Synergism of gut microbiota to double-stranded RNAs in RNA interference of a leaf beetle

Running title: Reinforcement of RNAi by gut bacteria

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The authors declare no conflict of interest.

Abstract

RNA interference (RNAi) has emerged as an efficient tool to control insect pests. When lethal double-stranded RNAs (dsRNAs) were ingested by the insects, strong gene silencing and mortality can be induced. To exert their function, dsRNA molecules must pass through insect's gut and enter epithelial cells and/or the hemolymph. Gut bacteria are known to inhabit on the epithelial surface to confer host new capabilities to counter both biotic and abiotic stress. Whether there is a crosstalk between gut bacteria and dsRNAs and the effects of the microbiome on RNAi efficiency remains unknown. Here, using a leaf beetle-gut microbiota system, we investigated whether and how gut bacteria interact with dsRNA molecules and its effects on host insects. We firstly showed that the leaf beetle *Plagiodera versicolora* (Coleoptera) is highly susceptible to RNAi. Then, we found that ingestion of dsRNAs by non-axenic *P. versicolora* larvae results in (i) significantly accelerated mortality compared to axenic larvae, and (ii) over-growth and dysbiosis of the gut microbiota. The latter is mainly caused by the bacterial utilization of the dsRNA degraded products initiated by the host insect. Furthermore, we found that *Pseudomonas putida*, a gut bacterium of *P. versicolora*, was a main commensal-to-pathogen strain that accelerated the death of *P. versicolora* larvae. Taken together, our findings reveal a synergistic role of gut microbiota to dsRNA-induced mortality of pest insects, which provides new insights in the ecological functions of insect gut bacteria, and also contributes to a better understanding of the RNAi mechanisms in insects.

Introduction

RNA interference (RNAi) is a conserved post-transcriptional gene-silencing process in which gene expression in eukaryotic cells is down-regulated by double-stranded RNAs (dsRNAs) and their degradation intermediates. RNAi has been widely used as a powerful experimental tool for determining gene functions, and more recently, was repurposed as a novel strategy for insect pest control. When dsRNAs that target essential insect genes are ingested by the pest insect, knock-down of the target genes by RNAi can result in reduced growth or even the death (1). RNAi-based pest control is, therefore, recognized as promising approach to develop new target-specific pesticides. Coleopteran insects, such as the red flour beetle (*Tribolium castaneum*), the western corn rootworm (*Diabrotica virgifera virgifera*), and the Colorado potato beetle (*Leptinotarsa decemlineata*, CPB) are highly susceptible to RNAi and can be efficiently killed, if lethal dsRNAs are orally delivered. Interestingly, the efficiency of RNAi-mediated pest control was shown to be highly variable among different groups of insects (2-5). A few factors influencing the RNAi efficiency in insects have been identified, including the stability of dsRNA in the digestive system, the cellular uptake efficiency of dsRNAs, endosomal entrapment, and the activity of the core RNAi machinery (6-8). However, a comprehensive understanding of the molecular mechanisms affecting RNAi efficiency is still lacking. Moreover, the complex network of molecular interactions underpinning the dsRNA-mediated killing mechanisms has received limited attention.

The gut of insects provides a distinctive and complex environment for microbial colonization. The gut microbiota of insects offers various beneficial services to the host, including multifarious contributions to food digestion, nutrient modification, pathogen defense, chemical detoxification, etc. (9). In addition to the positive contribution to the insect's fitness, the presence of gut bacteria has also been implicated in promoting the insecticidal activity of *Bacillus thuringiensis* (10-13). Furthermore, it was demonstrated that some commensal gut bacteria have the potential to translocate from the gastrointestinal tract into the hemolymph and induce a rapid death (14-16). Upon

ingestion of dsRNA by insects, the dsRNA molecules inevitably pass through the intestinal tract, where they get in contact with the diverse microbial community that thrives in the gut. Whether there is an interplay between the gut microbiota and exogenously applied dsRNAs, and whether or not the microbiome affects the RNAi response of host insect is largely unknown.

The willow leaf beetle, *Plagiodera versicolora*, is one of the most damaging pest species of Salicaceae plants such as willows and poplars (17). This pest is widely distributed across northern Africa, America, Europe and Asia. *P. versicolora* larvae and adults both feed on leaves of willows and poplars, especially during the summer months. If not controlled, the insects can completely skeletonize the leaves and cause serious economic damage (18).

In the present work, we have studied the response of *P. versicolora* to environmental RNAi. We found that, like many other Coleopteran insects, *P. versicolora* are susceptible to RNAi, and can be efficiently killed by feeding with dsRNAs against suitable essential genes. We further explored the role of the gut microbiota in the RNAi response in *P. versicolora*. Our results indicate that the gut microbiota can utilize the dsRNA degradation products for proliferation, and contribute substantially to insect mortality.

Materials and methods

Preparation of *P. versicolora* larvae and gut bacterial strains

P. versicolora insects used in the studies were reared by feeding with detached fresh willow leaves or axenic poplar leaves at 28 °C and 60% ± 5% relative humidity under a 16 h light /8 h dark photoperiod. Poplar plantlets was raised aseptically on a synthetic medium containing 4.4 g/L MS salts plus vitamins, 0.1 mg/L NAA and 3 % (w/v) sucrose (19). To obtain axenic larvae, egg masses were soaked in 40% NaOH for 1 min and subsequently in 70% ethanol for 5 min, and finally washed with sterilized water for 2 min. After air-drying, each egg was separately transferred onto Luria-Bertani (LB) solid medium. Removal of gut bacteria was confirmed by a colony forming unit assay

97 and PCR analysis using conserved primers for the 16S rRNA gene of gut bacteria. The
98 newly hatched larvae were fed with aseptic detached poplar leaves and maintained
99 axenically.

100 Strains of the gut bacteria *Enterobacter aerogenes, Pseudomonas putida* and
101 *Enterococcus faecalis* strains were isolated from *P. versicolora*. To identify the bacterial
102 species, samples of genomic DNA from the different bacterial isolates were obtained
103 by extraction with the High Pure PCR Template preparation kit (Roche), and used to
104 amplify 16S rRNA gene sequences by PCR using the universal 16S rRNA gene-specific
105 primers 8F and 1492R (Table S1). The purified PCR products were then sequenced
106 (Sangon Biotech, Shanghai), and the obtained sequences were aligned with the closest
107 relatives matching the 16S rRNA gene sequences by BLAST searches
108 (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

109

110 **Ingestion and injection of dsRNA in *P. versicolora* larvae**

111 *Srp54k*, *Actin*, *Snap*, *Shi*, and *EGFP* gene fragments were amplified by PCR using gene-
112 specific primer pairs (Table S1), and subsequently used as templates to synthesize
113 dsRNA (ds*Srp54k*, ds*Actin*, ds*Snap*, ds*Shi*, and ds*GFP*) with the T7 RiboMAX^{TM}
114 Express RNAi system (Promega, USA) following the manufacturer's instructions.
115 Integrity of dsRNAs was evaluated by electrophoresis in 1.5% agarose gels and the
116 amounts of dsRNA were quantified with a spectrophotometer (Nano-Drop 2000,
117 Thermo Scientific, USA).

118 For dsRNA feeding assays, 30 second instar larvae were fed with aseptic poplar
119 leaves that had been painted with 8 ng/cm^2 dsRNA. Survival was recorded daily. To
120 examine the efficiency of RNAi-mediated knock-down of target genes, five larvae were
121 randomly chosen each day after dsRNA feeding and total RNA was isolated for further
122 analysis.

123 For dsRNA injection assays, 60 second instar larvae were anaesthetized on ice before
124 injection, and samples of 36 ng ds*Srp54k* or 2 ng ds*Actin* in 5 nL were injected into
125 each larva using a micro-injector (World Precision Instruments, Sarasota, USA).

126 Negative controls received an equivalent amount of ds*GFP*. After injection, *P.*
127 *versicolora* larvae were reared on sterile poplar leaves, and survival rates were
128 monitored daily (n = 30). Five larvae were randomly chosen each day for RNA
129 extraction.

