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ORIGINAL ARTICLE

Effect of the rearing diet on gene expression of antimicrobial peptides in *Hermetia illucens* (Diptera: Stratiomyidae)

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Abstract Insect proteins have been proposed for human and animal food production. Safeguarding the health status of insects in mass rearing allows to obtain high-quality products and to avoid severe economic losses due to entomopathogens. Therefore, new strategies for preserving insect health must be implemented. Modulation of the insect immune system through the diet is one such strategy. We evaluated gene expression of two antimicrobial peptides (one defensin and one cecropin) in *Hermetia illucens* (L.) (Diptera: Stratiomyidae) reared on different diets. Analyses were performed on prepupae and 10-day-old larvae reared on cereal- and municipal organic waste-based diets and on only prepupae reared on a cereal-based diet supplemented with sunflower, corn, or soybean oil. The inclusion of sunflower oil at different points in the cereal-based diet was also evaluated. Moreover, diet-driven differences in the inhibitory activity of the hemolymph were tested against *Escherichia coli* DH5α and *Micrococcus yunnanensis* HI55 using diffusion assays in solid media. Results showed that a municipal organic waste-based diet produced a significant overexpression of antimicrobial peptides only in prepupae. Inclusion of vegetable oils caused an upregulation of at least one peptide, except for the corn oil. Higher expression of both genes was observed when sunflower oil was added 5 days before pupation. All hemolymph samples showed an inhibitory activity against bacteria colonies. Our results suggest that municipal organic waste-based diet and vegetable oil-added diet may successfully impact the immune system of *H. illucens*. Such alternatives may also exist for other species of economic interest.

Key words cecropin; cereal; defensin; hemolymph; organic municipal solid waste; vegetable oil

Introduction

Today's global food system is inadequate to meet current needs, let alone future projections (Vandermeer *et al.*, 2018). According to estimates compiled by the Food and Agriculture Organization (FAO), an increase of 70% in food production is required in order to supply the food demand expected for 2050 (FAO, 2009). To meet the protein demand, more efficient animal production, meat substitutes, and alternative protein sources are required (El-Chichakli *et al.*, 2016). Insect proteins have been proposed as high-quality, cost-effective, energy-efficient, and sustainable alternatives both for human and animal feed (van Huis, 2020; Meyer-Rochow *et al.*, 2022; van Huis, 2022). Coincidentally, some insect populations can successfully be grown on organic side streams, such as organic waste or low-value organic by-products, which

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offer an attractive approach within a circular economy (Gasco *et al.*, 2020; Jensen *et al.*, 2021).

Among the species of interest, *Hermetia illucens* (L.) (Diptera: Stratiomyidae), *Musca domestica* L. (Diptera: Muscidae), and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) have the highest potential for large-scale production (van Huis, 2020). It has been reported that the insect growth rates, chemical compositions, and their nutritional quality largely depend on the substrate used for the insect rearing (Harsányi *et al.*, 2020; Hopkins *et al.*, 2021). To date, researchers have focused their investigations on the effects of growth substrate on insects' nutrient composition (Barragan-Fonseca *et al.*, 2021; Fuso *et al.*, 2021) and on substrate reduction efficiency, bio-, and feed conversion rates (Ravi *et al.*, 2020; Parodi *et al.*, 2021; Veldkamp *et al.*, 2021). Moreover, great attention has been addressed on the qualitative/quantitative response of aquaculture and livestock animals fed insects-derived products (Gariglio *et al.*, 2019; Benzertiha *et al.*, 2020; Shariat Zadeh *et al.*, 2020; Bellezza Oddon *et al.*, 2021; Elahi *et al.*, 2022; Hong & Kim, 2022; Mohan *et al.*, 2022; Tran *et al.*, 2022). Nonetheless, little exists on the impact of rearing substrates on the insect immunological response.

The ability of insects to feed successfully on nutritionally unpredictable diets and/or those with high levels of bacterial contamination may lie in immune system adaptations (Vilcinskas, 2013). Humoral and cellular defenses produce insect immune responses (Lavine & Strand, 2002; Kanost *et al.*, 2004). Insect antimicrobial peptides/proteins (AMPs), produced in different organs and tissues, are key components for their humoral response (Tsakas & Marmaras, 2010), and may possess antibacterial, antiviral, and antifungal activity (Levy *et al.*, 2004). Even though AMPs have been characterized in different insect species (Chae *et al.*, 2012; Yi *et al.*, 2014; Elhag *et al.*, 2017), much remains to be understood about how to modulate their expression to improve the insect immune system. Stimulating the insect immune system through the diet could make a remarkable difference in insect mass rearing. Indeed, triggering the immune system could not only preserve healthy rearing conditions, but also increase insect "tolerance" to entomopathogens. Moreover, any reduction or avoidance of antibiotic use is beneficial in light of rising global resistance if the final use of the insect as for feed or food.

Our study builds on work that showed the impact on AMP expression from rearing diets containing high microbial loads, supplemented with cellulose, chitin, lignin, brewer's grains, protein, sunflower oil (Vogel *et al.*, 2018). Our aim was to investigate the diet-dependent expression of two genes coding for AMPs (one defensin and one cecropin) throughout the entire body of *H. illucens* 10-day-old larvae and prepupae reared on different diets. The inhibitory activity of the hemolymph, extracted from prepupae reared on the different diets, was also evaluated against one Gram-negative and one Gram-positive bacterium.

Materials and methods

Insect rearing

Hermetia illucens was reared at the experimental facility of the Department of Agricultural, Forest and Food Sciences (DISAFA; University of Torino, Carmagnola, Italy). Eggs were collected using a sticky-wood egg trap (Julita *et al.*, 2021) and with the help of a fine brush. Groups of eggs (1 g each) were positioned on a net placed above plastic containers (10 × 17.5 × 7 cm) filled with 400 g of a Gainesville diet (30% alfalfa, 50% wheat bran, 20% cornmeal, and a 70% moisture content; Tomberlin *et al.*, 2002) and maintained at 27 ± 1°C, 70% ± 5% RH, with a 14 : 10 h L : D photoperiod. After 6 days, all larvae were sieved, divided into groups of 10 larvae, weighed, and then used in the trials.

