The effects of dietary iron supplementation on bacterial infections in *Manduca sexta* larval hemolymph

Marilyn K. Reese, Michael R. Kanost, Maureen J. Gorman*

Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, Kansas, 66506, U.S.A.

*Corresponding author
E-mail: mgorman@ksu.edu (MG)

Abstract

Iron is an essential nutrient for all types of organisms, including insects and the microbes that infect them. We predicted that insects fed an iron-supplemented diet would accumulate more iron in their hemolymph, and, because infectious microbes acquire iron from their hosts, that this extra iron would increase the severity of bacterial infections. To test this hypothesis, we studied the effects of dietary iron supplementation on infection outcomes in *Manduca sexta* (tobacco hornworm). Larvae were fed an artificial diet, with or without antibiotics, or the same diets supplemented with 10 mM iron. Control and iron-treated larvae were inoculated with non-pathogenic *Escherichia coli* or the entomopathogenic *Enterococcus faecalis*, and bacterial load and larval survival were measured. We found that dietary iron supplementation increased the iron content of hemolymph by approximately 20 fold; however, contrary to our prediction, this increase in iron did not result in an increase in the bacterial load of either *E. coli* or *E. faecalis*. The effect of iron supplementation on survival was more complicated. As expected, for larvae inoculated with nonpathogenic *E. coli*, iron supplementation had no effect. For larvae inoculated with *E. faecalis*, the effect of iron supplementation depended on whether antibiotics were present in the diet. Without antibiotics, iron supplementation prolonged larval survival; with antibiotics, iron supplementation decreased larval survival. The results of this study do not support the hypothesis that dietary iron supplementation increases infection severity in *M. sexta*. Instead, the results support the viewpoint that the relationship between dietary iron and infection outcome is complex.

Introduction

Iron is an essential nutrient for all types of organisms, including insects and the microbes that infect them (1,2). This need for iron establishes a competition between microbes and their hosts for iron. Infectious microbes have evolved mechanisms for obtaining iron from their hosts, while hosts have evolved mechanisms that limit the ability of the infectious microbes to take up iron from their hosts, thereby limiting the severity of infections (3–5).

We were interested in the question of what happens to infection severity when a host insect consumes extra iron. Previous research has shown that dietary iron supplementation increases the amount of iron in insect bodies (6–8); therefore, one possible outcome would be that the additional host iron promotes growth of infectious agents, resulting in more severe infections. The number of studies testing this hypothesis are still somewhat limited. *Drosophila melanogaster* fed an iron-supplemented diet were more likely than those fed a control diet to die from the fungal pathogen *Rhizopus oryzae*, whereas flies fed the same diet containing an iron chelator that decreases iron bioavailability were less likely to die from the fungal infection (9). In contrast, the addition of iron or iron chelator to the diet had no effect on the number of the intracellular bacterium *Spiroplasma poulsonii* in infected flies, and dietary iron supplementation in flies did not increase the density of the intracellular bacterium *Wolbachia* (10,11). Oral administration of an iron chelator in the mosquito species *Anopheles albimanus* resulted in strain-specific differences in the severity of infections with the malaria parasite *Plasmodium berghei*: there were fewer oocysts (parasites) on the midguts of one strain of *A. albimanus*, but the opposite result occurred in a different strain (12). Taken together, these studies indicate that the effect of dietary iron on infection outcome can depend on the specific host, the specific infectious microbe, and even the host strain.

The goal of this study was to determine whether dietary iron supplementation increases the severity of extracellular bacterial infections in the hemolymph of larval stage *Manduca sexta*. We predicted that iron would accumulate in the hemolymph of *M. sexta* larvae fed an iron supplemented diet. Our hypothesis was that the extra iron would promote proliferation of bacteria injected into the hemolymph, possibly leading to decreased host survival. Because this

72 study is the first to focus on the effect of dietary iron on extracellular bacterial infections, we
73 anticipated that the results would improve our understanding of the relationship between
74 dietary iron and infections in insects. We also hoped the results would be useful for designing
75 insect diets that reduce susceptibility to infection.

76 Our overall experimental approach was to feed *M. sexta* larvae an artificial diet with or
77 without iron supplementation, inoculate larvae with bacteria, and measure the effects of iron
78 supplementation on bacterial load and larval survival. For these studies, the control diet
79 contained approximately 1.2 mM iron, and the iron-supplemented diet contained 11.2 mM iron.
80 The amount of iron in the iron-supplement diet is similar to that used in the studies involving *S.*
81 *poulsonii* and *Wolbachia* but less than the study involving *R. oryzae* (9–11). For comparison, the
82 amount of iron in tobacco leaves, a natural diet of *M. sexta* larvae, has been observed in the
83 range of 40-1000 mg/kg wet weight (assuming fresh tobacco leaves are 80-85% water), which
84 corresponds to 0.7-17.9 mM iron (13,14). We typically culture *M. sexta* larvae on diet containing
85 kanamycin and tetracycline to limit bacterial growth in the diet and, thus, decrease the
86 variability of lab-induced immune responses (15); however, antibiotics can affect bacterial load
87 and larval survival by inhibiting growth of inoculated bacteria and altering the microbiome of
88 the midgut (16–18). For this reason, we used both antibiotics-free and antibiotics-added diet for
89 this investigation.

90 We used two species of bacteria for this study. *Escherichia coli* was our choice of a
91 nonpathogenic species because iron uptake mechanisms in *E. coli* are well-studied, mutant
92 strains deficient in iron uptake are available, and it has been used it for previous studies of
93 immunity in *M. sexta* (19–22). *Enterococcus faecalis* was our choice of an entomopathogen
94 because multiple iron uptake mechanisms in *E. faecalis* have been identified, and it has been
95 used previously for initiating infections in *M. sexta* larvae via inoculation (23,24).

