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Assessment of symbiont-targeted control on non-target stink bugs and their associated egg parasitoids: Could this technique be suitable against *Halyomorpha halys* in New Zealand?

Sofia V. Prieto[1] · Gonzalo A. Avila[2] · Barbara Lima[2] · Elena Gonella[1] · Alberto Alma[1]

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Abstract

The potential arrival of *Halyomorpha halys* in New Zealand jeopardizes a vast range of crops. Therefore, different preparedness strategies are being assessed before its arrival. A symbiont-targeted control strategy might be used along with other control tactics such as biological control. Prior its implementation, it is necessary to assess its potential impact on non-target stink bug species and their associated egg parasitoids. In this study, the effect of symbiont-targeted control was evaluated on three stink bug species in New Zealand, *Oechalia schellenbergii* (a native predatory species), *Nezara viridula* (a cosmopolitan pest) and *Monteithiella humeralis* (a non-pest adventive species). The interference of anti-symbiont treatment of egg masses with their associated *Trissolcus* egg parasitoids, namely *T. basalis* and *T. oenone*, was also tested. A variable response to symbiont elimination was observed in stink bug species, with *N. viridula* and *M. humeralis* undergoing high mortality and no negative effect detected for *O. schellenbergii*. Parasitism of *N. viridula* by *T. basalis* declined on egg masses treated with an anti-symbiont biocomplex or water. Similar results were obtained for *T. oenone* parasitizing eggs of *M. humeralis*; while, a parasitism increase was observed for *O. schellenbergii* egg masses exposed to anti-symbiont treatment and treated with water. These results confirm previous evidence of species-specific response to anti-symbiont control and indicate a moderate and variable effect on egg parasitism. Such responses suggest that symbiont-targeted control would not significantly interfere with the native insect communities that may interact with *H. halys*, encouraging the future incorporation of symbiont-targeted control in pest management programs.

Keywords Biological control · Insect biocenosis · *Trissolcus* · Non-target species · Risk assessment

Key message

- *Halyomorpha halys* is a serious threat for New Zealand agricultural production.
- Symbiont-targeted control could be used in synergy with biological control with egg parasitoids.
- Anti-symbiont treatment on eggs increased the mortality of *N. viridula* and *M. humeralis*.
- No detrimental effect on egg parasitoids was detected after symbiont-targeted spray.
- Symbiont-targeted control is not expected to interfere with local insect communities in New Zealand.

Introduction

The superfamily Pentatomoidea is represented in New Zealand by three families encompassing a total of 18 species (Larivière 1995). Eight of these species and two subspecies belong to the family Pentatomidae (stink bugs). One species and the two subspecies are endemic, three are native, and the other four species are exotic. As for the native species, *Glaucias amyoti* Dallas is the only herbivorous species and feeds mostly on *Coprosma* (Rubiaceae) plants (Larivière 1995). *Cermatulus nasalis* Westwood and *Oechalia schellenbergii* Guérin are two predatory native species and widespread in

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✉ Elena Gonella
elena.gonella@unito.it

[1] Department of Agricultural, Forest and Food Sciences, University of Torino, Grugliasco, Italy

[2] The New Zealand Institute for Plant and Food Research Limited, Auckland, New Zealand

New Zealand. In particular, *O. schellenbergii* is considered one of the most important beneficial insects in New Zealand due to its role as a predator of pest insects, such as lepidopteran and chrysomelid larvae (Charles et al. 2019). In regard to the exotic Pentatomidae species, three of them were introduced from Australia (*Cuspicona simplex* Walker, *Dictyotus caenosus* Westwood and *Monteithiella humeralis* Walker), and the fourth is the cosmopolitan species *Nezara viridula* Linnaeus, widespread in New Zealand South to mid-Canterbury (Charles et al. 2019). The pittosporum shield bug *M. humeralis*, ubiquitous through the North and South Islands, is strictly associated with *Pittosporum* plants (Pittosporaceae) and is not considered a pest. On the contrary, *N. viridula* is a relevant cosmopolitan pest on vegetable crops such as beans, cauliflower, marrow, potato, pumpkin and tomato (Charles et al. 2019).

Biological control strategies targeting invasive stink bugs usually include the use of egg parasitoids (mostly in the Scelionidae family) as the main control agents, due to their high parasitism efficiency and their host specificity (Conti et al. 2021). In New Zealand, three parasitoids species are known to attack eggs of stink bugs of the superfamily Pentatomoidea: (1) *Trissolcus basalis*, intimately associated with *N. viridula* and deliberately introduced in New Zealand in 1948 after the first report of *N. viridula* as a pest (Cumber 1951); (2) the endemic *Trissolcus maori* Johnson, which mainly attacks species in the family Acanthosomatidae; (3) and the native *Trissolcus oenone* Dodd, parasitizing pentatomids (Cumber 1964; Johnson 1991). Except for *N. viridula*, the host range of *T. basalis* and *T. oneone* in New Zealand shows a certain level of overlap according to laboratory experiments (Saunders et al. 2022), though this might not completely reflect the behavior of these parasitoids in field.

Adopting a new pest control strategy implies being aware of its potential effects on non-target species. It is therefore advisable to perform a thorough experimental risk assessment of the strategy before proposing its implementation. In New Zealand, the Environmental Protection Authority (EPA) approved in 2018 the conditional release of *T. japonicus* (Charles et al. 2019; Avila et al. 2023), an egg parasitoid of the invasive stink bug *Halyomorpha halys* that shows high parasitism rates in the pest native area (Yang et al. 2009; Zhang et al. 2017). Prior to approval, a host range assessment was performed in quarantine conditions by Charles et al. (2019) to test the potential capacity of *T. japonicus* to parasitize egg masses of Pentatomidae species present in New Zealand. The results of this study indicate that *T. japonicus* was able to successfully parasitize almost all tested species, though parasitism rates varied between the pentatomid species tested (Charles et al. 2019).

It is common knowledge that the containment and long-term management of invasive pests should not rely on only one strategy. On the contrary, it is necessary to optimize the combination of different approaches to pest management that may work synergistically (i.e., Integrated Pest Management, IPM) (Prokopy 2003). In an IPM program against invasive stink bug species, the symbiont-targeted approach is a novel control strategy that may be useful against invasive pests such as *H. halys* (Gonella and Alma 2023). This strategy entails disrupting the acquisition of primary symbiotic bacteria by first instar stink bug nymphs in order to negatively affect their fitness and survivorship (Prado and Zucchi 2012). The development of this approach relies on the symbiont transmission strategy: during oviposition, female stink bugs apply a symbiont-containing substance that is incorporated by the nymphs upon hatching, eventually colonizing a particular gut ventricle (Prado et al. 2006). Therefore, the treatment of egg masses with anti-symbiont substances compromises the symbiont acquisition. Recent laboratory studies using an Italian population of *H. halys* and its egg parasitoids observed no interference on parasitism for the symbiotic control performed with an anti-symbiont biocomplex (Orrù et al. 2023).

