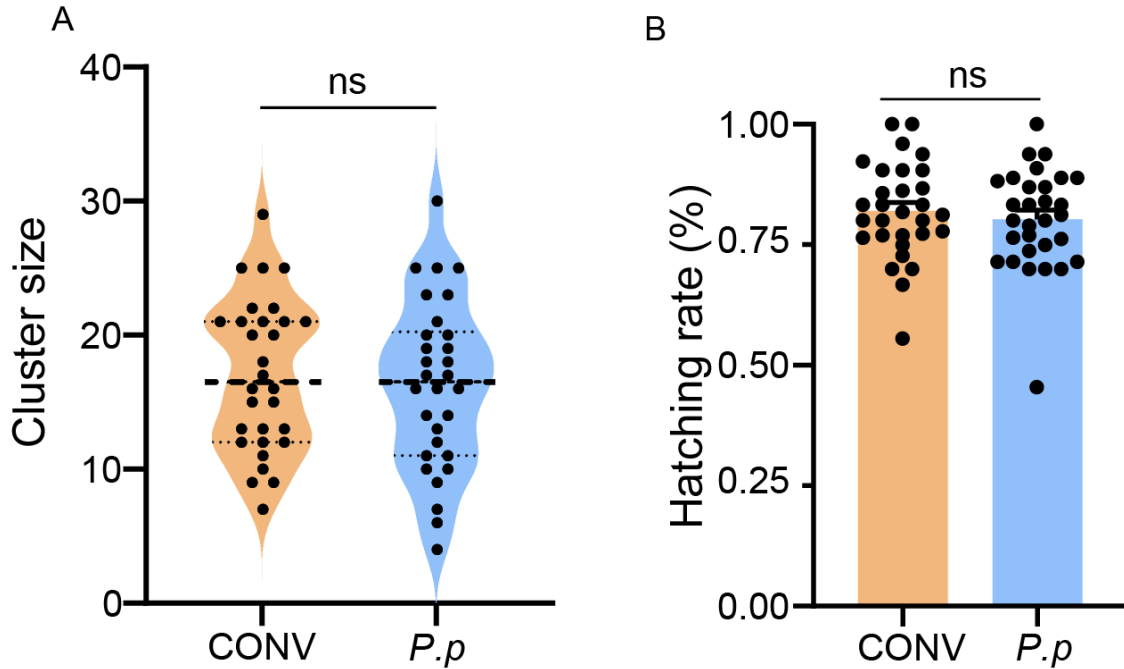


Figure S6. Impact of *Pseudomonas putida* exposure on adult fecundity and egg viability. (A–B) Comparison of reproductive performance between adults fed on detached leaves coated with *P. putida* (1×10^4 CFU/cm²) and conventional (CONV) controls (n=30). **(A)** Number of eggs laid per female, presented as a violin plot with individual data points and median/interquartile ranges indicated. **(B)** Hatching rate (%) of eggs from the respective treatment groups. No significant differences were observed between the *P. putida*-treated and CONV control groups for either parameter. Statistical significance was determined using two-sided independent samples *t*-tests. "ns" indicates not significant. All *p* values are listed in Supplementary Dataset 3. Bars represent means $\pm$ SEM.

