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

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Keywords:	cecropin, cereal, defensin, hemolymph, organic municipal solid waste, vegetable oil

Abstract:	Insect proteins have been proposed for human and animal food production. Ensure the rearing healthiness allows to obtain high-quality products and to avoid severe economic losses due to entomopathogens. Therefore, new strategies to safeguard 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 five days before pupation. All hemolymph samples showed high 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.

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1 **Effect of the rearing diet on gene expression of antimicrobial peptides in**

2 ***Hermetia illucens* (Diptera: Stratiomyidae)**

3 **Running title: Effect of diet on antimicrobial peptides**

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33 ## Abstract

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

54 ## Keywords:

55 Cecropin, cereal, defensin, hemolymph, organic municipal solid waste, vegetable oil.

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56 **1. Introduction**

57 Today's global food system is inadequate to meet current needs, let alone future
58 projections (Vandermeer *et al.,* 2018). According to estimates compiled by the Food and
59 Agriculture Organization (FAO), an increase of 70% in food production is required in
60 order to supply the food demand expected for 2050 (FAO, 2009). To meet the protein
61 demand, more efficient animal production, meat substitutes, and alternative protein
62 sources are required (El-Chichakli *et al.,* 2016). Insect proteins have been proposed as
63 high-quality, cost-effective, energy-efficient, and sustainable alternatives both for human
64 and animal feed (van Huis, 2020; Meyer-Rochow *et al.,* 2022; van Huis, 2022).
65 Coincidentally, some insect populations can successfully be grown on organic side
66 streams, such as organic waste or low-value organic by-products, which offer an attractive
67 approach within a circular economy (Gasco *et al,.* 2020; Jensen *et al.,* 2021).
68 Among the species of interest, *Hermetia illucens* (L.) (Diptera: Stratiomyidae), *Musca*
69 *domestica* L. (Diptera: Muscidae), and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae)
70 have the highest potential for large-scale production (van Huis, 2020). It has been reported
71 that the insect growth rates, chemical compositions, and their nutritional quality largely
72 depend on the substrate used for the insect rearing (Harsányi *et al.,* 2020; Hopkins *et al.,*
73 2021). To date, researchers have focused their investigations on the effects of growth
74 substrate on insects' nutrient composition (Barragan-Fonseca *et al.,* 2021; Fuso *et al.,*
75 2021) and on substrate reduction efficiency, bio- and feed conversion rates (Ravi *et al.,*
76 2020; Parodi *et al.,* 2021; Veldkamp *et al.,* 2021). Moreover, great attention has been
77 addressed on the qualitative/quantitative response of aqua culture and livestock animals
78 fed insects-derived products (Gariglio *et al.,* 2019; Benzertiha *et al.,* 2020; Shariat Zadeh
79 *et al.,* 2020; Bellezza Oddon *et al.,* 2022; Elahi *et al.,* 2022; Hong & Kim, 2022; Mohan

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80 *et al.,* 2022; Tran *et al.,* 2022). Nonetheless, little exists on the impact of rearing substrates
81 on the insect immunological response.
82 The ability of insects to feed successfully on nutritionally-unpredictable diets and/or those
83 with high levels of bacterial contamination may lie in immune system adaptations
84 (Vilcinskas, 2013). Humoral and cellular defences produce insect immune responses
85 (Lavine & Strand, 2002; Kanost *et al.,* 2004). Insect antimicrobial peptides/proteins
86 (AMPs), produced in different organs and tissues, are key components for their humoral
87 response (Tsakas & Marmaras, 2010), and may possess antibacterial, antiviral, and
88 antifungal activity (Levy *et al.,* 2004). Even though AMPs have been characterised in
89 different insect species (Chae *et al.,* 2012, Li *et al.,* 2014; Elhag *et al.,* 2017), much
90 remains to be understood about how to modulate their expression to improve the insect
91 immune system. Stimulating the insect immune system through the diet could make a
92 remarkable difference in insect mass rearing. Indeed, triggering the immune system could
93 not only preserve healthy rearing conditions, but also increase insect "tolerance" to
94 entomopathogens. Moreover, any reduction or avoidance of antibiotic use is beneficial in
95 light of rising global resistance if the final use of the insect as for feed or food.
96 Our study builds on work that showed the impact on AMP expression from rearing diets
97 containing high microbial loads, supplemented with cellulose, chitin, lignin, brewer's
98 grains, protein, sunflower oil (Vogel *et al.,* 2018). Our aim was to investigate the diet-
99 dependent expression of two genes coding for AMPs (one defensin and one cecropin)
100 throughout the entire body of *H. illucens* 10-day-old larvae and prepupae reared on
101 different diets. The inhibitory activity of the hemolymph, extracted from prepupae reared
102 on the different diets, was also evaluated against one Gram-negative and one Gram-
103 positive bacterium.

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104 ## 2. Material and methods

105 ### 2.1 *Insect rearing*

106 *Hermetia illucens* was reared at the experimental facility of the Department of
107 Agricultural, Forest and Food Sciences (DISAFA; University of Torino, Carmagnola,
108 Italy). For mating, adults were maintained in cages (1×1×2 m) in a climate-controlled
109 chamber equipped with SPR AGTECH Black Soldier Fly Breeding LED 150W (EVO
110 Conversion Systems, LLC, College Station, TX, USA) at 27±1°C, 70±5% RH, with a
111 14:10h L:D photoperiod. Eggs were collected using a sticky-wood egg trap (Julita *et al.,*
112 2021) and with the help of a fine brush. Groups of eggs (1 g each) were positioned on a
113 net placed above plastic containers (10×17.5×7 cm) filled with 400 g of a Gainesville diet
114 (30% alfalfa, 50% wheat bran, 20% cornmeal, and a 70% moisture content; Tomberlin *et*
115 *al.,* 2002). After six-days, all larvae were sieved, divided into groups of 10 larvae, and
116 then weighed.

