Cockroaches join the fight against infection: an improved insect host system for *Francisella tularensis*.

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1 Abstract

Francisella tularensis is a zoonotic bacterial pathogen that causes severe disease in a wide range of host animals, including humans. Well-developed murine models of *F. tularensis* pathogenesis are available, but they do not meet the needs of all investigators. Instead, researchers are increasingly turning to insect host systems to: (1) allow high-throughput that is cost-prohibitive or ethically-questionable in mammals; (2) enable studies of host-pathogen interactions when mammalian facilities are unavailable; and (3) provide valuable information about environmental persistence and transmission. However, the utility of previously-described insect hosts is limited because of temperature restriction, short lifespans, and concerns about the immunological status of insects mass-produced for other purposes. Here, we present a novel host species, the orange spotted (OS) cockroach (*Blaptica dubia*), that overcomes these limitations and is readily infected by *F. tularensis*. Intrahemocoel inoculation was accomplished using standard laboratory equipment and lethality was directly proportional to the number of bacteria injected. Disease progression differed in insects housed at low and high temperatures, a pattern indicative of a switch between virulence and transmission phenotypes. As in mammalian hosts, *F. tularensis* mutants lacking key virulence components were attenuated in OS cockroaches. Finally, antibiotics were delivered to infected OS cockroaches by systemic injection and controlled feeding; in the latter case, protection correlated with oral bioavailability in mammals. Collectively, these results demonstrate that this new host system should facilitate discovery of factors that control *F. tularensis* virulence, immune evasion, and transmission while also providing a platform for early stage drug discovery and development.

2 Importance

Invertebrate models of infection play a critical role in helping us understand host-pathogen interactions. Despite the availability of multiple invertebrate host species, we currently lack a robust, long-lived host that thrives at 37°C, has low background rates of mortality, and can be easily reared and manipulated in the laboratory without specialized equipment. In the work presented here, we establish that the OS cockroach meets these needs and is a permissive host for the model zoonotic pathogen *Francisella tularensis*. Further, relatively little is known regarding how *F. tularensis* survives and is transmitted by environmental arthropods. We show that *F. tularensis* virulence toward OS cockroaches varies according to temperature. Thus, investigators will be able to compare temperature-regulated virulence and transmission strategies within a single host. Finally, we demonstrate that the OS cockroach can be a cost-effective platform to test oral efficacy of antibiotic compounds.

3 Introduction

Francisella tularensis is a Gram-negative bacterial pathogen capable of causing disease in a remarkably diverse array of hosts; at least 190 different species of mammals, 23 birds, 3 amphibians and 88 invertebrates are recognized as being susceptible to *F. tularensis* infection [1]. In addition, *F. tularensis* utilizes a wide variety of environmental arthropod vectors for transmission [2-10]. In experimental animals, *F. tularensis* invades and replicates within both phagocytic and non-phagocytic cells [11-14] and several studies have demonstrated that *F. tularensis* survives engulfment by bacterivorous protists, often escaping from the food vacuole and replicating within the cytosol [15-17]. This ability to survive intracellularly is thought to contribute to the low infectious dose of *F. tularensis*, which is fewer than 10 bacteria for the highly-virulent strains [18]. Due to this high infectivity and an accompanying high rate of mortality and morbidity, *F. tularensis* is of particular concern as an agent of biological terrorism and is therefore classified as a Tier 1 select agent by the US Centers for Disease Control [19]. An attenuated live vaccine strain (LVS) originally was derived from a virulent isolate in the 1950s [20]. Desipte its name, LVS is not currently approved by the US Food and Drug Administration for standard

human use because of safety and efficacy concerns. However, the LVS strain can be manipulated in biosafety level two laboratories (BSL2) and still causes rapid and severe disease in many hosts, allowing for *F. tularensis* pathogenesis studies without the need for BSL3 containment.

While experimental models of infectious disease historically have been developed around mammalian host species, non-vertebrate hosts continue to gain attention as an alternative approach for studying pathogenic microorganisms [21-24]. In particular, studies using the fruit fly *Drosophila melanogaster* [25, 26] and Greater Wax Moth *Galleria mellonella* larvae [27-29] have significantly advanced our understanding of *F. tularensis* pathogenesis. *D. melanogaster* offers powerful host genetic tools but the small body size of this insect makes delivering an exact dose of bacteria difficult without specialized equipment and training. Moreover, *D. melanogaster* is temperature-restricted and cannot survive at typical mammalian body temperatures, making this host of limited use for analysis of pathogens with temperature-sensitive virulence patterns such as *F. tularensis* [30]. In contrast, *G. mellonella* survives well at 37°C and is large enough for confident dosing with a small-gauge syringe. *G. mellonella* larvae also are readily-available in large quantities from a number of commercial suppliers. However, this insect host also requires investigators to accept certain limitations and tradeoffs. Pupation, the process by which the larvae metamorphesize into adults, typically occurs within a short period of time when the larvae are kept at 37°C, thereby limiting the experimental window available to researchers. Immune function can vary widely before, during, and after pupation [31-33], thus making it difficult to standardize host immunological status in *G. mellonella*. When working with *G. mellonella* from commercial suppliers, we encountered tremendous shipment-to-shipment variability in experimental outcome, presumably due to differences in the general health status of the larvae. Other groups have observed similar trends and have addressed this concern by supplementing the insect meal with antibiotics [34] or setting a mortality threshold in control groups that, when surpassed, allows investigators to discard the results and repeat the experiment with a new batch of insects [35-37]. Disatisfied with these options, we began to rear *G. mellonella* in the laboratory so that we could better control their quality. We were surprised to find that, in contrast to larvae purchased from commercial sources, those reared in the lab quickly became encased in silk when transfered from the rearing vessel to a Petri plate for experimental manipulation (**Figure 1A** and **1B**). Others have reported a similar cocoon in laboratory-reared insects and recommend that larvae be mechanically removed from the structure prior to infection [24]. However, we found it difficult to perform this procedure without causing physical trauma to the larvae. Moreover, larvae would generally spin a new cocoon within a matter of hours, making it necessary to perform this manipulation each day of the study in order to observe the larvae for mortality. Thus when using laboratory-reared *G. mellonella* larvae, the throughput advantage of an insect model is compromised by this cumbersome procedure. In search of an explanation for this behavioral difference between commercially-obtained *G. mellonella* and those reared in the lab, we found two on-line forums for hobbyists that described the use of a brief freeze treatment to destroy the silk gland [53, 54]. Although we were unable to confirm that commercial suppliers of *G. mellonella* use this particular method, it is clear that they treat their insects in some way that prevents silk production. While this does aid in handling and improves experimental throughput, it is problematic for pathogenesis studies because the immunological consequences of a necrotic silk gland are unknown. On the one hand, necrosis could activate a generalized immune response [38]. Alternatively, the silk gland is an important component of the antibacterial immune response in wax worms [31] and its loss or dysregulation could impair functional immune responses in this host. Given these inherent problems with the *G. mellonella* model, we sought to identify another insect host that is simple and inexpensive to rear in the laboratory, survives well at 37°C for long periods of time, and is large enough to allow inoculation with known doses of bacteria without specialized equipment.

