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Article

Symbiotic control of *Halyomorpha halys* using a microbial biopesticide

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With 2 figures and 2 tables

Abstract: Microbial biocontrol agents are promising tools for sustainable crop protection, as many strains showed high potential against various pathogens and pests. A still underexplored field for their use is symbiotic control, i.e. insect pest suppression through targeting obligate bacterial symbionts. Symbiotic control has been proposed against the brown marmorated stink bug *Halyomorpha halys*, by interrupting acquisition of its symbiont '*Candidatus* Pantoea carbekii' by newborns from egg surface. We investigated the success of symbiotic control of *H. halys* by exposing various developmental stages to spray applications of the commercial formulation Amylo-X®, containing the biocontrol agent *Bacillus velezensis* (previously *B. amyloliquefaciens*). Along with treating egg masses, 2nd and 4th instar nymphs were submitted also to direct or residual contact exposures to explore the contribution of symbiont- and insect-targeted antagonistic action in short term. We demonstrated significant mortality induced in Amylo-X®-treated samples, regardless of the insect stage and type of contact. Quantitative PCR analysis indicated that death of neonates was related to the missed acquisition of *P. carbekii,* due to symbiont elimination from the egg surface. Nevertheless, nymphal mortality after direct contact with Amylo-X® seemed resulting from an unknown antagonistic activity exerted by *B. velezensis*. Residual contact induced a combination of anti-symbiont and anti-insect activities, depending on the exposed instar. Our results support the use of Amylo-X® for symbiotic control of *H. halys* through field applications targeting egg masses; still *B. velezensis* deserves further studies to explore its additional functions against insects.

Keywords: biological control, *Bacillus velezensis*, brown marmorated stink bug, Pentatomidae, *Candidatus* Pantoea carbekii

1 Introduction

Microbial pest control agents (MPCA) are bacteria and fungi that show a beneficial effect on plant health by direct or indirect antagonisms against pathogens and pests (Pirttilä et al. 2021). They are receiving growing attention due to the increased demand for environmentally friendly alternatives to chemical pest control in agriculture. Several microbial strains have been included in biopesticide formulations, which are active against bacterial, fungal, and animal pests (Köhl et al. 2019; Kumar et al. 2021). Their control mechanisms include parasitism, antibiosis (by producing lytic enzymes and secondary metabolites such as antibiotics and toxins), repellence and competition for resources (Pirttilä et al. 2021).

The family Bacillaceae includes several bacteria used as biopesticides, notably as insecticides (owing to production of entomopathogenic endotoxins), as well as against plant pathogens (Kachhawa 2017). The *Bacillus amyloliquefaciens* operational group is a cluster grouped in the *B. subtilis* species complex, which includes several recognized MPCA used as biostimulants, biofungides and antibacterials due to their production of secondary metabolites showing antimicrobial properties (Kumar et al. 2021; Ngalimat et al. 2021; Poveda 2021). Among *B. amyloliquefaciens*-related MPCAs, the *B. velezensis* strain D747 is the main ingredient of commercial formulations (e.g., Double Nickel 55™ and Amylo-X®) authorized in various countries against plant pathogens (Rotolo et al. 2018; Pethybridge et al. 2019). It prevents pathogen establishment by outcompeting during root colonization, and suppresses bacterial and fungal growth by producing a cyclic lipopeptide iturin (Highland et al. 2012).

Recent work focused on the involvement of different compounds, including MPCA-based products (MPCP), in complex multi-trophic interactions, highlighting differentially targeted co-occurring mechanisms resulting in synergistic beneficial effects to plant health (Jaber & Ownley

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2018; Batista et al. 2021). A similar outcome is obtained by exploiting the antimicrobial effect exerted by micronutrient-based compounds that are commercialized as fertilizers. A symbiont targeted control (i.e., symbiotic control) have been proposed against insect pests in the family Pentatomidae. The indirect insect-killing effect is mediated by the interruption of the vertical acquisition of primary symbiotic bacteria by newborns, which is naturally ensured by feeding on maternal smears laid on the egg surface (Gonella et al. 2020). A major pest in the Pentatomidae is the brown marmorated stink bug *Halyomorpha halys* (Stål). Native to Asia, *H. halys* was accidentally introduced in North America and Europe, where it attacks a broad range of plants, feeding preferentially on fruits and causing heavy economic losses. Moreover, it is considered a nuisance pest due to its aggregative behaviour, especially when overwintering adults invade houses and other buildings (Leskey & Nielsen 2018). The elimination from *H. halys* egg masses of its symbiont, '*Candidatus* Pantoea carbekii' (hereafter *P. carbekii*), results in high neonate mortality (Bansal et al. 2014; Kenyon et al. 2015). Anti-symbiont treatments of egg masses with micronutrient fertilizers showed high efficacy against *H. halys* nymphs (Gonella et al. 2019), allowing the provisional authorization of the commercial product Dentamet® (Diachem, Italy) – a zinc, copper and citric acid biocomplex – to control *H. halys* in Italy in 2021. However, despite the wide potential of this strategy for pest control, the symbiont killing activity of other antimicrobial products (including MPCPs) is still poorly investigated. Here we assess the anti-symbiont activity of the biofungicide-bactericide product Amylo-X® (Biogard, Italy), authorized in the EU for organic crop protection, on *H. halys* egg masses and nymphs under laboratory conditions. Moreover, to evaluate the potential usefulness of including Amylo-X® in symbiotic control protocols against *H. halys,* we measure nymphal mortality resulting from missed *P. carbekii* acquisition and/or induction of gut dysbiosis, i.e. any alteration of the microbial community composition that affects insect health (Hamdi et al. 2011).

