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 Songkla 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 chitinase and protease inhibitors led to a reduction in larvicidal activity, confirming that these two groups of enzymes are responsible for the macromolecule's entomopathogenicity. 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

42 **1. Introduction**

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

57 Mosquitoes live in constant interactions with microorganisms in their environment and
58 throughout their life cycle (Gendrin and Christophides, 2013; Wang et al., 2011), and in
59 particular, the aquatic stages of the mosquito larvae because they are submerged in water
60 (Dickson et al., 2017). Although microbiota is needed for the healthy development of the
61 mosquito larva (Chouaia et al., 2012; Coon et al., 2014; Dickson et al., 2017; Valzania et al.,
62 2018), certain microbes can be pathogenic to them (Baumann et al., 1991; Laurence et al.,
63 2011). These entomopathogenic microorganisms, such as *Bacillus thuringiensis* and *Bacillus*
64 *sphaericus* or their by-products, have long been used for mosquito population control
65 (Baumann et al., 1991; Laurence et al., 2011). In addition to these *Bacillus* species, recent
66 studies have identified entomopathogenic bacteria from the mosquito gut microbiota such as
67 *Chromobacterium* spp. and *Serratia marcescens* (Bahia et al., 2014; Ramirez et al., 2014;
68 Short et al., 2018). *Chromobacterium* spp. isolated from the mosquito gut from Panama
69 *(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.

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 works in this study were 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 protocol (BT-Animal 26/2560). Larvicidal activity assays were performed following the approved protocol (BT-Animal 20/2560). Both animal protocols were approved by the BIOTEC Committee for use and care of laboratory animals.

2.2 Mosquito rearing condition

Specimens of laboratory strain of *An. dirus* were originally collected in Mae Sod District, Tak Province, Thailand. A colony of *An. dirus* was maintained at the Department of Parasitology, Chiang Mai University and BIOTEC's insectary at 27 °C with 75% humidity and a 12-h day/night, 30-min dusk/ dawn lighting cycle. The larvae were fed a diet of powdered

96 fish food (Tetrabit, Germany). Adults were fed on a 10% sucrose solution supplemented with
97 5% multivitamin syrup (Sevenseas, UK) *ad libitum* (Choochote and Saeung, 2013).

98 *2.3 Bacterial isolation and identification*

99 Bacteria were isolated from two biological niches, larval rearing water and bacterial
100 biofilm at the bottom of the rearing pan. The rearing water was 10-fold serially diluted with
101 sterile 1X Phosphate buffered saline (PBS). The biofilm was gently rinsed five times with 1X
102 PBS then the biofilm was scraped with sterile plastic scraper, resuspended with 20 mL of
103 sterile PBS then 10-fold serially diluted. The serial dilutions were then plated on Luria-Bertani
104 (LB) and incubated at room temperature for 2 days. Bacterial colonies with distinct
105 morphologies from each ecological niche were then streaked on LB agar plate. A single colony
106 from each bacterial isolate was then propagated and used for molecular identification using
107 16s rRNA gene. Briefly, a colony of each isolate was resuspended in 50 µL of sterile water
108 then 1 µL of resuspended bacteria was used in a PCR reaction using Phusion® High-Fidelity
109 DNA Polymerase (New England Biolabs) with forward (16s-8F: 5'
110 AGAGTTTGATCCTGGCTCAG 3') and reverse (16s-1492R: 5'
111 CGGTTACCTTGTTACGACTT3') primers (Eden et al., 1991). PCR amplification was
112 performed with an initial denaturation of 2 minutes 30 seconds at 98°C, followed with 30 cycles
113 of denaturation at 98°C for 10 seconds, annealing at 52°C for 15 seconds and an extension
114 at 72°C for 1 minute. The amplicons were then purified using QIAquick PCR purification kit
115 then submitted for Sanger DNA sequencing. The obtained bacterial 16s rRNA gene
116 sequences from the forward and reverse primers were manually trimmed and assembled. The
117 assembled sequences were then searched on the NCBI's Basic Local Alignment Search Tool
118 (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990).

119

120 *2.4 Bacteria culture and bacterial spent media preparation*

121 All isolated bacteria were cultured in LB medium under specific conditions as
122 mentioned for each experiment. To start a culture, a colony of each bacterium was inoculated
123 in 10 mL of LB medium then cultured at 25°C, 200 rpm overnight. The absorbance at 600 nm

124 (OD_{600}) was measured for the overnight culture, then 1 mL of culture at OD_{600} = 1 was
125 inoculated into 5 mL of fresh LB medium. For static culture, 18 mL of inoculated culture was
126 added to a 90 mm diameter petri dish then incubated without shaking at specified temperature.
127 For planktonic culture, 18 mL of inoculated culture was added to a 50 mL conical tube then
128 agitated at 200 rpm at specified temperature. After incubation, bacterial cells were removed
129 from the spent media by centrifugation at 4500 rpm 4 ºC for 45 minutes. Supernatants were
130 then filtered through 0.22 µm syringe filter (Sartorius). The sterile spent media were then kept
131 at 4 ºC until further use.
132
133 *2.5 Characterization of physical property of secreted larvicidal molecules*
134 We first evaluated whether the secreted larvicidal molecules were proteins
135 (macromolecules) or small molecules because they would require different methods of
136 identification. Thus, the larvicidal molecules were characterized based on the heat stability
137 and molecular weight.
138 The heat stability of the secreted larvicidal molecules was determined by heating the
139 spent media at 45, 70, and 95 ºC for one hour. Heated spent media were then used to test for
140 remaining larvicidal activity.
141 Proteins (macromolecules) and small molecules were separated using Amicon® Ultra-
142 4 10K Centrifugal Filter Device following the manufacturer's instructions. Briefly, 4 ml of spent
143 media were added to the filter unit then centrifuged at 1500xg, 4 ºC for 30 minutes. The flow
144 through was collected as small molecule fraction while the molecules retained on the filter
145 device were resuspended in LB medium or PBS to dilution factors as indicated in each
146 experiment then used as macromolecule fraction.
147
148 *2.6 Larvicidal activity assay*
149 Instar 2 stage larvae of *An. dirus* were filtered and rinsed twice with 250 mL sterile
150 distilled water. Larvicidal assays were performed with 20 mosquitoes in 4 mL of total water
151 volume per treatment per replicate in each well of 6-well plate. Filtered bacteria spent media

152 were added to each well at 10% v/v and mortality was recorded for 72 hours. For the group
153 with antibiotics treatment, Penicillin/Streptomycin solution (Gibco) were added to the well at
154 final concentration of 100 U/mL and 100 µg/mL, respectively. For *Pseudomonas* spp.
155 challenge, the bacterium was cultured overnight at 25 ºC then the cells were collected by
156 centrifugation at 1500xg, 4 ºC for 15 minutes. Bacterial cells were then washed twice with 1X
157 PBS, diluted then added to larvicidal plate at a concentration as specified in the results section.
158

159 *2.7 Cytotoxicity in C6/36 insect cell line*

160 Cytotoxicity of *S. marcescens* spent medium in insect cell line was determined by
161 sulforhodamine B (SRB) assay according to previously published protocol (Vichai and
162 Kirtikara, 2006) with slight modification for C6/36 cell line. The SRB assay measured cellular
163 protein content to determine cell survival after spent medium treatment. Briefly, C6/36 cells
164 were seeded into each well of 96-well plate at 30,000 cells/well in 100 µl of 10% (v/v) *S.*
165 *marcescens* spent medium in complete L15 medium (L15 supplemented with 10% Fetal
166 bovine serum, 1% MEM non-essential amino acids, 2% Tryptose phosphate broth, 100 U/mL
167 Penicillin and 100 µg/mL Streptomycin) and incubated at 28 ºC for 72 hours. After incubation,
168 cell monolayers were fixed with 10% (w/v) trichloroacetic acid for 45 minutes at 4 ºC and rinsed
169 4 times with tap water. Fixed cell monolayer was then stained with 0.057% (w/v) SRB in 1%
170 acetic acid for 30 minutes at room temperature in the dark. Stained cell monolayers were then
171 washed with 100 µl 1% acetic acid for four times to remove unbound dye. To solubilize protein-
172 bound SRB, 200 µl of 10 mM Tris (pH 10.5) was added to each well then the plate was shaken
173 for 30 minutes at room temperature. Absorbance at 510 nm was then measured in a
174 microplate reader to determine cell viability. LB was used as an untreated control and 5 µM
175 cycloheximide was used as a positive control of the cytotoxicity assay.
176

177 *2.8 In-gel digestion of secreted S. marcescens macromolecules for LC-MS/MS analysis*

178 The cell-free macromolecules of *S. marcescens* static culture at 25 and 37 ºC were
179 mixed with Laemmli buffer, denatured at 95 ºC for 10 minutes then subjected to SDS-PAGE.