96 The results of this study do not support the hypothesis that dietary iron
97 supplementation increases the severity of extracellular bacterial infections in the hemolymph of
98 larval stage *M. sexta*. Although iron supplementation increased the iron content of hemolymph,
99 this increase did not result in an increase in bacterial load. Furthermore, the effect of iron
100 supplementation on larval survival depended on the species of bacteria used and the presence

101 or absence of antibiotics. Our results are consistent with previous studies that have shown a
102 complex relationship between dietary iron and infection outcome.
103

104 Materials and Methods

105

106 Insect culture

107

108 Our *M. sexta* colony was maintained at 26 °C as described previously, including the use
109 of a wheat germ-based larval diet containing two antibiotics (0.25 g/ L kanamycin monosulfate
110 and 0.25 g/ L tetracycline hydrochloride) (15). Larvae used for experiments were fed either the
111 standard wheat germ-based (control) diet with or without antibiotics, or iron-supplemented
112 diet with or without antibiotics. We estimated that the control diet contained approximately 1.2
113 mM iron (mainly from two diet components: Wesson's Salt Mixture and wheat germ). Iron-
114 supplemented diet contained an additional 10 mM iron from adding 3.15 g ferric ammonium
115 citrate (composed of 17.9% iron) per liter of diet.
116 The following procedure was used to rear larvae for experiments. Control diet was
117 added to cups of eggs. Newly hatched larvae were placed on control or iron-supplemented diet,
118 two per cup, for three days, and thereafter, larvae were reared individually as described
119 previously (15). All comparisons between control and iron-treated larvae were made between
120 larvae from the same batch of eggs. Regardless of diet type, a small percentage of larvae failed
121 to thrive and were excluded from our analyses.
122

123 Bacteria

124

125 *E. coli*

126 Two strains of *E. coli* from the Keio Collection of single-gene knockouts were obtained
127 from the Coli Genome Stock Center (20). Strain #7636 is the parent strain for the collection, and

128 we refer to it as wild-type. Strain #11768 is identical to the parent strain except that it has a
129 mutation in the entA gene (ΔentA734::kan); this mutation blocks the bacteria from synthesizing
130 enterobactin, a siderophore needed for good growth in low iron environments (25). To prepare
131 *E. coli* for inoculations, the following procedure was used. A fresh colony was added to 100 mL
132 Luria-Bertani (LB) broth in a 500 mL flask, and the culture was grown at 37 °C with shaking for
133 approximately 18 h. Bacteria were collected from 25 mL of culture by centrifugation at 2,612 × g
134 for 15 minutes, washed by resuspending the bacteria in 0.85% NaCl, and centrifuged at 2,612 ×
135 g for 15 minutes. The bacterial pellet was resuspended in 0.85% NaCl, and the density was
136 adjusted to an OD_{600} of 3, corresponding to approximately 1.2 x 10^6 colony forming units (CFU)
137 per µL. The expected CFU per µL was verified by plating dilutions on LB agar plates, growing
138 colonies overnight at 37 °C, and counting the resulting colonies.
139

140 ***E. faecalis***

141 The *E. faecalis* strain designated OG1RF was obtained from the American Type Culture
142 Collection. This strain was chosen because it is suitable for use in a biosafety level 1 lab and it
143 has been used previously to infect *M. sexta* larvae (23). To prepare *E. faecalis* for inoculations,
144 the following procedure was used. Unless otherwise noted, 100 mL Todd Hewitt (TH) broth in a
145 500 mL flask was inoculated with a fresh colony, and the culture was grown at 37 °C with
146 shaking for approximately 26 h. Three experiments were performed with LB broth rather than
147 TH, and these exceptions are noted in the relevant figure legend. Bacteria were collected by
148 centrifugation at 2,612 × g for 15 minutes, washed by resuspending the bacteria in 0.85% NaCl,
149 and centrifuged at 2,612 × g for 15 minutes. The bacterial pellet was resuspended in 0.85%
150 NaCl, and the density was adjusted to an OD_{600} of 2, 0.2, or 0.02, corresponding to
151 approximately 1.6 x 10^6, 1.6 x 10^5, or 1.6 x 10^4 colony forming units (CFU) per µL. The expected
152 CFU per µL was verified by plating dilutions on TH agar plates, growing colonies overnight at 37
153 °C, and counting the resulting colonies.
154 Two experiments involved inoculation of larvae with dead *E. faecalis*. For the first
155 experiment, the bacteria were grown and washed as usual, then resuspended in 0.37%
156 formaldehyde in 0.85% NaCl, and placed on a rotator at room temperature for 3 days. The

157 treated bacteria were washed three times and resuspended in 0.85% NaCl to an OD_{600} of 20,
158 and then tested for viability. Because viability was not 0%, the formaldehyde-treated bacteria
159 were heated to 70 °C for 1 h. To verify that the two-step killing process was effective, 50 µL
160 bacteria were spread on an LB agar plate and incubated overnight at 37 °C. No colonies were
161 observed. The killed bacteria were diluted 10 fold, corresponding to an OD_{600} of 2, before use.
162 For the second experiment, bacteria were grown, washed, resuspended in 20 mL 0.37%
163 formaldehyde in 0.85% NaCl, and placed on a rotator at room temperature for 7 days. The killed
164 bacteria were washed three times with 10 mL 0.85% NaCl and resuspended in 4.5 mL 0.85%
165 NaCl to correspond to an OD_{600} of 20. To verify that the bacteria were dead, 50 µL bacteria were
166 spread on an LB agar plate and incubated overnight at 37 °C. No colonies were observed.
167

168 ## Inoculations

169

170 Larvae fed antibiotics-free diet were inoculated when they were 11 days old,
171 corresponding to one-day-old fifth instar larvae. Larvae fed antibiotics-containing diet
172 developed more slowly, and so were inoculated at 12 days old, corresponding to a mix of one-
173 day- and two-day-old fifth instar larvae. To anesthetize larvae, they were buried in ice for at
174 least 8 minutes. Anesthetized larvae were injected with 50 µL bacteria with the use of a 1 mL
175 syringe attached to a 30 gauge needle. Inoculated larvae were reared as usual.
176