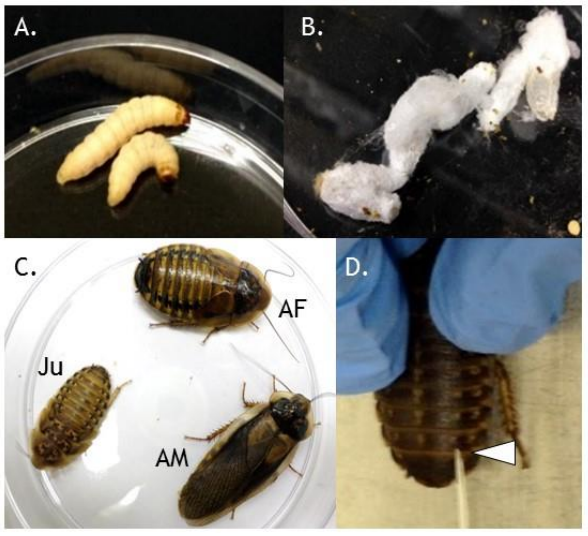

Figure 1. (A) Wax worms purchased commercially that fail to produce silk casing when transfered to a Petri dish. (B) Wax worms reared in the lab produce a thick silk casing when removed from the primary culture vessel. (C) Juvenile (Ju), adult female (AF), and adult male (AM) orange spotted cockroaches are easily distinguished from each other. Juveniles lack wings while adult females have vestigial forewings that cover the first few abdominal segments. Adult males have wings that extend the full length of their body, but they do not fly. Pictured here in a standard 100 mm Petri dish. (D) Typical site of injection to the right of the midline along the base of the third tergum (arrowhead) from the posterior. Note that sharpened pipette tips can be used for inoculation, instead of needles.

We previously determined that the Madagascar hissing (MH) cockroach (*Gromphadorhina laevigata*) met the above noted criteria and was a suitable surrogate host for the facultative intracellular pathogens *Burkholderia mallei*, *B. pseudomallei*, and *B. thailandensis* [39]. Here, we sought to determine if tropical cockroach species can serve as experimental hosts for another important bacterial pathogen, namely *F. tularensis*. The Orange Spotted (OS) cockroach (**Figure 1C**, *Blaptica dubia* Serville 1839) also meets the above criteria as an ideal insect host for pathogenesis studies but is more readily available from commercial suppliers and is more docile compared to the MH cockroach. The OS cockroach does not vocalize like the MH cockroach and it usually remains in an immobile defensive position when placed on its back. Of interest to researchers who desire to rear their own host animals, the OS cockroach does not climb vertical glass or plastic surfaces, which aids in containment of the cockroaches in their rearing containers. The body size and sclerotised cuticle of OS cockroach enables intrahemocoel inoculation using either a standard needle and syringe combination or a sharpened pipette tip (**Figure 1D**), which improves laboratory safety and decreases costs. We found the OS cockroach to be a permissive host for *F. tularensis* LVS. Lethality depended upon the dose of bacteria given, the temperature of incubation, and, interestingly, the developmental stage and gender of the cockroach. Intracellular and extracellular bacterial titers increased throughout the course of infection and several mutant strains lacking factors known to be involved in *Francisella* pathogenesis were attenuated. Infection could be rescued by systemic or peroral delivery of antibiotics, with protection by the peroral route correlating with known oral absorption profiles in mammals. These results extend our previous findings [39] and demonstrate that tropical cockroaches are a favorable alternative to mammals and other insect species for the study of multiple bacterial pathogens.

4 Results

4.1 Infection of OS cockroaches with *F. tularensis* LVS

To determine if OS cockroach survival was proportional to the number of bacteria present in the inoculum, serial dilutions of *F. tularensis* LVS were injected into the hemocoel of juvenile cockroaches that weighed between 0.7 and 1.0 grams. Following infection, cockroaches were housed at 37°C and survival was monitored over the course of 7 days. Overall, the percentage of cockroaches in each group that survived infection and the rates of death were dose-dependent. None of the cockroaches inoculated with 10^6 or 10^5 CFU of *F. tularensis* LVS survived beyond day 4 (**Figure 2**). In comparison, 5 out of 8 cockroaches infected with 10^4 CFU of *F. tularensis* LVS survived through day seven post-infection

(p=0.0141; compared to 10^5 CFU group) and 7 out of 8 cockroaches infected with 10^3 CFU of *F. tularensis* LVS survived through day seven post-infection (p<0.001; compared to 10^5CFU group). Injection of PBS alone did not result in any cockroach deaths.

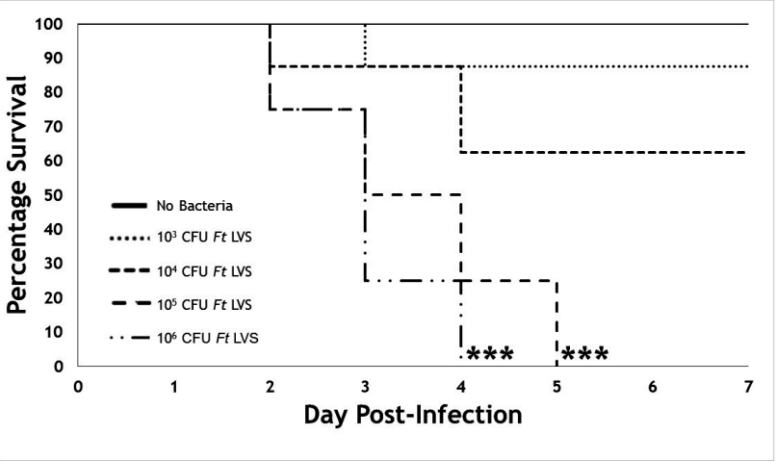

Figure 2. *B. dubia* survival is proportional to the *F. tularensis* LVS infection dose. Groups of juvenile OS cockroaches (n=8) were injected with either PBS (no bacteria) or serial dilutions of *F. tularensis* LVS (1.7 x 10^6 to 10^3 CFU), incubated at 37˚C, and monitored for survival over the course of 7 days. LVS was grown on CHOCII agar for 48 hours, harvested, and diluted in PBS. Statistical differences between groups were calculated by log-rank (Mantel-Cox) analysis. Asterisks indicate significant difference from the no bacteria control group (***=p<0.001).

