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1 **Evaluation of greater wax moth larvae, *Galleria mellonella,* as a novel *in vivo* model for non-
2 tuberculosis *Mycobacteria* infections and antibiotic treatments**

3 **Frances M. Entwistle**

4 Biomedical Sciences Research Complex, School of Biology, The North Haugh, University of St
5 Andrews, Fife, KY16 9ST, UK

6 **Peter J. Coote** (corresponding author - pjc5@st-andrews.ac.uk +44 (0)1334 463406)

7 Biomedical Sciences Research Complex, School of Biology, The North Haugh, University of St
8 Andrews, Fife, KY16 9ST, UK

9 **Key words:** *Galleria mellonella*; *Mycobacterium fortuitum*; *Mycobacterium marinum*; *Mycobacterium*
10 *tuberculosis*; invertebrate infection model; antibiotic susceptibility

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13 **Abbreviations:** AMK, amikacin; ADC, albumin dextrose catalase; CIP, ciprofloxacin; EMB, ethambutol;
14 INH, isoniazid; MDR-TB, multi-drug resistant tuberculosis; MIC, minimum inhibitory concentration;
15 PIP, piperacillin; RIF, rifampicin; TB, tuberculosis.

Abstract

Purpose: To evaluate the suitability of *Galleria mellonella* larvae as an *in vivo* model and drug-screening tool for mycobacteria infections.

Methodology: Larvae were infected using a range of inoculum sizes from a variety of rapid-growing mycobacteria, including strains of *M. fortuitum, M. marinum* and *M. aurum.* Larval survival, internal bacterial burden, and the effects of amikacin, ciprofloxacin, ethambutol, isoniazid and rifampicin treatment on larval survival were measured over 144h. The effects of these anti-mycobacterial drugs on phagocytosis and circulating hemocyte numbers were also examined using microscopy.

Results: Larval survival decreased after infection with *M. fortuitum* and *M. marinum* in a dose-dependent manner, but remained unaffected by *M. aurum*. Heat-killed bacteria did not cause larval death. Where antibiotic monotherapy was efficacious, larval survival post-infection increased in a dose-dependent fashion. However, efficacy varied between different antibiotics and species of infecting mycobacteria and, apart from rifampicin, efficacy *in vivo* correlated poorly with the *in vitro* MICs. Combinations of antibiotics led to higher survival of infected larvae than antibiotic monotherapy. Selected antibiotic treatments that enhanced larval survival reduced the overall internal burden of infecting mycobacteria but did not eradicate the pathogens. Administration of amikacin or ethambutol to uninfected larvae induced an initial transient increase in the numbers of circulating hemocytes and reduced the phagocytic rate of hemocytes in larvae infected with *M. marinum.*

Conclusions: This report demonstrates the potential of employing a wax moth larvae model for studying fast-growing mycobacteria infections, and as a cheap, effective system for initial screening of novel treatments.

38 **Introduction**

39 Mycobacteria species, primarily *Mycobacterium tuberculosis,* are major human pathogens
40 and a significant cause of morbidity and mortality. Multidrug resistant tuberculosis (TB) is now a global
41 challenge with around 480,000 new MDR-TB cases in 2015 [1]. Extensively drug resistant cases were
42 reported in 96 countries in 2012 [2] and "totally drug resistant" cases have been described in several
43 countries [3–6]. Using *M. fortuitum* [12, 13]*, M. marinum* [14, 15] and *M. aurum* [8, 11] as surrogates
44 for *M. tuberculosis* is well established, both *in vitro* and *in vivo*, but *M. fortuitum* and *M. marinum* are
45 important infectious bacteria in their own right. Infections with either bacteria have no reporting
46 requirements, but estimates of incidence are 4.65-5.99 cases per million persons for *M. fortuitum* [16]
47 and 0.05-0.27 case per 100,000 adult patients for *M. marinum* [17]*.* These mycobacteria have similar
48 host-pathogen interactions [7], cell wall profiles [8], membrane and efflux pump proteins [9], virulence
49 genes [10] and drug resistance profiles [11] compared with *M. tuberculosis*, but are much less capable
50 of infecting an immunocompetent human and generally have a much faster growth rate.

51 A rapid screening model is needed to assess effective treatment combinations and expedite
52 discovery of novel therapies for mycobacteria. Using wax moth larvae, *Galleria mellonella*, as an *in*
53 *vivo* model to assess the virulence of pathogens and the efficacy of antimicrobials is well established
54 (reviewed in [18, 19]). Their small size, low purchase and maintenance costs, and reduced ethical
55 controls means they are accessible to most laboratories. Many experiments require minimal
56 "specialised" equipment, and larvae can be incubated at 37˚C and easily inoculated with precise
57 volumes – a significant advantage over other invertebrate models such as fruit flies and nematodes
58 that are not viable at 37°C for extended incubations and are awkward to inoculate. The wax moth
59 larvae model can be a valuable screening tool to highlight treatment combinations, novel compounds
60 and alternative therapies that can then be assessed in mammalian trials.

61 Compared to mammals, *G. mellonella* lacks an adaptive immune system, yet the invertebrate
62 innate immune system is very robust, with complex cellular defences and production of at least 18

63 antimicrobial peptides [20]. Vertebrate white blood cells and invertebrate hemocytes are not
64 homologous, however they do have analogous roles including wound repair [21], cell clustering
65 around foreign bodies [22], innate immunity [23], phagocytosis [24], and production of reactive
66 oxygen species as a defence mechanism [23].

67 Using a cheap and rapid *in vivo* model to reliably model mycobacterial treatments is clearly
68 attractive. Considering the advantage of using a model organism which negates the requirement of
69 specialised laboratories and ethical approval, it is equally advantageous (for the reasons stated above)
70 to use non-tuberculosis mycobacteria species such as *M. fortuitum, M. marinum* and *M. aurum* , since
71 *M. tuberculosis* is a highly pathogenic bacterium capable of infecting immunocompetent individuals
72 [25] and requires weeks of incubation before cultures are ready to use. This report aims to
73 demonstrate the suitability of *G. mellonella* as a model for mycobacteria infections, allowing
74 application of the model as a cheap, efficient and rapid *in vivo* screen for antibiotic combinations and
75 novel treatments that may be effective against *M. tuberculosis* infections.

76 **Materials and Methods**

77 **Reagents and larvae**

78 Reagents were purchased from Sigma-Aldrich Ltd (Dorset, UK) unless stated otherwise.
79 Antibiotics were dissolved in sterile deionised water. Larvae were purchased from UK Waxworms Ltd
80 (Sheffield, UK), stored in the dark at 20˚C and used within 7 days of receipt. Injections were performed
81 with a 25 µL Hamilton syringe. Larvae were incubated at 37˚C in petri dishes in all experiments.

82 **Bacteria and growth media**

83 *M. fortuitum* NCTC 10394 and NCTC 8573, *M. marinum* Strain M and R356933F, and *M. aurum*
84 NCTC 10437 were purchased from the National Collection of Type Cultures (Porton Down, Salisbury).
85 *M. marinum* R356933F was kindly provided by Michael Smith (Scottish Mycobacteria Reference
86 Laboratory, Royal Infirmary of Edinburgh). Bacteria were cultured in a shaking incubator using
87 Mueller-Hinton M7H9 broth enriched with albumin dextrose catalase (ADC) overnight at 37˚C for *M.*
88 *aurum* and *M. fortuitum* strains, and at 27˚C for approximately 36h for *M. marinum* strains. Any agar
89 plates used were Mueller-Hinton M7H9 broth enriched with ADC supplement and 10% agar, except
90 for the selective plates used in the burden experiment, which included piperacillin (PIP) at 256 mg/L.

91 Prior to all inoculations, all bacteria were washed twice and diluted to the required
92 concentration using PBS. Heat-killing was performed in a 100˚C water bath for 10 minutes and loss of
93 viability confirmed by plating out as above.

94 **Antibiotic susceptibility testing**

95 The minimum inhibitory concentration (MIC) of each antibiotic against *M. fortuitum* NCTC
96 10394 and NCTC 8573, and *M. marinum* Strain M and R356933F was determined using MIC strips
97 (AMK and CIP, Oxoid, UK. EMB, INH and RIF, Liofilchem, Italy) according to manufacturer instructions.
98 Cells were plated at 2.5 x 10^6 c.f.u per 90mm plate and incubated at 27°C or 37°C as appropriate. The

99 MIC was performed in duplicate and determined as the point on the strip where bacteria no longer
100 grew when in contact with the paper.

101 **Infection of *G. mellonella* larvae**

102 Experiments were performed in duplicate (*n* = 15) and results pooled (*n* = 30). Larvae were
103 infected with varying inoculum sizes for each mycobacteria strain. Larvae injected with sterile PBS
104 were included as mock-infected controls. Non-viable-bacteria control larvae received injections of
105 heat-killed cells at a concentration equal to the largest viable cell inoculum tested. Larval survival was
106 assessed every 24h for 144h and recorded as dead when no longer responsive to touch. Survival was
107 plotted as Kaplan-Meir survival curves and comparisons made using log-rank tests. Holm's corrections
108 for multiple comparisons [26] was applied.

109 Antibiotic toxicity was assessed by injecting larvae with 100 mg/kg of each and recording
110 survival and levels of melanisation on the larval body for 144h. For antibiotic efficacy experiments,
111 larvae were infected then given single treatments of antibiotic or PBS (mock-treated control) 2h post-
112 infection. For combination experiments, antibiotics were prepared as a single 10 µL dose.
113 Comparisons of survival were made using log-rank tests on Kaplan-Meir survival curves. In all tests
114 $p \leq 0.05$ was considered significant and Holm's correction was always applied to account for multiple
115 comparisons.

116 **Measurement of the internal burden of mycobacteria inside infected larvae**

117 Forty larvae were infected with 10 µL of 4 x 10^8 c.f.u/mL *M. fortuitum* NCTC 10394 or 3 x 10^7
118 c.f.u/mL *M. marinum* Strain M. A single dose of antibiotic (10 mg/kg AMK or 50 mg/kg CIP for *M.*
119 *fortuitum,* and 25 mg/kg AMK or 50 mg/kg INH for *M. marinum*) or PBS (mock-treated control) was
120 administered 2h post-infection. Five larvae were randomly selected from each group and assessed for
121 internal bacterial burden at 5, 24, 48, 96 and 144h post-infection.

