Serratia marcescens secretes proteases and chitinases with larvicidal activity against *Anopheles dirus*

Natapong Jupatanakul[1,*], Jutharat Pengon[1], Shiela Marie Gines Selisana[2#], Waeowalee Choksawangkarn[3], Nongluck Jaito[4], Atiporn Saeung[5], Ratchanu Bunyong[1], Navaporn Posayapisit[1], Khrongkhwan Thammatinna[6], Nuttiya Kalpongnukul[7,8], Kittipat Aupalee[5], Trairak Pisitkun[8] and Sumalee Kamchonwongpaisan[1]

[1] Protein-Ligand Engineering and Molecular Biology Research Team, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani, Thailand

[2] Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, Laguna, Philippines

[3] Department of Biochemistry, Faculty of Science, Burapha University, Chonburi, Thailand

[4] Enzyme Technology Research Team, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani, Thailand

[5] Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

[6] Department of Biology, Faculty of Science, Prince of Songkhla University, 15 Karnjanavanich Rd., Hat Yai, Songkhla, Thailand

[7] Inter-Department Program of Biomedical Sciences, Faculty of Graduate, Chulalongkorn University, Bangkok, Thailand

[8] Center of Excellence in Systems Biology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

*Corresponding author: Email: natapong.jup@biotec.or.th

[#]Current address: School of Life Science and Technology, Tokyo Institute of Technology, Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8501, Japan.

ABSTRACT

Vector control, the most efficient tool to reduce mosquito-borne disease transmission, has been compromised by the rise of insecticide resistance. Recent studies suggest the potential of mosquito-associated microbiota as a source for new biocontrol agents or new insecticidal chemotypes. In this study, we identified a strain of *Serratia marcescens* that has larvicidal activity against *Anopheles dirus*, an important malaria vector in Southeast Asia. This bacterium secretes heat-labile larvicidal macromolecules when cultured under static condition at 25°C but not 37°C. Two major protein bands of approximately 55 kDa and 110 kDa were present in spent medium cultured at 25°C but not at 37°C. The Liquid Chromatography-Mass Spectrometry (LC-MS) analyses of these two protein bands identified several proteases and chitinases that were previously reported for insecticidal properties against agricultural insect pests. The treatment with protease and chitinase inhibitors led to a reduction in larvicidal activity, confirming that these two groups of enzymes are responsible for the macromolecule's toxicity. Taken together, our results suggest a potential use of these enzymes in the development of larvicidal agents against *Anopheles* mosquitoes.

***Keywords**: Anopheles*; microbiota; larvicidal; mass spectrometry; entomopathogen

1. Introduction

Aside from nuisances, mosquitoes are vectors of a wide range of human pathogens including *Plasmodium* parasites that cause malaria, filarial nematodes and viruses such as dengue, Zika, yellow fever, and chikungunya. These pathogens cause diseases that are major public health and economical burdens causing approximately 500,000 deaths each year (Shaw and Catteruccia, 2019), with malaria alone accounting for 90% of the deaths. *Anopheles dirus* has been incriminated as the primary vector of the malarial protozoa *P. falciparum* and *P. vivax* in Southeast Asia (Manguin et al., 2008). Although malaria cases and disease burden have significantly decreased over the past decades owing to an extensive effort in mosquito control and antimalarial drug treatment, malaria elimination faces obstacles such as the increasing insecticide resistance of mosquitoes as well as the emergence of antimalarial drug resistance of *Plasmodium* parasites (Whitty and Ansah, 2019). While there are several new classes of antimalarial drugs in the clinical pipelines ("MMV-supported projects | Medicines for Malaria Venture," 2020; Wells et al., 2015), insecticides with new mode of action are much less developed (Shaw and Catteruccia, 2019; Whitty and Ansah, 2019).

Mosquitoes live in constant interactions with microorganisms in their environment and throughout their life cycle (Gendrin and Christophides, 2013; Wang et al., 2011), and in particular, the aquatic stages of the mosquito larvae because they are submerged in water (Dickson et al., 2017). Although microbiota are needed for the healthy development of the mosquito larva (Chouaia et al., 2012; Coon et al., 2014; Dickson et al., 2017; Valzania et al., 2018), certain microbes can be pathogenic to them (Baumann et al., 1991; Laurence et al., 2011). These entomopathogenic microorganisms, such as *Bacillus thuringiensis* and *Bacillus sphaericus* or their by-products, have long been used for mosquito population control (Baumann et al., 1991; Laurence et al., 2011). In addition to these *Bacillus* species, recent studies have identified entomopathogenic bacteria from the mosquito gut microbiota such as *Chromobacterium* spp. and *Serratia marcescens* (Bahia et al., 2014; Ramirez et al., 2014; Short et al., 2018). *Chromobacterium* spp. isolated from the mosquito gut from Panama *(Csp_P)* has insecticidal properties and has potential to be developed as a mosquito larvicidal

agent (Caragata et al., 2020). This suggests that the natural microbial communities associated with mosquitoes are invaluable resources for the discovery of biocontrol agents with new mode of actions and the development of novel insecticides.

Our laboratory has recently set up a new insectary facility to expand our drug development pipeline to test antimalarial compounds against sexual stage of *Plasmodium* parasites. During the optimization of *Anopheles dirus* rearing condition, we found that if the larval food particle size was not fine enough, the food sedimented to the bottom of the pan. Due to the surface feeding nature of *An. dirus*, the sedimented food led to a bacterial growth and biofilm at the bottom of the pan. Mortality of the mosquito larva was observed a few days after the biofilm formed at the bottom of the pan. From this observation, we hypothesized that certain microbes in the larval rearing pan secrete molecule(s) that have larvicidal activity against *An. dirus* after the bacteria grow to a certain density when culture under static condition. In this study, we isolated and identified culturable bacteria with distinct morphology from the *An. dirus* larval rearing pan where larval death was being observed. Of seven isolates, we found a strain of *Serratia marcescens* that secreted heat-labile larvicidal macromolecules into spent medium when it was cultured under static condition at 25°C. We then used mass spectrometry to identify chitinases and proteases as potential larvicidal molecules. Chitinase and protease inhibitors were then used to confirm that these molecules contributed to larvicidal activity. This study demonstrates that the chitinases and proteases uncovered in this study could potentially be used as larvicides against *Anopheles* mosquito.

2. Materials and Methods

2.1 Ethics statement

Animal work in this study was carried out in accordance to the recommendations provided in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were used only as a blood source for mosquito rearing, according to the approved protocol (BT-Animal 26/2560). Larvicidal activity assays were performed following the

97 approved protocol (BT-Animal 20/2560). Both animal protocols were approved by the BIOTEC
98 Committee for use and care of laboratory animals.
99
100 *2.2 Mosquito rearing condition*
101 Specimens of laboratory strain of *An. dirus* were originally collected in Mae Sod
102 District, Tak Province, Thailand. A colony of *An. dirus* was maintained at the Department of
103 Parasitology, Chiang Mai University and BIOTEC's insectary at 27 °C with 75% humidity and
104 a 12-h day/night, 30-min dusk/ dawn lighting cycle. The larvae were fed a diet of powdered
105 fish food (Tetrabit, Germany). Adults were fed on a 10% sucrose solution supplemented with
106 5% multivitamin syrup (Sevenseas, UK) *ad libitum* (Choochote and Saeung, 2013). Female
107 ICR mice were anesthetized using 2% Tribromoethanol and used as a blood source to
108 maintain mosquito colony (approved protocol BT-Animal 26/2560)
109
110 *2.3 Bacterial isolation and identification*
111 Bacteria were isolated from two biological niches, larval rearing water and bacterial
112 biofilm at the bottom of the rearing pan. The rearing water was 10-fold serially diluted with
113 sterile 1X Phosphate buffered saline (PBS). The biofilm was gently rinsed five times with 1X
114 PBS then the biofilm was scraped with sterile plastic scraper, resuspended with 20 mL of
115 sterile PBS then 10-fold serially diluted. The serial dilutions were then plated on Luria-Bertani
116 (LB) and incubated at room temperature for 2 days. Bacterial colonies with distinct
117 morphologies from each ecological niche were then streaked on LB agar plate. A single colony
118 from each bacterial isolate was then propagated and used for molecular identification using
119 16s rRNA gene. Briefly, a colony of each isolate was resuspended in 50 µL of sterile water
120 then 1 µL of resuspended bacteria was used in a PCR reaction using Phusion® High-Fidelity
121 DNA Polymerase (New England Biolabs) with forward (16s-8F: 5'
122 AGAGTTTGATCCTGGCTCAG 3') and reverse (16s-1492R: 5'
123 CGGTTACCTTGTTACGACTT3') primers (Eden et al., 1991). PCR amplification was
124 performed with an initial denaturation of 2 minutes 30 seconds at 98°C, followed with 30 cycles

125 of denaturation at 98°C for 10 seconds, annealing at 52°C for 15 seconds and an extension
126 at 72°C for 1 minute. The amplicons were then purified using QIAquick PCR purification kit
127 then submitted for Sanger DNA sequencing. The obtained bacterial 16s rRNA gene
128 sequences from the forward and reverse primers were manually trimmed and assembled. The
129 assembled sequences were then searched on the NCBI's Basic Local Alignment Search Tool
130 (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990).
131
132 *2.4 Bacteria culture and bacterial spent media preparation*
133 All isolated bacteria were cultured in LB medium under specific conditions as
134 mentioned for each experiment. To start a culture, a colony of each bacterium was inoculated
135 in 10 mL of LB medium then cultured at 25°C, 200 rpm overnight. The absorbance at 600 nm
136 (OD_{600}) was measured for the overnight culture, then 1 mL of culture at OD_{600} = 1 was
137 inoculated into 5 mL of fresh LB medium. For static culture, 18 mL of inoculated culture was
138 added to a 90 mm diameter petri dish then incubated without shaking at specified temperature.
139 For planktonic culture, 18 mL of inoculated culture was added to a 50 mL conical tube then
140 agitated at 200 rpm at specified temperature. After incubation, bacterial cells were removed
141 from the spent media by centrifugation at 4,415 x *g* 4 ºC for 45 minutes. Supernatants were
142 then filtered through 0.22 µm syringe filter (Sartorius). The sterile spent media were then kept
143 at 4 ºC until further use.
144
145 *2.5 Larvicidal activity assay*
146 Second instar larvae of *An. dirus* were filtered and rinsed twice with 250 mL sterile
147 distilled water. Larvicidal assays were performed with 20 mosquitoes in 4 mL of total water
148 volume per treatment per replicate in each well of 6-well plate. Filtered bacteria spent media
149 were added to each well at 10% v/v and mortality was recorded for 72 hours. For the group
150 with antibiotics treatment, Penicillin/Streptomycin solution (Gibco) were added to the well at
151 final concentration of 100 U/mL and 100 µg/mL, respectively.

152 For *Pseudomonas* sp. challenge, the bacterium was cultured overnight at 25 ºC then
153 the cells were collected by centrifugation at 1500 x *g*, 4 ºC for 15 minutes. Bacterial cells were
154 then washed twice with 1X PBS, diluted then added to larvicidal plate at concentrations as
155 specified in the results section.
156
157 *2.6 Characterization of physical property of secreted larvicidal molecules and preparation of*
158 *concentrated macromolecule fraction*
159 We first evaluated whether the secreted larvicidal molecules were proteins
160 (macromolecules) or small molecules because they would require different methods of
161 identification. Thus, the larvicidal molecules were characterized based on the heat stability
162 and molecular weight.
163 The heat stability of the secreted larvicidal molecules was determined by heating the
164 spent media at 45, 70, and 95 ºC for one hour. Heated spent media were then used to test for
165 remaining larvicidal activity.
166 Proteins (macromolecules) and small molecules were separated using Amicon® Ultra-
167 4 10K Centrifugal Filter Device following the manufacturer's instructions. Briefly, 4 ml of spent
168 media were added to the filter unit then centrifuged at 1500 x *g*, 4 ºC for 30 minutes. The flow
169 through was collected as small molecule fraction while the molecules retained on the filter
170 device were used as macromolecule fraction. The concentrated macromolecule fraction was
171 reconstituted with either LB or 1X PBS to concentrations as indicated in each experiment.
172
173 *2.7 Cytotoxicity in C6/36 insect cell line*
174 Cytotoxicity of *S. marcescens* spent medium in insect cell line was determined by
175 sulforhodamine B (SRB) assay according to previously published protocol (Vichai and
176 Kirtikara, 2006) with slight modification for C6/36 cell line. The SRB assay measured cellular
177 protein content to determine cell survival after spent medium treatment. Briefly, C6/36 cells
178 were seeded into each well of 96-well plate at 30,000 cells/well in 100 µl of 10% (v/v) *S.*
179 *marcescens* spent medium in complete L15 medium (L15 supplemented with 10% Fetal