117 Three different growing trials were performed using 4 replicates of 300 larvae each. In
118 all trials, six-day-old larvae were reared in a plastic container (10×17.5×7 cm) and fed
119 with 400 g of the different tested diets in a climate-controlled chamber (T: 27°C; RH:
120 70%, 14:10h L:D photoperiod). In the first, two different diets were used: the Gainseville
121 diet (GAIN) and a diet composed of chopped organic fraction of municipals solid waste
122 (80% moisture content) (OFMSW).

123 For the second trial, three different vegetable oils (5% of the diet humid weight) were
124 individually added to the Gainesville diet 10 days before pupation (dbp). A total of four
125 different diets were prepared: i) Gainesville diet (GAIN) as the control, ii) Gainesville
126 diet added with sunflower oil (+SUNOIL), iii) Gainesville diet added with corn oil
127 (+CORNOIL), and iv) = Gainesville diet added with soybean oil (+SOYOIL).

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128 Finally, to assess if AMP encoded gene expression levels were affected by the timing of
129 the addition of oil into the rearing diet, another trial was conducted in which sunflower
130 oil was added to the Gainesville diet at 5, 4, 3, and 2 dbp. Again, the GAIN served as the
131 control diet. Groups of six-day-old larvae were partitioned into five allotments [one for
132 each time of oil inclusion (5, 4, 3 and 2 dbp) and one for the control diet] and reared on a
133 GAIN diet. Then, the sunflower oil (5% of the diet humid weight) was added at the
134 different time points. All experiments were stopped when 40% of the larvae reached the
135 prepupal stage.

136 **2.2 *Gene expression analysis***

137 For the gene expression analysis, at the end of the first trial, an average of 25 (18-26)
138 larvae (10-day-old) and 20 prepupae were collected, while at the end of the second trial,
139 20-25 prepupae were collected and used. In the third trial, for each treatment (GAIN,
140 +SUNOIL 5 dbp, +SUNOIL 4 dbp, +SUNOIL 3 dbp, +SUNOIL 2 dbp), an average of
141 17 (13-21) prepupae were used.

142 Larvae and pupae were sieved and washed in diethylpyrocarbonate (DEPC) water [Merck
143 KGaA, Darmstadt, Germany], 75% ethanol in DEPC water, and DEPC water for 2, 1 and
144 2 min, respectively in order to remove any diet residues. Insects were dried on filter paper
145 and frozen at -80°C until further analysis.

146 **2.2.1 *RNA isolation***

147 Total RNA extraction was performed following the TRI Reagent® protocol [Merck
148 KGaA, Darmstadt, Germany], according to the supplier's suggestions. Briefly, insects
149 were grounded to a fine powder under liquid nitrogen and lysed in 600 µl of TRI
150 Reagent®; then samples were incubated at room temperature for 5 min. Cleared lysate
151 solutions were obtained by centrifugation, and subsequently 60 µl of BCP (1-Bromo-3-

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152 chloropropane) [Merck KGaA, Darmstadt, Germany] were added, and samples were
153 incubated at room temperature for 15 min. After centrifugation, 300 µl of isopropanol
154 [Merck KGaA, Darmstadt, Germany] were added and incubated at room temperature for
155 10 min prior centrifugation. Finally, samples were washed once with 75% ethanol and
156 resuspended in 50 µl nuclease-free water.
157 After extraction, RNA quality and concentration were assessed with a ND-1000
158 spectrophotometer [NanoDrop Technologies, Wilmington, DE, USA]. First-strand cDNA
159 was synthesized using the iScriptTM cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA)
160 following the manufacturer's instructions.

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161 **2.2.2 *Quantitative real-time PCR***

162 AMPs coding gene expression levels were assessed by quantitative real-time PCR (RT-
163 qPCR) performed on a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad,
164 Hercules, CA, USA) using the SensiMix™ SYBR® No-Rox kit [Bioline Meridian
165 Bioscience, London, UK]. Reactions were conducted in clear HardShell® Low-Profile
166 96-Well PCR Plates (Bio-Rad, Hercules, CA, USA) with a 50 µl mixture containing 25
167 µl of SYBER® Green, 0.5 µl of each primer (25 µM), 5 µL of cDNA sample and 19 µl of
168 sterile H_2O, sealed with adhesive Microseal® PCR Plate Sealing Film (Bio-Rad, Hercules,
169 CA, USA); samples were analysed in triplicate. An initial denaturation at 95°C for 10 min
170 was followed by 40 cycles consisting of denaturation at 95°C for 15 sec, annealing at
171 58.5°C for 15 sec and for extension at 72°C for 15 sec. A final step for melting curve
172 analysis from 58.5 to 95°C, measuring fluorescence every 0.5°C, was added.
173 Primers for defensin (Hi-DEF) and cecropin (Hi-CEC) coding genes were designed on
174 AMP protein sequences predicted by transcriptome analysis by Vogel *et al.* (2018) (Table
175 1). For RT-qPCR, actin (Forward: 5′-TTCGAGCAGGAAATGGCCAC-3′ and Reverse

176 5′-TTGGAAGAGAGCCTCTGGAC-3′) was used as reference gene (Shin & Park, 2019).
177 Results were analysed using the CFX Manager™ Software (Bio-Rad, Hercules, CA,
178 USA) for Ct determination. Relative quantification of target genes was calculated using
179 the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and expressed as a fold change.

180 **2.3 *Hemolymph inhibitory activity***

181 **2.3.1 *Hemolymph extraction***

182 *Hermetia illucens* reared on different diets were collected and maintained without food
183 for one day before being washed in H_2O DEPC (2 min), in 75% ethanol (1 min) and
184 finally rinsed in H_2O DEPC (2 min). In order to extract the hemolymph, insect thorax was
185 gently injured with a scalpel and specimens were centrifuged individually by means of a
186 refrigerated centrifuge Z 326 K® [Hermle Labortechnik GmbH, Wehingen, Germany] for
187 5 min at 2,500 rpm at 4°C. The obtained supernatant was subsequently centrifuged for 10
188 min at 15,000 rpm at 4°C in order to precipitate the hemocytes and any impurities
189 previously collected. The new supernatant was collected and stored at -20°C until further
190 analyses. The hemolymph was extracted from prepupae reared on GAIN and OFWSW.
191 Moreover, the hemolymph was collected from prepupae reared on GAIN added with
192 sunflower oil at 10, 4, 3 and 1 dbp (+SUNOIL 10 dbp, +SUNOIL 4 dbp, +SUNOIL 3
193 dbp, +SUNOIL 1 dbp).

