Entomologia Generalis, Volume 43 (2023), Issue 6, 1151–1160
Published online November 21, 2023

The intrinsic diversity of *Nezara viridula* gut symbionts affects the host fitness decline promoted by primary symbiont elimination

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With 3 figures

Abstract: Pentatomid insects harbour an obligate gut symbiont, vertically transmitted via egg smearing by the females. The disruption of symbiont acquisition can be exploited for pest management, by spraying the egg surface with anti-symbiont agents, yet the insect response to symbiont deprivation remains a determining aspect of the success of this strategy. Here the effect of anti-symbiont egg masses treatment was assessed in an Italian population of the southern green stink bug *Nezara viridula* under field and laboratory conditions. In soybean fields, *N. viridula* was poorly affected by treatment, whereas in laboratory, nymphs deriving from treated egg masses underwent increased mortality and symbiont abundance decline. The primary symbiont dominated the microbiome of non-treated nymphs showing low mortality. Conversely, dysbiosis was recorded in treated samples as well as in control nymphs with high mortality. Nonetheless, dysbiosis induced by treatment was not in all cases associated with high mortality, and symbiont was missing in some non-treated samples as well. Five variants of a *Pantoea* symbiont were found, with higher variant diversity in untreated samples with low mortality. Altogether, our results provide information about the gut microbiota dynamics in the early nymphal instars of *N. viridula* and suggest a still incomplete dependence on the symbiont. However, egg masses anti-symbiont treatment increased mortality, therefore symbiont-targeted control may be used against *N. viridula* as a supplementary tool under high pest pressure.

Keywords: interaction; dysbiosis; soybean; pentatomid; 16SrRNA sequencing; *Pantoea*; microbiota dynamics

1 Introduction

Insects are undoubtedly the most species-rich group of animals on Earth, with more than a million described species. Their evolutionary success has been influenced by the symbiotic association with bacteria that positively affects the host in different ways, from upgrading nutrient-poor diets to even interfering in insect behaviour (Cornwallis et al. 2023). In addition to bacterial symbionts, a relevant beneficial role is played by the gut microbiome, where changes in its functionality and composition (gut dysbiosis) have been shown to affect the host health (e.g., Di Lelio et al. 2023; Hamdi et al. 2011).

Insects of the family Pentatomidae (Hemiptera: Heteroptera), commonly known as stink bugs, are worldwide distributed and several are relevant agricultural pests. Studies on the interaction between stink bugs and their gut microbiome showed that obligate bacterial symbionts inhabit a posterior region of the midgut (Prado et al. 2006; Taylor et al. 2014). This region (V4 ventricle or caeca) consists of small crypts that form four tubular out-growths (Hirose et al. 2006). The midgut crypts of most pentatomids are inhabited by symbionts of the genus *Pantoea* (Duron & Noël 2016). The vertical transmission of the symbiont occurs during oviposition: females smear on the egg surface a secretion produced by colleterial glands containing the symbiotic bacteria, thus allowing the immediate acquisition of these microorganisms by the newly hatched nymphs (Prado et al. 2006). Preventing symbiont acquisition by egg surface sterilization is harmful for many stink bugs species (Tada et al. 2011; Taylor et al.

DOI: 10.1127/entomologia/2023/2224
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2014). Delivering antibacterial agents on the egg surface to disrupt the vertical transmission of the symbiont is an innovative strategy of pest management (Gonella et al. 2023).

The southern green stink bug *Nezara viridula* (Linnaeus) (Hemiptera: Heteroptera: Pentatomidae) is a cosmopolitan, highly polyphagous insect (Todd 1989). Its strong preference for legumes makes *N. viridula* one of the most important pests in soybean culture, causing huge economic losses by yield reduction and, consequently, the use of pesticides, which ultimately brings adverse environmental impact (Hladik et al. 2014). Metagenomics revealed the presence of a non-cultivable symbiotic bacterium of the family Enterobacteriaceae in the gut of *N. viridula* (Hirose et al. 2006). The primary *Pantoea* symbiont of *N. viridula* is dominant in the V4 ventricle, while in the other gut regions other bacteria are present (Medina et al. 2018, 2022). The egg-associated microbiota is also dominated by the symbiont *Pantoea*, indicating its transmission modality (Geerinck et al. 2022).

Although detrimental for many stink bug species, the symbiont removal by egg surface sterilization showed variable effects on different populations of *N. viridula*. While Hawaiian populations managed to survive and reproduce equally well after symbiont deprivation, Japanese populations showed instead higher mortality with very few individuals reaching the adult stage (Prado et al. 2006; Tada et al. 2011).

Previous studies characterized the culturable fraction of the whole gut microbiota of *N. viridula* adults (Hirose et al. 2006; Medina et al. 2018). Conversely, little is known about the whole microbiota composition of *N. viridula* nymphs. At this stage, some bacteria other than the primary symbiont may be involved in the reported differential mortality observed when *N. viridula* symbiont is removed.

Here, we evaluated the response of an Italian *N. viridula* population and its microbiota to symbiont-targeted control. Specifically, we performed field and laboratory tests to evaluate: i. the efficiency of egg masses anti-symbiont treatments on insect survival; and ii. the changes induced by the treatment on first and second instar nymphs microbiota.

2 Material and methods

2.1 Field assessment of stink bug response to symbiont-targeted control

A preliminary assessment of *N. viridula* response to the interruption of the symbiont vertical transmission was done through symbiotic control experimental trials during 2021 and 2022 in a soybean field (var. P21T45) (Italy, Piedmont, Carmagnola – TO; (44°52′33.8″N, 7°45′45.8″E) where the occurrence of the species together with another pest pentatomid stink bug, *Halyomorpha halys* Stål, had been previously recorded. The symbiont targeted control was performed on both species using a commercially available micronutrient fertilizer (Dentamet®, Diachem S.p.A, Italy). This product, a biocomplex composed of zinc, copper and citric acid, had been previously shown to supress *H. halys* newborns via symbiont elimination (Gonella et al. 2019). The latter species thus can be considered as a suitable model to compare the effect of the treatment on *N. viridula*. Nine plots of 600 m^2 were selected in the field and assigned to three different treatments, with non-randomized replications (Table S1): 1) Dentamet®, 0,3 L/hL, 4 applications; 2) lambda-cyhalothrin (Karate Zeon 1.5, Syngenta, Italy), 0,2 L/hL, 1 application; 3) untreated used as control. Treatments with Dentamet® were conducted every 7–10 days, starting from the beginning of stink bug oviposition in the field, whereas the treatment with lambda-cyhalothrin was performed once the stink bug occurrence was observed. Seven days after each treatment, abundances of *N. viridula* and *H. halys* nymphs were evaluated visually in a single plant each 5 metres, in two internal lines of each plot.