130 To suppress dsRNase activity, larvae were fed with fresh poplar leaves painted with
131 40 ng/cm^2 ds*dsRNase* and 8 ng/cm^2 ds*GFP* or, as a control, the equivalent amount of
132 ds*GFP* (48 ng/cm^2). Five larvae were randomly chosen after four days of feeding for
133 RNA extraction.

134

135 **Analysis of gut microbiota**

136 After three days of dsRNA feeding (including H_2O as a negative control), larvae were
137 disinfected in 70% ethanol for 10 s, rinsed in sterile water several times, and then
138 dissected under sterile conditions. Four guts were pooled together as a replicate (n = 5).
139 DNA was extracted from gut samples with the High pure PCR template preparation kit
140 (Roche). A 16S rRNA gene fragment encompassing the V3 and V4 hypervariable
141 regions was amplified by PCR using the primer pair 338F and 806R (Table S1).

142 Sequencing of 16S rRNA amplicons was performed using an Illumina MiSeq
143 platform in MAJORBIO. Sequences were assigned to samples according to specific
144 barcodes, followed by removal of barcode and primer sequences. Paired-end reads were
145 assemble using FLASH (V1.2.7). High-quality data (clean reads) were obtained by
146 using the QIIME (Quantitative Insights Into Microbial Ecology) software packages
147 (V1.9.0) by filtering low-quality data with default parameters. Chimeric sequences
148 were checked and removed using the UCHIME algorithm. All effective reads from each
149 sample were initially clustered into Operational Taxonomic Units (OTUs) of 97%
150 sequence similarity with a UPARSE algorithm. The most abundant sequence in each
151 OTU was selected as the representative OTU and annotated by the RDP classifier
152 algorithm and reference data sets from the SILVA database under a confidence threshold
153 of 70%.

154

155 ***In vitro* incubation of dsRNA with hemolymph or gut juice**

156 Hemolymph fluid samples were collected with a 10 μL glass capillary tube from
157 abdomen of 10 fourth-instar larvae and were subsequently diluted with 100 μL ice-cold
158 Ringer solution (1 L: 8.766 g NaCl; 0.188 g CaCl2; 0.746 g KCl; 0.407 g $MgCl_2$; 0.336
159 g $NaHCO_3$; 30.807 g sucrose; 1.892 g trehalose; pH 7.2) (20). Samples were then
160 centrifuged at 12,000 *g* for 10 min to remove hemocytes and the supernatant was
161 collected. For gut juice preparation, whole guts from 10 fourth-instar larvae were
162 dissected and then crushed in 100 μL ice-cold Ringer solution in a centrifuge tube,
163 followed by centrifugation at 12,000 *g* for 10 min and collection of the supernatants.
164 The total protein concentrations of the fluid samples were determined using the BCA
165 Protein Assay kit following the manufacturer's instructions. 150 ng ds*GFP* (dissolved
166 in 5 μL nuclease-free water) were added to 5 μL hemolymph or gut juice and incubated
167 at 30 °C for the indicated times. Incubation in Ringer solution with the same amount of
168 ds*GFP* served as control. Subsequently, 2 μL of 6× loading dye (20% glycerol, 1.25mM
169 Na_2ETDA, 0.1% bromophenol blue, 0.1% xylene cyanol) was applied to the 10 μL
170 samples, and the RNAs were analyzed by electrophoreses in 1% agarose gels containing
171 Gelview.

172

173 **Bacterial growth promotion and inhibition assays**

174 To measure the effects of dsRNA degradation products on growth of gut bacteria,
175 bacterial cultures were grown in a modified minimal medium (21). For carbon source
176 determination, bacteria were grown in minimal medium supplemented with 24
177 nmol/mL ds*GFP*, NTP mix, uridine or inosine as sole carbon source and 0.4%
178 $(NH_4)_2SO_4$ as nitrogen source. For nitrogen source determination, bacteria were grown
179 in minimal medium supplemented with 4% glucose and 0.05% sodium citrate as carbon
180 source and 24 nmol/mL ds*GFP*, NTP mix, uridine or inosine as sole nitrogen source.
181 Saline-washed cells (approximately 10^7 cells) of each bacterial strain were used for
182 inoculation. Subsequently, the cultures were shaken for 24 h at 200 rpm and 30 °C. The
183 bacterial cell number was determined by counting colony-forming units (CFUs) on LB

184 medium.

185

186 **Reintroduction of bacteria into beetle guts**

187 After overnight culture, bacteria were collected by centrifugation at 4,000 rpm for 5
188 min to remove the supernatant. The bacterial cells were then washed with sterile PBS
189 3 times, re-suspended in PBS, and diluted to a final concentration of approximately $1\times$
190 10^6 cells/mL (22). The bacterial suspension was mixed with the indicated amounts of
191 dsRNA and painted onto aseptic poplar leaves. The leaves were then fed to axenic *P.*
192 *versicolora* larvae (n = 40). The aseptic poplar leaves coated with bacteria
193 (approximately 1×10^4 cells/cm^2) and dsRNA (8 ng/cm^2) were replaced every day.
194 Survival was recorded daily.

195

196 **Quantitative real-time PCR analysis**

197 RNA was extracted using RNAiso plus as described by the manufacturer (Takara,
198 Japan). cDNA was synthesized from 2 μg of total RNA using the Hifair$^{@}$ 1st Strand
199 cDNA Synthesis for qPCR Kit (YEASEN) with random primers. qPCRs were
200 performed in an Applied Biosystems 7300 Real-Time PCR system using SYBR premix
201 Ex Taq^{TM} (Takara, Japan). cDNA templates were denatured at 95°C for 2 min, followed
202 by 40 two-segment cycles of amplification at 95°C (5 s) and 60°C (30 s), where the
203 fluorescence was automatically measured. A melting curve analysis was performed
204 after the qPCR run (between 65°C and 95°C with 0.5°C increments). Prior to use in
205 qRT-PCR, cDNA was 1:9 diluted with H_2O. All data were normalized to levels of a
206 housekeeping gene (5S rRNA gene), and gene expression levels were calculated as fold
207 change values using the $2^{-\Delta\Delta Ct}$ method (23). Each experiment was carried out in
208 triplicate. All primers used were designed by Primer Premier 6. Primer sequences are
209 listed in Table S1.

210

211 **Histological analysis**

212 Larval tissues were immediately fixed in 10% neutral buffered formalin supplemented

213 with 2% dimethyl sulfoxide for 24 h, then dehydrated in a series of alcohol baths
214 (beginning with 50% and progressing to 100%), cleared in xylol for 4 h, and finally
215 embedded in paraffin. Cross-sections were prepared with a microtome (LEICA RM
216 2016, Leica Microsystems, USA) at a thickness of about 3 μm, stained with
217 hematoxylin and eosin, and analyzed with a fluorescence microscope (Nikon Eclipse
218 E-200 model, Tokyo, Japan).
219

220 **Statistical analysis of data**

221 Data comprising two groups were analyzed using Student's *t* test for unpaired
222 comparisons, and data comprising more than two groups were analyzed using one-way
223 ANOVA coupled with Bonferroni (equal variances) or Dunnett's T3 (unequal variances)
224 correction multiple comparison test. Survival curves were analyzed using the Kaplan–
225 Meier method, and the log-rank test was used to evaluate the significance of differences
226 between two groups. A value of $P<0.05$ was considered significantly different. Data
227 were statistically analyzed using SPSS version 19.0. Figures were drawn using Origin
228 8.5 and GraphPad Prism 7, and assembled in Adobe Illustrator CS6 and Photoshop CS6.
229

230 **Results**

231 **Gut microbiota accelerate dsRNA-induced mortality of *P. versicolora* larvae**

232 We first wanted to examine the sensitivity of *P. versicolora* to RNAi and identify
233 essential genes that represent suitable targets for RNAi-mediated pest control (24). To
234 this end, larvae were fed with *in vitro* synthetsized dsRNAs targeted against the *β-Actin*,
235 *Srp54k*, *Snap* and *Shi* genes. *β-Actin* encodes a multi-functional protein for
236 microfilament formation (25). *Srp54k* encodes the 54 kDa subunit of the signal
237 recognition particle which is involved in protein targeting to the endoplasmic reticulum
238 (26). *Snap* encodes the alpha-soluble N-ethylmaleimide-sensitive factor attachment
239 protein that is involved in the docking and fusion of vesicles to target membranes (27).
240 *Shi* encodes a dynamin-like protein associated with vesicular trafficking (28).
241 Compared to the negative control (feeding with *GFP* gene-derived dsRNA; ds*GFP*),

242 feeding of *P. versicolora* with dsRNAs derived from *Srp54k, Actin* and *Snap* resulted
243 in 100 % mortality of the larvae. The effects of RNAi were shown to be ds*Actin* >
244 ds*Srp54k* >ds*Snap* (Fig. S1; log-rank test, $P < 0.05$).

