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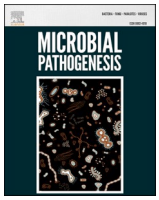

Development and evaluation of the *Galleria mellonella* (greater wax moth) infection model to study *Brucella* host-pathogen interaction

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ABSTRACT

Brucellosis is a zoonotic disease caused by Gram-negative bacteria of the genus *Brucella*. These pathogens cause long-lasting infections, a process in which *Brucella* modifications in the lipopolysaccharide (LPS) and envelope lipids reduce pathogen-associated molecular pattern (PAMP) recognition, thus hampering innate immunity activation. *In vivo* models are essential to investigate bacterial virulence, mice being the most used model. However, ethical and practical considerations impede their use in high-throughput screening studies. Although lacking the complexity of the mammalian immune system, insects share key-aspects of innate immunity with mammals, and *Galleria mellonella* has been used increasingly as a model. *G. mellonella* larvae have been shown useful in virulence analyses, including Gram-negative pathogens like *Klebsiella pneumoniae* and *Legionella pneumophila.* To assess its potential to study *Brucella* virulence, we first evaluated larva survival upon infection with representative *Brucella* species (*i.e. B. abortus* 2308W, *B. microti* CCM4915 and *B. suis* biovar 2) and mutants in the VirB type-IV secretion system (T4SS) or in the LPS-O-polysaccharide (O-PS). As compared to *K. pneumoniae*, the *Brucella* spp. tested induced a delayed and less severe mortality profile consistent with an escape of innate immunity detection. *Brucella* replication within larvae was affected by the lack of O-PS, which is reminiscent of their attenuation in natural hosts. On the contrary, replication was not affected by T4SS dysfunction and the mutant induced only slightly less mortality (not statistically significant) than its parental strain. We also evaluated *G. mellonella* to efficiently recognise *Brucella* and their LPS by quantification of the pro-phenoloxidase system and melanisation activation, using *Pseudomonas* LPS as a positive control. Among the brucellae, only *B. microti* LPS triggered an early-melanisation response consistent with the slightly increased endotoxicity of this species in mice. Therefore, *G. mellonella* represents a tool to screen for potential *Brucella* factors modulating innate immunity, but its usefulness to investigate other mechanisms relevant in *Brucella* intracellular life is limited.

1. Introduction

Bacteria of the genus *Brucella* are Gram-negative facultative intracellular coccobacilli responsible for brucellosis, a widespread zoonotic disease [1]. The genus *Brucella* includes different species that exhibit several preferential hosts: sheep and goats (*B. melitensis*), cattle (*B. abortus*) and swine (*B. suis*). Other brucellae infect marine mammals (*B. pinnipedialis* and *B. ceti*), the American woodrat (*B. neotomae*), the European common vole (*B. microti*), dogs (*B. canis*) and sheep (*B. ovis*) [2]. Recently, new species and several atypical strains (the so-called non-classical *Brucella*) have been described [3].

These brucellae exhibit modifications of PAMP (pathogen-associated molecular patterns) molecules that enable them to hamper and delay innate immunity activation. This is a key aspect of *Brucella* pathogenicity because, as a consequence, these bacteria can invade the cells of the mononuclear phagocyte system, resist intracellular killing and reach their replicative niche before activation of adaptive immunity [4]. Such PAMP modifications have been particularly documented in the case of the lipopolysaccharide (LPS), one of the main *Brucella* virulence determinants [5,6], whose structure departs markedly from that of classical Gram-negative bacteria and results in comparative low endotoxicity [6,7]. Nevertheless, there are subtle differences at this level

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among the brucellae: while the classical smooth species (*B. abortus, B. melitensis* and *B. suis*) display a marked delay in being detected by innate immunity, this feature is less conspicuous in *B. microti*, which results in a comparatively increased endotoxicity, in both cases far more reduced than that caused by bacteria carrying canonical PAMPs [8,9]. Also, these bacteria differ in growth rates, with *B. microti* multiplying faster than the classical smooth species. In addition, the brucellae possess a type IV secretion system (T4SS), named VirB, that translocates bacterial factors to the cytoplasm of the host cell [10–12], subverting cellular pathways and host immune response [12–14].

Whereas natural hosts are optimal to study *Brucella* pathogenicity and virulence, their use is restricted due to their size, containment infrastructure, maintenance costs and ethical aspects, and does not allow a refined analysis of the interaction of brucellae with the multiple components of mammalian immune system. Outbred and inbred mice of several genetic backgrounds remain as the main animal model for these purposes [15]. However, this model is not exempt of costs, which together with ethical concerns have highlighted the need to explore alternatives [16]. Invertebrates share with mammals central aspects of innate immunity [17,18] and, although *Drosophila melanogaster* and *Caenorhabditis elegans* have been used, *Galleria mellonella*, also known as greater wax moth, has emerged as another invertebrate model. *G. mellonella* is a honeybee parasitic species of *Lepidoptera* that has been widely studied due to its behaviour as a pest [19]. The advantages of this model include purchasing and maintenance costs, no current ethical concerns, the ability to be tested at 37 °C (the body temperature of mammals) and possibility to study innate immunity, especially relevant in the case of *Brucella*. *G. mellonella* has been investigated as a model for *Klebsiella pneumoniae* [20] and a variety of intracellular pathogens such as *Legionella pneumophila* [21,22], several Mycobacteria [23–25] and *Burkholderia* [26], or the fungus *Cryptococcus neoformans* [27]. Also, Sprynski et al. described some protocols to study several pathogens, including *B. melitensis* and *B. suis* (biovar 1), but the report does not include results suitable to assess the validity of the model [28].