122 Preparation of larval suspension for plating was performed as described previously [27].
123 Piperacillin (PIP) was selected from a range of antimicrobials to use in selective agar plates as it
124 inhibited the *in vitro* growth of microorganisms present in the larval gut but did not affect recovery of
125 colony forming units of any of the mycobacteria used in this study (data not shown). Larval
126 suspensions were serially diluted and plated on M7H9 + ADC + PIP selective agar plates then incubated
127 at 27°C or 37°C as appropriate for 24 – 72h until colonies were visible.

128 **Measuring phagocytosis of FITC-labelled mycobacteria**

129 *M. fortuitum* NCTC 10394 and *M. marinum* Strain M cultures were heat killed, resuspended in
130 0.1 mg/mL FITC (FITC dissolved in 0.2M Na_2CO_3, 0.2M $NaHCO_3$, pH 9), and incubated in a 27°C shaking
131 incubator for 30 minutes before washing three times with PBS.

132 Larvae received injections of PBS, AMK (50 and 5 mg/kg for *M. fortuitum,* 50 and 2.5 mg/kg
133 for *M. marinum*) or EMB (50 mg/kg) as required, 10 minutes before injection with FITC-stained
134 bacteria (4 x 10^{8} c.f.u/mL for *M. fortuitum,* 3 x 10^{7} c.f.u/mL for *M. marinum*). Larvae were incubated
135 for 1h then injected with 30 µL *Galleria* saline [28] saturated with phenylthiol urea (PTU) and bled into
136 individual sterile reaction tubes. The hemolymph was transferred to wells in a 24-well plate prefilled
137 with 300 µL ice-cold Grace's insect medium and 13mm round glass coverslips. The plate was incubated
138 for 1h in the dark in a 27°C low speed shaking incubator. The supernatant was removed and 1 mL 3.7%
139 formaldehyde added for 20 seconds, then 300 µL 0.4% trypan blue in PBS for 20 minutes. The
140 coverslips were washed three times with PBS then mounted for examination at 40X magnification
141 using a Delta Vision fluorescence microscope using differential interference contrast and filters for
142 FITC (excitation 490 nm/emission 528 nm). Hemocytes were imaged through an appropriately sized z-
143 stack to allow counting of internalised bacteria.

144 Ten larvae were examined for each condition, with 30 to 70 hemocytes counted per larvae.
145 Phagocytic rate was calculated as the percentage of total hemocytes that had engulfed one or more
146 bacteria. Multiple comparisons were assessed with Holm's correction.

147 **Determination of circulating hemocyte numbers**

148 Adapted from Harding *et al.* [13]. Fifteen larvae were injected per tested condition. Larvae
149 were infected with 10 µL of *M. fortuitum* NCTC 10394 or *M. marinum* Strain M at an inoculum size of
150 1.0 x 10^7 c.f.u/mL. This inoculum size was selected as it induced no lethality over the 48 h duration of
151 the experiment because the collection of haemolymph from dead larvae was impractical. Following
152 infection, larvae were inoculated 2h later with PBS, AMK (50 and 5 mg/kg for *M. fortuitum,* 50 and 2.5
153 mg/kg for *M. marinum*) or EMB (50 mg/kg for both species). Larvae were also mock-infected with 10
154 µL of PBS followed 2h later with AMK (50, 5 or 2.5 mg/kg) or EMB (50 mg/kg) to assess the effects of
155 these antibiotics on circulating hemocyte numbers in the absence of infection. Unmanipulated
156 controls were included. Three larvae were randomly selected and injected with 75 µL *Galleria* saline
157 [28] at 5, 18.5, 24 and 48 hours post-infection. Hemolymph was bled into individual sterile reaction
158 tubes and 10 µL was loaded onto an improved Neubauer haemocytometer and the hemocytes
159 counted with duplicates for each sample. This experiment was performed in triplicate to give n = 9 for
160 each experimental condition. Holm's correction was applied to multiple comparisons.

161 **Results**

162 **Infection of *G. mellonella* larvae with viable mycobacteria results in inoculum-size**
163 **dependent lethality.**

164 Larvae were infected with a range of inoculum sizes of different mycobacteria. In all cases,
165 except *M. aurum* NCTC 10437, as the inoculum size increased larval survival was reduced (Fig 1a, b, d,
166 e and f). *M. aurum* NCTC 10437 had no detrimental effect on larval survival at any of the inoculum
167 sizes tested (Fig. 1c). Notably, heat-killed bacteria had no detrimental effect on larval survival in all
168 experiments, indicating that larval death was caused by infection with viable *M. fortuitum* or *M.*
169 *marinum*. There were variations between *M. fortuitum* and *M. marinum* virulence – *M. marinum*
170 required fewer bacteria (~3 x 10^7 c.f.u/mL) to decrease larval survival substantially, compared to *M.*
171 *fortuitum* which required up to 30 times more bacteria to have the same effect.

172 *M. marinum* NCTC 2275 was not selected for continued experimentation as of the three *M.*
173 *marinum* strains used it had the weakest virulence. *M. aurum* NCTC 10437 was also not investigated
174 further as larvae were resistant to infection.

175 **Administration of anti-mycobacterial antibiotics enhances survival of *G. mellonella* larvae**
176 **infected with mycobacteria.**

177 The MICs of a range of anti-mycobacterial antibiotics (AMK, CIP, EMB, INH and RIF) were
178 measured for each of the mycobacteria to allow comparison of *in vitro* antibiotic susceptibility with
179 efficacy of the same drugs versus infected larvae *in vivo*. The *in vitro* MICs are described in Table 1.
180 For all antibiotics tested, the MICs varied widely even between strains of the same species.

181 Prior to studying the efficacy of the same antibiotics *in vivo*, larvae were administered a high
182 dose (100 mg/kg) of each antibiotic alone to determine if any were toxic. Compared to larvae
183 administered PBS alone, there was no evidence of toxicity to the larvae from any of the antibiotics -

184 survival was 100% 144h post-injection, and no melanisation was observed on the larval body beyond
185 the point of injection (data not shown).

186 The effects of a single dose 2h post-infection of the same antibiotics (Table 1) on survival of
187 *G. mellonella* larvae 144h post-infection with mycobacteria are shown in Fig. 2. Complete Kaplan–
188 Meier survival curves are available in Supplementary Data. To readily observe any therapeutic benefit
189 of antibiotic treatment, larvae were infected with an inoculum of each *Mycobacterium* known to result
190 in a high level of mortality (Fig 1). The antibiotic doses administered were selected on the basis of pilot
191 experiments that screened a wide range of doses for therapeutic benefit (data not shown). An upper
192 threshold dose of 100 mg/kg was selected for all the antibiotics tested.

193 AMK was the most successful antibiotic tested, conferring significant therapeutic benefit
194 against infections by all four mycobacteria tested (Fig. 2a). Comparison of the effectiveness of AMK *in*
195 *vivo* with the *in vitro* MICs of AMK for each bacterial strain (Table 1) revealed poor correlation. For
196 example, *M. marinum* R356933F and *M. marinum* Strain M had MICs for AMK of 2 and 1 mg/L
197 respectively. In contrast, the doses of AMK required to successfully treat the *in vivo* infections with
198 the same strains were reversed with a single-dose of only 10mg/kg required for the more resistant *M.*
199 *marinum* R356933F and a higher dose of 25 mg/kg for the more sensitive *M. marinum* Strain M.

200 CIP treatment (Fig. 2b) was effective for larvae infected with the *M. fortuitum* strains but
201 displayed little efficacy versus larvae infected with either strain of *M. marinum*. Unlike with AMK, this
202 observation did correlate with the MIC values for each strain (Table 1) as both *M. fortuitum* strains
203 were far more sensitive to CIP than the *M. marinum* strains.

204 EMB was the least effective antibiotic in these experiments – survival at 144h was almost
205 invariably indistinguishable from the mock-treated control (PBS) (Fig. 2c). The correlation between the
206 *in vitro* and *in vivo* efficacies of EMB was poor.

207 INH is largely ineffective for larvae infected with *M. fortuitum* NCTC 8573 (Fig. 2d), which is
208 supported by the *in vitro* data (Table 1). Survival of larvae infected with *M. fortuitum* NCTC 10394 is
209 statistically significant, and the MIC results suggest sensitivity to INH, but overall survival was low even
210 at the highest doses tested. This contrasts with larvae infected with *M. marinum* – both strains
211 responded positively to INH treatment and high doses conveyed a long-lasting survival advantage,
212 supported by the sensitivity to INH *in vitro*.

213 RIF significantly improved larval survival after infection with *M. marinum* (Fig. 2e) and this
214 correlated with the *in vitro* MICs (Table 1). However, the *M. fortuitum* strains had differing responses
215 to RIF. *Mycobacterium fortuitum* NCTC 10394 is unresponsive even at high doses, whereas larval
216 survival significantly improved when *M. fortuitum* NCTC 8573 was treated with similar doses. Notably,
217 this did correlate with the MICs because *M. fortuitum* NCTC 8573 was highly sensitive to RIF *in vitro*
218 whilst *M. fortuitum* NCTC 10394 had the highest MIC of any of the mycobacteria strains tested.

219 To conclude, in cases where the administered antibiotic enhanced larval survival *in vivo*, this
220 occurred in a dose-dependent manner with some doses resulting in near 100% survival compared to
221 mock-treated controls. However, not all antibiotics were efficacious *in vivo* and the degree of
222 correlation between the *in vitro* sensitivity of the mycobacteria to the drugs and their ability to
223 enhance larval survival *in vivo* was variable.

224 In subsequent experiments, *M. fortuitum* NCTC 10394 and *M. marinum* Strain M were used,
225 as they are the type strains for their species.

226 **Administration of AMK, CIP or INH to larvae infected with mycobacteria reduces the overall**
227 **bacterial burden within the larvae.**

228 The effect that efficacious doses of antibiotics had on the bacterial burden within the larvae
229 was measured by enumerating viable bacteria in homogenates of larvae that had been exposed to
230 bacteria and PBS or antibiotic. Doses of AMK, CIP and INH were selected that were previously shown

231 to confer almost full survival (Fig 2) on larvae infected with inoculum sizes of *M. fortuitum* NCTC 10394
232 or *M. marinum* Strain M that were shown to be lethal to untreated larvae over a period of 144 h (Fig
233 1).