177 ## Measuring bacterial load

178

179 To estimate the number of live bacteria in hemolymph, we used a track dilution method
180 that was based on previously published methods (26,27). Unless otherwise noted, this
181 procedure was performed 24 h post-inoculation. First, larvae were chilled on ice for at least 5
182 minutes, and the area around the horn was cleaned with 70% ethanol. A cut was made at the
183 base of the horn, and a few drops of hemolymph were collected in a sterile tube on ice. A 1:5
184 dilution was made by adding 20 µL hemolymph to 80 µL sterile 0.85% NaCl, and then up to five
185 additional dilutions, each 10-fold, were made. The 1:5 dilutions of hemolymph contained

186 antimicrobial activity that interfered with the assay; therefore, only the higher dilutions were
187 used for data analysis. Diluted hemolymph was analyzed by placing 20 µL of each dilution at the
188 top of square, gridded LB (for *E. coli*) or TH (for *E. faecalis*) agar plates, the plates were tilted
189 until the tracks ran the length of the plate, and then the plates were incubated at 37 °C
190 overnight. Colonies in tracks with suitable colony densities were counted, and data were
191 converted to colony forming units (CFUs) per µL hemolymph.
192

193 ## Monitoring larval survival

194

195 Feeding-stage larvae were observed each day, and any deaths were recorded. Once
196 larvae stopped feeding and entered the wandering stage, they were placed in groups in boxes
197 filled with vermiculite, and the number of larvae that reached the pupal stage was recorded. For
198 experiments designed to generate survival curves, all larvae were monitored each day until they
199 either died or pupated. Note that the larvae used for monitoring survival were not the same
200 larvae as those used for measuring bacterial load because the wounding procedure used for
201 collecting hemolymph can affect survival.
202

203 ## Iron content assay

204

205 The iron content of larval hemolymph was measured by a FerroZine-based method that
206 has been described previously (8) with several modifications. Larvae were fed control or iron-
207 supplemented diet without antibiotics. Hemolymph was collected from four control and four
208 iron-treated two-day-old fifth instar larvae by chilling them on ice, snipping off a proleg, and
209 collecting hemolymph into chilled tubes. Hemolymph was stored frozen. After thawing, 300 µL
210 hemolymph was mixed with 65 µL 12.1 N hydrochloric acid, and proteins were hydrolyzed at 95
211 °C for 20 minutes. Blanks were made by substituting hemolymph with deionized water. Samples
212 were centrifuged for 16,000 × g for 5 min, and 200 µL supernatant was transferred to a new
213 tube. To remove any remaining insoluble material, the samples were centrifuged for 16,000 × g
214 for 2 min, and then 150 µL supernatant was mixed with 60 µL 75 mM ascorbic acid to reduce

215 ferric ions to ferrous ions. After 10 min, the samples were mixed with 60 µL 10 mM FerroZine,
216 and 120 µL 10.4 M ammonium acetate was added to increase the pH, allowing iron-FerroZine
217 complex formation. To detect the iron-FerroZine complex, 300 µL of samples and blanks were
218 added to wells of a 96 well plate, and absorbance was measured at 562 nm. The concentration
219 of iron was calculated based on a molar extinction coefficient of the iron-FerroZine complex of
220 27,900 M^{-1} cm^{-1} (28).
221

222 ## Statistics

223

224 Statistical analyses were performed with GraphPad Prism version 5.04. An unpaired *t*-
225 test with Welch's correction was used to test for a statistically significant difference between
226 iron concentrations in hemolymph. Contingency tables containing percent survival data were
227 evaluated with a Fisher's exact test. Differences in survival curves were evaluated with a log-
228 rank test. Differences in the number of days to reach the wandering stage, which were based
229 on non-continuous data, were tested with a Mann Whitney test. Data representing CFU per µL
230 hemolymph were first tested for normality with a D'Agostino and Pearson omnibus normality
231 test. For data that passed the normality test, differences in means were evaluated with an
232 unpaired *t*-test, and means with standard deviations (SDs) are shown in graphs. For data that
233 did not pass the normality test, differences in medians were evaluated with a Mann Whitney
234 test, and medians with interquartile range (IQR) are shown in graphs. Samples sizes for all
235 experiments are indicated in the figure legends. We did not perform statistical analyses of
236 differences between experiments (for example, between experiments with and without
237 antibiotics) because of uncontrolled variables.
238

239 # Results and Discussion

240

241 ## Dietary iron supplementation increased the amount of iron in
242 hemolymph

We predicted that dietary iron supplementation would increase the amount of iron in hemolymph. To test this hypothesis, we used a FerroZine-based assay to measure the iron content of control and iron-treated larvae. We found that hemolymph from larvae fed iron-supplemented diet had approximately 20 times more iron than hemolymph from control larvae (with a mean ± SD of 680 ± 91 µM for iron-treated larvae and 33 ± 13 µM for control larvae) (Fig 1). Assuming that at least some of the additional iron is accessible to bacteria, this finding supports our hypothesis that iron supplementation could increase the proliferation of bacteria in hemolymph, leading to an increase in infection severity.

Fig 1. Iron supplementation increased iron content of hemolymph. Hemolymph was collected from fifth instar larvae that were reared on control (C) or iron-supplemented (Fe) diet without antibiotics, and a FerroZine-based assay was used to measure iron content. An unpaired *t*-test with Welch's correction was used to test for statistical significance, and means ± SD are shown (n = 4).

Dietary iron supplementation had little effect on naive larvae

To verify that the amount of iron in the iron-supplemented diet was not discernibly toxic to larvae, we fed larvae control or iron-supplemented diet, with or without antibiotics, and recorded growth rate, weight and survival. We found that in the absence of antibiotics, the iron-supplemented diet slowed the growth rate slightly, as demonstrated by the slightly longer time required for the iron-treated larvae to reach the wandering stage (Fig 2A); however, despite the slight developmental delay, the iron-treated larvae reached the same maximum weight as control larvae (Fig 2B). In the presence of antibiotics, neither growth rate nor maximum larval weight were affected by iron supplementation (Figs 2A and 2B). Regardless of the presence or absence of antibiotics, iron supplementation did not decrease larval survival (Fig 2C).

