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A novel *Galleria mellonella* experimental model for zoonotic pathogen *Brucella*

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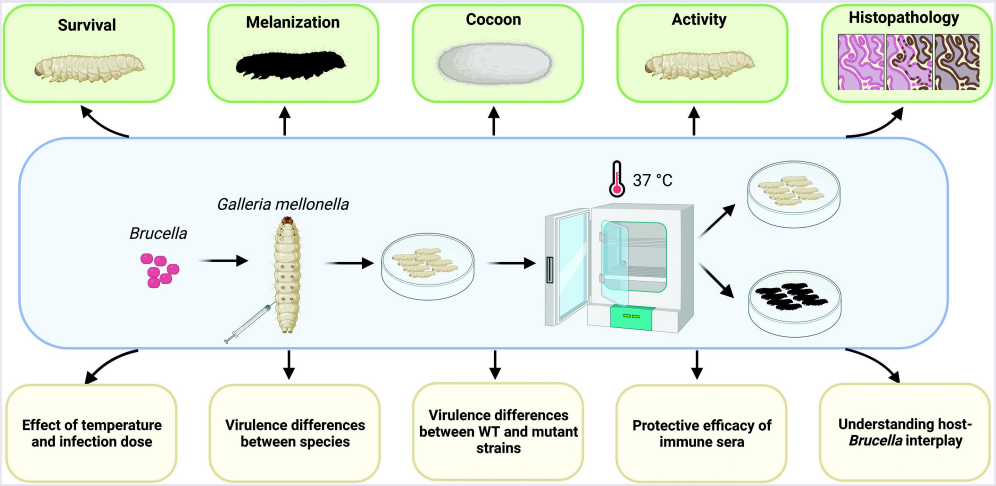
ABSTRACT

Brucellosis is a major threat to public health and animal husbandry. Several *in vivo* vertebrate models, such as mice, guinea pigs, and nonhuman primates, have been used to study *Brucella* pathogenesis, bacteria-host interactions, and vaccine efficacy. However, these models have limitations whereas the invertebrate *Galleria mellonella* model is a cost-effective and ethical alternative. The aim of the present study was to examine the invertebrate *G. mellonella* as an in vivo infection model for *Brucella*. Infection assays were employed to validate the fitness of the larval model for *Brucella* infection and virulence evaluation. The protective efficacy of immune sera was evaluated by pre-incubated with a lethal dose of bacteria before infection. The consistency between the mouse model and the larval model was confirmed by assessing the protective efficacy of two *Brucella* vaccine strains. The results show that *G. mellonella* could be infected by *Brucella* strains, in a dose- and temperature-dependent way. Moreover, this larval model can effectively evaluate the virulence of *Brucella* strains in a manner consistent with that of mammalian infection models. Importantly, this model can assess the protective efficacy of vaccine immune sera within a day. Further investigation implied that haemolymph played a crucial role in the protective efficacy of immune sera. In conclusion, *G. mellonella* could serve as a quick, efficient, and reliable model for evaluating the virulence of *Brucella* strains and efficacy of immune sera in an ethical manner.



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Galleria mellonella; Brucella; infection model; virulence evaluation; sera efficacy evaluation; rapid evaluation model

Introduction

Brucellosis is a zoonotic infectious disease caused by *Brucella* spp. It is viewed as a major threat to public health and animal husbandry, with annually about 500 thousand new reported cases worldwide, involving more than 170 countries and regions [1–5]. Vaccines are the most cost-effective means to prevent and control brucellosis. Commercial veterinary live attenuated vaccines, such as *Brucella abortus* S19/A19, *Brucella abortus* RB51, *Brucella melitensis* Rev.1, *Brucella melitensis* M5, and *Brucella Suis* S2, have played a substantial role in the prevention and control of brucellosis in livestock [6,7], but they still have deficiencies, such as residual virulence to the host, interfering conventional serological tests, and resulting infection in humans [8–12] (Table 1). Till now, the

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Table 1. Commercial *Brucella* attenuated vaccines and their virulence degree.

Vaccine name	Origin	Degree of Virulence
S19/A19	*Brucella abortus*	Cause abortion/orchitis [6,7] Virulent to humans [14,15]
RB51	*Brucella abortus*	Virulent to humans [16];
Rev.1	*Brucella. melitensis*	More virulent than S19 [15] Cause abortion in pregnant animals [17,18]; Virulent to humans [19]
M5	*Brucella. melitensis*	Virulent to humans [10]
S2	*Brucella. suis*	Less virulent than S19 and Rev-1 [20,21]
104M	*Brucella abortus*	Slightly virulent to humans [16]

Brucella abortus live attenuated vaccine 104M is the only human *Brucella* vaccine approved by the Chinese Food and Drug Administration, but it is not suitable for mass vaccination due to scratch inoculation and residual virulence [13] (Table 1). Rodents are the most commonly used animal models for *Brucella* infection, virulence comparison, and vaccine evaluation, with bacterial load and histopathological changes in the spleen defined as main indicators [6,8,22,23]. Nonetheless, the evaluation process is time-consuming, costly, requires specialized animal housing, and is ethically challenging. Therefore, the establishment of a simple and time-saving alternative *in vivo* infection and immune model of *Brucella* could not only greatly reduce the use of vertebrates but also speed up the evaluation process.

Galleria mellonella larvae, also known as wax worms, are invertebrates with a mammal-like innate immune system that consists of cellular and humoral immune responses [24,25]. In contrast to traditional models, *G. mellonella* larvae are an inexpensive and easily acquired resource that can be handled without difficulty, does not require feeding or specific caging, and thus could serve as a valuable tool for investigating microbial infections. Importantly, they can survive at 37°C, which is the optimum temperature for human pathogens. In addition, this model is ethically acceptable and does not require ethical approval. As a result, this model has been widely used in the evaluation of pathogen virulence, toxicity of chemicals, and rapid screening of novel antibacterial agents [24,26–30].

In this study, we described a *G. mellonella* larval infection and immune model for *Brucella* and validated its potential to evaluate the virulence of *Brucella* strains and the efficacy of immune sera in a fast, high-throughput, and ethical manner.

Materials and Methods

Bacterial strains and culture conditions

All strains were stored at −80°C in tryptone soya broth (TSB; Oxoid) containing 15% glycerol (SCR). Bacteria were cultured in TSB at 37°C with 220 rpm until the mid-log phase was reached. The samples were serially diluted and plated on tryptone soya agar (TSA, Oxoid) for CFU determination. *B. abortus* A19, A19Δ*VirB12* and *B. suis* S2 were obtained from Tecon Biology, and *B. abortus* 104M was obtained from Lanzhou Institute of Biological Products. The 104M:*Omp19* overexpression mutant was generated as previously described [31]. The 104MΔ*Omp19* deletion mutant was generated by amplification of the upstream and downstream flanking regions and the kanamycin resistance gene (*kanR* gene) using the primers Omp19-up-F, Omp19-up-R, Omp19-down-F, Omp19-down-R, *kanR*-F, and *kanR*-R (Additional file 1). Subsequently, the upstream, *kanR* and downstream regions of Omp19 were ligated by overlapping PCR, and the ligated product was inserted into *pEASY-Blunt* 3 vector (Transgen Biotech). The resulting vector was transformed into the genome of strain 104M by electroporation at 625 V for 10 ms and then transferred to selective TSA plates containing 100 μg/ml kanamycin..

G. mellonella *larvae*

Last-instar antibiotic-free *G. mellonella* larvae were purchased from Tianjin Huiyude Biotechnology Ltd. and were kept for one day before infection, without feeding during experimentation. Larvae with uniform colour, no markings, and high motility were deemed healthy and were used in subsequent experiments.

G. mellonella *larvae infection assay and virulence evaluation assay*

*Brucella*cultures were centrifuged for 5 min at 5 000 g. Bacteria pellets were washed once and resuspended in 1× PBS buffer (Gibco) to the desired density (5×10^4 CFU/ml to 5×10^9CFU/ml). To inactivate the bacteria, mid-log phase strains were suspended in PBS buffer (Gibco) heat inactivated at 100 °C for 10 min, or suspended in 4% paraformaldehyde (PFA, Leagene) for 1 h. Inactivity was confirmed by plating 100 μl of the suspension onto TSA plates and incubating for three

days. A 25 µl suspension of bacteria (1.25×10^3 CFU/larva to 1.25×10^8 CFU/larva) was injected into *G. mellonella* larvae through the lower left proleg using a sterile 29 G insulin syringe (BD). Control groups were left untreated or injected with 25 µl of sterile PBS (Gibco) or pooled sera from PBS-treated groups of mice (PBS sera). After infection, larvae were kept at 25°C or 37°C in a lidded 9-cm Petri-dish in the dark, monitored, and scored daily according to the "Health Index Scoring System (HISS) (Figure 1a) [10]. " for up to 5 days. The larvae were determined to be dead when they did not respond to stimulation. Pupae were excluded from the analysis.