180 After the SDS-PAGE fractionation, protein bands present in the 25 but not in 37 ºC culture
181 condition were excised from the gel for in-gel tryptic digestion followed by LC-MS/MS analyses
182 (Shevchenko et al., 2007). Briefly, the gel bands were excised and dehydrated in 100%
183 acetonitrile. Proteins were reduced by 10 mM dithiothreitol at 56°C for 30 min and alkylated
184 by 20 mM chloroacetamide at room temperature for 20 min. The gel bands were further
185 destained using 50 mM NH_4HCO_3 in 50% acetonitrile for 30 min, followed by dehydration in
186 neat acetonitrile. Digestion was carried out by adding 13 ng/μL trypsin in 50 mM NH_4HCO_3 to
187 cover the gel pieces, and the reaction was incubated at 37°C overnight. Extraction of cleaved
188 peptides was performed by adding extraction buffer (70% acetonitrile/1% formic acid) to the
189 digests at the ratio of 2:1 (v/v). Then the extracts were lyophilized and redissolved in 0.1%
190 formic acid ready for LC-MS/MS analyses.
191
192 *2.9 LC-MS/MS analyses of secreted S. marcescens macromolecules*
193 The digested peptides (200 ng) were analyzed by an EASY-nLC 1000 HPLC system
194 (Thermo Fisher Scientific) interfaced with a Q Exactive™ Hybrid Quadrupole-Orbitrap mass
195 spectrometer (Thermo Fisher Scientific) via an EASY-Spray ion source (Thermo Fisher
196 Scientific). The separation was performed for 60 min at the flow rate of 300 nL/min using C18-
197 reverse phase column (EASY-Spray™, Thermo Fisher Scientific). The stepwise gradient was
198 set from 5% solvent B (0.1% formic acid in acetonitrile)/ 95% solvent A (0.1% formic acid in
199 H_2O) to 20% solvent B/ 80% solvent A for 43 min and then to 40% solvent B within the next
200 10 min, followed by holding at 95% for 55 - 60 min. Data-dependent acquisition was performed
201 wherein the ten most abundant precursor ions were selected for fragmentation. The precursor
202 masses were scanned by the Orbitrap mass analyzer at the mass range of 400 – 1600 m/z
203 and the resolution of 70,000. Higher-energy collisional dissociation was employed for
204 fragmentation with an exclusion of +1 charged ions. All data were acquired using Thermo
205 Xcalibur 2.2 software.

206 Mass spectra were searched against NCBI database of *Serratia* sp. (August, 2018)
207 using Proteome Discoverer 2.2 connected to SEQUEST search engine (Eng et al., 1994).
208 Precursor mass tolerance was set to 10 ppm and the product ion mass tolerance was 0.02
209 Dalton. Trypsin was specified with maximum 2 missed cleavages. Carbamidomethylation was
210 chosen as a fixed modification; while oxidation of methionine was allowed as variable
211 modification. Decoy search was performed using the false discovery rate of 0.01 as a
212 threshold.
213
214 *2.10 Chitinase activity assay*
215 Chitinase activity of spent medium was determined by measuring the ability of the
216 spent medium to digest chitin into a reducing sugar, *N*-acetyl-*D*-glucosamine. To set up the
217 chitinase activity assay, 100 µL of cell-free spent culture media served as a crude enzyme
218 source was mixed with 100 µL of 1% (w/v) chitin powder (Sigma, USA) in 50 mM sodium
219 phosphate buffer (pH 6.5). The reaction mixture was incubated at 30ºC for 90 min. The amount
220 of reducing sugar was then determined by using *p*-hydroxybenzoic acid hydrazide (pHBAH)
221 assay (Lever, 1972). A stock solution of 5% (w/v) pHBAH (Sigma, USA) was prepared in acid
222 medium (0.5 M HCl) and diluted at the moment of analysis to 1% (w/v) in alkaline medium (0.5
223 M NaOH). Samples were mixed with 1% (w/v) pHBAH in alkaline solution (0.5 M NaOH) at a
224 ratio of 1:3 and the mixture was heated in the boiling bath for 5 min to develop the color. After
225 cooling, the solution was centrifuged at 12,000 rpm for 2 min. The absorbance at 410 nm of
226 the supernatant was then measured using spectrophotometer. *N*-acetyl-*D*-glucosamine
227 standard solution was prepared in the same manner at concentrations of 0, 0.2, 0.4, 0.6, 0.8,
228 and 1 mg/ml in distilled water. One unit of enzyme activity was defined as the amount of
229 enzyme which releases 1 µg of reducing sugar per hour under the assay conditions.
230
231 *2.11 Statistical analyses*

232 Statistical significance of the survival analyses was calculated using Log-rank test
233 followed by a Bonferroni post-hoc analysis using 'survminer ' package (Kassambara et al.,
234 2019) in R (R Core Team, 2020).
235 Statistical analysis comparing chitinase activity level and inhibition of chitinase activity
236 was performed using One-way ANOVA with Tukey multiple comparison test using
237 multcompView (Graves and Dorai-Raj, 2019) package in R (R Core Team, 2020).
238

239 **3. Results**

240 *3.1 Isolation of bacteria from An. dirus larval rearing pan*

241 Our laboratory has recently set up a new insectary facility to expand our drug
242 development pipeline to test antimalarial compounds against sexual stage of *Plasmodium*
243 parasites. During the optimization of *Anopheles dirus* rearing condition, we found that if the
244 larval food particle size was not fine enough, the food sedimented to the bottom of the pan.
245 Due to the surface feeding nature of *An. dirus*, the sedimented food led to a bacterial growth
246 and biofilm at the bottom of the pan. Mortality of the mosquito larva was observed a few days
247 after the biofilm formed at the bottom of the pan. From this observation, we hypothesized that
248 certain microbes in the larval rearing pan secrete molecule(s) that have larvicidal activity
249 against *An. dirus*.
250 A total of seven bacteria species were isolated and identified from the rearing pan by
251 16s rRNA gene sequencing. These include one Actinobacteria: *Leucobacter* spp. (Leu_spp),
252 two Bactereoidetes: *Sphingobacterium* spp. (Sph_spp) and *Sphingobacterium multivorum*
253 (Sch_m); and four proteobacteria: *Acinetobacter soli* (Aci_s), *Ochrobactrum* spp. (Och_spp),
254 *Serratia marcescens* (Ser_m), and *Stenotrophomonas maltophilia* (Ste_m) as summarized in
255 Table 1.
256 Table 1. Bacterial isolates from *An. dirus* larval rearing pan identified by 16s rRNA gene
257 sequencing

Abbreviation	Species	Phylum	Top known hit	Identities	Gaps

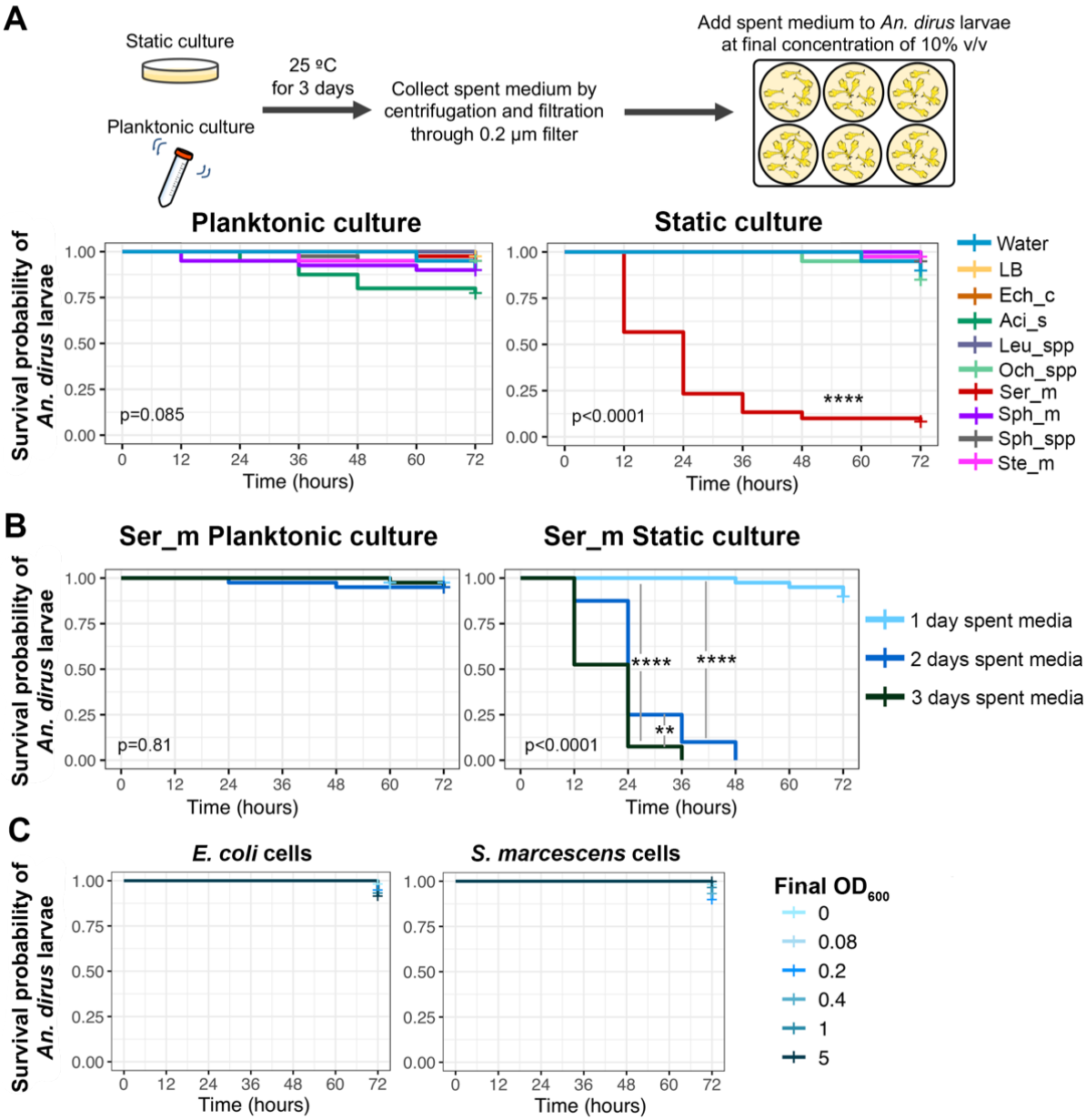
Leu_spp	*Leucobacter* spp.	Actinobacteria	*Leucobacter* sp. Re-6	1332/1332 (100%)	0/1332 (0%)
Sph_spp	*Sphingobacterium* spp.	Bacteroidetes	*Sphingobacterium* sp. strain JPB23	1305/1340 (97%)	0/1340 (0%)
Sph_m	*Sphingobacterium multivorum*	Bacteroidetes	*Sphingobacterium multivorum* strain GZT-2	1341/1342 (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%)