245 To evaluate whether the gut microbiota of *P. versicolora* is involved in determining
246 the efficiency of dsRNA-mediated killing, axenic larvae were obtained by surface
247 sterilization of eggs. The hatched larvae were then reared on aseptically grown poplar
248 leaves. The axenic status of the larvae was confirmed by two independent methods: (i)
249 absence of bacterial colony formation on LB agar plates, and (ii) absence of PCR
250 products from amplification reactions using primers for conserved regions of the
251 bacterial 16S rDNA (Fig. S2a,b). The axenic growth had no influence on insect survival
252 compared to conventionally raised larvae (Fig. S2c).

253 dsRNA feeding assays revealed no significant differences in survival of axenic
254 larvae compared to non-axenic larvae when fed with ds*GFP*. By contrast, non-axenic
255 larvae fed with ds*Srp54k* or ds*Actin* were killed significantly faster than axenic larvae
256 (Fig. 1a,c; log-rank test, $P<0.001$). Surprisingly, no significant differences in the level
257 of gene silencing were found between axenic and non-axenic larvae (Fig. 1b,d; log-
258 rank test, $P<0.05$). This finding suggests that the striking differences in mortality
259 between non-axenic and axenic larvae fed with lethal dsRNAs are not caused by
260 differences in the strength of the suppression of the targeted genes. Instead, these results
261 raise the interesting possibility that the gut microbiota confers the accelerated dsRNA-
262 mediated mortality in non-axenic *P. versicolora* larvae.

263 To examine whether breakdown of the barrier function of the gut epithelium is
264 responsible for the enhanced killing efficiency, we isolated hemolymph from non-
265 axenic larvae fed with dsRNAs and assayed the hemolymph samples for the presence
266 of bacteria by culturing them on LB medium. While numerous bacterial colonies
267 formed from non-axenic larvae fed with ds*Srp54k* or ds*Actin*, no colonies grew from
268 hemolymph samples obtained from larvae fed with ds*GFP*. This finding indicates
269 translocation of gut bacteria to the hemocoel in *P. versicolora* larvae fed with lethal
270 dsRNAs (ds*Srp54k* or ds*Actin*; Fig. S3). Moreover, histological analysis revealed that

271 the gut epithelia of non-axenic larvae fed with lethal dsRNAs were disrupted, while
272 they remained intact in the ds*GFP* control (Fig. S4). These observations provide further
273 evidence for gut bacteria promoting dsRNA-mediated killing.
274
275 **Feeding of dsRNAs results in alteration of the composition of the gut microbiota**
276 **of *P. versicolora***
277 We next investigated the composition of the gut bacterial community in *P. versicolora*
278 and its change after ingestion of lethal dsRNAs (ds*Srp54k* or ds*Actin*) or ds*GFP* as
279 control. Ingestion of sterilized water served as negative control. 878,019 sequences
280 (96.5% of the total number of 909,726 trimmed reads) were obtained from 16S rDNA
281 amplicon sequencing, and grouped into 122 operational taxonomic units (OTUs) using
282 a 97% similarity cut-off level. Alpha diversity was estimated using four measurements:
283 ACE index, Chao1 index, Shannon–Weiner index, and Simpson's index (Table S2). In
284 general, no significant differences were found for the α-diversity indexes between gut
285 samples of control animals and the three groups of larvae feeding on dsRNA-painted
286 leaves. However, the larvae fed with ds*Actin* had a significantly greater value for the
287 Shannon diversity index and a significantly lower Simpson's index than the other
288 treatments (Table S2; one-way ANOVA, $P<0.05$), possibly suggesting that the oral
289 administration of ds*Actin* can increase bacterial community evenness.
290 Principal co-ordinates analysis (PCoA) of Bray-Curtis distances of microbial
291 communities showed that bacterial communities from the control larvae clustered
292 independently and distinctly from the three dsRNA-fed larvae (Fig. 2a; PERMANOVA,
293 $P < 0.05$). Typing analysis of the four treatments indicated that they fall into three major
294 types (Fig. 2b). In all samples, the gut bacterial community was dominated by four
295 bacterial genera: *Enterobacter*, *Pseudomonas*, *Enterococcus* and *Serratia*, which
296 account for over 95% of the total sequences in the samples (Fig. 2c; Table S3). The
297 proportion of *Enterobacter* was significantly reduced in the three groups of dsRNA-fed
298 larvae compared to the control group (larvae feeding on dsRNA-free leaves) (Fig. 2c;
299 Kruskal-Wallis *H* test, $P < 0.05$), and the proportion of two major genera (*Pseudomonas*

300 and *Enterococcus*) increased after feeding with the dsRNAs. Remarkably, feeding with
301 lethal dsRNAs (i.e., ds*Srp54k* or ds*Actin*) significantly increased the proportion of
302 *Pseudomonas* in *P. versicolora* larvae compared to larvae fed with untreated or ds*GFP*-
303 treated leaves (Fig. 2c; Kruskal-Wallis *H* test, $P < 0.05$).
304

305 **Ingestion of dsRNA promotes growth of gut bacteria**

306 The total amounts of gut bacteria were significantly increased in all dsRNA-fed larvae
307 (2 days after feeding with ds*GFP* and ds*Actin*, 3 days after feeding with ds*Srp54k*)
308 compared to the control group of larvae feeding on leaves not painted with dsRNA (Fig.
309 3a-c; *T*-test, $P < 0.05$). This finding suggests that ingestion of dsRNA can promote
310 bacterial growth in the intestinal system. To further validate the effects of dsRNA
311 ingestion on the abundance of the three major genera of gut bacteria, we monitored the
312 relative quantities of the bacterial genera in the different groups of larvae. The
313 abundances of *Enterobacter* and *Pseudomonas* were found to be significantly increased
314 in larvae fed with ds*GFP*-treated leaves at day 3 and 4 compared to the control (Fig.
315 3d; *T*-test, $P < 0.05$), but dropped to similar abundance at day 5. The relative
316 abundances of both *Pseudomonas* and *Enterobacter* were significantly increased after
317 3 days of feeding on ds*Srp54k*, while no significant differences were found in relative
318 abundance of *Enterococcus* between the larvae fed with ds*Srp54k* and the control (Fig.
319 3e; *T*-test, $P < 0.05$). Similarly, the relative abundances of both *Pseudomonas* and
320 *Enterococcus* were significantly increased after 3 days of ds*Actin* feeding (Fig. 3f; *T*-
321 test, $P < 0.05$).
322

323 **Ingestion of dsRNA is confers accelerated mortality and increased gut bacterial**
324 **load in *P. versicolora***