Mouse immunization, sera collection and mouse infection

Six-to eight-week-old female BALB/c mice (Beijing Vital River Laboratory Animal Technology Co., Ltd.) were immunized subcutaneously with 1×10^5 CFU of strain 104M, strain 104MΔ*Omp19* or PBS on day 0, and infected with 1×10^8 CFU of strain A19 intraperitoneally on day 28. Sera were collected before infection, aliquoted, and stored at −80°C until use. On the 7th day post infection, the mice were euthanized, and their spleens were harvested. Mouse spleens from each group were fixed in 4% PFA, or homogenized, serially diluted, and spread on TSA plates for CFU determination.

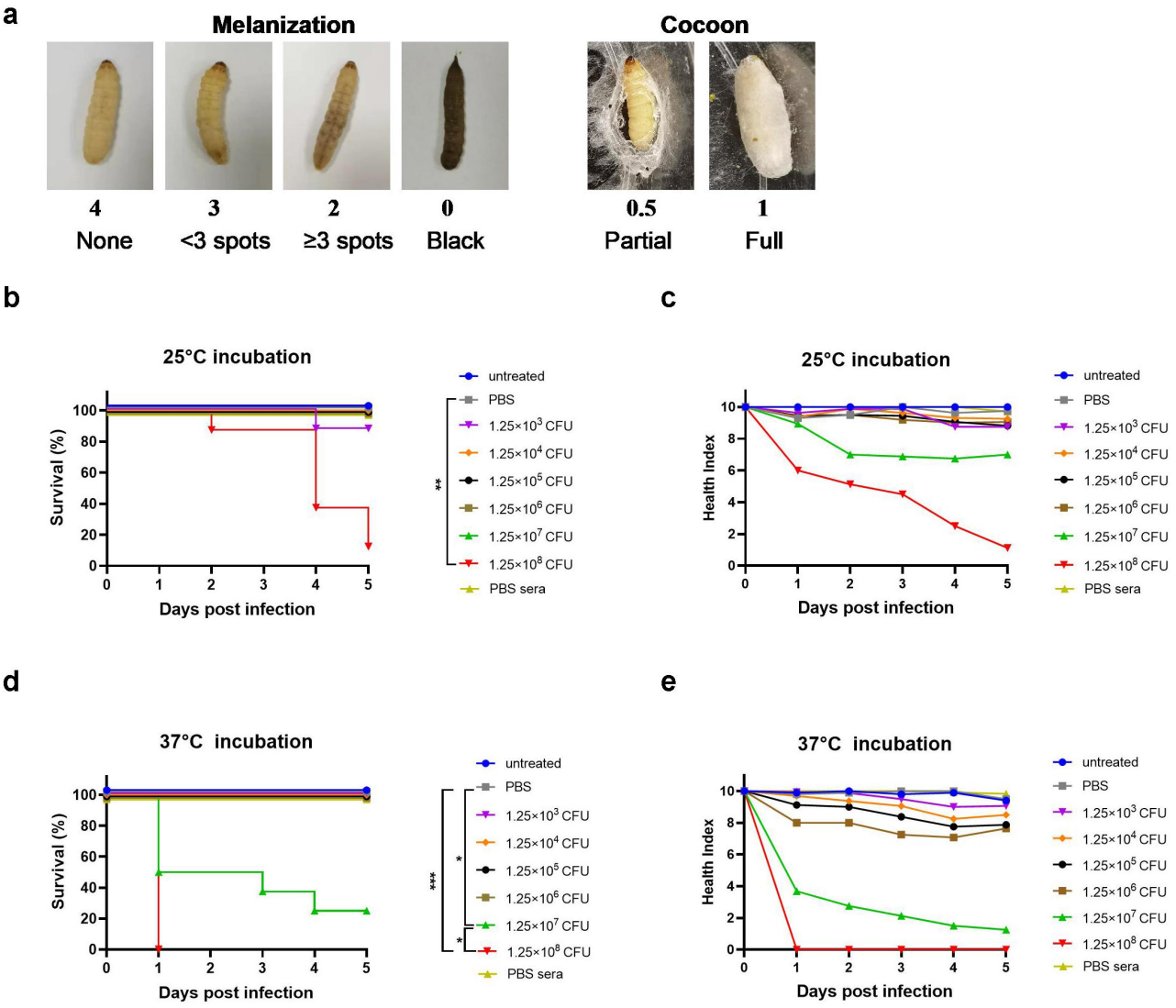

Figure 1. Establishment of the *G. mellonella* model of *Brucella* infection. *G. mellonella* larvae were injected with 25 µl bacteria suspension, incubated at 25°C or 37°C and scored daily according to the health index scoring system. a) Representative photographs of melanization scores and cocoon scores. b) Effect of different inoculum doses of *Brucella* strain A19 on *G. mellonella* larval survival at 25°C. c) Health index scores of larvae infected with different doses of *Brucella* strain A19 at 25°C. d) Effect of different inoculum doses of *Brucella* strain A19 on *G. mellonella* larval survival at 37°C. e) Health index scores of larvae infected with different doses of *Brucella* strain A19 at 37°C. Significant differences between the Kaplan-Meier curves were identified with the log-rank test (GraphPad Prism). *$P < 0.05$,**$P < 0.01$, ***$P < 0.001$.

Histopathology

Larvae were fixed in 4% PFA on day 1 and day 5 post infection, and mouse spleens were fixed on the 7th day post infection. Fixed samples were embedded and sectioned, followed by dewaxing and hydration. The tissue sections were stained using the Hematoxylin and Eosin (H&E) staining kit (Beyotime) or *Brucella* staining kit (Leagene), according to the manufacturer's instructions. Subsequently, dehydration, transparency, and neutral resin sealing were performed.

Evaluation of the protective efficacy of immune sera

B. abortus A19 was suspended in PBS buffer containing 4%-50% (v/v) pooled normal sera or heat-inactivated (56°C, 30 min) sera from mice immunized with strain 104M (hereafter referred to as 104M sera), strain 104MΔ*Omp19* (hereafter referred to as 104MΔ*Omp19* sera), or PBS control (hereafter abbreviated as PBS sera) and incubated at 37°C for 30, 45, or 60 min, evenly mixed every 10 min. Larvae were injected with a 25 µl mixture containing 1.25×10^8 CFU of bacteria, incubated at 37°C and scored daily according to HISS.

Depletion of G.mellonella hemocytes

Dichloromethylene-bisphosphonate (clodronate) has been reported to deplete the haemocytes of *G. mellonella* and *Anopheles gambiae* mosquitos [30,32]. Liposomes containing 5 mg/ml clodronate (CDLip) and control liposomes (Lip) in PBS were purchased from Liposoma Research and used in the assays. *G. mellonella* larvae were injected with 5 µL of Lip or CDLip in the last left proleg. After 24 h, the larvae were injected with 20 µL of *Brucella* A19 suspension or A19-sera pre-incubated mixture containing 1.25×10^8 CFU of bacteria in the last right proleg, and larval survival was monitored over a 5-day period.

Effect of priming G.mellonella with a non-lethal dose of Brucella

G. mellonella larvae were injected in the last left proleg with 5 µL of bacterial suspension containing 1.25×10^5 CFU of live or fixed *Brucell*a strains. After 24 h, a lethal dose of 10 µL of *Brucella* A19 was used to infect the larvae in the last right proleg. Larval survival and health status were evaluated for 5 days following infection.

Statistical analyses

Data were analysed for statistical significance using GraphPad Prism Version 9. Significant differences between the Kaplan-Meier curves were identified with the log-rank test (GraphPad Prism). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Significant differences in the bacterial load of mouse spleens were identified by one-way ANOVA (GraphPad Prism). $^*P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Results

G. mellonella larval survival was Brucella dose-dependent and temperature-dependent

First, we determined the virulence of *B.abortus* A19 to *G. mellonella* larvae at 25°C and 37°C using 37°C cultured strain A19, which were the optimum survival temperatures for larvae and bacteria, respectively. To begin with, larvae were injected with a series of doses of strain A19 at 25°C to verify whether *Brucella* could establish infection in *G. mellonella* larvae. Untreated larvae and larvae injected with PBS buffer both showed 100% survival when incubated at this temperature over a 5-day period, demonstrating that neither needle injection nor PBS buffer had any influence on larval survival. However, larvae infected with 1.25×10^8 CFU of bacteria showed significantly decreased survival ($P < 0.01$), with less than 20% survival on day 5; however, at lower infection doses, larvae showed almost 100% survival (Figure 1b). We also employed a health index scoring system to assess the health status of larvae, which enabled the observation of general health status and slight differences in larval activity, cocoon formation, melanization, and survival (Figure 1a) [24]. The daily health index scores of larvae infected with 1.25×10^8 CFU dropped with time, eventually falling below 2 on day 5. The daily scores of larvae infected with 1.25×10^7 CFU were always above 6, although they decreased gradually. On the contrary, the health index scores of groups treated with doses lower that 1.25×10^7 CFU were always above 8 (Figure 1c).