Three different growing trials were performed. For each growing trial and tested diet, 4 replicates of 300 larvae each were set up. In the first and the second trial, 6-day-old larvae were reared in a plastic container (10 × 17.5 × 7 cm) and fed with 400 g of the different tested diets in a climate-controlled chamber (T: 27°C; RH: 70%, 14 : 10 h L : D photoperiod) (Fig. 1). In order to evaluate the effect of the diet on the AMPs expression level and on the hemolymph inhibitory activity, in the first trial, two different diets were compared: the Gainseville diet (GAIN) as control diet, and a diet composed of chopped organic fraction of municipals solid waste (80% moisture content) (OFMSW) (R.g.l. Srl, Pilastro di Langhirano, Italy).

The second trial was set up to assess the effect of the addition of three different vegetable oils (5% of the diet humid weight) into the diet on AMP expression levels. Sunflower, corn and soybean oil were individually added to the Gainesville diet 10 days before pupation (dbp). So, totally, four different diets were prepared: (i) Gainesville diet (GAIN) as the control, (ii) Gainesville diet added with sunflower oil (+SUNOIL), (iii) Gainesville diet added with corn oil (+CORNOIL), and (iv) = Gainesville diet added with soybean oil (+SOYOIL).

Finally, to assess if AMP encoded gene expression levels and hemolymph inhibitory activity were affected by

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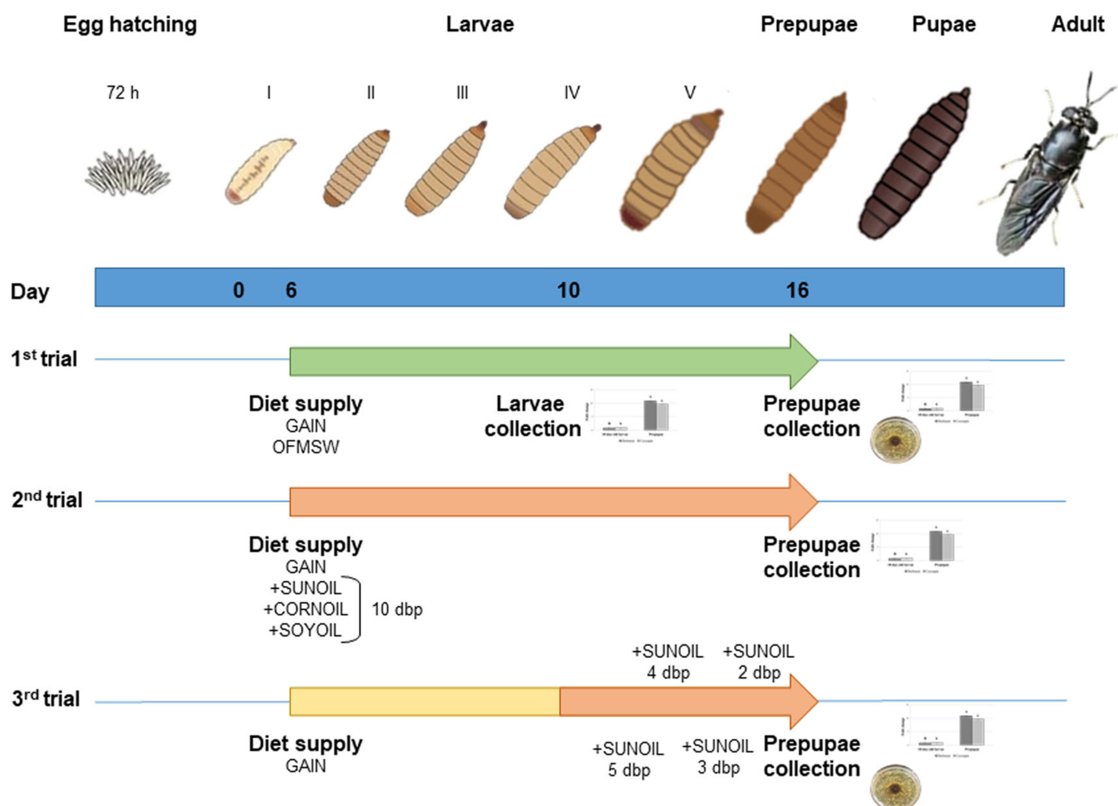

Fig. 1 Timeline of the first, second and third trial. For each trial, larvae were reared on GAIN diet from day 0 to day 6. Then, groups of homogeneous weight 6-day-old larvae were reared on the tested diets. Depending on the trial, 10-day-old larvae (4 days spent on the tested diet) and/or prepupae (10 days spent on the tested diet) were analysed. In the first trial, AMP expression levels were evaluated in larvae and prepupae while this analysis was conducted only in prepupae in the second and third trial. The inhibitory activity of the hemolymph extracted from the prepupae was investigated in the first and the third trial.

the timing of the addition of the oil into the rearing diet, another trial was conducted in which sunflower oil was added to the Gainesville diet at 5, 4, 3, and 2 dbp. Again, the GAIN served as the control diet. For this trial, the sunflower oil was selected given the results obtained in our research and by other authors (Vogel *et al.*, 2018). Groups of 6-day-old larvae were partitioned into five batches (one for each time of oil inclusion (5, 4, 3 and 2 dbp) and one for the control diet) and reared on 400 g of a GAIN diet in a plastic container (10 × 17.5 × 7 cm) in a climate-controlled chamber (T: 27°C; RH: 70%, 14 : 10 h L : D photoperiod). Then, the sunflower oil (5% of the diet humid weight) was added at the different time points (Fig. 1).

In the first trial, 10-day-old larvae, which spent 4 days on the tested diet, and prepupae, which spent 10 days on the tested diet, were collected and used for the gene expression analysis. Considering the results obtained in the first trial, only prepupae were collected from the second and the third trial and used to assess the diet-dependent expression of AMPs. The hemolymph inhibitory activity was evaluated only using hemolymph extracted from prepupae obtained in the first and third trial. In all trials, prepupae were collected and used for the analyses (gene expression and hemolymph inhibition assay) when 40% of the reared larvae reached the prepupal stage.