4.2 Effect of temperature on *F. tularensis* virulence

Transcriptional activation of *F. tularensis* virulence factors is at least partially controlled by temperature [30]. This is particularly interesting given that *F. tularensis* is well-known to be transmitted to mammals by ticks and other arthropod vectors [2-10] and, thus, the bacterium encounters dramatically different temperatures in its varied hosts. Since insects are ectothermic, taking on the ambient temperature of their surroundings, we sought to determine the impact of varied temperatures on *F. tularensis* pathogenesis in this system. Serial dilutions of *F. tularensis* were injected into the hemocoel of juvenile OS cockroaches and their survival rates were monitored over the course of 8 days at 22°C, 30°C, 37°C, or 40°C. Overall, we found that higher temperatures correlated with higher LVS virulence in the OS cockroach (**Figure 3, Table 1**). Following infection with 10^6 CFU, incubation at either 37°C or 40°C resulted in rapid OS cockroach death (mean time-to-death of 3.6 and 3.2 days, respectively), with no survivors at either temperature (**Figure 3**). By comparison, incubation at the lower temperatures of 22°C or 30°C resulted in delayed time-to-death and increased survival, despite slightly higher inoculums (**Figure 3**). At 30°C, 20 percent of cockroaches survived *F. tularensis* LVS infection with a mean time-to-death of 6.14 days. At 22°C, 30 percent of cockroaches survived *F. tularensis* LVS infection with a mean time-to-death of 6.33 days. There were no significance differences between cockroach survival at 22°C and 30°C or between cockroach survival at 37°C and 40°C. However, cockroach survival at both of the lower temperatures (22°C and 30°C) was significantly different from cockroach survival at both of the higher temperatures (37°C and 40°C; all p<0.001).

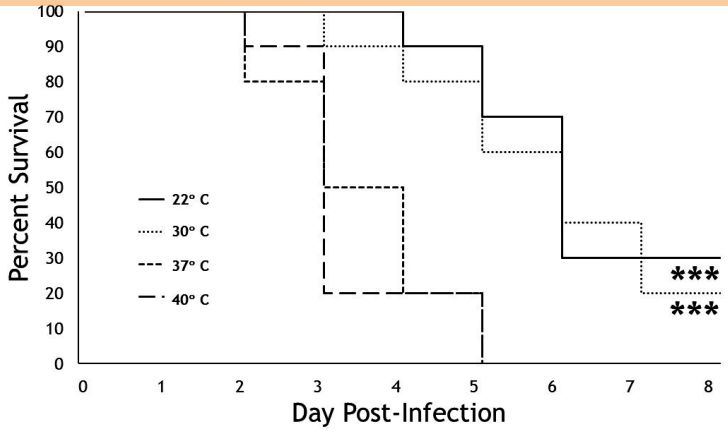

Figure 3. *F. tularensis* LVS lethality in *B. dubia* is temperature-dependent. Groups of juvenile *B. dubia* cockroaches (n=10) were injected with 6.0 x 10^6 CFU, 8.8 x 10^6 CFU, 4.5 x 10^6 CFU, or 3.0 x 10^6 CFU *F. tularensis* LVS (approx. 100 x LD_{50}) and incubated at 22°C, 30°C, 37°C, or 40°C, respectively. Statistical differences between groups were calculated by log-rank (Mantel-Cox) analysis. Asterisks indicate statistical differences from the 37°C and 40°C groups (***=p<0.001).

4.3 *F. tularensis* genes required for virulence in mammals are required for virulence in OS cockroaches

In order to determine if virulence factors known to be required for *F. tularensis* pathogenicity in mammals also are required in our cockroach model, we determined the LD_{50} value for several mutant strains of *F. tularensis* LVS. Juvenile OS cockroaches were infected by intrahemocoel injection followed by incubation at 37°C. Survival was monitored for 8 days post-infection and the LD_{50} value for each strain was determined by non-linear regression. Since the various LVS mutants were generated by two different laboratories, we excluded the possibility of inherent differences in LVS virulence by infecting cockroaches with either LVS parental strain, LVS-1 and LVS-2. Indeed, LVS-1 and LVS-2 did not exhibit any significant LD_{50} differences in juvenile cockroaches at 37°C (**Table 1**). In contrast, loss of *dsbA*, *dipA*, *iglC*, or *deoB* resulted in substantially decreased virulence in cockroaches (**Table 1**), similar to trends previously observed in mice [41-44] and chick embryos [30]. More specifically, *dipA* and *deoB* mutants were more than 1-log attenuated in cockroaches and *dipA* and *iglC* were nearly 2-log attenuated in cockroaches. These results suggest that, dispite obvious differences between mice and cockroaches, there are significant overlaps in how *F. tularensis* causes disease and death in hosts from diverse phyla.

Strain	Host	Temperature	LD_{50} (95% CI), CFU
F. tularensis LVS-1	Juvenile cockroaches	22°C	3.2 x 10^6 (5.6 x 10^5–1.2 x 10^7)
	Juvenile cockroaches	30°C	2.9 x 10^5 (4.6 x 10^4–1.8 x 10^6)
	Juvenile cockroaches	37°C	3.5 x 10^4 (1.1 x 10^4–1.0 x 10^5)
	Juvenile cockroaches	40°C	1.2 x 10^4 (4.2 x 10^3–3.3 x 10^4)
	Adult female cockroaches	37°C	5.4 x 10^4 (9.7 x 10^3–3.0 x 10^5)
	Adult male cockroaches	37°C	1.3 x 10^3 (4.0 x 10^2–4.2 x 10^3)
F. tularensis LVS-1 Δ*dsbA*	Juvenile cockroaches	37°C	2.2 x 10^6 (1.0 x 10^6–4.7 x 10^6)
F. tularensis LVS-1 Δ*dipA*	Juvenile cockroaches	37°C	8.1 x 10^5 (4.0 x 10^5–1.6 x 10^6)
F. tularensis LVS-2	Juvenile cockroaches	37°C	1.7 x 10^4 (6.7 x 10^3–4.4 x 10^4)
F. tularensis LVS-2 Δ*iglC*	Juvenile cockroaches	37°C	1.3 x 10^6 (2.8 x 10^5–5.7 x 10^6)
F. tularensis LVS-2 Δ*deoB*	Juvenile cockroaches	37°C	5.6 x 10^5 (2.1 x 10^5–1.8 x 10^6)
E. coli DH5α	Juvenile cockroaches	37°C	8.7 x 10^6 (3.0 x 10^6–2.5 x 10^7)

Table 1. Median lethal dose of *F. tularensis* LVS and derivatives in OS cockroaches and wax worms. The median lethal dose (LD_{50}) of *F. tularensis* LVS parental strains, LVS-1 or LVS-2, and deletion mutants of those LVS strains lacking known virulence factors, and *E. coli* DH5α. LD_{50} and corresponding 95% confidence intervals were estimated by non-linear regression using the 'drc' package [40] in the R programming environment (Version 3.2.1).