We subsequently conducted the larval infection assay at 37°C, as this was the optimal growth temperature for *Brucella*. As expected, both untreated larvae and larvae injected with PBS buffer also exhibited 100% survival at 37°C. Furthermore, larval survival was also dose-dependent at this temperature, with higher concentrations correlating with reduced survival. Larvae infected with less than 1.25×10^7 CFU of strain A19 all survived, while at higher infection doses, a concentration-dependent pattern on both survival

and health index scores was observed. All larvae died within 1 day and typically scored 0 or 2 when infected with 1.25×10^8 CFU of strain A19, whereas 50% mortality was reached on day 1 with 1.25×10^7 CFU of bacteria (Figure 1d,e). Similar results were obtained using 25°C cultured strain A19 (Fig. S1).

Overall, larval survival depends on both the infection dose of *Brucella* and the post infection incubation temperature. Consequently, a standard inoculum of 1.25×10^8 CFU/larva and a standard temperature of 37°C were determined for all subsequent experiments, which killed all larvae consistently and reproducibly within a day.

G. mellonella *developed Granuloma-like structures in response to non-lethal* Brucella *infection*

To examine histopathological changes, larvae were infected with 1.25×10^5 CFU or 1.25×10^8 CFU of A19 and processed at 1-day and 5-day post infection for H&E staining or Koster's staining. Larvae treated with the same volume of PBS were used as controls. Compared to the PBS control (Figure 2a), larvae infected with a non-lethal dose of A19 on day 1 and day 5 showed granuloma-like structures formed by the aggregation of haemocytes with melanin deposition, but no evidence of severe damage to the adipose body or total tissue structure (Figure 2b,c). Conversely, larvae infected with a lethal dose of A19 on day 1 showed extensive tissue destruction with severely disseminated basophils (Figure 2d).

To further confirm the distribution of bacteria, Koster's staining was performed. *Brucella* strains were stained magenta using this specific staining method, while non-*Brucella* strains and tissue structures were stained green. No *Brucella*-positive components were detected in the sections of the PBS control group (Figure 2e), whereas *Brucella*-positive elements

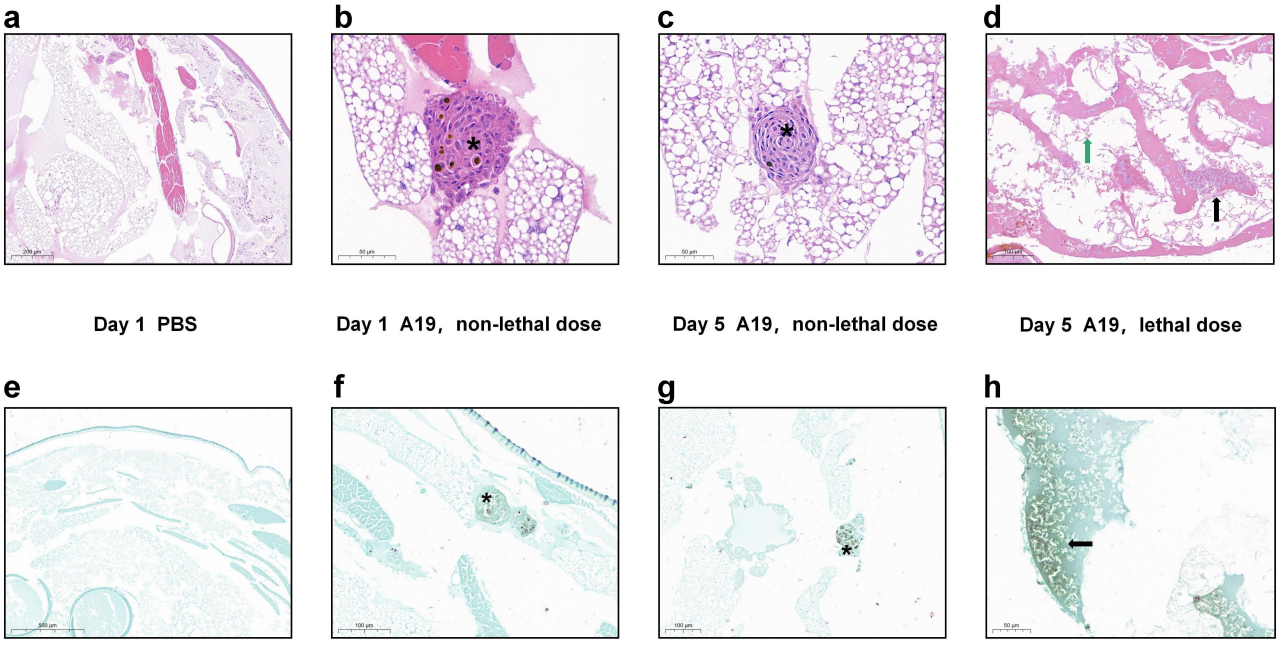

Figure 2. Histopathology examination of *G. mellonella* infected with *Brucella* strain A19. Larvae were infected with *Brucella* strain A19 (1.25×10^5 or 1.25×10^8 CFU) and histopathology were examined at 1- and 5-day post infection on fixed larval sections following H&E staining or Koster's staining. a) PBS control, H&E staining larval section on day 1. Normal histological structures, no haemocyte reaction or melanization was observed. b) Infected, non-lethal dose (1.25×10^5 CFU), H&E staining larval section on day 1. Granuloma-like structures formed by haemolymph were observed. Asterisks represented the granuloma-like structures formed by the aggregation of haemocytes. c) Infected, non-lethal dose (1.25×10^5 CFU), H&E staining larval section on day 5. d) Infected, lethal dose (1.25×10^8 CFU), H&E staining larval section on day 1. Extensive destruction of the adipose body with scattered melanin were observed. The black arrow represented disseminated *Brucella*. The green arrow represented damaged adipose body. e) PBS control, Koster's staining larval section on day 1. No bacteria were observed in the section. f) Infected, non-lethal dose (1.25×10^5 CFU), Koster's staining larval section on day 1. Granuloma-like structure containing individual *Brucella* or colonies of *Brucella* were observed. g) Infected, non-lethal dose (1.25×10^5 CFU), Koster's staining larval section on day 5. *Brucella*-positive components were observed within the nodules of haemocytes. h) Infected, lethal dose (1.25×10^8 CFU), Koster's staining larval section on day 1. Large numbers of *Brucella* were distributed in the haemocoel cavity.

were observed within the nodules of haemocytes in the sections of larvae infected with the non-lethal dose of strain A19 (Figure 2f,g). Moreover, in the sections of larvae infected with the lethal dose, large numbers of individual bacteria were stained magenta, indicating severe disseminated infection in this group (Figure 2h).

G. mellonella *as a model for rapid evaluation of* Brucella *strain virulence*

To further investigate whether killing *G. mellonella* requires live bacteria, *G. mellonella* larvae were injected with a dose of 1.25×10^8 CFU of live strain A19, live strain 104M, or with the same dose of heat-inactivated bacteria. Larvae infected with both live strains died by day 1, whereas those infected with heat-inactivated strains survived at all time points (Figure 3a), with average health score of 8.1 (A19-HI) and 8.4 (104M-HI) on day 5 (Figure 3b), indicating that only live strains had a significant effect on larval survival at this infection dose.