2.2 Insect rearing

For laboratory experiments, we used a stock of *N. viridula* collected during the summer 2021 from the same location were field experimental trials occurred. The colony was maintained at the DISAFA laboratories in mesh cages (930 × 475 × 475 mm) in a climatic chamber under controlled conditions, at 25 °C and 70% RH and under a long-day regimen (16 h light, 8 h dark). Insects were fed with dry soybean seeds, hazelnuts, apples and fresh green bean pods. Green bean plants were deposited inside the rearing cages to facilitate oviposition. Egg masses were collected daily and used for the egg mass exposure to anti-symbiont experiment.

2.3 Symbiont-targeted treatment of egg surface and measurement of pre-adult survival

The Dentamet® biocomplex was used to assess symbiont elimination from the egg surface. Egg masses 24 hours old ($n = 48$) were collected and randomly assigned to one of the treatments (Dentamet® or distilled water, as control). The egg masses contained an average of 70 eggs each (treated group: mean ± SE: 75 ± 3.3 eggs, control: 69 ± 3.4 eggs, unpaired *t* Student test, n.s.). Each egg mass was placed individually in a Petri dish with filter paper at the bottom. Treated egg masses were sprayed with Dentamet® using a 250 ml hand sprayer (Nalgene®, NY, USA). A single spray was applied under a fume hood at a concentration of 1% v/v as described in Gonella et al. (2019), while controls were sprayed with distilled water. Each egg mass was kept in a separate Petri dish with a wider lid respect to the base to provide ventilation.

Egg masses were individually maintained inside the Petri dish in a climatic chamber under the conditions described above. Time to hatching and hatching rate (calculated as the total of hatched eggs from the total number of eggs per egg mass) were recorded daily. Once hatched, nymphs were pro-

vided with a fresh bean pod and a wet cotton ball, that were changed every two days. Newly hatched nymphs from each egg mass were kept individually during the whole experiment. A subset of dead first instar and alive second instar nymphs were randomly collected from all replicates and stored separately at −80 °C in RNA later® (Sigma-Aldrich, MO, USA) for further molecular analysis and microbiome characterization. The remaining live specimens were reared, and their survival was monitored every 2 days until they either reached the adult stage or died. For all replicates, the total number of individuals observed for each nymphal instar was recorded. To evaluate development time, the day on which 50% of the total individuals reached the following instar was registered. The mortality rate in each nymphal instar was calculated as the total number of dead nymphs out of the total number of nymphs recorded for that instar.

2.4 RNA extraction and diagnostic Real Time q-PCR

Real Time PCR was used to determine the presence of *N. viridula* symbiont in nymphs deriving both from egg masses treated with Dentamet® and the control (hereafter D- and C-). Samples subject to symbiont screening were chosen based on the mortality rates observed during the first instar, considering that we observed two distinct sample groups with no overlap. The category 'low mortality' (LM) included replicates with less than 50% mortality while the category 'high mortality' (HM) stood for replicates with mortality higher than 50%. LM samples were taken among second instar nymphs found alive at the end of the experiments, whilst HM samples were collected among nymphs dead at the first instar. We followed the RNA-based approach proposed by Gonella et al. (2019) to avoid possible false positive detection due to amplification of the DNA related to dead symbiont cells. Selected specimens were individually subjected to total RNA extraction with SV Total RNA Isolation System (Promega, WI, USA) following the manufacturer's instructions. After extractions, RNA quality and concentration were assessed with a ND-1000 spectrophotometer (NanoDrop, DE, USA). First strand cDNA was synthesized by using the Reverse Transcription System (Promega) and random primers, following the manufacturer's instructions. cDNA was used as a template for Real Time PCR analysis with the symbiont-specific primers PNVir1F (5′-GGCTTGAGTCTCGTAGAGGG-3′) and PNVir1R (5′-CGGAAGCCACAGTTCAAGACTA-3′) designed in this study to amplify a 210 bp fragment of 16SrRNA gene. Primers were designed using NCBI Primer-BLAST, based on the symbiont sequences obtained through SMRT sequencing and sequences available in the NCBI database. PCR reactions were performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA) in 25 µl volume containing: 12.5 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 0.1 µl of 100 µM forward and reverse primer, 11.3 µl of sterile water, and 600 ng of cDNA template. PCR thermal conditions: 95 °C for 3 min followed by 40 cycles at 95 °C for 10 s and 56 °C for 30 s. A final step for melting curve analysis from 70 to 95 °C, measuring fluorescence every 0.5 °C, was added. To verify whether negative nymphs were truly deprived of the symbiont, rather than misdiagnosed due to cDNA quality, Real Time PCR targeting the insect 18S rRNA gene was used following Marzachì & Bosco (2005).

2.5 Full length 16S rRNA library preparation and sequencing

Four replicates from each treatment-mortality category, consisting of a pool of five nymphs obtained from the same egg mass, were selected. Total RNA was extracted and subject to retrotranscription as described above. One negative control with RNA extraction reagents alone and one sequencing service internal control were included. The SMRTbell libraries (Pacific Biosciences), targeting 16S rRNA primers 27F/1492R (Frank et al. 2008), were prepared and sequenced using PacBio Sequel following the manufacturer's guidelines by the Molecular Research DNA Labs, Inc (www.mrdnalab.com; Shallowater, TX, USA). After completion of initial DNA sequencing, each library underwent a secondary analysis, Circular Consensus Sequencing using PacBio's CCS algorithm (Larsen et al. 2014).