The imminent arrival of *H. halys* in New Zealand represents a severe threat to crops of great value to both domestic and export markets (Duthie 2012; Burne 2019). In recent years, adults have been regularly intercepted at the border of the country, mostly in association with cargo (e.g., shipping containers, imported cars). In this context, the release approval for *T. japonicus* will allow for a fast response in the event of the establishment of *H. halys* in New Zealand (Charles et al. 2019). Symbiont-targeted control has been reported to be compatible with *T. japonicus* (Orrù et al. 2023); therefore, it could be incorporated into an IPM program against *H. halys* in New Zealand. However, before the adoption of a symbiont-targeted control approach, it is necessary to assess its possible detrimental effects on local non-target pentatomid species and their associated egg parasitoids. To date, no information is available regarding potential symbiotic interactions with gut bacteria for *O. schellenbergii* and *M. humeralis*. It can be expected *M. humeralis* utilizes symbiotic relationships with vertical transmitted gut enterobacteria, as is the case of other stink bugs from the subfamily Pentatominae (Duron and Noël 2016). On the contrary, symbiont-containing gut ventricles are absent in strictly predaceous Heteroptera such as Asopinae stink bugs; and therefore, *O. schellenbergii* is not expected to display a stable symbiotic interaction with specific bacteria (Glasgow 1914). Egg masses treated with anti-symbiont substances could, however, be influenced by other aspects related to embryogenesis and egg hatching (Gonella et al. 2022). Finally, *N. viridula* shows an inconsistent response to symbiont absence among geographically distant populations, which implies the need to test each population independently (Tada et al. 2011; Prieto et al. 2023). A more complete risk assessment of a symbiont-targeted control approach can be achieved if the interaction with egg parasitoids is included, specifically the potential direct and indirect effects on female parasitoid host foraging behavior (Field 1998) and host parasitism success.

The aim of this study was to: (i) characterize the symbiotic bacteria residing the gastric caeca (V4 ventricle) of *M. humeralis* and the gut microbiome in *O. schellenbergii*; (ii) test the symbiont-targeted control on *O. schellenbergii*, *M. humeralis* and the New Zealand population of *N. viridula*; and (iii) assess the potential interference of symbiotic control with parasitism by two egg parasitoids, *T. oenone* and *T. basalis*, associated with the considered stink bug species.

Materials and methods

Laboratory rearing of pentatomids and preparation of egg masses

All the egg masses used were obtained from laboratory colonies maintained at the New Zealand Institute for Plant & Food Research Centre in Auckland (Mount Albert Research Centre). The colonies were established from field collections and reared as described by Charles et al. (2019). Egg masses used for all experiments were no older than 24 h. For *O. schellenbergii* and *M. humeralis*, individual egg masses were randomly allocated to the treatments. Experiments on *N. viridula* were performed with half egg masses to standardize the size of the masses between treatments (i.e., 26.7 ± 1.4 eggs per mass) (Tables 1 and 2), afterward each half was randomly assigned to one treatment.

Obtaining and maintaining egg parasitoids colonies for no-choice tests

Colonies of egg parasitoids were started from parasitized egg masses. *Trissolcus basalis* parasitoids were obtained from egg masses of *N. viridula* sourced from a colony maintained at the New Zealand Institute for Plant & Food Research Centre (Lincoln, NZ). *Trissolcus oenone* individuals were acquired from wild *M. humeralis* egg masses collected on *Pittosporum* spp. trees near the Plant & Food Research Centre (Auckland, NZ). Specimens of *T. oenone* were taxonomically identified by Joanne Poulton (New Zealand Institute for Plant & Food Research Centre, Mount Albert Research Centre). Egg masses were kept individually in separate vials (2.5 cm diameter, 5 cm high) with a smear of honey as a carbohydrate resource, in a controlled temperature room at 24 ± 1 °C, 60 ± 10% RH and 16L: 8D. *Trissolcus basalis* parasitoids were maintained on egg masses of *N. viridula*; while, the *T. oenone* colony was maintained on egg masses of both *M. humeralis* and *O. schellenbergii*. Females derived from these colonies were employed in no-choice tests.

Table 1 Effect of anti-symbiont treatment of stink bug egg masses on nymphal emergence and survival to the second instar

Species	Treatment	Replicates (n)	Eggs/egg mass (mean ± SE)	*df*	*F* value	*P* value	First instar nymphs (mean ± SE)	hatched eggs (%) (mean ± SE)	*df*	χ^2	*P* value	Second instar nymphs (mean ± SE)	Mortality first instar (%) (mean ± SE)	*df*	χ^2	*P* value
Nezara viridula	Control	11	28.2 ± 3.3	1	0.22	0.65	19.6 ± 2.3	75 ± 7	1	0.22	0.64	14.2 ± 2	23 ± 9	1	11.0	0.0009 ***
	Dentamet®	11	27.8 ± 2.1				21.9 ± 1.6	81 ± 5				10.9 ± 1.4	49 ± 9			
Montheithiella humeralis	Control	13	10.4 ± 0.8	1	0.44	0.52	9.0 ± 1.0	90 ± 10	1	1.31	0.25	7.8 ± 1.2	10 ± 10	1	5.1	0.027 *
	Dentamet®	13	9.7 ± 0.7				7.9 ± 0.9	80 ± 10				5.9 ± 1.0	30 ± 10			
Oechalia schellenbergii	Control	14	16.3 ± 2.5	1	1.26	0.27	10.1 ± 1.8	51 ± 10	1	3.04	0.08	7.4 ± 2.0	31 ± 10	1	1.0	0.312
	Dentamet®	14	19.9 ± 2.1				11.2 ± 1.6	60 ± 10				7.6 ± 1.9	35 ± 10			

The results of statistical comparison between biocomplex treatment and control are shown for the number of eggs per egg mass (degrees of freedom *df*, *F* and *P* value, one-way ANOVA analysis), and for the hatching and the mortality rates (*df*, χ^2 and *P* value, GLM binomial analysis). Asterisks indicate significance

SE standard error

Table 2 Results of no-choice tests with *Trissolcus basalis* and *Nezara viridula* egg masses ($N = 10$)