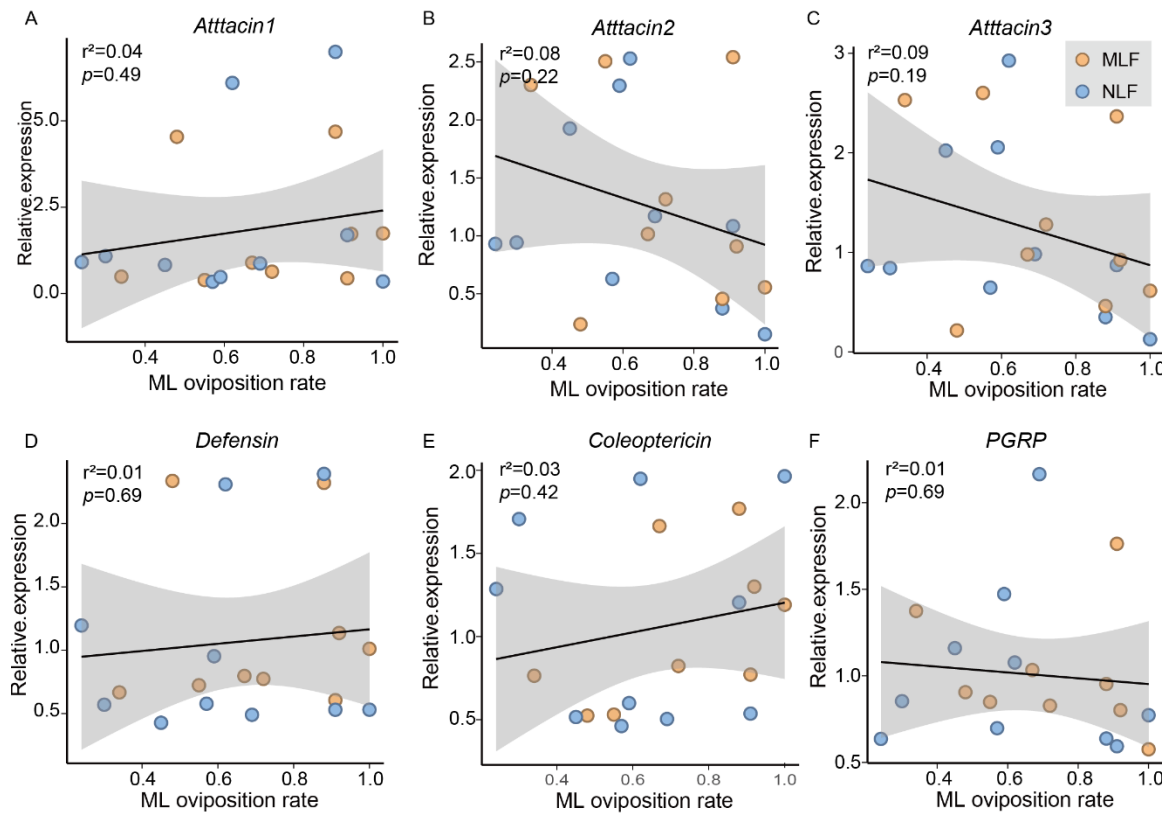


Figure S7. Pearson's correlations between the mature leaf (ML) oviposition rate and the relative expression levels of six immune-related genes in female heads: (A) *Attacin1*, (B) *Attacin2*, (C) *Attacin3*, (D) *Defensin*, (E) *Coleoptericin*, and (F) *PGRP* (n=19 total). Orange and blue circles represent CONV and GF females, respectively. No significant correlations were observed between the expression of these immune genes and oviposition behavior. Solid lines represent linear regression fits with 95% confidence intervals (shaded).