2.6 Bioinformatic analysis

Bioinformatic analyses of 16S rRNA sequencing results were performed using the R software (http://www.R-project.org) and the QIIME2 platform (Bolyen et al. 2019) as described in previous studies (e.g., Brunetti et al. 2022). Briefly, raw reads were denoised, filtered and checked for chimera presence using the dada2 algorithm (Callahan et al. 2016) aiming to obtain the bacterial Amplicon Sequence Variants (ASVs). The following parameters of the dada2 algorithm were used: minQ = 3, minLen = 1,000, maxLen = 1,600, maxEE = 2, minFoldParentOverAbundance = 3.5. The obtained ASVs were taxonomically annotated using the naïve Bayes classifier (Wang et al. 2007) implemented in the assignTaxonomy function of dada2 and using the release 138 of the SILVA database (Quast et al. 2012) as reference for sequences and taxonomy. Furthermore, ASVs assigned to the genus *Pantoea* were re-classified using the naïve Bayes classifier implemented in QIIME2 using a self-developed reference database consisting of more than 8,000 *Pantoea* 16S rRNA gene sequences obtained from the NCBI database and available at http://doi.org/10.6084/m9.figshare.23808015.

Rarefied tables (2,198 sequences per sample) were generated to normalise the datasets before the diversity analyses based on rarefaction curves inspection. Alpha diversity indices (richness, Pielou evenness, Shannon and Simpson index) were generated for all samples. Differences in the alpha-diversity estimates were visualized with boxplots and tested

with the Kruskal-Wallis test. To account for the sparse compositional nature of microbiome datasets a robust Aitchison PCA (Martino et al. 2019, q2-deicode) was applied using the tool DEICODE on QIIME2 to calculate log-ratios and differential ranks of ASVs associated with different groups.

2.7 Full length 16S rRNA gene tree

The 16S rRNA gene sequences obtained in this study and assigned to *Pantoea*, along with a selection of *Pantoea* and *Erwinia* sequences obtained from GenBank (including *N. viridula*'s primary symbiont, other stinkbugs symbionts and *Yersinia pestis* as outgroup) were included in the 16S rRNA full length dataset for phylogenomic analyses. Sequences were aligned using the MUSCLE algorithm (Edgar 2004). Hasegawa-Kishino-Yano substitution model with Gamma distribution and proportion of invariant sites was selected as best nucleotide substitution model. Maximum-Likelihood gene tree was inferred using MEGA-X software (Tamura et al. 2021) (selected option: gap treatment = partial deletions; bootstrap = 1,000 replicates; tree searching method = *Nearest-Neighbor-Interchange*).

2.8 Statistical analyses

To compare the effect of egg masses exposure to Dentamet®, hatching and mortality rates in each nymphal instar were assessed using a generalized linear model (GLM) with a binomial probability distribution and logit link function. Symbiont-positive nymphs according to treatment and mortality category were evaluated using the Fisher's Exact test. Development time in all five nymphal instars was evaluated by means of the Kruskal-Wallis test. The abundance of nymphs observed in the field trials, according to the treatment and throughout each survey, was evaluated using a two-way ANOVA test and means in each survey were separated through a Bonferroni's post hoc test. All statistical analyses were performed in R.

3 Results

3.1 Stink bug response to symbiont-targeted control in soybean field

The number of collected nymphs of both *N. viridula* and *H. halys* followed the phenology of the crop in the soybean field throughout the 2021 and 2022 growing seasons (Fig. 1A, B). Contrastive responses were recorded when comparing the nymphal abundance of the two species according to the treatment. As for *N. viridula*, no significant differences were observed between treatments, except from the third survey performed in 2021 (t3), where nymphal abundance was significantly higher in the control plots than in both the Dentamet® and the insecticide treatment (Bonferroni's test, $P < 0.001$). In contrast, significant differences were observed in the number of *H. halys* nymphs between treatments, excepting those in which a general low population was recorded (t1 and t2 2021, and t2 2022). In all other cases, the abundance of nymphs was higher in the control plots than in plots treated with lambda-cyhalothrin and with Dentamet® (Bonferroni's test, $P < 0.001$).

3.2 Mortality after egg masses symbiont-targeted treatment under laboratory conditions

The hatching rate did not change between egg masses sprayed with Dentamet® (82.1%) and control (85.7%), nor did the time to hatching (Dentamet®: mean ± SD 5.5 ± 1.96 days; control: 5.5 ± 2.06 days). During the first instar, nymphs from egg masses treated with Dentamet® showed significantly higher mortality than nymphs derived from control egg masses (Dentamet®: 33.4% ± 7.1%, n = 24; control 15.2% ± 4.2%, n = 24; $\chi^2 = 175.09$, df = 1, $P < 0.001$) (Fig. 1C). A similar trend was found for mortality at the fourth and fifth instars (fourth instar, control 24.7% ± 6, n = 18 and Dentamet® 35.3% % ± 9, n = 15; $\chi^2 = 21.4$, df = 1, $P < 0.001$; fifth instar, control 22.7% ± 5, n = 18; Dentamet® 31.8% ± 10, n = 13 $\chi^2 = 14.01$, df = 1, $P < 0.001$). On the contrary, no differences were observed in mortality rates during the second and third nymphal instars. A significant increase in the development time was observed only for the first instar from treated egg masses compared to the control (control: 5.1 ± 1.5 days, n = 24; Dentamet®: 6.7 ± 1.7 days, n = 23; $\chi^2 = 10.2$, df = 1, $P = 0.0013$) (Fig. 1D).

Despite the significant differences observed in mortality rates during the first instar, a remarkable number of egg masses showed low levels of mortality among newborns, irrespective of treatment. During the first instar, a mortality of 50% or higher was registered for 6 out of 24 egg masses treated with Dentamet®, as well as for 4 out of 24 from the control group.

3.3 Symbiont infection in *N. viridula* nymphs after egg masses treatment

Altogether, 19 D-LM, 20 C-LM, 8 D-HM, and 5 C-HM nymphs were used for the molecular detection of symbiont infection (Fig. S1). HM samples were less numerous, in fact, only 21% of replicates with high mortality were recorded. Regarding the C-LM group, 18 out of 20 nymphs were symbiont-positive. Conversely, only 1 out of 5 C-HM nymphs was symbiont positive. Only 4 of the 19 D-LM nymphs carried the symbiont, whilst 4 out of 8 D-HM samples were symbiont-positive. The symbiont presence in samples with low mortality was significantly different between treatment and control groups (Fisher's exact test, $P < 0.001$).