180 bovine serum, 1% MEM non-essential amino acids, 2% Tryptose phosphate broth, 100 U/mL
181 Penicillin and 100 µg/mL Streptomycin) and incubated at 28 ºC for 72 hours. After incubation,
182 cell monolayers were fixed with 10% (w/v) trichloroacetic acid for 45 minutes at 4 ºC and rinsed
183 4 times with tap water. Fixed cell monolayer was then stained with 0.057% (w/v) SRB in 1%
184 acetic acid for 30 minutes at room temperature in the dark. Stained cell monolayers were then
185 washed with 100 µl 1% acetic acid for four times to remove unbound dye. To solubilize protein-
186 bound SRB, 200 µl of 10 mM Tris (pH 10.5) was added to each well then the plate was shaken
187 for 30 minutes at room temperature. Absorbance at 510 nm was then measured in a
188 microplate reader to determine cell viability. LB was used as an untreated control and 5 µM
189 cycloheximide was used as a positive control of the cytotoxicity assay.
190
191 *2.8 In-gel digestion of secreted S. marcescens macromolecules for LC-MS/MS analysis*
192 The cell-free macromolecules of *S. marcescens* static culture at 25 and 37 ºC were
193 mixed with Laemmli buffer, denatured at 95 ºC for 10 minutes then subjected to SDS-PAGE.
194 After the SDS-PAGE fractionation, protein bands present in the 25 but not in 37 ºC culture
195 condition were excised from the gel for in-gel tryptic digestion followed by LC-MS/MS analyses
196 (Shevchenko et al., 2007). Briefly, the gel bands were excised and dehydrated in 100%
197 acetonitrile. Proteins were reduced by 10 mM dithiothreitol at 56°C for 30 min and alkylated
198 by 20 mM chloroacetamide at room temperature for 20 min. The gel bands were further
199 destained using 50 mM NH_4HCO_3 in 50% acetonitrile for 30 min, followed by dehydration in
200 neat acetonitrile. Digestion was carried out by adding 13 ng/µL trypsin in 50 mM NH_4HCO_3 to
201 cover the gel pieces, and the reaction was incubated at 37°C overnight. Extraction of cleaved
202 peptides was performed by adding extraction buffer (70% acetonitrile/1% formic acid) to the
203 digests at the ratio of 2:1 (v/v). Then the extracts were lyophilized and redissolved in 0.1%
204 formic acid ready for LC-MS/MS analyses.
205
206 *2.9 LC-MS/MS analyses of secreted S. marcescens macromolecules*

207 The digested peptides (200 ng) were analyzed by an EASY-nLC 1000 HPLC system
208 (Thermo Fisher Scientific) interfaced with a Q Exactive™ Hybrid Quadrupole-Orbitrap mass
209 spectrometer (Thermo Fisher Scientific) via an EASY-Spray ion source (Thermo Fisher
210 Scientific). The separation was performed for 60 min at the flow rate of 300 nL/min using C18-
211 reverse phase column (EASY-Spray™, Thermo Fisher Scientific). The stepwise gradient was
212 set from 5% solvent B (0.1% formic acid in acetonitrile)/ 95% solvent A (0.1% formic acid in
213 H_2O) to 20% solvent B/ 80% solvent A for 43 min and then to 40% solvent B within the next
214 10 min, followed by holding at 95% for 55 - 60 min. Data-dependent acquisition was performed
215 wherein the ten most abundant precursor ions were selected for fragmentation. The precursor
216 masses were scanned by the Orbitrap mass analyzer at the mass range of 400 – 1600 m/z
217 and the resolution of 70,000. Higher-energy collisional dissociation was employed for
218 fragmentation with an exclusion of +1 charged ions. All data were acquired using Thermo
219 Xcalibur 2.2 software.
220 Mass spectra were searched against NCBI database of *Serratia* sp. (August, 2018)
221 using Proteome Discoverer 2.2 connected to SEQUEST search engine (Eng et al., 1994).
222 Precursor mass tolerance was set to 10 ppm and the product ion mass tolerance was 0.02
223 Dalton. Trypsin was specified with maximum 2 missed cleavages. Carbamidomethylation was
224 chosen as a fixed modification; while oxidation of methionine was allowed as variable
225 modification. Decoy search was performed using the false discovery rate of 0.01 as a
226 threshold.
227
228 *2.10 Chitinase activity assay*
229 Chitinase secretion of *S. marcescens* when cultured in different culture temperature
230 was demonstrated by the ability of the bacterium to generate clear zone on LB agar
231 supplemented with 0.5% chitin. *S. marcescens* inoculated plates were cultured at 25°C or
232 37°C for 10 days before visualization of clear zone.

233 Chitinase activity of spent medium was determined by measuring the ability of the
234 spent medium to digest chitin into a reducing sugar, *N*-acetyl-*D*-glucosamine. To set up the
235 chitinase activity assay, 100 µL of cell-free spent culture media served as a crude enzyme
236 source was mixed with 100 µL of 1% (w/v) chitin powder (Sigma, USA) in 50 mM sodium
237 phosphate buffer (pH 6.5). The reaction mixture was incubated at 30ºC for 90 min. The amount
238 of reducing sugar was then determined by using *p*-hydroxybenzoic acid hydrazide (pHBAH)
239 assay (Lever, 1972). A stock solution of 5% (w/v) pHBAH (Sigma, USA) was prepared in acid
240 medium (0.5 M HCl) and diluted at the moment of analysis to 1% (w/v) in alkaline medium (0.5
241 M NaOH). Samples were mixed with 1% (w/v) pHBAH in alkaline solution (0.5 M NaOH) at a
242 ratio of 1:3 and the mixture was heated in the boiling bath for 5 min to develop the color. After
243 cooling, the solution was centrifuged at 13,800 x *g* for 2 min. The absorbance at 410 nm of
244 the supernatant was then measured using spectrophotometer. *N*-acetyl-*D*-glucosamine
245 standard solution was prepared in the same manner at concentrations of 0, 0.2, 0.4, 0.6, 0.8,
246 and 1 mg/ml in distilled water. One unit of enzyme activity was defined as the amount of
247 enzyme which releases 1 µg of reducing sugar per hour under the assay conditions.
248
249 *2.11 Statistical analyses*
250 Statistical significance of the survival analyses was calculated using Log-rank test
251 followed by a Bonferroni post-hoc analysis using 'survminer ' package (Kassambara et al.,
252 2019) in R (R Core Team, 2020).
253 Concentration of *Pseudomonas* sp. treatment resulting in 50% larval mortality (LC_{50})
254 and 90% larval mortality (LC_{90}) was calculated by Probit analysis with Abbot's modification
255 using 'ecotox' package (Scholefield et al., 2008) in R (R Core Team, 2020).
256 Statistical analysis comparing chitinase activity level and inhibition of chitinase activity
257 was performed using One-way ANOVA with Tukey multiple comparison test using
258 multcompView (Graves and Dorai-Raj, 2019) package in R (R Core Team, 2020).
259
260 **3. Results**

261 *3.1 Isolation of bacteria from An. dirus larval rearing pan*

262 A total of seven bacteria species were isolated and identified from the rearing pan by
263 16s rRNA gene sequencing. These include one Actinobacteria: *Leucobacter* spp. (Leu_spp),
264 two Bactereoidetes: *Sphingobacterium* spp. (Sph_spp) and *Sphingobacterium multivorum*
265 (Sch_m); and four proteobacteria: *Acinetobacter soli* (Aci_s), *Ochrobactrum* spp. (Och_spp),
266 *Serratia marcescens* (Ser_m), and *Stenotrophomonas maltophilia* (Ste_m) as summarized in
267 Table 1.

268 Table 1. Bacterial isolates from *An. dirus* larval rearing pan identified by 16s rRNA gene
269 sequencing

Abbreviation	Species	Phylum	Top known hit	Identities	Gaps
Leu_spp	*Leucobacter* spp.	Actinobacteria	*Leucobacter* sp. Re-6	1332/1332 (100%)	0/1332 (0%)
Sph_spp	*Sphingobacterium* spp.	Bacteroidetes	*Sphingobacterium* sp. strain JPB23	1305/1340 (97%)	0/1340 (0%)
Sph_m	*Sphingobacterium multivorum*	Bacteroidetes	*Sphingobacterium multivorum* strain GZT-2	1341/1342 (99%)	1/1342 (0%)
Ser_m	*Serratia marcescens*	Proteobacteria	Serratia sp. strain p2	1346/1346 (100%)	0/1346 (0%)
Aci_s	*Acinetobacter soli*	Proteobacteria	*Acinetobacter soli* strain GFJ2	1347/1349 (99%)	0/1349 (0%)
Ste_m	*Stenotrophomonas maltophilia*	Proteobacteria	*Stenotrophomonas maltophilia* strain D299	1259/1350 (93%)	1/1350 (0%)
Och_spp	*Ochrobactrum* spp.	Proteobacteria	*Ochrobactrum* sp. Azn6.2	1296/1307 (99%)	10/1307 (0%)

270

271 *3.2 S. marcescens secreted larvicidal molecule into spent medium when cultured under static*
272 *condition*

273 Because larval mortality was observed a few days after bacterial bloom, we
274 hypothesized that the isolated bacteria could secrete larvicidal molecule(s) after the bacteria
275 grow to a certain density; at a time when biofilm was observed at the bottom of the pan for a

276 few days. Therefore, the isolated bacteria were cultured under either static or planktonic
277 conditions for three days before spent media collection for the screening of larvicidal activity.
278 None of the cell-free spent media obtained from planktonic condition had any toxicity
279 against *An. dirus* larvae (Figure 1A). However, for the stationary culture, only the cell-free
280 spent media from *S. marcescens* had a detrimental effect on the survival of the larvae (Figure
281 1A). Next, spent medium of *S. marcescens* cultured under planktonic and static conditions
282 was collected over-time after 1, 2, or 3 days of incubation. Again, the larvicidal molecule was
283 secreted into the *S. marcescens* only under static culture condition and the larvicidal activity
284 of the spent medium increased over time starting from the second day after inoculation (Figure
285 1B).
286 To investigate if the *S. marcescens* cells are entomopathogenic to *An. dirus* larvae,
287 the bacterium was cultured under a static condition then washed with 1X PBS. Washed
288 bacterial cells were then added to larval culture at various concentrations (Figure 1C). Even
289 at OD_{600} of 5, *S. marcescens* cells did not cause significant mortality to *An. dirus* larvae. The
290 *Escherichia coli* cells used as a control also did not cause larval mortality. These results
291 suggested that *S. marcescens* only cause larval mortality through the secretion of
292 entomopathogenic molecules.