194 **2.3.2 *Inhibitory activity assays***

195 The hemolymph inhibitory activity was tested against one Gram-negative bacterium,
196 *Escherichia coli* DH5α, and one Gram-positive bacterium isolated from *H. illucens*
197 (Callegari *et al.,* 2020), *Micrococcus yunnanensis* HI55 in diffusion assays in solid media.
198 Bacteria were grown overnight in 5 ml of LB broth [Merck KGaA, Darmstadt, Germany]

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199 at 30°C in a thermostatic dome shaker VDRL 711/CT® [Asal srl, Cernusco sul Naviglio,
200 Italy]. The final concentration of the bacteria inoculum was adjusted to 5.68×10^{7} CFU
201 ml^{-1} of *E. coli* DH5α and 1.80×10^{7} CFU ml^{-1} of *M. yunnanensis* HI55 using phosphate-
202 buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4/KH2PO4, pH 7.4)
203 [Merck KGaA, Darmstadt, Germany]. Then, 50 µl of the bacteria solution were used to
204 inoculate Petri dishes (Ø 6 cm) [Sarstedt AG & Co., Nümbrecht, Germany] containing
205 13 ml of LBA media [Merck KGaA, Darmstadt, Germany]. Following the complete
206 absorption of the bacteria inoculum, 10 µl of hemolymph were released onto the agar
207 plates centre. For each diet, 3 repetitions were set up. Furthermore, for each bacterium, 3
208 inoculated plates without hemolymph were used as no-treated control while other 3
209 inoculated plates, without hemolymph but added with 26 µl of ampicillin on a disc of
210 bibulous paper (Ø 6 mm) [Biosigma SpA, Cona, Italy] placed in the centre of the plate,
211 were used as antibiotic-treated control. Plates inoculated with *E. coli* DH5α were
212 incubated at 37°C while the ones inoculated with *M. yunnanensis* HI55 were incubated
213 at 30°C. The hemolymph inhibitory activity was observed after 24h, 48h and 7 days of
214 incubation.

215 **2.4 *Statistical Analysis***

216 Statistical analyses were performed with SPSS Statistics 27 (IBM Corp. Released 2017,
217 Armonk, NY, United States). All the data were subjected to logarithmic (log10)
218 transformation for normality before statistical analysis. AMP expression levels recorded
219 from the analysis of insect reared on OFMSW were subject to pair-wise comparison of
220 the mean with Student's t-test. In the other AMP expression level analyses, data were
221 checked for homogeneity of variance (Levene test) and normality (Kolmogorov-Smirnov
222 test), and compared using a one-way analysis of variance (ANOVA); in the case of

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223 significant differences the means were separated by a Tukey's test. If the assumptions of
224 ANOVA were not met, the data were compared using Kruskal-Wallis test, and the means
225 were separated using a Mann-Whitney U test.

226 ## 3. Results

227 ### 3.1 *Gene expression analysis*

228 Different AMP encoding gene expression levels were recorded depending to the used
229 rearing diet.

230 #### 3.1.1 *Organic fraction of municipals solid waste diet*

231 All the analysed genes showed significant transcriptional differences depending on the
232 different phase of the life cycle (Defensin: t = -4.751; df= 44; p < 0.001; Cecropin: t = -
233 3.593; df= 43; p = 0.001). In 10-day-old larvae reared on OFMSW, both coding genes
234 were slightly down-regulated compared to insect reared on the control diet (GAIN) with
235 a fold change of 0.82 and 0.94 respectively (Figure 1). An up-regulation of defensin (fold
236 change: 10.92) and cecropin (fold change: 9,70) was recoded in prepupae reared on
237 OFMSW (Figure 1).

238 #### 3.1.2 *Gainesville diet added with vegetable oils*

239 Although not statistically significant, the inclusion of vegetable oils (sunflower, corn or
240 soybean oil) caused a variation of defensin and cecropin coding genes expression
241 following a different pattern according to the added oil (Figure 2). The expression level
242 of defensin was not influenced by the addition of the sunflower oil (fold change: 1.02)
243 while an up-regulation of the cecropin was observed (fold change:1.59) compared to the
244 control diet. The corn oil-added diet caused a slightly down-regulation of both defensin

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245 (fold change: 0.80) and cecropin (fold change: 0.77) while the soybean oil-added diet
246 determined an up-regulation of the defensin (fold change: 1.58) and a slightly down-
247 regulation of the cecropin (fold change: 0.93).

248 **3.1.3 *Evaluation of different inclusion time of the sunflower oil to the Gainesville diet***

249 The inclusion of sunflower oil at different time point before insect pupation determined
250 different expression level of both AMPs. Although significant differences were observed
251 only for defensin ($F_{3\text{-}65} = 5.708$; $p = 0.002$), the highest expression level of both AMPs
252 were recorded when sunflower oil was included in the rearing diet 5 dbp (fold change
253 defensin: 5.34; fold change cecropin: 3.33) then, AMPs expression levels markedly
254 decreased (Figure 3).

255 **3.2 *Evaluation of the hemolymph inhibitory activity***

256 All hemolymph samples showed high inhibitory activity against *E. coli* DH5α and *M.*
257 *yunnanensis* HI55 colonies.