3.4 Characterization of *N. viridula* microbiota

A total of 1,552,848 16S rRNA gene raw reads (mean per sample = 86,269.3) were obtained (project accession number PRJNA985561). After the filtering step, a total of 125

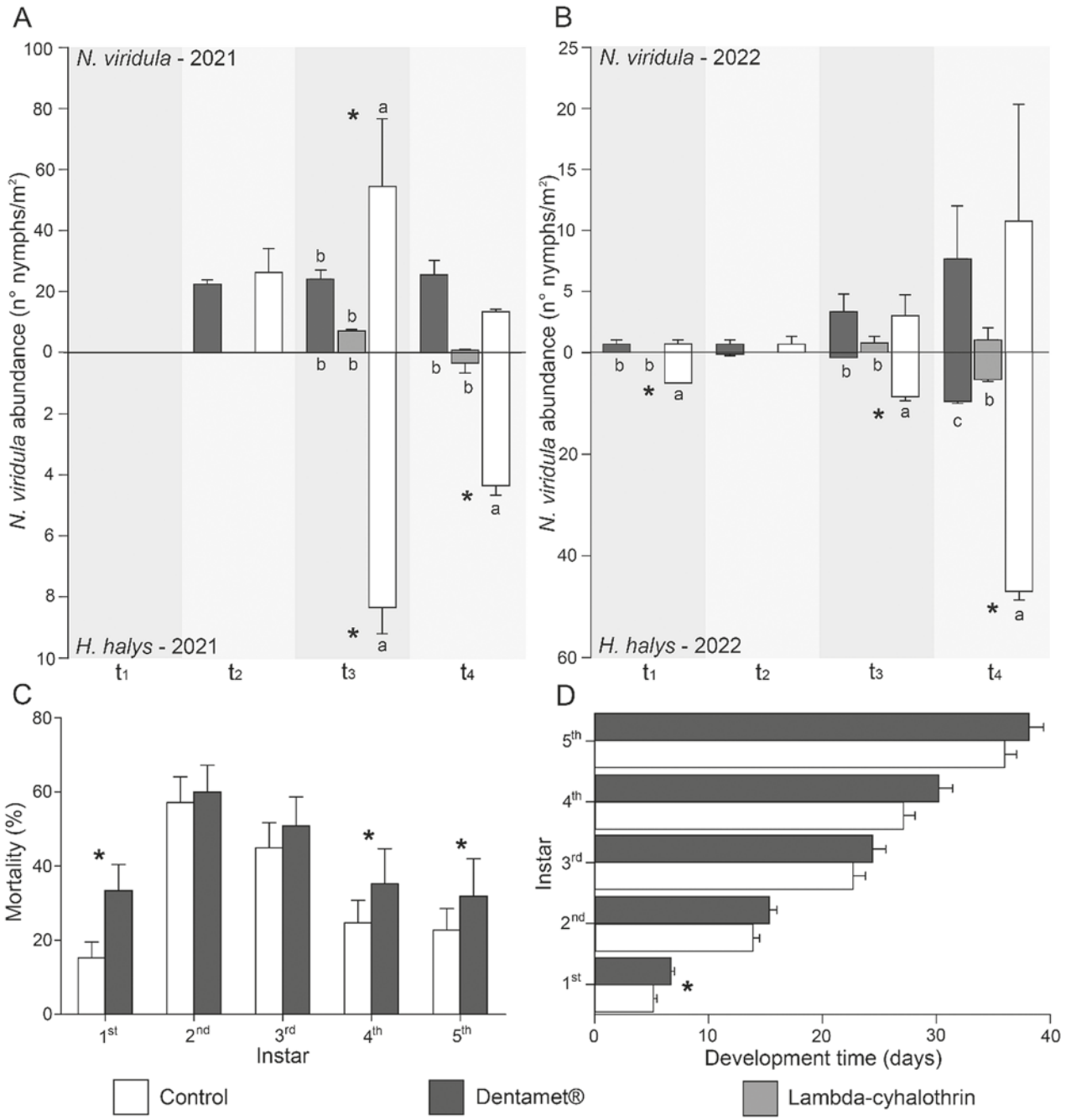

Fig. 1. *Nezara viridula* symbiont-targeted treatment. **A, B)** Evaluation of anti-symbiont treatment in soybean field trials occurred in 2021 **(A)** and 2022 **(B)**; number of *Halyomorpha halys* nymphs per m² are reported for comparison. Asterisk indicates statistically significant differences according to ANOVA with Bonferroni's post-hoc test ($P < 0.05$), while different letters indicate the significant differences between pairs of bars. Nymphal instars mean mortality rates **(C)** and development time **(D)** under laboratory conditions. Error bar indicates standard error; within the same instar, asterisk indicates statistically significant differences according to binomial GLM analysis ($P < 0.05$) **(C)** and Kruskal-Wallis test ($P < 0.05$) **(D)**.

ASVs were identified in the sixteen samples (average reads per sample = 39,430) (Table S2). One sample (D-HM2) was excluded from the analysis due to the low number of reads.

The ASVs were assigned to 21 bacterial genera belonging to 19 families (Fig. 2A). The most abundant bacterial genera (> 5% of the total number of reads) were: *Pantoea* (45.4%), *Serratia* (24.1%), *Cedecea* (7.4%), *Stenotrophomonas* (6.3%), *Pseudomonas* (6.0%) and *Staphylococcus* (5.5%). Approximately 90% of *Pantoea* ASVs were identified as *N. viridula* symbiont. Alpha diversity analysis indicated that