Gene expression analysis

For the gene expression analysis, at the end of the first trial, 18 reared on GAIN and 26 reared on OFMSW 10-day-old larvae and 20 prepupae were collected, while at the end of the second trial, 25 prepupae reared on +SUNOIL and +CORNOIL, and 20 prepupae reared on +SOYOIL were collected and used. In the third trial, 18 prepupae reared on GAIN, 21 prepupae reared on +SUNOIL 5 dbp, 17 prepupae reared on +SUNOIL 4 dbp, 13 prepupae reared on +SUNOIL 3 dbp and 16 prepupae reared on +SUNOIL 2 dbp were used.

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In order to work under sterile conditions, larvae and prepupae were sieved and washed in diethylpyrocarbonate (DEPC) water (Merck KGaA, Darmstadt, Germany), 75% ethanol in DEPC water, and DEPC water for 2, 1 and 2 min, respectively with the aim of removing any diet residues and any other possible contaminant present in it (e.g., insect frass, bacteria). Insects were dried on filter paper and frozen at −80°C until further analysis.

RNA isolation

Total RNA extraction was performed following the TRI Reagent® protocol (Merck KGaA, Darmstadt, Germany), according to the supplier's suggestions. Briefly, insects were grounded to a fine powder under liquid nitrogen and lysed in 600 μL of TRI Reagent®; then samples were incubated at room temperature for 5 min. Cleared lysate solutions were obtained by centrifugation, and subsequently 60 μL of BCP (1-Bromo-3-chloropropane) (Merck KGaA, Darmstadt, Germany) were added, and samples were incubated at room temperature for 15 min. After centrifugation, 300 μL of isopropanol (Merck KGaA, Darmstadt, Germany) were added and incubated at room temperature for 10 min prior centrifugation. Finally, samples were washed once with 75% ethanol and resuspended in 50 μL nuclease-free water.

After extraction, RNA quality and concentration were assessed with a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, 0.8–1 μg of RNA was used for cDNA synthesis by iScriptTM cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA). A 20 μL of reverse transcription reaction for each sample was performed according to the kit protocol. Subsequently, the cDNA was diluted (1 : 10) and used in quantitative real-time PCR (qPCR).

Quantitative real-time PCR

AMPs coding gene expression levels were assessed by qPCR performed on a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SensiMix™ SYBR® No-Rox kit (Bioline Meridian Bioscience, London, UK). Reactions were conducted in clear HardShell® Low-Profile 96-Well PCR Plates (Bio-Rad, Hercules, CA, USA) with a 50 μL mixture containing 25 μL of SYBER® Green, 0.5 μL of each primer (25 μmol/L), 5 μL of cDNA sample and 19 μL of sterile H_2O, sealed with adhesive Microseal® PCR Plate Sealing Film (Bio-Rad, Hercules, CA, USA); samples were analyzed in triplicate. An initial denaturation at 95°C for 10 min was followed by 40 cycles consisting of denaturation at 95°C for 15 s, annealing at 58.5°C for 15 s and for extension at 72°C for 15 s. A final step for melting curve analysis from 58.5°C to 95°C, measuring fluorescence every 0.5°C, was added.

Primers for defensin (Hi-DEF) and cecropin (Hi-CEC) coding genes were designed on AMP protein sequences predicted by transcriptome analysis by Vogel *et al.* (2018) (Table 1). For qPCR, actin (forward: 5′-TTCGAGCAGGAAATGGCCAC-3′ and reverse 5′-TTGGAAGAGAGCCTCTGGAC-3′) was used as reference gene (Shin & Park, 2019). Results were analyzed using the CFX Manager™ Software (Bio-Rad, Hercules, CA, USA) for Ct determination. Relative quantification of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and expressed as a fold-change.

Hemolymph inhibitory activity

Hemolymph extraction Prepupae reared on different diets were collected and maintained without food for 1 day before being washed in H_2O DEPC (2 min), in 75% ethanol (1 min) and finally rinsed in H_2O DEPC (2 min) with the aim of removing any diet residues and any other possible contaminant present in it, as previously described (Section 2.2). In order to extract the hemolymph, the protocol designed by Tabunoki *et al.* (2019) was modified and followed. Briefly, insect thorax was gently injured with a scalpel then all steps were performed maintaining samples directly on ice. Specimens were centrifuged individually by means of a refrigerated centrifuge Z 326 K® (Hermle Labortechnik GmbH, Wehingen, Germany) for 5 min at 590× *g* at 4°C. The obtained supernatant was subsequently centrifuged for 10 min at 21 380× *g* at 4°C in order to precipitate the hemocytes and any impurities previously collected. The new supernatant was collected and stored at −20°C until further analyses. Although the incision of the thorax should avoid contamination due to leaking intestinal material, prepupae were maintained without food for 1 day to empty the gut and thus ensure a greater collection of clean hemolymph.

The hemolymph was extracted from prepupae reared on GAIN and OFWSW (first trial). Moreover, the hemolymph was collected from prepupae reared on GAIN added with sunflower oil at 10, 4, 3, and 1 dbp (+SUNOIL 10 dbp, +SUNOIL 4 dbp, +SUNOIL 3 dbp, +SUNOIL 1 dbp) (third trial). For each trial and diet, 60 insects were totally collected (20 for each replicate) and the total extracted hemolymph was combined to obtain pooled samples.

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Table 1 Real-time quantitative PCR primers for defensin and cecropin encoding gene.