258 **3.2.1 Escherichia coli *DH5α***

259 After 24h of incubation, no colonies of *E. coli* DH5α developed in all samples in the area
260 affected by the presence of hemolymph showing a high inhibitory activity against this
261 bacterium (Figure 4). The inhibitory activity was still strongly evident in all the theses
262 after 48h (Figure 5) and 7 days (Figure 6) of incubation. Moreover, starting after 24h of
263 incubation, bacterial colonies deriving from the hemolymph itself were observed in all
264 the thesis. After 7 days of incubations, these bacterial colonies were more evident and
265 spread especially in the theses treated with the hemolymph extracted form insects reared
266 on OFMSW (Figure 6D), +SUNOIL 10 dbp (Figure 6E), +SUNOIL 4 dbp (Figure 6F)
267 and +SUNOIL 3 dbp (Figure 6G).

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268 **3.2.2 Micrococcus yunnanensis *HI55***

269 All hemolymph samples showed high inhibitory activity against *M. yunnanensis* HI55.
270 After 24h of incubation, bacterial colonies were still too small and in formation to easily
271 appreciate the inhibitory activity of the hemolymph (Figure 7). After 48h of incubation,
272 no colonies of *M. yunnanensis* HI55 developed in the area affected by the presence of the
273 hemolymph in all the theses (Figure 8). Moreover, a well-marked bacterial growth
274 inhibition zone was observed in the theses treated with the hemolymph extracted form
275 insects reared on OFMSW, +SUNOIL 10 dbp, +SUNOIL 4 dbp and +SUNOIL 1 dbp
276 (Figure 8D, E, F, H). After 7 days of incubation, the hemolymph inhibitory activity was
277 still strongly evident in all the theses (Figure 9).
278 Further, after 48h of incubation, bacterial colonies deriving from the hemolymph itself
279 were observed in all the thesis except for the plates treated with +SUNOIL 1 dbp
280 hemolymph (Figure 8H). After 7 days of incubations, these bacterial colonies were more
281 evident and spread (Figure 9C-G) while still absent in plates treated with hemolymph
282 collected form insect reared on +SUNOIL 1 dbp (Figure 9H).

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283 ## 4. Discussion

284 Thanks to its ability to recover and valorise a wide range of organic substrates, *H. illucens*
285 is taking a leading role in the recycling resources that are normally landfilled or that
286 cannot find a reallocation. The use of insects to bio-convert organic waste and low-value
287 organic by-products has become a great opportunity thanks to its short time, low carbon
288 footprint, high conversion rate (Gasco *et al.,* 2020; Smetana *et al.,* 2021). Nowadays
289 however, European regulations prohibit the insect rearing on manure or waste of animal
290 origin for food/feed purpose. Nevertheless, there is an increasing interest in the use of
291 these substrates for non-food uses such as the production of biodiesel and bioplastics or
292 even for the extraction of antimicrobial peptides. Variations in the diet not only influence
293 the insect growth rate but also affect its immune system (Lee *et al.,* 2006; Povey *et al.,*
294 2009; Vogel *et al.,* 2018; Cotter & Al Shareefi, 2022; Lee *et al.,* 2022). If managed
295 appropriately, the interaction among environment, rearing diet and insect immune system
296 could represents a valuable resource in order to enhance the insect healthiness. Beside the
297 final use of the insect-based products, it is interesting to evaluate any possible interaction
298 between the rearing substrate (authorized or not) and the immunological responses in
299 order to explore future use possibilities.

300 The results obtained in this study confirm the relation between the rearing substrate and
301 *H. illucens* immune responses. In our trials, the higher overexpression of both AMP
302 encoding genes was observed in prepupae fed a OFMSW diet. We also showed how the
303 transcription of AMPs differs during the developmental stages of *H. illucens* reared on
304 OFMSW. Indeed, defensin and cecropin transcripts were significantly up-regulated in
305 prepupae showing 13.65-fold and 10.32-fold upper transcript levels as compared to
306 larvae. Cause larvae remained on the substrate for only 4 days, this period could not be

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307 sufficient to influence the insect immune system. The up-regulation of both AMP
308 encoding genes observed in prepupae fed a OFMSW diet could be also related both to the
309 higher microbial load of the substrate (Choi *et al.,* 2018; Bruno *et al.,* 2021) and to its
310 richer nutrient composition (Vogel *et al.,* 2018) compared to the control diet. Moreover,
311 the diet may have positively influenced the transcription of different AMPs not
312 investigated in our trials.
313 Particular interesting are the results obtained adding different vegetable oils at the GAIN
314 diet. In this case, the stimulation of immune system may be due to the vegetable oil
315 composition and at the presence of phytosterols (Vogel *et al.,* 2018). Although the
316 addition of the vegetable oils did not lead to a synergic and homogeneous increase in the
317 gene expression of the AMPs considered in our trials, it contributed to upregulate of at
318 least one of them with the exception of corn oil. A wide range of expression level was
319 recorded in all samples fed a vegetable oil-added diet. That could be related to a non-
320 optimal homogenization of the oil within the substrate which may have resulted in a
321 different oil ingestion by larvae.
322 Moreover, we showed how the timing of the oil addition could play a crucial role on the
323 modulation of AMP transcriptions. In our trials, the most promising results were achieved
324 with the addition of the sunflower oil 5 dbp. Longer times (10 days) were not equally
325 effective probably due to the onset of oxidation. Shorter times could have been less
326 effective due to the different metabolic receptivity by the more mature larvae. Indeed,
327 mature larvae, following the achievement of the optimal mass to continue the biological
328 cycle, present a slowed metabolism as already observed by Gligorescu *et al.* (2019). In
329 our trials, we only investigated the diet-dependent expression level of two AMPs.