Fig 2. Iron supplementation had little effect on growth rate, weight and survival of naive larvae. Larvae were cultured on control (C) or iron-supplemented (Fe) diet, with or without antibiotics. A) Number of days from hatching to wandering stage. A Mann Whitney test was used to test for statistical significance (n = 43–47). B) Maximum larval weight. For the no-antibiotics diet, a Mann Whitney test was used to test for statistical significance, and medians ± IQR are shown (n = 48). For the antibiotics-containing diet, an unpaired *t*-test was used to test for statistical significance, and means ± SD are shown (n = 36). C) Percentage of larvae surviving to the pupal stage. A Fisher's exact test was used to test for statistical significance (without antibiotics, n = 48; with antibiotics, n = 36). For all experiments, the data presented were pooled from two or three biological replicates.

Dietary iron supplementation did not affect the outcome of *E. coli* infections

To test the hypothesis that dietary iron supplementation increases the severity of *E. coli* infections, we inoculated larvae with *E. coli* at an OD_{600} of 3 and measured larval survival and number of bacteria in hemolymph. As expected, infection with nonpathogenic *E. coli* had no effect on larval survival (Fig 3A); however, contrary to our prediction, iron supplementation did not lead to an increase in the number of bacteria in hemolymph, regardless of whether the diet contained antibiotics (Fig 3B). At 24 hours post-inoculation, the number of bacteria in hemolymph was low, with a median CFU per µL ranging from 0 to 15. Assuming a larval hemolymph volume of roughly 1 mL, the number of live bacteria decreased from 6×10^7 CFU per larva at the time of injection to a median of $\leq 1.5 \times 10^4$ CFU per larva 24 hours later. At 48 hours post-inoculation, even fewer bacteria remained. These results suggest that most of the *E. coli* injected into larvae were killed by the larval immune system.

Fig 3. Iron supplementation had no effect on the severity of *E. coli* infections. Larvae cultured on control (C) or iron-supplemented (Fe) diet, with or without antibiotics, were inoculated with *E. coli* at an OD_{600} of 3, and infection severity was assessed. A) Percentage of inoculated larvae

300 surviving to the pupal stage. A Fisher's exact test was used to test for statistical significance (n =
301 24). B) The number of CFU per µL hemolymph. A Mann Whitney test was used to test for
302 statistical significance; medians with upper IQR are shown (without antibiotics at 24 h, n = 48;
303 the other two experiments, n = 24). The data for the first experiment in panel B are pooled from
304 two biological replicates. C) Larvae fed the control diet without antibiotics were inoculated with
305 either wild-type (WT) or a mutant (Mut) strain of *E. coli* that grows poorly in low iron conditions,
306 and CFU per µL hemolymph 24 h after inoculation was determined. A Mann Whitney test was
307 used to test for statistical significance; medians with upper IQR are shown (n = 24).
308
309 A possible explanation for why iron supplementation did not increase bacterial load is
310 that *E. coli* in hemolymph may enter a quiescent state that does not require iron uptake. This
311 could happen, for example, if larval hemolymph did not support the growth of *E. coli*; however,
312 proliferation of *E. coli* in cell-free hemolymph in vitro suggests that hemolymph can support *E.*
313 *coli* growth in vivo (29). A second possible explanation is that the amount of iron in control
314 hemolymph may be sufficient for optimal bacterial growth. As an initial test of this hypothesis,
315 we took advantage of a mutant strain of *E. coli* that does not synthesize the siderophore
316 enterobactin and, thus, does not grow well in low iron environments (25). We inoculated
317 control larvae with either wild-type or mutant *E. coli*, and then measured bacterial load at 24
318 hours post-inoculation. The number of mutant *E. coli* was the same as the number of wild-type
319 *E. coli* (Fig 3C), suggesting that the amount of iron in hemolymph was not growth limiting.
320

321 The effects of dietary iron supplementation on the outcome of *E.* 322 *faecalis* infections was mixed

323
324 To test the hypothesis that iron supplementation would increase the severity of *E.*
325 *faecalis* infections, we used four experimental conditions: antibiotics-free diet with *E. faecalis* at
326 an OD_{600} of 0.02 or 0.2, and antibiotics-containing diet with *E. faecalis* at an OD_{600} of 0.2 or 2.
327 Because *E. faecalis* is an entomopathogen (23,30), we anticipated some larval death for both

328 diet treatment groups, but we predicted that the iron-supplemented diet would decrease
329 survival by promoting bacterial proliferation.
330 As expected, when fed an antibiotics-free diet, control and iron-treated larvae were
331 killed by *E. faecalis* infections. Whereas injection of *E. coli* at an OD_{600} of 3 was not lethal,
332 injection of *E. faecalis* at an OD_{600} of 0.2 killed nearly all larvae within two days (Fig 4A). The
333 number of *E. faecalis* increased from 8 x 10^6 to 10^8–10^9 bacteria per larva in the 24 hours after
334 inoculation, indicating that larval hemolymph can support the growth of *E. faecalis* (Fig 4B).
335 However, contrary to our hypothesis, we observed no significant difference in survival or
336 number of bacteria between control and iron-treated larvae (Figs 4A and 4B).
337
338 **Fig 4. Without antibiotics, iron supplementation prolonged survival of larvae infected with *E.***
339 ***faecalis* without decreasing bacterial load.** Larvae cultured on control (C) or iron-supplemented
340 (Fe) diet without antibiotics were inoculated with *E. faecalis*. A) Larvae were inoculated with *E.*
341 *faecalis* at an OD_{600} of 0.2, and mortality was monitored. The resulting survival curves were
342 compared by performing a log-rank test (n = 24). B) Larvae were inoculated with *E. faecalis* at an
343 OD_{600} of 0.2, and CFU per µL hemolymph was measured after 24 h. An unpaired *t*-test was used
344 to test for statistical significance, and means ± SD are shown (n = 24). C) Larvae were inoculated
345 with *E. faecalis* at an OD_{600} of 0.02, and mortality was monitored. The resulting survival curves
346 were compared by performing a log-rank test (n = 24). D) Larvae were inoculated with *E.*
347 *faecalis* at an OD_{600} of 0.02, and CFU per µL hemolymph was measured after 24 h. The results of
348 three biological replicates are presented. A Mann Whitney test was used to test for statistical
349 significance; medians ± IQR are shown (n = 24).
350
351 By injecting *E. faecalis* at an OD_{600} of 0.02, we were able to avoid the rapid, severe
352 infections produced by injecting *E. faecalis* at an OD_{600} of 0.2. Under these conditions, we saw a
353 difference in survival, but, surprisingly, instead of decreasing larval survival, iron
354 supplementation extended survival time (Fig 4C and S1 Fig). These data suggest that dietary iron
355 supplementation may temporarily protect larvae from infection with *E. faecalis*. To determine
356 whether the longer survival of iron-treated larvae corresponded to a reduced bacterial load, we