The insect-type immunity of *G. mellonella* involves cellular and humoral innate defences [29]. Haemocytes are insect specialised immune cells comprising several types located in the haemolymph, the fluid filling the internal cavity of *G. mellonella* larvae where the organs are located. Eight different types of haemocytes have been described, five of which are found in *G. mellonella* [30]: prohaemocytes, plasmatocytes, granular cells, spherulocytes and oenocytoids/coagulocytes [31,32]. Cellular immunity of insects comprises phagocytosis, shown to be very similar to that on mammals, and nodulation or encapsulation. Humoral immunity involves melanisation, coagulation and synthesis of antimicrobial peptides (AMPs). Melanisation consists in the synthesis and deposition of melanin, a series of pigmented phenolic-derived biopolymers, that work as a key defence in insects, whose synthesis is carried out by the phenoloxidase (PO) enzyme secreted by granulocytes upon pathogen recognition [33]. Through this process, different cytotoxic and opsonizing compounds are generated. As a result, melanisation performs a direct antimicrobial effect and triggers the rest of *G. mellonella* immune repertoire.

In this work, we evaluated the *G. mellonella* model to study *Brucella*. We investigated the ability of different *Brucella* species and attenuated mutants to induce larval death, to multiply intracellularly within haemocytes and to activate melanisation in comparison with *Klebsiella pneumoniae*, a pathogen previously studied in the model.

2. Material and methods

2.1. Bacterial strains and culture conditions

The different bacterial strains and LPS used in this study are listed in Table 1. All bacteria were grown on TSA (Tryptic Soy Broth, Scharlau; European Bacteriological Agar, Condalab) or BAB2 (Blood Agar Base No. 2, OXOID) plates at 37 °C and supplemented, when needed, with 50 μg/mL kanamycin and/or 25 μg/mL nalidixic acid and/or 5% sucrose. All strains were stored at −80 °C in skim milk (Scharlau) or TYSB-DMSO (TSB supplemented with 0.5% yeast extract [Pronadisa, Condalab] and 7% dimethyl sulphoxide [VWR]).

Table 1
Bacterial strains and LPS employed.

	Characteristics	Source or reference
Strains		
B. suis bv2	*Brucella suis* biovar 2 CITA198; wild-type strain, isolated from wild boar; smooth LPS.	[34]
B. abortus 2308W	*Brucella abortus* 2308 virulent biovar 1, smooth LPS, spontaneous nalidixic acid resistant. It is virulent in mice and has been sequenced and found to differ from 2308 to 2308A.	[35,36]
B. microti CCM4915	*Brucella microti* CCM4915 wild-type strain isolated from the common vole; smooth LPS.	[37]
*Ba*Δ*per*	*B. abortus* 2308W mutant lacking a functional perosamine-synthase gene. The *per* gene is involved in the synthesis of the O-polysaccharide of the LPS.	This work
*Ba*Δ*virB*	*B. abortus* 2308W mutant lacking a functional VirB system (non-polar mutant in *virB*10).	[12]
*Ba*2308W-GFP	*Brucella abortus* 2308W expressing GFP.	This work
*Ba*Δ*virB*-GFP	*B. abortus* 2308W mutant lacking a functional VirB and expressing GFP.	[12]
K. pneumoniae 52145	*Klebsiella pneumoniae* 52145 clinical isolate (serotype O1:K2); rifampin resistant.	[20]
E. coli S17 pJQKΔ*per*	*E. coli* S17 λpir bearing pJQK derivative for *per*-gene deletion containing kanamycin-resistance and SacB cassettes.	[38]
E. coli S17 pJC43	*E. coli* S17 λpir bearing pBBR1MCS-2 derivative expressing the *gfp-mut3* gene under the control of the lac promoter and containing a kanamycin-resistance cassette.	[39]
LPS		
*Ps*LPS	LPS extracted from *Pseudomonas aeruginosa* PAO1 by the phenol-water protocol.	UNAV collection
*Bm*LPS	LPS extracted from *B. melitensis* 16M by a modified phenol-water protocol [40].	UNAV collection
*Ba*LPS	LPS extracted from *B. abortus* 2308W by a modified phenol-water protocol [40].	UNAV collection
*Bmi*LPS	LPS extracted from *B. microti* CCM4915 by a modified phenol-water protocol [40].	UNAV collection

2.2. Generation of *B. abortus 2308W per* non-polar mutant and *B. abortus 2308W* expressing GFP

A *B. abortus* 2308W *per* non-polar mutant was generated using previously described methodology [41]. Briefly, *E. coli* S17 λpir pJQKΔ*per* (**Table 1**) was conjugated with *B. abortus* 2308W, the first recombination event was selected by nalidixic acid and kanamycin resistance, and the second recombination by nalidixic acid and sucrose resistance, and kanamycin sensitivity. Deletion of *per* was confirmed by PCR. The mutation resulted in the loss of codons 133–354, which represents the 60% of *per* ORF, and the mutant strain was called *Ba*Δ*per*. The expected LPS O-polysaccharide (O-PS) defect of *Ba*Δ*per* was confirmed by SDS-PAGE and Western Blot. A GFP-expressing *B. abortus* 2308W strain was generated by conjugation of *E. coli* S17 λpir pJC43 (**Table 1**) with *B. abortus* 2308W. Exconjugants bearing the GFP-expression plasmid were selected by nalidixic acid and kanamycin resistance.