Treatment	Dentamet®	Water	Control
Mean no. of eggs per mass ± SE	27.6 ± 5.6	24.1 ± 2.4	25.8 ± 3.1
% egg masses parasitized	90%	80%	80%
Parasitism	16.4 ± 4.9	12.8 ± 2.8	19.5 ± 4.8
%	66 ± 12.4 b	59 ± 11.3 b	68 ± 13.9 a
Emerged parasitoids	12.3 ± 3.3	10.3 ± 2.1	16.5 ± 4.4
%	52 ± 11.8 b	48 ± 8.9 b	56 ± 12.5 a
Failed emerged parasitoids	4.1 ± 1.6	2.5 ± 1.1	3.0 ± 1.2
%	14 ± 5.0 n.s	11 ± 4.4 n.s	11 ± 4.5 n.s
Hatched nymphs	3.5 ± 1.9	4.2 ± 2.8	2.8 ± 1.9
%	11 ± 6 n.s	14 ± 9 n.s	13 ± 9 n.s
Unhatched eggs	7.7 ± 3.6	7.1 ± 1.9	3.5 ± 1.5
%	23 ± 8.4 b	27 ± 5.8 b	20 ± 9.4 a

Egg parasitism, parasitoid emergence and failed emergence, and unhatched eggs, expressed as proportions of total eggs per egg mass (mean ± standard error), were compared between treatments. Different letters indicate significant differences between treatments (binomial GLM with Dunn post hoc test and Bonferroni's multiple comparison adjustment)

n.s. no significant differences observed

Symbionts identification through 16S rRNA gene sequencing

A total of 20 adult individuals (10 male, 10 female) of each stink bug species were collected and kept in absolute ethanol for molecular analysis. Twelve hours before dissection, samples were placed in a refrigerator (5 ± 1 °C) in sterile 0.9% NaCl solution. Using a dissecting scope and fine forceps, midgut dissections were performed inside a Petri dish (9 cm diameter, 2 cm high) with 500ul of a sterile 0.9% NaCl solution. In the case of *M. humeralis* and *N. viridula*, the V4 ventricle of the midgut (recognizable by its sac-like structures), was separated from the rest of the midgut. The V4 ventricle was not observed in *O. schellenbergii* individuals; therefore, the whole midgut was conserved. Midguts and V4 ventricles were kept separately in single 1.5 ml Eppendorf tubes at − 20 °C. Subsequently, samples were subjected to DNA extraction using a Phenol Chloroform protocol adapted from Doyle and Doyle (1990) as described in Gonella et al. (2012). The quality of the extracted DNA was checked using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific®). Symbiont DNA in *N. viridula* samples was tested with specific primers MMAO1f/MMAO1r designed by Tada et al. (2011). Polymerase chain reaction (PCR) was conducted with HOT FIREPol® DNA polymerase (Biosigma). After visualization of PCR products through electrophoresis in 1% agarose gel, positive samples were purified with the QIAquick® PCR purification Kit (Qiagen) and submitted to Sanger sequencing (Eurofins Genomics, Germany). Sequences were afterward manually checked and trimmed according to the quality scores using software Chromas version 2.6.6. Finally, sequences were confronted with the GenBank database of the NCBI, using the tool BLAST (Altschul et al. 1990).

A whole microbiome sequencing approach was used to characterize the bacteria composition in the V4 gut region of *M. humeralis* and in the whole gut of *O. schellenbergii*, by sequencing the V3 and V4 regions of the 16S rRNA gene in 16 and 15 samples of each species, respectively. Libraries were prepared with Herculase II Fusion DNA Polymerase and the Nextera XT Index V2 kit, based on manufacturer's guidelines, with primers forward 5′-CCTACGGGNGGCWGCAG-3′ and reverse 5′-GACTACHVGGGTATCTAATCC-3′, targeting the 16S rRNA gene V3 and V4 regions (Klindworth et al. 2013). Paired-end sequences (301 pb) were obtained with the Illumina NovaSeq platform. Library preparation and sequencing was performed by Macrogen, Inc. (Seoul, Republic of Korea).

Raw sequences analysis was performed with QIIME2 platform (Bolyen et al. 2019). Reads were trimmed based on an average Phred quality score threshold of 25, and then denoised, filtered and checked for chimera presence using the dada2 algorithm (Callahan et al. 2016). The taxonomy assignment of the obtained ASVs was held with the QIIME2 feature-classifier classify-sklearn plugin (Bokulich et al. 2018) using the release 138 of the SILVA database (Quast et al. 2013) as reference for sequences and taxonomy, previously trained with QIIME feature-classifier fit-classifier-naive Bayes plugin (Pedregosa et al. 2011).

To deepen into the taxonomy of ASVs assigned to the family Erwiniaceae, a database was built using the QIIME2 RESCRIPt plugin (Robeson et al. 2021), based on bacteria 16S rRNA sequences and taxonomy present in NCBI Genbank database (BioProjects 33,175 and 33,317; sequences minimum 1200 pb long). Erwiniaceae ASVs were taxonomically re-assigned using the self-created database and taxonomy with the QIIME2 plugin QIIME feature-classifier classify-sklearn. The database is available at https://doi.org/10.6084/m9.figshare.26935882.

Egg masses treatment targeting the stink bugs primary symbiont

A total of 22 halves of fresh egg masses (≤ 24 h) of *N. viridula* (each one composed of about 28 eggs), and 26 and 28 fresh egg masses of *M. humeralis* and *O. schellenbergii*, respectively, (10–20 eggs per mass) were used for the assessment of anti-symbiont treatment on newly emerged nymphs (Table 1). The micronutrient biocomplex Dentamet® (Diachem S.p.A., Italy), previously reported to effectively perform symbiont-targeted control of *H. halys*, was used as the anti-symbiont treatment (Gonella et al. 2019; Prieto et al. 2023). Egg masses were placed onto a filter paper in a Petri dish (9 cm diameter, 2 cm high) and randomly assigned to one treatment (sprayed with the anti-symbiont biocomplex or untreated control). The total number of eggs per replicate was recorded. Egg masses from the anti-symbiont treatment were sprayed with the biocomplex at the concentration recommended for field applications (300 ml/hl equivalent to a solution 0.3% v/v) following the protocol in Gonella et al (2019). Briefly, egg masses were sprayed under a fume hood with a 250 ml hand sprayer, applying one single spray from a distance of 20 cm from the egg mass. Sprayed egg masses were left for half an hour inside the fume hood for drying. Control egg masses were not treated in any way. Egg masses were kept in a controlled temperature room (24 ± 1 °C, 60 ± 10% RH and 16L: 8D) and the time to complete egg maturation was measured as the number of days between egg collection and egg hatching. After hatching, a piece of moistened cotton and a fresh bean was added to the Petri dish. The total number of first and second instar nymphs was recorded as well as the time (in days) it took for 50% of first instar nymphs to reach the second instar (i.e., first instar development time). Second instar nymphs derived from four replicates randomly chosen were stored in RNA later® (Sigma-Aldrich, MO, USA) at − 20 °C for further molecular analyses.