2 Materials and Methods

2.1 Insect material

A colony of *H. halys* was established in 2020 using field-collected adults from wild and cultivated host plants (Piedmont, Italy). The insects were reared on seedlings of broad bean, apples, and shelled hazelnuts, in net cages (930 × 475 × 475 mm) placed in climatic chambers (25 ± 1 °C, L:D 16:8). Egg masses were collected daily from the colony and used for the experiments. Egg masses dedicated to experiment 1 were directly exposed to treatments as explained below, within 24 hours from oviposition. Egg masses for experiments 2 and 3 were individually maintained in a climatic chamber in clear plastic Petri dishes with a wider lid with respect to the base to provide ventilation. After egg hatching, emerging nymphs were provided with an organic green bean as a food source and reared until the first moult. Newly emerged 2nd instar nymphs were collected daily and used for the experiments in batches made of five insects (= one replicate) until reaching required number of replicates. The remaining nymphs were further reared under the same conditions until they moulted to the 4th instar. Newly emerged 4th instar nymphs were collected daily to be used for the experiments until the final number of replicates was reached.

2.2 Evaluation of stage-specific lethal effects of *Bacillus velezensis* D747 on *Halyomorpha halys*

Different exposure routes to *B. velezensis* were tested to assess its anti-symbiont activity: 1) egg mass treatment with Amylo-X® to impair the acquisition of *P. carbekii* by newborns (Gonella et al. 2020); 2) direct exposure to Amylo-X® to allow active insect colonization by *B. velezensis* D747 followed by *in situ* anti-symbiont activity; 3) residual contact with Amylo-X® to allow MPCA colonization of the insect body and *in situ* anti-symbiont activity. In the trials included in experiment 1, an antibacterial activity, rather than insecticidal, was supposed. Therefore, the zinc, copper and citric acid biocomplex Dentamet® (2% water soluble Cu + 4% water soluble Zn) was used as a positive control since it was allowed for symbiotic control of *H. halys* in Italy, and it was previously demonstrated to cause high nymphal mortality (> 90%) due to symbiont suppression after egg mass treatment under the same conditions applied in this study (Gonella et al. 2019). Since this product has no insecticidal property, a different positive control was selected for experiments 2 and 3, where an insect-targeted effect (i.e., insect body colonization) was supposed. Accordingly, the microbiological insecticide Naturalis® (Biogard, Italy), containing the MPCA *Beauveria bassiana* strain ATCC 74040 (2.3×10^7 living spores/ml), was selected since this product is one of the few bioinsecticides recommended against hemipterans. Moreover, several strains of *B. bassiana* were previously demonstrated to cause high mortality in *H. halys* nymphs under laboratory conditions (Gouli et al. 2012; Parker et al. 2015; Tozlu et al. 2019).

2.2.1 Experiment 1: nymph suppression through egg mass treatment

One hundred-twenty egg masses laid by *H. halys* females within 24 hours were collected for this experiment, and the number of eggs per mass was recorded. Four groups of 30 egg masses with homogeneous egg number were randomly separated (average egg number per egg mass: 25.4 ± 0.36). The first group was treated with Amylo-X® (containing 5×10^{10} *B. velezensis* D747/g) at the label recommended dose of 2 g/L (water solution); moreover, to evaluate possible effects on egg masses related to co-formulants contained in the commercial product, a second group of egg masses was

treated with an unformulated suspension of *B. velezensis* D747 (Biogard) at the same dose (2 g/L). The third group of egg masses was dedicated to positive control and the fourth group was left untreated as a negative control. The positive control product Dentamet® was applied at the dose of 1% v/v (water solution) as reported by Gonella et al. (2019). Briefly, the egg masses were individually placed into Petri dishes covered with filter paper, and then treated using a 250 mL hand sprayer (Nalgene®, NY, USA) under a fume hood. A single spray (651 ± 7.42 µl) was applied with the hand sprayer held approximately 20 cm away from the Petri dish with the egg mass. After the treatment, egg masses were individually reared in climatic chamber as described above. Hatching percentages were checked daily; newly hatched nymphs were fed with green beans until they reached the 2nd instar. Mortality rates at the 1st instar were calculated; dead 1st instar nymphs were collected daily and stored at –80 °C in RNA later® (Sigma-Aldrich, MO, USA). As live nymphs moulted to the 2nd instar, they were collected and stored as well.