Primer pair	Target gene	Sequence (5′–3′)	Size (bp)	Source
Hi-DEF-F	*Defensin*	TCGTCCCATGGCAATACAAT	104	This study
Hi-DEF-R		TAGTGGAGCAGCATTATCGGG		
Hi-CEC-F	*Cecropin*	GGTCAAAGCGAAGCTGGTT	123	This study
Hi-CEC-R		TGCCAGAACATTGGCTCCTT		

Inhibitory activity assays The hemolymph inhibitory activity was tested against one Gram-negative bacterium, *Escherichia coli* DH5α, and one Gram-positive bacterium isolated from *H. illucens* (Callegari *et al.*, 2020), *Micrococcus yunnanensis* HI55 in diffusion assays in solid media. Bacteria were grown overnight in 5 mL of LB broth (Merck KGaA, Darmstadt, Germany) at 30°C in a thermostatic dome shaker VDRL 711/CT® (Asal srl, Cernusco sul Naviglio, Italy). The final concentration of the bacteria inoculum was adjusted to 5.68×10^7 CFU/mL of *E. coli* DH5α and 1.80×10^7 CFU/mL of *M. yunnanensis* HI55 using phosphate-buffered saline (PBS, 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4/KH_2PO_4, pH 7.4) (Merck KGaA, Darmstadt, Germany). Then, 50 μL of the bacteria solution were used to inoculate Petri dishes (Ø 6 cm) (Sarstedt AG & Co., Nümbrecht, Germany) containing 13 mL of LB Broth with agar (Lennox) (43% agar) (Merck KGaA, Darmstadt, Germany). Following the complete absorption of the bacteria inoculum, 10 μL of hemolymph were applied directly in the center of the agar surface. For each diet, 3 repetitions were set up. Furthermore, for each bacterium, 3 inoculated plates without hemolymph were used as no-treated control while other 3 inoculated plates, without hemolymph but added with a disc of bibulous paper (Ø 6 mm) (Biosigma SpA, Cona, Italy) soaked with 26 μL of ampicillin (50 mg/mL) placed in the center of the plate, were used as antibiotic-treated control. Plates inoculated with *E. coli* DH5α were incubated at 37°C while the ones inoculated with *M. yunnanensis* HI55 were incubated at 30°C. The hemolymph inhibitory activity was observed after 24 h, 48 h, and 7 days of incubation.

Statistical analysis

Statistical analyses were performed with SPSS Statistics 27 (IBM Corp. Released 2017, Armonk, NY, United States). All the data were subjected to logarithmic (log10) transformation for normality before statistical analysis. AMP expression levels recorded from the analysis of insect reared on OFMSW were subject to pair-wise comparison of the mean with Student's *t*-test. In the other AMP expression level analyses, data were checked for homogeneity of variance (Levene test) and normality (Kolmogorov–Smirnov test), and compared using a one-way analysis of variance (ANOVA); in the case of significant differences the means were separated by a Tukey's test. If the assumptions of ANOVA were not met, the data were compared using Kruskal–Wallis test, and the means were separated using a Mann–Whitney *U* test.

Results

Gene expression analysis

AMP encoding gene expression levels were differently modulated depending on the rearing diets and the insect developmental stage.

Organic fraction of municipals solid waste diet All the analyzed genes showed significant transcriptional differences depending on the different phase of the life cycle (Defensin: $t = -4.751$; df = 44; $P < 0.001$; Cecropin: $t = -3.593$; df = 43; $P = 0.001$). In 10-day-old larvae reared on OFMSW, both coding genes were slightly downregulated compared to insect reared on the control diet (GAIN) with a fold-change of 0.82 and 0.94, respectively (Fig. 2). An upregulation of defensin (fold-change: 10.92) and cecropin (fold-change: 9.70) was recoded in prepupae reared on OFMSW (Fig. 2).

Gainesville diet added with vegetable oils Although not statistically significant, the inclusion of vegetable oils (sunflower, corn, or soybean oil) caused a variation of defensin and cecropin coding genes expression following a different pattern according to the added oil (Fig. 3). The expression level of defensin was not influenced by the addition of the sunflower oil (fold-change: 1.02) while an upregulation of the cecropin was observed (fold-change: 1.59) compared to the control diet. The corn oil-added diet caused a slightly downregulation of both defensin (fold-change: 0.80) and cecropin (fold-change: 0.77) while the soybean oil-added diet determined an upregulation of the defensin (fold-change: 1.58) and a slightly downregulation of the cecropin (fold-change: 0.93).

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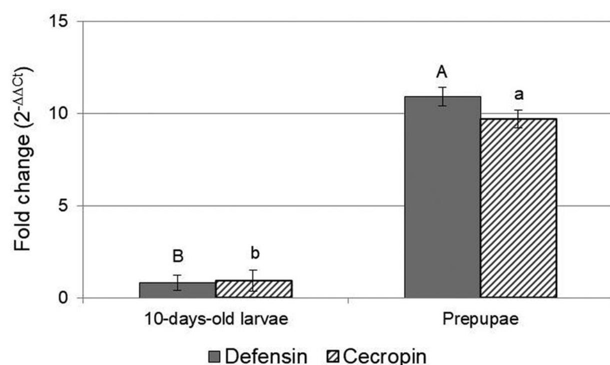

Fig. 2 Gene expression ($2^{-\Delta\Delta Ct}$) of defensin and cecropin in 10-day-old larvae and prepupae reared on OFMSW. Values are reported as average fold-change variation (mean ± SE). Samples were normalized against 10-day-old larvae or prepupae reared on GAIN respectively. Different letters indicate significantly different values (Student's *t*-test, $P < 0.05$).

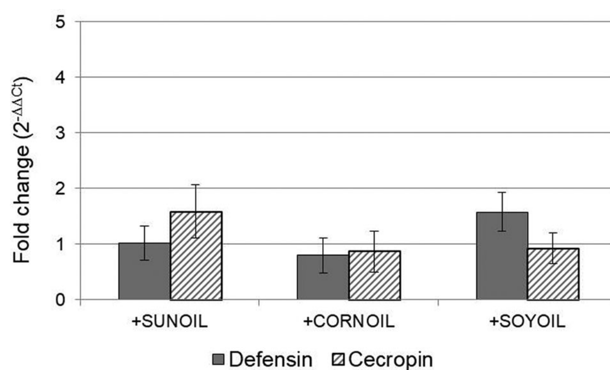

Fig. 3 Gene expression ($2^{-\Delta\Delta Ct}$) of defensin and cecropin in prepupae reared on Gainesville diet added with different vegetable oils: +SUNOIL, +CORNOIL, +SOYOIL. Values are reported as average fold-change variation (mean ± SE). Samples were normalized against prepupae reared on GAIN with no oil added.

Evaluation of different inclusion time of the sunflower oil to the Gainesville diet The inclusion of sunflower oil at different time point before insect pupation determined different expression level of both AMPs. Although significant differences were observed only for defensin ($F_{3–65} = 5.708$; $P = 0.002$), the highest expression level of both AMPs were recorded when sunflower oil was included in the rearing diet 5 dbp (fold-change defensin: 5.34; fold-change cecropin: 3.33) then, AMPs expression levels markedly decreased (Fig. 4).