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

Because larval mortality was observed a few days after bacterial bloom, we hypothesized that the isolated bacteria could secrete larvicidal molecule(s) after the bacteria grow to a certain density; at a time when biofilm was observed at the bottom of the pan for a few days. Therefore, the isolated bacteria were cultured under either static or planktonic conditions for three days before spent media collection for the screening of larvicidal activity.

None of the cell-free spent media obtained from planktonic condition had any toxicity against *An. dirus* larvae (Figure 1A). However, for the stationary culture, only the cell-free spent media from *S. marcescens* had a detrimental effect on the survival of the larvae (Figure 1A). Next, spent medium of *S. marcescens* cultured under planktonic and static conditions was collected over time after 1, 2, or 3 days of incubation. Again, the larvicidal molecule was secreted into the *S. marcescens* only under static culture condition and the larvicidal activity of the spent medium increased over time starting from the second day after inoculation (Figure 1B).

274 To investigate if the *S. marcescens* cells are entomopathogenic to *An. dirus* larvae,
275 the bacterium was cultured under a static condition then washed with 1X PBS. Washed
276 bacterial cells were then added to larval culture at various concentrations (Figure 1C). Even
277 at OD_{600} of 5, *S. marcescens* cells did not cause significant mortality to *An. dirus* larvae. The
278 *Escherichia coli* cells used as a control also did not cause larval mortality. These results
279 suggested that *S. marcescens* only cause larval mortality through the secretion of
280 entomopathogenic molecules.



281

282 Figure 1. Survival analyses of *An. dirus* mosquito larvae after treatment with bacterial spent
283 media at 10% (v/v) concentration. (A) Survival curve of *An. dirus* larvae after treatment with

284 bacterial spent media cultured under planktonic condition or static condition. The spent media
285 were collected at three days after incubation under planktonic or static state at 25 ºC Ech_c:
286 *Escherichia coli* strain XL10, Aci_s: *Acinetobactor soli*, Leu_spp: Leucobactor spp., Och_spp:
287 Ochrobatrum spp., Ser_m: *Serratia marcescens*, Sph_m: *Sphingobacterium multivorum,*
288 Sph_spp: *Sphingobacterium* spp., Ste_m: *Stenotrophomonas maltophilia*. LB medium or
289 water was used as a control at final concentration of 10% (v/v). The experiment was performed
290 in triplicate (B) Survival curve of *An. dirus* larvae after treatment with *S. marcescens* spent
291 medium cultured under planktonic or static condition and collected after 1, 2, or 3 days after
292 inoculation. The experiment was performed in duplicate (C) Survival curve of *An. dirus* larvae
293 after treatment with *E. coli* or *S. marcescens* cells at various concentration. Bacterial cells
294 were cultured under static condition at 25 ºC then washed twice with PBS. The experiment
295 was performed in triplicate. Statistical significance of the survival analyses was calculated
296 using Log-rank test followed by a Bonferroni post-hoc analysis using 'survminer 'package in
297 R. Significance level: **: $p<0.01$, ****: $p<0.0001$.

298 *3.3 Larvicidal molecules from S. marcescens are heat sensitive macromolecules*

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

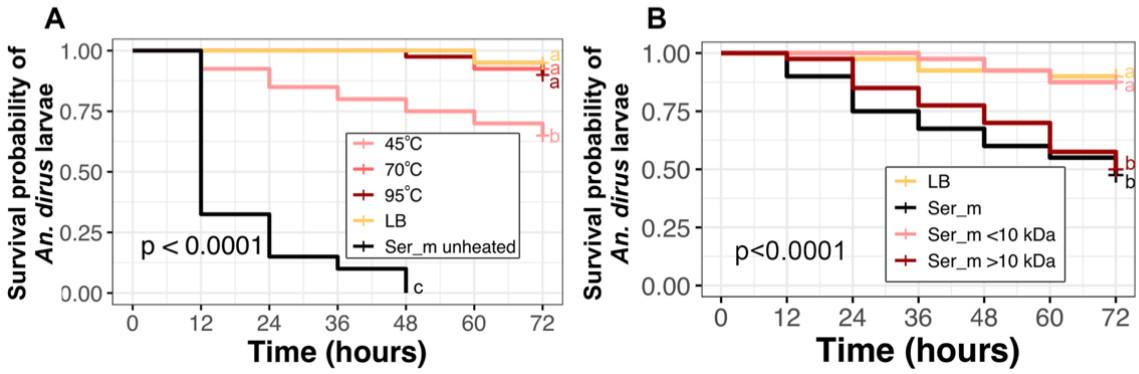
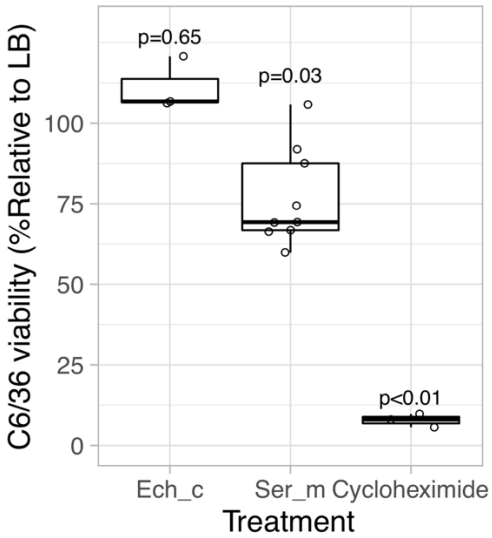


Figure 2. Larvicidal molecule(s) secreted by *S. marcescens* are heat sensitive macromolecule(s). (A) Heat sensitivity of larvicidal activity from *S. marcescens* spent medium. The *S. marcescens* spent medium was heated at 45, 70, and 95 ºC for one hour before testing for larvicidal activity at 10% (v/v) final concentration. LB medium was used as a control at a final concentration of 10% (v/v). The experiment was performed in duplicate (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 experiment was performed in duplicate. 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. marcescens*' entomopathogenic molecules. Another possible explanation is that the death of

332 *An. dirus* larvae is due to damage at the physiological level rather than direct toxicity to insect
333 cells.