325 To assess the contribution of ingestion of insecticidal dsRNA to the proliferation of gut
326 bacteria, we determined mortality and bacterial loads of *P. versicolora* larvae after
327 feeding and injection of dsRNAs. Upon injection of dsRNA, a significantly accelerated
328 mortality was observed in larvae treated with ds*Srp54k* compared to those injected with

ds*GFP* (Fig. 4a; log-rank test, $P < 0.05$). Moreover, we found that non-axenic larvae fed with ds*Srp54k* exhibit higher mortality and gut bacterial loads than larvae injected with ds*Srp54k* (Fig. 4a; log-rank test, $P < 0.05$; Fig. 4c, *T*-test, $P < 0.05$), although the silencing levels of the target gene (*Srp54k*) were similar in the two treatments (Fig. 4b). Ingestion of ds*Actin* by *P. versicolora* larvae lead to a similar morality as injection of ds*Actin* (Fig. 4d), even though the latter treatment caused a significantly higher efficiency of gene silencing (Fig. 4e; *T*-test, $P < 0.05$) and a lower gut bacterial load (Fig. 4f, *T*-test, $P < 0.05$). Taken together, these results indicate that ingestion of insecticidal dsRNA accelerates mortality of *P. versicolora* larvae.

dsRNA instability in *P. versicolora* gut juice

To obtain information on the stability of dsRNA in the *P. versicolora* digestive system and the hemolymph, ds*GFP* was incubated in gut juice or hemolymph extracted from insects. While ds*GFP* was rapidly degraded in the gut juice, it remained relatively stable in the hemolymph (Fig. 5a). To determine the possible cause of dsRNA instability in the digestive tract, we retrieved two putative dsRNase sequences from our *P. versicolora* RNA-seq datasets. Both sequences have high sequence similarities to previously identified gut dsRNases (Fig. S5). *dsRNase1* was found to be highly expressed in the gut tissue compared to the carcass, while expression of *dsRNase2* was relatively low in both gut and carcass (Fig. 5b). Expression of *dsRNase1* is induced upon feeding of *P. versicolora* larvae with ds*GFP* (Fig. 5c). Importantly, the degradation capability of dsRNA by the gut juice of *P. versicolora* was strongly reduced when *dsRNase1* was down-regulated by injection of double-stranded RNA (ds*dsRNase1*) (Fig. 5d,e). These results indicate that dsRNase1 is the major dsRNA-degrading enzyme activity in the gut juice of *P. versicolora*.

The degradation products of dsRNA can be utilized by gut bacteria for growth

To explore whether the instability of dsRNA contributes to the increased gut bacterial load, we fed larvae with dsRNA targeted against *dsRNase1* (ds*dsRNase1*) and dsGFP.

358 For comparison, dsGFP was fed as a control. Interestingly, the gut bacterial load was
359 significanly lower in the *dsRNase1*-silenced larvae than in larvae fed only with ds*GFP*
360 (Fig. 5f,g), suggesting that dsRNA degradation is associated with increased gut
361 bacterial load. To futher assess whether dsRNA or its degradation products promote gut
362 bacterial growth, we isolated and identified three bacterial species
363 (*Enterococcus aerogenes, P. putida,* and *E. faecalis*) from the gut of *P. versicolora*, and
364 used ds*GFP* and RNA degradation products or building blocks (NTP mix, uridine,
365 inosine) as either the sole carbon source or the sole nitrogen source for the growth of
366 these bacterial species. As expected, ds*GFP* does not promote growth of any of the
367 tested bacterial species. By contrast, all three species can utilize NTP mix, uridine and
368 inosine as sole nitrogen source. The growth of *P. putida* and *E. aerogenes* (but not *E.*
369 *faecalis*) also can be stimulated with uridine or inosine as sole carbon source (Fig. 5h;
370 Table S3).
371

372 **Reintroduction of gut bacteria into axenic *P. versicolora* larvae enhances mortality**
373 **upon administration of insecticidal dsRNA**

374 To ultimately confirm the impact of gut bacteria on dsRNA-induced killing, individual
375 gut bacterial species (*P. putida, E. aerogenes* or *E. faecalis*) were mixed with
376 insecticidal dsRNA and reintroduced into axenic larvae. Compared to axenic larvae,
377 mortality was significantly increased in larvae inoculated with bacteria. Upon feeding
378 of ds*Srp54k*, *P. putida* inoculated larvae were killed significantly faster than larvae
379 inoculated with *E. aerogenes* or *E. faecalis* (Fig. 6a; log-rank test, $P < 0.05$). Similar
380 results were obtained for ds*Actin* feeding, and the accelerating effect of the gut bacteria
381 was *P. putida* > *E. faecalis* > *E. aerogenes* (Fig. 6b; log-rank test, $P < 0.05$).
382

383 **Discussion**

384 In this work, we explored whether the gut microbiota plays a role in dsRNA-
385 mediated killing of pest insects by RNA interference (environmental RNAi). To this
386 end, we developed a method to obtain axenic larvae. The method uses surface

387 sterilization of eggs, which has several advantages compared to the antibiotic
388 treatments that are often used in microbiome research to eliminate bacteria, including
389 complete removal of microbes and absence of residual antibiotics that may affect larval
390 development upon hatching. The axenic status can be maintained by feeding with
391 aseptic leaves of poplar plants grown in a sterile envirionment. Feeding dsRNAs
392 targeted against two essential genes (*β-Actin* and *Srp54k*) to axenic and non-axenic
393 larvae of *P. versicolora*, we found that ds*Actin* is more effective in killing *P. versicolora*
394 than ds*Srp54k* in either axenic or non-axenic condition. The different killing efficiency
395 might due the different cellular functions of the two proteins and/or differences in
396 expression levels or transcript susceptibility to RNAi. Importantly, regardless of which
397 dsRNA was fed, non-axenic larvae were killed significantly faster than axenic larvae.
398 This effect was independent of the efficiency of gene knock-down (Fig. 1), excluding
399 the possibility that the different mortalities between axenic and non-axenic larvae are
400 caused by different levels of gene silencing of the targeted genes.

401 To pinpoint potential causes of the accelerated mortality of non-axenic larvae, we
402 determined abundance and composition of the gut microbiota in larvae after ingestion
403 of dsRNA. Compared to control larvae, feeding of dsRNAs (irrespective of RNAi
404 induction and target sequences, i.e., feeding with ds*GFP*, ds*Actin* or ds*Srp54k*) resulted
405 in significant alteration of the composition of the gut microbiota (Fig. 2) and excessive
406 growth of gut bacteria (Fig. 3).

407 The most abundant bacterial genus in the gut microbiota is *Enterobacter*, comprising
408 over 80% of the total microbiota (Fig. 2c). In previous studies, *Enterobacter* was also
409 found to be widely associated with leaf beetles and poplar pests (29, 30). Bacteria of
410 the genus *Rahnella* also could be readily isolated from *P. versicolora* using culture-
411 dependent methods (18). *Enterobacter* from the Colorado potato beetle was shown to
412 suppress plant defense (30); however, whether *Enterobacter* in *P. versicolora* has a
413 similar function remains to be investigated. Notably, we found that there was a drastic
414 increase in the abundance of *Pseudomonas* in larvae fed with ds*Srp54k* or ds*Actin*
415 compared to larvae fed with ds*GFP* (Fig. 2c). Together with our observation that

416 bacteria translocate to the hemocoel in *P. versicolora* larvae fed with insecticidal
417 dsRNA, these findings indicate that the gut microbiota is an important contributor to
418 dsRNA-mediated killing of pest insects.

