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1 **The contribution of melanization to *Drosophila* survival changes with *Enterococcus***
2 ***faecalis* V583 genomic content**

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13 **Running title**: *E. faecalis* virulence factors affect *Drosophila* melanization

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22 **ABSTRACT**

23 *Enterococcus faecalis* is a human opportunist pathogen able to infect and kill
24 *Drosophila*. Previous studies proved that *E. faecalis* carrying the Fsr quorum sensing
25 system are extremely virulent. Fsr is the regulator of two important virulence factors,
26 gelatinase and serine protease, which cause death of *Drosophila* adult flies by
27 decreasing its tolerance to infection. The exact mechanism underlying the toxicity of
28 these *E. faecalis* virulence factors is nevertheless not known, in particular the way they
29 interfere with the host immune response. In the present study, we investigated the
30 influence of Fsr-GelE-SprE bacterial factors on different immunity responses, namely
31 antimicrobial peptide production, phagocytosis and melanization. Using *E. faecalis*
32 V583 wild type and *E. faecalis* V583 Δ*fsrB*Δ*gelE*Δ*sprE* mutant we showed that both
33 drosomycin production and phagocytosis were activated to similar levels by the two
34 bacterial strains. However, fly pupae infected with the mutant strain showed less
35 melanization and higher survival rates when compared to pupae infected with wild type
36 bacteria. Using adult flies carrying the $PPO1^{\Delta}$, $PPO2^{\Delta}$ mutation, we found that absence
37 of melanization had a different impact in survival of the flies when infected with the
38 two *E. faecalis* strains. $PPO1^{\Delta}$, $PPO2^{\Delta}$ mutant flies were more tolerant to *E. faecalis*
39 deprived of its major virulence factors. By showing that the presence of the *E. faecalis*
40 proteases completely alters the impact of melanization activation on *Drosophila*
41 tolerance, this study provides new clues on the interactions between *E. faecalis*
42 virulence factors and the fly´s immune system. Future studies on *Drosophila* immunity
43 should consider the pathogen genomic content.

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46 **INTRODUCTION**

47 In order to cause disease and death, pathogens must overcome the host´s immune
48 defenses. Understanding how the host immune defense mechanisms react to pathogens
49 and how pathogens inflict disease on the host can therefore provide us with clues to
50 fight those more efficiently. Among the most challenging pathogens are the
51 opportunistic ones, namely *Enterococcus faecalis*, which are commensal to humans but
52 can cause disease in patients with impaired immune systems. Enterococci are natural
53 inhabitants of the oral cavity, intestinal tract and female genital tract of both human and
54 animals. In contrast to the beneficial role they play in intestinal homeostasis, these
55 organisms are becoming increasingly important to human health as leading causes of
56 nosocomial infections. They are prevalent in the nosocomial environment, causing
57 infections of the urinary tract, bloodstream, intra-abdominal and pelvic regions, surgical
58 sites and central nervous system (1). To do so, they rely on several mechanisms
59 including the *fsr* operon in the case of *E. faecalis*. The *fsr* (*Enterococcus faecalis*
60 regulator) two component system, a homologue of the *agr* system in *Staphylococcus*
61 *aureus*, is a quorum sensing-dependent regulatory system that regulates the expression
62 of two other important virulence factors, *gelE* and *sprE*. These genes encode,
63 respectively, gelatinase (GelE), an extracellular zinc metalloprotease, and SprE, a serine
64 protease (2-4).

65 Recently, our Lab provided evidence for their role, and also for Fsr function, in
66 *Drosophila melanogaster* mortality (5). *D. melanogaster* (fruit fly) is a powerful model
67 organism to understand both the molecular mechanisms regulating the activation of
68 innate immune response and to screen for bacterial effectors involved in virulence (6).
69 The fruit fly has a multilayered immune system consisting of at least seven defensive
70 mechanisms: regulation of the native microbiota in the gut through antimicrobial

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71 peptides (AMPs) and reactive oxygen species; the barrier epithelial response, which
72 recognizes infections and wounds, produces local AMPs and sends signals to the rest of
73 the body; the clotting response, which not only seals wounds and prevents bleeding, but
74 can physically trap bacteria; the phenoloxidase response, which deposits melanin at the
75 site of an immune reaction, releasing potentially antimicrobial reactive oxygen species;
76 the phagocytic response, through which phagocytes can kill microbes directly by either
77 encapsulation or phagocytosis, or indirectly by releasing systemic signals; the systemic
78 AMP response, which involves the release of massive quantities of AMPs from the fat
79 body (the liver analog) into the circulation (7); and the RNAi response, which is
80 required to fight viral infections.

81 The expression of AMPs, regulated by the Toll and Imd pathways (8), can take a few
82 hours to a few days to occur. In contrast, a more immediate immune response, induced
83 within a few minutes after infection, is melanization (9). This is considered to be the
84 earliest and most acute reaction of insects against pathogens upon injury (9) and is used
85 to encapsulate and sequester pathogens too large to be phagocytized (10). During
86 melanization reaction, phenols are oxidized to quinolones, which then polymerize to
87 form melanin that is deposited around intruding microorganisms to help sequester them
88 at the wound site. The quinolone substances and other reactive oxygen intermediates are
89 thought to be directly toxic to microorganisms. Melanin synthesis is the final product of
90 the proteolytic cascade leading to the cleavage of prophenoloxidase (proPO) to
91 phenoloxidase (PO).

92 We have shown that the *E. faecalis* virulence factors Fsr, GelE and SprE are necessary
93 to cause *Drosophila* mortality upon infection (5). However, it remains unclear how
94 these factors control this process. In the present study we asked how different aspects of
95 the immune response in *Drosophila* were affected upon infection with *E. faecalis* and

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96 how that depends on the Fsr, GelE and SprE machinery. We found that important
97 resistance mechanisms, such as drosomycin expression and phagocytosis, were not
98 altered in the absence of Fsr and the proteases. In contrast, the melanization response
99 was severely affected in flies infected with wild type but not with Fsr mutant bacteria.
100 Furthermore, we show that outcome of the impairment in the melanization reaction in
101 infected flies depends on the genomic content of the infecting *E. faecalis* strain.