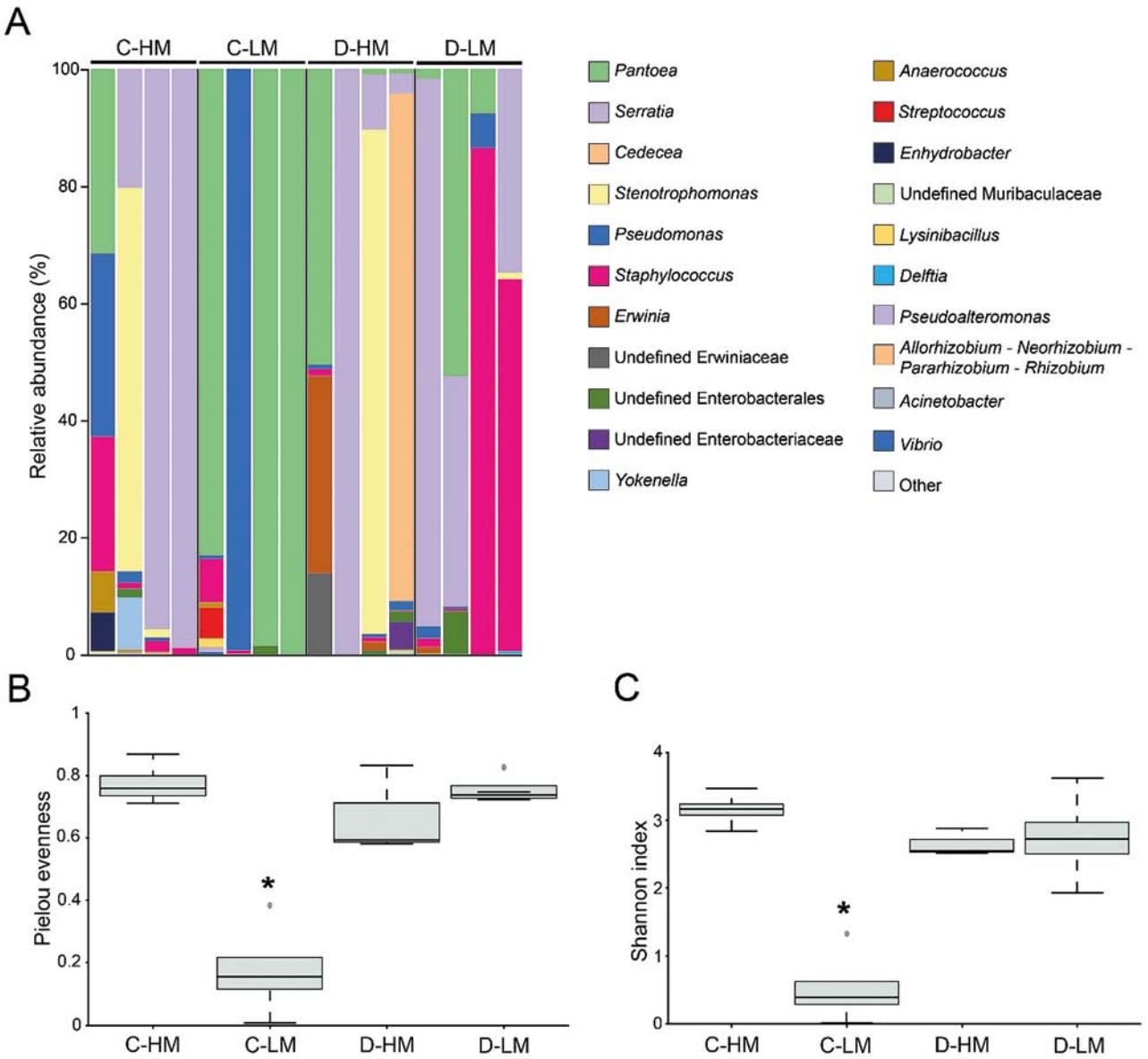

Fig. 2. *Nezara viridula* microbiota based on full length 16S rRNA. **A)** Bar plot reporting the microbiota composition of four *N. viridula* groups (C-LM: Control low mortality; C-HM: Control high mortality; D-LM: Dentamet® low mortality; D-HM: Dentamet® high mortality). **B, C)** Microbiota diversity measured by the Pielou's evenness **(B)** and the Shannon index **(C)**. Asterisk (*) indicates statistically significant difference according to Kruskal-Wallis test ($P < 0.05$).

all C-LM samples have significantly lower diversity than the others (Pielou, Shannon and Simpson index; $P < 0.05$; Fig. 2B, C).

3.5 16S rRNA gene variability and phylogenetic placement of *N. viridula* symbiont and other *Erwiniaceae*

Sequencing the full length of the 16S rRNA gene allowed the identification of five variants of *N. viridula* symbiont (hereafter named *Pantoea* NVir 1–5) with different distribution and relative abundance in the samples (Fig. 3A). *Pantoea* NVir1 was the most abundant, detected in samples from all treatment-mortality combinations, although it was more abundant in C-LM samples. Each one of the other variants was present in one C-LM sample except for *Pantoea* NVir3, detected in one C-HM. Furthermore, the log-ratios of *N. viridula* symbiont variants were relatively higher in the C-LM samples in respect to all other ASVs, compared to the other three groups (Fig. 3B; ANOVA: $F = 4.35$; $P = 0.027$ of C-LM *vs* D-HM).

The ASVs belonging to the *Erwiniaceae* family and not identified as *Pantoea* NVir were mostly recorded in samples