RNA extraction and diagnostic real time PCR of *Serratia marcescens*

After detection of high relative abundance of *Serratia marcescens* in the midgut of *O. schellenbergii*, a Real-Time PCR analysis was performed to assess if egg masses treatment enhanced the presence of *S. marcescens*. RNA was extracted from second instar nymphs derived from control and treated egg masses with the 'SV Total RNA Isolation System' (Promega, WI, USA), according to the manufacturer's indications. RNA quality and concentration were assessed with a ND-1000 spectrophotometer (NanoDrop, DE, USA). The 'Reverse Transcription System' (Promega) was used to synthesize first strand cDNA with Random Primers, following the manufacturer's instructions. cDNA was then used as a template for Real-Time PCR analysis with the newly designed primers SerQf (5′-TATTGCACAATGGGCGCAAG-3′)/SerR1 (5′-GGAGTTAGCCGGTGCTTCTT-3′), amplifying a ~ 150 bp fragment of the bacterial 16SrRNA gene. Primers were designed using NCBI Primer-BLAST based on sequences assigned to *S. marcescens* obtained in this study and from the NCBI GenBank database (accession numbers NR036886 and OR478422). PCR reactions were performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA) in 25 µl volume with 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 1 µl of cDNA template. As for the thermal conditions, an initial denaturation at 94 °C for 3 min was followed by 36 cycles of denaturation at 94 °C for 15 s and annealing at 55 °C for 30 s. A final step for melting curve analysis from 55 to 95 °C, measuring fluorescence every 0.5 °C, was added.

No-choice tests

Assays with egg parasitoids were performed in plastic Petri dishes (9 cm diameter, 2 cm high). Thirty halves of fresh egg masses of *N. viridula* and 30 complete fresh egg masses of *M. humeralis* and *O. schellenbergii* were assigned randomly to each treatment (anti-symbiont biocomplex, distilled water, or untreated control) (Tables 2, 3). The distilled water treatment was added in order to exclude any interference with the parasitoid foraging behavior due to spraying water on the egg mass, irrespective of the substance used. Egg masses were left to dry for 30 min under the fume hood. Subsequently one mated, naïve female wasp (5–10 days old) was randomly chosen and placed into each Petri dish containing the egg mass. Egg masses of *N. viridula* were exposed to *T. basalis* females; while, *M. humeralis* and *O. schellenbergii* eggs were exposed to females of *T. oenone*. The Petri dish contained a smear of honey and all replicates were kept in a controlled temperature room at (24 ± 1 °C, 60 ± 10% RH and 16L: 8D). Females were separated from the egg mass after 48 h.

Evaluation of behavior parameters in female parasitoids

Behavior patterns associated with the host foraging activity of females were assessed by direct observation during the first 20 minutes after the female was placed in the Petri dish. The following parameters were considered, based on the behavioral categories proposed by Field (1998): The approaching time indicated the time (seconds or minutes) it took the female to approach the egg mass for the first time; while, the approaching frequency referred to the quantity of times the female approached the egg mass (and went away) before standing on it; the drumming time was the total time (seconds or minutes) that the female spent examining the eggs with the antenna (i.e., drumming) before starting ovipositing, and the drumming frequency was the quantity of times the female stood on the egg mass exhibiting drumming behavior, and then walked off the egg mass; the probing starting time (minutes) indicated the moment the female started probing with the ovipositor on the eggs; whereas, the probing duration referred to the total amount of time (minutes) in which the female was observed probing before starting ovipositing; the start of oviposition was assumed

Table 3 Results of no-choice tests with *Trissolcus oenone* ($N = 10$)

Host	*Monteithiella humeralis*			*Oechalia schellenbergii*		
Treatment	Dentamet®	Water	Control	Dentamet®	Water	Control
Mean no. of eggs per mass ± SE	10.9 ± 1	10.6 ± 0.7	9.7 ± 0.7	15.8 ± 1.9	24.9 ± 2.7	14.7 ± 1.8
% egg masses parasitized	90%	100%	90%	100%	100%	80%
Parasitism	7.9 ± 1.4	10.6 ± 0.7	8.3 ± 1.3	14 ± 2.1	24.5 ± 2.7	9.4 ± 2.5
%	76 ± 11.6 a	100 ± 0 ab	84 ± 10.4 b	89 ± 7.3 a	99 ± 0.9 b	59 ± 12.5 c
Emerged parasitoids	7.9 ± 1.4	10.6 ± 0.7	8.3 ± 1.3	14 ± 2.1	22.9 ± 2.9	9.3 ± 2.5
%	76 ± 11.6 a	100 ± 0 ab	84 ± 10.4 b	89 ± 7.3 a	90 ± 6 a	58 ± 12 b
Failed emerged parasitoids	0	0	0	0	1.6 ± 0.8	0.1 ± 0.1
%	0	0	0	0	9 ± 26	1 ± 1
Hatched nymphs	0.2 ± 0.2	0	0	0.5 ± 0.3	0	0.5 ± 1.6
%	1 ± 1	0	0	4 ± 2	0	3 ± 3
Unhatched eggs	2.8 ± 1.6	0	1.4 ± 0.8	1.3 ± 0.8	0.4 ± 0.3	4.8 ± 1.4
%	23 ± 11.7 n.s	0 n.s	16 ± 10.4 n.s	8 ± 5.0 a	1 ± 1 b	38 ± 11.2 c

Ten egg masses were used for each host species—treatment combination. Egg parasitism, parasitoid emergence and failed emergence, and unhatched eggs, expressed as proportions of total eggs per egg mass (mean ± standard error), were compared between treatments. Different letters indicate significant differences between (binomial GLM with Dunn post hoc test and Bonferroni's multiple comparison adjustment)

n.s. no significant differences observed

when the female remained attached with the ovipositor onto the egg mass and when this event occurred the egg mass was considered accepted.

The initial time (i.e., time zero) was the moment in which the female was placed in the arena. After 48 h the females were removed from the arena and kept in separate vials for a week (provided with honey and in a controlled temperature room as described previously), to record any instances of mortality.

Egg categorization after no-choice test

A period of 20 days after removing the females from the arena was considered enough for both nymphs to hatch and/or parasitoids to emerge. After, all egg masses were observed under the microscope and the total number of emerged parasitoids from parasitized egg masses was recorded. Subsequently, the number of unemerged parasitoids (dead individuals that failed to exit the egg) and host nymphs was also recorded by conducting egg dissections of unhatched eggs. All egg outcomes were expressed as percentages with respect to the total number of eggs per egg mass.

Data analysis

The mean number of eggs per egg mass, where data showed a normal distribution and variance homogeneity, was compared through a one-way ANOVA, as well as the egg maturation time and the duration of the first instar. All response variables expressed as proportions (hatch and mortality rates and egg outcomes in no-choice tests), not respecting the assumption of homogeneity, were analyzed using a generalized linear model (GLM) with a binomial probability distribution and logit link function. For the multiple comparisons of specific treatments in no-choice tests with *T. basalis* and *N. viridula* egg masses, means were separated by performing a Tukey's post hoc test. All female parasitoid behavior parameters were assessed by means of the Kruskal–Wallis test with a Dunn post hoc test and a Bonferroni's multiple comparison adjustment. All statistical analyses were performed with software R (R Development Core Team 2010). Graphics were built using the ggplot2 package from the R software.