234 Infection with *M. fortuitum* NCTC10394 (4.0 x 10^8 c.f.u/mL) resulted in a large drop in viable
235 bacteria over the first 5 h p.i. (Fig. 3a). In contrast, infection with *M. marinum* Strain M (3.0 x 10^7
236 c.f.u/mL) led to only a minor decrease in bacterial numbers over the same time period (Fig 3b). This
237 obvious difference in the ability of the innate immune system to eliminate the two mycobacteria
238 strains could explain why larvae need a higher inoculum of *M. fortuitum* cells to cause significant larval
239 death compared to the *M. marinum* species (Fig. 1). Nonetheless, viable mycobacteria were recovered
240 from both mock (PBS) and antibiotic-treated larvae throughout the entire duration of the experiment
241 (Fig. 3) despite the fact that the doses of antibiotics administered permitted almost full survival of
242 infected larvae (Fig 2). Bacterial burden is mitigated by treatment with the selected antibiotics but
243 viable bacteria were still detected 144h post-infection with all antibiotics tested. At some time points,
244 the difference in internal mycobacterial burden between antibiotic-treated and mock-treated larvae
245 was significantly reduced, potentially accounting for the observed efficacy of these antibiotics (Fig. 2).

246 **Infecting mycobacteria are phagocytosed by *G. mellonella* hemocytes but phagocytosis is**
247 **reduced by exposure to antibiotics.**

248 To determine the fate of mycobacteria in *G. mellonella* larvae, and how this was influenced by
249 antibiotic therapy, bacterial phagocytosis was measured by microscopy (Fig 4). The procedure
250 required to label the mycobacteria with FITC meant that the infecting bacteria were dead (see
251 Methods). Nonetheless, FITC-labelling permitted the visualisation of internalised mycobacteria and a
252 representative image of larval hemocytes that have phagocytosed bacteria is shown in Fig 4a. Groups
253 of larvae were then administered a single 10 µL dose of either PBS, 2.5, 5 or 50 mg/kg AMK or 50
254 mg/kg EMB. These doses of AMK were selected because 2.5 and 5 mg/kg was shown to have no
255 therapeutic benefit on infected larvae compared to 50 mg/kg that resulted in almost complete survival

256 (Fig 2). Similarly, 50 mg/kg EMB was selected because this dose had little therapeutic benefit. In this
257 way the effect of therapeutic and non-therapeutic doses of antibiotics on phagocytosis could be
258 measured. After 10 min the larvae administered with either 10 µL PBS or antibiotics were then
259 inoculated with FITC-labelled mycobacteria and phagocytosis was measured after 1 h at 37°C (Fig 4b).

260 In larvae exposed to PBS only, over 40% of hemocytes compared to 21% had engulfed one or
261 more *M. fortuitum* NCTC 10394 or *M. marinum* Strain M cells respectively (Fig. 4b). This reflected the
262 higher infecting inoculum of *M. fortuitum* NCTC 10394 (4.0 x 10^8 c.f.u/mL) compared to *M. marinum*
263 Strain M (3.0 x 10^7 c.f.u/mL). For both strains, exposure to a dose of AMK that was shown to be fully
264 efficacious (50 mg/kg: Fig 2)) resulted in a significant reduction in phagocytosis compared to the PBS
265 treated controls. Phagocytosis dropped to 21% and 6.9% for *M. fortuitum* NCTC 10394 or *M. marinum*
266 Strain M cells respectively. The effect of non-efficacious doses of AMK and EMB on phagocytosis
267 differed between *M. fortuitum* NCTC 10394 and *M. marinum* Strain M cells. For *M. fortuitum* NCTC
268 10394, these doses had no impact on phagocytosis compared to larvae exposed to PBS. In contrast,
269 ineffective doses of 2.5 mg/kg AMK and 50 mg/kg EMB significantly reduced phagocytosis of *M.*
270 *marinum* Strain M cells to a similar extent as the efficacious dose of 50mg/kg AMK (Fig 4b).

271 In summary, the larval response in terms of phagocytosis was dependent on the infecting
272 species of mycobacteria and phagocytosis was reduced by exposure to both efficacious and non-
273 efficacious doses of antibiotics also in a species-dependent fashion.

274 **Administration of antibiotics to uninfected larvae and larvae infected with mycobacteria**
275 **induces a significant increase in the number of circulating hemocytes.**

276 The reduction in phagocytosis induced by exposure to antibiotics could perhaps be explained
277 by changes in the overall numbers of circulating hemocytes. Thus, the effect of exposure to the same
278 doses of AMK and EMB administered in Fig 4 on circulating hemocytes was measured using
279 microscopy (Fig 5 and 6).

280 Larvae were mock-infected with sterile PBS then injected 2 h later with PBS, AMK or EMB.
281 Relative to PBS treatment, exposure to all doses of each of the antibiotics resulted in a significant,
282 transient increase in the number of circulating hemocytes within the 3h after antibiotic administration
283 that disappeared by 24 h. The transient increase in hemocyte number induced by AMK after 5 h was
284 dose-dependent.

285 Similarly, when larvae were infected with a viable inoculum of *M. fortuitum* NCTC 10394 and
286 *M. marinum* Strain M cells followed by administration of PBS or the same antibiotic doses of AMK and
287 EMB used previously (Fig 4 and 5), circulating haemocyte numbers were again significantly higher after
288 5 h than in larvae mock-infected with PBS (Fig 6). Mock-treatment with PBS also resulted in an increase
289 in hemocytes but this was much less than that induced by antibiotic treatment. As before, the
290 antibiotic-induced increase in haemocyte numbers peaked at 5 h and declined noticeably thereafter
291 (Fig 6).

292 In summary, the previous data revealing that antibiotics reduced the phagocytic rate of FITC-
293 labelled, dead mycobacteria (Fig. 4) could perhaps be explained by the increase in circulating
294 hemocyte numbers that was induced by exposure to the antibiotics alone (Fig 5) or by antibiotics
295 administered to larvae infected with viable mycobacteria (Fig 6). For example, increased numbers of
296 hemocytes could mean that many may not encounter bacteria to phagocytose, thereby reducing the
297 overall quantity of hemocytes containing bacteria.

298 **Antibiotic combination treatments provide greater therapeutic benefit to larvae infected**
299 **with mycobacteria than antibiotic monotherapy.**

300 Treatment of *M. tuberculosis* infections usually involves administration of combinations of
301 antibiotics for optimal therapy. To mimic combination therapy with infected *G. mellonella* larvae, and
302 to observe whether typical combination treatments were also more efficacious in this model system,
303 three antibiotic combinations were selected using WHO treatment guidelines [29]. Thus, the

304 therapeutic benefit conferred by three multiple drug combinations was studied: one triple
305 combination (EMB+INH+RIF), and two quadruple combinations (AMK+EMB+INH+RIF and
306 CIP+EMB+INH+RIF) (Fig 7). The doses of the individual antibiotics administered within the combination
307 regimens were selected on the basis that they provided minimal therapeutic benefit when given as
308 monotherapy to larvae infected with mycobacteria (Fig. 2). When these non-efficacious doses were
309 combined, larval survival improved significantly compared to mock-treated groups (PBS) (Fig 7) and to
310 larvae administered with a doses of each drug individually (Fig. 2). Thus, larval model reflects the
311 enhanced efficacy of antibiotics administered in combinations as practiced in clinical settings.

312 **Discussion**

313 The data reported here shows that *G. mellonella* larvae could be employed as a valuable *in*
314 *vivo* infection model for several mycobacteria species and allow the therapeutic effects of antibiotic
315 treatments to be quantified.

316 The majority of the mycobacteria examined can kill *G. mellonella* larvae, and the numbers of
317 viable infecting bacteria correlates negatively with larval survival. The exception is *M. aurum* NCTC
318 10394, although *M. aurum* is exclusively reported as infectious in immuno-compromised humans [30,
319 31] (unlike *M. fortuitum* [32, 33] and *M. marinum* [17, 34]) so non-pathogenicity in immunocompetent
320 larvae is perhaps unsurprising.

321 Ethambutol, isoniazid and rifampicin were selected for their historic inclusion in standard TB
322 treatment regimens [35] and amikacin and ciprofloxacin for their use as second-line treatments for
323 MDR-TB [36]. All five have been used with *M. fortuitum* [32, 37–41] and *M. marinum* [17, 42, 43] *in*
324 *vitro* and *in vivo,* although *in vivo* experiments predominantly use combination treatments. When used
325 to treat human infections, amikacin can be used at 15 mg/kg for *M. fortuitum* infections [32] or 400
326 mg daily for *M. marinum* [44]. Ciprofloxacin has been used at approximately 8 mg/kg for *M. fortuitum*
327 infections [45] and 500 mg twice daily for *M. marinum* [46]. Ethambutol, isoniazid and rifampicin are
328 not prescribed for *M. fortuitum* infections, although daily doses of ethambutol at 25 mg/kg and
329 rifampicin at 600 mg is a well-established treatment for *M. marinum* [47] and isoniazid at 10 mg/kg
330 has been shown to be an effective treatment for *M. marinum* in zebrafish [48]. Whilst the efficacious
331 doses of these drugs in *G. mellonella* larvae were different, they were at least comparable and of a
332 similar order of magnitude.

333 The effective antibiotics generally improved larval survival in a dose-dependent manner
334 compared with mock-treated larvae (Fig. 2). However, varied sensitivity to each antibiotic was
335 observed between strains and several antibiotics had no therapeutic effect at the highest dose tested.

336 Combination therapy that included some of the ineffective antibiotics improved overall efficacy –
337 some combinations gave almost 100% larval survival across the 144h experiment. Considering the
338 necessity of employing antibiotic combinations when treating patients infected with mycobacteria,
339 this result is key to indicating the potential role of *G. mellonella* in testing further novel combination
340 treatments. In addition, measurement of the bacterial burden (Fig. 3) indicated that efficacious doses
341 of antibiotics that permitted almost full survival of the infected larvae only reduced the overall
342 numbers of bacteria within the larvae without eliminating them. This implies that a certain threshold
343 number of mycobacteria are required to cause larval death and that the larvae are readily able to
344 survive despite still being infected with bacterial numbers that are below this apparent lethal
345 threshold. It is not clear from this work what contribution to the measured efficacy of the antibiotics
346 is due to the direct inhibitory action of the drugs on the infecting bacteria, and what contribution is
347 due to the apparent stimulation of the innate immune system (in the form of increased hemocyte
348 numbers; Fig 5 and 6) that is triggered upon exposure to the antibiotics. It would seem likely that both
349 actions contribute to the measured efficacy of the drugs.