2.2.2 Experiment 2: nymph suppression through direct contact

A total of 90 2nd instar and 90 4th instar *H. halys* nymphs moulted within 24 hours were collected for this experiment. They were divided in batches made of five specimens moulted in the same day; batches were placed in Petri dishes covered with filter paper and randomly allocated to three different treatments. The experiment was replicated six times. The first group was treated with Amylo-X® spraying a 2 g/L water solution onto the nymph bodies using the same procedure as for egg masses; the second group was dedicated to positive control and the third group was left untreated as a negative control. For this experiment, Naturalis® was applied as a positive control, at the label recommended dose of 2 mL/L (water solution) as described above. The unformulated *B. velezensis* suspension was not applied in experiments 2 and 3 since a low efficiency was observed in experiment 1. After the treatment, nymphs were individually reared in climatic chamber until they moulted to the subsequent instar (3rd and 5th, respectively). Mortality rates at the 2nd or 4th instar were calculated; dead 2nd and 4th instar nymphs were collected daily and stored at –80 °C in RNA later® (Sigma-Aldrich, MO, USA). As live nymphs moulted to the 3rd or 5th instar, they were collected and stored as well.

2.2.3 Experiment 3: nymph suppression through residual contact

Treatments for this experiment were performed using the same products and concentrations as for experiment 2, but only the Petri dish, containing a leaf of *Vitis vinifera* L. (cv Barbera), was treated before introducing the insects. The grapevine leaves were isolated by placing petioles inside Eppendorf™ tubes containing a nutritive solution as described by Gonella et al. (2015) prior treatment, then the whole Petri dish was sprayed with a 2 g/L water solution of Amylo-X® or Naturalis® (as a positive control). Treated Petri dishes were maintained in climatic chamber for 48 hours. This period was selected as it is reported to allow the colonization of the grapevine leaf by *B. velezensis* and the production of antibacterial molecules, whereas after longer periods a drop of the MPCA load on the leaf surface was previously observed (Rotolo et al. 2016). Subsequently, 90 2nd instar and 90 4th instar nymphs were placed in the Petri dishes, divided in batches made of five specimens. Nymphs were randomly allocated to different treatments and maintained as described above until they moulted to the subsequent instar (3rd and 5th, respectively); the experiment was replicated six times. Mortality rates at the 2nd or 4th instar were calculated; dead 2nd and 4th instar nymphs were collected daily and stored at –80 °C in RNA later® (Sigma-Aldrich, MO, USA). As live nymphs moulted to the 3rd or 5th instar, they were collected and stored as well.

2.3 RNA extraction and Real Time PCR

To assess the involvement of *P. carbekii* suppression and dysbiosis induction in the lethal effect observed in *H. halys* nymphs, Real Time quantitative PCR (qPCR) diagnostic assays were conducted to measure the presence and density of *P. carbekii* and *B. velezensis*. To quantify the active bacterial load, RNA extraction was performed from up to 10 surface-sterilized nymphs for each experiment, treatment, instar and live/dead status, using the "SV Total RNA Isolation System" (Promega, WI, USA), according to the supplier's suggestions. 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, WI, USA) and Random Primers, following the manufacturer's instructions. cDNA was used as a template for Real Time qPCR analyses with the *P. carbekii*-specific primers PcarQF/R, as described in Gonella et al. (2019), and with the primer pair Bsub5F/3R (Wattiau et al. 2001), which efficiently amplify the 16SrRNA gene of *B. velezensis* D747, as described in Rotolo et al. (2016) (Table S1). All 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 1 µl of cDNA template. Further qPCR tests were conducted to target the insect's 18S rRNA gene (MqFw/MqRv, Marzachì & Bosco 2005) at the conditions described in Gonella et al. (2019), to i) verify whether the target bacteria were actually absent in negative nymphs rather than misdiagnosed due to sample quality, and ii) normalize the absolute bacterial densities. Normalized *P. carbekii* and *B. velezensis* cell numbers were calculated per pg of insect 18Sr RNA gene. Standard curves for the Real

Time PCRs were constructed with cloned PCR-amplified 16S rRNA gene of *P. carbekii* and *B. velezensis,* obtained using the pGEM T-easy Vector Cloning Kit (Promega, WI, USA). For each reaction, the detection limit was calculated as the lowest concentration of cloned amplicons used for determining the standard curves that were successfully amplified.