419 A previous study showed that dsRNA-mediated gene knock-down in the migratory
420 locust can induce overgrowth of opportunistic pathogens and intestinal atrophy (31),
421 but the reasons remained unclear. To clarify how RNAi influences gut bacteria, we
422 analyzed bacterial growth in *P. versicolora* after ingestion versus injection of
423 insecticidal dsRNA. We found that the knock-down of an essential gene has limited
424 relevance to the increase in gut bacterial abundance (Fig. 4), in that *Srp54k* knock-down
425 triggered by injection of ds*Srp54k* had no statistically significant effect on total
426 bacterial growth. Similarly, the increase in bacterial abundance in larvae injected with
427 ds*Actin* was lower than that in larvae fed with ds*Actin*. Thus, the direct comparison
428 between ingestion and injection indicates that ingestion was required for accelerated
429 mortality (Fig. 4). This finding raises the interesting question why oral administration
430 leads to proliferation of the gut microbiota. As bacteria do not possess an RNAi
431 machinery and no dsRNA uptake systems have been found in bacteria (32), we
432 hypothesized that the dsRNA itself cannot trigger overgrowth of gut bacteria. A dsRNA-
433 degradating enzyme (dsRNase) in the gut was first identified and characterized in
434 *Bombyx mori*, and related enzymes were subsequently found in almost all orders of
435 insects, including the beetle family (Coleopteran). Differences in the expression levels
436 and activities of intestinal dsRNases are likely, at least in part, responsible for the
437 observed differences in susceptibility to RNAi between different groups of insects (2,
438 7, 33-36). In this work, we found that a gut dsRNase (dsRNase1) is chiefly response
439 for dsRNA degradation in the digestive tract of *P. versicolora* (Fig. 5a-e). Moreover,
440 we observed that ingestion of dsRNA upregulated *dsRNase1* expression (Fig. 5c).
441 Suppressed expression of *dsRNase1* strongly reduced dsRNA degradation and
442 decreased the abundance of gut bacteria (Fig. 5f-g), suggesting a contribution of *P.*
443 *versicolora* dsRNase1 to growth control of gut bacteria. This finding also raised the
444 interesting possibility that gut bacteria utilize the degradation products of dsRNAs as

445 nutrient source (Fig. 5a). Evidence in support of this idea was supplied by bacterial
446 growth assays that revealed that NTP mix, uridine and inosine, but not dsRNA, can be
447 utilized as carbon and nitrogen sources to promote the growth of three dominant
448 bacterial strains in the gut microbiome of *P. versicolora* (Fig. 5h). Thus, ingested
449 dsRNA can suffer one of two fates in the gut of *P. versicolora* larvae: (i) it can be taken
450 by midgut cells by a SID-type channel (putative dsRNA-selective transport channel)
451 and/or the endocytosis pathway and subsequently trigger the RNAi response in the
452 insect (37), or (ii) it undergoes degradation by dsRNase1 in the midgut (Fig. 5a) and its
453 breakdown products are subsequently utilized by gut bacteria as carbon and nitrogen
454 source for their growth (Figs. 5g and 7). However, it seems reasonable to assume that
455 dsRNA is not be the only nutrient that controls gut bacterial growth and there are likely
456 other factors that contribute to the observed gut bacterial dysbiosis.

457 It seems noteworthy that the capabililty of *P. putida* to utilize dsRNA degradation
458 products as both carbon and nitrogen source for its growth is more pronounced than
459 that of the other two abundant gut bacterial species tested (Fig. 5h; Table S3). This
460 finding may, at least in part, explain why the proportion of *P. putida* was drastically
461 increased in larvae fed with insecticidal dsRNAs (Fig. 2c). Moreover, re-introduction
462 of gut bacteria into axenic larvae increased their mortality after feeding with lethal
463 dsRNAs, and *P. putida* showed the strongest effect on acceleration of larval mortality
464 (Fig. 6). *P. putida* was previously shown to be a nitrogen-fixing strain that can promote
465 the growth of plants (38, 39), but whether the *P. putida* population in the gut
466 microbiome of *P. versicolora* was originally acquired from its host plants and now
467 functions as mildly pathogenic strain to *P. versicolora* remains to be investigated.

468 In conclusion, the present study illuminates the complex interplay between
469 insecticidal dsRNA, the insect host, and its gut microbiota. Our work provides new
470 insight into these interactions and suggests an important role of host sepsis in the
471 multifaceted killing mechanism that underlies environmental RNAi.

472

473 **Acknowledgements**

This work was supported by grants from the National Key Research and Development Program of China (2017YFD0600101), the National Natural Science Foundation of China (31572071, 31971663) and the Recruitment Program of Global Experts (China) to J.Z.

Author contributions

LX and JZ conceived the project; LX, SX, LS, YZ, and JL performed the research; LX, SX, and JZ analysed the data. LX, RB and JZ wrote the article with contributions of all the authors.

Supplementary information is available at The ISME Journal's website

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599 1987;33:390-395
600
601

Figure legends

Fig. 1 Gut bacteria accelerate mortality of *P. versicolora* in lethal dsRNA-mediated killing process. (**a** and **c**) Kaplan-Meier survival curves of axenic and non-axenic larvae (n=30) after feeding with ds*Srp54k* (**a**) and ds*Actin* (**c**). *Srp54k* (**b**) and *Actin* (**d**) relative expression in axenic and non-axenic larvae upon fed on ds*Srp54k* and ds*Actin*. Gene expression levels were set as one in larvae fed with ds*GFP* control. Kaplan-Meier survival curve was analyzed by the log-rank test. Gene expression differences between two groups were calculated using the independent-samples *t*-test. Data are presented as mean ± SE, *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS, not significant.

Fig. 2 Ingestion of dsRNA alters gut microbiota composition of *P. versicolora*. (**a**) Principal co-ordinates analysis (PCoA) of Bray-Curtis distances at the genus level for 16S rRNA gene sequences. (**b**) Typing analysis of Jensen-Shannon divergence distance at the OTU level for 16S rRNA gene sequence data. (**c**) Kruskal-Wallis *H* test bar plot analysis of the relative proportions of major genera of gut bacteria. The relative abundances of the identified microbial taxa were determined in gut samples collected from larvae fed with ds*GFP,* ds*Srp54k*, ds*Actin*, or H_2O. *P*-values were calculated using the Kruskal-Wallis *H* test. ** $P < 0.01$; * $P < 0.05$.

Fig. 3 Ingestion of dsRNA promotes the growth of gut bacteria in *P. versicolora* larvae. qRT-PCR analyses were performed to determine the relative abundance of gut bacteria in *P. versicolora* larvae fed with ds*GFP* (**a**), ds*Srp54k* (**b**) and ds*Actin* (**c**) (n = 5), and the abundances of three major bacterial genera in non-axenic *P. versicolora* larvae fed with ds*GFP* (**d**), ds*Srp54k* (**e**) and ds*Actin* (**f**). The measurements were performed at different time points (after 2-5 days of feeding). The qRT-PCR value obtained for gut bacterial 16S rRNA in *P. versicolora* control larvae (fed with water-treated leaves) was set as 1.0. *P*-values were calculated using the independent-samples *t*-test. Data are presented as means ± SE, *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS, not significant.