Results

Assessment of symbiotic control on New Zealand laboratory populations of *N. viridula, M. humeralis* and *O. schellenbergii*

Symbiont characterization through 16S rRNA gene sequencing

Regarding *N. viridula*, 15 samples out of 20 showed amplification with the symbiont-specific primers. Sequencing of the amplicons (average length 730 bp) showed that all sequences were identical to each other and to sequences previously obtained from individuals of *N. viridula* collected elsewhere, and specifically to *Pantoea* Nvir1 sequence variant of the symbiont (Duron and Noël 2016; Prieto et al. 2023).

As for the two other stink bugs, after V3-V4 16S rRNA gene sequencing of bacteria present in the caeca of *M. humeralis* and the gut of *O. schellenbergii*, a total of 3,231,755 raw reads were obtained (mean per sample = 104,250) (project accession number PRJNA1156616). Denoising and filtering steps led to a total of 1,047 ASVs (average reads per sample = 76,795) taxonomically assigned to 175 bacteria families and 323 genera. Mitochondria and Chloroplast assigned ASVs were filtered from further analysis.

The relative abundance of different bacteria at the family or genera level in each sample is presented in Fig. 1. Taxa with relative abundance lower than 5% (considering all samples) were collapsed into 'Other'. In general, the microbiome associated with each sample was dominated by only a few taxa (between 1 and 5), although with differences in the taxonomical composition according to each stink bug species. In the V4 ventricle of *M. humeralis*, a strong presence of ASVs assigned to the family Erwiniaceae was detected, with 9 samples out of 16 having 60% or more ASVs assigned to this family. Moreover, when confronting all ASVs assigned to the Erwiniaceae family in *M. humeralis* caeca to a second taxonomy approach using a self-made database, all of them were assigned to the genera *Pantoea*. Other bacteria in samples of this species were assigned to the genera *Serratia* and *Yokenella* and to the family Enterobacteriaceae. On the contrary, the presence of Erwiniaceae ASVs was lower or inexistent at all in the gut microbiome of *O. schellenbergii*, except for some ASVs assigned to *Pantoea* (3 out of 15 samples, relative abundance of 2.38% in one sample and lower than 1% in the other two) and *Rosenbergiella* (1 sample, less than 1%). The gut microbiome associated to this species was mainly integrated by bacteria of the genera *Serratia* and *Yokenella* and other Enterobacteriaceae.

Egg masses treatment targeting the primary symbiont

To evaluate the effect of interrupting symbiont acquisition on stink bugs, a comparison was made between egg masses sprayed with an anti-symbiont biocomplex and untreated egg masses (Table 1). The number of eggs per egg mass did not change between treatments nor did the hatching rates (Table 1). On the contrary, egg mass treatment with biocomplex had a variable effect in the first instar mortality rates of the species evaluated (Table 1). In *N. viridula*, a significant increase in the mortality rate during the first nymphal instar was detected for nymphs from egg masses treated with the biocomplex (treated: 49 ± 9%; control: 23 ± 9%; P value < 0.001) (Table 1). An increase in the first instar mortality rate was also observed for *M. humeralis* (treated: 30 ± 10%; control: 10 ± 10%; P value = 0.027) (Table 1). Conversely, no significant differences were observed in the mortality during the first instar in *O. schellenbergii* (treated: 35 ± 10%; control: 31 ± 10%; P value = 0.31) (Table 1). Egg maturation time did not differ significantly for any species-treatment combination (*N. viridula*: $F = 2.22$, $df = 1$, P value = 0.15; *M. humeralis*: $F = 0.95$, $df = 1$, P value = 0.34; *O. schellenbergii*: $F = 0.005$, $df = 1$, P value = 0.94) (Fig. 2a).

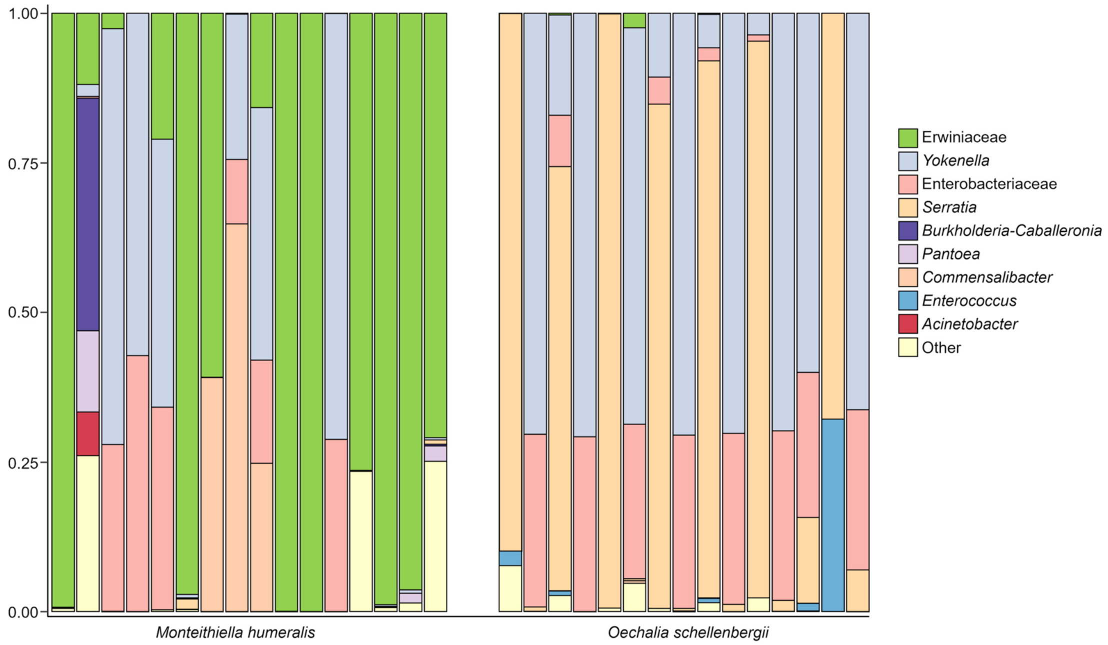

Fig. 1 Barplot representing the relative abundance of bacteria in the V4 ventricle of *Montheithiella humeralis* and the midgut of *Oechalia schellenbergii*