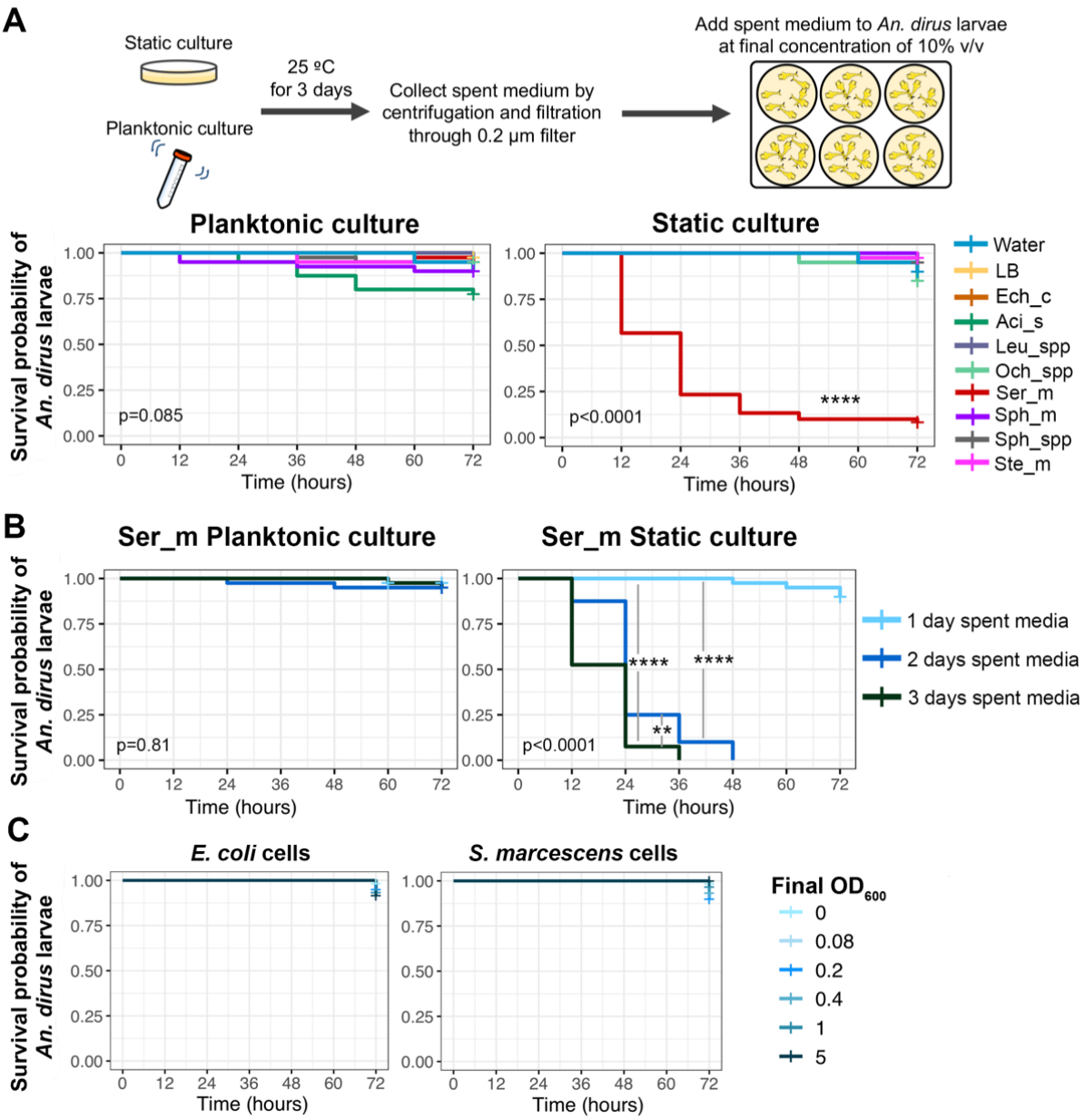


Figure 1. Survival analyses of *An. dirus* mosquito larvae after treatment with bacterial spent media at 10% (v/v) concentration. (A) Survival curve of *An. dirus* larvae after treatment with bacterial spent media cultured under planktonic condition or static condition. The spent media were collected at three days after incubation under planktonic or static state at 25 ºC Ech_c: *Escherichia coli* strain XL10, Aci_s: *Acinetobactor soli*, Leu_spp: Leucobactor spp., Och_spp: Ochrobatrum spp., Ser_m: *Serratia marcescens*, Sph_m: *Sphingobacterium multivorum,* Sph_spp: *Sphingobacterium* spp., Ste_m: *Stenotrophomonas maltophilia*. LB medium or water was used as a control at final concentration of 10% (v/v). The graphs represent data from three independent biological replicates (B) Survival curve of *An. dirus* larvae after

303 treatment with *S. marcescens* spent medium cultured under planktonic or static condition and
304 collected after 1, 2, or 3 days after inoculation. The graphs represent data from two
305 independent biological replicates. (C) Survival curve of *An. dirus* larvae after treatment with *E.*
306 *coli* or *S. marcescens* cells at various concentration. Bacterial cells were cultured under static
307 condition at 25 ºC then washed twice with PBS. The graphs represent data from three
308 independent biological replicates. Statistical significance of the survival analyses was
309 calculated using Log-rank test followed by a Bonferroni post-hoc analysis using 'survminer
310 'package in R. Significance level: **: $p<0.01$, ****: $p<0.0001$.

311 *3.3 Larvicidal molecules from S. marcescens are heat sensitive macromolecules*

312 Because the nature of larvicidal molecule would dictate subsequent methods for
313 identification and characterization, the larvicidal molecule was then next broadly characterized
314 based on heat sensitivity and molecular weight. The *S. marcescens* filtered spent medium was
315 heated at 45, 70 and 95 ºC for one hour then used to determine larvicidal activity at 10% (v/v)
316 (Figure 2A). The larvicidal molecules secreted by *S. marcescens* are heat-sensitive as the
317 heat treatment at 45 ºC for 1 hour significantly reduced the 3-day mortality rate of treated
318 larvae from 100% to 35%. Heat treatment at 70 and 95 ºC completely destroyed the larvicidal
319 activity. To further characterize if the heat-labile active larvicidal elements were small or large
320 macromolecules, we fractionated the active spent media by size exclusion using Amicon®
321 Ultra-4 10K filter device and tested their larvicidal activity. The result shows that only the
322 macromolecule fraction with molecular weight larger than 10 kDa possesses larvicidal activity
323 (Figure 2B).

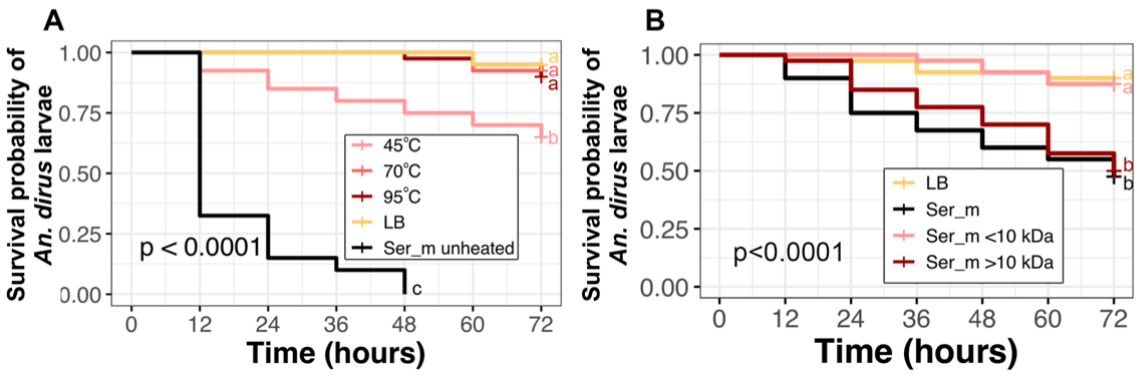
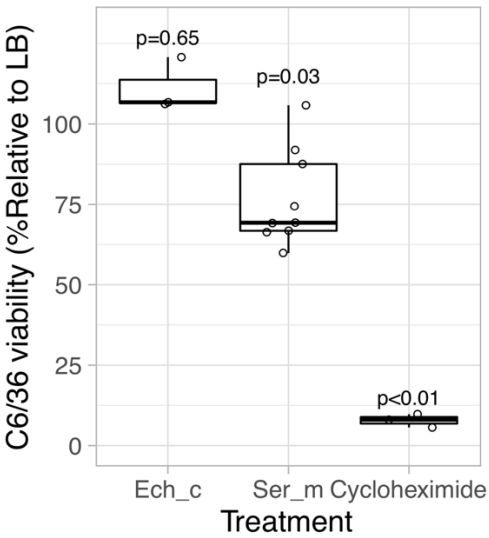


Figure 2. Larvicidal molecule(s) secreted by *S. marcescens* are heat sensitive macromolecule(s). (A) Heat sensitivity of larvicidal activity from *S. marcescens* spent medium. The *S. marcescens* spent medium was heated at 45, 70, and 95 ºC for one hour before testing for larvicidal activity at 10% (v/v) final concentration. LB medium was used as a control at a final concentration of 10% (v/v). The graphs represent data from two independent biological replicates (B) Larvicidal activity of *S. marcescens* spent medium after separation of small (<10 kDa) and macromolecules (>10 kDa) using Amicon® Ultra-4 10K filter device at 10% (v/v). LB medium was used as a control at final concentration of 10% (v/v). The graphs represent data from two independent biological replicates. Statistical significance of the survival analyses was calculated using Log-rank test followed by a Bonferroni post-hoc analysis using 'survminer' package in R.

3.4 Larvicidal activity of S. marcescens spent medium is not likely to be caused by cytotoxicity.

To test the cytotoxicity of *S. marcescens* spent medium against insect cells, the spent medium was used to treat C6/36 cells at the final concentration of 10% (v/v) spent medium in complete L15 medium at 28 ºC for 72 hours. When compared to LB medium treatment, the cell viability of C6/36 treated with *E. coli* spent medium, *S. marcescens* spent medium, and cycloheximide were 111.25±8.23%, 76.79±15.02%, and 7.82±2.06%, respectively (Figure 3). Although statistically significant, the low cytotoxicity level of *S. marcescens* spent medium against C6/36 cells could not support the strong larvicidal activity as shown in our experiments. This could be because the cell does not carry the essential molecule/pathway targeted by *S.*

345 *marcescens*' entomopathogenic molecules. Another possible explanation is that the death of
346 *An. dirus* larvae is due to damage at the physiological level rather than direct toxicity to insect
347 cells.



348

349 Figure 3. C6/36 cell viability assay of *E. coli* spent medium (Ech_c), *S. marcescens* spent
350 medium (Ser_m), and 5 μM cycloheximide. The cell viability was shown relative to LB medium
351 treatment. Spent medium and LB were used at 10% (v/v) final concentration. P-values were
352 calculated using raw data from sulforhodamine B assay by One-way ANOVA with TukeyHSD
353 post hoc test with multcompView package in R software.

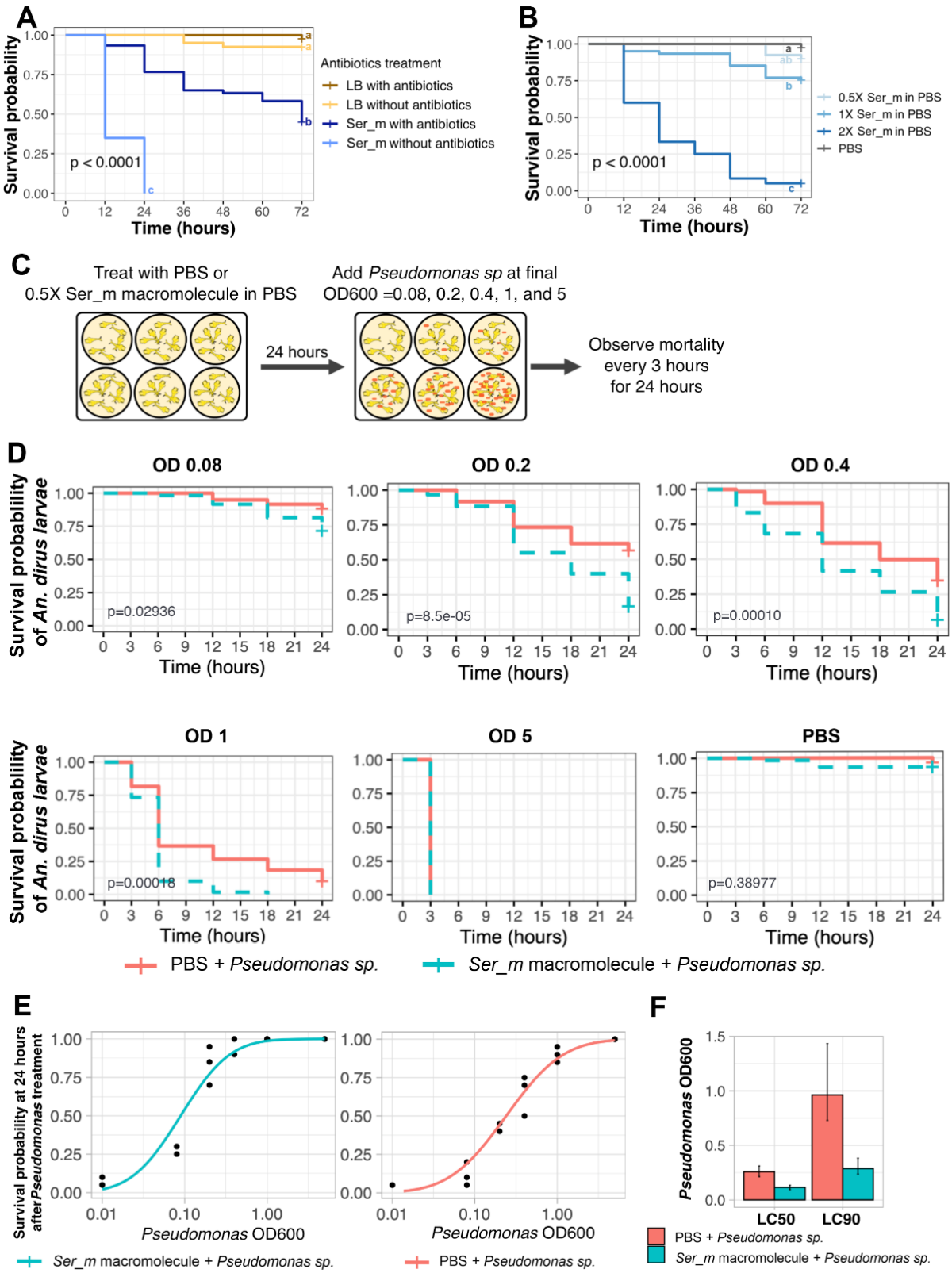
354 *3.5 S. marcescens spent medium can directly kill An. dirus larvae and also enhance larvae's*
355 *susceptibility to bacterial infection.*

356 In previous experiments, LB medium was used as a control in the larvicidal assays.
357 We observed that the presence of LB or filtered spent medium allowed bacteria in the
358 environment to grow in these treatments (Figure 1 and 2). Although these bacteria did not
359 cause larval death in the LB-treated group, they might interfere with the larvicidal activity
360 caused by *S. marcescens* spent medium treatment.