102

103 **MATERIALS AND METHODS**

104 **Bacterial Strains**: Strains used in this study are listed in Table 1. Enterococcal strains
105 were grown in BHI (Brain Heart Infusion) medium at 37ºC, and *Micrococcus luteus*
106 strain was grown in LB medium at 37ºC with agitation.

107 **RNA extraction and Real-Time PCR for AMP expression**: *E. faecalis* and *M. luteus*
108 strains were grown in BHI and LB, respectively, at 37ºC, until OD (600nm) 0, 02. The
109 bacterial strains were injected into W^{1118} flies. At 6h and 24h after infection 10 flies
110 were collected and homogenized to proceed to RNA extraction. Total RNA extraction
111 was prepared using a TRIzol (Life Technologies) extraction protocol and purified with
112 RNA Clean-up & Concentration from Zymo Research Company. SYBR Green
113 quantitative real-time PCR analysis was performed using 1st Strand cDNA Synthesis kit
114 RT-PCR (AMV) and LightCycler® 96 System from Roche Company. The primers used
115 are listed in Table 1. The amount of mRNA detected was normalized to control rp49
116 mRNA values. Normalized data were used to quantify the relative levels of a given
117 mRNA according to cycling threshold analysis (ΔCt). Relative ΔCt gene/ΔCt rp49
118 ratios of unchallenged wild-type controls were anchored in 1 to indicate fold induction.

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119 Graphs represent the mean and SD of relative ratios detected in three independent
120 biological repetitions.

121 ***Drosophila* infection**: W^{1118} *Drosophila* male flies (Table 2) were injected with 50 nl of
122 bacteria at OD (600 nm) 0.02 from one of the strains: V583, V583*ΔfsrBΔgelEΔsprE* and
123 *M. luteus*. As control, flies were injected with the same volume of BHI medium. Male
124 flies were anesthetized with CO_2 and injections were carried out with a pulled glass
125 capillary needle using a nano-injector (Nanoliter 2000, World Precision Instruments).
126 Reproducibility was measured by determining the number of bacteria injected at time
127 zero. Injected flies were placed at 29ºC, 65% humidity. Seventy-five flies were assayed
128 for each survival curve, and they were placed in three vials of 25 flies each. Each
129 experiment was repeated three times, making a total of 75 flies tested per strain in each
130 set of three replicates, to ensure high confidence results. Death was recorded at 0, 4, 6,
131 8, 10, 12, and 24 h hours post-injection. All experiments were performed at least three
132 times. Following challenge with bacteria, six individual flies were collected (at 0 h, 4 h,
133 8 h, 12 h and 24 h), homogenized, diluted serially, and plated onto Enterococcel agar
134 (Quilaban). *E. faecalis* CFUs (colony forming units) were determined by testing three
135 groups of six flies for each time point.

136 ***Drosophila* melanization**: *Drosophila* W^{1118}, in pre-pupa stage, was injected with 50 nl
137 of bacteria at OD (600 nm) 0.02 from one of the strains: V583, V583*ΔfsrBΔgelEΔsprE*
138 and *M. luteus*. As control, flies were injected with the same volume of BHI medium.
139 Injections were carried out with a pulled glass capillary needle using a nano-injector
140 (Nanoliter 2000, World Precision Instruments). The melanization process was recorded
141 at 0, 6, 24 and 48 h hour's post-injection using the stereoscope Lumar V12 (Zeiss
142 company). All experiments were performed at least three times.

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143 **Statistical analysis:** Statistical analysis of *Drosophila* survival was performed using
144 GraphPad Prism software version 5.03. Survival curves were compared using Log-rank
145 and Gehan-Breslow-Wilcoxon tests. Statistical analysis of *Drosophila* survival was
146 performed using Student's *t-test*.

147

148 **RESULTS**

149 In order to understand the mechanisms by which the Fsr-GelE-SprE factors in *E.*
150 *faecalis* induce fast death of *Drosophila* upon infection, we tested whether known
151 innate immune system pathways are differentially regulated in two *E. faecalis* strains,
152 V583 (wild type) and its isogenic mutant devoid of *fsr*, *gelE* and *sprE* genes.

153

154 **Drosomycin expression is similar during *Drosophila* infection with either V583 or**
155 **V583*ΔfsrBΔgelEΔsprE* strains**

156 It is known that Gram positive bacteria activate the Toll pathway and that Drosomycin
157 is one of the AMPs produced to kill this group of bacteria (6). One way bacteria use to
158 hamper the immune system of the host is by inhibiting these peptides. Indeed, Park *et al*
159 demonstrated that gelatinase from *E. faecalis* is able to degrade Gm cecropin, an
160 inducible AMP in the insect *Galleria mellonela* (11). We were therefore interested to
161 know whether the presence of Fsr-GelE-SprE influenced the expression levels of
162 AMPs. For that we measured the expression of Drosomycin by qRT-PCR at 6h and 24h
163 post-infection in both V583 and V583Δ*fsrB*Δ*gelE*Δ*sprE* strains and in the control strain
164 *M. luteus*. Interestingly, we found that all strains induced drosomycin expression to
165 similar levels in both time points analyzed, over the period of 24h (Figure 1). These

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166 results suggest that the *E. faecalis* virulence factors tested do not regulate AMP
167 production in *Drosophila*.

168

169 **_E. faecalis_ Fsr, GelE and SprE do not interfere with _Drosophila_ phagocytosis**

170 Phagocytosis is an important defense mechanism that has been conserved during
171 evolution. In *Drosophila* the circulating phagocytic cells are the plasmocytes, which are
172 part of the innate immune system. This complex cellular process is initiated by the
173 recognition of the particles or pathogens to be ingested, followed by cytoskeletal
174 remodeling and signaling events leading to their engulfment and destruction (12). It is
175 known that *E. faecalis* can survive for a prolonged period in mouse peritoneal, human
176 and zebrafish macrophages after being phagocyted (13-15). To investigate whether *E.*
177 *faecalis* Fsr-GelE-SprE perturb phagocytosis in the fruit fly, we used a *Drosophila* line
178 genetically modified to lack all hemocytes (*W^{1118}HmlΔ>GFP/UAS-Bax)*. We found that
179 flies without hemocytes (HmlΔ>GFP/UAS-Bax) show only slightly increased survival
180 rates upon infection with V583 when compared with control flies
181 (W^{1118}HmlΔ>GFP/UAS) (Figure 2A). The same was observed when the two
182 *Drosophila* lines were infected with the *E. faecalis* mutant strain
183 V583*ΔfsrBΔgelEΔsprE* (Figure 2B). The flies died at the same rate with or without
184 hemocytes and regardless of the presence of the *E. faecalis* virulence factors studied.
185 These data suggest that the role of the *E. faecalis* virulence factors tested in host death
186 does not seem to occur through changes in phagocytosis by the hemocytes.