350 Human phagocytic cells have been assessed against a multitude of antimicrobials and
351 pathogens to determine if phagocytosis is enhanced, supressed or unaffected (reviewed in [49]), and
352 is it well established that a number of medications can reduce [50] or increase [51] the number of
353 circulating immune cells in humans. A reduction in the number of circulating white blood cells is a very
354 rare side-effect when using anti-tuberculosis medications [52] – there is no suggestion that any of the
355 drugs in this study cause a reliable change in the number of circulating immune cells in humans.
356 Interactions between antibiotics and the larval immune system may explain why antibiotics cause such
357 a significant increase in the number of circulating hemocytes in the hours post-injection (Fig. 5). A
358 similar response using antifungals has been shown with *G. mellonella* elsewhere [53, 54]. Considering
359 the increase is not seen when larvae are mock-treated with PBS, this suggests that antimycobacterials
360 trigger a non-specific immune response. Increased numbers of circulating hemocytes has been shown
361 to correlate with improved larval survival for fungal infections [55], yet here, the antibiotic EMB

362 increased hemocyte density but did little to improve larval survival so this correlation may not be
363 universally applicable.

364 The lack of correlation between the *in vitro* efficacy of the antibiotics (Table 1) and the *in vivo*
365 data (Fig. 2) is not necessarily detrimental to the conclusions of this study. A useful application of this
366 model is to test drugs which have only been examined *in vitro* to determine possible variances in the
367 *in vivo* results, and it is vital not to overly extrapolate *in vitro* data to predict *in vivo* results as there
368 are often discrepancies between the two.

369 Existing whole-organism models for mycobacterial, particularly *M. tuberculosis,* infections
370 include mice, rats, guinea pigs, rabbits, cattle, and primates). However, all present problems with cost,
371 housing requirements, operating regulations, and ethical concerns. Non-mammalian models can also
372 be a useful tool. For example, zebrafish are not natural hosts of *M. tuberculosis*, but *M. marinum*
373 infections eventually produce lipid-rich and necrotic granulomas [56] in adult and larval fish, despite
374 the immature fish larvae having no adaptive immune system at that developmental stage [15].

375 *Drosophila melanogaster* (fruit fly) is becoming a well-established model for *M. marinum* [57].
376 Infected flies show evidence of "wasting" [58], a key symptom in humans. Fly hemocytes engulf *M.*
377 *marinum* which replicate inside the hemocytes and are eventually released, then found extracellularly
378 to the host cells [59] similar to *M. tuberculosis* with human immune cells. Unfortunately, fruit flies are
379 reared at 18-29°C and are difficult to inoculate with specific volumes. Circulating hemocytes can only
380 be collected from fly larvae, [60], meaning that the short term effects of infection on circulating
381 hemocytes can only be examined in larval flies – long term experiments must use adult flies [59].

382 For these reasons, *Galleria mellonella* larvae are an attractive model organism for screening novel
383 compounds against mycobacterial infections. They thrive at 37°C, can be easily and consistently
384 inoculated with specific quantities of bacteria and drugs, and hemocytes can be collected from their
385 hemolymph long after infection. Similarly to *D. melanogaster*, experiments are limited to the life cycle

386 of the larvae, and larvae are unlikely to be of use in persistence models of latent TB infections.
387 However, considering the pressure to locate new and novel compounds to treat mycobacterial
388 infections, especially drug-resistant strains, having access to a convenient *in vivo* model early in the
389 drug discovery and development timeline could be invaluable. We suggest that *G. mellonella* would
390 be a valuable tool for testing compounds with efficacy against mycobacteria and may provide useful
391 evidence to support further work with clinically relevant cell lines or small-mammal trials.

392

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396 **Conflicts of interest:** None to declare

397

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550 **Figure legends**

Figure 1. Effect of infection with a range of mycobacteria species and strains on survival of *G. mellonella* larvae. The data is presented as Kaplan-Meir survival curves. The number of cells injected (10 µL) into each larva is shown beside each panel as colony forming units (c.f.u) per mL (highest cell concentrations, black filled squares; middle concentration, grey filled triangles; lowest concentration, black filled circles). Controls of larvae mock-infected with PBS (uninfected, grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat killed, black open squares) are also shown. $n = 30$.

Figure 2. Effect of administration of a single dose of a range of antimycobacterial antibiotics on survival of *G. mellonella* larvae 144h post-infection with four mycobacteria species and strains. Larvae were infected with viable bacteria then treated 2 hours post-infection (*M. fortuitum* NCTC 10394, 4 x 10^8 c.f.u/mL; *M. fortuitum* NCTC 8573, 5 x 10^8 c.f.u/mL; *M. marinum* Strain M, 3 x 10^7 c.f.u/mL; *M. marinum* R356933F, 9 x 10^8 c.f.u/mL). Larval survival was monitored for 144h post-infection. The doses of antibiotic used are indicated below each bar in mg/kg. Each bar represents mean survival ± SEM after 144h. Mock-treated larvae were injected with PBS 2 hours post-infection. Any doses which conveyed a significant ($p < 0.05$, Log-Rank test on complete Kaplan-Meier curves) increase in larval survival across the full 144h observation are indicated with a star (*). Multiple comparisons were corrected with Holm's correction. $n = 30$. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

Figure 3. The effect of infection with mycobacteria and subsequent mock-treatment with PBS or antibiotics on the bacterial burden within *G. mellonella* larvae. Larvae were infected with *M. fortuitum* NCTC 10394 (4 x 10^8 c.f.u/mL) (a) and *M. marinum* Strain M (3 x 10^7 c.f.u/mL) (b). Antibiotics or PBS were administered 2h post-infection at the doses indicated. Mock-treated larvae are represented by grey filled triangles. Larvae treated with amikacin, ciprofloxacin and isoniazid are represented with grey circles, open black squares and black hatched diamonds respectively. Data shows mean ± SEM with each marker representing the average c.f.u/mL for five larvae in each condition at each time point. Asterisks indicate significant differences between groups treated with an antibiotic and those that received PBS ($p < 0.05$, Mann-Whitney *U*-test). Multiple comparisons were corrected with Holm's correction. AMK, amikacin; CIP, ciprofloxacin; INH, isoniazid.

Figure 4. Effect of exposure to PBS or antibiotics on phagocytosis of FITC-labelled mycobacteria by *G. mellonella* hemocytes. Panel (a) represents a typical captured image of an optical slice through *G. mellonella* haemocytes showing internalised, fluorescent *M. marinum*. The proportion of larval hemocytes with phagocytosed *M. fortuitum* NCTC 10394 or *M. marinum* Strain M after 1h of incubation in the presence of efficacious or non-efficacious doses of AMK (50 mg/kg; 2.5 or 5 mg/kg, respectively) or a non-efficacious dose of EMB (50 mg/kg) and compared to a mock-treated control (PBS) is shown in (b). Data is from 10 larvae, 30 – 70 hemocytes examined per larvae, with mean ± SEM. $n = 10$. ($p < 0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons). AMK, amikacin; EMB, ethambutol.

Figure 5. Effect of exposure to antibiotics on overall numbers of circulating *G. mellonella* hemocytes. Larvae were mock-infected with PBS followed 2 hours later by administration of PBS or doses of AMK (2.5, 5 or 50 mg/kg) or EMB (50 mg/kg). Numbers of hemocytes in the larval hemolymph were then counted at 5, 18.5, 24 and 48 h post the initial mock-infection with PBS. The number of hemocytes is presented relative to larvae mock-infected and treated with PBS. Data is from 9 larvae per time-point showing mean ± SEM. ($p < 0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons) AMK, amikacin; EMB, ethambutol.

Figure 6. Effect of infection with mycobacteria and subsequent treatment with PBS or antibiotics on overall numbers of circulating *G. mellonella* hemocytes. Larvae were infected with *M. fortuitum* NCTC 10394 or *M. marinum* Strain M (1.0×10^7 c.f.u/mL) then 2 h post-infection were treated with efficacious or non-efficacious doses of AMK (50 mg/kg; 2.5 or 5 mg/kg, respectively) or a non-efficacious dose of EMB (50 mg/kg) or PBS as a mock-treated control. Numbers of hemocytes in the larval hemolymph were then counted at 5, 18.5, 24 and 48 h post-infection. The number of hemocytes is presented relative to larvae mock-infected and treated with PBS. ($p < 0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons). Data is from 9 larvae per time-point showing mean ± SEM. AMK, amikacin; EMB, ethambutol.

Figure 7. Effect of antibiotic monotherapy or combination therapy on survival of *G. mellonella* larvae 144 h post-infection with mycobacteria. Larvae were infected with *M. fortuitum* NCTC 10394 (4×10^8 c.f.u/mL) (a) or *M. marinum* Strain M (3×10^7 c.f.u/mL) (b) followed by treatment 2 h post-infection with a single dose of either PBS, antibiotic monotherapy or antibiotic combinations. Antibiotic monotherapy doses for *M. fortuitum* were 5 mg/kg AMK, 2.5 mg/kg CIP, 100 mg/kg EMB, 5 mg/kg INH and 30 mg/kg RIF, and for *M. marinum* were 2.5 mg/kg AMK, 50 mg/kg CIP, 100 mg/kg EMB, 5 mg/kg INH and 10 mg/kg RIF. The combination treatments consisted of the same drug concentrations as above but pooled to a make a single, combination dose. Data shows mean survival ± SEM after 144 h. * indicates a significant increase in survival of combination therapy compared to each individual monotherapy ($p < 0.05$, Log-rank test on complete Kaplan-Meier curves with Holm's correction applied for multiple comparisons).