361 To test whether these naturally growing bacteria have any impact on larvicidal activity
362 by *S. marcescens*, penicillin (100 U/mL) and streptomycin (100 μg/mL) were added in the

363 larvicidal assay experiment to prevent bacterial growth. The addition of antibiotics significantly
364 inhibited bacterial contamination because water in each well of the larvicidal assay remained
365 clear throughout the experiment. In the absence of natural bacterial growth, the
366 macromolecules secreted by *S. marcescens* can still kill *An. dirus* larvae confirming its intrinsic
367 toxicity (Figure 4A). Interestingly, the observed mortality in the antibiotics-treated group was
368 significantly lower than the group without antibiotics treatment. In addition to antibiotics
369 treatment, resuspension of *S. marcescens* macromolecules in PBS instead of LB also reduced
370 natural bacterial growth in larvicidal assays. Even without obvious natural bacterial growth, *S.*
371 *marcescens* macromolecule resuspended in PBS could kill *An. dirus* larvae in a concentration-
372 dependent manner (Figure 4B). These results suggest that the larvicidal molecule has intrinsic
373 larvicidal activity, and also increases larvae's susceptibility to bacterial infection.

374 To confirm the hypothesis that *S. marcescens* secreted macromolecules increases
375 larvae's susceptibility to bacterial infection, we pretreated *An. dirus* larvae with a sublethal
376 concentration of *S. marcescens* secreted macromolecule (0.5X diluted in PBS; see Figure 4B)
377 for 24 hours then added an entomopathogenic strain of *Pseudomonas* sp. at the final bacterial
378 density of OD_{600} = 5, 1, 0.4, 0.2, and 0.08 (Figure 4C). *An. dirus* larvae pretreated with *S.*
379 *marcescens* secreted macromolecules significantly increased the mortality rate caused by
380 *Pseudomonas* sp. in all treatment except at OD 5, which the entomopathogenicity was too
381 high to observe the difference (Figure 4D). The *Pseudomonas* sp. LC_{50} of the *S. marcescens*
382 macromolecule-treated group was 0.115 OD_{600}, a 55% decrease from 0.258 OD_{600} in the PBS-
383 treated group. Similarly, the *Pseudomonas* sp. LC_{90} of the *S. marcescens* macromolecule-
384 treated group was 0.287 OD_{600}, a 70% decrease from 0.962 OD_{600} in the PBS-treated group
385 (Figure 4E and 4F). These results confirm significant effect of *S. marcescens* macromolecules
386 on susceptibility of *An. dirus* larvae to bacterial infection, even at sublethal concentration.



387

388 Figure 4. Intrinsic larvicidal activity of *S. marcescens* macromolecule and its enhancing effect

389 on larvae's susceptibility to bacterial infection. (A) Survival of *An. dirus* larvae treated with 2X

390 concentration of macromolecules from *S. marcescens* spent medium diluted in LB with or
391 without antibiotics. LB with or without antibiotics were used as a control. The graphs represent
392 data from two independent biological replicates (B) *An. dirus* larvae treated with *S.*
393 *marcescens* macromolecules reconstituted to the final concentration of 0.5X, 1X, and 2X in
394 PBS. The graphs represent data from two independent biological replicates. (C) Schematic
395 diagram of the treatment of *An. dirus* larvae with sublethal concentration of *S. marcescens*
396 secreted macromolecule followed by *Pseudomonas* sp. treatment. The larvae were treated for
397 24 hours with 0.5x concentration of macromolecules from *S. marcescens* diluted in PBS buffer
398 before addition of *Pseudomonas* sp. at final concentration of OD_{600} = 0.08, 0.2, 0.4, 1, and 5.
399 Mortality of the larvae was monitored every 3 hours for 24 hours. The graphs represent data
400 from three independent biological replicates. (D) Kaplan-Meier curve of *An. dirus* larvae from
401 *Pseudomonas* sp. after treatment with sublethal concentration of *S. marcescens*
402 macromolecules comparing to the PBS control. (E) Probit analysis of *An. dirus* survival
403 probability at 24 hours after treatment of *S. marcescens* macromolecules. (F) LC_{50} and LC_{90}
404 of *Pseudomonas* sp. treatment in *An. dirus* calculated from Probit analysis.

405 *3.6 S. marcescens* secreted different sets of macromolecules when cultured under different
406 conditions

407 Because we observed different levels of larvicidal activity from *S. marcescens* spent
408 medium when cultured under different conditions, the comparison of macromolecules from
409 these culture conditions would help us to pinpoint macromolecule(s) that has larvicidal activity.
410 Although the planktonic condition did not induce secretion of a larvicidal molecule, *S.*
411 *marcescens* barely secreted proteins under planktonic condition. Further comparison between
412 these two conditions would yield several protein candidates for further studies.

413 To identify other culture conditions that might result in differential larvicidal activity, *S.*
414 *marcescens* was cultured under static condition at different temperatures, 25 ºC and 37 ºC.

415 Here, *S. marcescens* spent medium from 25 ºC incubation could kill *An. dirus* larvae while
416 those cultured at 37 ºC could not (Figure 5A).

417 Pattern of secreted protein from *S. marcescens* cultured at 25 ºC and 37 ºC was then
418 compared by SDS-PAGE (Figure 5B). Two major protein bands (approximately 110 and 55
419 kDa) were observed in the spent medium from 25ºC culture but not 37ºC (Figure 5B). These
420 two protein bands were then excised from the gel then subjected to LC-MS/MS for
421 identification.

422 The 110 kDa protein band consists of several protease enzymes including
423 aminopeptidase N, serralysin, and peptidase M60 viral enhancin protein; chitinase enzymes
424 including chain A chitobiase; cell surface protein; DUF4214 domain-containing protein;
425 hemolysin; DUF4214 domain-containing protein; peptide ABC transporter substrate-binding
426 protein; aconitate hydratase; DNA-directed RNA polymerase; formate dehydrogenase-N
427 subunit alpha; glycine dehydrogenase; translation elongation factor Tu; phosphoenolpyruvate
428 carboxykinase; LysR family transcriptional regulator; short-chain dehydrogenase/reductase
429 SDR; phosphorylcholine phosphatase; acyl-CoA dehydrogenase (Supplementary table 1).

430 The 55 kDa protein band also consists of proteases including serralysin, proline
431 dipeptidase, extracellular serine protease precursor, secreted alkaline metalloproteinase, and
432 Xaa-Pro aminopeptidase; chitinase enzyme; serine 3-dehydrogenase; DUF4214 domain-
433 containing protein; arginine-tRNA ligase; succinylarginine dihydrolase; cell surface protein;
434 histidine ammonia-lyase; capsule assembly Wzi family protein; glutathione reductase;
435 phosphoenolpyruvate carboxykinase; flagellin; F0F1 ATP synthase subunit beta; and
436 hypothetical protein (Supplementary table 2).

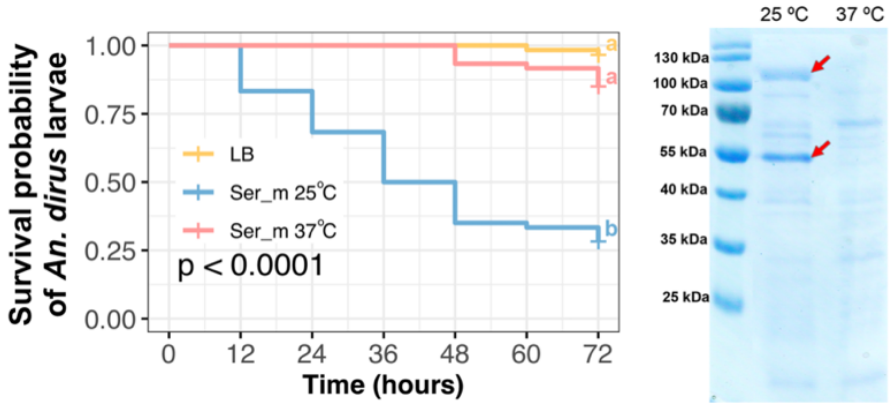


Figure 5. *S. marcescens* secreted larvicidal molecules when cultured under static condition at 25 ºC but not 37 ºC. (A) Survival curve of *An. dirus* larvae after treatment with *S. marcescens* spent media cultured under static condition at 25 ºC or 37 ºC. The graphs represent data from three independent biological replicates. Statistical significance of the survival analyses was calculated using Log-rank test followed by a Bonferroni post-hoc analysis using 'survminer 'package in R. (B) SDS-PAGE of *S. marcescens* spent media cultured under static condition at 25 ºC or 37 ºC. Two red arrows indicate major protein bands that were expressed when the bacterium was cultured at 25 ºC but not 37 ºC.

Supplementary Table 1. LC-MS analysis of 110 kDa protein band showing the NCBI accession number, protein description, percentage of the protein sequence covered by identified peptides (% sequence coverage), the number of identified peptides (# Peptides), total number of identified peptide spectra matched for each identified protein (#PSM), number of amino acid residues in each identified protein (#AA), molecular weight of proteins (MW), calculated pI of proteins (Calc. pI) and the identification score of proteins calculated by Sequest HT algorithms (Protein score).

Accession number	**Protein description**	**% Sequence coverage**	**# Peptides**	**# PSMs**	**# AAs**	**MW [kDa]**	**Calc. pI**	**Protein score**

1081143318	cell surface protein [*Serratia* sp. HMSC15F11]	27	18	84	998	100.9	4.79	210.67
1333427106	DUF4214 domain-containing protein [*Serratia marcescens*]	10	7	65	1000	101.1	4.83	191.57
1047610669	aminopeptidase N [*Serratia marcescens* subsp. marcescens]	15	10	11	872	98.1	5.49	16.76
1184111806	peptidase M60 viral enhancin protein [*Serratia marcescens*]	11	8	9	855	94.3	6.39	16.36
983153020	peptidase M60 viral enhancin protein [*Serratia marcescens*]	11	8	9	855	94.2	6.32	16.29
1091769957	hemolysin [*Serratia marcescens*]	11	13	15	1608	165	8.73	10.8
1027975265	peptide ABC transporter substrate-binding protein [*Serratia marcescens*]	4	2	2	535	59.9	7.2	4.13
542019733	protease [*Serratia marcescens* EGD-HP20]	4	3	3	964	107.6	6.2	3.55
944700465	aconitate hydratase [*Serratia* sp. Leaf50]	2	1	1	891	97.3	5.85	2.77
291423462	DNA-directed RNA polymerase, beta subunit [*Serratia* odorifera DSM 4582]	5	5	6	1365	153	5.2	2.73

1194684911	MULTISPECIES: formate dehydrogenase-N subunit alpha [*Serratia*]	1	1	1	1015	112.7	7.14	2.7
1340368250	glycine dehydrogenase (aminomethyl-transferring), partial [*Serratia* sp. SSNIH2]	4	1	1	246	26.4	8.73	2.45
1047638873	hypothetical protein AN660_0220605 [*Serratia marcescens* subsp. *marcescens*]	2	1	1	718	81.3	5	2.45
1000823267	Translation elongation factor Tu *[Serratia rubidaea*]	2	1	1	394	43.2	5.31	2.3
1047650606	phosphoenolpyruvate carboxykinase (ATP) [*Serratia marcescens* subsp. *marcescens*]	7	4	4	539	59.3	5.5	1.97
1001786477	LysR family transcriptional regulator *[Serratia rubidaea*]	4	1	1	306	33.2	9	1.92
1006552443	DNA-directed RNA polymerase subunit beta' [*Serratia ficaria*]	3	3	3	1408	155	6.74	1.78
1195419500	short-chain dehydrogenase/reductase SDR [*Serratia marcescens*]	2	1	1	297	31.7	7.15	1.71

363988229	succinyl-CoA synthetase alpha subunit [*Serratia symbiotica* str. *'Cinara cedri'*]	3	1	1	291	30.5	7.72	1.7
640696690	MULTISPECIES: phosphorylcholine phosphatase [Serratia]	2	1	1	353	39.8	6.58	1.69
1048498251	Serralysin precursor [*Serratia marcescens*]	9	3	3	487	52.2	4.89	1.67
408489453	Chain A, 1 Chitobiase	6	4	5	858	95.7	6.57	1.64
977285585	Chitobiase precursor [*Serratia marcescens*]	8	5	6	885	98.3	6.52	1.64
948122997	acyl-CoA dehydrogenase [*Serratia* sp. Leaf51]	1	1	1	822	90.2	7.06	1.64

Supplementary Table 2. LC-MS analysis of 55 kDa protein band showing the NCBI accession number, protein description, percentage of the protein sequence covered by identified peptides (% sequence coverage), the number of identified peptides (# Peptides), total number of identified peptide spectra matched for each identified protein (#PSM), number of amino acid residues in each identified protein (#AA), molecular weight of proteins (MW), calculated pI of proteins (Calc. pI) and the identification score of proteins calculated by Sequest HT algorithms (Protein score).