187

188 **Fsr-GelE-SprE leads to increased melanization in pre-pupae**

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189 One of the key immune reactions in *Drosophila* is the activation of tyrosinase-type
190 phenoloxidases (POs), which catalyze several reactions leading to the crosslinking of
191 proteins, the production of reactive intermediates with potential cytotoxic activity and
192 ultimately to the production of melanin (16). Melanization is the earliest reaction
193 against the evasion of pathogen and it is visible by the blackening of wound site. To
194 determine if melanization is affected by the presence of Fsr-GelE-SprE in infecting *E.*
195 *faecalis*, we injected wild type pre-pupae, a stage that allows the easy detection of
196 melanized dark spots, with *E. faecalis* V583 and *E. faecalis* V583Δ*fsrB*Δ*gelE*Δ*sprE*
197 strains. At 6h post-infection, melanized spots are only around the site of injection in all
198 strains analyzed (Figure 3).

199 After 24h it is clear that the pre-pupae infected with V583 strain have an exacerbated
200 melanization, which is observed all over the body. In pre-pupae infected with *E. faecalis*
201 triple mutant, however, melanization remains restricted to the wound site, similar to pre-
202 pupae infected with the *M. luteus* control strain. Moreover, pre-pupae infected with wild
203 type bacteria were dead after 24h whereas those infected with the mutant bacteria were
204 still alive after 48h. These results indicate that the presence of the Fsr-GelE-SprE *E.*
205 *faecalis* virulence factors interferes with the melanization process during infection
206 through which it contributes to host death.

207

208

209 **_E. faecalis_ virulence factors modulate melanization effect on _Drosophila_ survival**

210 We thus asked if the excessive melanization was responsible for the fast and massive
211 death of the infected hosts. To answer this, we infected flies mutated in two
212 prophenoloxidases (PPO1 and PPO2), which makes them unable to produce melanin

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213 (9). Figure 4 shows the survival rates of $W^{1118}PPO1^{\Delta}PPO2^{\Delta}$ mutant and wild type flies
214 infected with V583 and mutant strains. When we compare PPO mutant and control flies
215 infected with the same wild type bacteria, survival rates are similar. However, they have
216 different shapes. During the first 12h of infection $PPO1^{\Delta}$, $PPO2^{\Delta}$ flies were affected in
217 their capacity to survive infection by V583 strain while the bacterial counts were
218 slightly higher during this same period, compared to wild type *Drosophila*. Similar
219 survival behavior was observed in $PPO1^{\Delta}$, $PPO2^{\Delta}$ flies infected with OG1-RF *E.*
220 *faecalis* strain (17), which carries the same Fsr-GelE-SprE virulence factors (18). This
221 decreased survival was not observed when $PPO1^{\Delta}$, $PPO2^{\Delta}$ flies were infected with the
222 bacterial mutant strain (Figure 4B). Taken together, these results (Figures 4 and 5)
223 suggest that melanization may be involved both in resistance and tolerance to *E.*
224 *faecalis*, and that its role depends on the presence of the Fsr-GelE-SprE virulence
225 factors. The two bacterial proteases present in V583, GelE and SprE, are able to degrade
226 host structural proteins, thus causing tissue damage, which must be healed in order for
227 the fly to maintain its healthy status. In the absence of melanization, which contributes
228 to tissue healing, it is possible that the flies tolerate less the presence of V583 carrying
229 Fsr-GelE-SprE factors.

230 When we compare the survival rates of PPO mutant and control flies infected with the
231 mutant bacteria major differences were observed. 24 hours after infection, 80% of the
232 $PPO1^{\Delta}PPO2^{\Delta}$ mutant flies infected with V583Δ*fsrB*Δ*gelE*Δ*sprE* triple mutant were still
233 alive (Figure 4B). In contrast, all wild type flies were dead when infected with the wild
234 type bacteria (Figure 4A). These results clearly indicate that when both melanization
235 and Fsr-GelE-SprE are absent almost all flies survive infection.

236

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237 **DISCUSSION**

238 The fly mechanisms responsible for protection against bacterial infections are not
239 clearly understood yet. *Drosophila* has four distinct pathways implicated in regulation
240 of genes induced upon septic injury, namely Toll, Imd, JNK and JAK-STAT (19).
241 Previous studies have shown that *E. faecalis* induces both cellular and humoral immune
242 response mechanisms in *Drosophila*. Toll seems to be the crucial pathway in the
243 defense against *E. faecalis* (20): whereas Toll pathway mutants are susceptible to *E.*
244 *faecalis*, Imd mutants are not (19). The Toll pathway is responsible for production of
245 several AMPs: diptericin, cecropin, drosocin and attacin are active against Gram-
246 negative bacteria and drosomycin, metchnikowin and defensin to fungi and Gram-
247 positive bacteria. Except for defensin, *E. faecalis* is resistant to the bactericidal activity
248 of all AMPs produced by *Drosophila*, and even from *G. mellonella* (21). It is thus not
249 clear how the Toll pathway confers protection against Gram-positive bacteria, as it is
250 known that defensin is not necessary to mediate protection (20). Previous *in vitro*
251 studies showing that the proteases GelE and/or SprE may degrade insect AMPs, have
252 led researchers suggest that *E. faecalis* success in insect species could be attributed to
253 the degradation of the host innate immune AMPs by the proteases. However, in a
254 previous study, our findings suggest otherwise (5). In fact, as we observed no difference
255 in growth inside the host between any of the mutants and wild type V583, we conclude
256 that neither the Fsr system nor the proteases it controls affect bactericidal action by the
257 fly. This implies that none of the proteases provides self-protection against any AMP in
258 the fly immune system (5). In the present study we showed that the presence of Fsr-
259 GelE-SprE does not affect the levels of drosomycin expression, further supporting the
260 likely irrelevant role of *Drosophila* AMPs on *E. faecalis* infection progression.
261 However, this does not exclude the possibility that the presence of these proteases in

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262 high amounts may turn the host more fragile to other bacteria due to AMPs degradation.
263 In fact, previous work has shown that GelE is able to degrade host AMP´s and that this
264 is responsible for insects getting less able to deal with *Escherichia coli* strains (11).