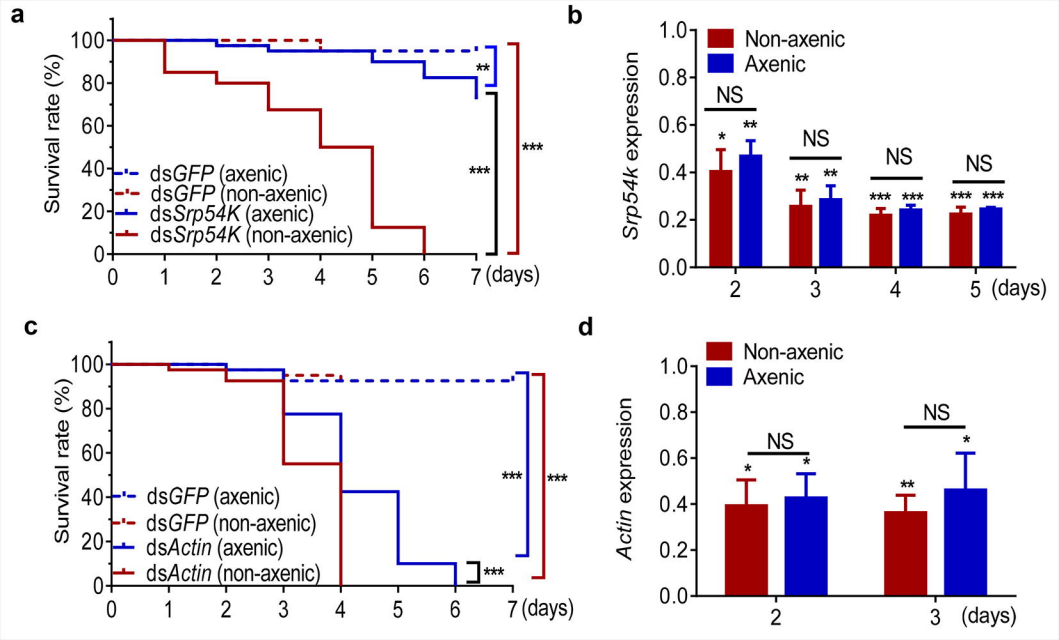
632

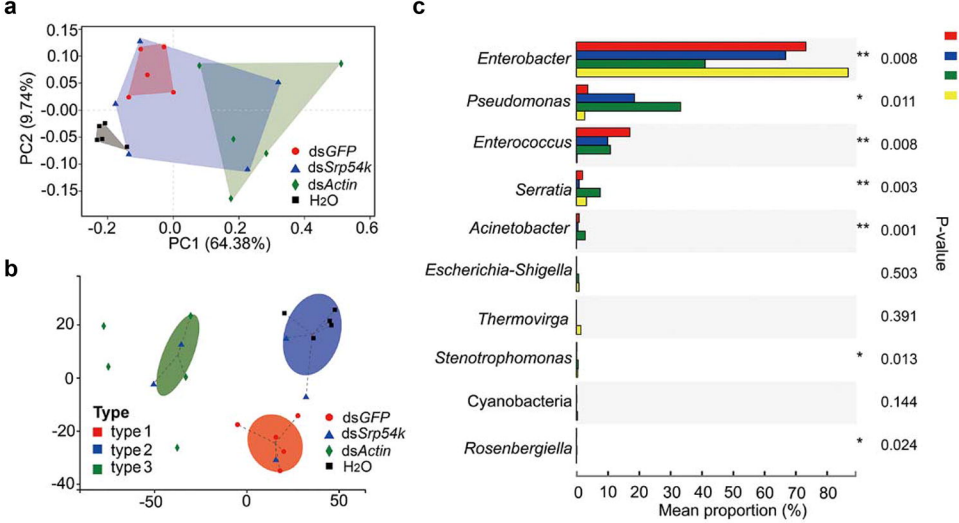
633 **Fig. 4** Comparison of mortality of *P. versicolora* in response to insecticidal dsRNA
634 upon administration by injection (injected dsRNA, I-dsRNA) or ingestion (fed dsRNA,
635 F-dsRNA). (**a, d**) Kaplan–Meier survival curves of *P. versicolora* larvae (n = 30) after
636 injection and ingestion of ds*Srp54k* (**a**) or ds*Actin* (**d**). Ingestion or injection of ds*GFP*
637 were performed as controls. The survival curves were analyzed by the log-rank test. (**b,**
638 **e**) Expression levels of *Srp54k* and *Actin* in *P. versicolora* larvae fed or injected with
639 ds*Srp54k* (**b**) or ds*Actin* (**e**). Relative gene expression levels were determined by qRT-
640 PCR assays and expression in control larvae treated with ds*GFP* were set as one. (**c,f**)
641 qRT-PCR analysis of the relative abundance of whole gut bacteria in *P. versicolora*
642 larvae after injection with ds*Srp54k* (**c**) or ds*Actin* (**f**) compared to ds*GFP* as control.
643 *P*-values were calculated using the independent-samples *t*-test. *** $P < 0.001$; ** $P <$
644 0.01; * $P < 0.05$; NS, not significant.

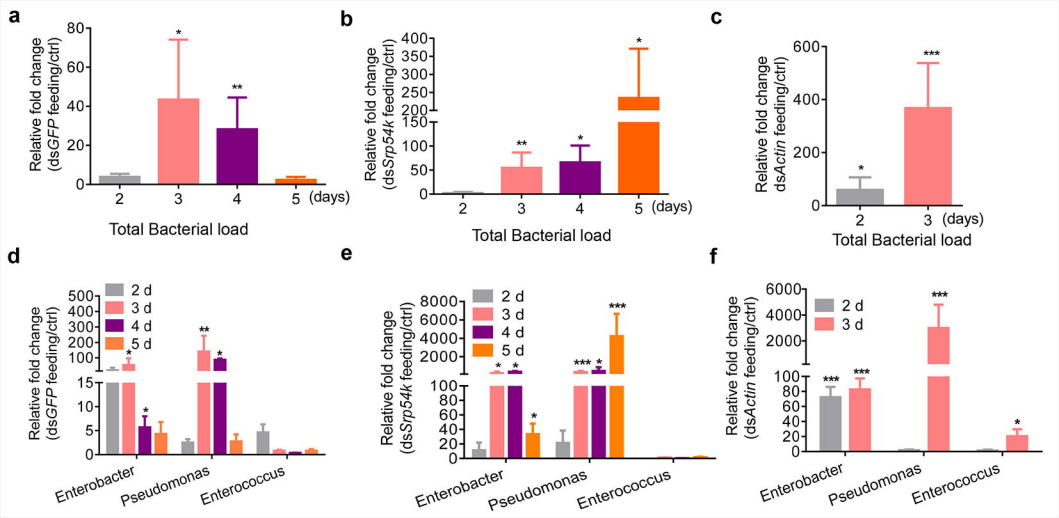
645

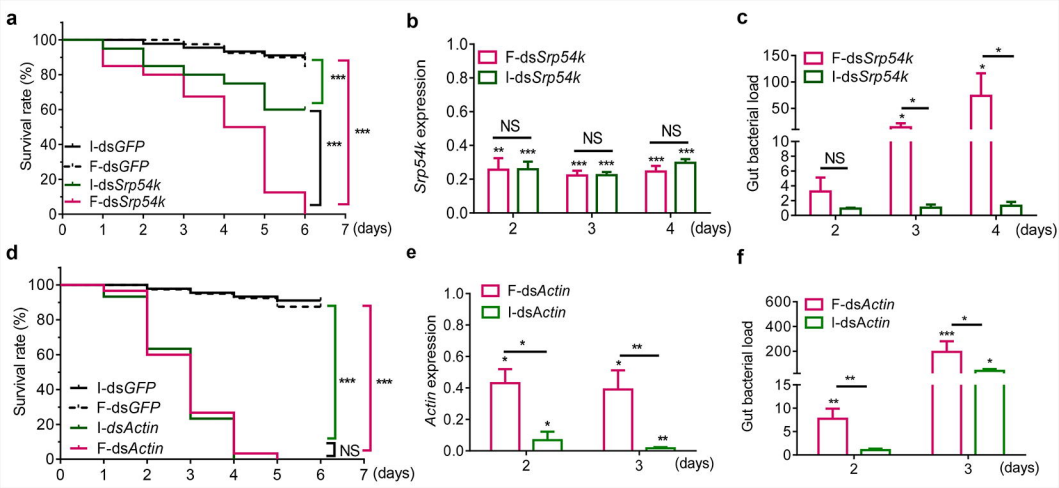
646 **Fig. 5** Degradation of dsRNA promotes gut bacterial growth in *P. versicolora* larvae.
647 (**a**) ds*GFP* incubation with hemolymph or gut fluid of *P. versicolora* third instar larvae.
648 Samples of 5 μL gut juice or hemolymph (adjusted to 5 μg of total protein) were
649 incubated with 400 ng of ds*GFP* for the indicated times at 28 ℃. G: gut juice sample
650 without dsRNA added; H: hemolymph sample without dsRNA added. (**b**) Expression
651 patterns of two putative *dsRNase* genes in third instar larvae. Each sample comprises
652 five pooled guts or carcasses from dissected larvae. Error bars represent the mean of
653 five independent samples ± SEM. (**c**) Relative expression changes of *dsRNase1* in
654 larvae fed with ds*GFP*-painted leaves (8 ng/cm^2) at the indicated times compared to
655 untreated control larvae. Gene expression levels were set to 1 in larvae fed with H_2O-
656 treated control leaves (ctrl). (**d**) ds*RNase1* silencing efficiency over time in larvae (n =
657 5) injected with 40 ng of ds*dsRNase*. Injection of larvae with the same amount of
658 ds*GFP* served as control. (**e**) ds*GFP* incubation in gut fluid of *P. versicolora* larvae with
659 or without suppression of *dsRNase1* expression. Samples of 5 μL gut fluid (adjusted to
660 5 μg of total protein) were incubated with 400 ng of ds*GFP* for the indicated times at