2.4 Statistical analyses

In experiment 1, egg hatching rate was calculated as the ratio between the number of emerged nymphs and the total number of laid eggs per egg mass; nymphal mortality at the 1st instar was measured as the percentage of dead specimens with respect to the number of emerged nymphs per egg mass, and combined suppression was estimated as the percentage of unborn + dead nymphs at the 1st instar with respect to the total number of laid eggs for each egg mass. Corrected mortality and combined suppression rates were calculated according to the Abbott's formula (Abbott 1925). Percentages were compared by generalized linear model (GLM) with binomial (untransformed data) or normal (Abbott-corrected data) distribution and log link followed by a post hoc sequential Bonferroni procedure. In experiment 2 and 3, mortality rates in each replicate (N = 5) were calculated as the percentage of dead nymphs at the 2nd or 4th instar; corrected rates were obtained using the Abbott's formula. Statistical analysis of untransformed rates was conducted by GLM with binomial distribution and log link followed by a post hoc sequential Bonferroni procedure. Abbott-corrected rates were compared by Student's t test. In all experiments, for each treatment, instar and live/dead status, bacterial infection rates were calculated as the ratio of qPCR-positive nymphs for *P. carbekii* or *B. velezensis* among analysed samples. Statistical analysis was done using a GLM with binomial probability distribution and logit link function and means were separated with a sequential Bonferroni post hoc test ($P < 0.05$). Bacterial concentrations resulting from qPCR were submitted to log transformation and analysed by generalized linear model (GLM) with normal distribution followed by a sequential Bonferroni post hoc test ($P < 0.05$). Statistical analyses were performed with SPSS Statistics 27 (IBM Corp. Released 2020, Armonk, NY, USA).

3 Results

3.1 Nymphal mortality after product application and effects on infection with '*Ca.* Pantoea carbekii'

3.1.1 Nymph suppression through egg mass treatment (experiment 1)

To assess if the applications of tested products on *H. halys* egg masses led to higher mortality of neonates with respect to control, mortality rates of 1st instars were compared prior to Abbott correction. There was a significant increase of nymphal mortality for all treatments (Table 1). As a further step, to compare the impact of different products for the symbiotic control on *H. halys* egg masses, we analysed mortality rates of 1st instars by GLM after Abbott correction. Amylo-X® induced a significantly lower mortality

Table 1. Mortality rates recorded in *H. halys* nymphs during the experiments. Mean values (± SE); in each line, different letters indicate significantly different values according to binomial GLM + sequential Bonferroni (at $P < 0.05$); N.A. = not applicable. E: experiment number.

E	Measured value	Amylo-X®	Unformulated *B. velezensis*	Dentamet®	Naturalis®	Control	df	$\chi 2$	*P*
1	% of newborns dead before II instar	70.14 ± 6.92**c**	42.37 ± 8.17**b**	90.01 ± 2.07**d**	N.A.	10.95 ± 1.37**a**	3	2286.77	<0.001
1	% of egg mortality	47.18 ± 4.96**d**	42.17 ± 5.54**c**	25.50 ± 3.26**b**	N.A.	13.91 ± 1.81**a**	3	414.88	<0.001
1	% of unborn + dead nymphs before 2nd instar (combined suppression)	83.23 ± 4.67**c**	61.50 ± 6.61**b**	92.03 ± 1.86**d**	N.A.	23.09 ± 2.30**a**	3	1385.25	<0.001
2	% of nymphs dead before 3rd instar	43.33 ± 6.15**b**	N.A.	N.A.	56.67 ± 6.15**b**	16.67 ± 6.15**a**	2	13.82	<0.001
2	% of nymphs dead before 5th instar	53.33 ± 6.67**b**	N.A.	N.A.	46.67 ± 6.67**b**	6.67 ± 4.22**a**	2	27.13	<0.001
3	% of nymphs dead before 3rd instar	46.67 ± 4.22**b**	N.A.	N.A.	26.67 ± 6.67**ab**	13.33 ± 6.67**a**	2	9.23	0.010
3	% of nymphs dead before 5th instar	46.67 ± 6.67**b**	N.A.	N.A.	23.33 ± 6.15**ab**	10.00 ± 6.83**a**	2	11.63	0.003

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with respect to positive control, treated with Dentamet®, but caused higher mortality than the unformulated *B. velezensis* suspension (normal GLM: df 2, $\chi^2 = 32.164$, $P < 0.001$; Fig. 1). Treatment with Amylo-X® (or with the unformulated *B. velezensis* suspension) induced significant reduction of egg hatching, both when compared to the untreated control prior Abbott correction (Table 1) and when compared to Dentamet® after correction (normal GLM: df 2, $\chi^2 = 13.343$, $P = 0.001$; Fig. 1). The combined suppression rate (unhatched eggs + dead 1st instar nymphs) of all treatments was higher than in control (Table 1), and after Abbott correction Amylo-X® reached values that were not significantly divergent from Dentamet®. Both commercial products were more suppressive than unformulated *B. velezensis* (normal GLM: df 2, $\chi^2 = 24.197$, $P < 0.001$; Fig. 1). A missed acquisition of the primary symbiont was observed in neonates that died before reaching the 2nd instar, as confirmed also by the significance of the comparison between *P. carbekii* infection rates obtained by qPCR (binomial GLM: df 7, $\chi^2 = 974.132$, $P < 0.001$; Fig. 1). No *P. carbekii*-positive samples from the 1st instar dead nymphs were detected by qPCR from egg masses treated with Amylo-X®. However, 50% of live 2nd instars from Amylo-X® treatment were infected by *P. carbekii*, as opposed to Dentamet® treatment, where the total symbiont elimination was observed also in live specimens, and to the untreated control, where 100% infection was found irrespective of the nymph live/dead status. When compared to both 1st and 2nd instar nymphs from the