265 The way the fly is able to fight invading microorganisms also includes a cellular
266 immune response that can result in the phagocytosis of relatively small organisms like
267 bacteria or the encapsulation of larger parasites (22). However, little is known about
268 how *Drosophila* phagocytes affect the course of infections (23). On the other hand,
269 bacteria that are specialized in growing inside phagocytes have developed ways to fight
270 these cells from within. Moreover, previous studies have demonstrated that the
271 pathogenesis mechanisms developed by *Mycobacterium marinum* and *Listeria*
272 *monocytogenes* to fight vertebrate phagocytes also function in the fly (24, 25). In the
273 case of extracellular pathogens, such as *E. faecalis*, it is known that these bacteria are
274 able to stand macrophages defense mechanisms for hours and days (26). Although some
275 *E. faecalis* defense mechanisms have been implicated in its prolonged life inside
276 macrophages (27-29), neither Fsr nor the two proteases it regulates seem to play a role
277 in bacterial survival inside these defensive cells. Recently, macrophages in zebrafish
278 were shown to phagocytize bacteria in blood circulation being only able to engulf
279 surface-associated microbes (15). It is also known that homolog of tumor necrosis factor
280 (TNF) encoded by *eiger* is required for innate immune responses that are effective at
281 fighting extracellular pathogens but are wasteful or simply ineffective when fighting
282 intracellular pathogens (30). In our model, despite being phagocytized by *Drosophila*
283 hemocytes (results not shown), neither Fsr nor GelE or SprE were found to affect the
284 cellular immune response of *Drosophila*.

285 Melanization is another *Drosophila* immune response. It is visible by the blackening of
286 a wound site or the surface of pathogens, which results from the synthesis and

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287 deposition of melanin. In addition to being important for wound healing, melanin can
288 encapsulate and sequester pathogens, and the reaction intermediates appear to be
289 directly toxic to microbes as well (31). In our study, the effect of the presence of *E.*
290 *faecalis* virulence factors Fsr-GelE-SprE in melanization was evaluated. Pre-pupa
291 infected with *E. faecalis* V583 strain showed a different melanization pattern from that
292 shown by pre-pupa infected with the triple mutant strain. Consistent with the excessive
293 and all over body melanization observed with V583 infection, pre-pupa died earlier with
294 this virulent strain. The phenotype of these pre-pupa at 24h post-infection resembles
295 that of pupa devoid of serpin27 and PPO2 proteins (17), which present high levels of
296 constitutive PO activity. Activation of melanization is strictly regulated. Uncontrolled
297 melanization generates excessive toxic intermediates that can kill the host (9).
298 Recognition of pathogens and injury leads to the activation of a serine protease cascade
299 that culminates in proteolytic cleavage of inactive PPO to active PO. Serine protease
300 inhibitors, called serpins, are responsible for keeping the melanization strictly localized
301 at the site of injury or infection (9). Manipulation of the PO activity, through interfering
302 with the proteolytic activation of the melanization cascade, is a strategy developed by
303 some pathogens (32). The bacterial proteases studied GelE and SprE, are known to
304 degrade host proteins and cause tissue injury, and although their ability to interfere with
305 the PO activation was never evaluated. Our pre-pupa results suggest one of two
306 hypotheses: that the *E. faecalis* virulence factors are involved in melanization de-
307 repression and massive release of PO activity from crystal cells in the hemolymph; or
308 that the proteases induce such a high degree of tissue injury that the massive activation
309 of the melanization for tissue healing becomes overwhelming and deleterious to the host
310 itself.

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311 If the first hypothesis was to be true, infection of *Drosophila* *PPO1*$^{\Delta}$, *PPO2*$^{\Delta}$ mutant
312 with V583, which carries the proteases, would result in reduced host death. However,
313 the proteases, and the Fsr system that regulates their expression, were found not to play
314 a part in death by melanization in adult flies. A possible explanation for these results
315 could be related with a different role and impact of crystal cells in pre-pupae and adults.
316 As reported by Binggeli *et al* (2014), crystal cells could have evolved as an adaptation
317 to release a large quantity of PO activity in the hemolymph of pupa(17). Therefore, a
318 role for the bacterial proteases in induced PO activity, which would cause host death,
319 cannot be ruled out in the pre-pupa developmental stage, and is likely through activation
320 of melanization through massive tissue injury.

321 In adult flies, infection with the V583 mutant did not lead to increased survival. This
322 was unexpected as we previously reported that Oregon flies showed increased tolerance
323 to the same V583 mutant (5). This result shows that, within the same host species, the
324 host genetic background plays an important role in the outcome of an infection by an
325 opportunistic pathogen, such as *E. faecalis*. The difficulty in studying *E. faecalis*
326 virulence is, once again, evidenced with this result. Interestingly, the host genetic
327 background is not the only caveat of *E. faecalis* virulence studies. The bacterial
328 genomic content is also important. In fact, the *PPO1*$^{\Delta}$, *PPO2*$^{\Delta}$ mutation had a different
329 impact in survival of the flies when infected with the two *E. faecalis* bacterial strains.
330 *PPO1*$^{\Delta}$, *PPO2*$^{\Delta}$ mutant flies were more tolerant to *E. faecalis* deprived of its major
331 virulence factors than the wild type *Drosophila*. The presence of the proteases
332 completely altered the PO activation impact on *Drosophila* tolerance to *E. faecalis*. The
333 current knowledge on *Drosophila* immunity and on *E. faecalis* virulence, and on their
334 interaction as host and pathogen, is not enough to provide solid explanation for this
335 finding. Although knowledge of the effects of having no PO activity is scarce, some

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336 reports have highlighted the facts that affecting one particular immune pathway, leads to
337 changes in other *Drosophila* functions. The *Drosophila* mutant strain used in this study
338 has been partially, and recently, characterized and found to show increased Toll
339 activation by Gram-positive bacteria, when compared to its wild type (17). Activation
340 of Toll leads to induction of immunity, but also to reallocation of host resources, by
341 suppressing insulin signaling throughout the organism, leading to a decrease in both
342 nutrient stores and host growth (33). Immune and metabolic rearrangements in the
343 *Drosophila* PPO mutant flies may be the cause of the observed increased tolerance to
344 infection by the *E. faecalis* mutant strain. Future studies should clarify this.