4.4 *In vivo* growth of *F. tularensis* LVS

In order to monitor the kinetics of *F. tularensis* LVS growth during infection, we harvested hemolymph from infected OS cockroaches at various time points post-infection. The antibiotic gentamicin was used to distinguish between intracellular and total bacterial loads. Since gentamicin does not penetrate eukaryotic host cells, this antibiotic is active only toward bacteria that are located extracellularly during the exposure period [25, 45]. After an initial inoculum of 10^6 CFU, *F. tularensis* LVS quickly replicated, reaching 10^9 CFU per ml of hemolymph by 96 hours (**Figure 4**). Interestingly, most bacteria in the hemolymph were sensitive to gentamicin, indicating that they were exposed to the extracellular environment at some time during the 2 hours prior to harvest.

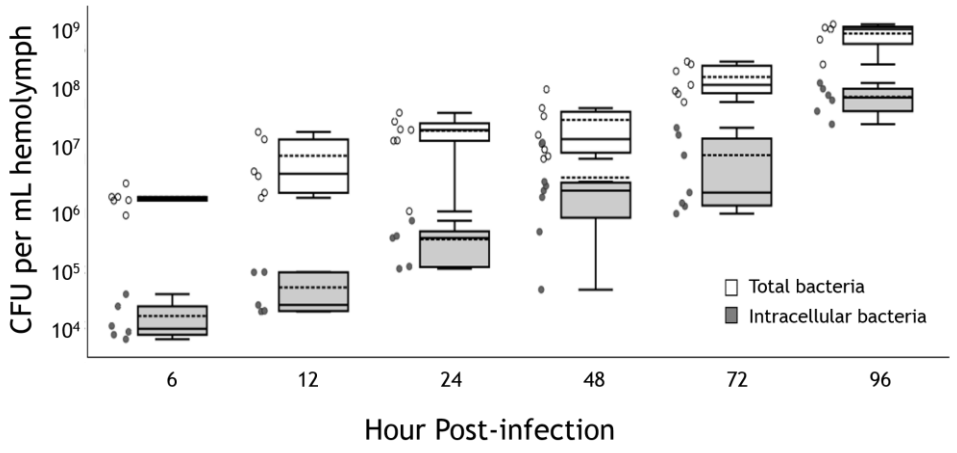

Figure 4. *In vivo* growth of intracellular and total *F. tularensis* LVS in OS cockroaches. Total (open symbols) and gentamicin-protected (intracellular; shaded symbols) *F. tularensis* LVS from at least 5 infected OS cockroaches per time point. Bacterial CFU were determined by serial dilution of hemolymph and enumeration on CHOCII agar plates. Intracellular bacterial numbers were determined by injecting gentamicin into infected cockroaches 2 hours prior to each time point. Results from individual insects are shown as open (total CFU) and closed (intracellular CFU) circles. Boxes indicate the median (solid line), mean (dotted line) and interquartile ranges (IQR; box boundaries) for each group. Upper and lower whiskers correspond with the largest and smallest data points, respectively, within 1.5 x IQR for each group.

4.5 Effect of OS cockroach development on *F. tularensis* virulence

Because insect immune responses are known to vary by age and developmental stage [46-48], we sought to determine if there were any differences in susceptibility to *F. tularensis* LVS in juvenile and adult cockroaches. Injections in adults were performed using the same method described for the juveniles. Cockroaches cannot be separated by gender as juveniles, but anatomical differences (**Figure 1C**) make gender determination possible in adults. We, therefore, analyzed survival in adult female and adult male cockroaches separately. We found that although the overall percentages of surviving cockroaches differed slightly between adult female (50 percent survival) and juvenile (30 percent

survival) cockroaches, both groups were equally susceptible to *F. tularensis* LVS infections (juvenile mean time-to-death 4.7 days; adult female mean time-to-death 4.8 days; no statistical difference between groups). By comparison, adult male cockroaches were more sensitive to killing by *F. tularensis* LVS, with rapid death rates (mean time-to-death 3.8 days) and 100% mortality by day 6 post-infection ($p=0.0169$ compared with juveniles; $p<0.001$ compared with adult females; **Figure 5**). The LD_{50} of adult males was determined to be 1.3×10^3 CFU, which is a 1.4- and 1.6-log decrease from that of juvenile and adult female cockroaches, respectively (**Table 1**).

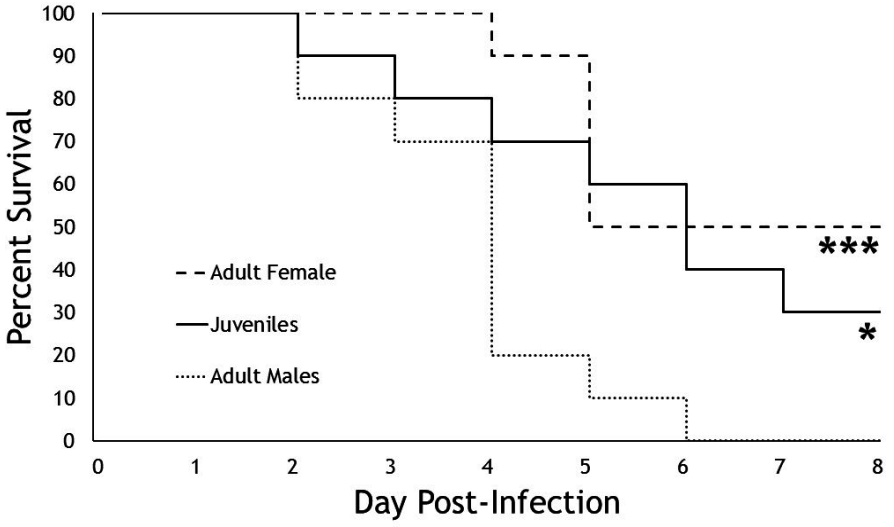

Figure 5. OS cockroach susceptibility to *F. tularensis* infection varies by life stage. Groups of juvenile, adult male, or adult female cockroaches (n=10) were injected with 3.4×10^4 CFU *F. tularensis* LVS prior to incubation at 37˚C. Statistical differences between groups were calculated by log-rank (Mantel-Cox) analysis. Asterisks indicate statistical differences from the adult male group (*=$p<0.05$; ***=$p<0.001$). Survival in the adult female and juvenile groups was not significantly different ($p>0.05$).