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330 However, it is important to highlight that the tested diets may have also influenced the
331 transcription of other AMPs that could also be involved in hemolymph inhibitory assay.
332 In our trials, the hemolymph inhibitory activity was strongly evident. The hemolymph is
333 well-defended by hemocytes and by various soluble molecules with anti-microbial
334 function (Blow *et al.,* 2019). These soluble effectors include AMPs, thio-ester proteins as
335 well as the prophenoloxidase cascade products (Cerenius *et al.,* 2008; Zhang & Gallo,
336 2016; Shokal & Eleftherianos, 2017). In our trials, hemocytes were immediately
337 discarded after the hemolymph collection by precipitation. Studies reported that is also
338 possible avoid melanisation adding few crystals of phenylthiourea or ascorbic acid to the
339 collected hemolymph (Shelby & Popham, 2006; Mak *et al.,* 2010). However, our
340 preliminary tests showed an inhibitory activity against bacterial colonies caused by these
341 two reagents (data not shown). In order to avoid any other possible inhibitory activity not
342 due to the hemolymph itself we did not treat the collected hemolymph. Therefore, the
343 inhibitory activity against bacterial colonies observed in our trials is only due to the
344 hemolymph different humoral immune responses and to their synergistic action.
345 The hemolymph inhibitory activity was observed in all treatments against *E. coli* DH5α
346 and *M. yunnanensis* HI55 colonies. The growth inhibition assays showed that the rearing
347 diet has a significant impact on the hemolymph antimicrobial activities. A higher
348 inhibition was assessed against the Gram-positive bacteria. In particular, a well-marked
349 inhibition zone was observed with the hemolymph of prepupae reared on OFMSW and
350 on +SUNOIL 10 and 4 dbp. While in other trials the inhibition activity against Gram-
351 negative bacteria was observed to persist only for 24 h (Choi *et al.,* 2018), in our study
352 the hemolymph inhibitor activity against both Gram types was still present after 7 days
353 of incubation. The results suggest that the rearing diet strongly influenced the humoral

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354 responses. Moreover, in our trial the inhibition zone was determinate only by the diet and
355 not following the immunization of insects with pathogens as reported in other studies
356 (Park *et al.,* 2015; Lee *et al.,* 2020a, 2020b and 2020c).
357 Healthy insect hemoplymph has long been considered a hostile environment for
358 microorganisms and therefore microbiologically sterile (Lemaitre & Hoffmann, 2007).
359 Recently, various researches overturned this conventional wisdom, and there are now
360 evidences that various non-pathogenic microorganisms can stably or transiently inhabit
361 hemolymph in a diversity of insects (Blow *et al.,* 2019). However, hemolymph
362 microbiota-insect host interactions, as well as the function of hemolymph microbiota, are
363 still unclear. In our trials, we observed the growing of different bacterial colonies deriving
364 from the hemolymph itself. The diet composition and the substrate quality enhance the
365 immune system, but they could also affect the hemolymph microbiota composition.
366 Indeed, in our trials, morphologically different colonies were observed in the hemolymph
367 extracted from insects fed different substrates. The identification of these microorganisms
368 could open new prospective in order to better understand the relation between the
369 hemolymph microbiota, the diet and the *H. illucens* immune system. Moreover, further
370 investigations are required in order to clarify also if the hemolymph microbiota could
371 have an active role against entomopathogens not only due to space and nutrition
372 competitions.
373 *Hermetia illucens* as well as other insects rearing are becoming interesting for large-scale
374 production. In this contest, a greater attention is dedicated to the nature of the rearing
375 substrate and to optimise diets for ensure higher quality of the final products and cheapest
376 mass production. However, the impact of the diet on the insect immune system is rarely
377 taken in to account during diet formulation. Enhance the insect immune responses

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378 through the diet and in particular the transcription of AMPs is a valuable opportunity.
379 This allows to increase insect resistance pathogens and optimize health status during mass
380 rearing. Moreover, insect AMPs have potential applications in agriculture and
381 pharmaceutical fields (Wu *et al.,* 2018; Azmier *et al.,* 2022) and have been also suggested
382 to be used as food and feed additives. Today, the identification of novel antibacterial
383 therapeutics represents an auspicious perspective and the possibility of producing new
384 generation antimicrobials from a sustainable supply chain involving insect rearing
385 contributes to green economy policies and reduce antibiotic resistance risks.

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390 **6. Disclosure**

391 The authors declare that they have no conflict of interest.

392 **7. References**

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8. Tables

562 **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		

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565 ## 9. Figure legends

566 **Figure 1.** Gene expression ($2^{-\Delta\Delta Ct}$) of defensin and cecropin in 10-day-old larvae and prepupae reared on
567 OFMSW. Values are reported as average fold change variation (mean±SE). Samples were normalized
568 against 10-day-old larvae or prepupae reared on GAIN respectively. Different letters indicate significantly
569 different values (Student's t-test, $p < 0.05$).

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

574 **Figure 3.** Gene expression ($2^{-\Delta\Delta Ct}$) of defensin and cecropin in prepupae reared for different period on
575 sunflower oil-added diet. Values are reported as average fold change variation (mean±SE). Samples were
576 normalized against prepupae reared on GAIN with no oil added. Different letters indicate significantly
577 different values (ANOVA, $p < 0.05$).

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

583 **Figure 5.** Growth inhibition of *E. coli* DH5α after 48h of incubation. Radial diffusion assay: A) antibiotic-
584 treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D) hemolymph of
585 prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F) hemolymph of
586 prepupae reared on +SUNOIL 4 dbp, G) hemolymph of prepupae reared on +SUNOIL 3 dbp, H)
587 hemolymph of prepupae reared on +SUNOIL 1 dbp.

588 **Figure 6.** Growth inhibition of *E. coli* DH5α after 7 days of incubation. Radial diffusion assay: A)
589 antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D)
590 hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F)

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591 hemolymph of prepupae reared on +SUNOIL 4 dbp, G) hemolymph of prepupae reared on +SUNOIL 3
592 dbp, H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

593 **Figure 7.** Growth inhibition of *M. yunnanensis* HI55 after 24h of incubation. Radial diffusion assay: A)
594 antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D)
595 hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F)
596 hemolymph of prepupae reared on +SUNOIL 4 dbp, G) hemolymph of prepupae reared on +SUNOIL 3
597 dbp, H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

598 **Figure 8.** Growth inhibition of *M. yunnanensis* HI55 after 48h of incubation. Radial diffusion assay: A)
599 antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D)
600 hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F)
601 hemolymph of prepupae reared on +SUNOIL 4 dbp, G) hemolymph of prepupae reared on +SUNOIL 3
602 dbp, H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