357 measured the number of bacteria in hemolymph 24 hours after inoculation. For one biological
358 replicate, iron-treated larvae had fewer bacteria, but for the other two replicates there was no
359 significant difference between diet treatments (Fig 4D). These results suggest that the cause of
360 the extended survival of iron-treated larvae is unrelated to bacterial load. The finding that iron
361 supplementation may temporarily protect larvae from *E. faecalis*-induced death was
362 unexpected. Given that dietary iron supplementation can affect the gut microbiome of humans
363 (31,32), a potential explanation is that iron supplementation may affect the gut microbiome in a
364 way that influences infection tolerance (17).

365 Because our standard larval diet includes kanamycin and tetracycline, we wanted to
366 know how iron supplementation in the presence of these antibiotics affects infection severity.
367 We found that the addition of antibiotics considerably altered the effects of iron
368 supplementation. As described above, when injected with *E. faecalis* at an $OD6_{00}$ of 0.2, most
369 larvae fed antibiotics-free diet developed a high bacterial load and died (Fig 4A); in contrast,
370 larvae fed the antibiotics-containing diet survived the infection and had a very low bacterial
371 load, with no significant difference between control and iron-treated larvae (Figs 5A and 5B).
372 These results suggest that antibiotics can be transported from the diet to the larval hemolymph
373 where they can kill *E. faecalis* (a phenomenon we did not observe with *E. coli*).
374

375 **Fig 5. With antibiotics, iron supplementation decreased survival of larvae infected with *E.***
376 ***faecalis* without increasing bacterial load.** Larvae cultured on control (C) or iron-supplemented
377 (Fe) antibiotics-containing diet were inoculated with *E. faecalis*. A) Larvae were inoculated with
378 *E. faecalis* at an OD_{600} of 0.2, and survival to the pupal stage was monitored. A Fisher's exact
379 test was used to test for statistical significance (n = 24). B) Larvae were inoculated with *E.*
380 *faecalis* at an OD_{600} of 0.2, and CFU per µL hemolymph was measured after 24 h. A Mann
381 Whitney test was used to test for statistical significance; medians with upper IQR are shown (n =
382 24). C) Larvae were inoculated with *E. faecalis* at an OD_{600} of 2, and mortality was monitored.
383 The percentage surviving at two days post-inoculation, the wandering stage, and the pupal
384 stage are shown. The results of two biological replicates are presented. A Fisher's exact test was
385 used to test for statistical significance (n = 24). D) Larvae were inoculated with *E. faecalis* at an

386 OD_{600} of 2, and CFU per µL hemolymph was measured after 24 h. The results from two
387 biological replicates are shown. A Mann Whitney test was used to test for statistical significance;
388 medians ± IQR are shown (n = 24). E) Larvae were inoculated with dead *E. faecalis* at an OD_{600} of
389 2 or 20, and survival to the pupal stage was monitored. A Fisher's exact test was used to test for
390 statistical significance (n = 24). *E. faecalis* was cultured in LB broth for experiments presented in
391 A, B, and C replicate 1, whereas *E. faecalis* was cultured in TH broth for experiments presented
392 in C replicate 2 and D.
393
394 For the next set of experiments, we fed larvae antibiotics-containing diet and injected *E.*
395 *faecalis* at an OD_{600} of 2. Under these experimental conditions, the iron-treated larvae were
396 significantly more likely than control larvae to die; this effect was significant at 2 days post-
397 inoculation and was even more dramatic by the end of the larval period (Fig 5C). This result
398 supported our hypothesis that iron supplementation may increase infection severity; however,
399 surprisingly, decreased survival did not correspond to an increased bacterial load (Fig 5D).
400 Excess iron, immune reactions, and antibiotics (including tetracyclines) can all cause
401 physiological stress (33–35), and it appears that the inoculated larvae that were exposed to
402 antibiotics and extra iron may have died from a lethal combination of these stresses. We
403 wondered whether live bacteria were necessary for the high mortality or if an infection-free
404 immune response (36,37) would be sufficient. To answer this question, we inoculated larvae
405 with killed *E. faecalis* at an OD_{600} of 2 and then monitored larval survival. We found that killed
406 bacteria did not significantly decrease the survival of iron-treated larvae (Fig 5E). Even a ten fold
407 increase in dead bacteria did not result in a significant decrease in survival (Fig 5E). These
408 results suggest that live *E. faecalis* were needed for high larval mortality. Our experiments
409 involving the injection of a large number of *E. faecalis* into larvae fed antibiotics-containing diet
410 demonstrate that iron supplementation can increase larval mortality, but this outcome is
411 independent of bacterial load, and the mechanism underlying the phenomenon is unknown.
412

413 # Conclusions

414

The central hypothesis of this study was that dietary iron supplementation would increase the amount of iron in larval hemolymph, promoting the growth of infectious microbes, possibly resulting in larval death. Although we verified that iron supplementation increased the iron content of hemolymph, we saw no evidence that additional iron increased proliferation of either *E. coli* or *E. faecalis* under any of our experimental conditions. Different bacterial species or different experimental conditions (e.g., more or less iron in the diet) could produce different outcomes; however, the results of this study support the conclusion that dietary iron supplementation does not promote bacterial proliferation in *M. sexta* hemolymph.