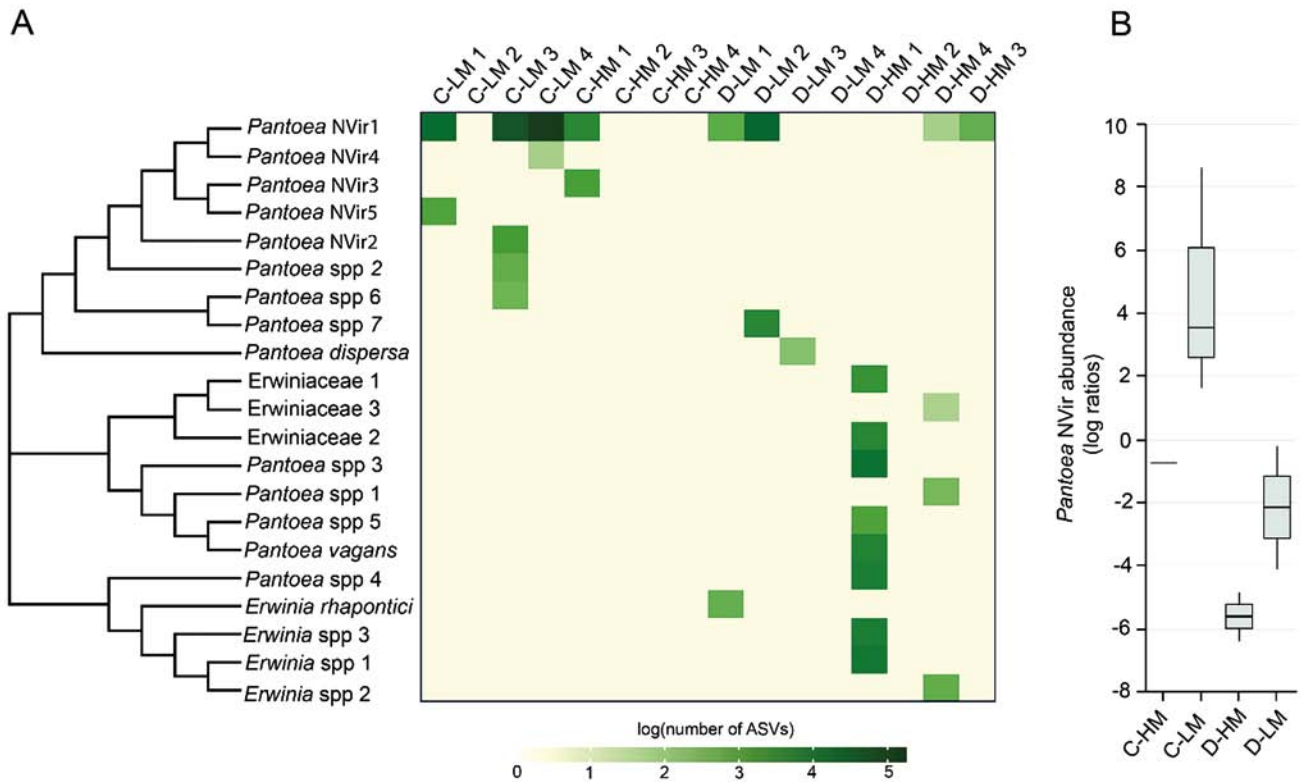

Fig. 3. Diversity and abundance of *Pantoea* NVir and other Erwinaceae bacteria found in *N. viridula* according to treatment and mortality level. **A)** 16S rRNA UPGMA dendrogram and heatmap of the total number of reads assigned to each ASVs after log transformation. **B)** Boxplots showing the log-ratio of the number of *Pantoea* NVir ASVs over the total number of ASVs for each treatment-mortality category.

treated with Dentamet®. Only two *Erwiniaceae* that were not identified as *Pantoea* NVir were present in control samples (*Pantoea* spp. 2 and 6), although both were closer to *Pantoea* NVir variants than to the other *Pantoea* based on sequence identity.

Two main groups can be observed in the full length 16S rRNA gene tree (Fig. S2). One includes the sequences assigned to *N. viridula* symbiont obtained in this study, along with sequences belonging to *N. viridula* symbiont from different geographical regions (bootstrap value of 98%). Within this group, a slight divergence of *Pantoea* NVir 2 from the other *Pantoea* NVir can be observed, the former is phylogenetically closer to *Pantoea* spp. variants 2, 5 and 6 from this study. Also the symbiont sequences of *Nezara antennata* fell in this group, while the sequences belonging to other pentatomids symbionts clustered in a separate group, that included also some other *Pantoea* sequences from this study.

4 Discussion

The first evidence reported in this study is the low susceptibility of *N. viridula* to anti-symbiont treatment as observed in field trials, where an opposite response was recorded for *N. viridula* and *H. halys* after treatments with Dentamet®. The abundance of *N. viridula* nymphs was not affected by Dentamet® but only by lambda-cyalothrin, suggesting a limited effect of symbiont deprivation compared to *H. halys* (Gonella et al. 2019, 2023). However, in 2021, in one single sampling date a significant reduction of nymph abundance with respect to control was detected. This event corresponded to the highest *N. viridula* density recorded in our experiments, suggesting that, under high pest pressure, the anti-symbiont treatment may support other pest control tools to increase the success of insect containment.

To decipher the cause of the observed limited susceptibility to symbiont-targeted treatment in the field, the response of *N. viridula* to interruption of symbiont acquisition was assessed under laboratory conditions. Mortality and development time increased during the first instar in nymphs of the group treated with Dentamet®, indicating an immediate effect of interrupting the symbiosis on the host fitness; mortality was higher in the treated group also during the late instars. On the other hand, many treated egg masses resulted in low mortality rates, and altogether, the mortality rates were always lower than those recorded for *H. halys* after treatment with the same product (Gonella et al. 2019).

Molecular analyses confirmed a decreased symbiont acquisition by *N. viridula* nymphs in Dentamet® samples, since the nymphs natural infection rate of 80% dropped to ~20% in the treated ones (Fig. S1). Besides, the fitness defects observed after the treatment were not completely referable to impairment of symbiont acquisition, considering that no significant difference was found in the *Pantoea* NVir abundance between D-LM and D-HM samples. The number of symbiont-negative nymphs of D-LM was higher than in C-HM, indicating that symbiont deprivation does not necessarily imply higher mortality. On the other hand, Dentamet® did not eliminate completely the symbiont from the egg surface, as confirmed by treated-positive samples. This last result was highly divergent from what was reported for *H. halys* where a complete elimination of the symbiont was observed (Gonella et al. 2019). Additionally, several control nymphs were *Pantoea*-negative, suggesting that the substantial variability in the response of *N. viridula* to symbiont elimination may be also related to imperfect vertical transmission, in agreement with previous reports (Prado et al. 2006; Tada et al. 2011).

Accordingly, *N. viridula* symbiont was the most abundant species in three out of four samples from C-LM, proving its dominance within the insect microbiota in the absence of perturbative events, but its abundance decreased strongly in samples from all other three treatment-mortality combinations. We can argue that multiple factors are involved in inducing the microbiota dysbiosis that leads to the loss of dominance of the main symbiont, including the anti-symbiont activity (in the nymphs derived from egg masses treated with Dentamet®) and the incomplete vertical transmission, with a consequent intrinsic mortality. Accordingly, C-HM samples displayed a lower abundance of the *N. viridula* symbiont both in qPCR and targeted metagenomics experiments. Such baseline mortality was slightly increased by anti-symbiont treatment, at least in the first nymphal instar, but the effect was scarce suggesting a limited dependence on the symbiont.