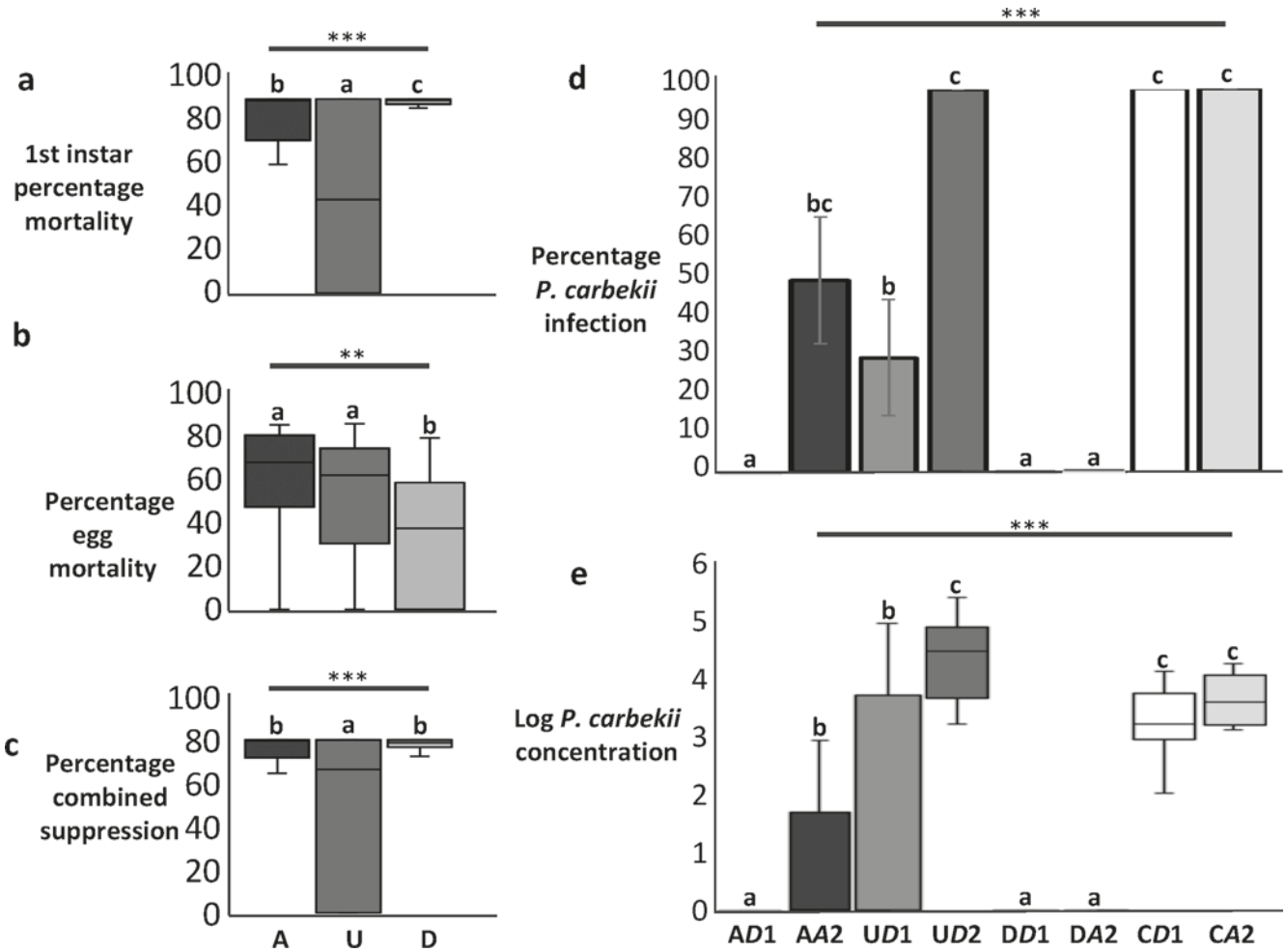

Fig. 1. Effects of Amylo-X® application onto *H. halys* egg masses (experiment 1). a) Abbott-corrected mortality rates recorded among newborns emerging from egg masses submitted to different treatments; b) Abbott-corrected percentage of hatched eggs; c) Abbott-corrected rates of total suppression, expressed as the percentage of unborn + dead nymphs; d) Percentage of *P. carbekii*-positive nymphs among tested dead and live specimens for each treatment; e) Average density of live *P. carbekii*, indicated as the log-transformed number of symbiont expressed 16S rRNA gene copies per nymph. A = Amylo-X®; U = unformulated *B. velezensis* suspension; D = Dentamet®; C = negative control. Italic letters *A* and *D* indicate the nymph status (alive / dead); 1 = 1st instar, 2 = 2nd instar. Bars indicate standard error; asterisks indicate significant different values according to normal GLM (a-c, e) or binomial GLM (d), with $P < 0.01$ (**), $P < 0.001$ (***). Different letters refer to significantly different values according to sequential Bonferroni test.