334

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

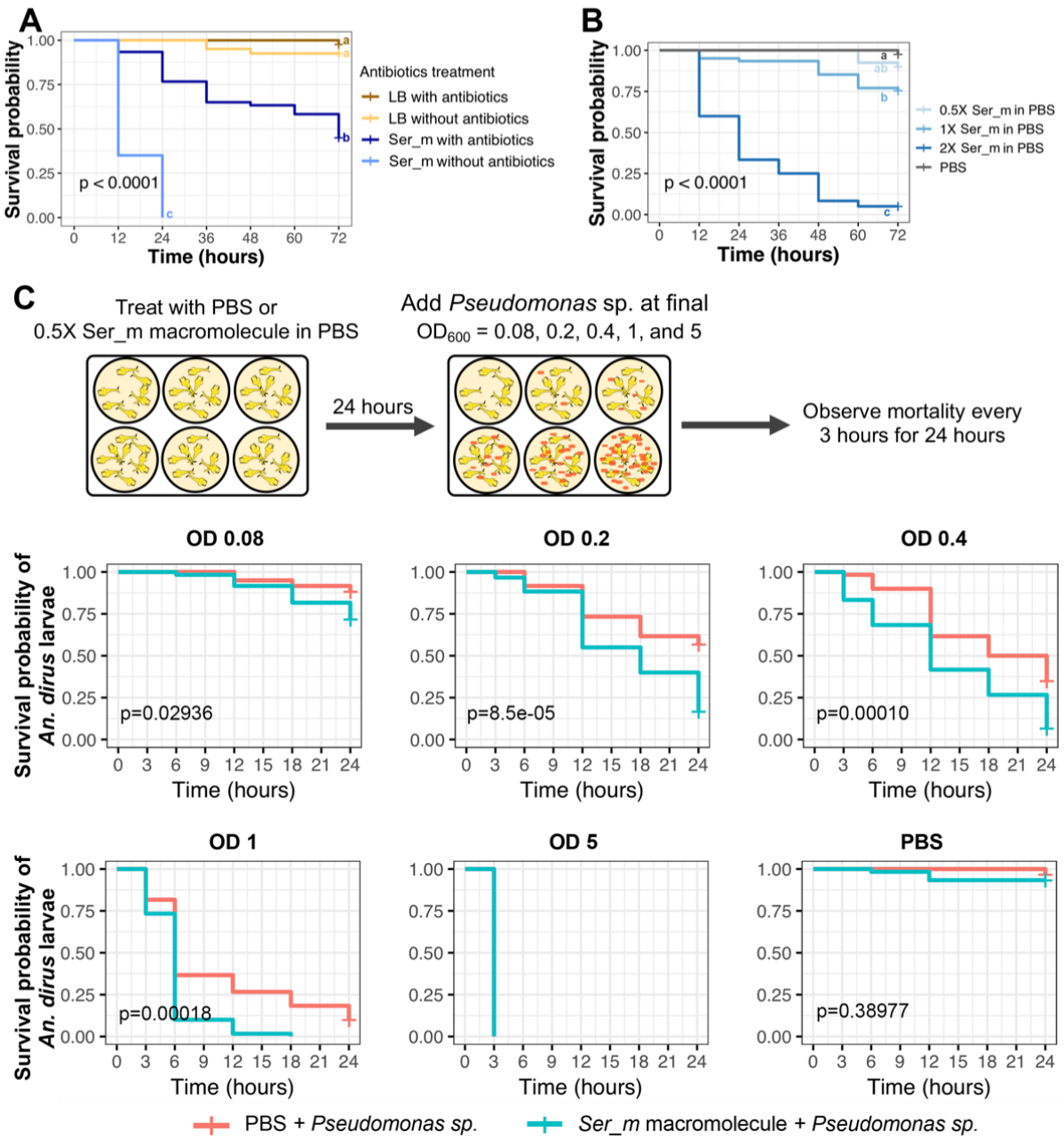
340 *3.5 S. marcescens spent medium can directly kill An. dirus larvae and also enhance larvae's*
341 *susceptibility to bacterial infection.*

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

347 To test whether these naturally growing bacteria have any impact on larvicidal activity
348 by *S. marcescens*, penicillin (100 U/mL) and streptomycin (100 μg/mL) were added in the
349 larvicidal assay experiment to prevent bacterial growth. The addition of antibiotics significantly

350 inhibited bacterial contamination because water in each well of the larvicidal assay remained
351 clear throughout the experiment. In the absence of natural bacterial growth, the
352 macromolecules secreted by *S. marcescens* can still kill *An. dirus* larvae confirming its intrinsic
353 entomopathogenic property (Figure 4A). Interestingly, the observed mortality in the antibiotics-
354 treated group was significantly lower than the group without antibiotics treatment. In addition
355 to antibiotics treatment, resuspension of *S. marcescens* macromolecules in 1X PBS instead
356 of LB also reduced natural bacterial growth in larvicidal assays. Even without obvious natural
357 bacterial growth, *S. marcescens* macromolecule resuspended in 1X PBS could kill *An. dirus*
358 larvae in a dose dependent manner (Figure 4B). These results suggest that the larvicidal
359 molecule has intrinsic larvicidal activity, and also increases larvae's susceptibility to bacterial
360 infection.

361 To confirm the hypothesis that *S. marcescens* secreted macromolecules increases
362 larvae's susceptibility to bacterial infection, we next pretreated *An. dirus* larvae with sublethal
363 dose of *S. marcescens* secreted macromolecule (0.5X diluted in PBS; see Figure 4B) for 24
364 hours then added an entomopathogenic strain of *Pseudomonas* sp. at the final bacterial
365 density of OD_{600} = 5, 1, 0.4, 0.2, and 0.08 (Figure 4C). *An. dirus* larvae pretreated with *S.*
366 *marcescens* secreted macromolecules significantly increased the mortality rate caused by
367 *Pseudomonas* sp. in all treatment except at OD5, which the entomopathogenicity was too high
368 to observe the difference. These results confirm significant effect of *S. marcescens*
369 macromolecules on susceptibility of *An. dirus* larvae to bacterial infection, even at sublethal
370 dose.



371

372 Figure 4. Intrinsic larvicidal activity of *S. marcescens* macromolecule and its enhancing effect
373 on larvae's susceptibility to bacterial infection. (A) Survival of *An. dirus* larvae treated with 2X
374 concentration of macromolecules from *S. marcescens* spent medium diluted in LB with or
375 without antibiotics. LB with or without antibiotics were used as a control. The experiment was
376 performed in duplicate. (B): *An. dirus* larvae treated with *S. marcescens* macromolecules
377 reconstituted to the final concentration of 0.5X, 1X, and 2X in PBS. The experiment was
378 performed in duplicate. (C): Treatment of sublethal dose of *S. marcescens* secreted
379 macromolecule enhances *An. dirus* larvae susceptibility to bacterial infection. The larvae were

380 treated for 24 hours with 0.5x concentration of macromolecules from *S. marcescens* diluted in
381 PBS buffer before addition of *Pseudomonas* sp. at final concentration of OD_{600} = 0.08, 0.2,
382 0.4, 1, and 5. Mortality of the larvae was monitored every 3 hours for 24 hours. The experiment
383 was performed in triplicates. Statistical significance of the survival analyses was calculated
384 using Log-rank test followed by a Bonferroni post-hoc analysis using 'survminer 'package in
385 R.

386 *3.6 S. marcescens* secreted different sets of macromolecules when cultured under different
387 conditions

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

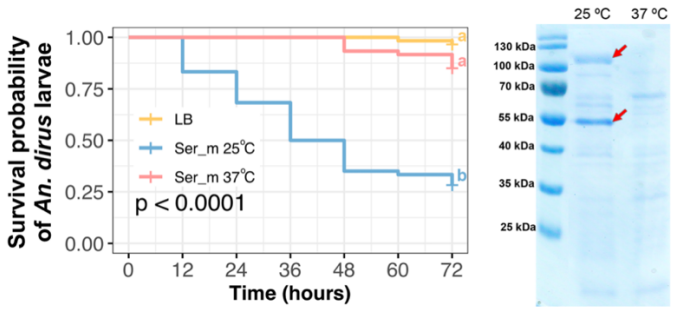
394 To identify other culture conditions that might result in differential larvicidal activity, *S.*
395 *marcescens* was cultured under static condition at different temperatures, 25 ºC and 37 ºC.
396 Here, *S. marcescens* spent medium from 25 ºC incubation could kill *An. dirus* larvae while
397 those cultured at 37 ºC could not (Figure 5A).

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

403 The 110 kDa protein band consists of several protease enzymes including
404 aminopeptidase N, serralysin, and peptidase M60 viral enhancin protein; chitinase enzymes
405 including chain A chitobiase; cell surface protein; DUF4214 domain-containing protein;

406 hemolysin; DUF4214 domain-containing protein; peptide ABC transporter substrate-binding
407 protein; aconitate hydratase; DNA-directed RNA polymerase; formate dehydrogenase-N
408 subunit alpha; glycine dehydrogenase; translation elongation factor Tu; phosphoenolpyruvate
409 carboxykinase; LysR family transcriptional regulator; short-chain dehydrogenase/reductase
410 SDR; phosphorylcholine phosphatase; acyl-CoA dehydrogenase (Supplementary table 1).

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



418

419 Figure 5. *S. marcescens* secreted larvicidal molecules when cultured under static condition at
420 25 ºC but not 37 ºC. (A) Survival curve of *An. dirus* larvae after treatment with *S. marcescens*
421 spent media cultured under static condition at 25 ºC or 37 ºC. The experiment was performed
422 in triplicate. Statistical significance of the survival analyses was calculated using Log-rank test
423 followed by a Bonferroni post-hoc analysis using 'survminer 'package in R. (B) SDS-PAGE of
424 *S. marcescens* spent media cultured under static condition at 25 ºC or 37 ºC. Two red arrows
425 indicate major protein bands that were expressed when the bacterium was cultured at 25 ºC
426 but not 37 ºC.