345 In humans melanization does not occur, however PPO activation mediated by a serine
346 protease cascade is somewhat analogous to the coagulation pathway and complement
347 system (CS) in human plasma (34). Like the melanization, the fast activation of the
348 complement system after a microorganism infection of a potential host is a crucial step
349 in clearance of many pathogens. For example, anaphylatoxins like C3a and C5a,
350 products of the CS cascade, are commonly involved in exacerbated inflammatory
351 reactions that can cause direct harm to the host following infections (35). We know that
352 GelE destroys the C3a complement of human cells and AMPs of *G. mellonella* (11, 36).
353 Taking into account that the serine protease cascade during melanization is analogous to
354 the complement system, we hypothesize that in humans, Fsr regulated components
355 interfere with the complement response. Future studies should investigate this
356 hypothesis.

357 Our study shows that the outcome of *Drosophila* infection with *E. faecalis* depends both
358 on the bacteria gene content (in particular, the presence of Fsr and the proteases it
359 regulates) and on the host immunity status (in particular, on the PO activity). This
360 stresses the need to know more about how the host reaction is altered when the

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361 pathogen changes. As pointed out by others (10), a systematic genome-wide exploration
362 of pathogen mutants and their interaction with fruit fly immunity is important. The
363 present study points out some interesting facts that should orient future studies aiming at
364 finding new ways to diminish the mortality associated with *E. faecalis* infections. It may
365 not be sufficient to shut off the bacterial virulence, namely through interference with the
366 quorum-communication. An efficient control of *E. faecalis* infection outcome should
367 also include the host immune manipulation. The conservation between the innate
368 immune system of humans and *Drosophila* will allow future studies to develop new
369 targets to control *E. faecalis* infections in humans.

370

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376 *Drosophila* lines; and to Luis Teixeira from Instituto Gulbenkian de Ciência, Oeiras –
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381

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532

533 **Table 1 – Bacterial strains and primers used in this study.**

Strains	Relevant Characteristics	References
***E. faecalis* V583**	Clinical Isolate, TIGR sequenced strain; Va^R	(37)
MG03	*E. faecalis* V583Δ*fsrB*Δ*gelE*Δ*sprE*; $GelE^-$, $SprE^-$, $GBAP^-$	(5)
Micrococcus luteus	Gram positive bacteria	(#)
Primers for RT-PCR	**Sequence (5´-3´)**	
Drosomycin R	TCCCAGGACCACCAGCAT	(38)
Drosomycin F	CGTGAGAACCTTTTCCAATATGATG	(38)
Ribosomal protein 49 F	GACGCTTCAAGGGACAGTATCTG	(38)
Ribosomal protein 49 R	AAACGCGGTTCTGCATGAG	(38)

534 (#) strain provide by Luís Teixeira laboratory, IGC

535

536 **Table 2 – Flies used in this study.**

Flies	Relevant Characteristics
W^{1118}	Wild type fly
$W^{1118}PPO1^{\Delta}PPO2^{\Delta}$	Flies without PPO1 and PPO2 (17)
$W^{1118}Hml\Delta$>GFP/UAS	W^{1118} flies with hemocytes labeled with GFP
$W^{1118}Hml\Delta$>GFP/UAS-Bax	W^{1118} flies without hemocytes

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545

546 **Figure legends**

547 **Figure 1- Drosomycin relative expression (scale log_{10}) measured by qRT-PCR.**
548 *W^{1118}* flies were challenged by septic injury with Gram positive bacteria: *M. luteus*
549 (black), *E. faecalis* V583 (white) and *E. faecalis* V583Δ*fsrB*Δ*gelE*Δ*sprE* (grey). Total
550 RNAs were extracted at 6h and 24h post-infection. Results were normalized to *rpo49*
551 expression levels. *M. luteus* was used as a positive control of Drosomycin expression
552 and of the Toll pathway activation. Normalized data were used to quantify the relative
553 levels of a drosomycin according to cycling threshold analysis (ΔCt).

554

555 **Figure 2 – Survival curves of *Drosophila*, with and without phagocytes, infected**
556 **with *E. faecalis* V583 and *V583ΔfsrBΔgelEΔsprE.*** (A) *W^{1118}HmlΔ>GFP/UAS-Bax*
557 survival to septic injury with V583wt (B) *W^{1118}HmlΔ>GFP/UAS-Bax* survival to septic
558 injury with *V583ΔfsrBΔgelEΔsprE*. As a control *Drosophila* *W^{1118}HmlΔ>GFP/UAS*
559 flies were used. For each survival curve, 75 male adult flies, rose at 25ºC, where divided
560 in tubes 25 flies each, and infected, by septic injury onto the thorax with thin needle.
561 Data is representative of three independent experiments. Statistical analysis of
562 *Drosophila* survival was performed using GraphPad Prism software version 5.03.
563 Survival curves were compared using Log-rank and Gehan-Breslow-Wilcoxon tests and
564 they were not statistically different.

565

566 **Figure 3 – Melanization in wild type fly pre-pupae after infection.** *W^{1118}* pre-pupae
567 were infected by septic injury with 50nl of *M. luteus* at 0, 02 OD; V583wt and
568 V583Δ*fsrB*Δ*gelE*Δ*sprE* at 0, 02 OD, and placed at 29ºC. Injection with BHI medium

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569 and *M. luteus* are controls of this experiment. Pictures were taken with stereoscope
570 Zeiss Lumar V12 after 0h, 6h, 24h and 48h post-infection. This procedure was made at
571 least in 10 pre-pupae and the results were always the same. After 24h hours an
572 exacerbated melanization in the pre-pupae infected with V583wt was observed. All the
573 other pre-pupae showed only the normal black dots around the injection site.

