1 ***Serratia marcescens* secretes proteases and chitinases with larvicidal activity**

2 **against *Anopheles dirus***

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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

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 vectors 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 is 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

70   agent (Caragata et al., 2020). This suggests that the natural microbial communities associated

71   with mosquitoes are invaluable resources for the discovery of biocontrol agents with new mode

72   of actions and the development of novel insecticides.

73   In this study, we isolated and identified culturable bacteria with distinct morphology

74   from the *An. dirus* larval rearing pan where larval death was being observed. Of seven isolates,

75   we found a strain of *Serratia marcescens* that secreted heat-labile larvicidal macromolecules

76   into spent medium when it was cultured under static condition at 25°C. We then used mass

77   spectrometry to identify chitinases and proteases as potential larvicidal molecules. Chitinase

78   and protease inhibitors were then used to confirm that these molecules contributed to larvicidal

79   activity. This study demonstrates that the chitinases and proteases uncovered in this study

80   could potentially be used as larvicides against *Anopheles* mosquito.

81

82   **2. Materials and Methods**

83   *2.1 Ethics statement*

84   Animal works in this study were carried out in accordance to the recommendations

85   provided in the Guide for the Care and Use of Laboratory Animals of the National Institutes of

86   Health. Mice were used only as a blood source for mosquito rearing, according to the protocol

87   (BT-Animal 26/2560). Larvicidal activity assays were performed following the approved

88   protocol (BT-Animal 20/2560). Both animal protocols were approved by the BIOTEC

89   Committee for use and care of laboratory animals.

90

91   *2.2 Mosquito rearing condition*

92   Specimens of laboratory strain of *An*. *dirus* were originally collected in Mae Sod

93   District, Tak Province, Thailand. A colony of *An. dirus* was maintained at the Department of

94   Parasitology, Chiang Mai University and BIOTEC's insectary at 27 °C with 75% humidity and

95   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%)

258

259 *3.2 S. marcescens secreted larvicidal molecule into spent medium when cultured under static*

260 *condition*

261   Because larval mortality was observed a few days after bacterial bloom, we

262 hypothesized that the isolated bacteria could secrete larvicidal molecule(s) after the bacteria

263 grow to a certain density; at a time when biofilm was observed at the bottom of the pan for a

264 few days. Therefore, the isolated bacteria were cultured under either static or planktonic

265 conditions for three days before spent media collection for the screening of larvicidal activity.

266   None of the cell-free spent media obtained from planktonic condition had any toxicity

267 against *An. dirus* larvae (Figure 1A). However, for the stationary culture, only the cell-free

268 spent media from *S. marcescens* had a detrimental effect on the survival of the larvae (Figure

269 1A). Next, spent medium of *S. marcescens* cultured under planktonic and static conditions

270 was collected over time after 1, 2, or 3 days of incubation. Again, the larvicidal molecule was

271 secreted into the *S. marcescens* only under static culture condition and the larvicidal activity

272 of the spent medium increased over time starting from the second day after inoculation (Figure