Accession number	**Protein description**	**% Sequence coverage**	**# Peptides**	**# PSMs**	**# AAs**	**MW [kDa]**	**Calc. pI**	**Protein score**
1048498251	Serralysin precursor [*Serratia marcescens*]	36	15	76	487	52.2	4.89	166.7

1205048185	serine 3-dehydrogenase [*Serratia nematodiphila*]	31	13	43	472	50.4	4.75	96.43
452850059	insecticidal serralysin-like protein, partial [*Serratia marcescens*]	52	14	69	320	35	5.33	144.33
1333426919	serine 3-dehydrogenase [*Serratia marcescens*]	25	12	31	472	50.4	4.74	71.38
1268850421	serine 3-dehydrogenase [*Serratia* sp. BW106]	18	8	29	487	51.9	4.82	67.01
1122396880	DUF4214 domain-containing protein [*Serratia marcescens*]	4	3	12	1000	101	4.87	39.48
1047617935	arginine--tRNA ligase [*Serratia marcescens* subsp. marcescens]	22	12	12	576	63.9	5.55	18.71
1039033801	succinylarginine dihydrolase [*Serratia marcescens*]	27	12	12	446	49.4	6.77	11.96
1081143318	cell surface protein [*Serratia* sp. HMSC15F11]	10	6	8	998	100.9	4.79	7.17
1334464241	histidine ammonia-lyase [*Serratia marcescens*]	19	7	8	514	54.9	6	12.68
1127886605	Proline dipeptidase [*Serratia marcescens* SMB2099]	18	7	9	444	50.2	6.38	11.32
977423541	Extracellular serine protease precursor [*Serratia marcescens*]	12	8	9	1034	107.8	5.39	4.24
1280179082	capsule assembly Wzi family protein [*Serratia* sp. TKO39]	17	6	6	478	52.7	5.41	6.61
1027970958	Xaa-Pro aminopeptidase [*Serratia marcescens*]	11	4	5	438	49	5.38	7.85

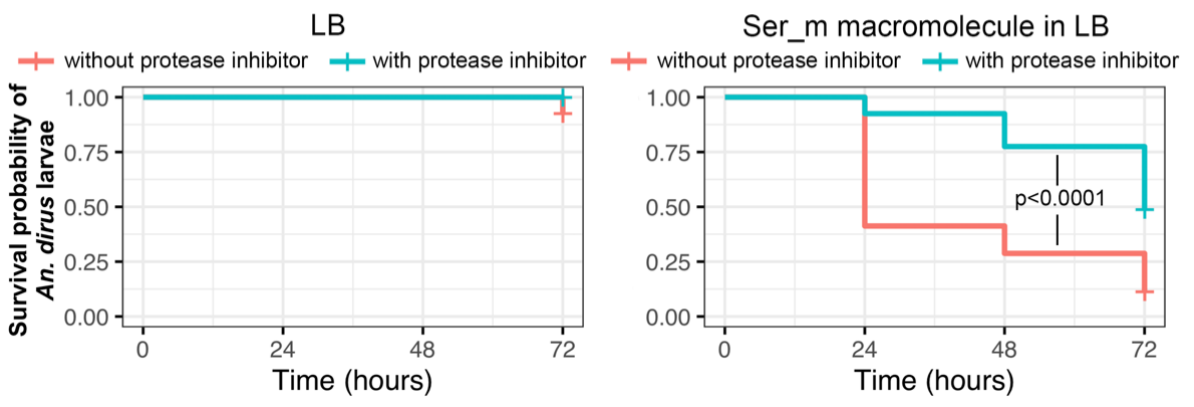
1027968317	glutathione reductase [*Serratia marcescens*]	16	5	5	450	48.5	6.38	6.32
1001783318	phosphoenolpyruvate carboxykinase (ATP) [*Serratia rubidaea*]	5	2	2	539	59	5.25	1.95
1216656901	flagellin [Serratia marcescens]	11	4	5	423	44.4	5.24	1.99
493760228	F0F1 ATP synthase subunit beta [*Serratia symbiotica*]	7	2	2	460	50.2	5.12	2.55
1141208419	serine 3-dehydrogenase [*Serratia oryzae*]	6	3	7	522	55.7	4.54	11.77
1028171175	hypothetical protein [*Serratia* sp. YD25]	4	2	2	568	60.8	4.65	2.17
676309841	N-succinylglutamate 5-semialdehyde dehydrogenase [*Serratia* sp. SCBI]	2	1	1	639	68.5	9.8	2.13
383294472	glucose-6-phosphate isomerase *[Serratia* sp. M24T3]	4	2	2	549	61.2	6.09	1.64
1000823334	Secreted alkaline metalloproteinase [*Serratia rubidaea*]	4	2	6	497	53.2	4.7	9.45
1057964136	chitinase *[Serratia* sp. YD25]	3	2	2	480	51.8	5.62	2
1195419500	short-chain dehydrogenase/reductase SDR [*Serratia marcescens*]	2	1	3	297	31.7	7.15	5.5
1142792582	serine 3-dehydrogenase [*Serratia* sp. S119]	3	1	1	479	52	4.81	1.8

1006546552	NADP-dependent isocitrate dehydrogenase *[Serratia ficaria]*	3	1	1	417	45.6	5.68	2.03

461

462 *3.7 Protease inhibitors reduced larvicidal activity of S. marcescens spent medium.*

463 The LC-MS results indicated that *S. marcescens* secreted several proteases when
464 cultured at 25 ºC under a static condition. Some of these proteases have been reported for
465 insecticidal activity in other insects (Baumann, 2013; Wang and Granados, 1997). Therefore,
466 we added the Complete protease inhibitor (Roche) supplemented with 5 mM EDTA in the
467 larvicidal assays to determine whether these proteases contributed to *S. marcescens* larvicidal
468 activity. We found that protease inhibitors significantly reduced the three-day mortality of the
469 larvae to 50% as compared to 87.5% of the control group without protease inhibitors. This
470 suggests that the larvicidal activity of proteases was partially involved in the observed larval
471 mortality (Figure 6).



472

473 Figure 6. Protease inhibitor reduced larvicidal activity of *S. marcescens* secreted
474 macromolecule. The graphs represent data from four independent biological replicates.
475 Statistical significance of the survival analyses was calculated using Log-rank test followed by
476 a Bonferroni post-hoc analysis using 'survminer 'package in R.

477 *3.8 Chitinase inhibitor reduced larvicidal activity of S. marcescens spent medium.*

478 Because the LC-MS analysis suggested that our strain of *S. marcescens* expressed
479 chitinase and chitobiose enzymes when cultured under static condition at 25 ºC but not 37 ºC,
480 we then inoculated the bacterium on LB agar supplemented with 0.5% chitin then incubated
481 at 25 ºC or 37 ºC to determined chitinase secretion. After ten days of incubation, *S.*
482 *marcescens* cultured at 25 ºC produced a clear zone while the incubation at 37 ºC did not
483 (Figure 7A). Chitinase activity was also determined in the liquid static culture by pHBAH assay.
484 Chitinase activity of *S. marcescens* spent medium increased overtime during the incubation
485 under static condition at 25 ºC and saturated after 3 days of incubation (Figure 7B). This result
486 coincides well with the increase in larvicidal activity during the first three days of *S.*
487 *marcescens* incubation (Figure 1B).

488 Cyclic dipeptides such as Cyclo-Gly-Pro have previously been reported to inhibit
489 chitinase enzyme activity (Houston et al., 2004). This compound was then used to confirm that
490 the larvicidal activity was due to chitinase secretion. The results in Figure 6C show that Cyclo-
491 Gly-Pro can inhibit chitinase activity of *S. marcescens* spent medium in a concentration-
492 dependent manner. At 8 mM, Cyclo-Gly-Pro inhibited chitinase activity by 53.36±12.22%,
493 which was not significantly different from 56.81±8.34% inhibition by 10 mM Cyclo-Gly-Pro
494 (Figure 7C). Thus, 8 mM Cyclo-Gly-Pro was used in the following larvicidal assays. Larvicidal
495 activity was significantly reduced when 8 mM Cyclo-Gly-Pro was added to the spent medium
496 compared to the group without the chitinase inhibitor, albeit not to the same level as the LB
497 control (Figure 7D). This might be due to the incomplete inhibition of the chitinase activity or
498 other enzymes might be contributing to the larvicidal activity in addition to chitinases.

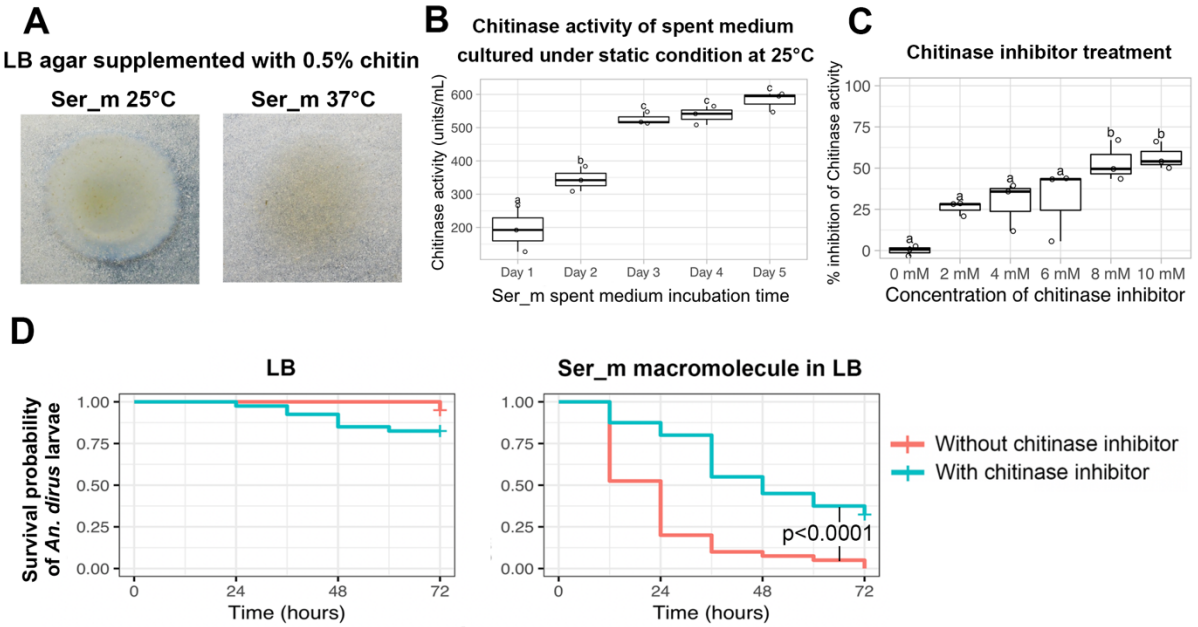
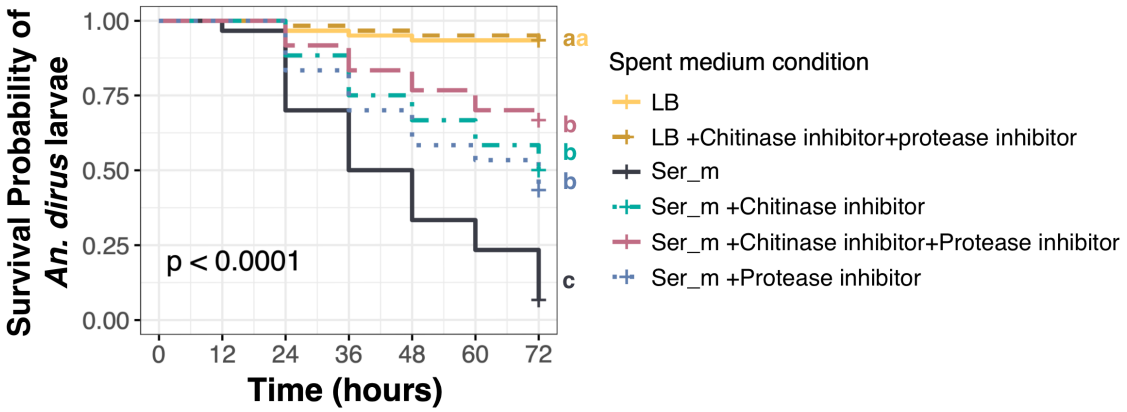


Figure 7. *S. marcescens* secreted macromolecules have chitinase activity and chitinase inhibitor reduced larvicidal activity. (A) *S. marcescens* secreted chitinase that resulted in a clear zone around bacterium colony when cultured at 25 ºC but not 37 ºC. (B) *S. marcescens* spent medium accumulated chitinase activity over time and saturated after three days of incubation. One unit of chitinase activity was defined as the amount of enzyme which releases 1 µg of reducing sugar per hour as measured by pHBAH assay. (C) Cyclo-Gly-Pro inhibit chitinase activity in a concentration-dependent manner. Statistical analyses comparing chitinase activity and inhibition of chitinase activity were performed using One-way ANOVA with Tukey multiple comparison test using multcompView package in R. (D) Chitinase inhibitor reduced larvicidal activity of the *S. marcescens* secreted macromolecule. The graphs represent data from two independent biological replicates. Statistical significance of the survival analyses was calculated using Log-rank test followed by a Bonferroni post-hoc analysis using ‘survminer ‘package in R.