2.3. G. mellonella larvae inoculation and survival assessment

G. mellonella larvae TruLarv™ were obtained from BioSystems Technology (Exeter, United Kingdom) or from animal feeding companies, as these animals are typically used as reptile food (see Results). Larvae were kept at 15 °C under starvation in the dark prior to use. For

the experiments, larvae with homogeneous parameters of length (2–3 cm) and weight (180–250 mg) were selected.

Bacterial inocula were prepared from bacteria grown on TSA (*K. pneumoniae* and *B. abortus*) or BAB2 (*B. suis* and *B. microti*) plates in sterile PBS (0.14 M NaCl, 0.0027 M KCl and 0.010 M Phosphate buffer pH 7.4; Medicago). Bacterial concentrations were adjusted to an optical density at 600 nm (OD_{600}) of 0.25 for *Brucella* and 1.00 for *Klebsiella*, equivalent to approximately 1×10^9 CFU/mL. Finally, serial 10-fold dilutions were obtained and the exact doses were assessed retrospectively by plating.

For larva inoculation, a Hamilton syringe (25 μL Microliter Syringe Model 802 N, Cemented Needle, 22s gauge, 2 in, point style 2; Hamilton) was disinfected by taking and ejecting 70% ethanol several times and cleaned with sterile PBS. Then, larvae were injected with 10 μL of inoculum at the level of the posterior second pro-leg. Once injected, each experimental group (n = 10) was placed in a Petri dish containing a circular filter paper, incubated at 37 °C in the dark and larva survival was monitored daily by the observation of no movement of the larvae when rolled over.

2.4. G. mellonella homogenisation and CFU counts

After immersing in 70% ethanol for surface disinfection, the larvae were homogenised in a PTU (phenylthiourea; Sigma-Aldrich, P7629)-saturated PBS solution with 0.1% Triton-100X (Sigma, T8787) with an organ dissociator (gentleMACS Dissociator, MACS Miltenyi Biotec). PTU has routinely been used as an inhibitor of insect haemolymph melanisation [20]. The homogenates were 10-fold diluted and plated on selective media, CITA and Farrell's for brucellae [42,43], and TSA supplemented with rifampin (50 μg/mL) for *K. pneumoniae* 52145, which is resistant to this antibiotic [20].

2.5. Extraction and ex vivo infection of G. mellonella haemocytes and fluorescence processing

Haemocytes extraction was performed by a modification of the protocol from Senior et al., 2020 [44]. Larvae were placed in ice for 5–10 min, immersed in 70% ethanol for surface disinfection and placed in a Petri dish to make a fine cuticular incision with a sterile surgical blade at the level of the two posterior segments to avoid damaging the gut and contamination of the sample. The haemolymph was collected with a 200 μL pipette and 50 μL of each sample were 10-fold diluted in ice-cold Grace's Insect Medium (Gibco, 11605045) supplemented with 10% foetal bovine serum (FBS; Gibco, 10270), Pen-Strep solution (100 U/mL penicillin, 100 μg/mL streptomycin; Gibco, 15140122), 2.5 μg/mL amphotericin B (Sigma, A4888), and 16% PTU-saturated PBS solution. The diluted samples (approximately 1–3 x 10^6 haemocytes/mL) were plated on a 24-well cell culture cluster (Corning, Costar) containing a 12 mm diameter glass coverslip (VWR, 631–1577), and maintained at 37 °C with a 5% CO_2 atmosphere overnight prior to infection.

Infections were performed as described elsewhere [45,46]. Briefly, cells were infected at an approximate multiplicity of infection (MOI) of 100. After a centrifugation step at 400×*g* for 10 min at 4 °C, cells were incubated for 30 min at 37 °C with 5% CO_2. Then, cells were washed three times with fresh Grace's Insect Medium to remove extracellular bacteria and incubated for 1 h with medium without penicillin-streptomycin and amphotericin B but supplemented with 100 μg/mL of gentamicin. After this, the cells were maintained with culture medium containing 25 μg/mL of gentamicin.

At 2 and 48 h after infection, cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS, no Ca, no Mg, 14190-240), fixed for 30 min at room temperature with 3.7% paraformaldehyde (Merck, 818715) in 0.2 M HEPES at pH 7.4 and washed again three times with DPBS. Permeabilisation of cells was performed by incubation with 0.1% Triton X-100 (Sigma, T8787) in DPBS for 5 min at room temperature followed by a wash with DPBS. Cover glasses were then incubated with TRITC-phalloidin (1 μg/mL in PBS) for 30 min at room temperature and washed again three times with PBS. Finally, coverslips were placed on microscope slides using a DAPI-based mounting solution (DAPI I Counterstain 1000 ng/mL, Abbott Molecular).

Fluorescence imaging was carried out by a LED epi-fluorescence Axiolab 5 microscope (Carl Zeiss, 430037-9021-000) and images were processed with the program Zen 3.5 (blue edition) (Carl Zeiss Microscopy GmbH, 2011).

2.6. ProPO system activation assessment by spectrophotometry

G. mellonella haemolymph was extracted at 1-, 3- and 6-h post-infection as described above and 10-fold diluted in PBS. Afterwards, samples were centrifuged (Centrifuge 5415 R, Eppendorf) at 15,700×*g*, at 4 °C for 5 min to remove cells and debris and the absorbance of the supernatant was determined at 400 nm with a Genesis 20 (Thermo Scientific) spectrophotometer.