427 Supplementary Table 1. LC-MS analysis of 110 kDa protein band.

Accession	Description	Coverage [%]	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI	Score Sequest HT
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

428

429 **Supplementary Table 2**

430 LC-MS analysis of 55 kDa protein band

Accession	**Description**	**Coverage [%]**	**# Peptides**	**# PSMs**	**# AAs**	**MW [kDa]**	**calc. pI**	**Score Sequest HT**
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

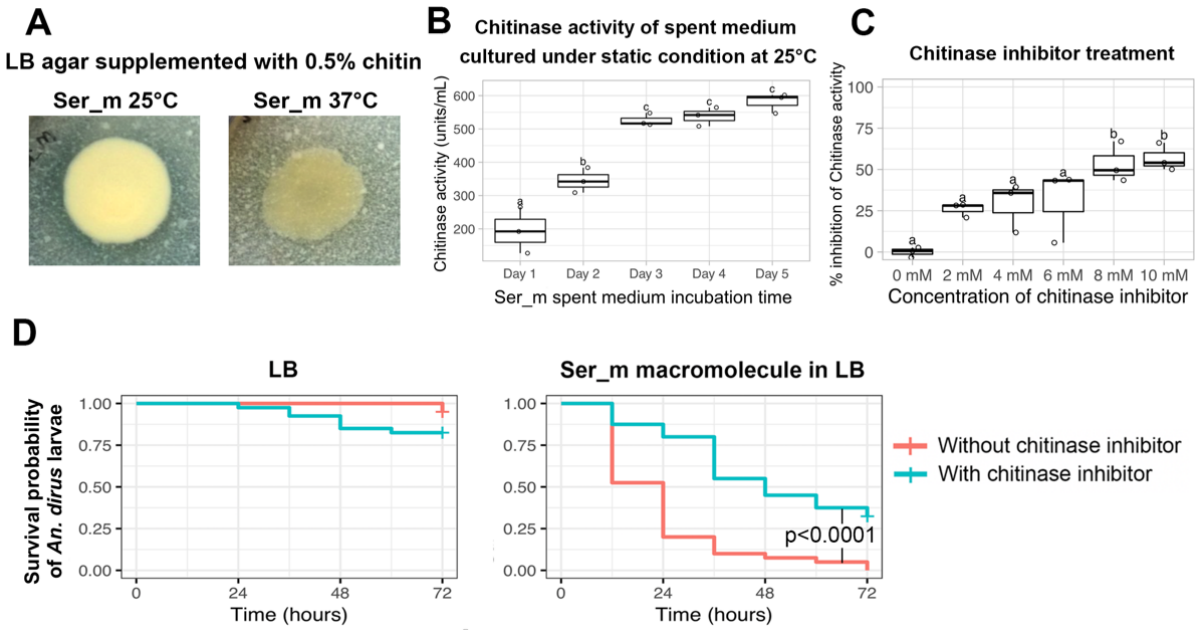
1280179082	capsule assembly Wzi family protein [*Serratia* sp. TKO39]	17	6	6	478	52.7	5.41	6.61
1027970958	Xaa-Pro aminopeptidase [*Serratia marcescens*]	11	4	5	438	49	5.38	7.85
1027968317	glutathione reductase [*Serratia marcescens*]	16	5	5	450	48.5	6.38	6.32
1001783318	phosphoenolpyruvate carboxykinase (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

3.7 Chitinase inhibitor reduced larvicidal activity of S. marcescens spent medium.

Because the LC-MS analysis suggested that our strain of *S. marcescens* expressed chitinase and chitobiose enzymes when cultured under static condition at 25 ºC but not 37 ºC, we then inoculated the bacterium on LB agar supplemented with 0.5% chitin then incubated at 25 ºC or 37 ºC to determined chitinase secretion. After five days of incubation, *S. marcescens* cultured at 25 ºC produced a clear zone while the incubation at 37 ºC did not (Figure 6A). Chitinase activity was also determined in the liquid static culture by pHBAH assay. Chitinase activity of *S. marcescens* spent medium increased overtime during the incubation under static condition at 25 ºC and saturated after 3 days of incubation (Figure 6B). This result coincides well with the increase in larvicidal activity during the first three days of *S. marcescens* incubation (Figure 1B).

443 Cyclic dipeptides such as Cyclo-Gly-Pro have previously been reported to inhibit
444 chitinase enzyme activity (Houston et al., 2004). This compound was then used to confirm that
445 the larvicidal activity was due to chitinase secretion. The results in Figure 6C show that Cyclo-
446 Gly-Pro can inhibit chitinase activity of *S. marcescens* spent medium in a dose-dependent
447 manner. At 8 mM, Cyclo-Gly-Pro inhibited chitinase activity by 53.36±12.22%, which was not
448 significantly different from 56.81±8.34% inhibition by 10 mM Cyclo-Gly-Pro (Figure 6C). Thus,
449 8 mM Cyclo-Gly-Pro was used in the following larvicidal assays. Larvicidal activity was
450 significantly reduced when 8 mM Cyclo-Gly-Pro was added to the spent medium compared to
451 the group without the chitinase inhibitor, albeit not to the same level as the LB control (Figure
452 6D). This might be due to the incomplete inhibition of the chitinase activity or other enzymes
453 might be contributing to the larvicidal activity in addition to chitinases.



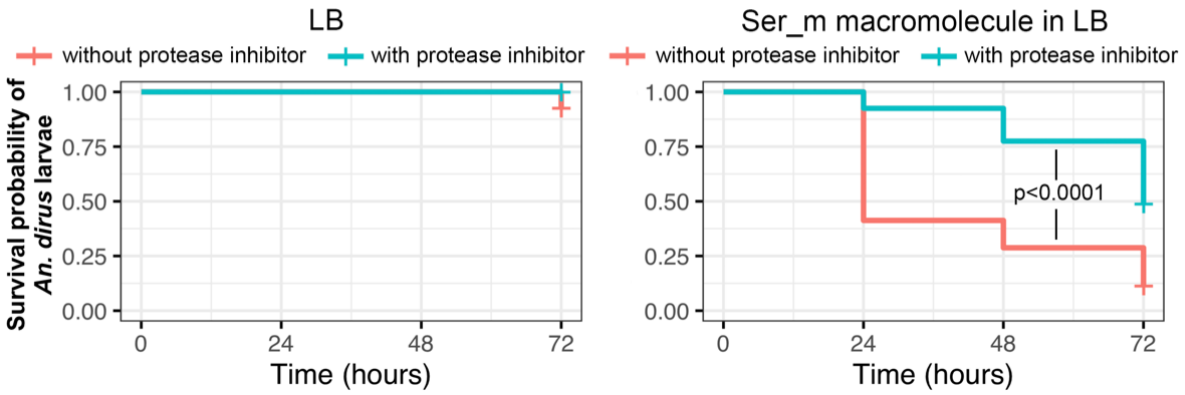
454

455 Figure 6. *S. marcescens* secreted macromolecules have chitinase activity and chitinase
456 inhibitor reduced larvicidal activity. (A) *S. marcescens* secreted chitinase that resulted in a
457 clear zone around bacterium colony when cultured at 25 ºC but not 37 ºC. (B) *S. marcescens*
458 spent medium accumulated chitinase activity over time and saturated after three days of
459 incubation. One unit of chitinase activity was defined as the amount of enzyme which releases
460 1 µg of reducing sugar per hour as measured by pHBAH assay. (C) Cyclo-Gly-Pro inhibit

461 chitinase activity in a dose-dependent manner. Statistical analyses comparing chitinase
462 activity and inhibition of chitinase activity were performed using One-way ANOVA with Tukey
463 multiple comparison test using multcompView package in R. (D) Chitinase inhibitor reduced
464 larvicidal activity of the *S. marcescens* secreted macromolecule. The experiment was
465 performed in duplicate. Statistical significance of the survival analyses was calculated using
466 Log-rank test followed by a Bonferroni post-hoc analysis using 'survminer 'package in R.

467 *3.8 Protease inhibitors reduced larvicidal activity of S. marcescens spent medium.*

468 The LC-MS results indicated that *S. marcescens* secreted several proteases when
469 cultured at 25 ºC under a static condition. Some of these proteases have been reported for
470 insecticidal activity in other insects (Baumann, 2013; Wang and Granados, 1997). Therefore,
471 we added the Complete protease inhibitor (Roche) supplemented with 5 mM EDTA in the
472 larvicidal assays to determine whether these proteases contributed to *S. marcescens* larvicidal
473 activity. We found that protease inhibitors significantly reduced the three-day mortality of the
474 larvae to 50% as compared to 87.5% of the control group without protease inhibitors. This
475 suggests that the larvicidal activity of proteases was partially involved in the observed larval
476 mortality (Figure 7).



477

478 Figure 7. Protease inhibitor reduced larvicidal activity of *S. marcescens* secreted
479 macromolecule. The experiment was performed in four replicates. Statistical significance of
480 the survival analyses was calculated using Log-rank test followed by a Bonferroni post-hoc
481 analysis using 'survminer 'package in R.

482 **4. Discussion**

483 In this study, a strain of non-prodigiosin producing *S. marcescens* was identified and
484 found to secrete molecules with larvicidal activity against *Anopheles dirus*, a malaria vector in
485 Southeast Asia. *S. marcescens* is a Gram negative bacterium commonly found in soil and
486 water but not as a human fecal microbiome (Donnenberg, 2015). With such natural habitats,
487 it is often associated with insects including mosquitoes (Gendrin and Christophides, 2013;
488 Wang et al., 2017, 2011). Interestingly, different strains of *S. marcescens* can have different
489 relationship with *Anopheles* mosquitoes raging from symbiotic (Wang et al., 2017) to
490 entomopathogenic (Bahia et al., 2014). The cells of *S. marcescens* isolated in the present
491 study did not kill *An. dirus* larvae directly, but it can secrete larvicidal molecules into the culture
492 medium when cultured under static condition for at least 2-3 days. The results were in
493 accordance with our original observations that the larval death occurred a few days after larval
494 water became turbid. The fact that the larvicidal molecules were secreted is an advantage for
495 application purposes, given that the secreted factors can be applied instead of the bacterium.
496 Thus preventing or limiting the risk of nosocomial infections (Golemi-Kotra, 2008). The
497 secreted factors can also be expressed heterologously in other expression systems that are
498 more suited for industrial scale production.