3.9 Combination of protease inhibitors and chitinase inhibitor could not completely inhibit larvicidal activity.

516 Since both the protease inhibitors and chitinase inhibitors could partially reduced
517 larvicidal activity, the combination of both inhibitors rewas used to determine if simultaneous
518 inhibition of proteases and chitinases could completely inhibit the larvicidal activity (Figure 8).
519 The combination of protease and chitinase inhibitors significantly reduced larvae mortality to
520 33.33% from 93.33% in the no inhibitor control. Although not significantly different, the
521 combination of protease and chitinase inhibitors has a trend to further reduce larvicidal activity
522 compared to the treatment with single class of inhibitors (56.66% in the protease inhibitor
523 treatment and 48.33% in the chitinase inhibitor treatment).



524

525 Figure 8: Combination of protease inhibitor and chitinase inhibitor could not completely inhibit
526 larvicidal activity. The graph represents data from three independent biological replicates.
527 Statistical significance of the survival analyses was calculated using Log-rank test followed by
528 a Bonferroni post-hoc analysis using 'survminer 'package in R.

529 **4. Discussion**

530 In this study, a strain of non-prodigiosin producing *S. marcescens* was identified and
531 found to secrete molecules with larvicidal activity against *Anopheles dirus*, a malaria vector in
532 Southeast Asia. *S. marcescens* is a Gram-negative bacterium commonly found in soil and
533 water but not as a human fecal microbiome (Donnenberg, 2015). With such natural habitats,
534 it is often associated with insects including mosquitoes (Gendrin and Christophides, 2013;
535 Wang et al., 2017, 2011). Interestingly, different strains of *S. marcescens* can have different
536 relationship with *Anopheles* mosquitoes raging from symbiotic (Wang et al., 2017) to

537 entomopathogenic (Bahia et al., 2014). The cells of *S. marcescens* isolated in the present
538 study did not kill *An. dirus* larvae directly, but instead can secrete larvicidal molecules into the
539 culture medium when cultured under static condition for at least 2-3 days. The results were in
540 accordance with our original observations that the larval death occurred a few days after larval
541 water became turbid. The fact that the larvicidal molecules were secreted is an advantage for
542 application purposes, given that the secreted factors can be applied instead of the bacterium.
543 Thus preventing or limiting the risk of nosocomial infections (Golemi-Kotra, 2008). The
544 secreted factors can also be expressed heterologously in other expression systems that are
545 more suited for industrial scale production.

546 *S. marcescens* has been extensively studied for its potential as insect pest control
547 agent in agriculture due to its insecticidal property (Bidari et al., 2017; Li et al., 2011; Miller et
548 al., 2008; Pineda-Castellanos et al., 2015; Tu et al., 2010). The bacterium was known to
549 secrete many insect virulence factors including several enzymes, as well as secondary
550 metabolite including a red pigment prodigiosin. Prodigiosin is a metabolite possessing several
551 bioactivities including antimalarial and insecticidal/mosquito larvicidal properties (Bidari et al.,
552 2017; Darshan and Manonmani, 2015; Patil et al., 2011; Suryawanshi et al., 2015). The fact
553 that our *S. marcescens* isolate doesn't produce red pigment suggested that the bacterium
554 secretes other mosquito larvicidal molecules. This finding is in line with a previous study
555 reporting that level of prodigiosin production did not correlate with *S. marcescens* insecticidal
556 activity against silk worm larva (Zhou et al., 2016).

557 The heat-labile larvicidal macromolecules were secreted only when *S. marcescens*
558 was cultured under static condition at lower temperature of 25 ºC but not 37 ºC nor at 25 ºC
559 under planktonic condition. These results suggested that the metabolic activity of this
560 bacterium was influenced by culture conditions, consequently affecting the subsets of
561 molecules secreted by this strain of *S. marcescens*. Comparison of protein expression profiles
562 between the 25 ºC and 37 ºC static cultures helped us to identify potential proteins with
563 larvicidal activity which include many proteases and chitinases. Protease and chitinase

564 activities were then associated with larvicidal activity against *An. dirus* as supplementation of
565 chitinase or protease inhibitors diminished the larvicidal activity. These proteases and
566 chitinases are likely to damage the larvae at physiological levels rather than at the cellular
567 level because the secreted macromolecules lack cytotoxicity against mosquito cells.

568 Serralysin, one of the proteases identified from the mass spectrometry analysis of *S.*
569 *marcescens* secreted macromolecules, has previously been extensively characterized.
570 Serralysin is a metalloprotease first discovered in the culture medium of *Serratia* sp. (Miyata
571 et al., 1970) then later found in other Gram negative bacteria (Baumann, 2013). The protease
572 is secreted as an inactive enzyme and autocatalytically activated in the presence of divalent
573 cations (Baumann, 2013). The enzyme was reported to have entomopathogenic property
574 against larvae of scarab beetle (Pineda-Castellanos et al., 2015). Interestingly, serralysin was
575 found to have immunosuppression property in silk worm and considered as a virulence factor
576 (Ishii et al., 2014). The mechanism of immunosuppression in the study was through the
577 degradation of hemocyte adhesion molecule. Another study demonstrated that a serralysin-
578 type metalloprotease could cleave other proteins involved in insect immunity found in
579 *Manduca sexta* hemolymph (Felföldi et al., 2009)**.** The immune suppression by serralysin
580 could be one of the underlying mechanisms by which *An. dirus* larvae were rendered more
581 susceptible to bacterial infection.

582 One of the proteases that was detected in our LC-MS analysis is Peptidase M60
583 enhancin protein, which is a metalloprotease that requires zinc for its function. Enhancin was
584 first described in baculovirus (Hashimoto et al., 1991) then later found in several bacteria and
585 fungi (Nakjang et al., 2012; Slavicek, 2012). Mucin is an important protein component of insect
586 peritrophic matrix including *Anopheles* mosquito (Dinglasan et al., 2009). Enhancin from
587 baculoviruses could degrade intestinal mucin of the insects thus making them more
588 susceptible to viral infection (Toprak et al., 2012; Wang and Granados, 1997) and Bt toxin
589 (Granados et al., 2001). Bacterial enhancin could also digest mucin (Nakjang et al., 2012), but

590 the impact on baculovirus and Bt toxin toxicity was inconclusive (Fang et al., 2009; Galloway
591 et al., 2005; Zhao et al., 2011).

592 Our results indicate that chitinase and protease inhibitors could only partially reduce
593 the larvicidal activity of the spent medium against *An. dirus* larvae. This suggests that the
594 effect was likely to be a result from a combination of both chitinase and protease enzymes.
595 Although larvicidal activity caused by macromolecules was further reduced when protease
596 inhibitors and chitinase inhibitors were used in combination, the combinatorial effect was
597 marginal and could not completely inhibited the larvicidal activity. Therefore, it is possible that
598 macromolecules other than proteases and chitinases also contributed to the larvicidal activity.
599 This could also be explained by the incomplete inhibition of proteases and chitinases by the
600 inhibitors. As discussed above, several previous studies suggested that larvicidal activity of
601 the identified enzymes were marginal on their own. Contribution of each individual enzyme on
602 larvicidal activity should therefore be further investigated. This can be done by heterologous
603 expression of each enzyme to determine lethal concentrations individually and in combination.

604 Chitin, a polymer of *N*-acetylglucosamine, is the major component of mosquito
605 extracellular structures including the cuticle of the exoskeleton and the peritrophic matrix (PM)
606 of the midgut in arthropods (Liu et al., 2019). These exoskeletal structures provide strength to
607 the body structure, and serve as muscle attachment sites which allow locomotion and flight
608 (Liu et al., 2019). They are also important barriers for the protection of arthropods from the
609 surrounding environment, mechanical damage, heat, desiccation, and invasion of
610 microorganisms (Liu et al., 2019). With all these important functions, damage to the
611 exoskeleton can be fatal to mosquitoes. Several techniques have been developed to
612 compromise the insects' exoskeleton including inhibition of chitin synthesis (Belinato et al.,
613 2013; Doucet and Retnakaran, 2012; Zhang et al., 2010), and degradation of the chitin
614 structure (Berini et al., 2019; Singh and Arya, 2019). The later strategy has been exploited
615 using chitinase enzymes or chitinase-producing microorganisms including those from *S.*
616 *marcescens* (Aggarwal et al., 2015; Binod et al., 2007). However, many studies suggested

617 that chitinases might not be ideal to be used as a stand-alone insecticide for some insects
618 because the larvicidal activity from chitinase can be marginal (Bahar et al., 2012; Zhong et al.,
619 2015). Rather, the enzymes could be used to enhance the insecticidal activity of other control
620 agents including Bt toxin, entomopathogenic virus and chemical insecticides (Kelkenberg et
621 al., 2015; Ramírez-Suero et al., 2011; Zhong et al., 2015). Previous research has
622 demonstrated that chitinases can also help overcome any resistance phenotype developed
623 against a biocontrol strategy. Cai et al. showed that *Bacillus sphaericus* (Bs) toxin-resistant
624 *Culex quinquefasciatus* became susceptible to the Bs treatment when Chitinase AC was
625 overexpressed in *B. sphaericus* (Cai et al., 2007). In addition to the insecticidal property,
626 chitinases can also inhibit fungus by destroying chitinous cell wall structure (Berini et al., 2017;
627 Singh and Arya, 2019; Zarei et al., 2011). *S. marcescens* has been tested to be used against
628 plant fungal pathogen due to its fungicidal properties (Purkayastha et al., 2018) making it an
629 interesting candidate to be used against several classes of agricultural diseases.

630 Peritrophic matrix (PM) is an acellular structure inside the mosquito gut that
631 compartmentalize gut content from the midgut epithelium (Richards and Richards, 1977;
632 Terra, 2001). It serves as a barrier to protect the insect from the digestive enzymes, abrasion
633 from food particles, and invading parasites (Terra, 2001). PM structure consist of chitin and
634 proteins network, with peritrophins as major protein components (Terra, 2001). Several
635 previous studies have shown the insecticidal activity of microbial chitinases and proteases,
636 and their ability to degrade the exoskeleton and perithrophic matrix (Berini et al., 2019, 2018;
637 Chang et al., 2013; Li et al., 2008; Singh and Arya, 2019). Thus, the larvicidal acticity of our
638 *Serratia* culture-derived fractions in combination to the reduction of larvicidal activity when
639 exposed to chitinase and protease inhibitors hints that this is one if the underlying mechanisms
640 of larval killing. However, more histological-based studies would be needed to concretely
641 confirm this assertion. In addition to direct killing, the compromised peritrophic matrix can be
642 responsible for enhanced susceptibility to bacterial infection observed in our study. For
643 instance, in *Drosophila*, disruption of the peritrophic matrix formation renders the fruit fly more

susceptible to bacterial infection (Buchon et al., 2013). The disruption of PM may be the underlying mechanism of the increased larvae's susceptibility to *Pseudomonas* sp. after *S. marcescens* spent medium treatment.

Since chitin and protein are fundamental building blocks of several organisms, the secreted larvicidal chitinase and proteases can have unintended effects to non-target organisms in the same ecological niches. When chitinases are used for agricultural insect pest management, the enzymes can have additional benefit in fungal disease control as chitin also serve as exoskeleton of fungi (Singh and Arya, 2019). However, *An. dirus* used in this study reside in forestry areas (Obsomer et al., 2007). The application of non-specific chitinase and protease larvicides in such area can affect biodiversity in the forestry environment. On the contrary to the *An. dirus* in Southeast Asia, several reports found adaptation of *Anopheline* mosquitoes in urban environment in Africa (Mattah et al., 2017; Takken and Lindsay, 2019). These urban *Anopheline* mosquitoes might be better targets for the protease- or chitinase-based insecticide due to less potential impact on biodiversity but the efficiency of larvicidal activity has to be confirmed in these species. The shorter duration of the larvicidal effect from enzymatic-based larvicidal molecules suggests environmentally friendly alternative to long-lasting chemical pesticides (Winding et al., 2004).

In summary, this study has identified a strain of *S. marcescens* from a larval rearing habitat in the insectary, that secretes chitinases and proteases that are pathogenic to *An. dirus* larvae. In addition to their intrinsic toxicity, these molecules have potential to increase the larvae's susceptibility to natural entomopathogenic microorganisms, and other mosquito control strategies currently in use. Although the secreted larvicidal proteases and chitinases have potential for *Anopheles* mosquito control, further studies on concentration optimization, formulation of enzyme mixtures, and enzyme modifications to enhance heat stability are needed for its development into field applications.

Funding

This work was supported by the National Center for Genetic Engineering and Biotechnology (P1751424) given to N. Jupatanakul. The funders had no role in study design, data collection and interpretation.