603 **Figure 9.** Growth inhibition of *M. yunnanensis* HI55 after 7 days of incubation. Radial diffusion assay: A)
604 antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D)
605 hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F)
606 hemolymph of prepupae reared on +SUNOIL 4 dbp, G) hemolymph of prepupae reared on +SUNOIL 3
607 dbp, H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

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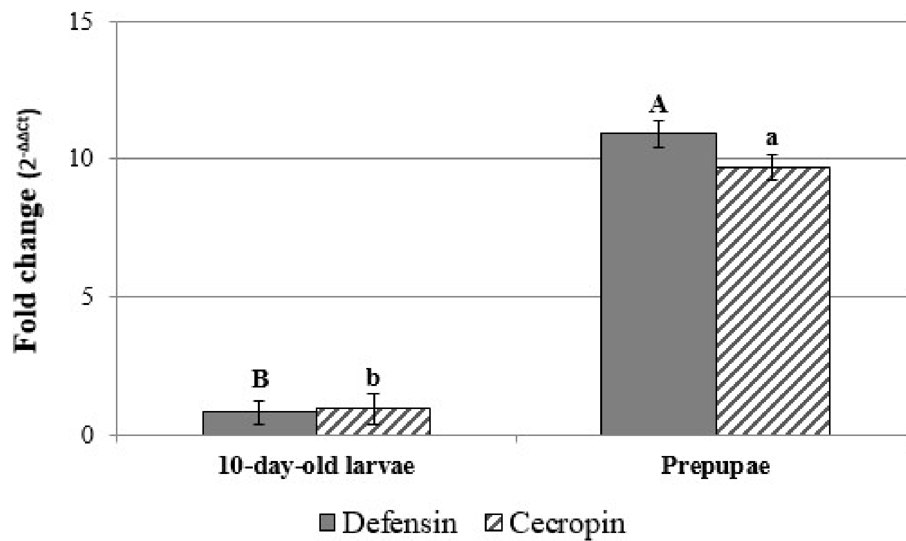

Figure 1. Gene expression (2-ΔΔCt) of defensin and cecropin in 10-day-old larvae and prepupae reared on OFMSW. Values are reported as average fold change variation (mean±SE). Samples were normalized against 10-day-old larvae or prepupae reared on GAIN respectively. Different letters indicate significantly different values (Student's t-test, $p < 0.05$).

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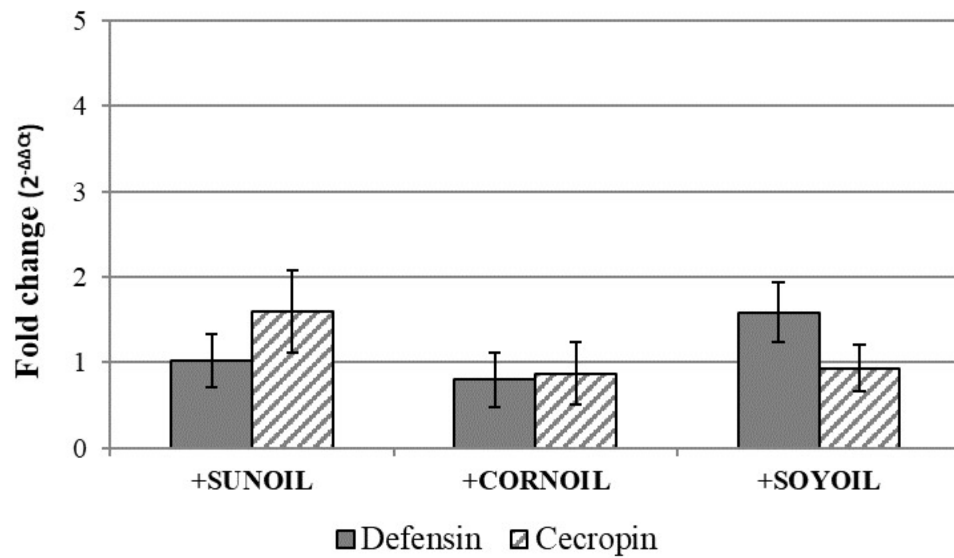

Figure 2. Gene expression (2-ΔΔCt) of defensin and cecropin in prepupae reared on Gainesville diet added with different vegetable oils: +SUNOIL, +CORNOIL, +SOYOIL. Values are reported as average fold change variation (mean±SE). Samples were normalized against prepupae reared on GAIN with no oil added.

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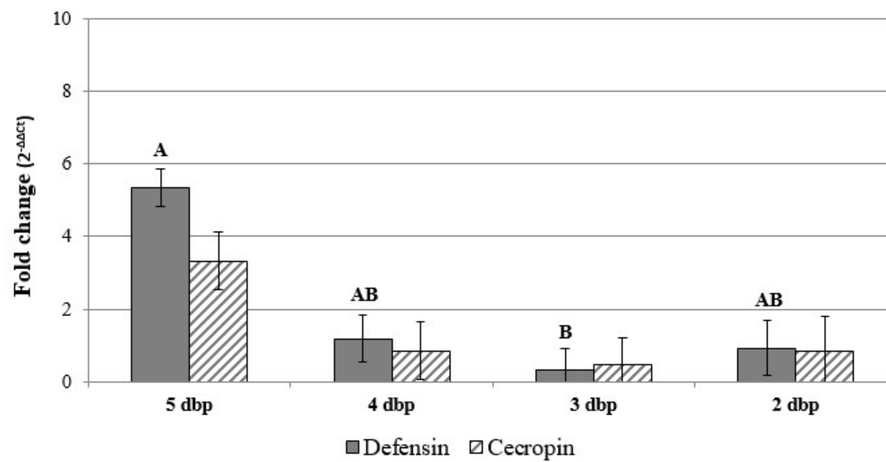

Figure 3. Gene expression (2-ΔΔCt) of defensin and cecropin in prepupae reared for different period on sunflower oil-added diet. Values are reported as average fold change variation (mean±SE). Samples were normalized against prepupae reared on GAIN with no oil added. Different letters indicate significantly different values (ANOVA, $p < 0.05$).