273 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.
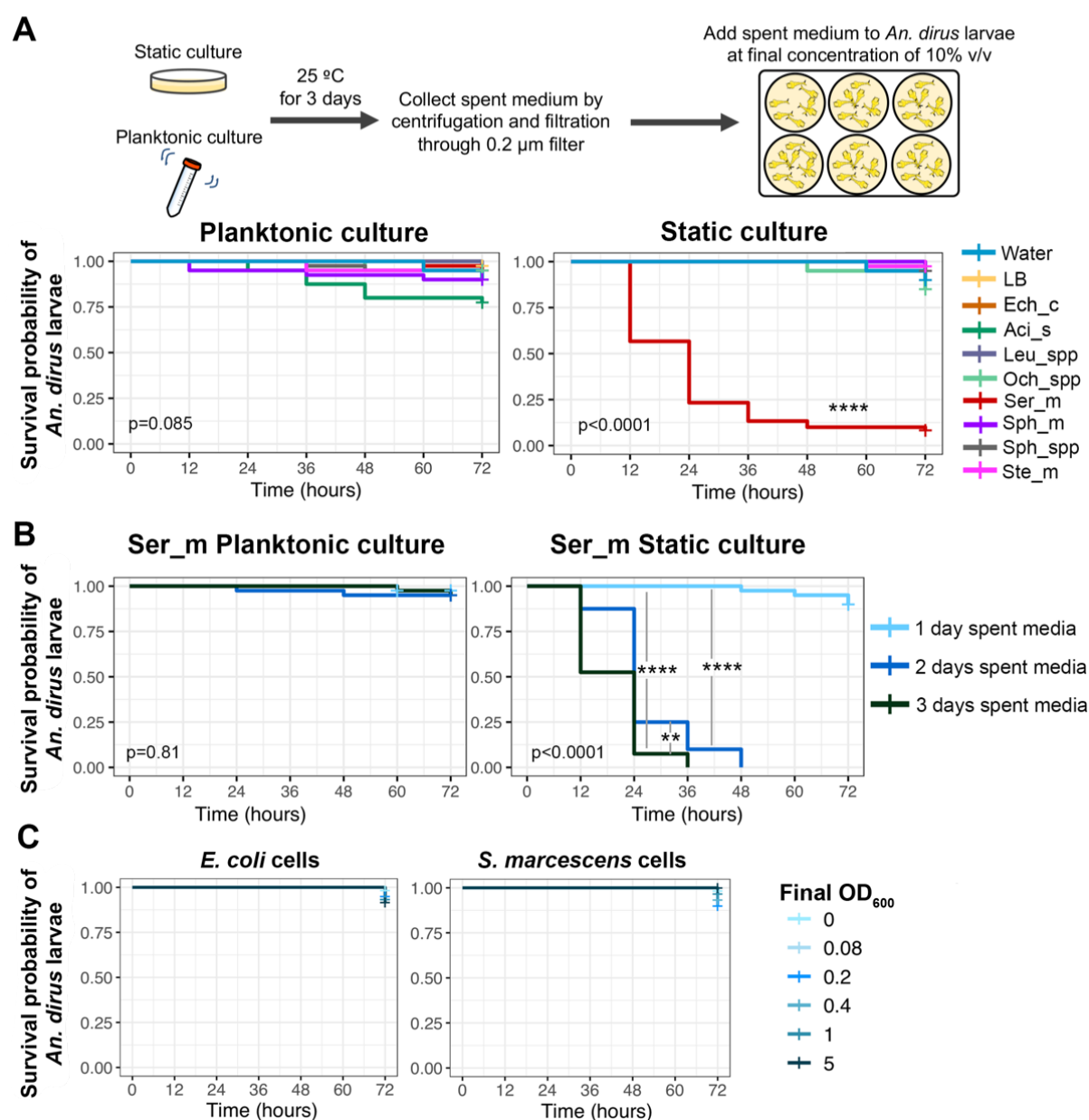

281

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: *Acinetobacter soli*, Leu_spp: Leucobacter 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
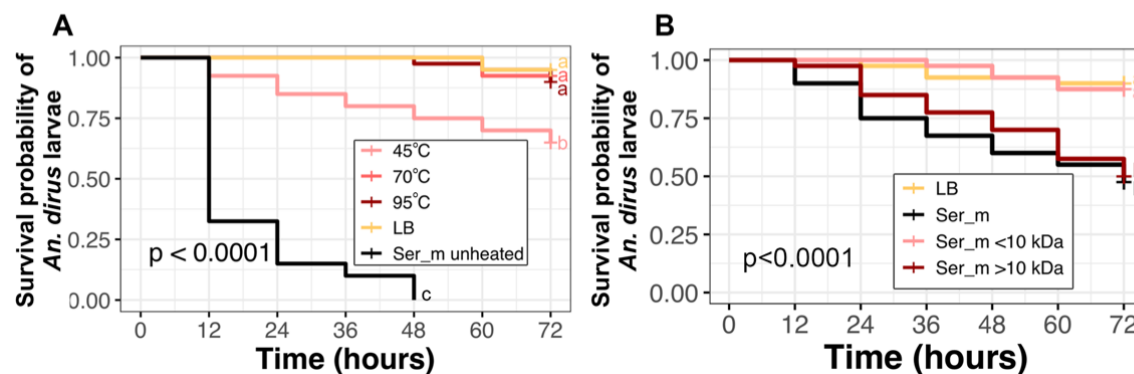
310 (Figure 2B).