4.6 Antibiotic rescue of infected OS cockroaches

To explore whether OS cockroaches may be useful in the drug development pipeline, we infected cockroaches with a high dose (1.3×10^6 CFU to 3.4×10^6 CFU) of *F. tularensis* LVS by intrahemocoel injection and then administered antibiotics either by injection or by controlled feeding (**Figure 6**). All cockroaches in the vehicle only control groups died by day 7 post-infection (**Table 2**). Doxycycline, an antibiotic known to absorb well through mucus membranes, effectively prevented cockroach death when delivered by either route (**Table 2**, $p<0.001$). *F. tularensis* LVS is resistant to azithromycin [49] and this antibiotic failed to protect OS cockroaches from infection, illustrating the specificity of protection in the assay (**Table 2**). Streptomycin and gentamicin, which have poor oral bioavailbility in mammals, were effective at preventing cockroach mortality when injected directly into the hemocoel (80 percent survival with streptomycin; 90 percent survival with gentamicin; $p<0.001$ for both antibiotics compared to no antibiotic treatment; **Table 2**). However, neither of these antibiotics rescued OS cockroaches when delivered perorally ($p=0.00199$ for injection of streptomycin compared to forced feeding; $p<0.001$ for injection of gentamicin compared to forced feeding). Finally, Resazurin, an experimental drug candidate that has anti-*F. tularensis* activity *in vitro* [50], failed to protect OS cockroaches from infection (no survival by either delivery route; **Table 2**).

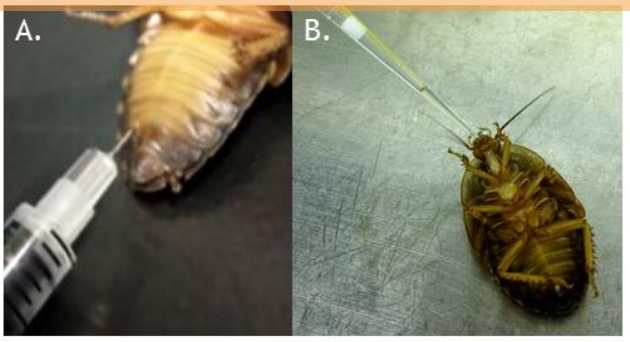

Figure 6. Antibiotic delivery to OS cockroaches. (A) For systemic delivery, a 20 µl aliquot of each antibiotic or PBS vehicle control was injected at the base of the 3rd (shown) or 4th tergum on the ventral side of the abdomen. (B) For oral delivery, cockroaches were placed on their backs and were fed a solution containing either 50% sucrose or 50% sucrose containing the specified antibiotic. In both cases, antibiotics were delivered at 2-, 48-, and 96-hours post infection.

Antibiotic	Route	Dose[a]	Survival Rate	Log-rank Comparison to Vehicle Control	Log-rank Comparison between Routes
Vehicle only	i.h.		0/18		n.s.
	p.o		0/10		
Doxycycline	i.h.	32 µg	9/10	***	n.s.
	p.o	32 µg	10/10	***	
Streptomycin	i.h.	32 µg	8/10	***	**
	p.o	32 µg	0/10	n.s.	
Gentamicin	i.h.	32 µg	7/8	***	***
	p.o	32 µg	0/10	n.s.	
Azithromycin	i.h.	100 µg	0/10	n.s.	n.s.
	p.o	100 µg	0/10	n.s.	
Resazurin	i.h.	11 µg	0/10	n.s.	n.s.
	p.o	11 µg	0/10	n.s.	
Additional control groups					
Bacteria only	No manipulation		0/10	n.s.[b]	
No Bacteria	No manipulation		10/10		
	i.h. vehicle		18/18		
	p.o. vehicle		6/6		

Table 2. Rescue of OS cockroaches with antibiotics after lethal dose of *F. tularensis* LVS. Groups of OS cockroaches (n=8-18) were infected with approximately 100 x LD_{50} (between 1.3 x 10^6 CFU and 3.4 x 10^6 CFU) *F. tularensis* LVS using sharpened pipette tips. Then at 2, 48, and 96 hours post-infection, infected cockroaches were treated by intrahemocoel injection (i.h.) or peroral feeding (p.o.) of the antibiotics indicated. Statistical differences in survival endpoints were determined by log-rank (Mantel-Cox) analysis. Asterisks indicate significant differences compared to the corresponding vehicle control group or alternative route of delivery (**=p<0.01, ***=p<0.001). [a]=this dose was administered three times during the study. [b]=survival outcome in the no manipulation group was not statistically different than survival in either vehicle control group (i.h. or p.o.; p>0.05 for all comparisons).

5 Discussion

F. tularensis is a highly-virulent zoonotic pathogen that causes significant morbidity and mortality globally. To facilitate future advances in our understanding of this important bacterium, we sought to develop an improved insect host system that eliminates undesirable biological and logistical trade-offs that accompany other popular host species such as *D. melanogaster* and *G. mellonella*. While insects lack adaptive immune functions, their innate immune systems share similar regulation and effector mechanisms with mammalian innate immune systems [21-24]. Because of this, insects can provide investigators with a host-pathogen interaction system capable of high-throughput that would be either financially or ethically unacceptable in mammals. Importantly, insects also provide scientists at institutions that lack access to mammalian housing facilities an alternative means by which to assess *in vivo* host-pathogen interactions. Finally, insects and other arthropods can be important environmental reservoirs and vectors for numerous zoonotic pathogens, including *F. tularensis*. Thus, insect host systems also aid in illuminating how these microorganisms evade arthropod immune systems during this part of their lifecycle without the necessity of rearing sanguinivorous arthropods in the lab. Here, we sought to identify an experimental host for *F. tularensis* that is (1) readily-available, (2) simple to rear in the laboratory, (3) tolerant of mammalian body temperatures, (4) large enough in size to allow consistent delivery of bacterial inoculations using standard needle-syringe combinations, (5) long-lived with low background mortality, and (6) hardy enough to withstand multiple injections of bacteria and/or antibiotics. We found that the *B. dubia* OS cockroach satisfied all of these requirements.