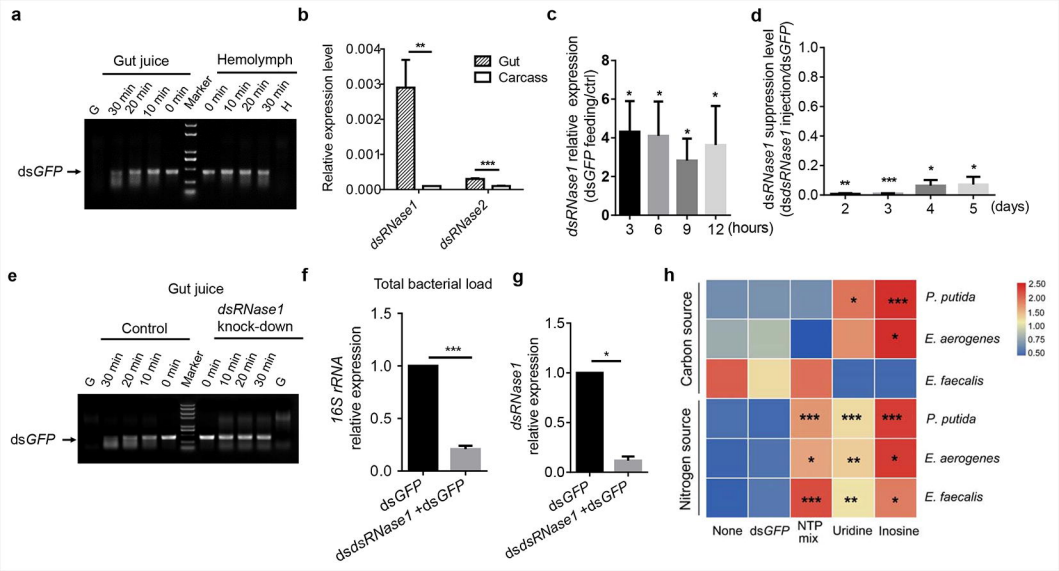
661 28 °C. (**f**) qRT-PCR analysis of the relative abundance of gut bacteria in *P. versicolora*
662 second instar larvae fed on young poplar leaves painted with ds*dsRNase1* (40 ng/cm^2)
663 and ds*GFP* (8 ng/cm^2) or ds*GFP* (48 ng/cm^2). Guts were sampled at day 4 (n = 7). (**g**)
664 Relative expression levels of *dsRNase1* in the larvae assayed in (f). (**h**) dsRNA
665 degradation products promote the growth of three gut bacterial species. Bacteria were
666 grown for 24 h in either carbon-free or nitrogen-free liquid medium supplemented with
667 24 nmol/mL of the indicated compounds (dsGFP, NTP mix, inosine, uridine). Glucose
668 (0.4%) and sodium citrate (0.05%) served as carbon source in nitrogen-free media.
669 $(NH_4)_2SO_4$ (0.4%) served as nitrogen source in carbon-free media. *** $P < 0.001$, ** P
670 < 0.01, * $P < 0.05$.
671
672 **Fig. 6** Kaplan–Meier survival curves of axenic *P. versicolora* larvae (n = 30) fed with
673 ds*Srp54k* (**a**) or ds*Actin* (**b**) after reintroduction of bacterial species (*E. aerogenes*, *E.*
674 *faecalis* or *P. putida*). The bacteria were mixed with either ds*Srp54k* or ds*Actin* and
675 introduced into axenic larvae via spreading the bacteria onto aseptic poplar leaves. The
676 log-rank test was used to assess the significance of differences between two survival
677 curves. ***$P < 0.001$, * $P < 0.05$.
678
679 **Fig. 7** Working model of dsRNA-mediated insect killing. In the gut of the insect,
680 dsRNA can be taken by midgut epithelial cells and loaded into the RNAi machinery,
681 thus suppressing expression of the target gene. dsRNA can also induce expression of a
682 gene encoding dsRNase, thereby causing degradation of dsRNA retained in the gut. Gut
683 bacteria can utilize the degradation products of dsRNAs for their growth and translocate
684 from the gut lumen into the hemocoel, thus resulting in accelerated mortality of their
685 host insect.

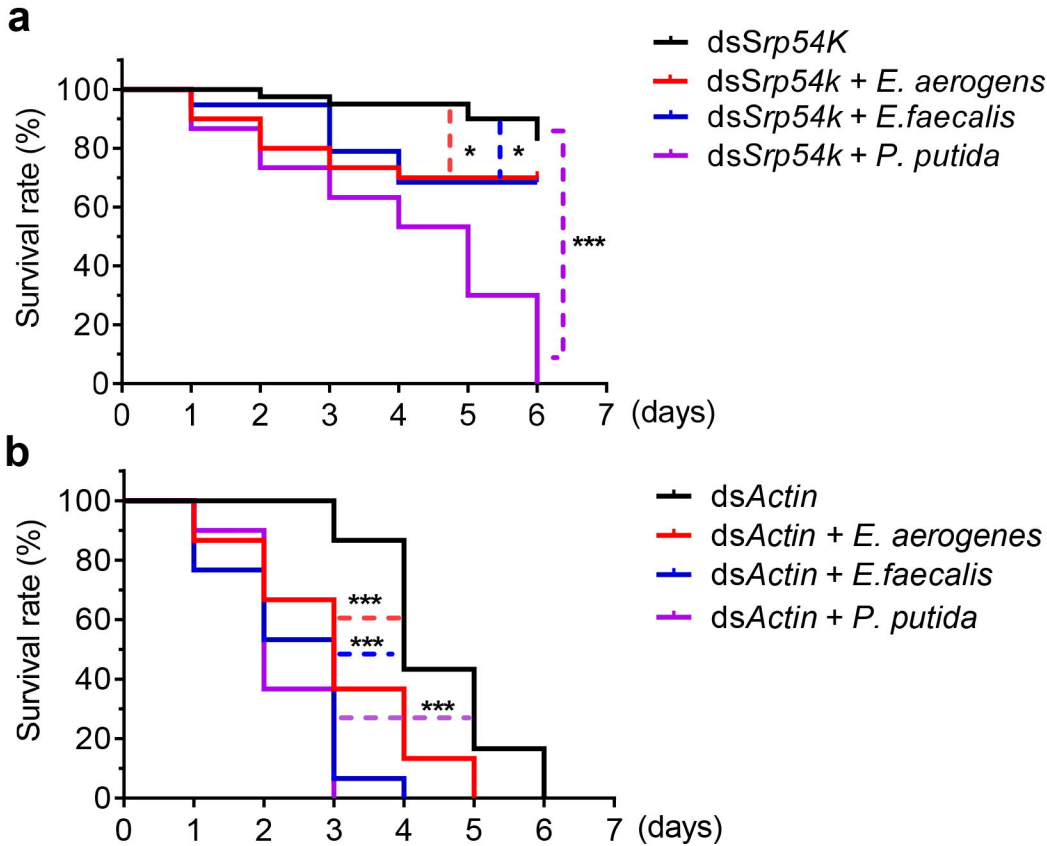

a
Survival rate (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 (days)
dsGFP (axenic)
dsGFP (non-axenic)
dsSrp54K (axenic)
dsSrp54K (non-axenic)
**
***
***
b
Srp54k expression
1.0
0.8
0.6
0.4
0.2
0.0
Non-axenic
Axenic
NS
NS
NS
NS
2 3 4 5 (days)
c
Survival rate (%)
dsGFP (axenic)
dsGFP (non-axenic)
dsActin (axenic)
dsActin (non-axenic)
***
***
***
0 1 2 3 4 5 6 7 (days)
d
Actin expression
Non-axenic
Axenic
NS
NS
2 3 (days)