499 *S. marcescens* has been extensively studied for its potential as insect pest control
500 agent in agriculture due to its insecticidal property (Bidari et al., 2017; Li et al., 2011; Miller et
501 al., 2008; Pineda-Castellanos et al., 2015; Tu et al., 2010). The bacterium was known to
502 secrete many insect virulence factors including several enzymes, as well as secondary
503 metabolite including a red pigment prodigiosin. Prodigiosin is a metabolite possessing several
504 bioactivities including antimalarial and insecticidal/mosquito larvicidal properties (Bidari et al.,
505 2017; Darshan and Manonmani, 2015; Patil et al., 2011; Suryawanshi et al., 2015). The fact
506 that our *S. marcescens* isolate doesn't produce red pigment suggested that the bacterium
507 secrete other mosquito larvicidal molecules. This finding is in line with a previous study

508 reporting that level of prodigiosin production did not correlate with *S. marcescens* insecticidal
509 activity against silk worm larva (Zhou et al., 2016).

510 The heat-labile larvicidal macromolecules were secreted only when *S. marcescens*
511 was cultured under static condition at lower temperature of 25 ºC but not 37 ºC nor at 25 ºC
512 under planktonic condition. These results suggested that the metabolic activity of this
513 bacterium was influenced by culture conditions, consequently affecting the subsets of
514 molecules secreted by this strain of *S. marcescens*. Comparison of protein expression profiles
515 between the 25 ºC and 37 ºC static cultures helped us to identify potential proteins with
516 larvicidal activity which include many proteases and chitinases. Protease and chitinase
517 activities were then associated with larvicidal activity against *An. dirus* as supplementation of
518 chitinase or protease inhibitors diminished the larvicidal activity. These proteases and
519 chitinases are likely to damage the larvae at physiological levels rather than at the cellular
520 level because the secreted macromolecules lack cytotoxicity against mosquito cells.

521 Chitin, a polymer of N-acetylglucosamine, is the major component of mosquito
522 extracellular structures including the cuticle of the exoskeleton and the peritrophic matrix (PM)
523 of the midgut in arthropods (Liu et al., 2019). These exoskeletal structures provide strength to
524 the body structure, and serve as muscle attachment sites which allow locomotion and flight
525 (Liu et al., 2019). They are also important barriers for the protection of arthropods from the
526 surrounding environment, mechanical damage, heat, desiccation, and invasion of
527 microorganisms (Liu et al., 2019). With all these important functions, damage to the
528 exoskeleton can be fatal to mosquitoes. Several techniques have been developed to
529 compromise the insects' exoskeleton including inhibition of chitin synthesis (Belinato et al.,
530 2013; Doucet and Retnakaran, 2012; Zhang et al., 2010), and degradation of the chitin
531 structure (Berini et al., 2019; Singh and Arya, 2019). The later strategy has been exploited
532 using chitinase enzymes or chitinase-producing microorganisms; however, many studies
533 suggested that chitinases might not be ideal to be used as a stand-alone insecticide because
534 the larvicidal activity from chitinase can be marginal. Rather, the enzymes could be used to

535 enhance the insecticidal activity of other control agents including Bt toxin, entomopathogenic
536 virus and chemical insecticides (Kelkenberg et al., 2015; Ramírez-Suero et al., 2011; Zhong
537 et al., 2015). Previous research has demonstrated that chitinases can also help overcome any
538 resistance phenotype developed against a biocontrol strategy. Cai et al. showed that *Bacillus*
539 *sphaericus* (Bs) toxin-resistant *Culex quinquefasciatus* became susceptible to the Bs
540 treatment when Chitinase AC was overexpressed in *B. sphaericus* (Cai et al., 2007). In
541 addition to the insecticidal property, chitinases can also inhibit fungus by destroying chitinous
542 cell wall structure (Berini et al., 2017; Singh and Arya, 2019; Zarei et al., 2011). *S. marcescens*
543 has been tested to be used against plant fungal pathogen due to its fungicidal properties
544 (Purkayastha et al., 2018) making it an interesting candidate to be used against several
545 classes of agricultural diseases.

546 Peritrophic matrix (PM) is an acellular structure inside the mosquito gut that
547 compartmentalize gut content from the midgut epithelium (Richards and Richards, 1977;
548 Terra, 2001). It serves as a barrier to protect the insect from the digestive enzymes, abrasion
549 from food particles, and invading parasites (Terra, 2001). PM structure consist of chitin and
550 proteins network, with peritrophins as major protein components (Terra, 2001). The secreted
551 chitinase and proteases could damage the structure of peritrophic matrix thus destroying the
552 larvae's homeostasis and result in larval death. In addition to direct killing, the compromised
553 peritrophic matrix can be responsible for enhanced susceptibility to bacterial infection
554 observed in our study. For instance, in *Drosophila*, disruption of the peritrophic matrix
555 formation renders the fruit fly more susceptible to bacterial infection (Buchon et al., 2013).

556 Serralysin, one of the proteases identified from the mass spectrometry analysis of *S.*
557 *marcescens* secreted macromolecules, has previously been extensively characterized.
558 Serralysin is a metalloprotease first discovered in the culture medium of *Serratia* sp. (Miyata
559 et al., 1970) then later found in other Gram negative bacteria (Baumann, 2013). The protease
560 is secreted as an inactive enzyme and autocatalytically activated in the presence of divalent
561 cations (Baumann, 2013). The enzyme was reported to have entomopathogenic property

562 against larvae of scarab beetle (Pineda-Castellanos et al., 2015). Interestingly, serralysin was
563 found to have immunosuppression property in silk worm and considered as a virulence factor
564 (Ishii et al., 2014). The mechanism of immunosuppression in the study was through the
565 degradation of hemocyte adhesion molecule. Another study demonstrated that a serralysin-
566 type metalloprotease could cleave other proteins involved in insect immunity found in
567 *Manduca sexta* hemolymph. Examples of these proteins are β-1,3 glucan recognition protein
568 2 (immune recognition), hemocyte aggregation inhibitor protein (HAIP), serine proteinase,
569 serpin-1 (immune signaling and regulation), and scolexins A and B (coagulation cascade
570 effector function) (Felföldi et al., 2009). The immune suppression by serralysin could be one
571 of the underlying mechanisms by which *An. dirus* larvae were rendered more susceptible to
572 bacterial infection.

573 Peptidase M60 enhancin protein is a metalloprotease that requires zinc for its function.
574 Enhancin was first described in baculovirus (Hashimoto et al., 1991) then later found in several
575 bacteria and fungi (Nakjang et al., 2012; Slavicek, 2012). Mucin is an important protein
576 component of insect peritrophic matrix including *Anopheles* mosquito (Dinglasan et al., 2009).
577 Enhancin from baculoviruses could degrade intestinal mucin of the insects thus making them
578 more susceptible to viral infection (Toprak et al., 2012; Wang and Granados, 1997) and Bt
579 toxin (Granados et al., 2001). Bacterial enhancin could also digest mucin (Nakjang et al.,
580 2012), but the impact on baculovirus and Bt toxin toxicity were inconclusive (Fang et al., 2009;
581 Galloway et al., 2005; Zhao et al., 2011).

582 Our results indicate that chitinase and protease inhibitors could only partially reduce
583 the larvicidal activity of the spent medium against *An. dirus* larvae. This suggests that the
584 effect was likely to be a result from a combination of both chitinase and protease enzymes. As
585 discussed above, several previous studies suggested that larvicidal activity of the identified
586 enzymes were marginal on their own. Contribution of each individual enzyme on larvicidal
587 activity should therefore be further investigated. This can be done by heterologous expression
588 of each enzyme to determine lethal doses individually and in combination. The partial

reduction of larvicidal activity could also be a result from incomplete inhibition of chitinases and proteases.

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 entomopathogenicity, these molecules have potential to increase the larvae's susceptibility to natural entomopathogenic microorganisms, and other mosquito control strategies currently in use. Further studies on dose 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

615 **Ratchanu Bunyong:** Investigation

616 **Navaporn Posayapisit:** Investigation

617 **Khrongkhwan Thammatinna:** Investigation

618 **Nuttiya Kalpongnukul:** Investigation

619 **Kittipat Aupalee:** Investigation

620 **Trairak Pisitkun:** Methodology, Resources,

621 **Sumalee Kamchonwongpaisan:** Resources, Writing - Review & Editing

622

623 **Declaration of Competing Interest**

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

625 **Acknowledgements**

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

630 **References:**

631 Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment
632 search tool. J. Mol. Biol. 215, 403–410.