196x99mm (96 x 96 DPI)

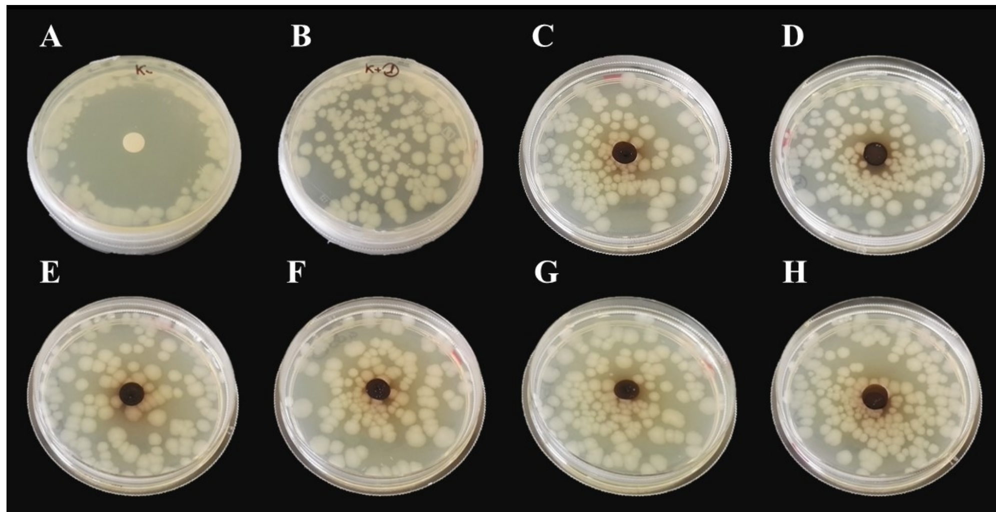

Figure 4. Growth inhibition of *E. coli* DH5α after 24h of incubation. Radial diffusion assay: A) antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D) hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F) hemolymph of prepupae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

198x100mm (150 x 150 DPI)

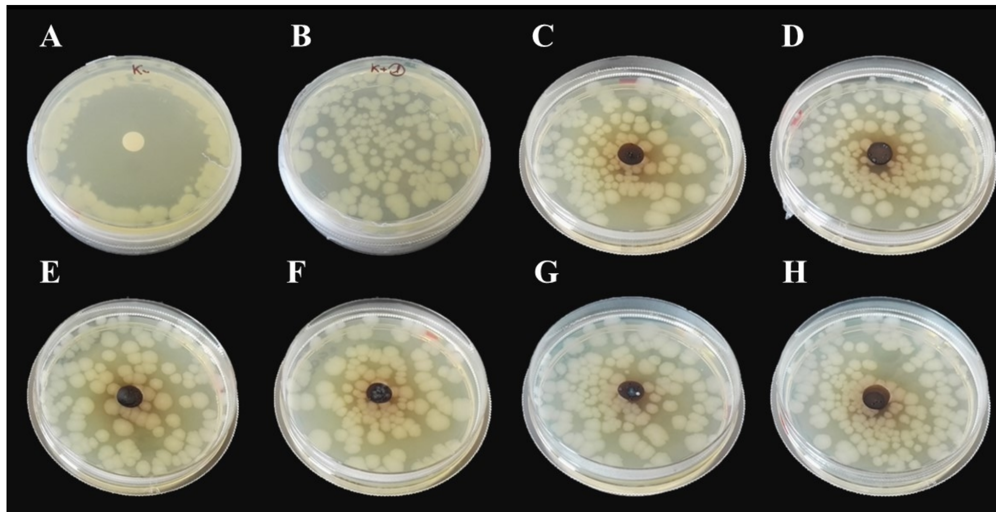

Figure 5. Growth inhibition of *E. coli* DH5α after 48h of incubation. Radial diffusion assay: A) antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D) hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F) hemolymph of prepupae reared on +SUNOIL 4 dbp, G) hemolymph of prepupae reared on +SUNOIL 3 dbp, H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

198x100mm (150 x 150 DPI)

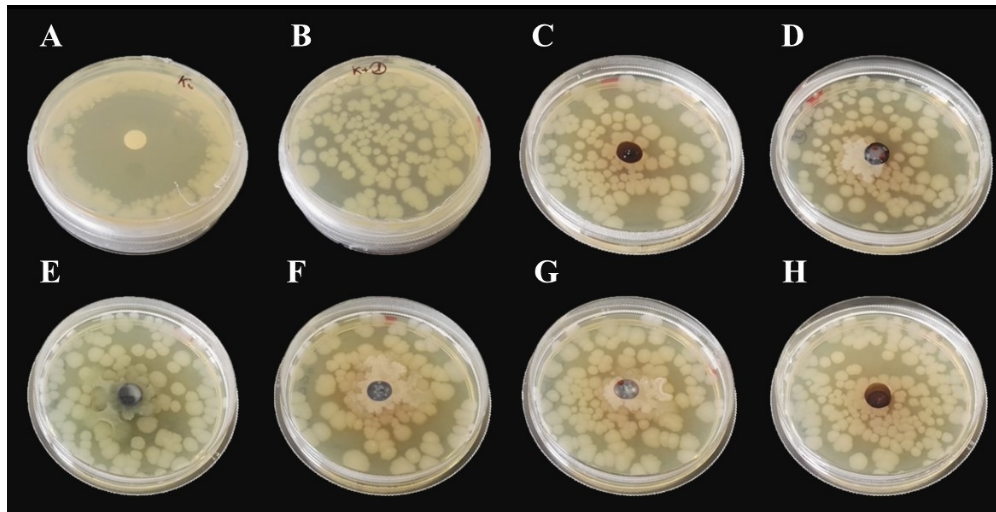

Figure 6. Growth inhibition of E. coli DH5α after 7 days of incubation. Radial diffusion assay: A) antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D) hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F) hemolymph of prepupae reared on +SUNOIL 4 dbp, G) hemolymph of prepupae reared on +SUNOIL 3 dbp, H) hemolymph of prepupae reared on +SUNOIL 1 dbp.