311

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
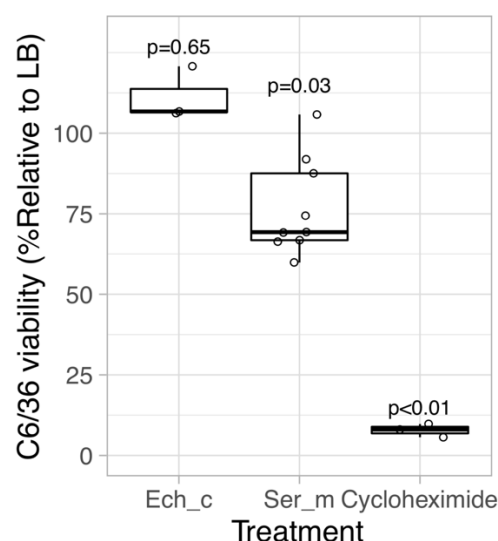
333   cells.

334

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
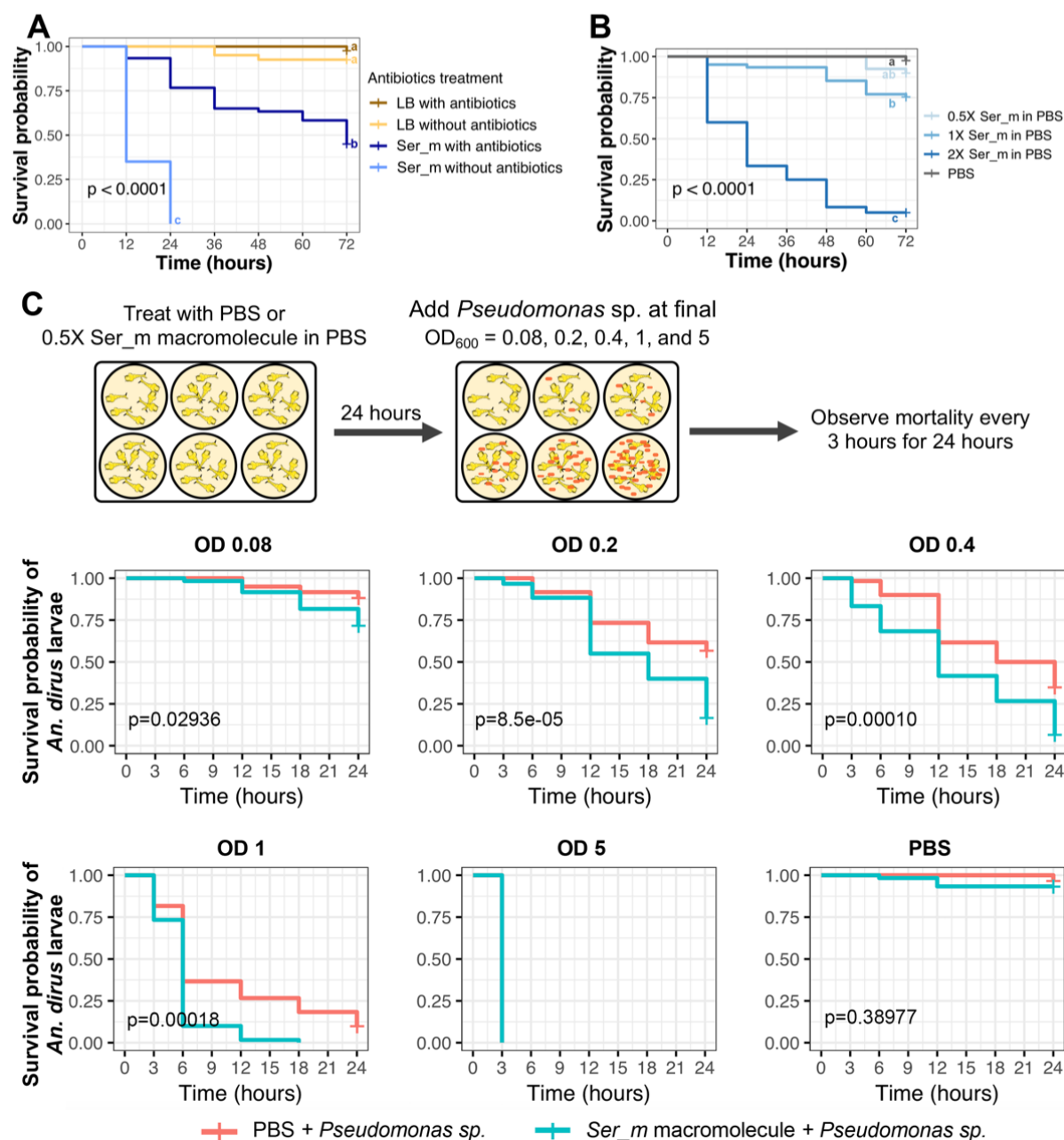
370   dose.