We subsequently evaluated the presence of virulence factors on larval survival by inoculating larvae with 1.25×10^8 CFU of different *Brucella* strains or their deletion/over-expression mutants. *Brucella abortus* A19 and A19Δ*VirB12* are both commercially available live vaccines that show similar virulence in mice [33]. Also, we generated the deletion mutant and overexpression mutant of strain 104M, as recent evidence suggests that Omp19 is a crucial virulence factor of *Brucella* [34–36]. Consistent with a previous report in the mouse model, strain A19 and strain A19Δ*VirB12* showed comparable pathogenicity in the larval model. As anticipated, larvae infected with strain 104MΔ*Omp19* showed prolonged survival compared to the parental strain, with all larvae surviving on day 1 (Figure 3c). However, there was no significant difference in the survival rate and health index scores between the 104M-infected group and the 104M:*Omp19*-infected group (Figure 3c,d), implying that overexpression of the virulence factor Omp19 did not increase the virulence of the strain. Finally, we compared the differences in virulence between *Brucella* species. Strain S2 showed lower virulence in the larval model than strain A19 and strain 104M, which is consistent with previous reports [37–39]. Taken

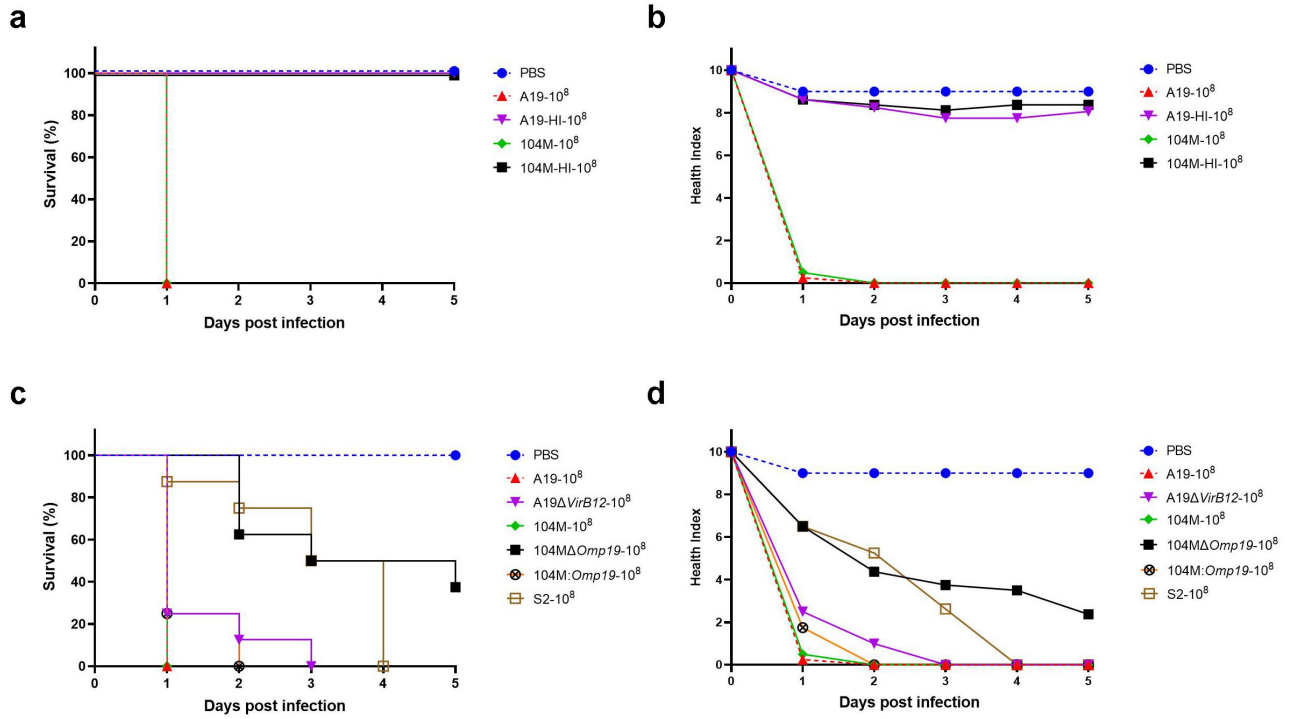

Figure 3. *G. mellonella* as a model for rapid evaluation of *Brucella* strain virulence. Groups of larvae were infected with 1.25×10^8 CFU of live or heat-inactivated *Brucella* strain A19, strain 104M, strain 104MΔ*Omp19* deletion mutant, strain 104M:*Omp19* over-expression mutant or strain S2, incubated at 37°C and monitored for 5 days. a) Kaplan-Meier curves representing daily percentage of survived larvae infected with live or heat-inactivated *Brucella* strains. b) Daily health index scores of larvae infected with live or heat-inactivated *Brucella* strains. c) Kaplan-Meier curves representing daily percentage of survived larvae infected with *Brucella* strains. d) Daily health index scores of larvae infected with *Brucella* strains.

together, these data indicate that the *G. mellonella* model is suitable for the rapid virulence evaluation of *Brucella* strains.

G. mellonella *as a model for rapid evaluation of* Brucella *vaccine immune sera efficacy*

G. mellonella larvae have been used for the evaluation of monoclonal antibodies [40,41]. We therefore made an effort to develop the *G. mellonella* larvae as a rapid evaluation model for *Brucella* vaccine immune sera. To begin with, we determined whether the serum components affected the survival of *G. mellonella* larvae. PBS sera had no effect on larval survival (Figure 4a), indicating that serum components were not toxic to *G. mellonella* larvae. Likewise, the health index scores of the larvae injected with PBS sera were always above 9, proving that the larvae were healthy (Figure 4b).

Next, as a proof of concept, we applied this model to examine the efficacy of 104M sera, since 104M live attenuated vaccine has already been proven to be highly protective against brucellosis [13,42]. To study the inhibitory effect of the antibody, strain A19 was pre-treated with pooled sera before inoculation. To optimize the pre-incubation time, bacteria were suspended in PBS buffer containing 50% (v/v) 104M immune sera or PBS sera and incubated at 37°C for 30, 45, or 60 min. Larvae were then inoculated with a 25 µl mixture containing 1.25×10^8 CFU of bacteria. It is not surprising that PBS sera had no protective effect on the larvae, regardless of the pre-incubation time. Contrastingly, 104M sera showed an excellent protective effect. The survival rates of groups pre-incubated with 104M sera for 30, 45, or 60 min were 62.5%, 75%, and 87.5% on day 1, respectively (Figure 4a). The protective efficacy of the 60-minute pre-incubated group outperformed the other

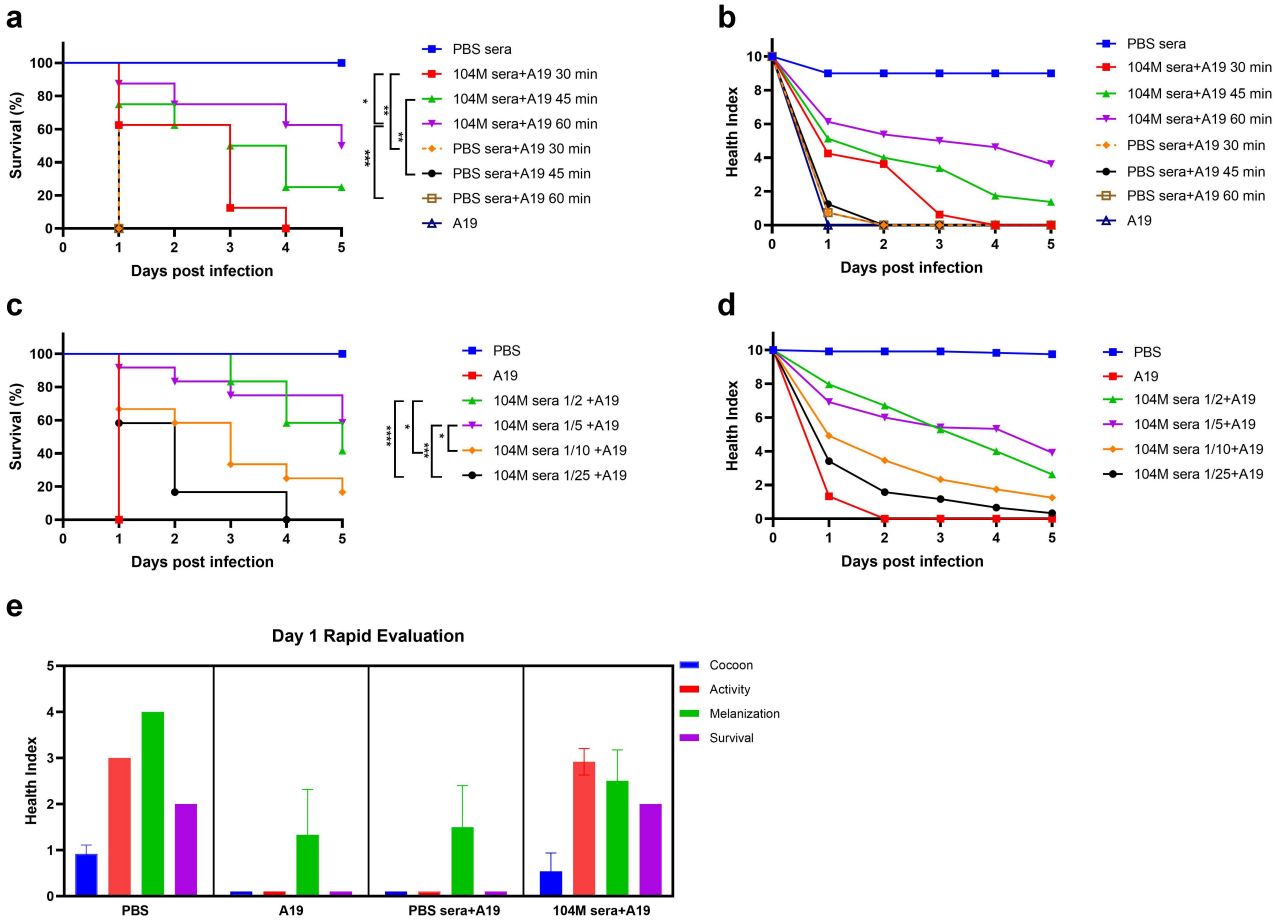

Figure 4. *G. mellonella* as a model for rapid evaluation of *Brucella* vaccine immune sera efficacy. a) & b) The effect of pre-incubation time of immune sera and bacteria on *G. mellonella* larval survival. a) Survival rate and b) health index scores of larvae infected with *Brucella* strain A19 pre-treated with sera for 30,45 or 60 minutes. c) & d) The effect of immune sera concentration on *G. mellonella* larval survival. c) Survival rate and d) health index scores of larvae infected with A19 pre-treated with different concentrations of sera. e) Cocoon formation, activity, melanization and survival of *G. mellonella* larvae on day 1. Significant differences between the Kaplan-Meier curves were identified with the log-rank test (GraphPad Prism). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

two groups, as reflected in both the daily survival rates and health index scores (Figure 4b). Thus, 60 min was employed as the pre-incubation time in subsequent studies.