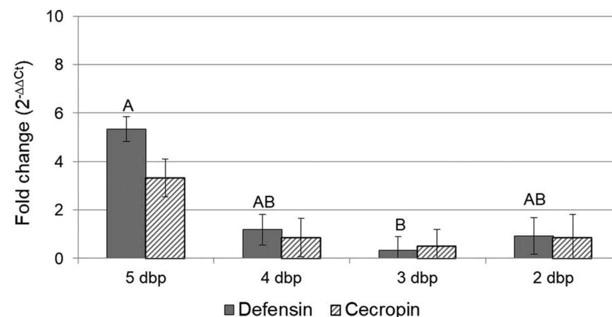

Fig. 4 Gene expression ($2^{-\Delta\Delta Ct}$) of defensin and cecropin in prepupae reared for different period on sunflower oil-added diet. Values are reported as average fold-change variation (mean ± SE). Samples were normalized against prepupae reared on GAIN with no oil added. Different letters indicate significantly different values (ANOVA, $P < 0.05$).

Evaluation of the hemolymph inhibitory activity

All hemolymph samples showed high inhibitory activity against *E. coli* DH5*α* and *M. yunnanensis* HI55 colonies.

Escherichia coli **DH5*α*** After 24 h of incubation, no colonies of *E. coli* DH5*α* developed in all samples in the area affected by the presence of hemolymph showing inhibitory activity against this bacterium (Fig. 5). The inhibitory activity was still evident in all the theses after 48 h (Fig. 6) and 7 days (Fig. 7) of incubation. Moreover, starting after 24 h of incubation, bacterial colonies deriving from the hemolymph itself were observed in all thesis. After 7 days of incubations, these bacterial colonies were more evident and spread especially in the theses treated with the hemolymph extracted form insects reared on OFMSW (Fig. 7D), +SUNOIL 10 dbp (Fig. 7E), +SUNOIL 4 dbp (Fig. 7F) and +SUNOIL 3 dbp (Fig. 7G).

Micrococcus yunnanensis HI55 After 24 h of incubation, bacterial colonies were still too small and in formation to easily appreciate the inhibitory activity of the hemolymph (Fig. 8). After 48 h of incubation, no colonies of *M. yunnanensis* HI55 developed in the area affected by the presence of the hemolymph in all the theses (Fig. 9). Moreover, a well-marked bacterial growth inhibition zone was observed in the theses treated with the hemolymph extracted form insects reared on OFMSW, +SUNOIL 10 dbp, +SUNOIL 4 dbp, and +SUNOIL 1 dbp (Fig. 9D, E, F, H). After 7 days of incubation, the hemolymph inhibitory activity was still evident in all the theses (Fig. 10).

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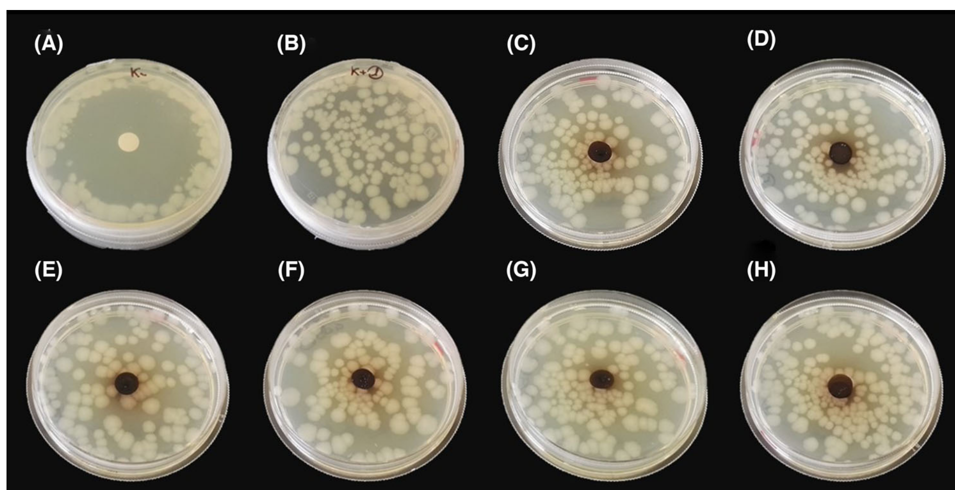

Fig. 5 Growth inhibition of *E. coli* DH5α after 24 h of incubation. Radial diffusion assay: (A) antibiotic-treated control, (B) no-treated control, (C) hemolymph of prepupae reared on GAIN, (D) hemolymph of prepupae reared on OFMSW, (E) hemolymph of prepupae reared on +SUNOIL 10 dbp, (F) hemolymph of prepupae reared on +SUNOIL 4 dbp, (G) hemolymph of prepupae reared on +SUNOIL 3 dbp, (H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

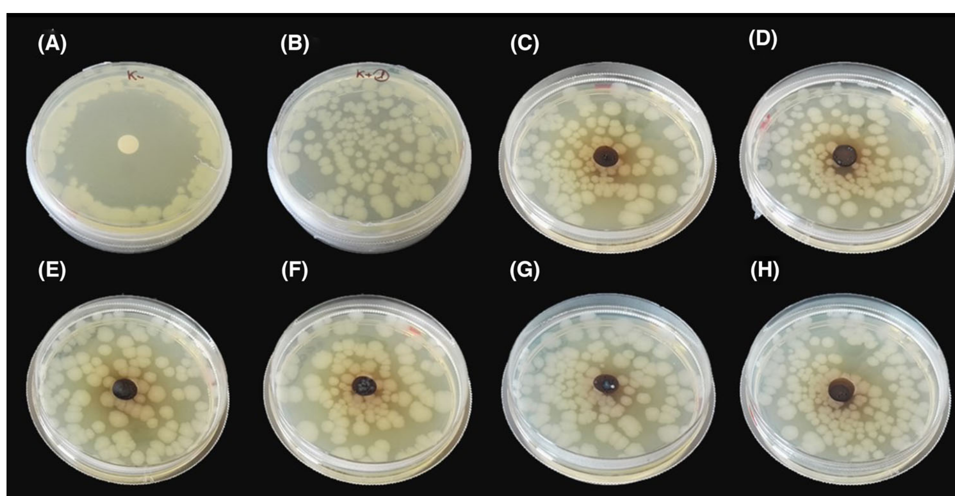

Fig. 6 Growth inhibition of *E. coli* DH5α after 48 h of incubation. Radial diffusion assay: (A) antibiotic-treated control, (B) no-treated control, (C) hemolymph of prepupae reared on GAIN, (D) hemolymph of prepupae reared on OFMSW, (E) hemolymph of prepupae reared on +SUNOIL 10 dbp, (F) hemolymph of prepupae reared on +SUNOIL 4 dbp, (G) hemolymph of prepupae reared on +SUNOIL 3 dbp, (H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