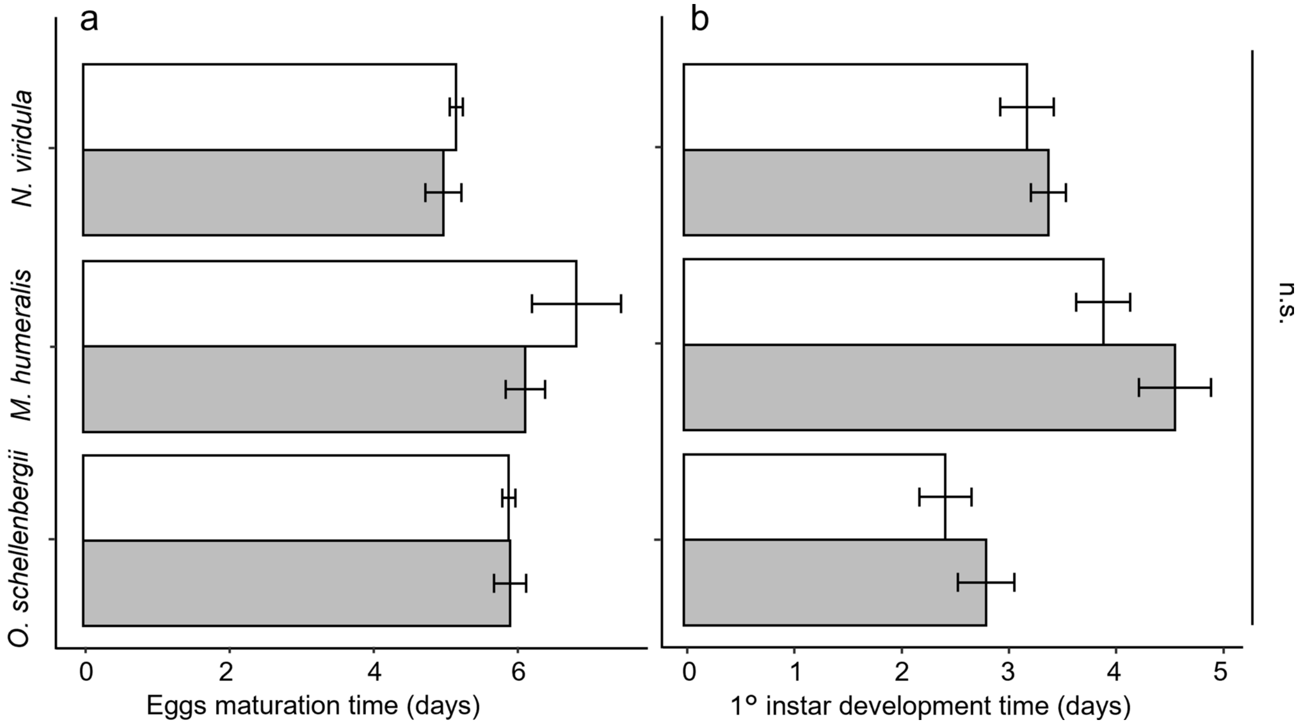

Fig. 2 Egg maturation time (**a**) and first instar duration (**b**), measured in days, for each species according to the treatment (white bars: untreated control; gray bars: biocomplex Dentamet®). n.s. represents no significant differences between treatments (one-way ANOVA)

Similar results were obtained regarding the first instar development time, although slightly prolonged in nymphs from biocomplex-treated eggs for all species (*N. viridula*: $F = 0.45$, $df = 1$, P value $= 0.51$; *M. humeralis*: $F = 2.51$, $df = 1$, P value $= 0.13$; *O. schellenbergii*: $F = 0.45$, $df = 1$, P value $= 0.51$) (Fig. 2b). PCR reactions on *O. schellenbergii* nymphs using *Serratia*-specific primers revealed the absence of positive samples either in treated and control specimens, indicating that this bacterium is not acquired at this stage.

No-choice tests with *T. basalis* and *T. oenone* after anti-symbiont treatment of host egg masses

Assessment of egg parasitoids behavior parameters

All the expected behavior parameters were observed and therefore recorded in *T. basalis* females exposed to eggs of *N. viridula* (Table 2, Fig. 3a, b). In contrast, no particular behavior was observed in females of *T. oenone* during the first twenty minutes, for either host species, so no assessment of behavior parameters was performed.

When recording *T. basalis* approaching time, the start of probing and the probing duration, no significant differences were detected (approaching time: $\chi^2 = 3.15$, $df = 2$, P value $= 0.21$; start of probing: $\chi^2 = 3.97$, $df = 2$, P value $= 0.14$; probing duration: $\chi^2 = 0.16$, $df = 2$, P value $= 0.92$) (Fig. 3a). Conversely, differences were detected in the time *T. basalis* females spent drumming on the egg masses ($\chi^2 = 6.64$, $df = 2$, P value $= 0.036$). The longest drumming time was spent on egg masses treated with the biocomplex (2.3 ± 0.3 min, $n = 10$), followed by the water and the control treatment (Water: 1.36 ± 0.23 min, $n = 7$; Control: 1.25 ± 0.36 min, $n = 10$). According to multiple comparison analysis, significant differences were detected between the biocomplex and the control treatment (P value $= 0.034$); while, the water treatment was statistically equal to the biocomplex and the control (biocomplex *vs* water: P value $= 0.34$; control *vs* water P value $= 1.00$) (Fig. 3a).

Significant differences were observed in the frequency of approaching and drumming for *T. basalis* (Approaching frequency: $\chi^2 = 6.64$, $df = 2$, P value $= 0.03$; Drumming frequency: $\chi^2 = 9.03$, $df = 2$, P value $= 0.01$) (Fig. 3b). For both parameters, the lowest frequency was observed for the egg masses treated with the anti-symbiont biocomplex (Approaching frequency: biocomplex: 2.6 ± 0.9 times, $n = 10$; Control 6.5 ± 1.6 times, $n = 10$; Water: 4.1 ± 0.55. Drumming frequency: biocomplex: 3.7 ± 6.8 times, $n = 10$; Control: 4.5 ± 0.8 times, $n = 10$; Water: 3 ± 0.8 times, $n = 7$) (Fig. 3b). Frequency of approaching and drumming in egg masses treated with water were not different from both the biocomplex and the untreated treatment, showing an intermediate condition (Approaching frequency: biocomplex *vs* water P value $= 0.20$, water *vs* control P value $= 1.00$;