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

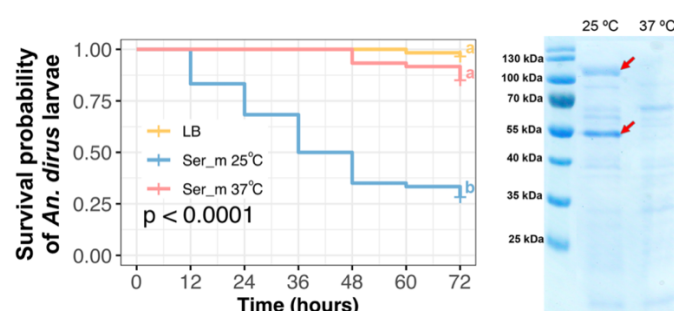417   hypothetical protein (Supplementary table 2).

418

419   Figure 5. *S. marcescens* secreted larvicidal molecules when cultured under static condition at

420   25 °C but not 37 °C. (A) Survival curve of *An. dirus* larvae after treatment with *S. marcescens*

421   spent media cultured under static condition at 25 °C or 37 °C. 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 °C or 37 °C. Two red arrows

425   indicate major protein bands that were expressed when the bacterium was cultured at 25 °C

426   but not 37 °C.

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

431

432   *3.7 Chitinase inhibitor reduced larvicidal activity of S. marcescens spent medium.*

433   Because the LC-MS analysis suggested that our strain of *S. marcescens* expressed

434   chitinase and chitobiose enzymes when cultured under static condition at 25 ºC but not 37 ºC,

435   we then inoculated the bacterium on LB agar supplemented with 0.5% chitin then incubated

436   at 25 ºC or 37 ºC to determined chitinase secretion. After five days of incubation, *S.*

437   *marcescens* cultured at 25 ºC produced a clear zone while the incubation at 37 ºC did not

438   (Figure 6A). Chitinase activity was also determined in the liquid static culture by pHBAH assay.

439   Chitinase activity of *S. marcescens* spent medium increased overtime during the incubation

440   under static condition at 25 ºC and saturated after 3 days of incubation (Figure 6B). This result

441   coincides well with the increase in larvicidal activity during the first three days of *S.*