Further, after 48 h of incubation, bacterial colonies deriving from the hemolymph itself were observed in all the thesis except for the plates treated with +SUNOIL 1 dbp hemolymph (Fig. 9H). After 7 days of incubations, these bacterial colonies were more evident and spread (Fig. 10C–G) while still absent in plates treated with hemolymph collected form insect reared on +SUNOIL 1 dbp (Fig. 10H).

Discussion

Thanks to its ability to recover and valorize a wide range of organic substrates, *H. illucens* is taking a leading role in the recycling resources that are normally landfilled or that cannot find a reallocation. The use of insects to bio-convert organic waste and low-value organic by-products has become a great opportunity thanks to its

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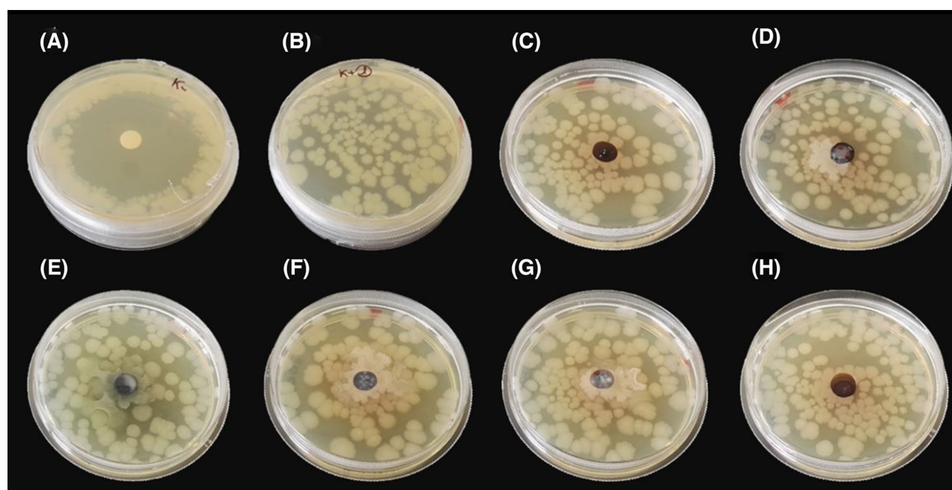

Fig. 7 Growth inhibition of *E. coli* DH5α after 7 days of incubation. Radial diffusion assay: (A) antibiotic-treated control, (B) no-treated control, (C) hemolymph of prepupae reared on GAIN, (D) hemolymph of prepupae reared on OFMSW, (E) hemolymph of prepupae reared on +SUNOIL 10 dbp, (F) hemolymph of prepupae reared on +SUNOIL 4 dbp, (G) hemolymph of prepupae reared on +SUNOIL 3 dbp, (H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

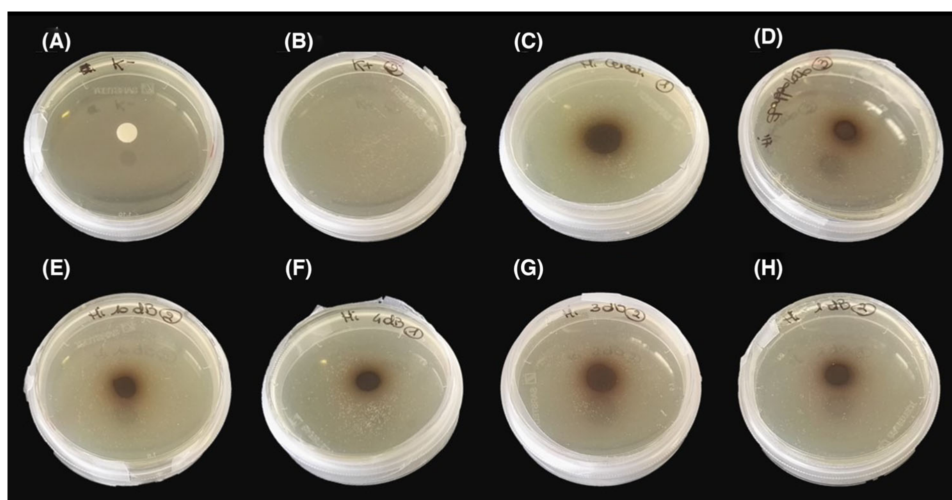

Fig. 8 Growth inhibition of *M. yunnanensis* HI55 after 24 h of incubation. Radial diffusion assay: (A) antibiotic-treated control, (B) no-treated control, (C) hemolymph of prepupae reared on GAIN, (D) hemolymph of prepupae reared on OFMSW, (E) hemolymph of prepupae reared on +SUNOIL 10 dbp, (F) hemolymph of prepupae reared on +SUNOIL 4 dbp, (G) hemolymph of prepupae reared on +SUNOIL 3 dbp, (H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

short time, low carbon footprint and high conversion rate (Gasco *et al.*, 2020; Smetana *et al.*, 2021). Nowadays however, European regulations prohibit the insect rearing on manure or waste of animal origin for food/feed purpose. Nevertheless, there is an increasing interest in the use of these substrates for nonfood uses such as the production of biodiesel and bioplastics or even for the extraction of antimicrobial peptides. Variations in the diet not only influence the insect growth rate but also affect its immune system (Lee *et al.*, 2006; Povey *et al.*, 2009; Vogel *et al.*, 2018; Cotter & Al Shareefi, 2022; Lee *et al.*, 2022). If managed appropriately, the interaction among environment, rearing diet and insect immune system could represents a valuable resource in order to enhance insect health. Beside the final use of the insect-based products,