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untreated control, live 2nd instars from the Amylo-X® treatment showed a significantly reduced concentration of *P. carbekii* (normal GLM: df 7, $\chi^2 = 621.909$, $P < 0.001$; Fig. S1, Table S2). Treatment with the unformulated *B. velezensis* suspension produced only a partial reduction of *P. carbekii* acquisition by neonates. One third of dead 1st instar nymphs were still infected with the symbiont, and the survived 2nd instars showed an infection rate and symbiont concentration similar to the untreated control.

3.1.2 Nymph suppression through direct contact (experiment 2)

When *H. halys* nymphs were exposed to direct contact with the MPCPs (both at the 2nd and the 4th instar), a significantly high mortality was observed with respect to untreated control (Table 1). The comparison of Abbott-corrected percentages showed no difference between the mortality induced by Amylo-X® and Naturalis®, used as a bioinsecticidal positive control (Student's t test, 2nd instars: df 10, t = 1.408, P = 0.189; 4th instars: df 10, t = 0.848, P = 0.416; Fig. 2). However, no treatment effect was observed when the infection with *P. carbekii* was measured, either in terms of infection or bacterial concentration (binomial GLM, *P. carbekii* infection rate, 2nd instars: df 5, $\chi^2 = 2.254$, P = 0.813; 4th instars: df 5, $\chi^2 = 3.611$, P = 0.607; normal GLM, *P. carbekii* concentration, 2nd instars: df 5, $\chi^2 = 2.603$, P = 0.761; 4th instars: df 5, $\chi^2 = 4.856$, P = 0.434; Fig. 2, Fig. S1, Table S2).

3.1.3 Nymph suppression through residual contact (experiment 3)

The residual contact with Amylo-X® caused an increment of nymphal mortality at both the examined instars compared to the untreated control (Table 1). No significant difference was observed in the induced mortality between Amylo-X® and Naturalis® after Abbott correction (Student's t test, 2nd instars: df 5.44, t = 2.283, P = 0.067; 4th instars: df 10, t = 2.023, P = 0.071; Fig. 2). Insects exposed to Amylo-X® underwent the alteration of infection with *P. carbekii*, but only when 2nd nymphal instar was treated. Comparing dead 2nd instar nymphs with juveniles survived to the 3rd instar within each treatment, only in the Amylo-X® treatment dead 2nd instars had significant smaller infection than the respective live 3rd instars (binomial GLM: df 5, $\chi^2 = 29.467$, $P < 0.001$; Fig. 2). The symbiont concentration was significantly