Author contributions

Natapong Jupatanakul: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition

Jutharat Pengon: Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing

Shiela Marie Gines Selisana: Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing

Waeowalee Choksawangkarn: Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing

Nongluck Jaito: Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft

Atiporn Saeung: Methodology, Resources, Writing - Review & Editing

Ratchanu Bunyong: Investigation

Navaporn Posayapisit: Investigation

Khrongkhwan Thammatinna: Investigation

Nuttiya Kalpongnukul: Investigation

Kittipat Aupalee: Investigation

Trairak Pisitkun: Methodology, Resources,

Sumalee Kamchonwongpaisan: Resources, Writing - Review & Editing

Declaration of Competing Interest

All authors declare that we have no conflict of interest, financial or otherwise.

Acknowledgements

We thank Jose Luis Ramirez (USDA) for the suggestions and editorial help with the manuscript. We are grateful to Asst. Prof. Dr. Patchara Sriwichai from Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, who kindly provided *An. dirus* adult used to establish the colonies used in this study to A. Saeung.

References:

Aggarwal, C., Paul, S., Tripathi, V., Paul, B., Khan, Md.A., 2015. Chitinolytic activity in *Serratia marcescens* (strain SEN) and potency against different larval instars of Spodoptera litura with effect of sublethal doses on insect development. BioControl 60, 631–640. https://doi.org/10.1007/s10526-015-9674-3

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bahar, A.A., Sezen, K., Demirbağ, Z., Nalçacioğlu, R., 2012. The relationship between insecticidal effects and chitinase activities of Coleopteran-originated entomopathogens and their chitinolytic profile. Ann Microbiol 62, 647–653. https://doi.org/10.1007/s13213-011-0301-y

Bahia, A.C., Dong, Y., Blumberg, B.J., Mlambo, G., Tripathi, A., BenMarzouk-Hidalgo, O.J., Chandra, R., Dimopoulos, G., 2014. Exploring *Anopheles* gut bacteria for *Plasmodium* blocking activity. Environmental Microbiology 16, 2980–2994. https://doi.org/10.1111/1462-2920.12381

Baumann, P., Clark, M.A., Baumann, L., Broadwell, A.H., 1991. *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxins. Microbiology and Molecular Biology Reviews 55, 425–436.

Baumann, U., 2013. Serralysin and Related Enzymes, in: Handbook of Proteolytic Enzymes. Elsevier, pp. 864–867.

Belinato, T.A., Martins, A.J., Lima, J.B.P., Valle, D., 2013. Effect of triflumuron, a chitin synthesis inhibitor, on *Aedes aegypti, Aedes albopictus* and *Culex quinquefasciatus* under laboratory conditions. Parasites & Vectors 6, 83. https://doi.org/10.1186/1756-3305-6-83

Berini, F., Casartelli, M., Montali, A., Reguzzoni, M., Tettamanti, G., Marinelli, F., 2019. Metagenome-Sourced Microbial Chitinases as Potential Insecticide Proteins. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.01358

726 Berini, F., Katz, C., Gruzdev, N., Casartelli, M., Tettamanti, G., Marinelli, F., 2018. Microbial and viral
727 chitinases: Attractive biopesticides for integrated pest management. Biotechnology Advances,
728 Prospects in Biotechnology 36, 818–838. https://doi.org/10.1016/j.biotechadv.2018.01.002
729 Berini, F., Presti, I., Beltrametti, F., Pedroli, M., Vårum, K.M., Pollegioni, L., Sjöling, S., Marinelli, F.,
730 2017. Production and characterization of a novel antifungal chitinase identified by functional
731 screening of a suppressive-soil metagenome. Microbial Cell Factories 16, 16.
732 https://doi.org/10.1186/s12934-017-0634-8
733 Bidari, F., Shams-Bakhsh, M., Mehrabadi, M., 2017. Isolation and characterization of a *Serratia*
734 *marcescens* with insecticidal activity from *Polyphylla olivieri* (Col.: Scarabaeidae) 142, 162–
735 172. https://doi.org/10.1111/jen.12421
736 Binod, P., Sukumaran, R.K., Shirke, S.V., Rajput, J.C., Pandey, A., 2007. Evaluation of fungal culture
737 filtrate containing chitinase as a biocontrol agent against *Helicoverpa armigera*. Journal of
738 Applied Microbiology 103, 1845–1852. https://doi.org/10.1111/j.1365-2672.2007.03428.x
739 Buchon, N., Osman, D., David, F.P.A., Fang, H.Y., Boquete, J.-P., Deplancke, B., Lemaitre, B., 2013.
740 Morphological and Molecular Characterization of Adult Midgut Compartmentalization in
741 *Drosophila*. CellReports 3, 1725–1738. https://doi.org/10.1016/j.celrep.2013.04.001
742 Cai, Y., Yan, J., Hu, X., Han, B., Yuan, Z., 2007. Improving the Insecticidal Activity against Resistant
743 *Culex quinquefasciatus* Mosquitoes by Expression of Chitinase Gene *chiAC* in *Bacillus*
744 *sphaericus*. Applied and Environmental Microbiology 73, 7744–7746.
745 https://doi.org/10.1128/aem.01510-07
746 Caragata, E.P., Otero, L.M., Carlson, J.S., Dizaji, N.B., Dimopoulos, G., 2020. A non-live preparation
747 of *Chromobacterium* sp. Panama (Csp_P) is a highly effective larval mosquito biopesticide.
748 Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.00240-20
749 Chang, Y.-T., Hsieh, C., Wu, L.-C., Chang, H.C., Kao, S.-S., Meng, M., Hsieh, F.-C., 2013.
750 Purification and Properties of an Insecticidal Metalloprotease Produced by *Photorhabdus*
751 *luminescens* Strain 0805-P5G, the Entomopathogenic Nematode Symbiont. International
752 journal of molecular sciences 14, 308–321. https://doi.org/10.3390/ijms14010308
753 Choochote, W., Saeung, A., 2013. Systematic Techniques for the Recognition of *Anopheles* Species
754 Complexes, in: Manguin, S. (Ed.), *Anopheles* Mosquitoes - New Insights into Malaria Vectors.
755 InTech. https://doi.org/10.5772/54853

756 Chouaia, B., Rossi, P., Epis, S., Mosca, M., Ricci, I., Damiani, C., Ulissi, U., Crotti, E., Daffonchio, D.,
757 Bandi, C., Favia, G., 2012. Delayed larval development in *Anopheles* mosquitoes deprived of
758 *Asaia* bacterial symbionts. BMC Microbiology 12, S2. https://doi.org/10.1186/1471-2180-12-
759 S1-S2
760 Coon, K.L., Vogel, K.J., Brown, M.R., Strand, M.R., 2014. Mosquitoes rely on their gut microbiota for
761 development. Molecular Ecology 23, 2727–2739. https://doi.org/10.1111/mec.12771
762 Darshan, N., Manonmani, H.K., 2015. Prodigiosin and its potential applications. Journal of Food
763 Science and Technology 52, 5393–5407. https://doi.org/10.1007/s13197-015-1740-4
764 Dickson, L.B., Jiolle, D., Minard, G., Moltini-Conclois, I., Volant, S., Ghozlane, A., Bouchier, C., Ayala,
765 D., Paupy, C., Moro, C.V., Lambrechts, L., 2017. Carryover effects of larval exposure to
766 different environmental bacteria drive adult trait variation in a mosquito vector. Science
767 Advances 3, e1700585. https://doi.org/10.1126/sciadv.1700585
768 Dinglasan, R., Devenport, M., Florens, L., Johnson, J., McHugh, C., Donnelly-Doman, M., Carucci, D.,
769 Yates, J., Jacobs-Lorena, M., 2009. The *Anopheles gambiae* Adult Midgut Peritrophic Matrix
770 Proteome. Insect Biochem Mol Biol 39, 125–134. https://doi.org/10.1016/j.ibmb.2008.10.010
771 Donnenberg, M.S., 2015. Enterobacteriaceae, in: Principles and Practice of Infectious Diseases. p.
772 2503.
773 Doucet, D., Retnakaran, A., 2012. Insect Chitin: Metabolism, Genomics and Pest Management, in:
774 Dhadialla, T.S. (Ed.), Advances in Insect Physiology, Insect Growth Disruptors. Academic
775 Press, pp. 437–511. https://doi.org/10.1016/B978-0-12-391500-9.00006-1
776 Eden, P.A., Schmidt, T.M., Blakemore, R.P., Pace, N.R., 1991. Phylogenetic Analysis of
777 *Aquaspirillum magnetotacticum* Using Polymerase Chain Reaction-Amplified 16S rRNA-
778 Specific DNA. International Journal of Systematic Bacteriology 41, 324–325.
779 https://doi.org/10.1099/00207713-41-2-324
780 Eng, J.K., McCormack, A.L., Yates, J.R., 1994. An approach to correlate tandem mass spectral data
781 of peptides with amino acid sequences in a protein database. J Am Soc Mass Spectr 5, 976–
782 89. https://doi.org/10.1016/1044-0305(94)80016-2
783 Fang, S., Wang, L., Guo, W., Zhang, X., Peng, D., Luo, C., Yu, Z., Sun, M., 2009. *Bacillus*
784 *thuringiensis* Bel Protein Enhances the Toxicity of Cry1Ac Protein to *Helicoverpa armigera*

785 Larvae by Degrading Insect Intestinal Mucin. Appl. Environ. Microbiol. 75, 5237–5243.
786 https://doi.org/10.1128/AEM.00532-09
787 Felföldi, G., Marokházi, J., Képiró, M., Venekei, I., 2009. Identification of Natural Target Proteins
788 Indicates Functions of a Serralysin-Type Metalloprotease, PrtA, in Anti-Immune Mechanisms.
789 Appl. Environ. Microbiol. 75, 3120–3126. https://doi.org/10.1128/AEM.02271-08
790 Galloway, C.S., Wang, P., Winstanley, D., Jones, I.M., 2005. Comparison of the bacterial Enhancin-
791 like proteins from *Yersinia* and *Bacillus* spp. with a baculovirus Enhancin. Journal of
792 Invertebrate Pathology 90, 134–137. https://doi.org/10.1016/j.jip.2005.06.008
793 Gendrin, M., Christophides, G.K., 2013. The *Anopheles* Mosquito Microbiota and Their Impact on
794 Pathogen Transmission, in: Manguin, S. (Ed.), Anopheles Mosquitoes - New Insights into
795 Malaria Vectors. InTech. https://doi.org/10.5772/55107
796 Golemi-Kotra, D., 2008. *Serratia, Edwardsiella* and *Morganella* Infections, in: Enna, S.J., Bylund, D.B.
797 (Eds.), XPharm: The Comprehensive Pharmacology Reference. Elsevier, New York, pp. 1–6.
798 https://doi.org/10.1016/B978-008055232-3.63827-9
799 Granados, R.R., Fu, Y., Corsaro, B., Hughes, P.R., 2001. Enhancement of *Bacillus thuringiensis*
800 Toxicity to *Lepidopterous* Species with the Enhancin from *Trichoplusia* ni Granulovirus.
801 Biological Control 20, 153–159. https://doi.org/10.1006/bcon.2000.0891
802 Graves, S., Dorai-Raj, H.-P.P. and L.S. with help from S., 2019. multcompView: Visualizations of
803 Paired Comparisons.
804 Hashimoto, Y., Corsaro, B.G., Granados, R.R., 1991. Location and nucleotide sequence of the gene
805 encoding the viral enhancing factor of the *Trichoplusia* ni granulosis virus. Journal of General
806 Virology 72, 2645–2651. https://doi.org/10.1099/0022-1317-72-11-2645
807 Houston, D.R., Synstad, B., Eijsink, V.G.H., Stark, M.J.R., Eggleston, I.M., Aalten, D.M.F. van, 2004.
808 Structure-based exploration of cyclic dipeptide chitinase inhibitors. 47, 5713–5720.
809 https://doi.org/10.1021/jm049940a
810 Ishii, K., Adachi, T., Hamamoto, H., Sekimizu, K., 2014. *Serratia marcescens* Suppresses Host
811 Cellular Immunity via the Production of an Adhesion-inhibitory Factor against
812 Immunosurveillance Cells. J. Biol. Chem. 289, 5876–5888.
813 https://doi.org/10.1074/jbc.M113.544536