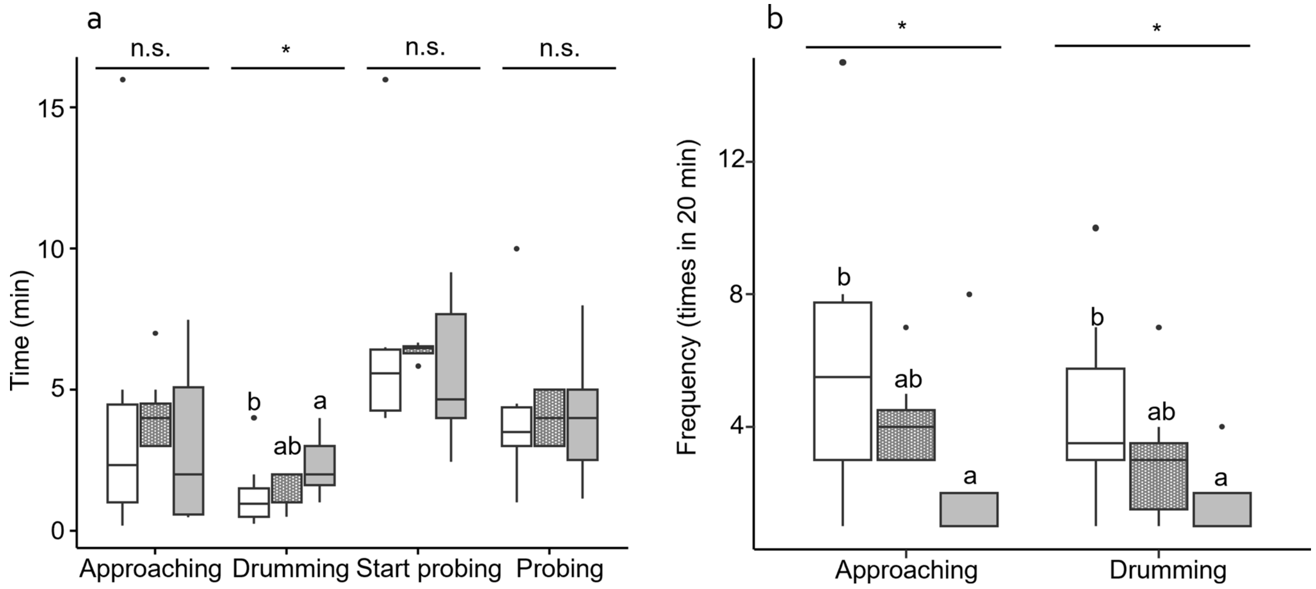

Fig. 3 Box-plots showing the variation on the behavior parameters evaluated on *T. basalis* females offered with egg masses of *N. viridula* treated with the biocomplex Dentamet® (gray), with distilled water (gray dotted pattern) and left untreated (white). **a** Approaching time, drumming duration, start probing time and probing duration, measured in minutes (min). **b** Approaching and drumming frequency (measured as the number of times that the event took place in a total of 20 min of observation). Different letters indicate significant differences for each behavioral parameter between treatments (Kruskal–Wallis test with Dunn post hoc test and Bonferroni's multiple comparison adjustment)

drumming frequency: biocomplex *vs* water *P* value = 0.36, water *vs* control *P* value = 0.66). Significant differences were detected when comparing the biocomplex treatment with the control (Approaching frequency: biocomplex *vs* control *P* value = 0.042; drumming frequency: biocomplex *vs* control *P* value = 0.008) (Fig. 3b).

Parasitism recorded in no-choice tests

For all stink bug parasitoid combinations, the number of parasitized egg masses ranged between 8 and 10 from a total of 10 egg masses per treatment (i.e.., between 80 and 100% of egg masses were successfully parasitized) (Tables 2, 3). Regarding *T. basalis* parasitism of *N. viridula* egg masses, significant differences were recorded according to the treatment performed on the egg mass ($\chi^2 = 30.0$, $df = 2$, P value < 0.001) (Table 2). The highest parasitism rate was observed in control egg masses (68%), followed by the biocomplex (66%) and the water-sprayed treatment (59%). Despite the average percentages were only slightly divergence between control and biocontrol treatments, statistically the parasitism rate was significantly higher in control egg masses; while, no significance was detected between the biocomplex and the water-sprayed egg masses (biocomplex vs control *P* value = 0.0002; water vs control *P* value < 0.001; water vs biocomplex *P* value = 0.32) (Table 2). A similar pattern was recorded when evaluating the percentage of parasitoids emerged from the egg masses ($\chi^2 = 28.7$, $df = 2$, P value < 0.001) (Table 2), with 56% emergence from the control, 52% from the biocomplex and 48% from the water-treated egg masses. Significant differences were recorded only in the control egg masses (biocomplex vs control *P* value < 0.001; water vs control: *P* value < 0.001; water vs biocomplex *P* value > 0.91) (Table 2). In contrast, no differences were detected in the number of parasitoids that failed emerging ($\chi^2 = 2.6$, $df = 2$, P value $= 0.27$) (Table 2). Differences were as well detected in the number of unhatched eggs ($\chi^2 = 23.4$, $df = 2$, P value < 0.001) (Table 2), with lower rates in the untreated control (20%) compared to biocomplex (23%) and the water-sprayed egg masses (27%) (biocomplex vs control *P* value = 0.001; water vs control: *P* value < 0.001; water vs biocomplex *P* value = 0.92) (Table 2).

In no-choice tests performed with *T. oenone* and egg masses of *M. humeralis* no failed emergence of parasitoids was observed (Table 3). Different parasitism rates were detected according to the egg mass treatment ($\chi^2 = 45.5$, $df = 2$, P value < 0.001) (Table 3). The lowest parasitism rates were observed in the biocomplex-treated egg masses (76%), followed by the control (84%) and the water-sprayed egg masses (100%). Significant differences were detected only between the biocomplex and the control, and no differences were recorded between the biocomplex and water and water and untreated control (Biocomplex vs control *P* value = 0.0476; water vs control = 0.99; water vs biocomplex

P value = 0.99) (Table 3). No differences were detected in the unhatched eggs rates ($\chi^2 = 4.23$, $df = 2$, *P* value = 0.12) (Table 3).

In egg masses of *O. schellenbergii* the parasitism rate varied significantly with the treatment ($\chi^2 = 94.1$, $df = 2$, *P* value < 0.001) (Table 3). The highest parasitism rate was observed in egg masses treated with water (99%), followed by the egg masses treated with the biocomplex (89%) and by the untreated control (59%). All three treatments differed significantly in terms of parasitism rates (Biocomplex vs control *P* value < 0.001; water vs control: *P* value < 0.001; water vs biocomplex *P* value < 0.001) (Table 3). The proportion of unhatched eggs per egg mass also differed according to the treatment ($\chi^2 = 84.1$, $df = 2$, *P* value < 0.001). The highest rates were observed in untreated egg masses (38%), followed by biocomplex-sprayed egg masses (8%) and water-treated egg masses (1%) (Biocomplex vs control *P* value < 0.001; water vs control: *P* value < 0.001; water vs Biocomplex *P* value = 0.009) (Table 3). The occurrence of failed emerged parasitoids was observed for this species in four egg masses from the water treatment and one egg mass from the control treatment (Table 3).