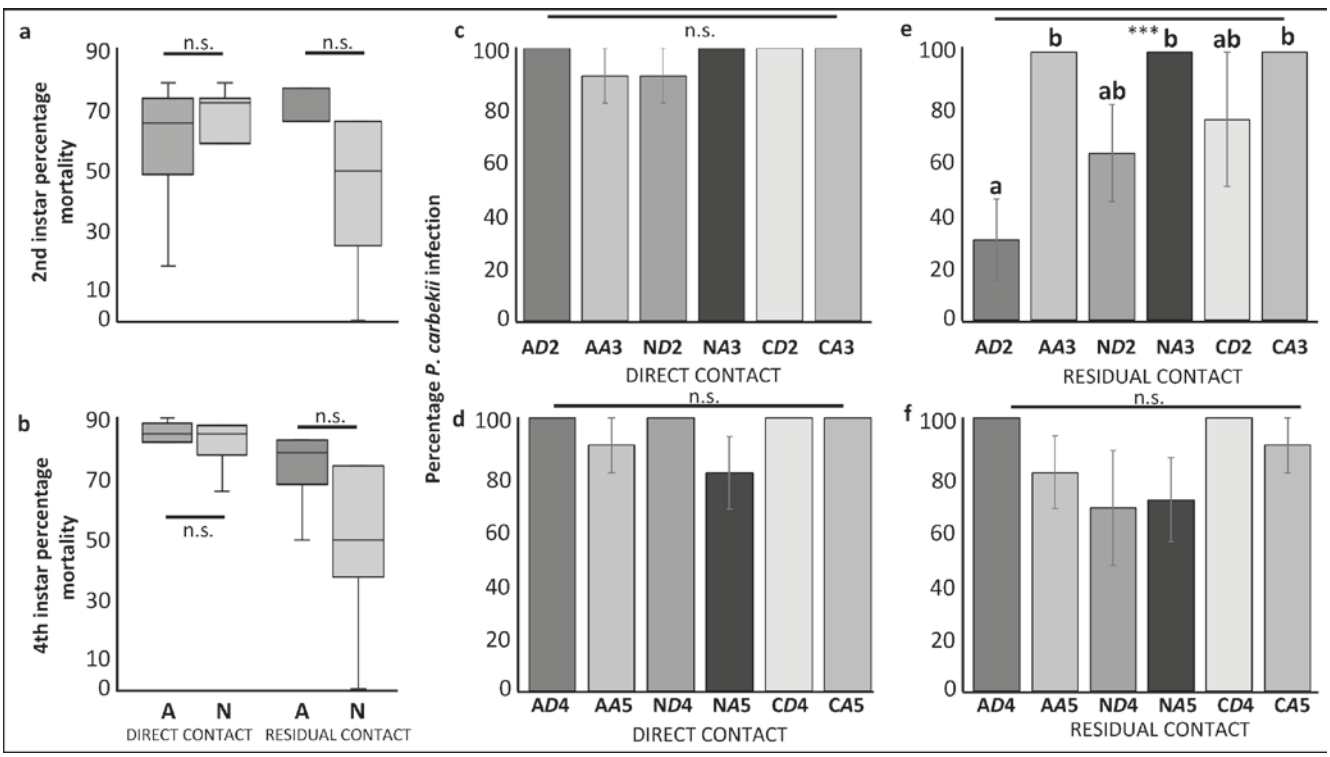

Fig. 2. Effects of Amylo-X® treatment to 2nd and 4th instar nymphs of *H. halys*. a-b) Abbott-corrected percentages of nymphal mortality after direct or residual contact to Amylo-X® (A) and Naturalis® (N); c-f) Percentage of *P. carbekii*-positive nymphs among tested dead and live specimens for each treatment and instar, in direct and residual contact experiments. A = Amylo-X®; N = Naturalis®; C= negative control. Italic letters *A* and *D* indicate the nymph status (alive / dead); 2 = 2nd instar, 3 = 3rd instar, 4 = 4th instar. Bars indicate standard error; asterisk indicates significant different values according to binomial GLM, with $P < 0.001$. Different letters refer to significantly different values according to sequential Bonferroni test; n.s. = not significant.

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reduced in dead 2nd instar nymphs after both treatments, compared to the values found in treated insects surviving to the 3rd instar and those from the untreated group, irrespective of the live/dead status (normal GLM: df 5, $\chi^2 = 45.192$, $P < 0.001$; Fig. S1). In contrast, the residual contact with Amylo-X® or Naturalis® had no effect on the infection with *P. carbekii* in older nymphs, i.e. 4th instars, both in terms of infection percentage and symbiont density (binomial GLM, *P. carbekii* infection rate: df 5, $\chi^2 = 10.897$, $P = 0.053$; normal GLM, *P. carbekii* concentration: df 5, $\chi^2 = 2.245$, $P = 0.814$; Fig. 2, Fig. S1, Table S2).

3.2 Insect colonization by *Bacillus velezensis* D747

The infection rate of *B. velezensis* following Amylo-X® treatment varied according to the tested exposure route; hence the results from treatment of 2nd and 4th instars (both for direct and residual contact) were pooled to increase their statistical reliability. Moreover, infection data from the unformulated *B. velezensis* suspension in experiment 1 were pooled with those obtained from the Amylo-X® treatment. The colonization percentage by *B. velezensis* showed significant differences among experiments (binomial GLM: df 5, $\chi^2 = 118.985$, $P < 0.001$); treatment of egg masses caused the highest infection rate, whereas residual contact resulted in lowest infection rate (Table 2). Comparing the infection percentages between dead and live nymphs in the same experiment, a significant increase of *B. velezensis* was recorded only in dead 1st instar specimens from egg mass treatment (Table 2). Moreover, a difference was found also regarding the average concentration of *B. velezensis* (normal GLM: df 5, $\chi^2 = 45.933$, $P < 0.001$). The higher values were detected in nymphs from all direct contact experiments; high bacterial density was found also in dead 1st instar nymphs from egg mass treatment, though very low bacterial concentration was found in all the tested insects that experienced residual exposure (Fig. S1, Table S2).

Table 2. Percentage of *B. velezensis*-positive nymphs among tested dead and live specimens for each experiment. Average values ± standard errors are presented; different letters indicate significantly different values according to binomial GLM + sequential Bonferroni (at $P < 0.05$).