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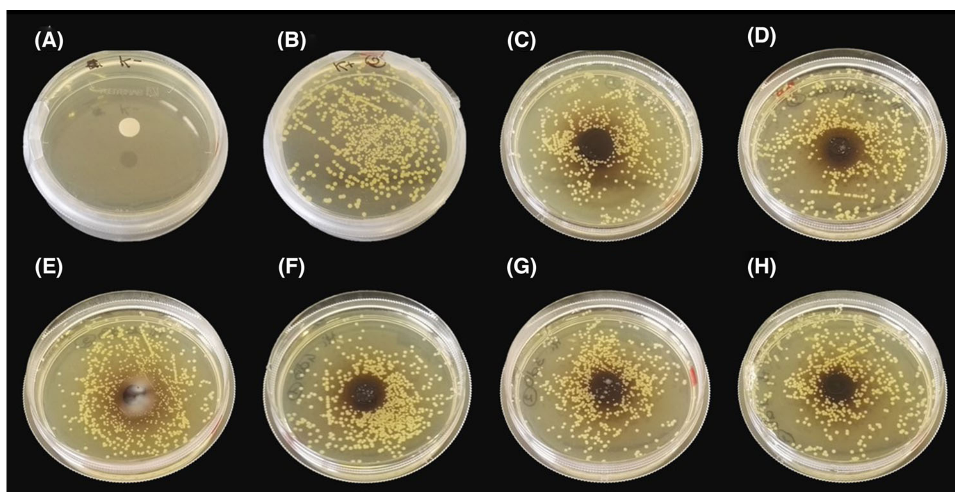

Fig. 9 Growth inhibition of *M. yunnanensis* HI55 after 48 h of incubation. Radial diffusion assay: (A) antibiotic-treated control, (B) no-treated control, (C) hemolymph of prepupae reared on GAIN, (D) hemolymph of prepupae reared on OFMSW, (E) hemolymph of prepupae reared on +SUNOIL 10 dbp, (F) hemolymph of prepupae reared on +SUNOIL 4 dbp, (G) hemolymph of prepupae reared on +SUNOIL 3 dbp, (H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

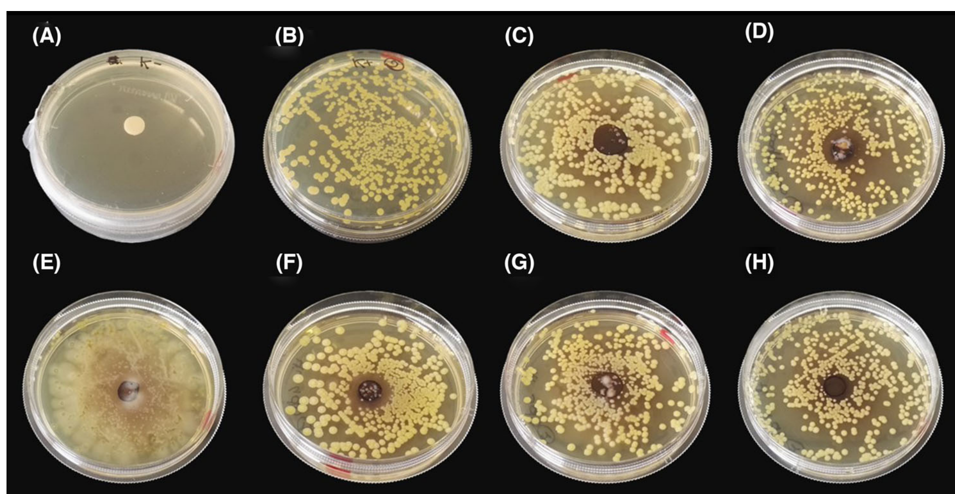

Fig. 10 Growth inhibition of *M. yunnanensis* HI55 after 7 days of incubation. Radial diffusion assay: (A) antibiotic-treated control, (B) no-treated control, (C) hemolymph of prepupae reared on GAIN, (D) hemolymph of prepupae reared on OFMSW, (E) hemolymph of prepupae reared on +SUNOIL 10 dbp, (F) hemolymph of prepupae reared on +SUNOIL 4 dbp, (G) hemolymph of prepupae reared on +SUNOIL 3 dbp, (H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

it is interesting to evaluate any possible interaction between the rearing substrate (authorized or not) and the immunological responses in order to explore future use possibilities.

The results obtained in this study confirm the relation between the rearing substrate and *H. illucens* immune responses. In our trials, the higher overexpression of both AMP encoding genes was observed in prepupae fed an OFMSW diet. We also showed how the transcription of AMPs differs during the developmental stages of *H. illucens* reared on OFMSW. Indeed, defensin and cecropin transcripts were significantly upregulated in prepupae showing 13.65 fold and 10.32 fold upper transcript levels as compared to larvae. A different AMPs expression level among the developmental stages was recorded also in other insect species. Higher AMPs expression levels

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in preupae as compared with larvae where recorded both for *T. molitor* (Dennis, 2020) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Critchlow *et al.*, 2019).

The upregulation of both AMP encoding genes observed in prepupae fed a OFMSW diet could be also related both to the higher microbial load of the substrate (Choi *et al.*, 2018; Bruno *et al.*, 2021) and to its richer nutrient composition (Vogel *et al.*, 2018) compared to the control diet. Indeed, in our trial, the OFMSW diet was used as "extreme" diet cause its presumed and inevitable high microbial load. Moreover, the diet may have positively influenced the transcription of different AMPs not investigated in our trials. Therefore, further analyses are require in order to clarify these aspects.