442   *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.
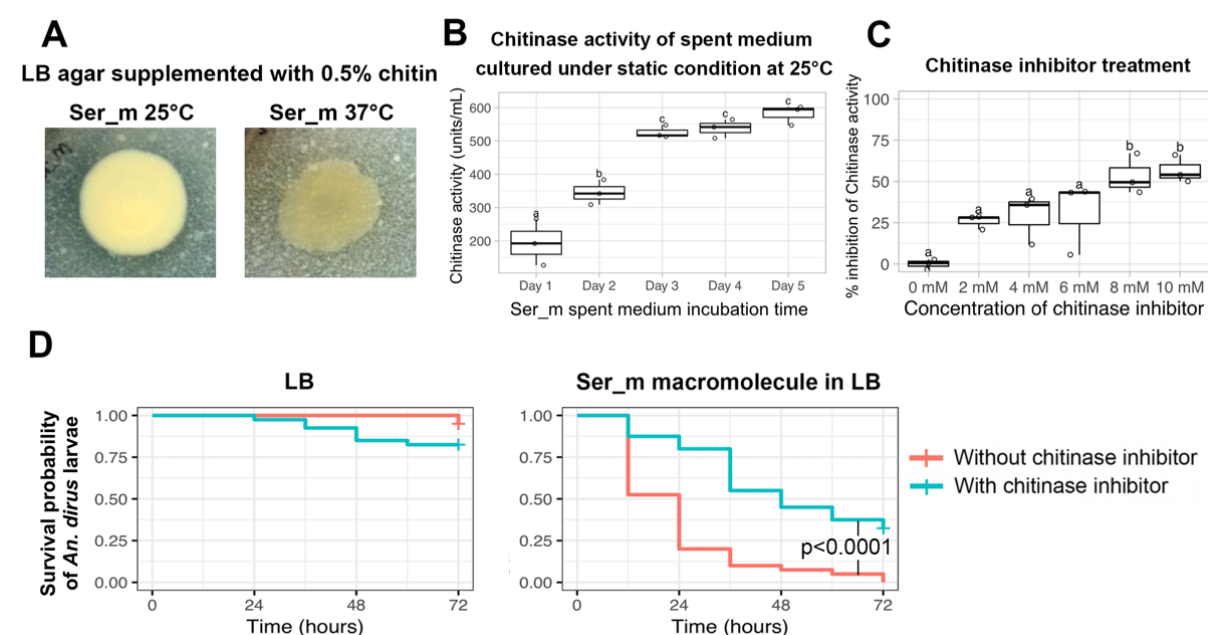

454

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
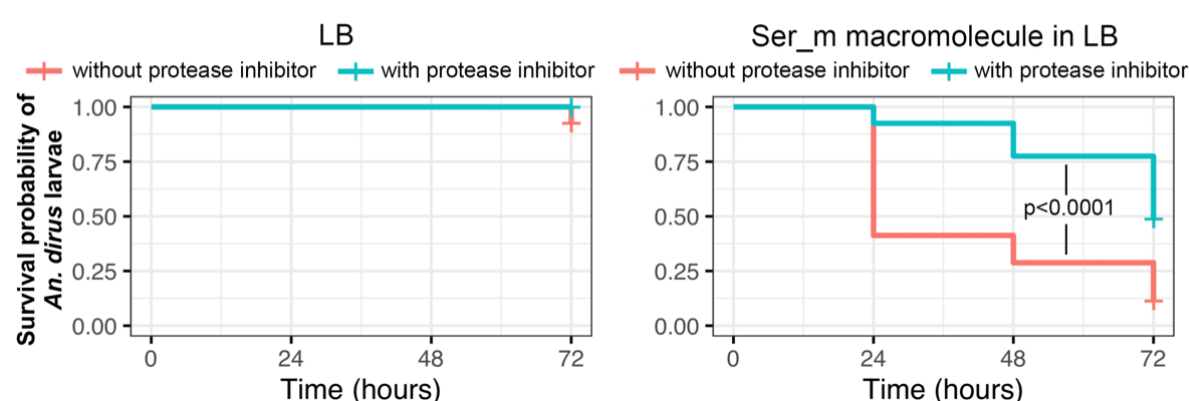
476   mortality (Figure 7).

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.

4. Discussion

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

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

589   reduction of larvicidal activity could also be a result from incomplete inhibition of chitinases

590   and proteases.

591   In summary, this study has identified a strain of *S. marcescens* from a larval rearing

592   habitat in the insectary, that secretes chitinases and proteases that are pathogenic to *An. dirus*

593   larvae. In addition to their intrinsic entomopathogenicity, these molecules have potential to

594   increase the larvae's susceptibility to natural entomopathogenic microorganisms, and other

595   mosquito control strategies currently in use. Further studies on dose optimization, formulation

596   of enzyme mixtures, and enzyme modifications to enhance heat stability are needed for its

597   development into field applications.

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622

Declaration of Competing Interest

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

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