Exp. No.	Alive/dead status	% infection by *B. velezensis*
1	Dead (1st instar)	95 ± 5.00**c**
1	Alive (2nd instar)	20 ± 9.18**a**
2	Dead (2nd + 4th instar)	60 ± 11.24**b**
2	Alive (3rd + 5th instar)	40 ± 11.24**ab**
3	Dead (2nd + 4th instar)	25 ± 9.93**ab**
3	Alive (3rd + 5th instar)	15 ± 8.19**a**

4 Discussion

We demonstrated a high lethal effect of Amylo-X® on *H. halys* eggs and nymphs. The suppressive activity was strong after treatment of egg masses, and was related to induced dysbiosis in neonates; it resulted from missed acquisition of *P. carbekii* and colonization of insects by *B. velezensis* once newborns ingested the bacterial biofilm on egg surface. The high fitness cost induced by hampering the establishment of the primary symbiont in the *H. halys* midgut after birth is widely documented (Kenyon et al. 2015; Gonella et al. 2020). The presence of *B. velezensis* in dead nymph emerged from egg masses treated with Amylo-X® suggests that the gut niche made available by the missed acquisition of the primary symbiont was occupied by this bacterium (and/or other microbial strains) as an opportunistic pathogen. Nonetheless, whether *B. velezensis* D747 displays an actual entomopathogenic effect against *H. halys* nymphs – for example through the production of secondary metabolites – remains unknown, although several bacteria were previously reported to show both antimicrobial and insecticidal activity, including many strains in the *B. amyloliquefaciens* operational group (Dimkić et al. 2022). Another outcome of treatment with Amylo-X® is a modest colonization by *B. velezensis*, in surviving 2nd instar nymphs as well. Experiments involving the rearing of *H. halys* lines obtained from egg masses sprayed with this biopesticide may clarify whether these insects experience a long-term treatment effect.

The insect containment resulting from egg masses treatment with the *B. velezensis* – based product was partly due to the reduction of egg hatching, which was higher than after exposure to micronutrient-based products. High egg mortality was recorded also in egg masses treated with the unformulated *B. velezensis* suspension, suggesting that the ovicidal effect is related to the pathogen itself rather than to co-formulants occurring in the commercial product. However, these additives seem to be involved in improving the MPCA coverage of the egg masses, supporting a more efficient elimination of *P. carbekii* and consequent neonate mortality.

When *H. halys* – as 2nd or 4th instar nymphs – were provided with Amylo-X® by direct contact, insects were colonized by *B. velezensis* (as indicated by qPCR results) and relatively high percentages of dead insects were observed. Nonetheless, nymph suppression was not accompanied by the elimination of *P. carbekii* or any apparent microbiome alteration. Rather, *B. velezensis* D747 may produce virulence factors with a pathogenic role for *H. halys* nymphs. Entomopathogenic bacteria in the *Bacillus* genus are known to enter the insect by ingestion only (Melo et al. 2016); however, the production of hydrolytic enzymes by *B. velezensis* D747 may support the active penetration of the soft cuticle of newly-moulted nymphs used in our experiments. Similar hydrolytic enzymes, such as chitinase, are commonly produced by strains in the *B. amyloliquefaciens* operational

group (Subbanna et al. 2018; Malovichko et al. 2019). Alternatively, accidental ingestion after body contamination could be the major penetration route, or a mixed colonization modality may occur.

Nymphs exposed to Amylo-X® by residual contact showed a higher disturbance of the gut colonization by *P. carbekii* than those treated by direct contact. Our results suggest indeed a contact between *B. velezensis* and the symbiont, resulting in the elimination of *P. carbekii* from the insect body. However, the antagonistic effect seems to be stage-related. In effect, a significant reduction of *P. carbekii* was documented in 2nd instar nymphs used in the residual contact experiments, but not in 4th instars. Moreover, in latter group, also nymphs in the control group showed less than 100% symbiont infection, in agreement with previous observations (Gonella et al. 2019), suggesting that some individuals may survive even if they naturally fail in acquiring the symbiont. In the closely related pentatomid *Plautia stali* Scott, a complex morphogenesis of the terminal midgut portion hosting the primary symbiont (symbiotic organ) has been reported (Oishi et al. 2019). During the juvenile development, stink bugs undergo a modification of the gut conformation, with the progressive formation of crypts, which are colonized by the symbiont. From the late 2nd instar on, the symbiont organ is structurally separated from the previous midgut regions by a constriction of gut walls (Oishi et al. 2019). If the same process occurs also in *H. halys*, the accidental ingestion of *B. velezensis* cells or metabolites by early 2nd instars may be still harmful for *P. carbekii* before the isolation of the symbiotic organ. In contrast, when insects are exposed to residual treatment as 4th instars, the complete midgut compartmentalization protects *P. carbekii* from virulence factors; therefore, at this stage, the lethal effect exerted by *B. velezensis* must be due to a different process.

Taken together, our results support the use of Amylo-X® for symbiotic control programs targeting egg masses of the stink bug. A field validation of the effectiveness of control treatments using this strategy is required, since a reduced effect of bacteria-based products is sometimes observed, depending on the post-application conditions. Moreover, a possible insecticidal activity against *H. halys* nymphal stages may be supposed for the MPCA, since it affected the survival of nymphs similarly to *B. bassiana* – whose entomopathogenic activity is well-known after treatments with Naturalis®. Further scientific efforts could investigate the processes inducing the suggested antagonistic activity of *B. velezensis*, also assessing the long-term interaction with *P. carbekii* and its host, to unravel the contribution of anti-symbiont and anti-insect molecular factors in determining the death of *H. halys*.

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