The sequencing of the full-length 16S rRNA allowed detection of five closely related ASVs referable to the genus *Pantoea*, which can be regarded as the main *N. viridula* symbionts. According to our phylogenetic analysis, all five sequences clustered together, except for one variant (*NVir 2*) that showed slight divergence from the others. However, based on these results we cannot state with certainty that the observed ASVs correspond to different variants of the *N. viridula* symbiont or to the nucleotide variability of the different 16S rRNA gene copies present in the symbiont genome. Indeed, up to seven copies of the 16S rRNA gene are present in the genome of *Pantoea* bacteria (Stoddard et al. 2015); three 16S rRNA gene copies are present in the symbiont of *H. halys* '*Candidatus* Pantoea carbekii' (Kenyon et al. 2015). Based on the obtained results, it is not possible to know the exact number of 16S rRNA copies owned by the *N. viridula* symbiont, but interestingly the variants were found either individually or in pairs, with *Nvir1* coupled with *Nvir2, Nvir3, Nvir4*, or *Nvir5*. Therefore, *Nvir1* may correspond to a single symbiont variant (either with one or more identical 16S rRNA gene copies), whereas *Nvir2-Nvir5* seem to correspond to different symbiont variants. However, we cannot argue if they are separated from *Nvir1* or they correspond to a symbiont with at least two 16S rRNA gene copies, one of which is the same as *Nvir1*. Among the variants, *Nvir1* was the most abundant and was recorded in nymphs from both the treated and untreated groups, suggesting it is the main symbiont in the analysed population. All other variants were present in the control samples only, especially C-LM. According to these results, in the absence of anti-symbiont treatment, *N. viridula* low mortality rates during the first instar might be linked to a richer diversity of symbiont variants. The occurrence of several symbiotic variants of *Pantoea*-allied symbiont has been previously reported in other pentatomids, such as *Plautia stali*, where six known symbiotic lineages were reported to equally maintain the host fitness (Hosokawa et al. 2016). In contrast, in *H. halys*, which is highly susceptible to anti-symbiont treatment, no '*Candidatus* Pantoea carbekii' variants are reported (Kenyon et al. 2015). Treatment with Dentamet® resulted in the elimination of the symbiont-related variants with low abundance, which in turn caused the mortality increase.

The estimates of the overall bacterial diversity were consistent with the previous observations on the main symbiont variants. Significant differences were detected in alpha diversity between the C-LM and all the other treatment-mortality combinations. The lower alpha diversity in C-LM is explained by the dominant role of *N. viridula* symbiont(s) over the microbial community. Nymphs where the symbiont is dominant showed low mortality rates during the first instar, confirming that such a condition correlates with the healthy status of the host (Hosokawa et al. 2016; Kenyon et al. 2015). As for the other treatment-mortality combinations, *N. viridula* symbiont was completely absent or its abundance significantly reduced, due to exposure to anti-symbiont treatment or to other unknown factors (e.g., a natural imperfect vertical transmission). Consequently, alpha diversity increases with no complete dominance of other species, describing a dysbiotic scenario.

Several *Pantoea* or *Erwinia*-related sequences, phylogenetically divergent from the symbiont, were recorded mostly in treated samples. Bacteria from both genera are often considered environmental microorganisms and may become either beneficial or opportunistic (Hosokawa et al. 2016; Mikonranta et al. 2015). In this study, *Pantoea* and *Erwinia* were recorded both in D-LM and D-HM, so no conclusions can be stated regarding the effect of these bacteria on insect fitness.

In conclusion, our results showed that anti-symbiont treatment has a moderate effect on the survival of *N. viridula*, and the symbiont deprivation cannot be straightforwardly connected with the effect on insect fitness. Such a

complex scenario suggests incomplete insect dependence on the symbiont *Pantoea* Nvir; moreover, we cannot exclude the symbiont replacement through environmental acquisition in the later nymphal instars. The genomic investigation of the symbiont variants may better clarify if multiple strains colonize the same individual host, as well as their role on the host fitness (e.g., enhancing its nutritional capability on poor substrates).

Acknowledgements: The authors are grateful to SAGEA s.r.l. and DIACHEM S.p.A. for technical support and funding of field experiments.

References

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology, 37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Brunetti, M., Magoga, G., Gionechetti, F., De Biase, A., & Montagna, M. (2022). Does diet breadth affect the complexity of the phytophagous insect microbiota? The case study of Chrysomelidae. *Environmental Microbiology, 24*(8), 3565–3579. https://doi.org/10.1111/1462-2920.15847

Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cornwallis, C. K., van 't Padje, A., Ellers, J., Klein, M., Jackson, R., Kiers, E. T., … Henry, L. M. (2023). Symbioses shape feeding niches and diversification across insects. *Nature Ecology & Evolution, 7*(7), 1022–1044. https://doi.org/10.1038/s41559-023-02058-0

Di Lelio, I., Forni, G., Magoga, G., Brunetti, M., Bruno, D., Becchimanzi, A., … Pennacchio, F. (2023). A soil fungus confers plant resistance against a phytophagous insect by disrupting the symbiotic role of its gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America, 120*(10), e2216922120. https://doi.org/10.1073/pnas.2216922120

Duron, O., & Noël, V. (2016). A wide diversity of *Pantoea* lineages are engaged in mutualistic symbiosis and cospeciation processes with stinkbugs. *Environmental Microbiology Reports, 8*(5), 715–727. https://doi.org/10.1111/1758-2229.12432

Edgar, R. C. (2004). MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics, 5*(1), 113. https://doi.org/10.1186/1471-2105-5-113

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., & Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and Environmental Microbiology, 74*(8), 2461–2470. https://doi.org/10.1128/AEM.02272-07

Geerinck, M. W. J., Van Hee, S., Gloder, G., Crauwels, S., Colazza, S., Jacquemyn, H., … Lievens, B. (2022). Diversity and composition of the microbiome associated with eggs of the Southern green stinkbug, *Nezara viridula* (Hemiptera: Pentatomidae). *MicrobiologyOpen, 11*(6), e1337. https://doi.org/10.1002/MBO3.1337