633 Bahia, A.C., Dong, Y., Blumberg, B.J., Mlambo, G., Tripathi, A., BenMarzouk-Hidalgo, O.J.,
634 Chandra, R., Dimopoulos, G., 2014. Exploring *Anopheles* gut bacteria for *Plasmodium*
635 blocking activity. Environ. Microbiol. 16, 2980–2994.

636 Baumann, P., Clark, M.A., Baumann, L., Broadwell, A.H., 1991. *Bacillus sphaericus* as a
637 mosquito pathogen: properties of the organism and its toxins. Microbiol Mol Biol Rev.
638 55, 425–436.

639 Baumann, U., 2013. Serralysin and Related enzymes, in: handbook of proteolytic enzymes.
640 Elsevier, pp. 864–867.

641 Belinato, T.A., Martins, A.J., Lima, J.B.P., Valle, D., 2013. Effect of triflumuron, a chitin
642 synthesis inhibitor, on *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus*
643 under laboratory conditions. Parasit. Vectors 6, 83.
644 Berini, F., Casartelli, M., Montali, A., Reguzzoni, M., Tettamanti, G., Marinelli, F., 2019.
645 Metagenome-sourced microbial chitinases as potential insecticide proteins. Front.
646 Microbiol. 10, 1358.
647 Berini, F., Presti, I., Beltrametti, F., Pedroli, M., Vårum, K.M., Pollegioni, L., Sjöling, S.,
648 Marinelli, F., 2017. Production and characterization of a novel antifungal chitinase
649 identified by functional screening of a suppressive-soil metagenome. Microb. Cell Fact.
650 16, 16.
651 Bidari, F., Shams-Bakhsh, M., Mehrabadi, M., 2017. Isolation and characterization of a
652 *Serratia marcescens* with insecticidal activity from Polyphylla olivieri (Col.:
653 Scarabaeidae). J. Appl. Entomol. 2142, 162–172.
654 Buchon, N., Osman, D., David, F.P.A., Fang, H.Y., Boquete, J.-P., Deplancke, B., Lemaitre,
655 B., 2013. Morphological and molecular characterization of adult midgut
656 compartmentalization in *Drosophila*. Cell Reports 3, 1725–1738.
657 Cai, Y., Yan, J., Hu, X., Han, B., Yuan, Z., 2007. Improving the Insecticidal activity against
658 resistant *Culex quinquefasciatus* mosquitoes by expression of chitinase gene chiAC in
659 *Bacillus sphaericus*. Appl. Environ. Microbiol. 73, 7744–7746.
660 Caragata, E.P., Otero, L.M., Carlson, J.S., Dizaji, N.B., Dimopoulos, G., 2020. A non-live
661 preparation of Chromobacterium sp. Panama (Csp_P) is a highly effective larval
662 mosquito biopesticide. Appl. Environ. Microbiol. AEM.00240-20.
663 Choochote, W., Saeung, A., 2013. Systematic techniques for the recognition of *Anopheles*
664 species complexes, in: Manguin, S. (Ed.). *Anopheles* mosquitoes-new insights into
665 malaria vectors. Intech, pp. 57–79.
666 Chouaia, B., Rossi, P., Epis, S., Mosca, M., Ricci, I., Damiani, C., Ulissi, U., Crotti, E.,
667 Daffonchio, D., Bandi, C., Favia, G., 2012. Delayed larval development in Anopheles

668 mosquitoes deprived of Asaiabacterial symbionts. BMC Microbiol. 12 Suppl 1(Suppl
669 1):S2.
670 Coon, K.L., Vogel, K.J., Brown, M.R., Strand, M.R., 2014. Mosquitoes rely on their gut
671 microbiota for development. Mol. Ecol. 23, 2727–2739.
672 Darshan, N., Manonmani, H.K., 2015. Prodigiosin and its potential applications. J. Food Sci.
673 Technol. 52, 5393–5407.
674 Dickson, L.B., Jiolle, D., Minard, G., Moltini-Conclois, I., Volant, S., Ghozlane, A., Bouchier,
675 C., Ayala, D., Paupy, C., Moro, C.V., Lambrechts, L., 2017. Carryover effects of larval
676 exposure to different environmental bacteria drive adult trait variation in a mosquito
677 vector. Sci. Adv. 3, e1700585.
678 Dinglasan, R., Devenport, M., Florens, L., Johnson, J., McHugh, C., Donnelly-Doman, M.,
679 Carucci, D., Yates, J., Jacobs-Lorena, M., 2009. The *Anopheles gambiae* Adult midgut
680 peritrophic matrix proteome. Insect Biochem. Mol. Biol. 39, 125–134.
681 Donnenberg, M.S., 2015. Enterobacteriaceae, in: Principles and Practice of Infectious
682 Diseases. Elsevier Inc; Philadelphia, PA: 2010. p. 2503.
683 Doucet, D., Retnakaran, A., 2012. Insect Chitin: Metabolism, Genomics and Pest
684 Management, in: Dhadialla, T.S. (Ed.), Advances in Insect Physiology, Insect Growth
685 Disruptors. Academic Press, pp. 437–511.
686 Eden, P.A., Schmidt, T.M., Blakemore, R.P., Pace, N.R., 1991. Phylogenetic Analysis of
687 Aquaspirillum magnetotacticum using polymerase chain reaction-amplified 16S rRNA-
688 Specific DNA. Int. J. Syst. Bacteriol. 41, 324–325.
689 Eng, J.K., McCormack, A.L., Yates, J.R., 1994. An approach to correlate tandem mass
690 spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc.
691 Mass Spectr. 5, 976–89.
692 Fang, S., Wang, L., Guo, W., Zhang, X., Peng, D., Luo, C., Yu, Z., Sun, M., 2009. *Bacillus*
693 *thuringiensis* Bel protein enhances the toxicity of Cry1Ac protein to *Helicoverpa*
694 *armigera* larvae by degrading insect intestinal mucin. Appl. Environ. Microbiol. 75,
695 5237–5243.

696 Felföldi, G., Marokházi, J., Képiró, M., Venekei, I., 2009. Identification of natural target proteins
697 indicates functions of a serralysin-type metalloprotease, PrtA, in anti-immune
698 mechanisms. Appl. Environ. Microbiol. 75, 3120–3126.
699 Galloway, C.S., Wang, P., Winstanley, D., Jones, I.M., 2005. Comparison of the bacterial
700 Enhancin-like proteins from *Yersinia* and *Bacillus* spp. with a baculovirus Enhancin. J.
701 Invertebr. Pathol. 90, 134–137.
702 Gendrin, M., Christophides, G.K., 2013. The *Anopheles* Mosquito microbiota and their impact
703 on pathogen transmission, in: Manguin, S. (Ed.), *Anopheles* Mosquitoes - New Insights
704 into Malaria Vectors. InTech.
705 Golemi-Kotra, D., 2008. Serratia, Edwardsiella and Morganella Infections, in: Enna, S.J.,
706 Bylund, D.B. (Eds.), XPharm: The comprehensive pharmacology reference. Elsevier,
707 New York, pp. 1–6.
708 Granados, R.R., Fu, Y., Corsaro, B., Hughes, P.R., 2001. Enhancement of *Bacillus*
709 *thuringiensis* toxicity to *Lepidopterous* Species with the Enhancin from Trichoplusia ni
710 Granulovirus. Biol. Control 20, 153–159.
711 Graves, S., Dorai-Raj, H.-P.P. and L.S. with help from S., 2019. multcompView: Visualizations
712 of Paired Comparisons.
713 Hashimoto, Y., Corsaro, B.G., Granados, R.R., 1991. Location and nucleotide sequence of
714 the gene encoding the viral enhancing factor of the Trichoplusia ni granulosis virus. J.
715 Gen. Virol. 72, 2645–2651.
716 Houston, D.R., Synstad, B., Eijsink, V.G.H., Stark, M.J.R., Eggleston, I.M., Aalten, D.M.F. van,
717 2004. Structure-based exploration of cyclic dipeptide chitinase inhibitors. J. Med.
718 Chem. 47, 5713–5720.
719 Ishii, K., Adachi, T., Hamamoto, H., Sekimizu, K., 2014. *Serratia marcescens* suppresses host
720 cellular immunity via the production of an adhesion-inhibitory factor against
721 immunosurveillance cells. J. Biol. Chem. 289, 5876–5888.
722 Kassambara, A., Kosinski, M., Biecek, P., 2019. survminer: Drawing Survival Curves using
723 “ggplot2” [WWW Document]. URL https://CRAN.R-project.org/package=survminer.