574

575 **Figure 4 –Survival curves of *Drosophila* with and without melanization.** *Drosophila*
576 W^{1118} and $PPO1^{\Delta}PPO2^{\Delta}$ survival to septic injury, with V583wt (A); and
577 V583Δ*fsrB*Δ*gelE*Δ*sprE* (B) strains. For each survival curve, 75 male adult flies, rose at
578 25ºC, where divided in tubes 25 flies each, and infected, by septic injury onto the thorax
579 with a thin needle. Data is representative of three independent experiments. Statistical
580 analysis of *Drosophila* survival was performed using GraphPad Prism software version
581 5.03. Survival curves were compared using Log-rank and Gehan-Breslow-Wilcoxon
582 tests and they were not statistically different. Survival rates at time point 12h and 24h
583 are marked with (*) to represent statistically different results (calculated using the
584 Student's *t-test*) from the respective wild-type ($*p < 0.05$; $**p < 0.005$).

585

586 **Figure 5 - *E. faecalis* growth curves in injected flies.** (A) *E. faecalis* V583 growth
587 rates in injected fly W^{1118} and $W^{1118}PPO1^{\Delta}PPO2^{\Delta}$. (B) *E. faecalis*
588 V583*ΔfsrBΔgelEΔsprE* growth rates in injected fly W^{1118} and $W^{1118}PPO1^{\Delta}PPO2^{\Delta}$ Male
589 adult flies (5- to 7-day-old), raised at 25°C, were divided in tubes of 25 flies each, and
590 infected, by septic injury onto the thorax with a thin needle, with V583 strains. Flies
591 were collected at 0, 6, 12, and 24 h. Three groups of six flies for each time point were
592 homogenized and plated in Enterococcel agar and *E. faecalis* CFUs were determined.

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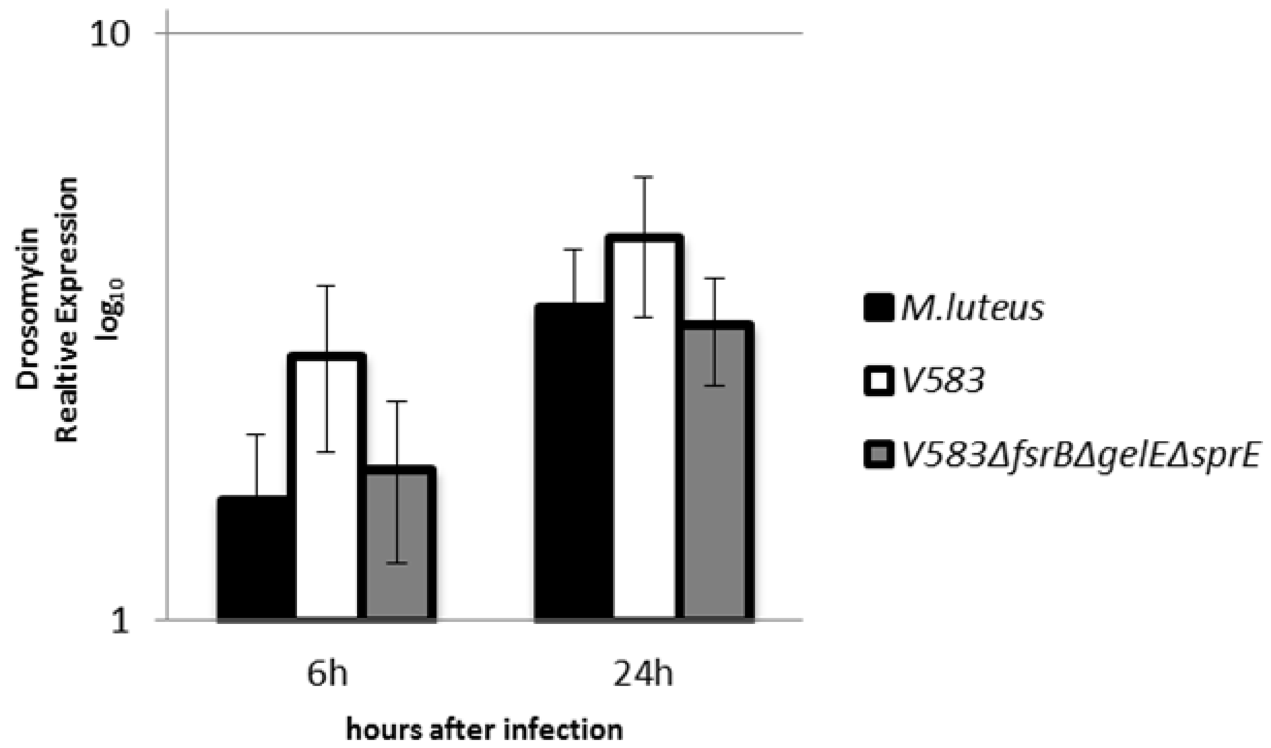
593 Student's t-test was used for statistical analysis. Asterisks (*) indicate the statistical
594 significance (*$p < 0.05$; **$p < 0.005$).
595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Drosomycin
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hours after infection
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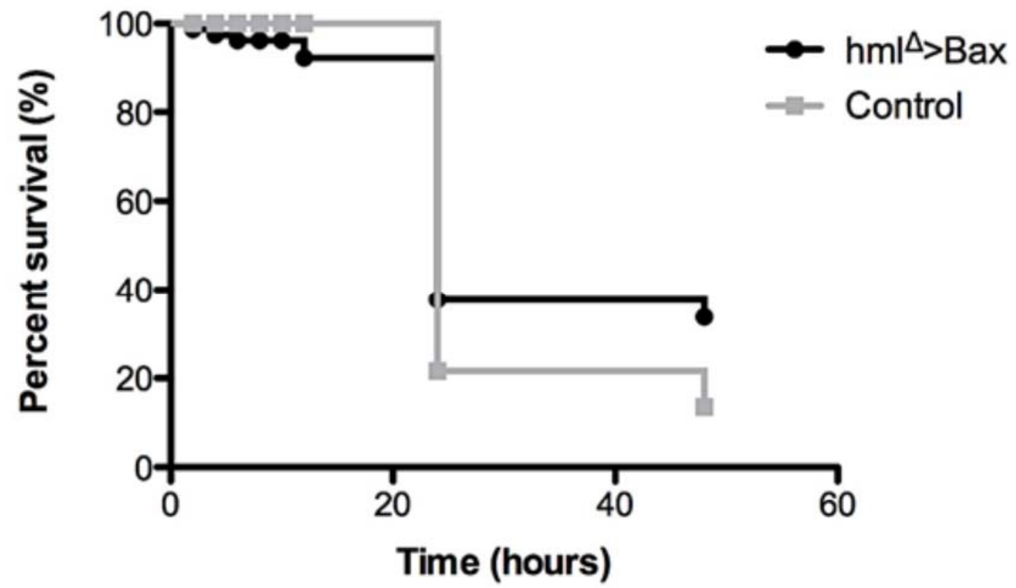
V583ΔfsrBΔgelEΔsprE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Percent survival (%)
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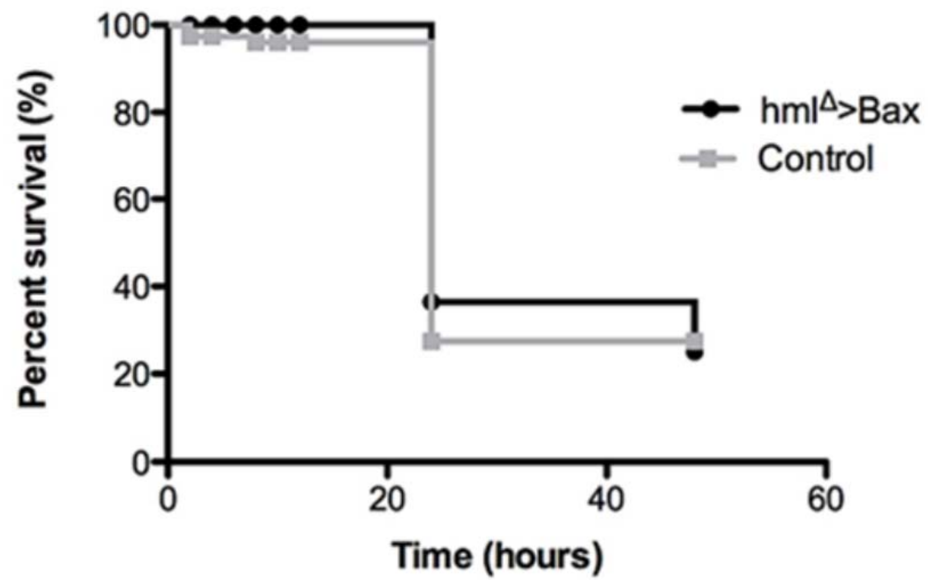
Time (hours)
hml$^{\Delta}$>Bax
Control