Like fruit flies and wax moth larvae, OS cockroaches are readily available. Several strain repositories supply the scientific community with *D. melanogaster* seed stocks and in-house rearing is easily accomplished using well-established protocols [51, 52]. Unfortunately, *D. melanogaster* does not tolerate incubation at mammalian body temperatures and quantitative infection requires highly specialized equipment. Thus, we did not consider *D. melanogaster* for our studies. In contrast, both *G. mellonella* and *B. dubia* survive at mammalian body temperatures. Critically, both of these hosts are able to mount effective immune responses against non-pathogenic microorganisms while infection by *F. tularensis* results in dose-dependent mortality ([29]; **Figure 1** and **Table 1**). Thus, *F. tularensis* LVS is able to evade active immune functions and establish a lethal infection in both of these experimental hosts.

Unfortunately, suppliers of wax worms and OS cockroaches are generally focused on non-scientific audiences. Wax worms are a popular choice for fishing bait and both wax worms and OS cockroaches are used as food for captive reptiles. In these markets, easy handling by consumers is critically important. This has led commercial suppliers to inactivate the wax worm silk gland by some unknown procedure (possibly using a freeze treatment as described by hobbyists in online forums [53, 54]). The physiological and immunological impacts of silk gland dysfunction are unknown, but it is clear that this organ is an import part of the antibacterial response in wax worms [31]. To avoid this serious complication for pathogenesis studies, wax worms can be reared in the laboratory [24] but we found it difficult to consistently do so without microbial contamination, a factor that might contribute to unpredictable rates of background mortality in our and others' studies [34-37]. In contrast, maintenance of a cockroach breeding colony in the laboratory is simple and straightforward [55, 56]. Compared to other cockroach species, OS cockroaches are docile and easy to handle. They are relatively slow, remain immobile when placed on their back, do not climb vertical glass or plastic surfaces, and they do not fly. It is unclear if these are characteristics of wild OS cockroaches or if they have been selected during captive breeding. Importantly, a minimal amount of maintenance is required to prevent microbial contamination (and odor) in OS cockroach breeding colonies. As a result, we rarely observe mortality in uninfected control groups of OS cockroaches (**Figure 1** and **Table 2**).

Both wax worms and cockroaches can be infected with known doses of microorganisms using needle and syringe combinations [24, 39]. But unlike wax worms, OS cockroaches can also be infected using sharpened gel-loading pipette tips, which increases the safety and decreases the cost associated with pathogenesis studies in this host. After infection, wax worms can survive at least one subsequent administration of antibiotics [29, 35]. Here, we established that OS cockroaches can tolerate at least 3 injections following infection without an increase in background mortality (**Table 2**). Importantly, the experimental window available to investigators is substantially different between wax worms and OS cockroaches. OS cockroaches undergo incomplete metamorphosis, with each developmental stage (or instar) lasting between 20 and 45 days. In total, it takes approximately 6 months for OS cockroaches to reach adulthood. The juvenile cockroaches used in this study were infected during the 6th instar (next to last), leaving between 30 and 60 days of experimental observation before they would have molted into the adulthood. In contrast, we often observed that considerable fractions (>25% in some cases, data not shown) of wax moth larvae pupated during a typical 7 day survival experiment. This is troublesome for studies of host-pathogen interactions because wax worm immune responses are known to vary throughout the period leading up to pupation [57]. Thus, small differences in individual age may impact the immune status of wax worms. While we did observe that adult male cockroaches were more susceptible to *F. tularensis* (**Figure 5**), the similarity between mortality in juvenile and adult female cockroaches indicates that small differences in age are unlikely to effect experimental results in this system. Collectively, these differences demonstrate that OS cockroaches offer important improvements compared to wax worms for studies of microbial pathogenesis. Thus, we went on to characterize several relevant factors in this model, including the impact of temperature on virulence, intracellular versus extracellular growth, genetic requirements for bacterial virulence, and the usefulness of the model for pharmacological screening.

Temperature is known to regulate expression of *F. tularensis* virulence factors [30]. One of the advantages of insect models, in comparison with mammals, is the ability to experimentally manipulate the temperature at which host-pathogen interactions occur. When we varied the temperature at which infection took place, we observed that higher temperatures correlated with higher mortality (**Figure 2, Table 1**). Others have shown that temperature can effect insect immune pathways [58-62], and there may be some differences in the immune response of OS cockroaches infected at different temperatures. However, it is intriguing to consider that, since *F. tularensis* can be spread by environmental arthropods [2-10], temperature may provide an important environmental cue that allows *F. tularensis* to dampen virulence pathways that would otherwise kill these vectors before they have an opportunity to transmit the bacterium to a subsequent host mammal. Thus, the OS cockroach system is an attractive new platform with which to interrogate the important but understudied environmental stage of the *F. tularensis* lifecycle and the switch between mammalian and arthropod hosts.

We took a genetic approach to test the hypothesis that virulence toward OS cockroaches at 37°C mimics virulence toward mammals. We examined the virulence of a small panel of *F. tularensis* LVS mutants that are attenuated in other model systems. DsbA and DipA are both associated with the normal structure and function of the *F. tularensis* membrane and their loss results in severe attenuation in mammals [42, 43]. DeoB is a metabolic protein (a phosphopentomutase) required for cellular invasion and virulence toward macrophages, dendritic cells, and chick embryos in *F. tularensis* [30, 63] and virulence toward mice in the closely related *Francisella novicida* [64]. Finally, IglC is a virulence factor encoded on the *Francisella* pathogenicity island that is required for intracellular survival and virulence toward mice [65, 66, 44]. We found that mutants lacking each of these proteins are also attenuated in OS cockroaches (**Table 1**). Since these proteins play essential roles in distinct components of the *F. tularensis* virulence program, this finding supports the idea that *F. tularensis* uses similar mechanisms to evade immune clearance and cause disease in extremely diverse host organisms. Thus, the OS cockroach model should be useful in identifying additional regulators and effectors of *F. tularensis* pathogenesis.