Discussion

This study aimed to assess the outcome of introducing symbiont-targeted control as a complementary strategy to *T. japonicus* releases against an eventual establishment of *H. halys* in New Zealand. While the compatibility between anti-symbiont treatment and parasitism by *T. japonicus* has been already demonstrated (Orrù et al. 2023), here native non-target species were taken into account along with two egg parasitoid species present in the country in order to assess the ecological risk of integrated approaches in complex agroecosystems where *H. halys* may establish.

The suppressive effect observed after symbiont disruption in a New Zealand laboratory population of *N. viridula* confirmed previous observations in other populations elsewhere (Prieto et al. 2023; Tada et al. 2011). Therefore, symbiont-targeted control could eventually be incorporated as a containment tool for this species, considering that in New Zealand, *N. viridula* is regarded as a pest in vegetable crops (Larivière 1995). Before such an approach would be initiated, it is crucial to exclude the possibility of anti-symbiont treatment interfering with *T. basalis* parasitism, which was introduced into New Zealand for classical biocontrol. When examining the host foraging activity of *T. basalis* (Field 1998), a limited impact of anti-symbiont spray was suggested, as no differences were detected in the time females took to approach the egg masses of *N. viridula.* Probing was not impacted either. However, females were observed to begin drumming sooner on untreated egg masses compared to egg masses treated with the biocomplex, but a similar behavior was recorded for water-sprayed eggs. We speculate that wetting the surface of egg masses, irrespective of the substance used, might affect the balance of volatile substances contained on the egg surface, resulting in an interference with the behavior of the female in natural conditions. It is known that volatile compounds released by *N. viridula* during oviposition affect the activity of egg parasitoids (Colazza et al. 1999). Conversely, the number of times that the females of *T. basalis* approached the egg masses, and the number of times that they jumped onto the egg mass and started drumming, was lower in the egg masses treated with the anti-symbiont biocomplex than either control treatment (water and untreated). This pattern might be related to specific changes occurring on the egg surface because of the biocomplex composition. Nevertheless, lower frequency means that the female acceptance of the egg mass happened faster, and suggests that the treatment of the egg mass might enhance parasitism.

No-choice tests with *T. basalis* showed that parasitism and emergence rates decreased in egg masses treated with the anti-symbiont biocomplex and with water versus the untreated control. This mainly coincides with the pattern observed for the drumming time, and the drumming and approaching frequency behavior parameters. The occurrence of visibly developed wasps that failed emergence from the egg was observed only in *N. viridula* egg masses parasitized by *T. basalis*. According to Saunders et al (2022), this may be related to a condition of low humidity in the arena that hardens the egg chorion. This hypothesis, however, still needs to be experimentally tested.

Contrasting results were detected when assessing the impact of symbiont-targeted control on the two non-pest pentatomids. For *M. humeralis*, a negative effect was detected in first instar nymphs after spraying egg masses with the anti-symbiont biocomplex; while, no differences were observed for *O. schellenbergii*. The presence of a *Pantoea* bacterium in the V4 ventricle of *M. humeralis*, and the negative impact of its elimination on the insect, agree with previous studies reporting an obligated symbiosis between pentatomids and *Pantoea*-like bacteria (Duron and Noël 2016; Tada et al. 2011). Although potentially detrimental for *M. humeralis,* the exposure to anti-symbiont treatment would be unlikely for this species, with a minimum impact on nymphal development, since it is rarely found in crops in New Zealand (Larivière 1995). Interestingly, no effect on mortality rates during the first nymphal instar were observed on the predative stink bug *O. schellenbergii*. This is relevant due to the role of this species as a biological control agent against highly impacting pests in New Zealand (Larivière 1995). Equally, in this case the lack of associations with *Pantoea* gut bacteria matches the absence of an extracellular vertical transmission of obligate bacteria through egg smearing, resulting in non-lethality of treatments for nymphs. No sac-like gut ventricle was observed in dissected adults of *O. schellenbergii*, confirming the absence of a specialized structure that hosts symbiotic bacteria in other pentatomids. The absence of the V4 ventricle was noticed in other predaceous Asopinae such as *Podisus maculiventris* (Glasgow 1914). Bacteria detected in the gut of *O. schellenbergii* usually inhabit other stink bugs midgut compartments (Medina et al. 2018). Among them, the potentially entomopathogenic *S. marcescens* was found with high frequency in the midgut of *O. schellenbergii* adults (McQuade and Stock 2018; Zeng et al. 2020). Its absence in both treated and control nymphs derived from our experimental egg masses suggests that the treatment should not benefit the outgrowth of this bacterium in *O. schellenbergii* first instar nymphs, and is unlikely to induce the establishment of opportunistic or pathogenic species.

Another important result of this study is the absence of detrimental effects of symbiont-targeted control on parasitism by the native species *T. oenone*. Moreover, symbiont-targeted treatment did not affect the success of parasitism by *T. oenone*, as parasitism rates of biocomplex-treated egg masses was either no different from rates of water-treated egg masses or statistically intermediate between water-treated and untreated egg masses. Therefore, it is not possible to conclude whether the differences in observations of *T. oenone* are related to the use of anti-symbiont biocomplex or simply to the action of wetting the egg mass. Additionally, no differences were observed in total unhatched eggs. Surprisingly, *O. schellenbergii* egg masses subjected to anti-symbiont and water treatments exhibited greater parasitism rates than untreated egg masses. The possible change of the volatile compounds composition of the egg surface in this species after both treatment with water and with the biocomplex deserves further studies, as it could be exploited to develop synthetic blends that may find applications as stimulants for parasitoid wasps.

Egg parasitoids are a key part in current integrative pest management strategies against stink bug species (Conti et al. 2021). In the context of an IPM strategy, complementary strategies to biological control should not interfere with parasitism by biological control agents, especially those occurring in the same agroecosystem of a target pest. This study reveals a variable effect of anti-symbiont control in non-target species, consistent with previous work performed on *H. halys* and several parasitoids (Orrù et al. 2023). The lack of negative impact on the predatory stink bug *O. schellenbergii* suggests this strategy should not interfere with its role as a biological control agent of phytophagous larvae. Likewise, the negative impact on *M. humeralis* is expected to be mitigated by the low probability of direct exposure of this pest to anti-symbiont field treatments. The significant effect on *N. viridula* supports the possible application of this strategy against other pentatomid pest species along with *H. halys*. As to parasitism by egg parasitoids, the slight reduction often observed on the parasitism rates does not suggest a significant interference with biological control agents for two reasons: (1) The total number of dead eggs was not affected; and (2) the parasitism reduction was also detected when treating the egg masses with water. Therefore no assumptions can be stated on the reduction of parasitism rates as a result of symbiont-targeted control. If this strategy was to be incorporated in pest management programs in New Zealand, the evaluation at a field-level is recommended to confirm the suggested limited impact of anti-symbiont substances on natural enemies.

Authors contribution

SVP, GA, EG and AA conceived the ideas and designed the research. SVP and BL conducted the experiments. SVP analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Ethical approval This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

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