724 Kelkenberg, M., Odman-Naresh, J., Muthukrishnan, S., Merzendorfer, H., 2015. Chitin is a
725 necessary component to maintain the barrier function of the peritrophic matrix in the
726 insect midgut. Insect Biochem. Mol. 56, 21–28.
727 Laurence, D., Christophe, L., Roger, F., 2011. Using the Bio-Insecticide *Bacillus thuringiensis*
728 *israelensis* in Mosquito Control, in: Stoytcheva, M. (Ed.), Pesticides in the Modern
729 World - Pests Control and Pesticides Exposure and Toxicity Assessment. InTech.
730 Lever, M., 1972. A new reaction for colorimetric determination of carbohydrates. Anal.
731 Biochem. 47, 273–279.
732 Li, B., Yu, R., Liu, B., Tang, Q., Zhang, G., Wang, Y., Xie, G., Sun, G., 2011. Characterization
733 and comparison of *Serratia Marcescens* Isolated from edible cactus and from silkworm
734 for virulence potential and chitosan susceptibility. Braz. J. Microbiol. 42, 96–104.
735 Liu, X., Zhang, J., Zhu, K.Y., 2019. Chitin in Arthropods: Biosynthesis, Modification, and
736 Metabolism, in: Yang, Q., Fukamizo, T. (Eds.), Targeting Chitin-Containing Organisms,
737 Advances in Experimental Medicine and Biology. Springer, Singapore, pp. 169–207.
738 Manguin, S., Garros, C., Dusfour, I., Harbach, R.E. & Coosemans, M., 2008. Bionomics,
739 taxonomy, and distribution of major malaria vector taxa of *Anopheles* subgenus *Cellia*
740 in Southeast Asia: an updated review. Infect. Genet. Evol. 8, 489–503.
741 Miller, T.A., Lauzon, C.R., Lampe, D.J., 2008. Technological advances to enhance agricultural
742 pest management. Adv. Exp. Med. Biol. 627, 141–150.
743 Miyata, K., Maejima, K., Tomoda, K., Isono, M., 1970. Serratia Protease. Agric. Biol. Chem.
744 34, 310–318.
745 MMV-supported projects | Medicines for Malaria Venture [WWW Document], 2020. URL
746 https://www.mmv.org/research-development/mmv-supported-projects (accessed
747 4.5.20).
748 Nakjang, S., Ndeh, D.A., Wipat, A., Bolam, D.N., Hirt, R.P., 2012. A Novel extracellular
749 metallopeptidase domain shared by animal host-associated mutualistic and
750 pathogenic microbes. PLOS ONE 7, e30287.

751 Patil, C.D., Patil, S.V., Salunke, B.K., Salunkhe, R.B., 2011. Prodigiosin produced by *Serratia*
752 *marcescens* NMCC46 as a mosquito larvicidal agent against *Aedes aegypti* and
753 *Anopheles stephensi*. Parasitol. Res. 109, 1179–1187.
754 Pineda-Castellanos, M.L., Rodríguez-Segura, Z., Villalobos, F.J., Hernández, L., Lina, L.,
755 Nuñez-Valdez, M.E., 2015. Pathogenicity of Isolates of *Serratia Marcescens* towards
756 larvae of the Scarab *Phyllophaga Blanchardi* (Coleoptera). Pathogens 4, 210–228.
757 Purkayastha, G.D., Mangar, P., Saha, A., Saha, D., 2018. Evaluation of the biocontrol efficacy
758 of a Serratia marcescens strain indigenous to tea rhizosphere for the management of
759 root rot disease in tea. PLOS One 13, e0191761.
760 R Core Team, 2020. R: The R Project for Statistical Computing. R Foundation for Statistical
761 Computing, Vienna, Austria.
762 Ramirez, J.L., Short, S.M., Bahia, A.C., Saraiva, R.G., Dong, Y., Kang, S., Tripathi, A.,
763 Mlambo, G., Dimopoulos, G., 2014. Chromobacterium Csp_P Reduces malaria and
764 dengue infection in vector mosquitoes and has entomopathogenic and in vitro anti-
765 pathogen activities. PLoS Pathog. 10, e1004398-13.
766 Ramírez-Suero, M., Valerio-Alfaro, G., Bernal, J.S., Ramírez-Lepe, M., 2011. Synergisitic
767 effect of chitinases and B*acillus thuringiensis israelensis* spore-toxin complex against
768 *Aedes aegypti* larvae. Can. Entomol. 143, 157–164.
769 Richards, A.G., Richards, P.A., 1977. The Peritrophic Membranes of Insects. Annu. Rev.
770 Entomol. 22, 219–240.
771 Shaw, W.R., Catteruccia, F., 2019. Vector biology meets disease control: using basic research
772 to fight vector-borne diseases. Nat. Microbiol. 4, 20–34.
773 Shevchenko, A., Tomas, H., Havli, J., Olsen, J.V., Mann, M., 2007. In-gel digestion for mass
774 spectrometric characterization of proteins and proteomes. Nat. Protoc. 1, 2856–2860.
775 h
776 Short, S.M., Tol, S. van, MacLeod, H.J., Dimopoulos, G., 2018. Hydrogen cyanide produced
777 by the soil bacterium *Chromobacterium* sp. Panama contributes to mortality in
778 *Anopheles gambiae* mosquito larvae. Sci. Rep. 8, 1–13.

779 Singh, G., Arya, S.K., 2019. Antifungal and insecticidal potential of chitinases: A credible
780 choice for the eco-friendly farming. Biocatal. Agric. Biotechnol. 20, 101289.
781 Slavicek, J.M., 2012. Baculovirus enhancins and their role in viral pathogenicity, in: Molecular
782 Virology. InTech. pp. 147–155.
783 Suryawanshi, R.K., Patil, C.D., Borase, H.P., Narkhede, C.P., Salunke, B.K., Patil, S.V., 2015.
784 Mosquito larvicidal and pupaecidal potential of prodigiosin from Serratia marcescens
785 and understanding its mechanism of action. Pestic. Biochem. Phys. 123, 49–55.
786 Terra, W.R., 2001. The origin and functions of the insect peritrophic membrane and peritrophic
787 gel. Arch. Insect Biochem. Physiol. 47, 47–61.
788 Toprak, U., Harris, S., Baldwin, D., Theilmann, D., Gillott, C., Hegedus, D.D., Erlandson, M.A.,
789 2012. Role of enhancin in Mamestra configurata nucleopolyhedrovirus virulence:
790 selective degradation of host peritrophic matrix proteins. J Gen Virol, 93, 744–753.
791 Tu, S., Qiu, X., Cao, L., Han, R., Zhang, Y., Liu, X., 2010. Expression and characterization of
792 the chitinases from *Serratia marcescens* GEI strain for the control of Varroa destructor,
793 a honey bee parasite. J. Invertebr. Pathol. 104, 75–82.
794 Valzania, L., Martinson, V.G., Harrison, R.E., Boyd, B.M., Coon, K.L., Brown, M.R., Strand,
795 M.R., 2018. Both living bacteria and eukaryotes in the mosquito gut promote growth of
796 larvae. PLoS Negl. Trop. Dis. 12, e0006638.
797 Vichai, V., Kirtikara, K., 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening.
798 Nat. Protoc. 1, 1112–1116.
799 Wang, P., Granados, R.R., 1997. An intestinal mucin is the target substrate for a baculovirus
800 enhancin. PNAS 94, 6977–6982.
801 Wang, S., Dos-Santos, A.L.A., Huang, W., Liu, K.C., Oshaghi, M.A., Wei, G., Agre, P., Jacobs-
802 Lorena, M., 2017. Driving mosquito refractoriness to *Plasmodium falciparum* with
803 engineered symbiotic bacteria. Science 357, 1399–1402.
804 Wang, Y., Iii, T.M.G., Kukutla, P., Yan, G., Xu, J., 2011. Dynamic Gut Microbiome across Life
805 History of the Malaria Mosquito *Anopheles gambiae* in Kenya. PLOS One 6, e24767.

806 Wells, T.N.C., van Huijsduijnen, R.H., Van Voorhis, W.C., 2015. Malaria medicines: a glass
807 half full? Nat. Rev. Drug Discov. 14, 424–442.
808 Whitty, C.J.M., Ansah, E., 2019. Malaria control stalls in high incidence areas. BMJ 365.
809 Zarei, M., Aminzadeh, S., Zolgharnein, H., Safahieh, A., Daliri, M., Noghabi, K.A., Ghoroghi,
810 A., Motallebi, A., 2011. Characterization of a chitinase with antifungal activity from a
811 native *Serratia marcescens* B4A. Braz. J. Microbiol. 42, 1017–1029.
812 Zhang, X., Zhang, J., Zhu, K.Y., 2010. Chitosan/double-stranded RNA nanoparticle-mediated
813 RNA interference to silence chitin synthase genes through larval feeding in the African
814 malaria mosquito (*Anopheles gambiae*). Insect Mol. Biol. 19, 683–693.
815 Zhao, D., Guo, W., Sun, W., Xu, D., Liu, D., 2011. Identification of a novel enhancin-like gene
816 from *Bacillus thuringiensis*. Front. Agric. China 5, 423–429.
817 Zhong, W., Ding, S., Guo, H., Zhong, W., Ding, S., Guo, H., 2015. The chitinase C gene
818 PsChiC from *Pseudomonas* sp. and its synergistic effects on larvicidal activity. Genet
819 Mol Biol. 38, 366–372.
820 Zhou, W., Li, J., Chen, J., Liu, X., Xiang, T., Zhang, L., Wan, Y., 2016. The red pigment
821 prodigiosin is not an essential virulence factor in entomopathogenic *Serratia*
822 *marcescens*. J. Invertebr. Pathol. 136, 92–94.
823