Since *F. tularensis* is considered a facultative intracellular pathogen, we sought to determine the proportion of bacteria that were located in intracellular and extracellular compartments throughout infection of OS cockroaches. As seen in **Figure 4**, intracellular bacteria can be recovered as early as six hours post injection, indicating successful invasion of OS cockroach cells. The intracellular population continues to grow throughout the infection process, as does the total bacterial population. Initially, we were surprised that the majority of the bacterial population at each time point was located in the extracellular environment, as judged by sensitivity to gentamicin. However, our results are similar to what others have observed for *F. novicida* in *D. melanogaster* [25]. Since hemocoel-injected gentamicin rescued OS cockroaches from lethality (**Table 2**), the extracellular *F. tularensis* population appears to be essential to the infection process, as has been recently suggested elsewhere [67]. While the intracellular phase of *F. tularensis* pathogenesis is well-appreciated, our findings suggest that the OS cockroach may be a useful model for elucidating the mechanisms by which *F. tularensis* survives, grows, and moves within the extracellular environment.

Host immune function is not static; it can vary dramatically across developmental stages in wax worms and other lepidopterans [57, 46, 68] and fruit flies [69, 70]. Importantly, Meylaers, et al, found that wax worm immunity dramatically increases as larvae progress through the wandering stage, in which they are typically used, and enter pupation [57]. We therefore sought to determine if OS cockroach susceptibility to *F. tularensis* LVS varied by developmental stage. We determined the killing kinetics and LD_{50} s of *F. tularensis* LVS against juvenile, adult female, and adult male OS cockroaches. The susceptibility pattern of juveniles (which we used for all other experiments reported here) and adult females were highly similar. In comparison, adult males showed enhanced susceptibility, with a shorter mean time-to-death (**Figure 4**) and a lower LD_{50} (**Table 1**). The reason for increased susceptibility in adult males is currently unknown and could result from either decreased resistance to *F. tularensis* or decreased tolerance to damage that occurs during infection. Interestingly, Horn, et al, found that the phagocytic ability of *D. melanogaster* hemocytes, migratory cells similar in function to mammalian macrophages, decreases with age [69] and it will be interesting to examine this and other possible causes of the increased susceptibility that we observed in adult males. Revealing these causes may illuminate key host factors that differentiate protective and unprotective immune responses to *F. tularensis* infection, information that could aid in developing a much-needed safe and effective vaccine.

Finally, we tested the ability of five different antibiotics to protect OS cockroaches from *F. tularensis* LVS infection (**Table 2**). Doxycycline is readily absorbed orally and was able to protect OS cockroaches from infection when delivered by either route. This protection was specific to antibiotics with anti-*Francisella* activity since azithromycin, to which *F. tularensis* LVS is resistant, failed to protect from lethality. Streptomycin and gentamicin are aminoglycoside antibiotics with poor oral bioavailability in mammals. Interestingly, these antibiotics only protected OS cockroaches when delivered by systemic injection and not when provided perorally. These findings indicate that oral absorption of antibiotics is similar in both mammals and insects and that OS cockroaches can provide a preliminary screening platform for identification of new antibiotics with anti-*Francisella* activity. As an example, we examined the ability of resazurin, which has been shown to have potent anti-*F. tularensis* LVS activity *in vitro* [50], to rescue OS cockroaches from lethality. Unfortunatley, resazurin failed to protect OS cockroaches from infection. Thus, we hypothesize that further modifications of the resazurin chemical backbone, something we are currently pursuing, will be required in order establish *in vivo* anti-*Francisella* activity. It is thought-provoking to consider how screening for *in vivo* activity in insects prior to substantial investments in mammalian models could change the cost profile of early stage antibiotic development efforts.

We suggest that, collectively, these results show that the OS cockroach offers significant biological and logistical advantages compared to other experimental host choices. Further, the OS cockroach will allow for characterization of virulence and transmission pathways within a single host including identification of *F. tularensis* genes required for these processes, dissection of effective and ineffective immune responses within a single host species, and pre-mammalian screening of therapeutic

candidates. Thus, the OS cockroach model is an important new addition to the repertoire of invertebrate hosts for mammalian pathogens.

6 Methods

All work described in this manuscript was approved by the appropriate institutional review board prior to completion.

6.1 Bacterial Strains and Knockout Construction

F. tularensis LVS strains used in this study were provided by two different laboratories. The strain designated as *F. tularensis* LVS-1 was provided by the J.F. Huntley laboratory at the University of Toledo (from ATCC). LVS-1 was the parental strain use to generate both *ΔdsbA* and *ΔdipA* mutants. The strain designated as *F. tularensis* LVS-2 was provided by the J. Horzempa laboratory at West Liberty University (originally provived by Karen Elkins). LVS-2 was the parental strain used to generate both *ΔdeoB* and *ΔiglC* mutants.

Generation of isogenic deletion mutants of *dsbA*, *dipA*, and *deoB* was described previously [42, 71, 72]. Markerless deletion *iglC* was accomplished by sequentially deleting codons 71-140 of both copies of *iglC*. The entire open reading frame was not deleted to preserve elements presumed to be required for expression of neighboring genes [73]. A 0.5 kb region of the *F. tularensis* LVS chromosome consisting of DNA upstream of *iglC* and a portion of the N-terminal coding sequence as was amplified using primer 1 (CATGGCATGCTAAGATTGGTAGTATTGTGGATGTCGAGTCG) and primer 2 (GTCGACGGTACCACCGGTTTATTATTAACTAGCAGCAGCTGTAGCCG). An additional region of the *F. tularensis* LVS chromosome consisting of the C-terminal coding sequence of *iglC* as well as downstream DNA was amplified using primer 3 (TAATAATAAACCGGTGGTACCGTCGACCTATCTAATTTAGAGTTATATCCAATAAGTGC) and primer 4 (CATGCTGCAGCTTATCAGTCATTATTTGTAAAGATAACGG). The two 0.5 kb amplicons were cloned into pJH1 [72] adjacent to each other to generate pJH1ΔiglC. This plasmid was mobilized into *F. tularensis* LVS using triparental mating as we have done previously [72, 63]. Isolated merodiploids were electroporated with pGUTS to force resolution . Deletion of a single *iglC* allele was confirmed by PCR (data not shown). After a strain was isolated in which a single copy of *iglC* was deleted, pGUTS was cured as previously described [72], and pJH1ΔiglC was subsequently re-introduced by triparental mating. PCR was used to confirm the recombination of this plasmid into DNA neighboring the intact *iglC* allele (data not shown). Again, pGUTS was introduced into isolated merodiploids by electroporation [72]. Deletion of the second *iglC* allele was confirmed by PCR and western blotting (data not shown). Subsequently, pGUTS was cured as was described previously [72, 63]. The resulting *iglC*-null mutant strain is referred to as *ΔiglC*.