814 Kassambara, A., Kosinski, M., Biecek, P., 2019. survminer: Drawing Survival Curves using “ggplot2”
815 [WWW Document]. URL https://CRAN.R-project.org/package=survminer
816 Kelkenberg, M., Odman-Naresh, J., Muthukrishnan, S., Merzendorfer, H., 2015. Chitin is a necessary
817 component to maintain the barrier function of the peritrophic matrix in the insect midgut.
818 Insect Biochemistry and Molecular Biology 56, 21–28.
819 https://doi.org/10.1016/j.ibmb.2014.11.005
820 Laurence, D., Christophe, L., Roger, F., 2011. Using the Bio-Insecticide *Bacillus Thuringiensis*
821 Israelensis in Mosquito Control, in: Stoytcheva, M. (Ed.), Pesticides in the Modern World -
822 Pests Control and Pesticides Exposure and Toxicity Assessment. InTech.
823 https://doi.org/10.5772/17005
824 Lever, M., 1972. A new reaction for colorimetric determination of carbohydrates. Analytical
825 Biochemistry 47, 273–279. https://doi.org/10.1016/0003-2697(72)90301-6
826 Li, B., Yu, R., Liu, B., Tang, Q., Zhang, G., Wang, Y., Xie, G., Sun, G., 2011. Characterization and
827 Comparison of *Serratia marcescens* Isolated from Edible Cactus and from Silkworm for
828 Virulence Potential and Chitosan Susceptibility 42, 96–104. https://doi.org/10.1590/s1517-
829 83822011000100013
830 Li, H., Tang, H., Sivakumar, S., Philip, J., Harrison, R.L., Gatehouse, J.A., Bonning, B.C., 2008.
831 Insecticidal activity of a basement membrane-degrading protease against *Heliothis virescens*
832 (Fabricius) and *Acyrthosiphon pisum (*Harris). Journal of Insect Physiology 54, 777–789.
833 https://doi.org/10.1016/j.jinsphys.2008.02.008
834 Liu, X., Zhang, J., Zhu, K.Y., 2019. Chitin in Arthropods: Biosynthesis, Modification, and Metabolism,
835 in: Yang, Q., Fukamizo, T. (Eds.), Targeting Chitin-Containing Organisms, Advances in
836 Experimental Medicine and Biology. Springer, Singapore, pp. 169–207.
837 https://doi.org/10.1007/978-981-13-7318-3_9
838 Manguin, S., Garros, C., Dusfour, I., Harbach, R.E., Coosemans, M., 2008. Bionomics, taxonomy,
839 and distribution of the major malaria vector taxa of *Anopheles* subgenus Cellia in Southeast
840 Asia: An updated review. Infection, Genetics and Evolution 8, 489–503.
841 https://doi.org/10.1016/j.meegid.2007.11.004
842 Mattah, P.A.D., Futagbi, G., Amekudzi, L.K., Mattah, M.M., de Souza, D.K., Kartey-Attipoe, W.D.,
843 Bimi, L., Wilson, M.D., 2017. Diversity in breeding sites and distribution of *Anopheles*

844 mosquitoes in selected urban areas of southern Ghana. Parasites & Vectors 10, 25.
845 https://doi.org/10.1186/s13071-016-1941-3
846 Miller, T.A., Lauzon, C.R., Lampe, D.J., 2008. Technological advances to enhance agricultural pest
847 management. 627, 141–150. https://doi.org/10.1007/978-0-387-78225-6_12
848 Miyata, K., Maejima, K., Tomoda, K., Isono, M., 1970. *Serratia* Protease. Agricultural and Biological
849 Chemistry 34, 310–318. https://doi.org/10.1080/00021369.1970.10859598
850 MMV-supported projects | Medicines for Malaria Venture [WWW Document], 2020. URL
851 https://www.mmv.org/research-development/mmv-supported-projects (accessed 4.5.20).
852 Nakjang, S., Ndeh, D.A., Wipat, A., Bolam, D.N., Hirt, R.P., 2012. A Novel Extracellular
853 Metallopeptidase Domain Shared by Animal Host-Associated Mutualistic and Pathogenic
854 Microbes. PLOS ONE 7, e30287. https://doi.org/10.1371/journal.pone.0030287
855 Obsomer, V., Defourny, P., Coosemans, M., 2007. The *Anopheles dirus* complex: spatial distribution
856 and environmental drivers. Malaria Journal 6, 26–16. https://doi.org/10.1186/1475-2875-6-26
857 Patil, C.D., Patil, S.V., Salunke, B.K., Salunkhe, R.B., 2011. Prodigiosin produced by *Serratia*
858 *marcescens* NMCC46 as a mosquito larvicidal agent against *Aedes aegypti* and *Anopheles*
859 *stephensi*. Parasitology Research 109, 1179–1187. https://doi.org/10.1007/s00436-011-2365-
860 9
861 Pineda-Castellanos, M.L., Rodríguez-Segura, Z., Villalobos, F.J., Hernández, L., Lina, L., Nuñez-
862 Valdez, M.E., 2015. Pathogenicity of Isolates of *Serratia marcescens* towards Larvae of the
863 Scarab *Phyllophaga blanchardi* (Coleoptera). Pathogens 4, 210–228.
864 https://doi.org/10.3390/pathogens4020210
865 Purkayastha, G.D., Mangar, P., Saha, A., Saha, D., 2018. Evaluation of the biocontrol efficacy of a
866 *Serratia marcescens* strain indigenous to tea rhizosphere for the management of root rot
867 disease in tea. PLOS ONE 13, e0191761. https://doi.org/10.1371/journal.pone.0191761
868 R Core Team, 2020. R: The R Project for Statistical Computing. R Foundation for Statistical
869 Computing, Vienna, Austria.
870 Ramirez, J.L., Short, S.M., Bahia, A.C., Saraiva, R.G., Dong, Y., Kang, S., Tripathi, A., Mlambo, G.,
871 Dimopoulos, G., 2014. *Chromobacterium Csp*_P Reduces Malaria and Dengue Infection in
872 Vector Mosquitoes and Has Entomopathogenic and In Vitro Anti-pathogen Activities. PLoS
873 Pathogens 10, e1004398-13. https://doi.org/10.1371/journal.ppat.1004398

874 Ramírez-Suero, M., Valerio-Alfaro, G., Bernal, J.S., Ramírez-Lepe, M., 2011. Synergisitic effect of
875 chitinases and *Bacillus thuringiensis* israelensis spore-toxin complex against *Aedes aegypti*
876 larvae. The Canadian Entomologist 143, 157–164. https://doi.org/10.4039/n10-051
877 Richards, A.G., Richards, P.A., 1977. The Peritrophic Membranes of Insects. Annu. Rev. Entomol. 22,
878 219–240. https://doi.org/10.1146/annurev.en.22.010177.001251
879 Scholefield, R.J., Slaght, K.S., Stephens, B.E., 2008. Seasonal Variation in Sensitivity of Larval Sea
880 Lampreys to the Lampricide 3-Trifluoromethyl-4-Nitrophenol. North American Journal of
881 Fisheries Management 28, 1609–1617. https://doi.org/10.1577/M06-178.1
882 Shaw, W.R., Catteruccia, F., 2019. Vector biology meets disease control: using basic research to fight
883 vector-borne diseases. Nature Microbiology 4, 20–34. https://doi.org/10.1038/s41564-018-
884 0214-7
885 Shevchenko, A., Tomas, H., Havli, J., Olsen, J.V., Mann, M., 2007. In-gel digestion for mass
886 spectrometric characterization of proteins and proteomes. Nat Protoc 1, 2856–2860.
887 https://doi.org/10.1038/nprot.2006.468
888 Short, S.M., Tol, S. van, MacLeod, H.J., Dimopoulos, G., 2018. Hydrogen cyanide produced by the
889 soil bacterium *Chromobacterium* sp. Panama contributes to mortality in *Anopheles gambiae*
890 mosquito larvae. Nature Publishing Group 8, 1–13. https://doi.org/10.1038/s41598-018-
891 26680-2
892 Singh, G., Arya, S.K., 2019. Antifungal and insecticidal potential of chitinases: A credible choice for
893 the eco-friendly farming. Biocatalysis and Agricultural Biotechnology 20, 101289.
894 https://doi.org/10.1016/j.bcab.2019.101289
895 Slavicek, J.M., 2012. Baculovirus Enhancins and Their Role in Viral Pathogenicity, in: Molecular
896 Virology. IntechOpen.
897 Suryawanshi, R.K., Patil, C.D., Borase, H.P., Narkhede, C.P., Salunke, B.K., Patil, S.V., 2015.
898 Mosquito larvicidal and pupaecidal potential of prodigiosin from *Serratia marcescens* and
899 understanding its mechanism of action. Pesticide Biochemistry and Physiology 123, 49–55.
900 https://doi.org/10.1016/j.pestbp.2015.01.018
901 Takken, W., Lindsay, S., 2019. Increased Threat of Urban Malaria from *Anopheles stephensi*
902 Mosquitoes, Africa. Emerg. Infect. Dis. 25, 1431–1433.
903 https://doi.org/10.3201/eid2507.190301

904 Terra, W.R., 2001. The origin and functions of the insect peritrophic membrane and peritrophic gel.
905 Arch. Insect Biochem. Physiol. 47, 47–61. https://doi.org/10.1002/arch.1036
906 Toprak, U., Harris, S., Baldwin, D., Theilmann, D., Gillott, C., Hegedus, D.D., Erlandson, M.A., 2012.
907 Role of enhancin in *Mamestra configurata* nucleopolyhedrovirus virulence: selective
908 degradation of host peritrophic matrix proteins. Journal of General Virology, 93, 744–753.
909 https://doi.org/10.1099/vir.0.038117-0
910 Tu, S., Qiu, X., Cao, L., Han, R., Zhang, Y., Liu, X., 2010. Expression and characterization of the
911 chitinases from Serratia marcescens GEI strain for the control of Varroa destructor, a honey
912 bee parasite 104, 75–82. https://doi.org/10.1016/j.jip.2010.02.002
913 Valzania, L., Martinson, V.G., Harrison, R.E., Boyd, B.M., Coon, K.L., Brown, M.R., Strand, M.R.,
914 2018. Both living bacteria and eukaryotes in the mosquito gut promote growth of larvae. PLoS
915 Neglected Tropical Diseases 12, e0006638. https://doi.org/10.1371/journal.pntd.0006638
916 Vichai, V., Kirtikara, K., 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nature
917 Protocols 1, 1112–1116. https://doi.org/10.1038/nprot.2006.179
918 Wang, P., Granados, R.R., 1997. An intestinal mucin is the target substrate for a baculovirus
919 enhancin. PNAS 94, 6977–6982. https://doi.org/10.1073/pnas.94.13.6977
920 Wang, S., Dos-Santos, A.L.A., Huang, W., Liu, K.C., Oshaghi, M.A., Wei, G., Agre, P., Jacobs-
921 Lorena, M., 2017. Driving mosquito refractoriness to *Plasmodium falciparum* with engineered
922 symbiotic bacteria. Science 357, 1399–1402. https://doi.org/10.1126/science.aan5478
923 Wang, Y., Iii, T.M.G., Kukutla, P., Yan, G., Xu, J., 2011. Dynamic Gut Microbiome across Life History
924 of the Malaria Mosquito *Anopheles gambiae* in Kenya. PLOS ONE 6, e24767.
925 https://doi.org/10.1371/journal.pone.0024767
926 Wells, T.N.C., van Huijsduijnen, R.H., Van Voorhis, W.C., 2015. Malaria medicines: a glass half full?
927 Nature Reviews Drug Discovery 14, 424–442. https://doi.org/10.1038/nrd4573
928 Whitty, C.J.M., Ansah, E., 2019. Malaria control stalls in high incidence areas. BMJ 365.
929 https://doi.org/10.1136/bmj.l2216
930 Winding, A., Binnerup, S.J., Pritchard, H., 2004. Non-target effects of bacterial biological control
931 agents suppressing root pathogenic fungi. FEMS Microbiol Ecol 47, 129–141.
932 https://doi.org/10.1016/S0168-6496(03)00261-7

933 Zarei, M., Aminzadeh, S., Zolgharnein, H., Safahieh, A., Daliri, M., Noghabi, K.A., Ghoroghi, A.,
934 Motallebi, A., 2011. Characterization of a chitinase with antifungal activity from a native
935 *Serratia marcescens* B4A. Braz J Microbiol 42, 1017–1029. https://doi.org/10.1590/S1517-
936 838220110003000022
937 Zhang, X., Zhang, J., Zhu, K.Y., 2010. Chitosan/double-stranded RNA nanoparticle-mediated RNA
938 interference to silence chitin synthase genes through larval feeding in the African malaria
939 mosquito (*Anopheles gambiae*). Insect Molecular Biology 19, 683–693.
940 https://doi.org/10.1111/j.1365-2583.2010.01029.x
941 Zhao, D., Guo, W., Sun, W., Xu, D., Liu, D., 2011. Identification of a novel enhancin-like gene from
942 *Bacillus thuringiensis*. Front. Agric. China 5, 423–429. https://doi.org/10.1007/s11703-011-
943 1117-z
944 Zhong, W., Ding, S., Guo, H., Zhong, W., Ding, S., Guo, H., 2015. The chitinase C gene PsChiC from
945 *Pseudomonas* sp. and its synergistic effects on larvicidal activity. Genetics and Molecular
946 Biology 38, 366–372. https://doi.org/10.1590/S1415-475738320140320
947 Zhou, W., Li, J., Chen, J., Liu, X., Xiang, T., Zhang, L., Wan, Y., 2016. The red pigment prodigiosin is
948 not an essential virulence factor in entomopathogenic *Serratia marcescens*. Journal of
949 Invertebrate Pathology 136, 92–94. https://doi.org/10.1016/j.jip.2016.03.011
950