Contrary to our model, there were differences in survival between control and iron-treated larvae that were independent of the number of bacteria in the hemolymph. Surprisingly, when larvae were injected with *E. faecalis*, the effect of iron supplementation on survival depended on whether the diets contained antibiotics. In the absence of antibiotics, iron-treated larvae survived longer than control larvae, whereas, in the presence of antibiotics, iron-treated larvae were less likely to survive than control larvae. Given that antibiotics in the diet probably alter the gut microbiome, changes in the microbiome may underlie the difference in outcomes. But less obvious explanations are also possible. For example, the effect of antibiotics on the survival of iron-treated larvae may be mediated by chemical interactions between iron and the tetracycline in the diet (38,39). More studies are needed to explore these possibilities.

The previously published research on the effects of dietary iron on immunity suggest that the relationship between dietary iron and infection outcome is complex (5,9–12). The results of our study support this viewpoint.

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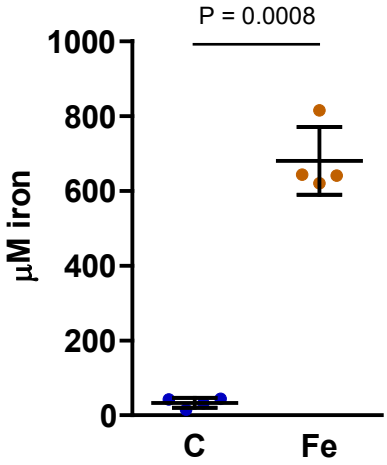
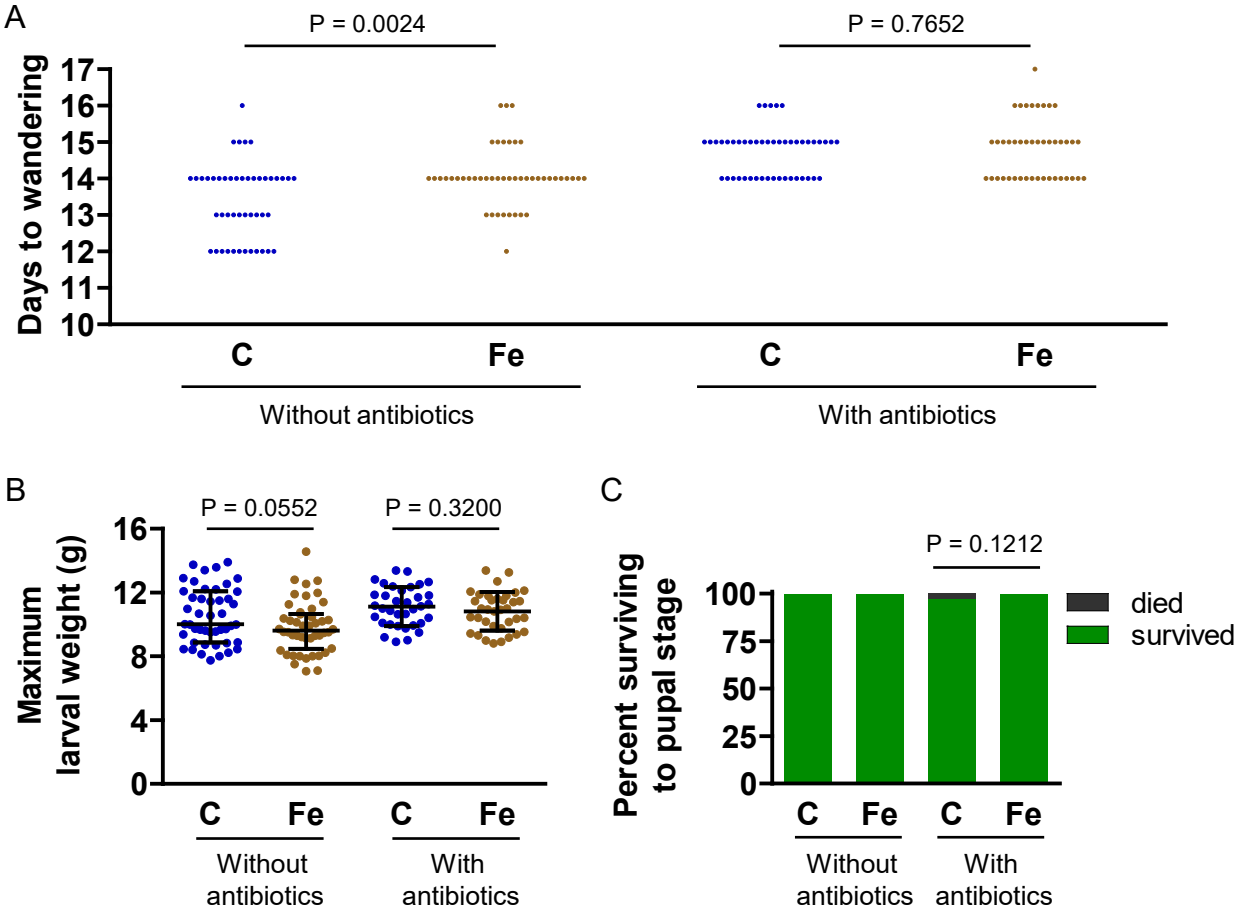
562

563 # Supporting information

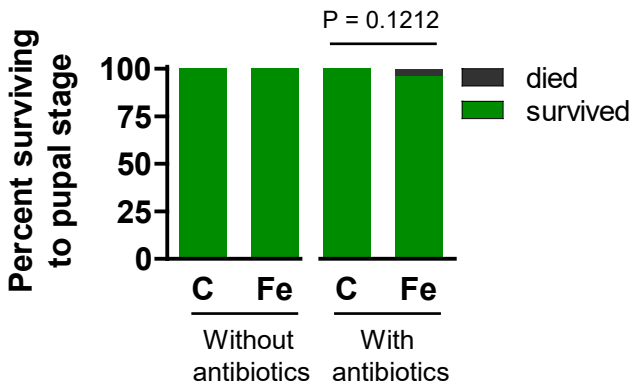
564

565 **S1 Fig. In the absence of antibiotics, iron supplementation decreased *E. faecalis*-induced**
566 **mortality of feeding stage larvae.** Larvae cultured on control (C) or iron-supplemented (Fe) diet
567 without antibiotics were inoculated with *E. faecalis* at an OD_{600} of 0.02, and mortality was
568 monitored. The percentage of larvae surviving at three days post-inoculation, the wandering
569 stage, and the pupal stage are shown. A Fisher's exact test was used to test for statistical
570 significance (n = 24).