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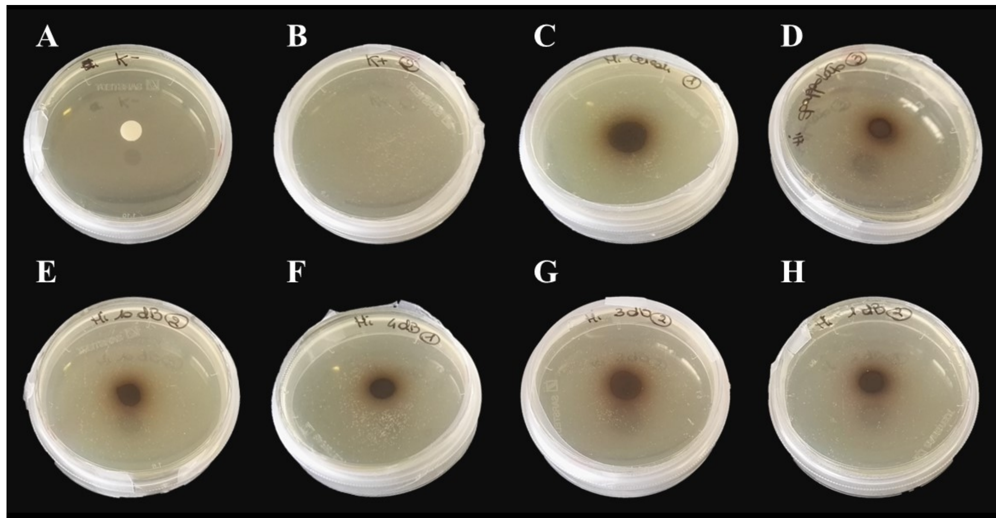

Figure 7. Growth inhibition of *M. yunnanensis* HI55 after 24h of incubation. Radial diffusion assay: A) antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D) hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F) hemolymph 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.

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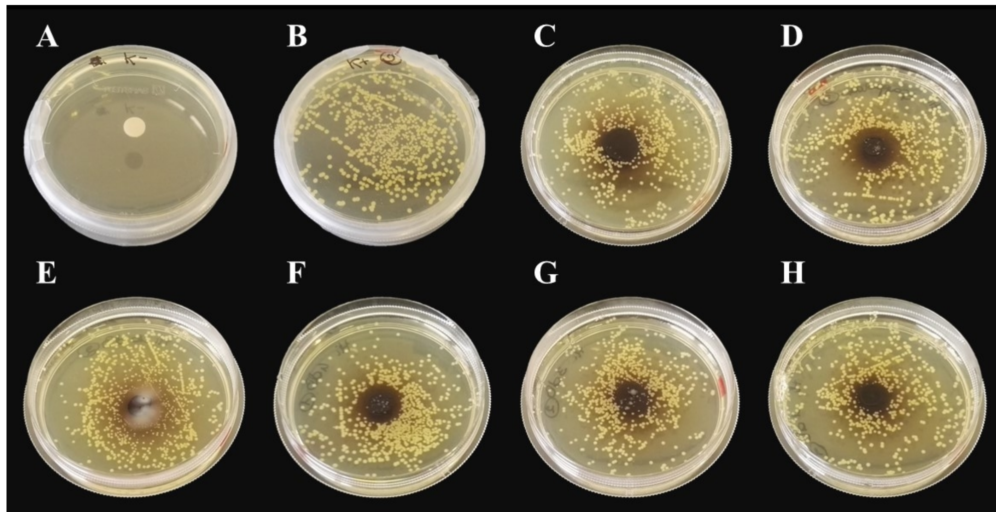

Figure 8. Growth inhibition of *M. yunnanensis* HI55 after 48h of incubation. Radial diffusion assay: A) antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D) hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F) hemolymph 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.

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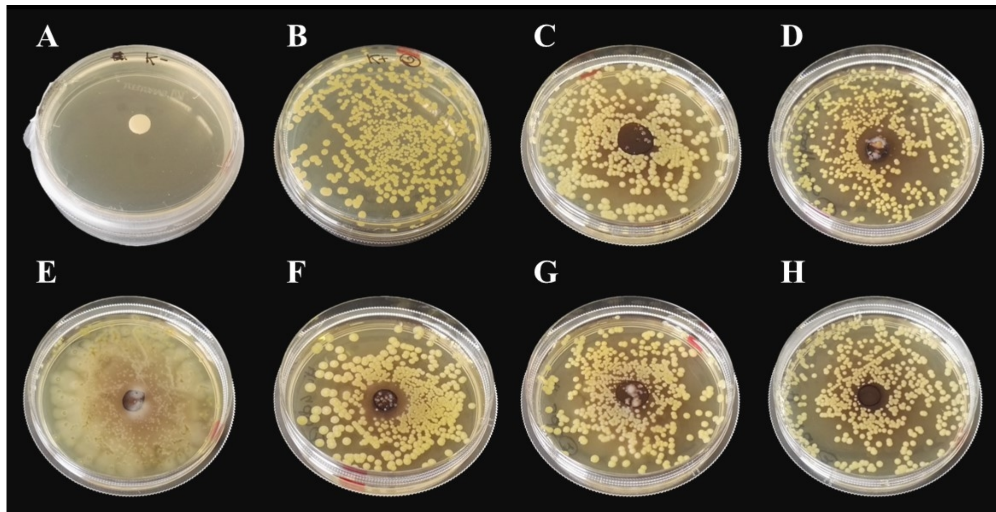

Figure 9. Growth inhibition of *M. yunnanensis* HI55 after 7 days of incubation. Radial diffusion assay: A) antibiotic-treated control, B) no-treated control, C) hemolymph of prepupae reared on GAIN, D) hemolymph of prepupae reared on OFMSW, E) hemolymph of prepupae reared on +SUNOIL 10 dbp, F) hemolymph 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.

198x100mm (150 x 150 DPI)