B
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80
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Percent survival (%)
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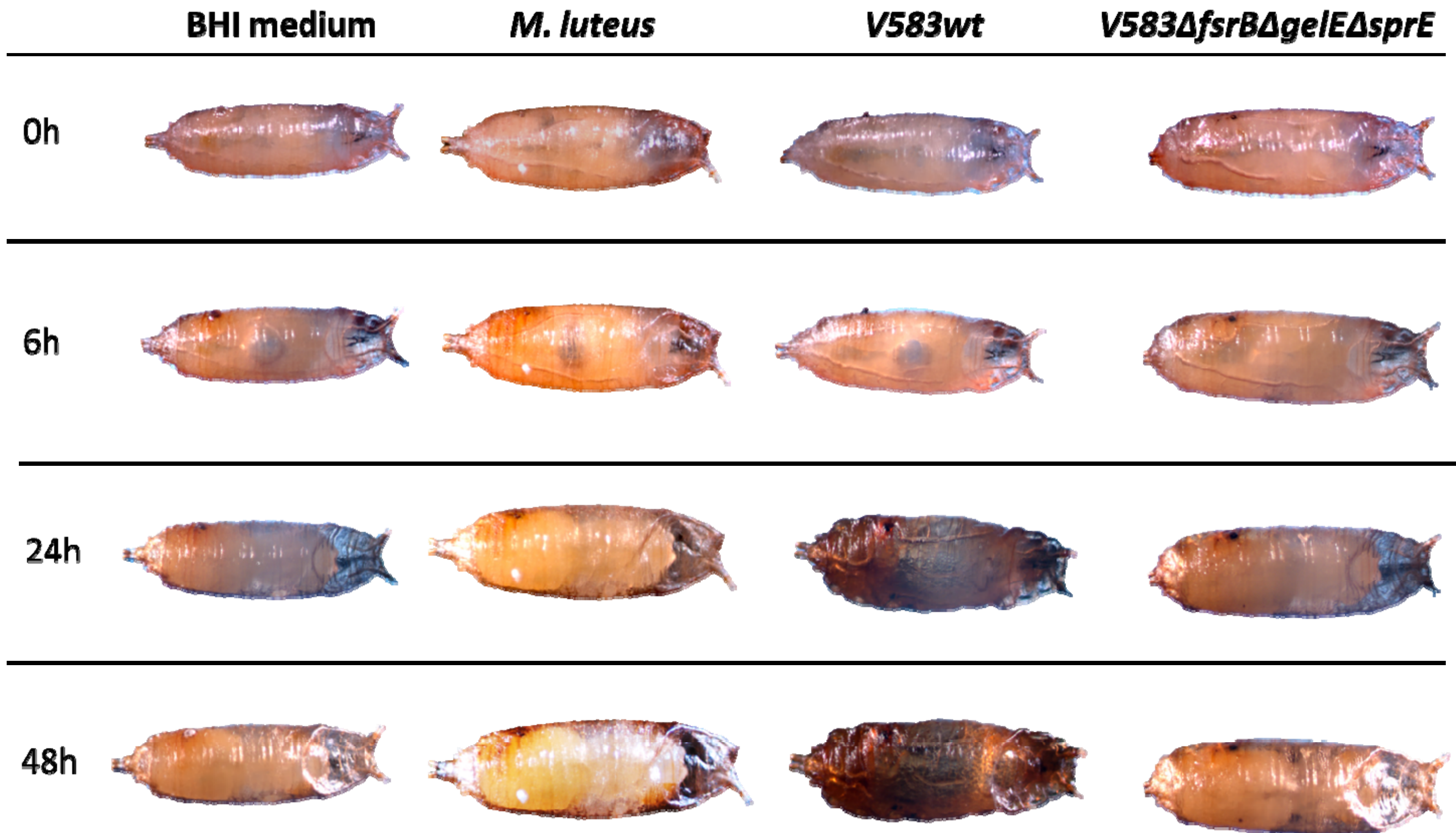
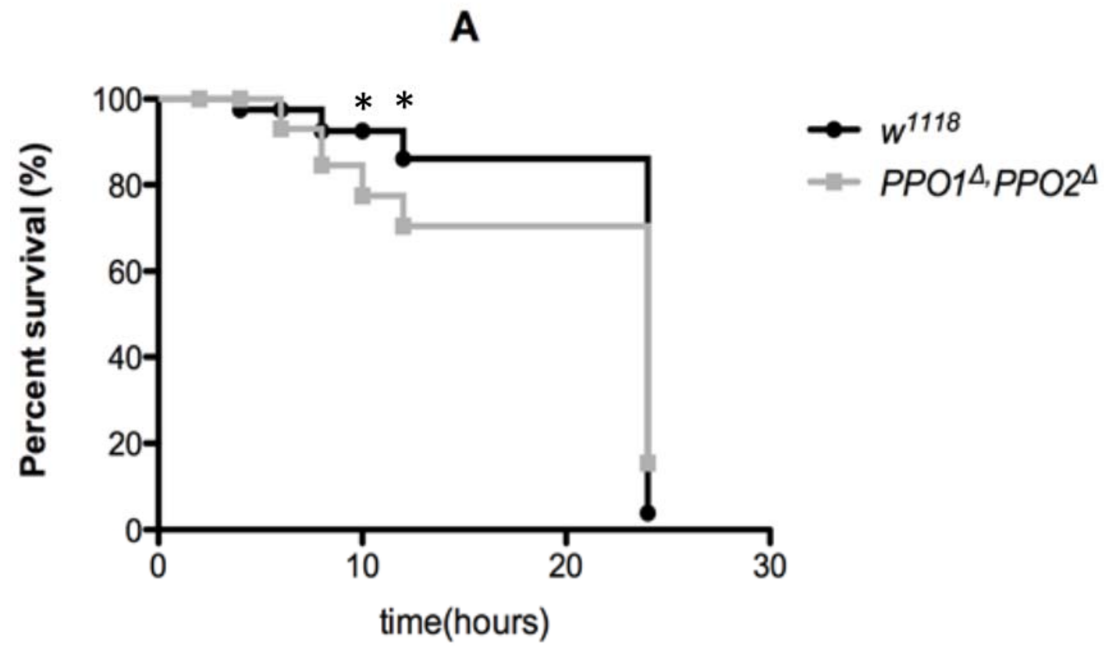
20
40
60
Time (hours)
hml$^{\Delta}$>Bax
Control