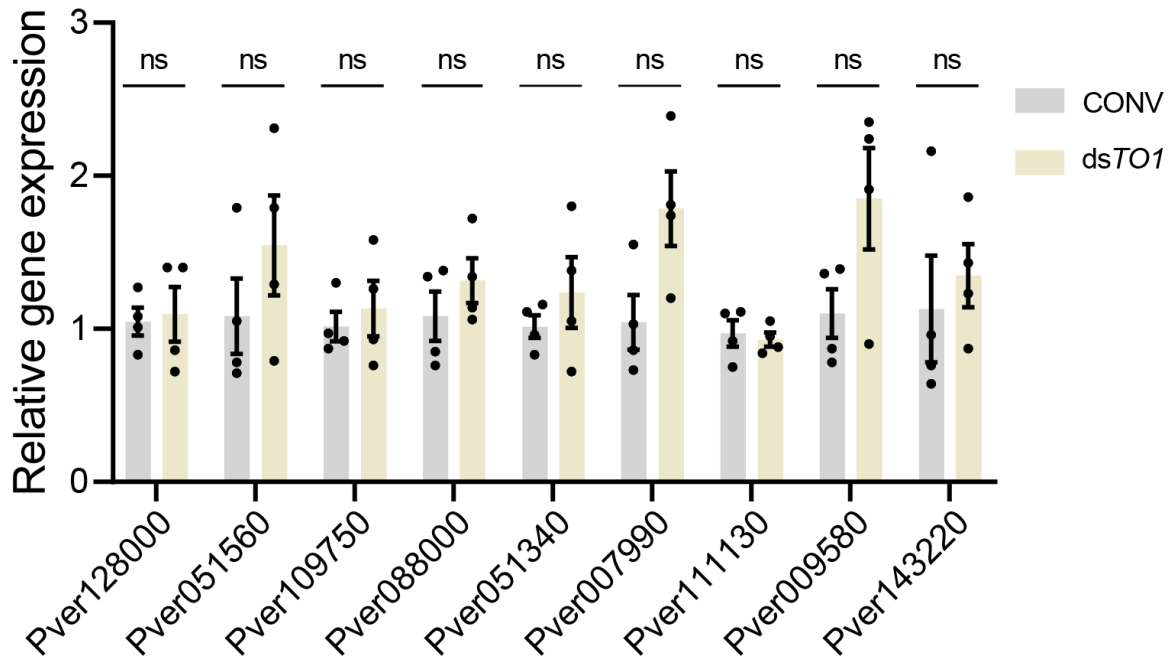


Figure S8. Assessment of potential off-target effects of *dsTO1* on other *takeout* homologs. To verify the specificity of RNA interference, potential off-target genes were predicted using the TBtools tool. The *dsTO1* sequence was blasted against a local database built from the *P. versicolora* genome-predicted transcripts. Sequences with continuous matches ≥ 19 bp were identified as high-risk off-target candidates. Only genes with an average expression level of TPM > 10 in the head transcriptome were included. The relative expression levels of nine identified *takeout* homologs (*Pver128000*, *Pver051560*, *Pver109750*, *Pver088000*, *Pver051340*, *Pver007990*, *Pver111130*, *Pver009580*, and *Pver143220*) were quantified in the heads of CONV and *dsTO1*-treated females ($n = 4$, 10 heads per replicate). No significant differences in the expression of these homologs were observed between the two groups, confirming the high specificity of the *dsTO1* treatment. Statistical significance was determined using two-sided independent samples *t*-tests. "ns" indicates not significant ($p > 0.05$). All *p* values are listed in Supplementary Dataset 3. Data are presented as mean $\pm$ SEM.

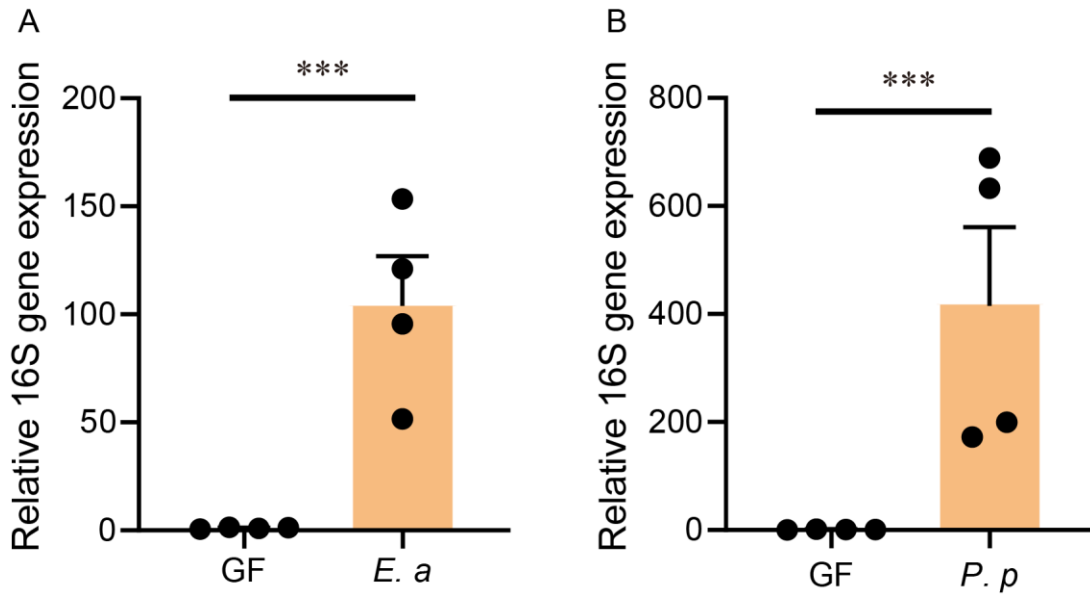


Figure S9. Confirmation of successful colonization of *Pseudomonas putida* and *Enterobacter aerogenes* in germ-free *Plagioderma versicolora* adults. Relative abundance of the bacterial 16S rRNA gene, measured by qPCR, in germ-free (GF) adults compared to those colonized with either *Enterobacter aerogenes* (*E.a*) in panel (A) or *Pseudomonas putida* (*P.p*) in panel (B). For all 16S rRNA qPCR analyses, CT values greater than 38 were considered below the detection limit and were assigned a value of 0. Data represents independent biological replicates (n = 4, 3 guts per replicate). Bars show means ± SEM. Statistical significance was assessed using two-sided independent samples *t*-tests on log2-transformed values. ****p* < 0.001. All *p* values are listed in Supplementary Dataset 3.