Table 1. MICs of five antibiotics for *M. fortuitum* NCTC 10394, *M. fortuitum* NCTC 8573, *M. marinum* Strain M and *M. marinum* R356933F using three independent biological replicates.

	mg/L			
	M. fortuitum		*M. marinum*	
	NCTC 10394	NCTC 8573	Strain M	R356933F
AMK	0.12 – 0.25	0.12 – 0.25	1.0	2.0
CIP	0.008	0.05	0.5	0.25 – 0.5
EMB	2.0	>256	0.125 – 0.19	0.19 – 0.25
INH	1.0 – 2.0	>256	4.0 – 6.0	2.0 – 3.0
RIF	12.0	<0.016	6.0 – 8.0	0.38 – 0.5

AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

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575 Figure 1

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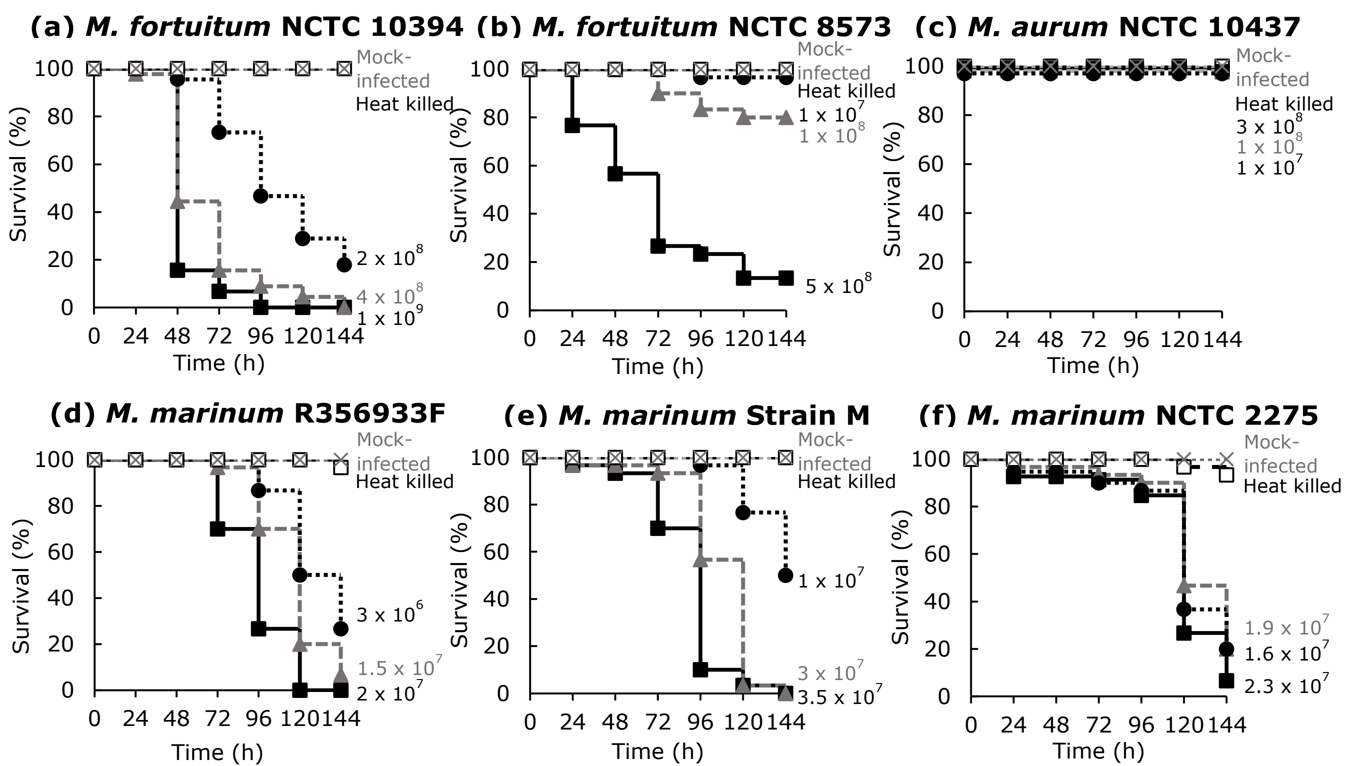

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584 Figure 2

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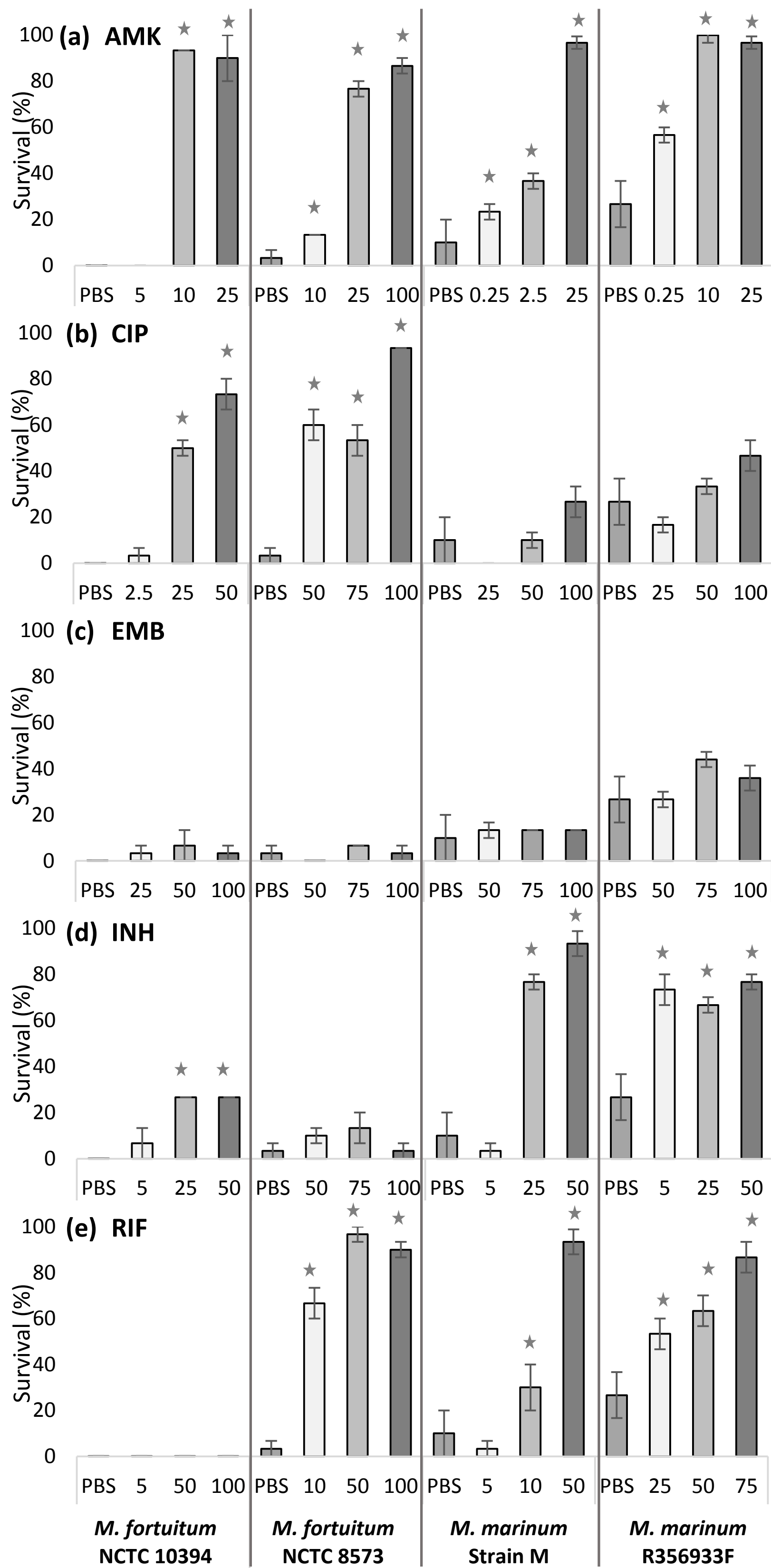

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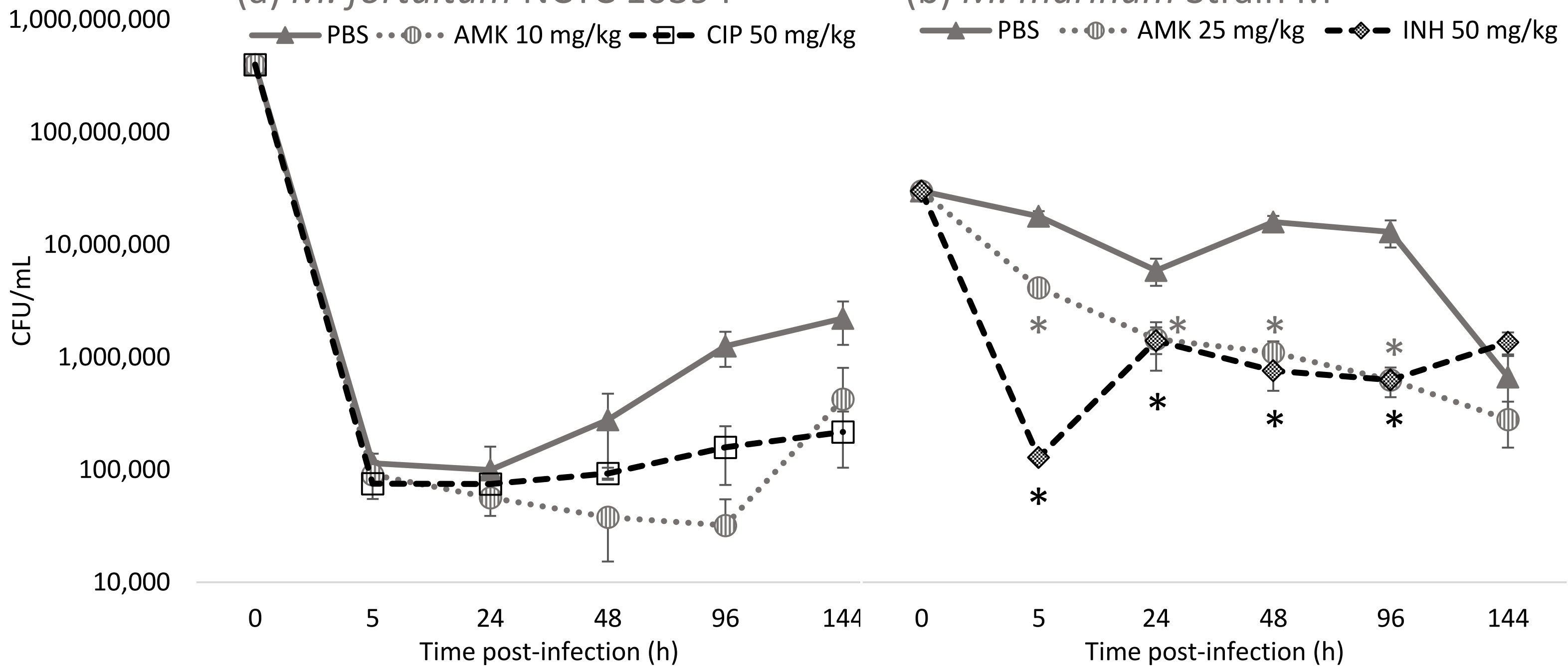