6.2 Bacterial Growth Conditions

All *F. tularensis* isolates and mutant derivatives were maintained as permanent frozen stocks at -80°C in BHI broth supplemented with 15% glycerol. For each experiment, bacteria were recovered by streak plating onto Chocolate II agar (GC base with 1% isovitalex and 1% Hemoglobin) and incubated at 37°C for 48 hours. Since the mutant strains used are genetically stable, antibiotics were not added to the growth media prior to infections.

6.3 Cockroach Housing

The original laboratory stock of *Blaptica dubia* cockroaches was purchased from Backwater Reptiles (www.backwaterreptiles.com). Subsequent generations that were reared in the lab were used for most experiments, although we did supplement our stock with additional orders from Backwater Reptiles, as necessary. Cockroaches were stored in vented 18- or 32-gallon plastic containers and kept at 30°C

in the dark. Since *B. dubia* cockroaches do not climb vertical glass or slick plastic surfaces, no chemical or physical restrains are needed to keep the insects in the rearing containers. Pressed paper egg cartons or paper-based cat litter (www.yesterdaysnews.com) was used as a substrate in the rearing containers. Rearing containers were cleaned and cockroaches were sorted into new containers according to their developmental stage at least monthly, or more frequently as needed. Dry dog chow (Purina) was provided *ad libitum* and fresh fruits or vegetables including carrots, oranges, bananas and apples were given periodically as a source of water. After infection, groups of 4 or 5 cockroaches were kept in 100 mm Petri dishes with dry dog food and carrot slices, which were changed daily.

6.4 Bacterial Infection

Newly molted, sixth-instar cockroaches weighing 0.7-1.0 g were transfered to the challenge temperature (usually 37°C) at least seven days prior to infection in order to allow them to acclimate. We found this acclimation step to be critical–cockroaches that experienced a simultaneous temperature shift and injection trauma tended to have unpredictable deaths in control groups and dose-independent deaths in experimental groups (data not shown). All data reported here is from temperature-acclimated insects. Bacterial suspensions of approximately 10^8 colony forming units (CFU) per mL were created by suspending 3-4 colonies from a Chocolate II agar plate incubated at 37°C for 48 hours in PBS. These initial suspensions were serial diluted in PBS and aliquots (20 µl) of each dilution were delivered by intrahemoceol injection to the right of the midline at the base of the third tergum (**Figure 1D**). A 28-gauge needle-syringe combination was used for all experiments except those testing for antibiotic rescue. Those experiments used a sharpened pipette tip, made by cutting a 60° bevel into a gel-loading pipette with a razor blade prior to sterilization, for inoculation of bacteria. The rate of mortality in groups receiving 10^6 CFU of *F. tularensis* LVS by each method was not significantly different ($p>0.05$), but we have not yet compared the two methods at lower doses. Using sharpened pipette tips for bacterial delivery is advantageous because is lowers cost and increases safety compared to the use of needle and syringe. In either case, the abdomen was swabbed with 70% isopropanol prior to injection in order to lessen the risk of external contamination. For each experiment, a control group was injected with PBS to observe effects of trauma alone. Groups of 8 to 10 cockroaches were used for each experiment. Cockroaches were stored at the temperature indicated in **Table 1** and observed for survival up to 10 days post-inoculation. Cockroaches were considered dead when they displayed no response to touch.

6.5 Antibiotic Administration

Groups of 10 cockoaches were injected with *F. tularensis* LVS using sharpened gel-loading pipette tips and treated with antibiotics at 2, 48, and 96 hours post infection. Two methods of delivery were used for antibiotic administration; systemic injection and controlled feeding. For systemic injection, 20 µl of each antibiotic suspension was injected into the base of the third terga (abdominal plate) on the ventral side of the body, on the right side halfway between the midline and the spiracle (**Figure 6A**) using a needle and syringe combination. For the second and third injections, the left side of the same tergum and the right side of the next anterior tergum were used, respectively. For controlled feeding, antibiotics were prepared and diluted to the appropriate concentration in a sterile 50% sucrose solution. Cockroaches were placed on their back and a 10 µl aliquot of the sucrose solution containing antibiotic was slowly dispensed onto the mouth (**Figure 6B**). This resulted in the rapid consumption of the entire dose. Antibiotics were delivered at the following total doses, regardless of route: Streptomycin, 32 µg; Gentamicin, 32 µg; Doxycycline, 32 µg; Azithromycin, 100 µg; Resazurin; 11 µg.

6.6 Enumeration of bacteria in hemolymph

B. dubia roaches were inoculated with 10^6 *F. tularensis* LVS cells as described previously. Cockroaches were incubated at 37°C with access to dry dog chow and carrot slices (to prevent dehydration). Hemolymph was extracted from insects at each designated time point of 6, 12, 24, 50,

72, and 96 hours post initial infection. Two groups were used for each extraction time point. One group of cockroaches received a 16 µg dose of gentamicin 2 hours prior to hemolymph extraction, and a second group received an equal volume of sterile PBS in parallel. At the time of harvest, cockroaches were cleansed with 70% isopropyl alcohol and decapitated using sterile surgical scissors. Hemolymph was immediately drained into a 1.5 mL Eppendorf tube containing 10 µl chilled PBS with anticoagulant (0.05% N-Phenylthiourea). Tubes were weighed before and after addition of hemolymph in order to estimate the volume of hemolyph collected from each cockroach. In order to quantify the number of *F. tularensis* LVS CFU present in the hemolymph, the harvested samples were serial diluted 1:10 in PBS and aliquots of each dilution were plated on Chocolate II agar plates supplemented with ampicillin and trimethoprim. The total number of *F. tularensis* LVS per mL of hemolymph was determined based on the number of CFU observed from PBS-treated cockroaches and the number of intracellular *F. tularensis* LVS per mL of hemolymph was determined based on the number of CFU observed from gentamicin-treated cockroaches.

6.7 Statistial analysis

The R programming environment (version 3.2.1; [74]), accessed via the RStudio interactive development environment [75], was used for all comparisons. Lethal doses 50% and corresponding 95% confidence intervals were estimated by non-linear regression using the 'drc' package [40]. Differences in survival outcome between groups that received equivalent doses of *F. tularensis* with or without antibiotics were determined by log-rank (Mantel-Cox) analysis using the 'survival' package [76, 77]. For analysis of *in vivo* growth patterns of *F. tularensis* LVS, the mean, median, and interquartile range of each group was calculated using plot.ly [78].

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