2.7. Statistical analysis

Statistical analyses were done with the GraphPad Prism (version 8) software. Kaplan Meyer survival curves significance was analysed by the Log-rank Mantel-Cox test. For CFU counts and PO activity assessment, the Kolmogorov-Smirnov test was applied to assess the normal distribution of data. Accordingly, statistical comparisons were made by non-parametric Kruskal-Wallis test or by parametric unpaired Student's *t*-test or one-way ANOVA and Dunnett's multiple comparison post-hoc test. All values are expressed as mean ± standard deviation (S.D.).

3. Results

3.1. Brucella induces a low and delayed mortality profile in G. mellonella model

For the evaluation and setting up of *G. mellonella* model to study *Brucella* virulence, we initially used larvae purchased from reptile feeding companies. However, we obtained highly variable experimental outputs. For this reason, we ruled out this type of larvae and used only research grade larvae in the experiments presented in this work.

G. mellonella larva survival is considered a good indicator of virulence for different pathogens [20,22,26,47–50]. Thus, we first compared the survival of larvae infected with *K. pneumoniae* 52145 (from 10^3 to 10^6 CFU/larva), a bacterium with a canonical LPS lipid A readily detected by innate immunity, and *B. abortus* 2308W (10^3–10^6 CFU/-larva), a typical zoonotic *Brucella*. Although a time- and dose-dependent effect on larva survival was observed in both cases, the mortality induced by *K. pneumoniae* was noticeably higher than that induced by *B. abortus* 2308W (Fig. 1A and B). In fact, a considerable larva mortality was already noticed at the first day of infection for *K. pneumoniae*, with an 80% mortality for the highest dose (Fig. 1A). In contrast, the mortality caused by *B. abortus* 2308W was markedly delayed and lower, with only one death recorded on the first day of infection in the group inoculated with the highest dose (Fig. 1B). In fact, statistical differences with the PBS-control were obtained for the *K. pneumoniae* dose of 10^3 CFU/larva ($p = 0.0039$), whereas only the *B. abortus* 2308W dose of 10^6 CFU/larva showed statistical differences ($p = 0.0291$). Similar results were obtained with the non-zoonotic *B. suis* bv2 representative strain **(Supplemental Fig. 1A).**

Then, we evaluated larva survival upon infection with mutants in known key virulence factors of *Brucella*: *BaΔper* (defective in the LPS O-PS) and *BaΔvirB* (defective in T4SS). The attenuation of *Brucella* O-PS defective mutants has been known for decades and it relates to changes in the bacterial surface that affect the interaction with the cells and soluble effectors of the immune system [51,52]. VirB-T4SS mutants are also known to be strongly attenuated in different models and in the

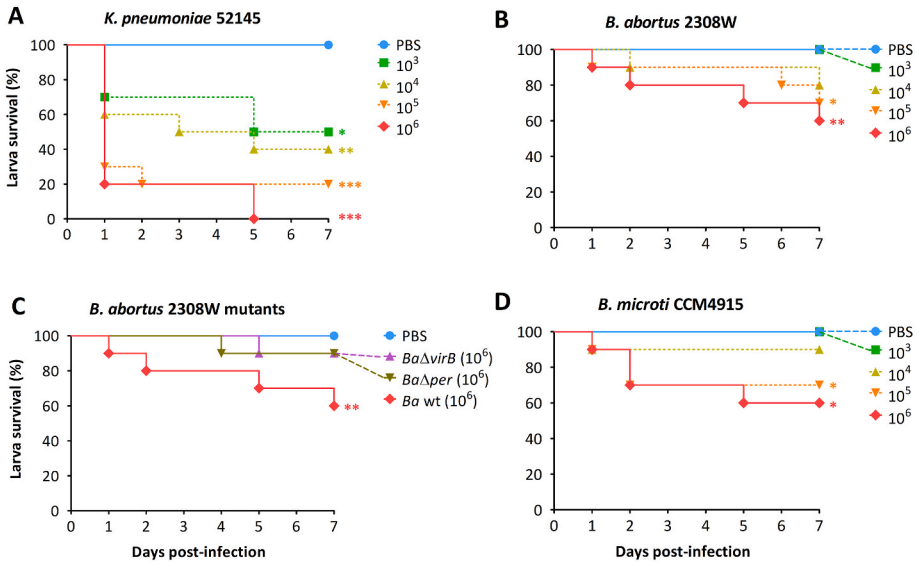


Fig. 1. *Brucella* induce a low and delayed *G. mellonella* mortality. Larvae (n = 10) were inoculated with doses ranging from 10^3-10^6 CFU/larva of *K. pneumoniae* 52145 (A), *B. abortus* 2308W (B), Δ*virB10* and Δ*per B. abortus* 2308W mutants (C) and *B. microti* CCM4915 (D); as well as a PBS-injected control. Kaplan Meyer survival curves significance was analysed by the Log-rank Mantel-Cox test; differences between values of each sample *versus* PBS were considered significant at $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.0005$ (***).

natural host [53], as this protein complex is instrumental in *Brucella* intracellular-trafficking-hijack to reach their intracellular replicative niche [12,54]. Since it was the *B. abortus* 2308W dose showing a significant effect, we selected 10^6 CFU/larva for these experiments. Despite any mutant showed statistical differences with the wild-type parental strain, neither any of them showed significant differences with PBS, with only one death (*i.e.,* 90% survival) at 4- and 5-days after infection for *BaΔvirB* and *BaΔper*, respectively (Fig. 1C). Finally, we expanded our work to *B. microti* CCM4915, a *Brucella* species that exhibits increased growth rate in macrophages and lethality in mice in comparison with *B. abortus* and other classical smooth *Brucella* species [8]. *B. microti* CCM4915 showed a larva mortality similar to that of *B. abortus* 2308W, with a 60% survival at term (Fig. 1D) and significant differences ($p = 0.0291$) at 10^6 CFU/larva with PBS control.