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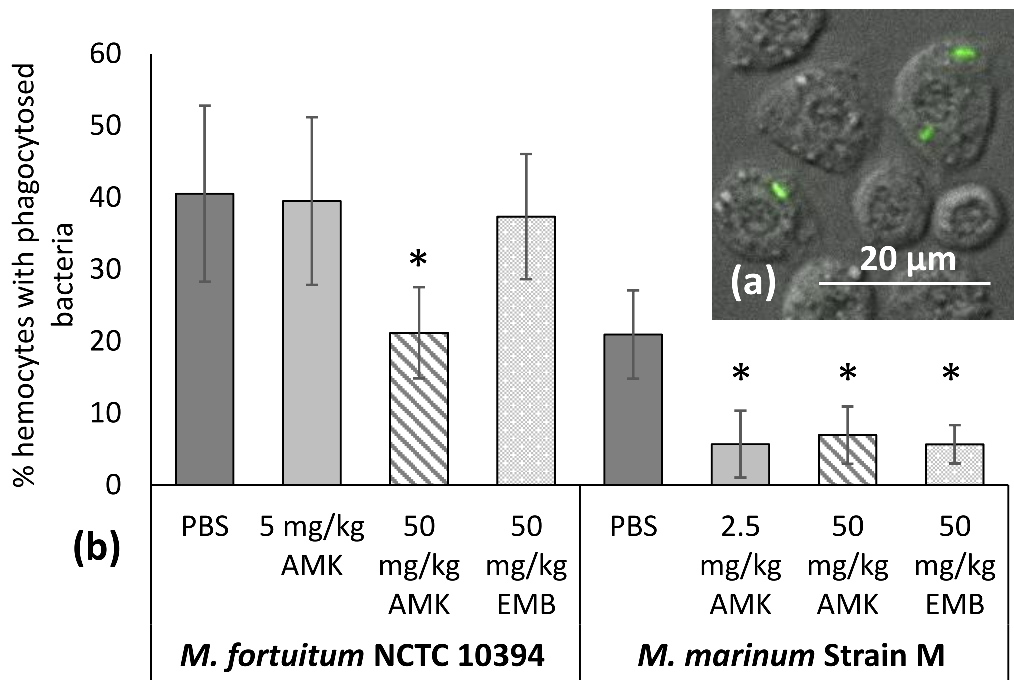

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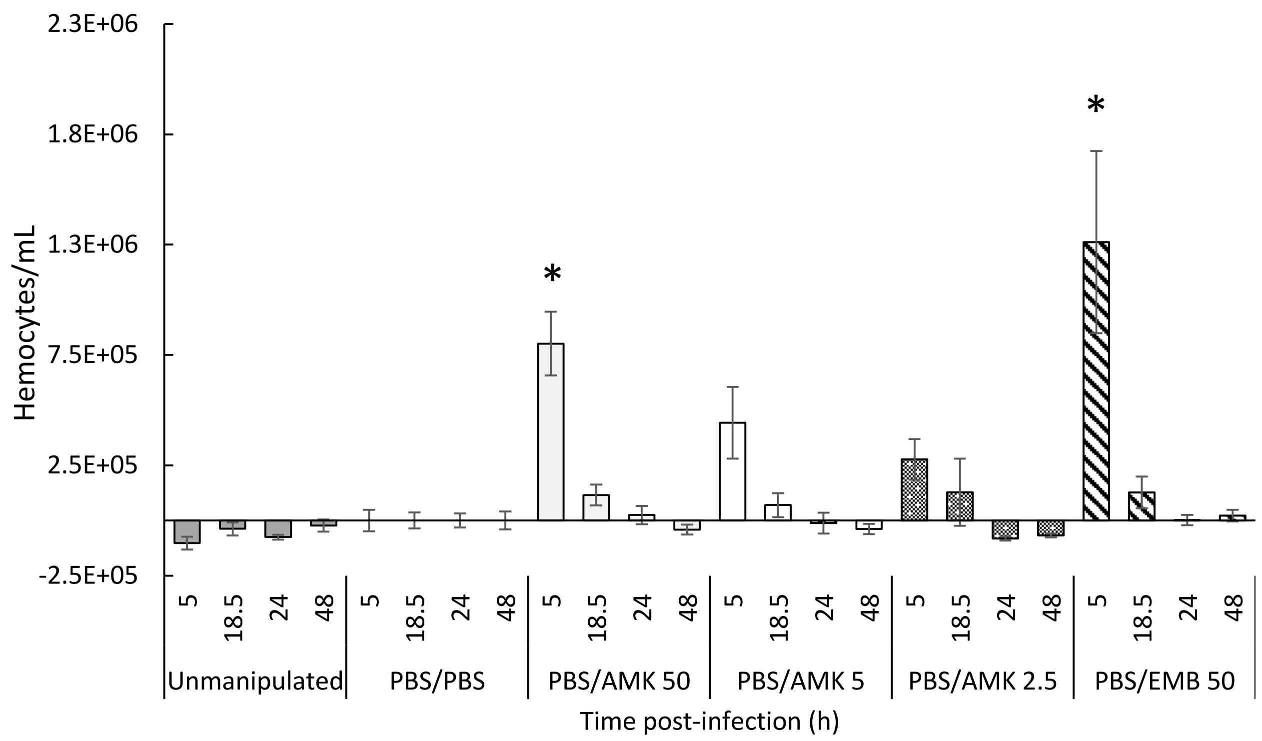

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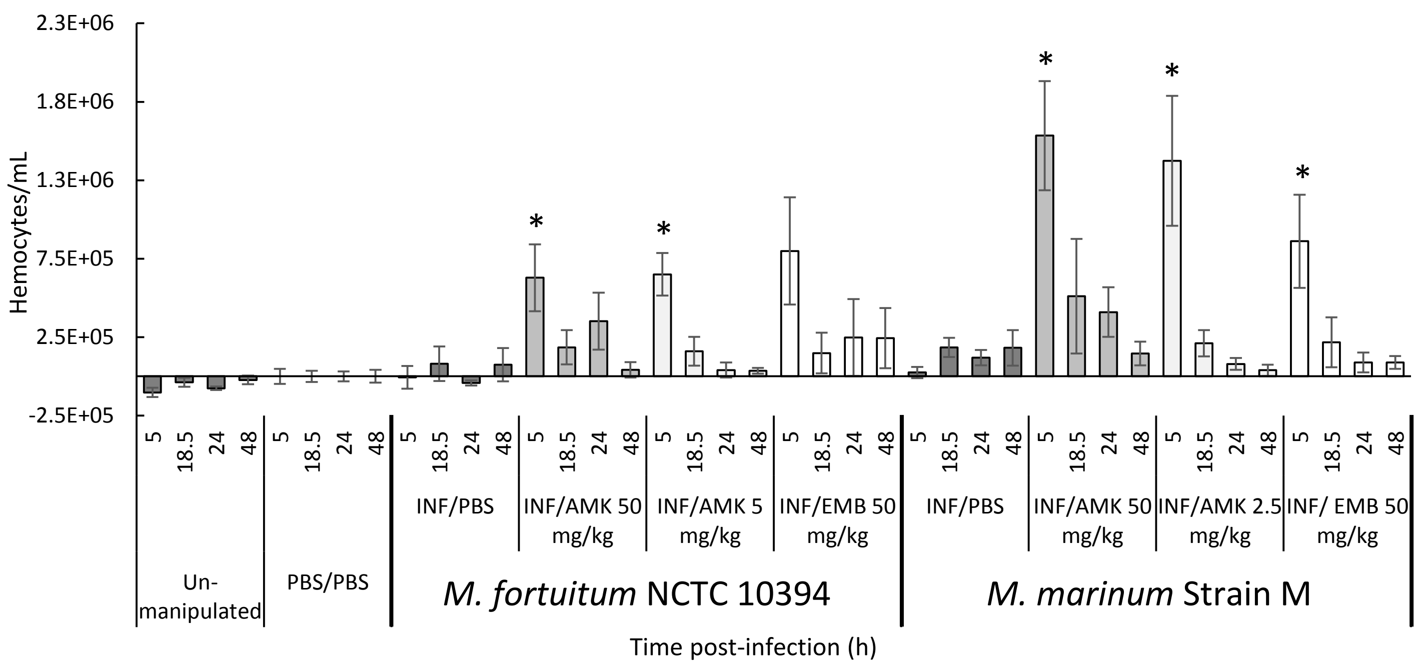

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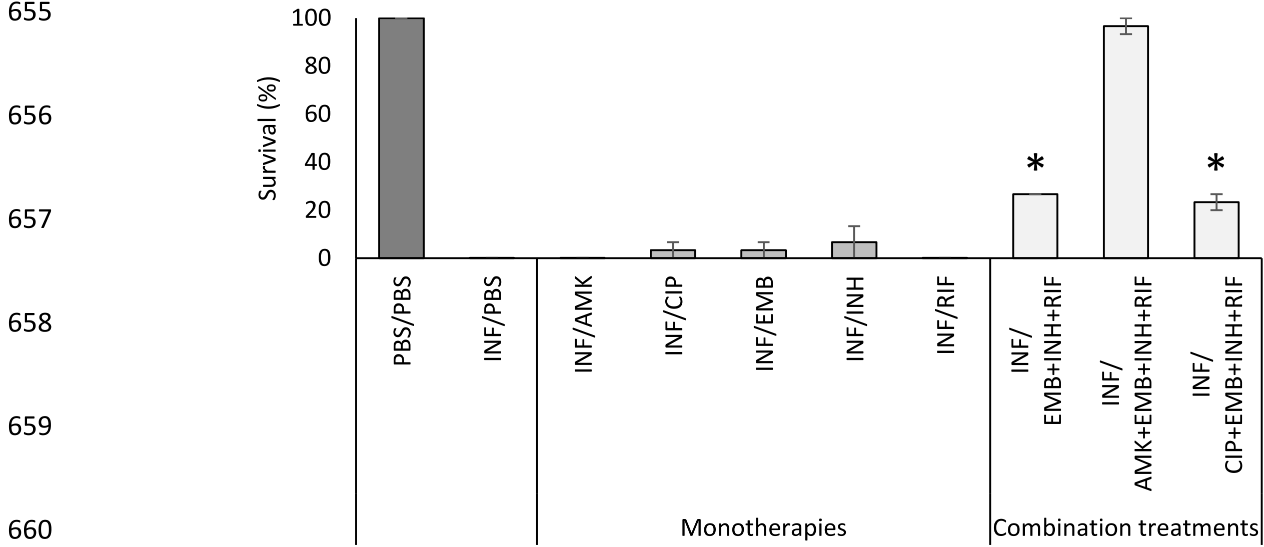