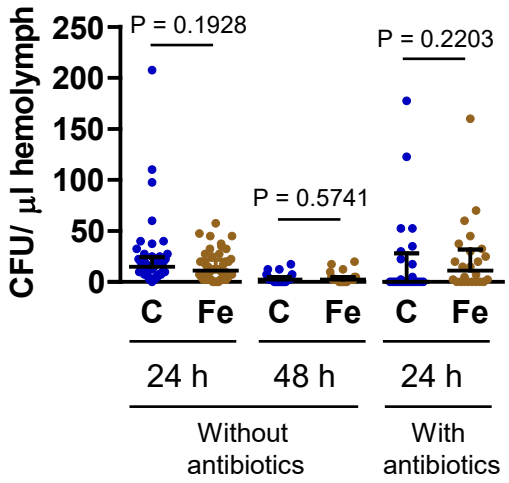

P = 0.0008
1000
800
600
400
200
0
µM iron
C
Fe


A
P = 0.0024
P = 0.7652
Days to wandering
17
16
15
14
13
12
11
10
C
Fe
C
Fe
Without antibiotics
With antibiotics
B
P = 0.0552
P = 0.3200
Maximum larval weight (g)
16
12
8
4
0
C
Fe
C
Fe
Without antibiotics
With antibiotics
C
P = 0.1212
Percent surviving to pupal stage
100
75
50
25
0
died
survived
C
Fe
C
Fe
Without antibiotics
With antibiotics

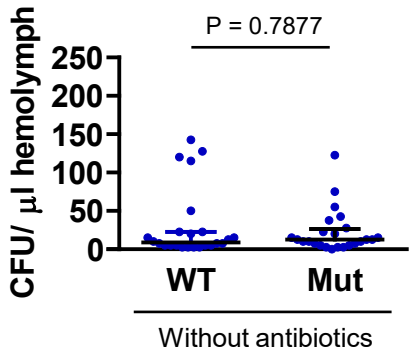
A

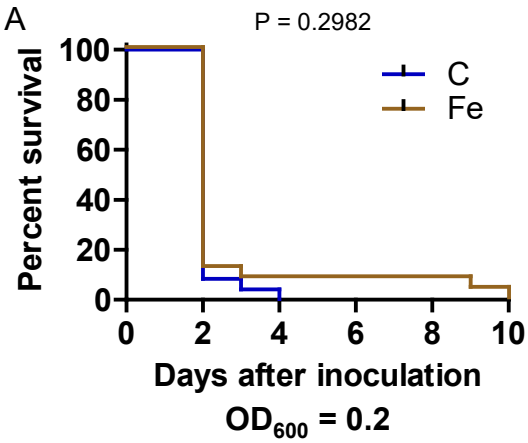

P = 0.1212
Percent surviving to pupal stage
100
75
50
25
0
died
survived
C
Fe
C
Fe
Without antibiotics
With antibiotics

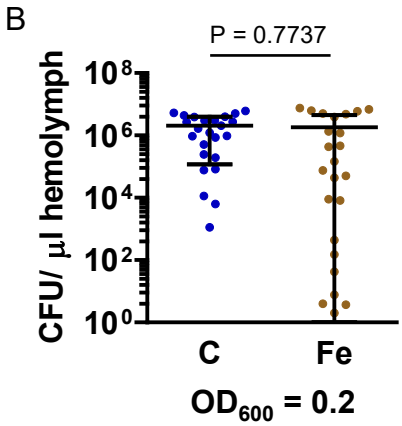
B

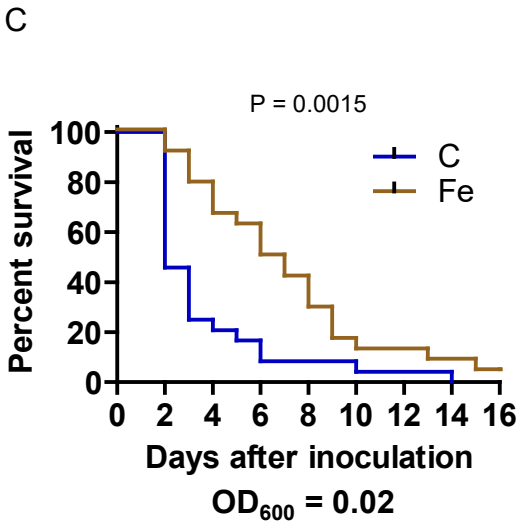

CFU/ µl hemolymph
250
200
150
100
50
0
P = 0.1928
P = 0.5741
P = 0.2203
C
Fe
C
Fe
C
Fe
24 h
48 h
24 h
Without antibiotics
With antibiotics