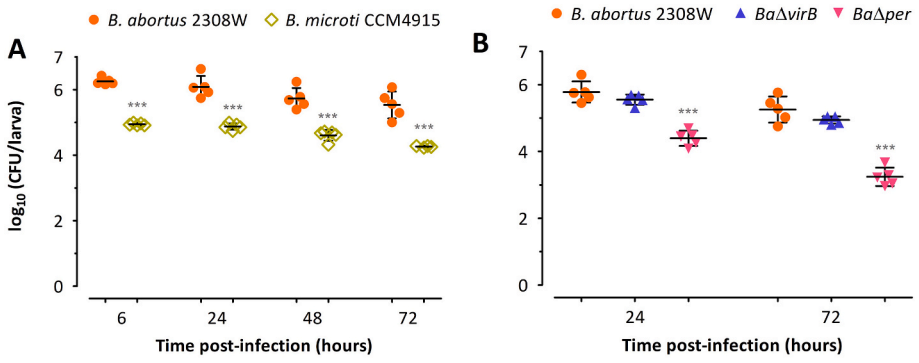
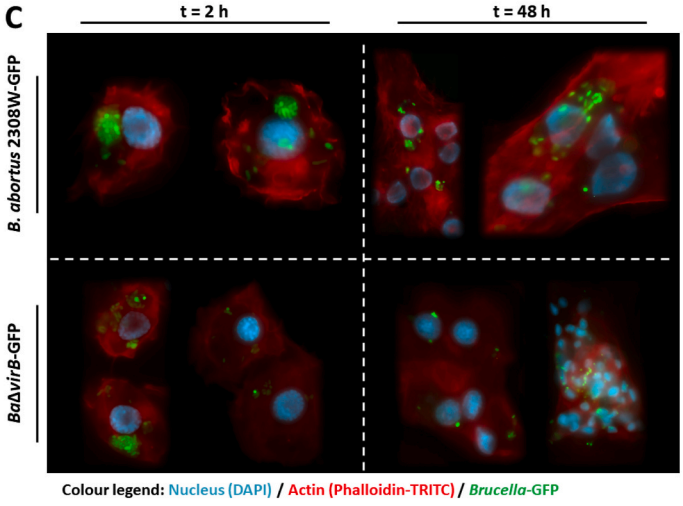


Fig. 2. Brucellae develop a persistent infection dependent on an effective early immune-recognition evasion. Larvae (n = 5) were inoculated with 10^6 CFU/larva of *B. abortus* 2308W and *B. microti* CCM4915 (A) (left panel), and *BaΔvirB* and *BaΔper* (B). Larvae were homogenised at given time-points and bacterial load assessed by CFU counting. Results are expressed as mean ± standard deviation and differences between values of each sample *versus B. abortus* 2308W were considered significant at $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.0005$ (***). (C) Primary isolated *G. mellonella* haemocytes were infected with *B. abortus* 2308W-GFP and *BaΔvirB*-GFP, and intracellular bacterial load was screened at 2 and 48 h after infection.



3.2. *Brucella* is able to persist within *G. mellonella*

To assess if the low and delayed larva mortality induced by *Brucella* was associated with an ability to persist undetected or with the capability of the larvae to clear bacteria, larvae were infected with 10^6 CFU/larva of *B. abortus* 2308W, *B. suis* bv2, *B. microti* CCM4915 or *K. pneumoniae* 52145 as a control, and multiplication was assessed by CFU-counting. As reported previously [20], *K. pneumoniae* 52145 showed a time-dependent increase in bacterial load (Supplemental Fig. 2), while CFU values of *B. abortus* 2308W, *B. suis* bv2 and *B. microti* CCM4915 were almost stable (barely a half log decrease) through the 3 days of tracking (Fig. 2A and Supplemental Fig. 1A). *B. microti* CCM4915 showed an initial 1-log decline in bacterial counts at 6 h post-infection, not seen for *B. abortus* 2308W (Fig. 2A), suggesting that *B. microti* is more efficiently recognised by *Galleria* immunity. Similarly, we evaluated if the increased larva survival observed with VirB and Per mutants with respect to the parental strain was related with the clearance of bacteria. To this end, we carried out infections with 10^6 CFU/larva. The *per* defective mutant showed a remarkable drop in CFUs at 24 h post-infection, even sharper at the 72 h-time-point (Fig. 2B). On the contrary, *Ba*Δ*virB* did not exhibit such decline in bacterial load (Fig. 2B) with CFU-counts that, although lower, were parallel to those of *B. abortus* 2308W, suggesting a VirB-independent persistence.