Melanization in W^{1118} pre-pupae

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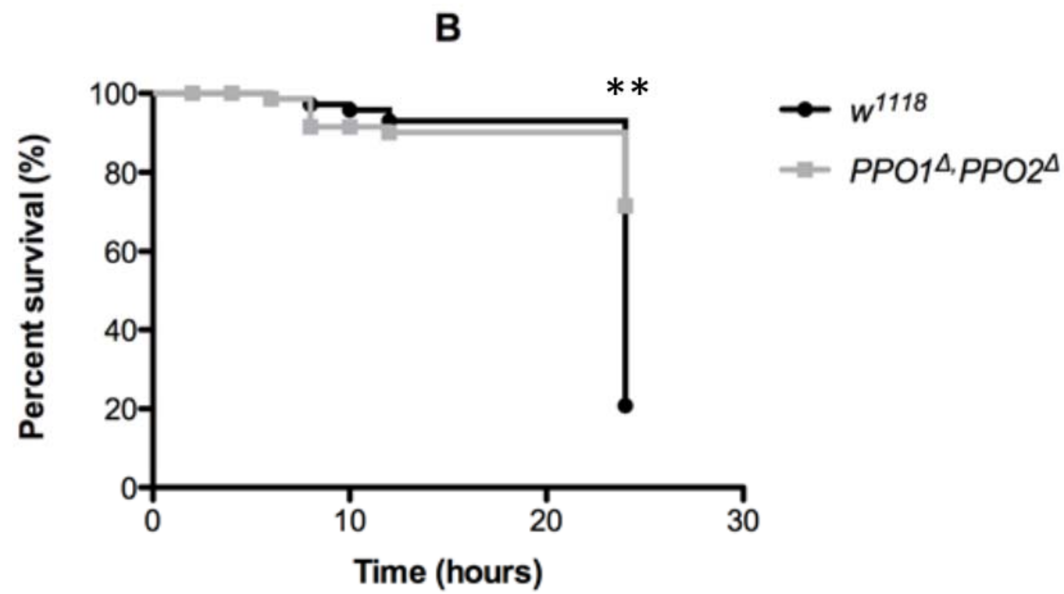
* *
Percent survival (%)
time(hours)
w1118
PPO1Δ,PPO2Δ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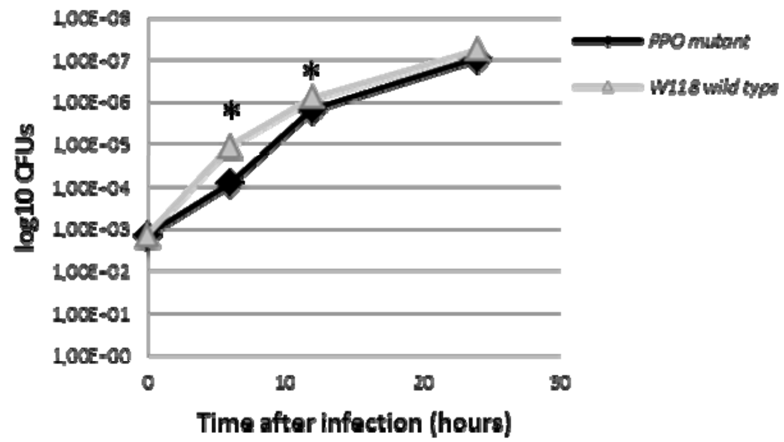
**
Percent survival (%)
Time (hours)
w1118
PPO1Δ,PPO2Δ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1,00E-06
1,00E-05
1,00E-04
1,00E-03
1,00E-02
1,00E-01
1,00E-00
log10 CFUs
0
10
20
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Time after infection (hours)
*
*
PPO mutant
W118 wild type

B
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
log10 CFUs
0
10
20
30
Time after infection (hours)