Particularly interesting are the results obtained adding different vegetable oils at the GAIN diet. In this case, the stimulation of immune system may be due to the vegetable oil composition and to the presence of phytosterols (Vogel *et al.*, 2018). Although the addition of the vegetable oils did not lead to a synergic and homogeneous increase in the gene expression of the AMPs considered in our trials, it contributed to upregulate of at least one of them with the exception of corn oil. In a very small number of samples (2–3) analyzed for each vegetable oil tested, 5–10 fold upper transcript levels as compared with the control diet were observed. That could be related to a nonoptimal homogenization of the oil within the substrate. Indeed, the oil addition at a later time made it difficult to mix it into the diet. Within the same diet, areas where the presence of oil could be more or less concentrated may have formed and thus resulted in a different oil ingestion by larvae, and consequentially in a different AMPs transcript modulation.

Considering the results obtained in the second trials and the results reported by Vogel *et al.* (2018) on the sunflower oil, the possible effect by the timing of the oil inclusion into the diet was further investigated for the sunflower oil only. In our trials, we showed how the timing of the oil addition plays a crucial role on the modulation of AMP transcription. The most promising results were achieved with the addition of the sunflower oil 5 dbp. In this last case, defensin and cecropin transcripts were upregulated showing a 5 fold and a 2 fold upper transcript level as compared with the oil addition at 10 dbp; longer times (10 days) were not equally effective probably due to the onset of oxidation. Shorter times could have been less effective due to the different metabolic receptivity by the more mature larvae. It has been reported that, after reaching the IV larval instar, the metabolic rate of *H. illucens* decreases as the following developmental stages (V larval instar, prepupa, and pupa) increasingly focus on building body mass and storing the energy reserves required for metamorphosis and for reproduction in the adult life stage (Schmolz & Lamprecht, 2000; Gligorescu *et al.*, 2019).

The obtained results have a remarkable and useful impact on the practical applications in insect mass rearing. Indeed, the temporal change of the diet modulation may have an impact on the insect immune system not only when vegetable oil could be integrated in the diet, but also with the use of other additives.

In our trials, we only investigated the diet-dependent expression level of two AMPs. However, it is important to highlight that the tested diets may have also influenced the transcription of other AMPs that could also be involved in hemolymph inhibitory assay.

In our trials, the hemolymph inhibitory activity was evident. The hemolymph is well-defended by hemocytes and by various soluble molecules with antimicrobial function (Blow & Douglas, 2019). These soluble effectors include AMPs, thio-ester proteins as well as the prophenoloxidase cascade products (Cerenius *et al.*, 2008; Zhang & Gallo, 2016; Shokal & Eleftherianos, 2017). In our trials, hemocytes were immediately discarded after the hemolymph collection by precipitation. Studies reported that is also possible to avoid melanization adding few crystals of phenylthiourea or ascorbic acid to the collected hemolymph (Shelby & Popham, 2006; Mak *et al.*, 2010). However, our preliminary tests showed an inhibitory activity against bacterial colonies caused by these two reagents (data not shown). In order to avoid any other possible inhibitory activity not due to the hemolymph itself we did not treat the collected hemolymph. Therefore, the inhibitory activity against bacterial colonies observed in our trials is only due to the hemolymph different humoral immune responses and to their synergistic action.

The hemolymph inhibitory activity was observed in all treatments against *E. coli* DH5α and *M. yunnanensis* HI55 colonies. The growth inhibition assays showed that the rearing diet has a significant impact on the hemolymph antimicrobial activities. A more evident inhibition was assessed against the Gram-positive bacteria. In particular, a well-marked inhibition zone was determined by the hemolymph of prepupae reared on OFMSW and on +SUNOIL 10 and 4 dbp. While in other trials the inhibition activity against Gram-negative bacteria was observed to persist only for 24 h (Choi *et al.*, 2018), in our study the hemolymph inhibitor activity against both Gram types was still present after 7 days of incubation. The results suggest that the rearing diet strongly influenced the humoral responses. Moreover, in our trial the inhibition zone was determined only by

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the diet and not following the inoculation of insects with entomopathogens as reported in other studies (Lee *et al.*, 2020b, 2020c; Park *et al.*, 2015; Lee *et al.*, 2020a). Healthy insect hemoplymph has long been considered a hostile environment for microorganisms and therefore microbiologically sterile (Lemaitre & Hoffmann, 2007). Recently, various researches overturned this conventional wisdom, and there are now evidences that various nonpathogenic microorganisms can stably or transiently inhabit hemolymph in a diversity of insects (Blow & Douglas, 2019). The diet composition and its microbiological profile may affect both the hemolymph composition and the insect immune system. Indeed, the rearing diet could firstly affect the content and/or the variety of nutrients circulating in the hemolymph; which in turn could influence (being favorable or not) some of the few microorganisms that normally reside in the hemolymph with possible repercussions on immune responses.

The hemolymph microbiota-insect host interactions, as well as the function of hemolymph microbiota, are still unclear. In our trials, we observed the growing of morphologically different bacterial colonies deriving from the hemolymph itself extracted from insects fed different substrates. The identification of these microorganisms could open new prospective in order to better understand the relation among the hemolymph microbiota, the diet and *H. illucens* immune system. Moreover, further investigations are required in order to clarify also if the hemolymph microbiota could have an active role against entomopathogens not only due to space and nutrition competitions.

Hermetia illucens as well as other insects rearings are becoming interesting for large-scale production. In this contest, a greater attention is dedicated to the nature of the rearing substrate and to optimize diets to guarantee higher quality of the specific products of interest (e.g., proteins for feed, biodiesel, nutraceuticals). However, the impact of the diet on the insect immune system is still poorly investigated by the scientific community and rarely taken into account during diet formulation in large-scale productions. Modulate the insect immune responses through the diet and, in particular, the transcription of AMPs is a valuable opportunity in order to optimize health status during mass rearing. Further investigations are required in order to find a diet that allows both the optimal insect quality production (e.g., protein, lipids) and increases insect resistance to pathogens. Moreover, considering that insect AMPs have potential applications in agriculture and pharmaceutical fields (Wu *et al.*, 2018; Azmier *et al.*, 2022) and have been also suggested to be used as food and feed additives, holdings may be interested to optimize their insect rearing diet with the purpose to modulate the AMPs production only. To date, in fact, the identification of novel antibacterial therapeutics represents an auspicious perspective and the possibility of producing new generation antimicrobials from a sustainable supply chain involving insect rearing contributes to green economy policies and reduce antibiotic resistance risks.

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Disclosure

The authors declare that they have no conflict of interest.

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