These results made us wonder whether *Brucella* are able to persist and/or replicate intracellularly within *G. mellonella* haemocytes. Insect haemocytes include phagocyte populations, mainly plasmatocytes [55]. For this reason, we infected *G. mellonella* haemocytes with *B. abortus* 2308W-GFP and evaluated the intracellular burden. Infected cells typically exhibited large clusters of intracellular bacteria with around 20–40 bacteria at 2 h post-infection (Fig. 2C), without differences between wild-type and *Ba*Δ*virB*. At 48 h after infection, the numbers of infected cells diminished slightly and the intracellular bacteria also decreased, typically to 10–15 intracellular brucellae (Fig. 2C). In both cases, intracellular brucellae showed perinuclear location, as in mammalian cellular models [56]. Again, this phenomenon was observed both in wild-type and *Ba*Δ*virB* infected cells. Intriguingly, *Ba*Δ*virB*-GFP-infected haemocytes aggregated in a mesh of nodular structures at 48 h post-infection, suggesting an aggregative haemocyte response. Consistent with this interpretation, most *Ba*Δ*virB*-GFP-infected cells were located in these aggregates, while non-aggregated haemocytes contained none or few (<5 bacteria/cell) intracellular brucellae.

3.3. The non-canonical *Brucella* LPS does not activate the proPO system

Melanisation consists in the synthesis and deposition of melanin catalysed by PO, which is rapidly activated by pathogen recognition [21]. In the above-described survival experiments (Fig. 1A), we observed that *K. pneumoniae* 52145 infected larvae displayed an intense dose-dependent melanisation even after few hours of infection for the highest dose, while brucellae-infected larvae showed no melanisation during the whole week of survival assessment, with no visual differences with the PBS-injected control (Fig. 3A). We carried out a more precise analysis by quantification of the increase in haemolymph absorbance due to the production of melanin [20,21,27]. Because of biosafety issues regarding the haemolymph extraction procedure, we selected the virulent but non-zoonotic *B. suis* bv2 for this experiment. Infection with 10^6 CFU/larva of *K. pneumoniae* 52145 and *B. suis* bv2 showed that, while the former induced a rapid and time-dependent melanisation, *B. suis* bv2 induced no significant increase at any time tested (Fig. 3B). These results confirmed further that live *Brucella* are not efficiently recognised and do not properly activate innate immunity upon *G. mellonella* infection.

To study whether the low induction of *G. mellonella* immunity by *Brucella* was related to the modified PAMP of its LPS, we assessed PO activity after injecting larvae with 5 or 10 μg/larva of the highly endotoxic *P. aeruginosa* PAO1 LPS (*Ps*LPS), *B. abortus* 2308W LPS (*Ba*LPS), *B. melitensis* 16M (*Bm*LPS), or *B. microti* CCM4915 (*Bmi*LPS) (Table 1). While *Ps*LPS induced a significant increase in PO activity, *Ba*LPS did not, in accordance with the non-stimulatory effect of infection (Fig. 3C). Similar results were obtained with *Bm*LPS. Interestingly, *Bmi*LPS induced an increase in haemolymph absorbance (Fig. 3C), which is in agreement with the slightly increased endotoxicity of this species in mammalian models. These results showed that haemolymph absorbance quantification assay distinguished the non-canonical *Brucella* LPSs from the endotoxic LPSs commonly found in gram-negative bacteria and is

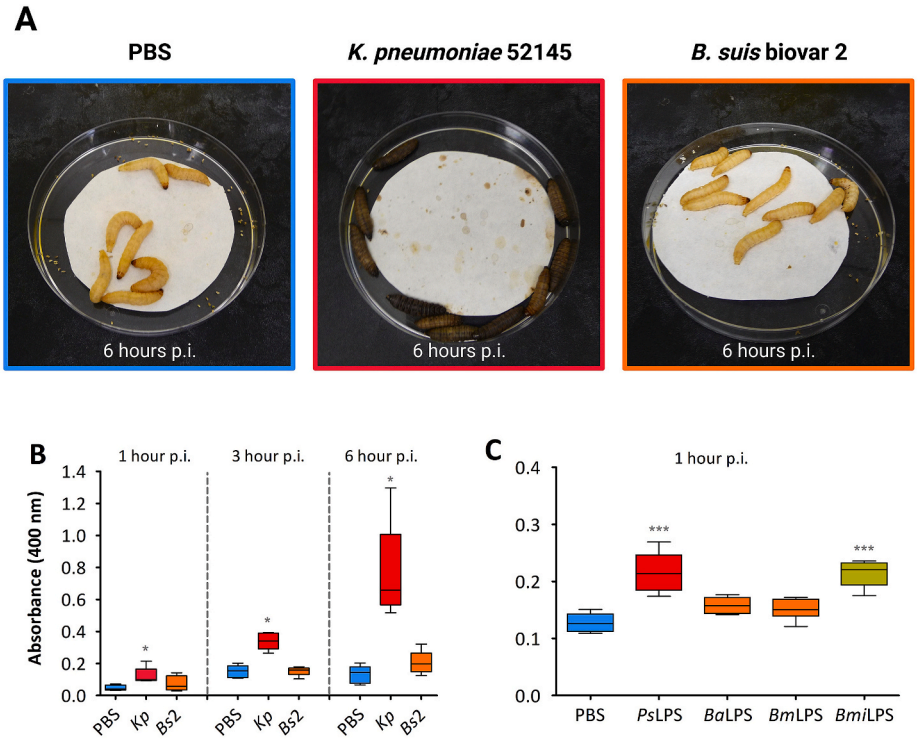


Fig. 3. The non-canonical *Brucella* LPS does not activate the proPO system. *G. mellonella* larvae (n = 5) were inoculated with live bacteria (A and B): 10^6 CFU/larva of *K. pneumoniae* 52145 (*Kp*) and *B. suis* bv2 (*Bs*2); or purified LPS (C): 5 μg/larva of LPS from *P. aeruginosa* PAO1 (*Ps*LPS) and 10 μg/larva of LPS from *B. abortus* 2308W (*Ba*LPS), *B.melitensis* 16M (*Bm*LPS) and *B. microti* CCM4915 (*Bmi*LPS); both experiments including a PBS-injected group. Larva haemolymph was extracted at 1, 3 and/or 6 h post-inoculation and their absorbance measured at 400 nm wavelength. Differences between values of each sample *versus* PBS were considered significant at $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.0005$ (***).

able to detect small differences in the endotoxicity of LPSs from different *Brucella* species.