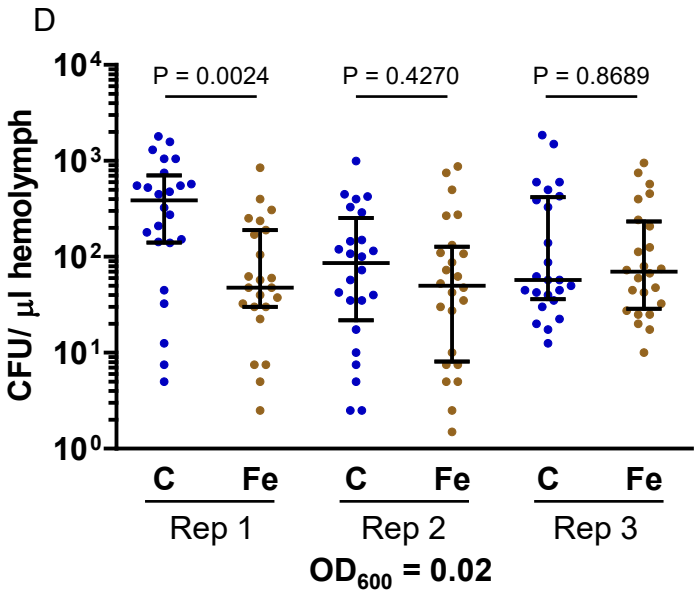
C

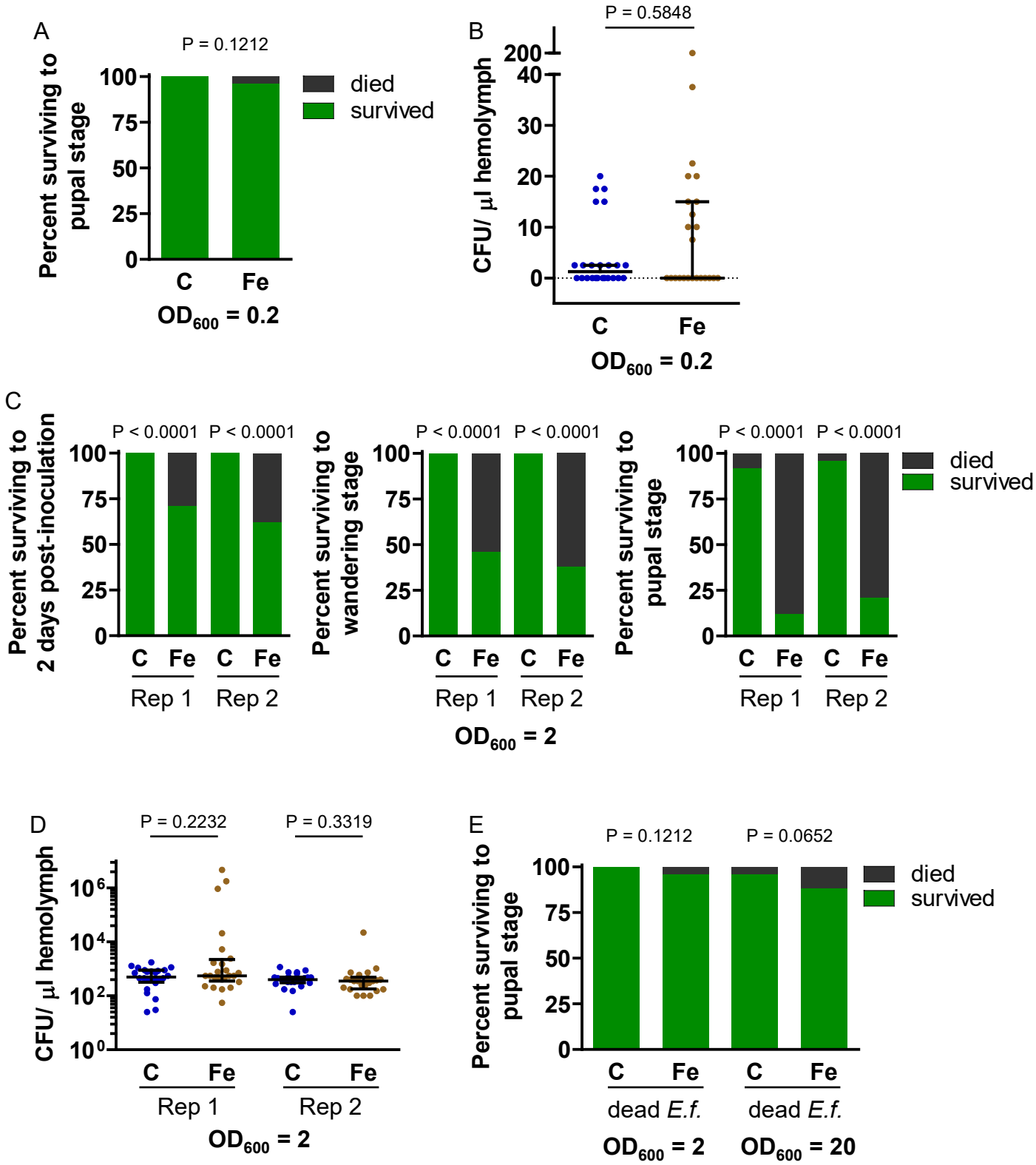

P = 0.7877
CFU/ µl hemolymph
250
200
150
100
50
0
WT
Mut
Without antibiotics


A
P = 0.2982
Percent survival
100
80
60
40
20
0
C
Fe
0
2
4
6
8
10
Days after inoculation
OD600 = 0.2


B
P = 0.7737
CFU/ µl hemolymph
10^8
10^6
10^4
10^2
10^0
C
Fe
OD600 = 0.2


C
P = 0.0015
Percent survival
100
80
60
40
20
0
C
Fe
0
2
4
6
8
10
12
14
16
Days after inoculation
OD600 = 0.02


D
P = 0.0024
P = 0.4270
P = 0.8689
CFU/ µl hemolymph
10^4
10^3
10^2
10^1
10^0
C
Fe
C
Fe
C
Fe
Rep 1
Rep 2
Rep 3
OD600 = 0.02


A
P = 0.1212
Percent surviving to pupal stage
100
75
50
25
0
died
survived
C
Fe
OD600 = 0.2
B
P = 0.5848
CFU/ µl hemolymph
200
40
30
20
10
0
C
Fe
OD600 = 0.2
C
P < 0.0001
P < 0.0001
Percent surviving to 2 days post-inoculation
Percent surviving to wandering stage
Percent surviving to pupal stage
C
Fe
Rep 1
Rep 2
OD600 = 2
died
survived
D
P = 0.2232
P = 0.3319
CFU/ µl hemolymph
10^6
10^4
10^2
10^0
C
Fe
Rep 1
Rep 2
OD600 = 2
E
P = 0.1212
P = 0.0652
Percent surviving to pupal stage
dead E.f.
OD600 = 2
OD600 = 20