To determine the optimal sera concentration, strain A19 was suspended in PBS containing 1/2 diluted to 1/25 diluted [4%-50% (v/v)] 104M sera and incubated at 37°C for 60 min before being inoculated into the larvae. Larval survival rates were all improved in the 104M sera pre-incubated groups by 58.3%-100% on day 1, compared with the PBS sera pre-treated group. All larvae inoculated with 1/25 diluted sera died by day 4, whereas those injected with 1/2 or 1/5 diluted sera showed more than 40% survival by day 5 (Figure 4c). Similar observations were apparent when comparing the health index scores of these different treated groups. Larvae in the 1/25 and 1/10 diluted 104M sera pre-incubated groups scored consistently lower than those in the 1/2 and 1/5 diluted 104M sera pre-incubated groups (Figure 4d). Therefore, these data indicate that 104M sera provide good protection against a lethal *Brucella* challenge when pre-incubated with bacteria for 60 min, with 1/2, 1/5, and 1/10 diluted sera outperforming 1/25 diluted sera.

Except for the total health index scores, individual indicator (activity, cocoon formation, melanization, and survival) scores also reflected that 104M sera had a good protective effect against A19 during the infection course. It is worth mentioning that differences in the protective efficacy of immune sera were already rather noticeable on the first day of infection (Figure 4e), suggesting that the *G. mellonella* model offers the possibility to rapidly evaluate the efficacy of serum antibodies.

Haemocytes play a major role in the protective effect of 104M sera

To better understand the bacteria-larvae interaction and compare the histopathological differences between the 104M sera pre-incubated group and the PBS sera pre-incubated group, transverse sections of larvae were examined following H&E or Koster's staining. Consistent with our expectations, larvae in the PBS sera pre-incubated group showed extensive tissue destruction, with *Brucella* scattered within the coelom (Figure 5a,b). In contrast, in the 104M sera pre-incubated group, bacteria were confined to the granuloma-like structures, thus preventing diffuse infection of the organism on day 1 (Figure 5c,d). Additionally, an increase in the size of granulomatous-like lesions was observed on day 5 (Figure 5e,f).

One possible explanation for our observations is that the pre-incubation of bacteria and immune sera may contribute to the recognition, phagocytosis, and encapsulation of bacteria by larval haemocytes. To test this hypothesis, we injected larvae with CDLip to deplete haemocytes, or Lip as a control; after 24 h, we exposed the larvae to the 104M sera pre-incubated bacteria, and measured larval survival over 5 days. Lip and CDLip alone showed no significant toxicity towards larvae (data not shown). In line with our speculation, larval survival was significantly reduced in the CDLip pre-treated larvae, compared with the larvae pre-treated with Lip, indicating that the protective effect of 104M sera is dependent on larval haemocytes. However, it should be noted that pretreatment with CDLip only decreased, but did not completely inhibit, the protective effect of 104M sera, suggesting that several mechanisms may play a role in the protective effect of 104M sera (Figure 6a,b).

To verify whether the protective effect of 104M sera is complement-related, we further inactivated the serum complement. Immune sera were first heat-inactivated at 56°C for 30 min and then co-incubated with 1.25×10^8 CFU of strain A19. The protective effect of the 104M sera was not affected by complement inactivation, suggesting that the serum complement is not relevant to the protective effect of the 104M sera in the larval model (Figure 6c,d).

Priming with a non-lethal dose of Brucella *strains protects* G.mellonella *larvae against a subsequent lethal challenge*

Several studies have shown that priming low-dose injections of microorganisms shielded the larvae from repeated exposure to the same or other microorganisms. To verify whether pre-exposure to a non-lethal dose of *Brucella* strains protects the larvae from a subsequent lethal challenge, groups of larvae were first primed with 1.25×10^5 CFU of live or fixed strain A19 through the last left proleg, followed by infection with 1.25×10^8 CFU of the same strain 24 h later via the last right proleg. As expected, priming with a non-lethal dose of either live or fixed strains protected the larvae from a subsequent lethal challenge, with the live strain outperforming the fixed strain (Figure 7a).

We simultaneously explored whether priming with heterologous *Brucella* strains could also protect against a subsequent lethal challenge. To this end, larvae were primed with 1.25×10^5 CFU of strain 104M or strain 104MΔOmp19 24 h before confronted with 1.25×10^8 CFU of strain A19. The protective efficacy of the 104M primed group was not statistically different from that of