a
PC2 (9.74%)
PC1 (64.38%)
dsGFP
dsSrp54k
dsActin
H2O
b
Type
type 1
type 2
type 3
dsGFP
dsSrp54k
dsActin
H2O
c
Enterobacter
Pseudomonas
Enterococcus
Serratia
Acinetobacter
Escherichia-Shigella
Thermovirga
Stenotrophomonas
Cyanobacteria
Rosenbergiella
P-value
Mean proportion (%)


a
Relative fold change
(dsGFP feeding/ctrl)
80
60
40
20
0
*
**
2
3
4
5
(days)
Total Bacterial load
b
Relative fold change
(dsSrp54k feeding/ctrl)
400
350
300
250
200
150
100
50
0
**
*
*
2
3
4
5
(days)
Total Bacterial load
c
Relative fold change
dsActin feeding/ctrl)
600
400
200
0
***
*
2
3
(days)
Total Bacterial load
d
Relative fold change
(dsGFP feeding/ctrl)
2 d
3 d
4 d
5 d
500
400
300
200
100
15
10
5
0
*
**
*
*
Enterobacter
Pseudomonas
Enterococcus
e
Relative fold change
(dsSrp54k feeding/ctrl)
2 d
3 d
4 d
5 d
8000
6000
4000
2000
100
80
60
40
20
0
*
*
*
***
*
***
Enterobacter
Pseudomonas
Enterococcus
f
Relative fold change
(dsActin feeding/ctrl)
2 d
3 d
6000
4000
2000
100
80
60
40
20
0
***
***
***
*
Enterobacter
Pseudomonas
Enterococcus


a
Survival rate (%)
I-dsGFP
F-dsGFP
I-dsSrp54k
F-dsSrp54k
***
***
***
7 (days)
b
Srp54k expression
F-dsSrp54k
I-dsSrp54k
NS
NS
NS
(days)
c
Gut bacterial load
F-dsSrp54k
I-dsSrp54k
NS
(days)
d
Survival rate (%)
I-dsGFP
F-dsGFP
I-dsActin
F-dsActin
***
***
NS
7 (days)
e
Actin expression
F-dsActin
I-dsActin
(days)
f
Gut bacterial load
F-dsActin
I-dsActin
(days)


a
Gut juice
Hemolymph
G
30 min
20 min
10 min
0 min
Marker
0 min
10 min
20 min
30 min
H
dsGFP
b
Relative expression level
0.004
0.003
0.002
0.001
0.000
**
***
Gut
Carcass
dsRNase1
dsRNase2
c
dsRNase1 relative expression (dsGFP feeding/ctri)
8
6
4
2
0
*
3
6
9
12 (hours)
d
dsRNase1 suppression level (dsdsRNase1 injection/dsGFP)
1.0
0.8
0.6
0.4
0.2
0.0
**
***
*
2
3
4
5 (days)
e
Gut juice
Control
dsRNase1 knock-down
G
30 min
20 min
10 min
0 min
Marker
0 min
10 min
20 min
30 min
G
dsGFP
f
Total bacterial load
16S rRNA relative expression
1.5
1.0
0.5
0.0
***
dsGFP
dsdsRNase1 +dsGFP
g
dsRNase1 relative expression
1.0
0.5
0.0
*
dsGFP
dsdsRNase1 +dsGFP
h
Carbon source
Nitrogen source
P. putida
E. aerogenes
E. faecalis
P. putida
E. aerogenes
E. faecalis
None
dsGFP
NTP mix
Uridine
Inosine
2.50
2.00
1.50
1.00
0.75
0.50


a
dsSrp54K
dsSrp54k + E. aerogens
dsSrp54k + E.faecalis
dsSrp54k + P. putida
Survival rate (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
(days)
*
*
***
b
dsActin
dsActin + E. aerogenes
dsActin + E.faecalis
dsActin + P. putida
Survival rate (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
(days)
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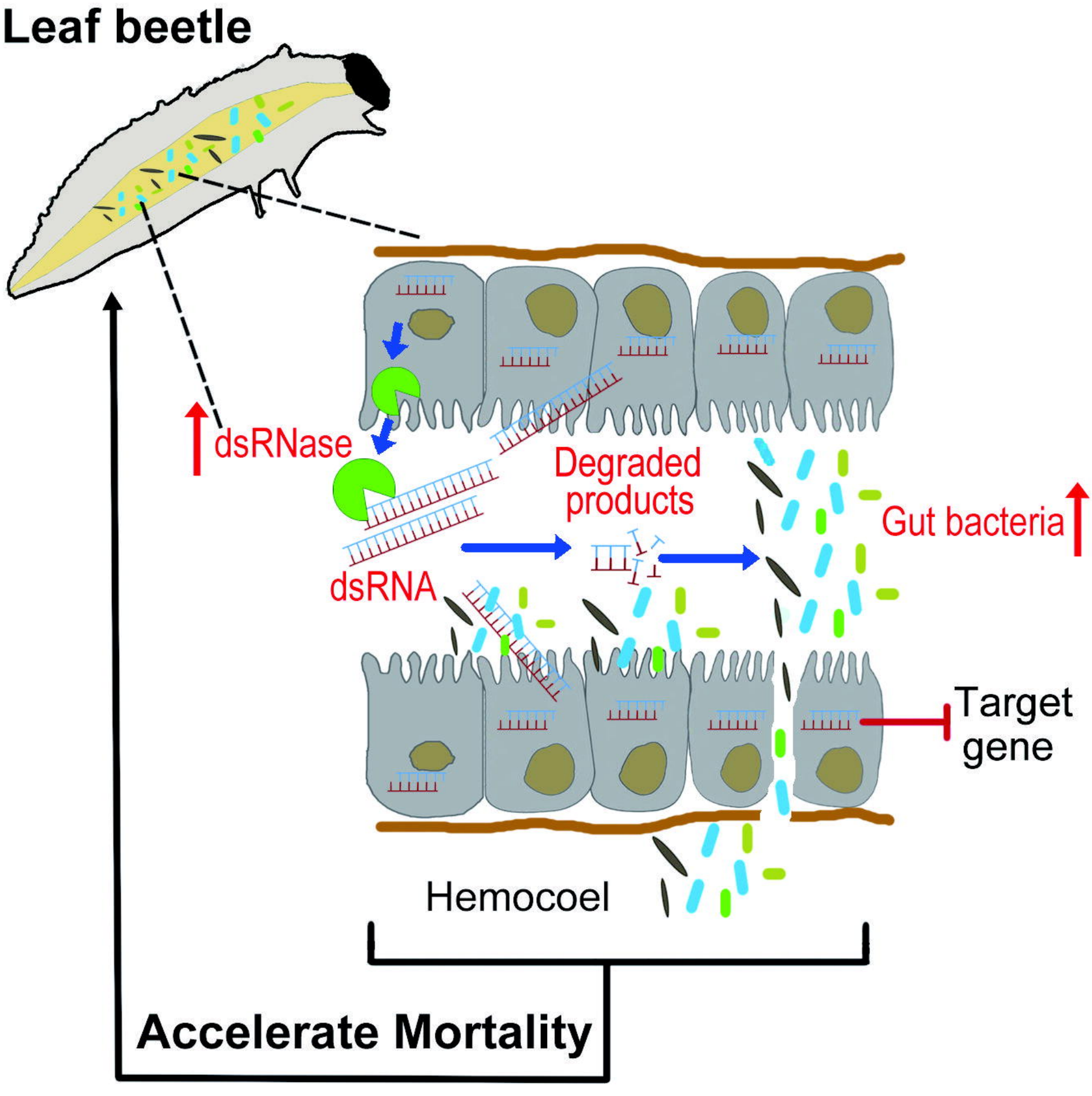
Leaf beetle
dsRNase
Degraded products
Gut bacteria
dsRNA
Target gene
Hemocoel
Accelerate Mortality