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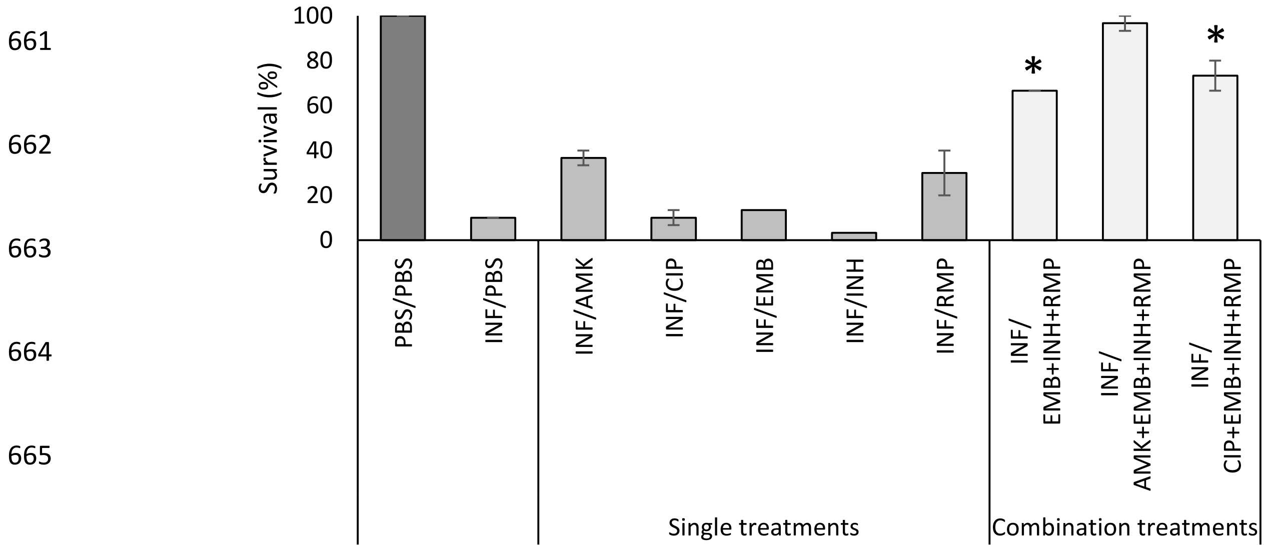

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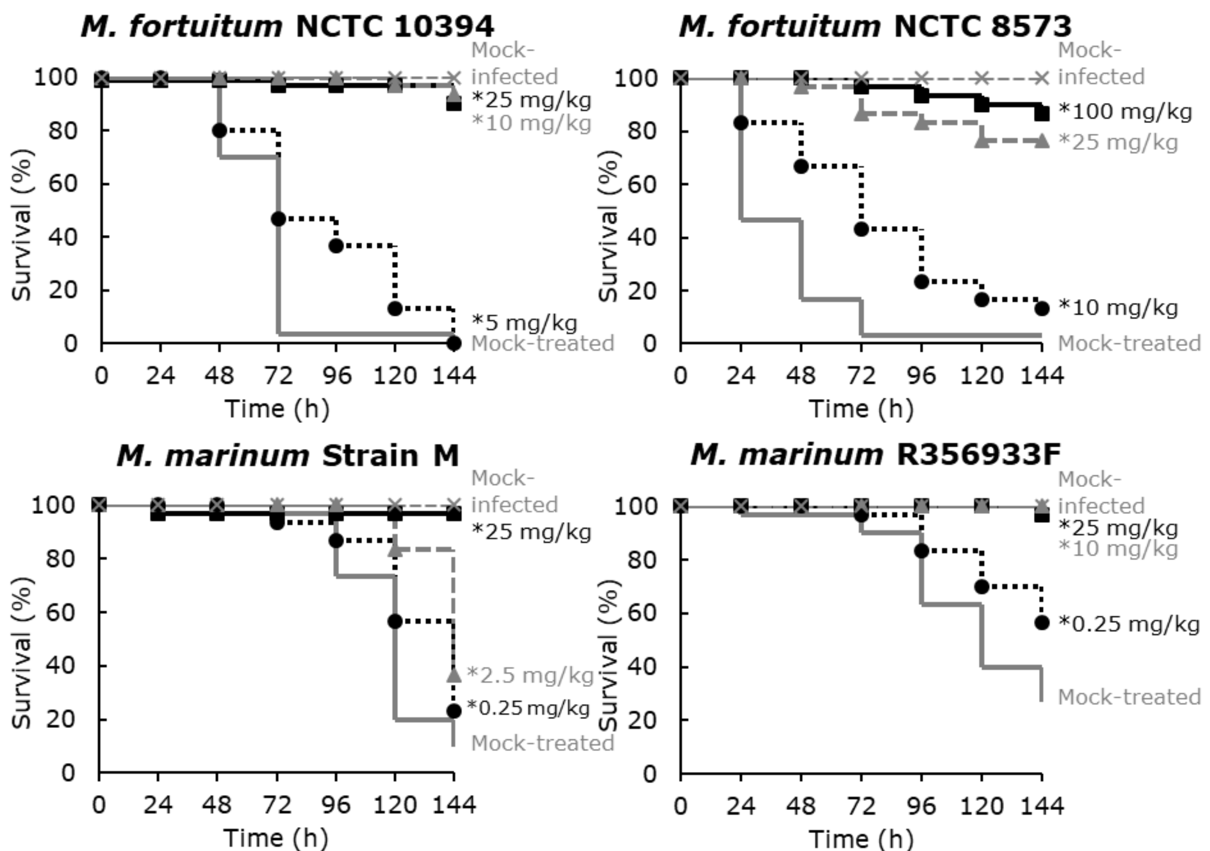
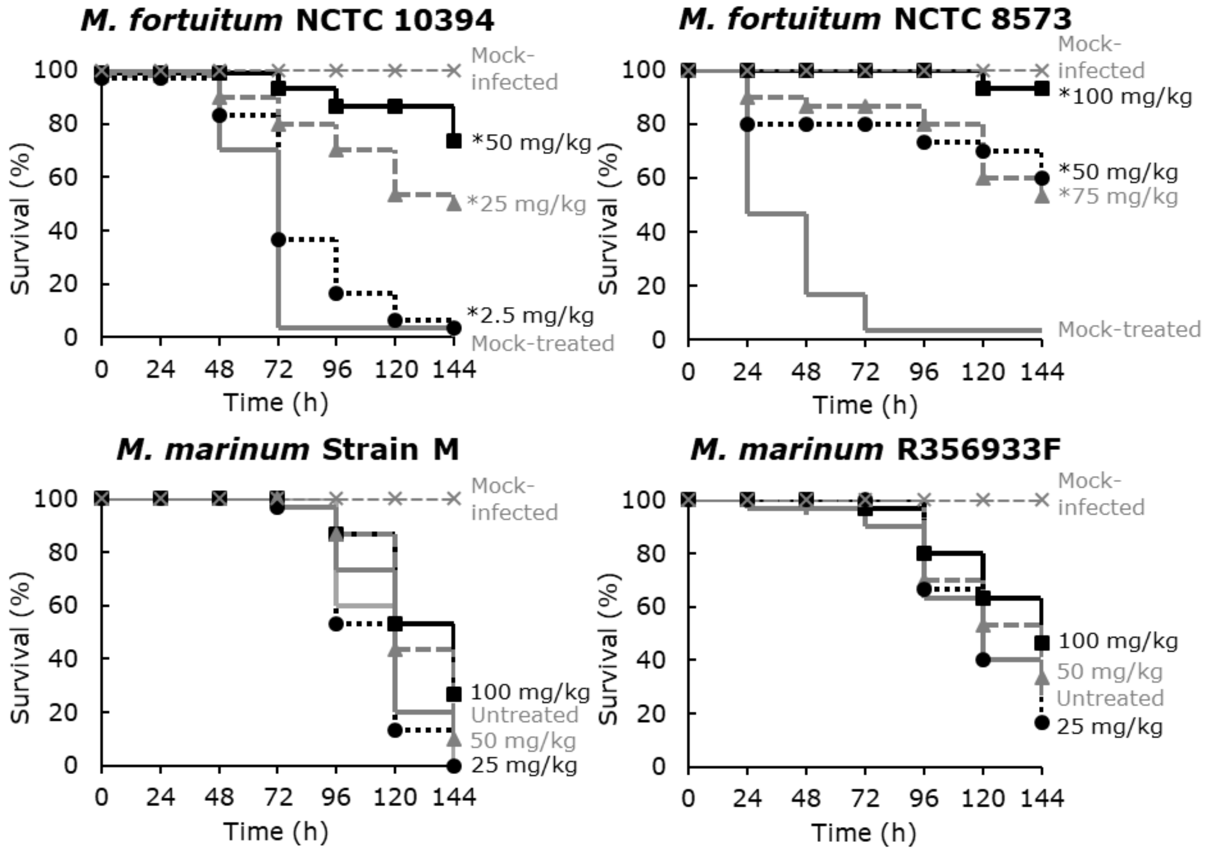
669 Supplementary data – Kaplan-Meier survival curves for antibiotic susceptibility experiments (pages
670 1-3) and antibiotic combinations (page 4)

671 The antibiotic used is at the top of each grouping, and the species and strain of *Mycobacteria* used at
672 the top of each curve. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RMP,
673 rifampicin.

674 The mock-treated larvae are indicated with a grey line without line markers. The highest antibiotic
675 dose is an unbroken black line with black, filled square markers. Middle doses have a dashed grey line
676 and filled grey triangle markers, and the lowest dose have a dotted black line with a filled black circle
677 marker. A mock-infected control is shown with grey crossed markers and a dashed grey line.

678 The doses used in mg/kg are shown to the right of each curve. Any doses that increased larval survival
679 significantly above the survival of the mock-treated control have an asterisk by the dose.