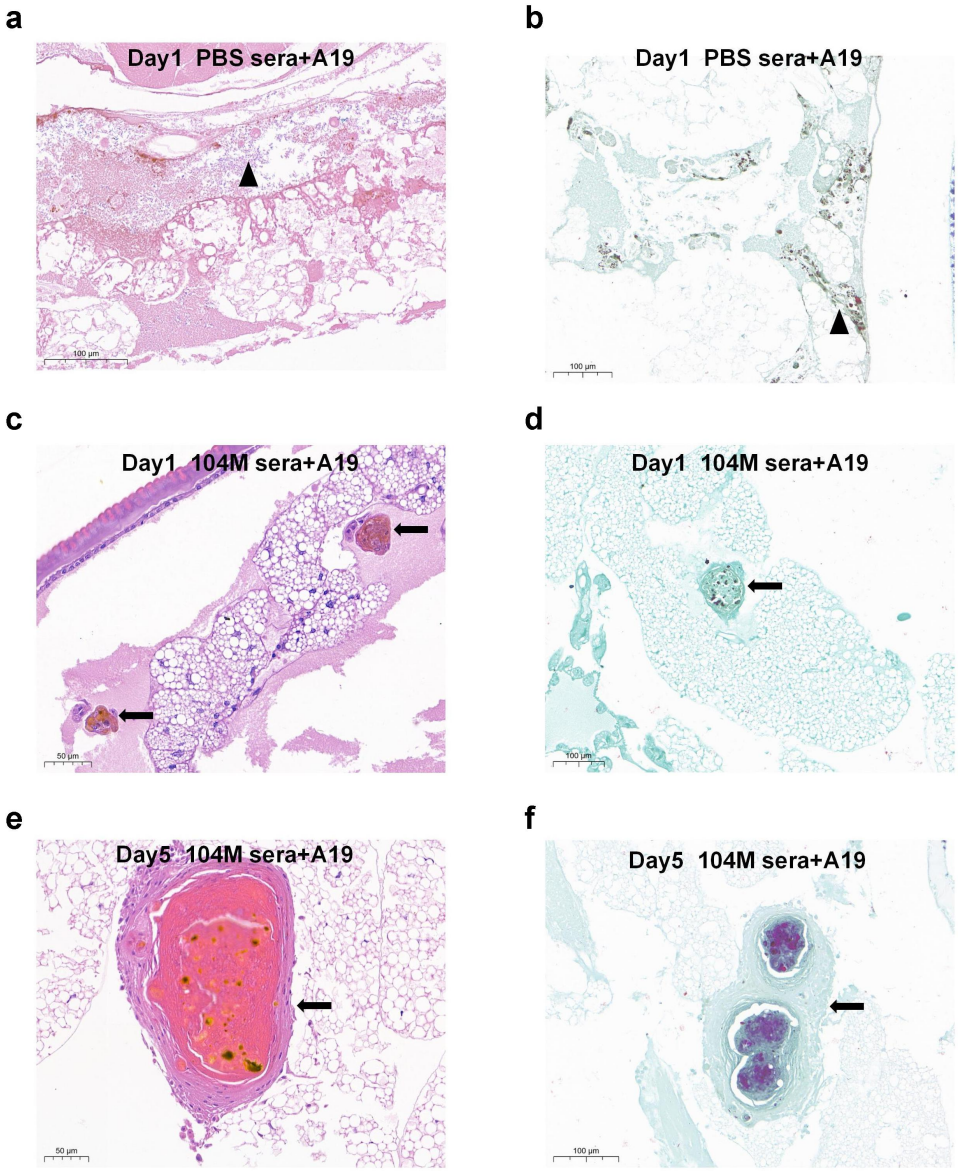

Figure 5. Histological analysis of *G. mellonella* larvae infected with sera-pre-treated *Brucella* strain A19. Groups of larvae were infected with 1.25×10^8 CFU of live *Brucella* strain A19 pre-treated with 104M sera or PBS sera for 1 hour. a) Larvae infected with PBS sera pre-treated bacteria on day 1, H&E staining. Black triangles represented disseminated *Brucella*. b) Larvae infected with PBS sera pre-treated bacteria on day 1, Koster's staining. c) Larvae infected with 104M sera pre-treated bacteria on day 1, H&E staining. d) Larvae infected with 104M sera pre-treated bacteria on day 1, Koster's staining. e) Larvae infected with 104M sera pre-treated bacteria on day 5, H&E staining. f) Larvae infected with 104M sera pre-treated bacteria on day 5, Koster's staining. Black arrows represented the granuloma-like structures formed by the aggregation of haemocytes.

the A19 primed group, while the 104MΔ*Omp19* strain primed group showed significantly decreased protective efficacy (Figure 7b,c), demonstrating that the presence of Omp19 is of great importance for the 104M vaccine.

G. mellonella *model and the mouse model are comparable in evaluation of vaccine efficacy*

To verify whether the *G. mellonella* and mouse models were comparable in evaluation of vaccine efficacy, strain 104M and strain 104MΔ*Omp19* were employed. The protective efficacy of the two strains was firstly tested in a mouse infection model. Groups of mice were immunized with 1×10^5 CFU of strain 104M, strain 104MΔ*Omp19*, or the PBS control on day 1. Four weeks after immunization, the mice were infected intraperitoneally with 1×10^8 CFU of strain A19. The protective efficacy of 104M was far superior to that of the 104MΔ*Omp19* deletion mutant, with a 15-fold difference in the spleen bacterial load ($P < 0.001$) between these two groups. (Figure 8a).

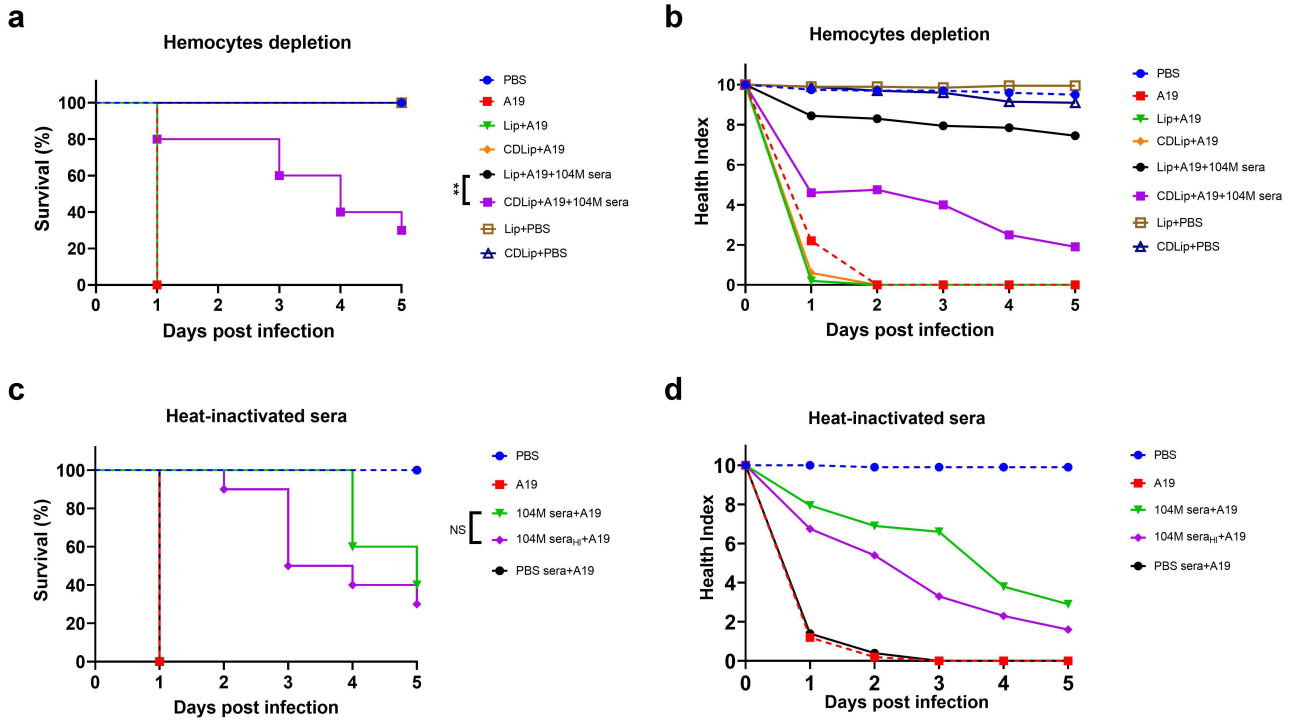

Figure 6. The effect of haemocytes depletion and complement inactivation on larval survival. Larvae were either injected with CDLip to deplete haemocytes or injected with Lip as a control 24 hours before infection (a&b). Sera were untreated or heat-inactivated at 56°C for 30 minutes before co-incubated with bacteria (c&d). a) The effect of haemocytes depletion on *G. mellonella* larval survival. b) Health index scores of haemocytes depletion larvae infected with a lethal dose of *Brucella*. c) The effect of complement inactivation on *G. mellonella* larval survival. d) Health index scores of larvae infected with a lethal dose of *Brucella* strain A19 pre-incubated with complement inactivated immune sera. Significant differences between the Kaplan-Meier curves were identified with the log-rank test (GraphPad Prism). $^{**}P < 0.01$.

We then compared the protective efficacy of 104M and 104MΔ*Omp19* immune sera from the two groups in the *G. mellonella* model. Compared with PBS sera, 104MΔ*Omp19* immune sera did have some protective effects, as reflected in the 20% survival rate on day 1 and prolonged survival time (Figure 8b and Additional file 2). However, this protective effect was rather limited because all larvae died on day 4 (Additional file 2). In contrast, the 104M sera pre-incubated group, consistent with the results from the mouse model, demonstrated a significant protective effect. 90% of the larvae survived on day 1 (Figure 8b) and the survival rate remained at 30% on day 5 (Additional file 2). Meanwhile, the daily health index scores of the 104M sera pre-incubated group were consistently higher than that of the 104MΔ*Omp19* sera pre-incubated group (Additional file 2).

Histopathological changes in mouse spleens (Figure 8c) and *G. mellonella* larvae (Figure 8d) were then investigated by H&Estaining sections. No abnormalities were observed in the spleen of the PBS-immunized, uninfected group, while in the PBS-immunized, challenged group and 104MΔ*Omp19* immunized, challenged group, the number of splenic nodules was decreased, and hyperplasia of connective tissue around splenic nodules (red arrows) was observed, with punctate neutrophil infiltration (blue arrows). Large areas of extramedullary haematopoiesis can be observed in the red pulp (black arrows), accompanied by an increase in the number of multinucleated giant cells (yellow arrows). In contrast, fewer severe lesions in the spleen section were observed in the 104M immunized group, with small extramedullary haematopoietic foci (black arrows) and a slight increase in the number of multinucleated giant cells (yellow arrows). Similar observations were discovered when comparing lesions in the larval sections. Larval sections in the PBS sera pre-treated group showed extensive disruption of the tissue structure and diffuse distribution of bacteria, while those in the 104MΔ*Omp19* sera pre-treated group only showed partial disruption. A portion of the bacteria was distributed in the haemocoel, while the remainder was encapsulated in the haemolymph nodes. Notably, in the 104M serum pre-treated group, almost all bacteria were encapsulated in the