Gonella, E., & Alma, A. (2023). The Role of Symbiont-Targeted Strategies in the Management of Pentatomidae and Tephritidae Pests under an Integrated Vision. *Agronomy (Basel), 13*(3), 868. https://doi.org/10.3390/agronomy13030868

Gonella, E., Orrù, B., & Alma, A. (2019). Egg masses treatment with micronutrient fertilizers has a suppressive effect on newly-emerged nymphs of the brown marmorated stink bug *Halyomorpha halys*. *Entomologia Generalis, 39*(3–4), 231–238. https://doi.org/10.1127/entomologia/2019/0819

Hamdi, C., Balloi, A., Essanaa, J., Crotti, E., Gonella, E., Raddadi, N., … Cherif, A. (2011). Gut microbiome dysbiosis and honeybee health. *Journal of Applied Entomology*, 135(7), 524–533. https://doi.org/10.1111/j.1439-0418.2010.01609.x

Hirose, E., Panizzi, A. R., De Souza, J. T., Cattelan, A. J., & Aldrich, J. R. (2006). Bacteria in the gut of southern green stink bug (Heteroptera: Pentatomidae). *Annals of the Entomological Society of America, 99*(1), 91–95. https://doi.org/10.1603/0013-8746(2006)099[0091:BITGOS]2.0.CO;2

Hladik, M. L., Kolpin, D. W., & Kuivila, K. M. (2014). Widespread occurrence of neonicotinoid insecticides in streams in a high corn and soybean producing region, USA. *Environmental Pollution, 193*, 189–196. https://doi.org/10.1016/j.envpol.2014.06.033

Hosokawa, T., Ishii, Y., Nikoh, N., Fujie, M., Satoh, N., & Fukatsu, T. (2016). Obligate bacterial mutualists evolving from environmental bacteria in natural insect populations. *Nature Microbiology, 1*(1), 15011. https://doi.org/10.1038/nmicrobiol.2015.11

Kenyon, L. J., Meulia, T., & Sabree, Z. L. (2015). Habitat visualization and genomic analysis of "*Candidatus Pantoea carbekii*," the primary symbiont of the brown marmorated stink bug. *Genome Biology and Evolution, 7*(2), 620–635. https://doi.org/10.1093/gbe/evv006

Larsen, P. A., Heilman, A. M., & Yoder, A. D. (2014). The utility of PacBio circular consensus sequencing for characterizing complex gene families in non-model organisms. *BMC Genomics, 15*(1), 720. https://doi.org/10.1186/1471-2164-15-720

Martino, C., Morton, J. T., Marotz, C. A., Thompson, L. R., Tripathi, A., Knight, R., & Zengler, K. (2019). A Novel Sparse Compositional Technique Reveals Microbial Perturbations. *mSystems, 4*(1), 1–13. https://doi.org/10.1128/mSystems.00016-19

Marzachí, C., & Bosco, D. (2005). Relative quantification of chrysanthemum yellows (16Sr I) phytoplasma in its plant and insect host using real-time polymerase chain reaction. *Molecular Biotechnology, 30*(2), 117–128. https://doi.org/10.1385/MB:30:2:117

Medina, V., Rosso, B. E., Soria, M., Gutkind, G. O., Pagano, E. A., & Zavala, J. A. (2022). Feeding on soybean crops changed gut bacteria diversity of the southern green stinkbug (*Nezara viridula*) and reduced negative effects of some associated bacteria. *Pest Management Science, 78*(11), 4608–4617. https://doi.org/10.1002/ps.7080

Medina, V., Sardoy, P. M., Soria, M., Vay, C. A., Gutkind, G. O., & Zavala, J. A. (2018). Characterized non-transient microbiota from stinkbug (*Nezara viridula*) midgut deactivates soybean chemical defenses. *PLoS One, 13*(7), e0200161. https://doi.org/10.1371/journal.pone.0200161

Mikonranta, L., Mappes, J., Laakso, J., & Ketola, T. (2015). Within-host evolution decreases virulence in an opportunistic bacterial

pathogen. *BMC Evolutionary Biology, 15*(1), 165. https://doi.org/10.1186/s12862-015-0447-5

Prado, S. S., Rubinoff, D., & Almeida, R. P. P. (2006). Vertical transmission of a Pentatomid caeca-associated symbiont. *Annals of the Entomological Society of America, 99*(3), 577–585. https://doi.org/10.1603/0013-8746(2006)99[577:VTOAPC]2.0.CO;2

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research, 41*(D1), 590–596. https://doi.org/10.1093/nar/gks1219

Stoddard, S. F., Smith, B. J., Hein, R., Roller, B. R. K., & Schmidt, T. M. (2015). rrnDB: Improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Research, 43*(D1), 593–598. https://doi.org/10.1093/nar/gku1201

Tada, A., Kikuchi, Y., Hosokawa, T., Musolin, D. L., Fujisaki, K., & Fukatsu, T. (2011). Obligate association with gut bacterial symbiont in Japanese populations of the southern green stinkbug *Nezara viridula* (Heteroptera: Pentatomidae). *Applied Entomology and Zoology, 46*(4), 483–488. https://doi.org/10.1007/s13355-011-0066-6

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution, 38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120

Taylor, C. M., Coffey, P. L., DeLay, B. D., & Dively, G. P. (2014). The importance of gut symbionts in the development of the brown marmorated stink bug, *Halyomorpha halys* (Stål). *PLoS One, 9*(3), e90312. https://doi.org/10.1371/journal.pone.0090312

Todd, J. W. (1989). Ecology and behavior of *Nezara viridula. Annual Review of Entomology, 34*(1), 273–292. Retrieved from www.annualreviews.org https://doi.org/10.1146/annurev.en.34.010189.001421

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology, 73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07

Manuscript received: July 31, 2023
Revisions requested: September 14, 2023
Revised version received: October 2, 2023
Manuscript accepted: October 9, 2023

The pdf version (Adobe JavaScript must be enabled) of this paper includes an electronic supplement:
Table S1, S2, Figure S1, S2