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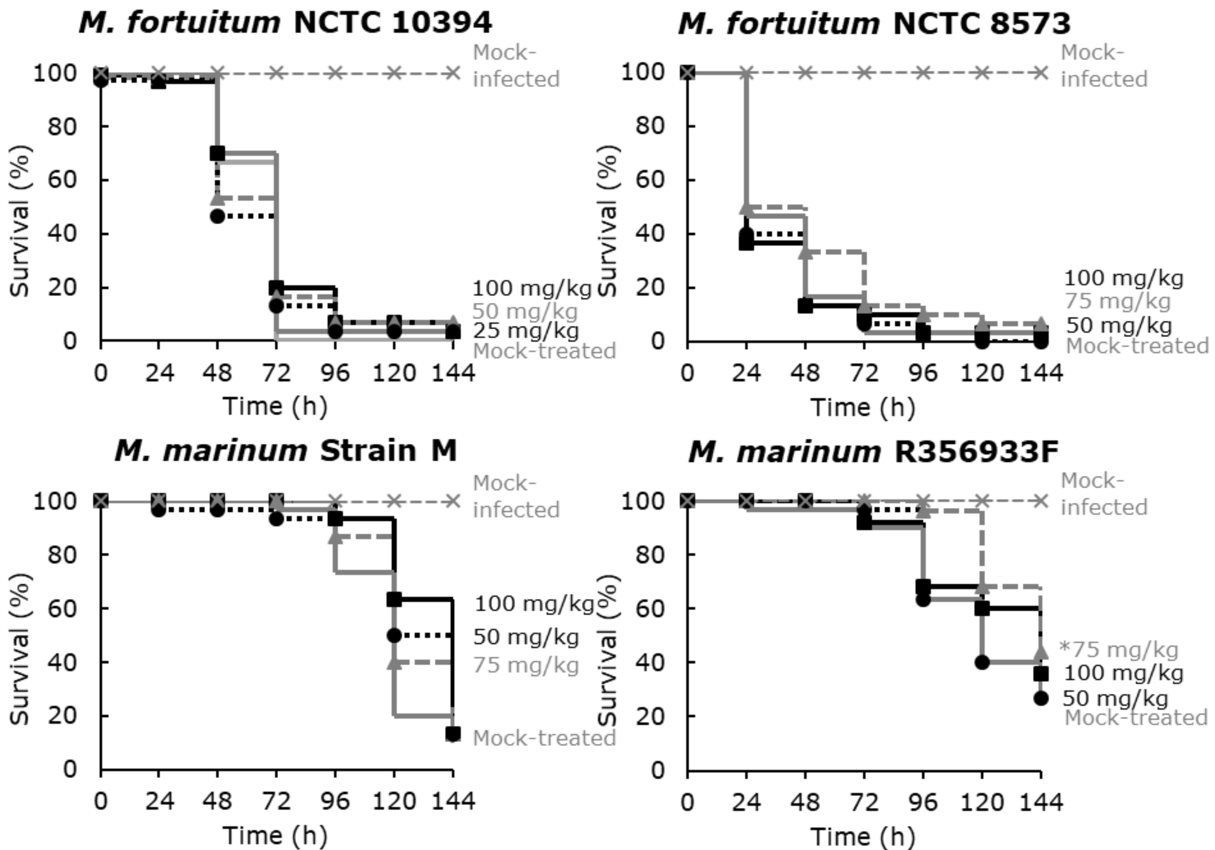

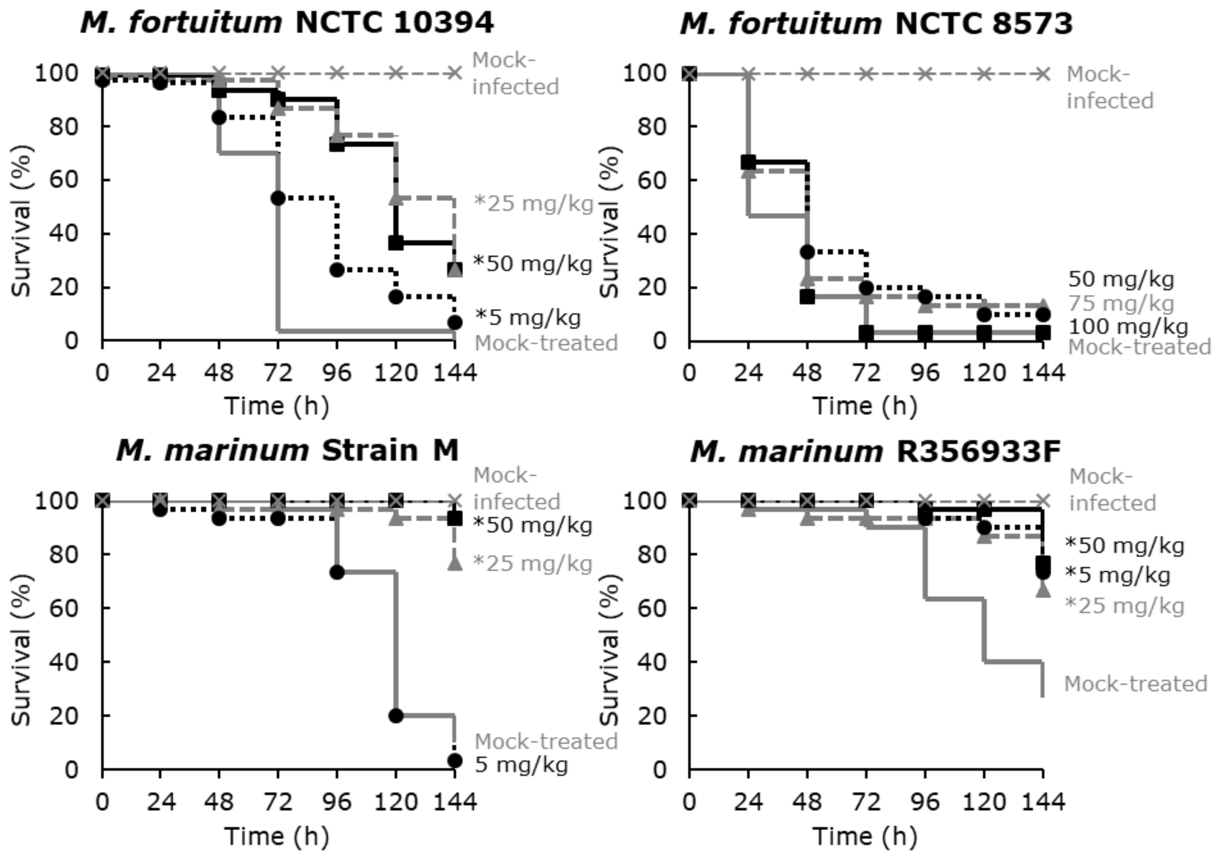

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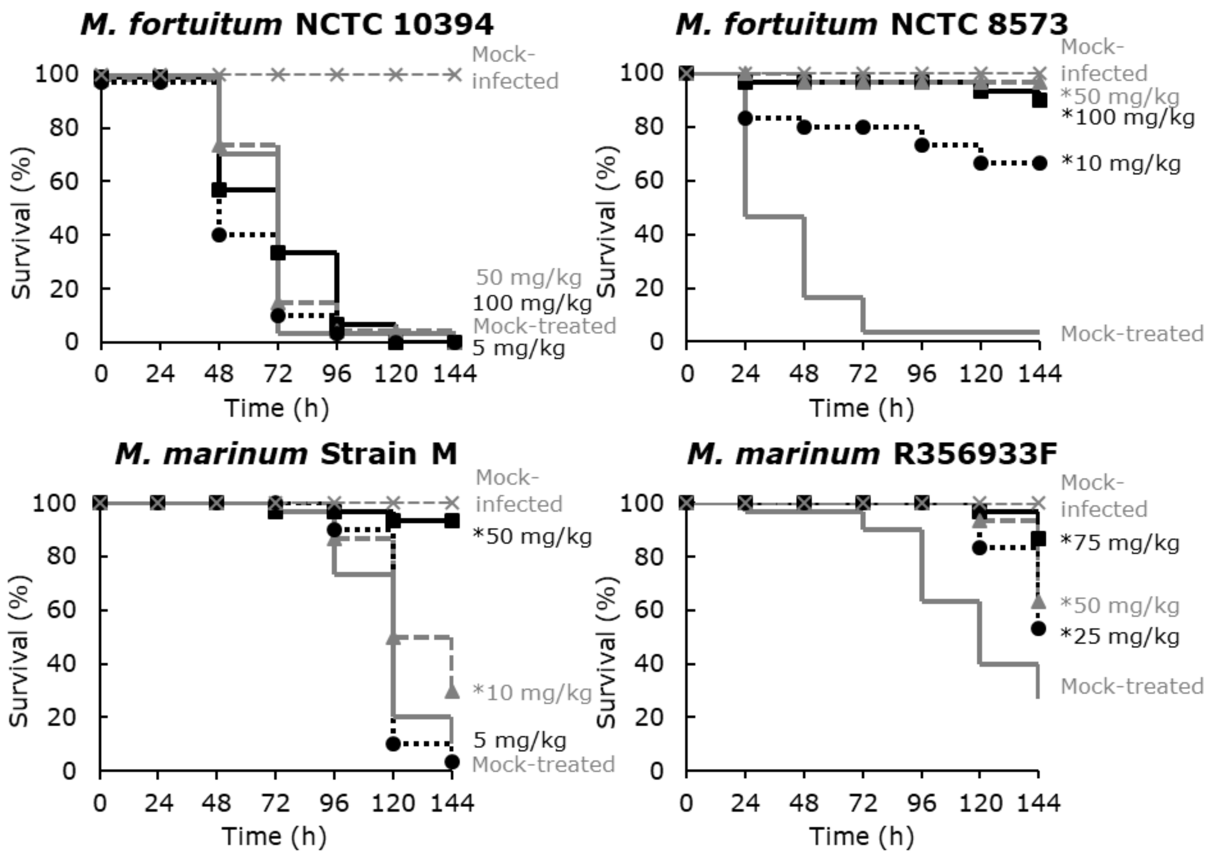

682 Kaplan-Meir survival curves for the antibiotic combination experiments. Each of the single antibiotic
683 doses are shown in pale grey, with the initial of the antibiotic as the point marker. A, amikacin; C,
684 ciprofloxacin; E, ethambutol; I, isoniazid; R, rifampicin. The combinations are shown in black, with the
685 combinations used listed on the right hand side of the figure. The mg/kg are included (eg. A5 describes
686 a 5 mg/kg dose of amikacin). An asterisk next to this label means that the survival is significantly higher
687 than the mock-treated control (log rank test, Holm's correction applied).

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