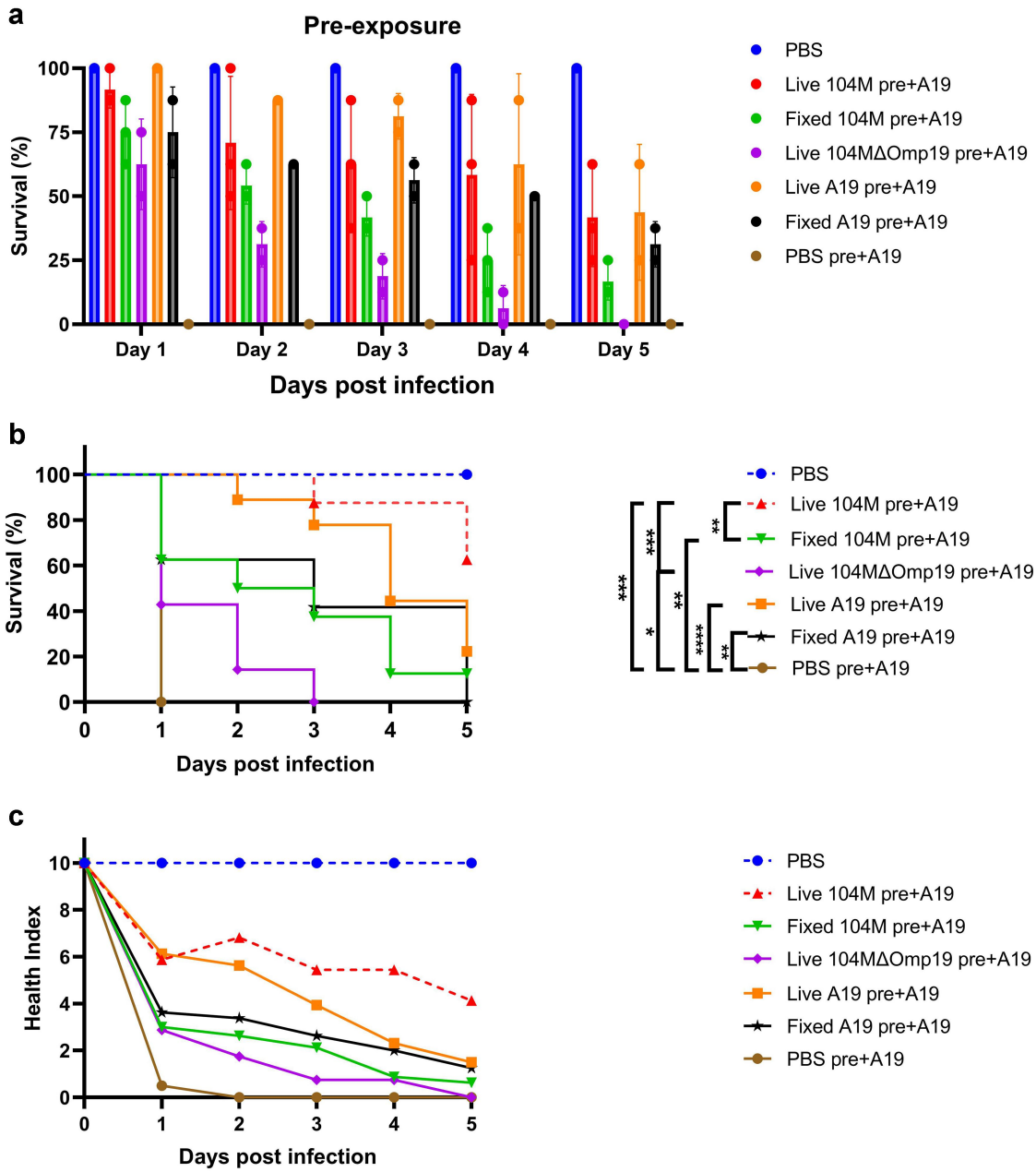

Figure 7. The effect of priming *G. mellonella* larvae with a non-lethal dose of *Brucella* strains. Larvae were primed with a non-lethal dose of *Brucella* or PBS control 24 hours before infection with a lethal dose of strain A19. a) & b) Survival rate of larvae primed with non-lethal dose of bacteria. c) Health index scores of larvae primed with non-lethal dose of bacteria. Significant differences between the Kaplan-Meier curves were identified with the log-rank test (GraphPad Prism). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

granuloma-like nodes formed by haemocytes. Consequently, the tissue structure was relatively intact. Taken together, these data indicate that comparable results to murine studies can be obtained using the *G. mellonella* model when 104M and 104MΔ*Omp19* are used as model vaccines. Consequently, as a bridge between the *in vitro* and *in vivo* models, *G. mellonella* model could be used as a simple, rapid, and high-throughput screening tool to evaluate the protective efficacy of immune sera.

Discussion

Although there is an urgent demand for new strategies and therapies to control, combat, and eradicate brucellosis, this process is extremely slow. Animal models

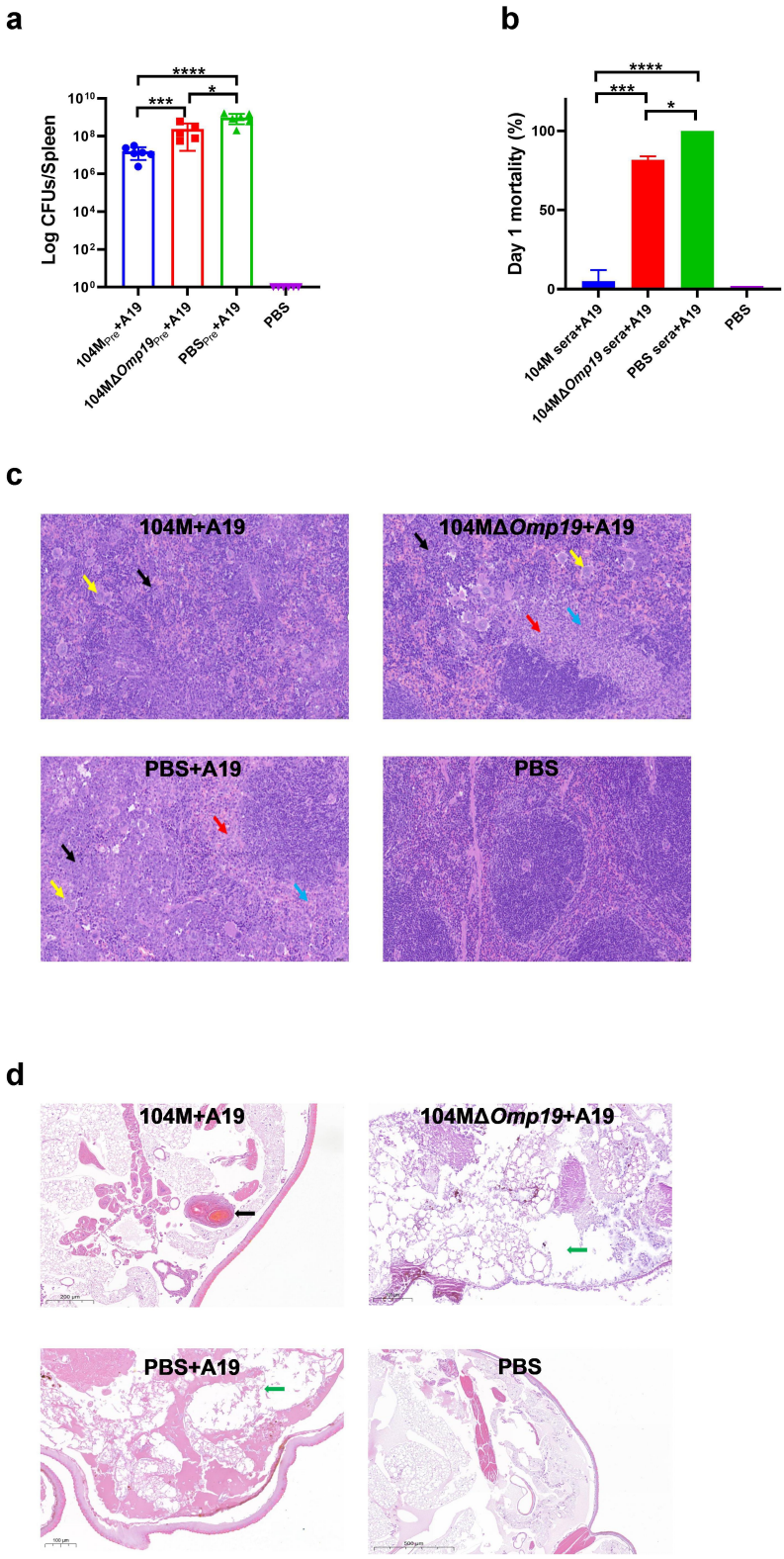

Figure 8. Evaluation of the consistency between the invertebrate *G. mellonella* model and the mouse model. Groups of mice were immunized with 1×10^5 CFU of strain 104M, strain 104MΔ*Omp19*, or the PBS control on day 1, and 4 weeks after immunization, the mice were infected intraperitoneally with 1×10^8 CFU of strain A19. Sera were collected on day 28. a) CFU counts from 104M and 104MΔ*Omp19* immunized mice spleens in the challenge study. b) Day 1 mortality of larvae infected with 1.25×10^8 CFU of *Brucella* strain A19 pre-treated with 104M sera, 104MΔ*Omp19* sera or PBS sera for 1 hour. c) Representative images of H&E staining mouse spleens in different treatment groups on day 35. Red arrows represented hyperplasia of connective tissue around splenic nodules, blue arrows represented punctate neutrophil infiltration, black arrows represented large areas of extramedullary haematopoiesis in the red pulp, and yellow arrows represented multinucleated giant cells. d) Representative images of H&E staining larvae sections in