4. Discussion

In vivo research in brucellosis has been carried out mainly in mammals, both classical laboratory models (*i.e.,* mouse and guinea pig) and natural hosts (*i.e.*, ruminants). However, the need to reduce the use of mammals in biomedical research has been in the spotlight over the last decades. This idea is integrated in the principles of the 3Rs: Replacement (direct avoidance or replacement of the use of animals), Reduction (number of animals used) and Refinement (minimisation of animal suffering and welfare improvement) [57]. Invertebrate models may be an alternative for studies regarding the interaction of *Brucella* with conserved aspects of innate immunity. Nonetheless, there is a dearth of publications employing any of the current invertebrate animal models (*i.e., C. elegans* and *D. melanogaster*). To the best of our knowledge, *D. melanogaster* has never been used as a model for *Brucella* and the nematode *C. elegans* has been used only once [58]. In *C. elegans* work, the authors referred no sickness due to *B. pinnipedialis* infection and reported that survival could not be efficiently monitored due to confinement issues. In this context, *G. mellonella* is an emerging model that may represent a robust alternative for microbiological virulence and pathogenicity studies. Due to the size and housing conditions, the larvae of this insect can be more easily and safely handled and followed than a nematode or a fly.

One of the main advantages of *G. mellonella* as a model is the possibility and ease to administer larvae with controlled doses of pathogens and evaluate their survival. Brucellae display a stealthy-infection strategy, with poor activation of early mechanisms of defence, such as the complement system, pro-inflammatory cytokines and early response of innate-immunity cells [4]. Hence, we first assessed whether this low and retarded immuno-induction could be replicated in *G. mellonella*. For this purpose, the use of healthy larvae is instrumental, and even though larvae from animal feeding companies have been used in research [25,26,50,59,60], we only obtained homogenous results with research grade larvae reared in controlled conditions. We observed that *B. abortus* 2308W showed a lower and delayed larva mortality when compared to a typical endotoxic gram-negative bacterium such as *K. pneumoniae* 52145. The high and rapid mortality observed for *K. pneumoniae* 52145, together with the high activation of the PO system and subsequent melanisation, can be related to its endotoxicity. In fact, the time-dependent levels of PO activation seen in the *K. pneumoniae* 52145 infection might be a consequence of bacterial replication and accumulative stimulation of *G. mellonella* immunity by a pathogen endowed with canonical PAMPs. Similarly, the low endotoxicity of *Brucella* is replicated in the low and retarded mortality and no significant activation of melanisation (Fig. 4).

When we evaluated larva survival and CFU counts upon infection with the *Ba*Δ*per* O-PS mutant, we observed not only a reduced larva mortality but also a 3-log decrease in bacterial load at 72 h post-infection with respect to the parental strain, a strong bacterial killing consistent with the role of *Brucella* O-PS-shielding to avoid an efficient recognition by innate immunity. However, the absence of a functional VirB, that intensely impairs the ability of *Brucella* to develop an effective intracellular pathogenesis in mammal models, resulted only in reduced