are indispensable for studying virulence, pathogenic mechanisms, bacteria-host interactions, and vaccine development of *Brucella*. However, commonly used vertebrate evaluation models require feeding and housing, are time-consuming and costly. On the other hand, the *in vitro* evaluation methods, such as the serum bactericidal activity assay and the minimum inhibitory concentration assay, are not recommended because of the potential for creating aerosols that are hazardous to laboratory personnel. As such, the *G. mellonella* larvae *in vivo* evaluation model is of great value, as it offers a bridge between *in vitro* cell assays and final vertebrate studies. The use of the *G. mellonella* larvae model in pathogen-host interaction, virulence comparison, and antibiotic screening has been broadly investigated, but few studies have focused on this model for *Brucella* infection or evaluation of immune serum efficacy. In 2014, Nicolas et al. presented a protocol to analyse the virulence of select agents, including two highly virulent *Brucella* strains [43]. Here, we report that *G. mellonella* larvae could be used as a complementary *in vivo* model for infection and virulence comparison of *Brucella* strains. Also, we showed that this simple invertebrate model exhibited promise as a novel, fast, and efficient evaluation model for the efficacy of immune sera, which, to our knowledge, is a brand new finding.

To characterize the *Brucella* infection model, we first evaluated the virulence of A19, an attenuated strain of *B. abortus*, to the larvae using a range of doses at 25°C and 37°C, and determined the survival of the larvae over a 5-day infection course. Also, the infection process was monitored by the health index scores of larvae. Larval survival occurred in a temperature- and dose-dependent manner. A dose of 1.25×10^8 CFU of *Brucella* A19 killed almost all the larvae by the end of infection regardless of the temperature, and a delay in larval death was observed when they were maintained at a lower temperature. This difference may be related to the growth disadvantage of bacteria at lower temperatures or the expression of temperature-dependent virulence factors. Similarly, health index scores correlated well with infection doses, with lower doses leading to higher scores. As a result, the infection dose of 1.25×10^8 CFU and the incubation temperature of 37°C were used in subsequent studies. In addition, the histopathological sections showed that *Brucella* was restricted to granuloma-like structures formed by haemocytes during non-lethal dose of infection, whereas bacteria were scattered throughout the course of the lethal dose of infection, indicating that larval death may be related to haemocyte depletion.

Next, since the *G. mellonella* larval model has been reported for the virulence evaluation of bacteria and fungi [24,26,27,44,45], we compared the virulence differences between live bacteria and heat-inactivated bacteria. Live strain 104M and live strain A19 both killed the larvae completely at an inoculation dose of 1.25×10^8 CFU, indicating that there was probably little difference in virulence between these two *Brucella* strains in the *G. mellonella* model. Meanwhile, the killing activity could be annulled by heat inactivation, which agreed with the results of Dijokaite et al. [30], implying that inactivated bacterial lysate and endotoxin did not play a significant role in larval survival. Strain A19 and strain A19Δ*VirB12* showed equal pathogenicity in the larval model, which is in keeping with an earlier report in the mouse model [33]. Importantly, the survival rate of larvae inoculated with the 104MΔ*Omp19* deletion mutant was greatly increased at the same infection dose, proving that Omp19 is indeed a key virulence factor of strain 104M. In addition, strain S2 was less virulent in the larval model than strain A19, which is consistent with the results of previous studies [37,39]. Together, the *G. mellonella* model could detect virulence differences of *Brucella* strains, and a good correlation has been established between the *G. mellonella* model and the murine model. Therefore, the larval model might be a good substitute for the early evaluation of potential virulence genes when using a mammalian model, which might not be morally or practically appropriate. While preparing this article, a study was published by Aitor et al., which focused on utilizing the larval model to screen for potential *Brucella* factors modulating innate immunity and yielded similar findings to our own [46].

After successfully established the larval model of *Brucella* infection and virulence evaluation, we determined the possibility of using this model to assess the efficacy of immune sera. As expected, the serum components did not affect the survival of *G. mellonella*, which is the prerequisite to the *G. mellonella* model for evaluation of immune serum efficacy. Then, the

different treatment groups on day 1. The black arrow represented the granuloma-like structures formed by the aggregation of haemocytes, and green arrows represented damaged adipose body. Significant differences were identified by one-way ANOVA (GraphPad Prism). $^*P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

protective efficacy of 104M immune sera was determined. Unsurprisingly, 104M sera provided good protection against a *Brucella* lethal challenge when incubated with bacteria in advance, while PBS sera showed no effect on preventing *Brucella* induced larval death. This protective effect depends on both the concentration of immune sera and the co-incubation time of the bacteria and immune sera. These data highlight the potential of the *G. mellonella* larvae model for the evaluation of immune serum efficacy. Notably, the differences in the survival rates and health index scores between the 104M sera and PBS sera were quite clear by day 1. Accordingly, the survival rates and health index scores on day 1 could be used as predictors of the overall protective effect without monitoring the larvae for 5 days, which greatly shortens the experimental cycle.

To explore the mechanisms underlying the protective efficacy of 104M sera in the *G. mellonella* model, histopathological differences between the 104M sera and PBS sera pre-treated groups were analysed. Strain A19 caused severe disseminated infection and tissue destruction in the PBS sera pre-treated group, whereas in the 104M sera pre-treated group, bacteria were confined to the granulomatous structures formed by haemocytes, thus preventing disseminated infection.

We then depleted larval haemocytes to verify whether they cooperated with 104M sera. As expected, the protective effect of 104M sera decreased but did not completely disappear, indicating that multiple mechanisms may be involved in the protective effect of 104M immune sera. Additionally, we heat-inactivated the serum complement to determine whether it caused a difference in the serum protective efficacy. As the outcome demonstrates, complement is not important for understanding how 104M sera protects. Omp19 plays a significant role in bacterial adhesion, invasion, colonization, and intracellular survival; thus, it is considered a leading target for *Brucella* vaccines. To verify whether the protective efficacy of strain 104M decreases after deletion of Omp19, groups of larvae were primed with a non-lethal dose of *Brucella* strain 104M and strain 104MΔ*Omp19*, followed by a lethal dose of strain A19 challenge. Indeed, priming with strain 104M or 104MΔ*Omp19* could protect larvae from the lethal challenge, with strain 104M outcompeting strain 104MΔ*Omp19*, as reflected in the daily survival rate and health index scores of different groups. In line with our expectations, the protective effect of pre-immunization with a non-lethal dose of live vaccine was superior to that of the corresponding inactivated vaccine, demonstrating the reliability of the model.

Finally, we compared the consistency between the larval and mouse models. A prophylactic study was performed in the mouse model, and the protective efficacy of strain 104M, 104MΔ*Omp19* and PBS control were determined. As predicted, strain 104MΔ*Omp19* showed a much lower protective efficacy than strain 104M, reflecting the difference in bacterial load. Subsequently, the protective efficacy of 104M sera, 104MΔ*Omp19* sera, and PBS sera from the mouse study was determined using the larval model. Similarly, 104MΔ*Omp19* sera showed much lower protective efficacy than 104M sera, following the same pattern as the mouse model. Further analysis of the histopathological changes also showed that strain 104M provided better protection than strain 104MΔ*Omp19* in the mouse and *G. mellonella* models. Combined, these data highlight the correlation between the larval model and the mouse model, implying the potential of the larval model in immune sera evaluation.

In conclusion, in the present study, we characterized a *G. mellonella* larval model for *Brucella* infection and virulence evaluation. Moreover, we report for the first time a *G. mellonella* larval model for potential evaluation of the protective efficacy of immune sera, which shortens the evaluation cycle to 1 day and therefore greatly accelerates the evaluation process. Future research will concentrate on the cellular, proteomic, and metabolomic responses to *Brucella* infection in the *G. mellonella* larval model. We anticipate that the *G. mellonella* infection and immune model will lessen our reliance on mammalian infection models, while enabling us to gain a better understanding of *Brucella* virulence factors, bacteria-host interactions, and vaccine development.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data Availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

Ethics approval

The animal studies and procedures were approved by the Animal Care and Use Committee of Beijing Institute of Biotechnology under project license number IACUC-SWGCYJS-2022-004. The animal procedures followed the guidelines of the Administration of Affairs Concerning Experimental Animals.

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