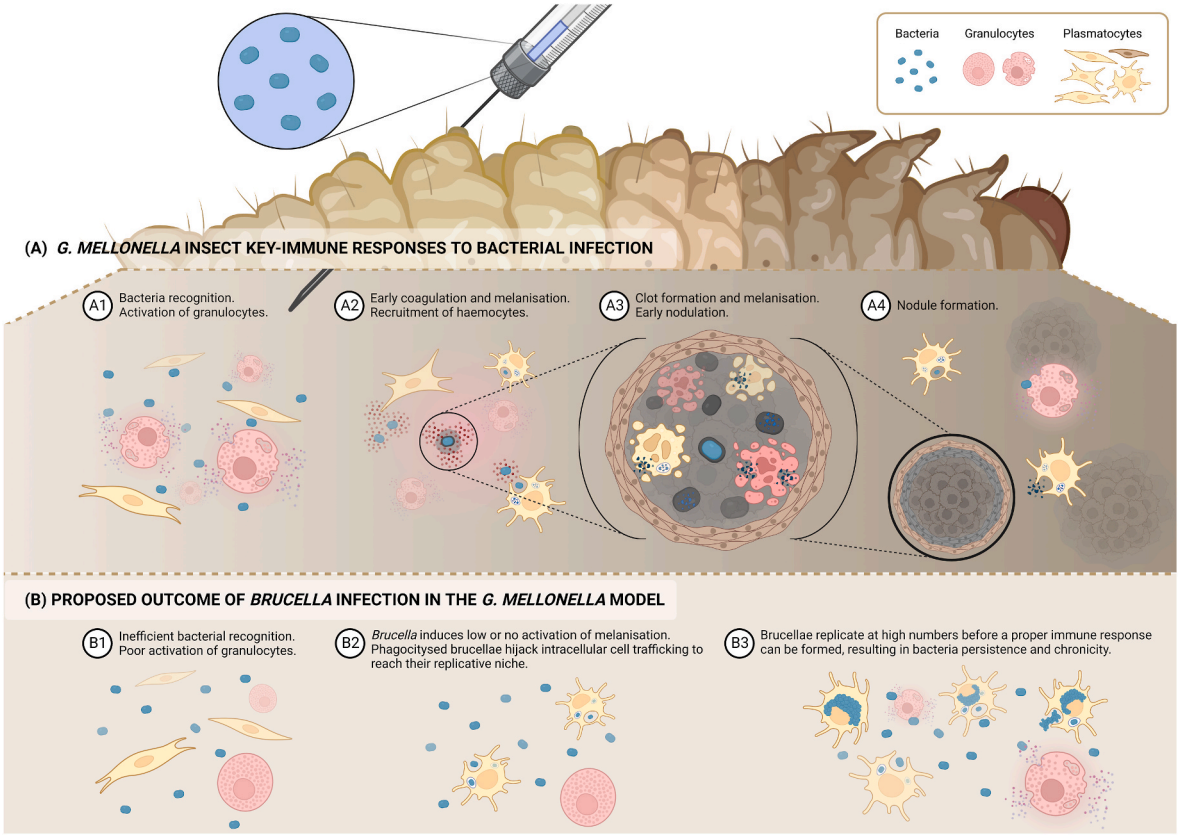


Fig. 4. Schematic representation of *G. mellonella* insect key-immune responses to bacterial infection (A) and the proposed outcome of *Brucella* infection in this model (B). Infecting bacteria are recognised by soluble pathogen recognition receptors (PRRs) that trigger granulocyte activation and secretion of a wide repertoire of antimicrobial peptides (AMPs). Phenoloxidase enzyme activates upon its release and induces the synthesis and polymerization of melanin around invading bacteria, generating cytotoxic compounds and entrapping bacteria. On top of that, plasmatocytes are attracted to the infection site and phagocytise and further decimate the bacteria. The melanised clots are finally surrounded by recruited plasmatocytes forming a nodule, further trapping bacteria and controlling the infection (Illustration created with Biorender.com).

larva mortality and a half-log decrease of CFU counts. The infection of *G. mellonella* haemocyte primary cell-cultures with *B. abortus* 2308W wild-type and the VirB mutant resulted in no differences 2- and 48-h after post-infection. This phenomenon is not anecdotal, as previous works have also described an absence of attenuation, or even a slight replication of VirB-defective brucellae in eukaryotic cell models [45]. Moreover, the observation of haemocytes aggregation following infection with the VirB mutant leaves unclear the role of T4SS in this model and it is tempting to speculate that some previously described T4SS-dependent *Brucella* effectors involved in modulating immune response (e.g. BtpA/B, VceC or BPE005) [61,62] could be responsible for the haemocyte aggregation observed for the VirB-mutant. These aspects are intriguing and warrant further investigation. Indeed, studies with *L. pneumophila*, another intracellular pathogen, have shown the involvement of T4SS in the virulence of these bacteria in *G. mellonella,* similar to that observed in mammalian macrophages [21,22,63].

We also evaluated *B. microti* CCM4915 in the *G. mellonella* model since this non-classical *Brucella* species exhibits unusual lethality in mice [8]. *B. microti* CCM4915-infected larvae exhibited a similar survival phenotype than that of the larvae infected with *B. abortus* 2308W at all the doses tested (10^3–10^6 CFU/larva). These results do not parallel those obtained in mice, in which 10^5 (Balb/c) and 10^6 CFU/mouse (CD1 or C57BL/6) are enough to induce lethality. The authors hypothesised that the slight increase in mouse mortality caused by *B. microti* might be related to an LPS-induced endotoxicity. In fact, our results in the PO activity assay support this idea because we observed a significant induction of melanisation after inoculation with *Bmi*LPS, but not with *Ba*LPS or *Bm*LPS. In line with these results, *B. microti* did show an initial drop in the CFU-counts at 6 h post-infection, suggesting a higher sensitivity of this *Brucella* species to *G. mellonella* immunity, consistent with the more-rapid clearing of *B. microti* CCM4915 by innate immunity in the mouse model.

Our results are consistent with the usefulness of *G. mellonella* model as an alternative tool for the screening of potential *Brucella* factors modulating innate immunity (Fig. 4B). However, the suitability of this model for the study of other aspects of *Brucella* pathogenesis, such as *Brucella* intracellular life including the role of *Brucella* VirB-system, remains to be investigated and might help to gain an insight into how *Brucella* alter their intracellular trafficking in eukaryotic cells. Hence, *G. mellonella* represents a novel model to expand the already available models to study *Brucella* biology (e.g. interaction with innate immunity), although findings in this model will not be exempt of further validation in other stablished mammalian models.

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CRediT authorship contribution statement

Aitor Elizalde-Bielsa: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Beatriz Aragón-Aranda:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Maite Loperena-Barber:** Investigation, Methodology, Writing – review & editing. **Miriam Salvador-Bescós:** Data curation, Investigation, Methodology, Writing – review & editing. **Ignacio Moriyón:** Conceptualization, Writing – original draft, Writing – review & editing. **Amaia Zúñiga-Ripa:** Investigation, Methodology, Writing – review & editing. **Raquel Conde-Álvarez:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2022.105930.

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