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Original article

Galleria Mellonella as A Surrogate Model System to Study Pathogenicity of Salmonella Enterica Serovar Typhimurium Strain ST4/74 and Enterohaemorrhagic E. Coli (EHEC) O157:H7

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Abstract
In this research, Galleria mellonella larvae served as an in vivo model to examine the virulence of two pathogenic bacteria, Salmonella Typhimurium and Escherichia coli, along with their quorum-sensing receptor mutants, qseC and qseE. The most widely used inoculation route for Galleria is intra-haemocoelic injection through the last left pro-leg (the route used in this study). To determine the optimum bacterial dose, a range of inocula was used (103,104, 105, 106, and 107 CFU/10 µl injection volume); these were injected using a Hamilton syringe (26-gauge, 10 µl capacity). For control purposes, 10 larvae were inoculated with only Phosphate Buffered Saline (PBS) buffer. Lethal dose (LD50) was a useful tool for differentiating between high and low pathogenicity, but not for intermediate pathogenicity. The larvae were subsequently incubated at 37°C in a dark environment, and the culture dilution that resulted in the mortality of 50% of the larvae (LD50) for each replicate was assessed after 24 hours. For each experiment, a minimum of 10 larvae (located in 100 mm Petri dishes) were used per strain to be tested. Likewise, melanization could function as a criterion for evaluating the pathogenicity of the isolates, but it is essential to correlate it with other factors. Throughout the experiment, individual larvae were consistently assessed for indications of melanization and viability by observing their reflexive responses to physical contact. Mortality rates and survival curves were useful tools for characterizing non-pathogenic and highly pathogenic isolates. The precise numbers of inoculated bacteria into the larvae were carried out by plating serial dilutions of the inoculum onto Luria agar. The findings are expressed in terms of percent survival and are derived from at least three independent experimental trials.
Keywords. Bacterial Virulence, Galleria Mellonella, Infection, *In vivo* Study, Melanization.

Introduction

Understanding the intricate relationship between the host and the bacterium is a crucial step for revealing the pathogenicity of a certain strain. The role of animals in experimental research is essential for exploring the pathogenicity of bacteria. However, during the preclinical phase, in vivo models using mammals, particularly mice and rats, face certain challenges, including the need for adequate facilities and lengthy experimental processes. In recent years, there has been an increasing trend towards the use of insects in in vivo studies, which make up approximately 90% of all animal species. This preference is largely due to the similarities between the insect immune system and human innate immunity [1,2], which can be referred to as the "evolutionary roots of human innate immunity [3]. Consequently, insects serve a dual purpose in research; they are utilized not only to examine their interactions with natural pathogens but also to investigate the virulence factors associated with human pathogens and to evaluate the efficacy of antimicrobial drugs in vivo [4-6]. The larvae of Galleria mellonella, commonly known as wax moth larvae, are notable for their numerous benefits. They are regarded as one of the most prevalent and favoured models for serving as an alternative surrogate host in the investigation of microbial infections [6]. This type of mini-host offers economic and ethical advantages compared to mammals, and its short lifespan makes it suitable for high-throughput studies [7,8].

The scientific community has shown a significant rise in interest in employing larvae for the research of pathogenic bacteria, especially in the past few years [9]. Galleria mellonella has been a valuable model for investigating host-pathogen interactions in several organisms, including Pseudomonas aeruginosa [10]. In recent studies, it has been specifically utilized to analyze the virulence factors of Vibrio parahaemolyticus [11]. It is noteworthy that a clear correlation has been established between the virulence of an organism in G. mellonella and its virulence in mammalian models [12]. The Galleria mellonella insect serves as an in vivo model and belongs to the Galleriinae subfamily within the Pyralidae family of the Lepidoptera order, where it is known to infest bee hives. This greater wax moth progresses through four distinct life stages: egg, larva, pupa, and adult. The larvae of Galleria are characterized by their opaque white coloration, reaching approximately 3 cm in length and weighing between 0.3 and 0.5 grams, before undergoing metamorphosis into grey moths. Temperature plays a vital role in the insect's development, with optimal conditions ranging from 29 to 33°C; additionally, the larvae are capable of surviving at the physiological temperature of mammals, which is 37°C [13,14].

G. mellonella has characteristic advantages valuable to infection studies, such as they are cheaper than mouse models, they do not need special laboratory equipment, and can adapt and live at 37˚C temperature [15], preferred by human pathogens. Moreover, G. mellonella's short lifespan positions it as an optimal

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choice for high-throughput infection investigations. In most cases, experimental evaluations of the response to infection are restricted to analyzing the melanization process and quantifying the death rate of larvae. In this investigation, G. mellonella larvae served as an in vitro model to assess the virulence of the pathogenic bacteria Salmonella typhimurium and E. coli, including their two-component system (TCS) mutants.

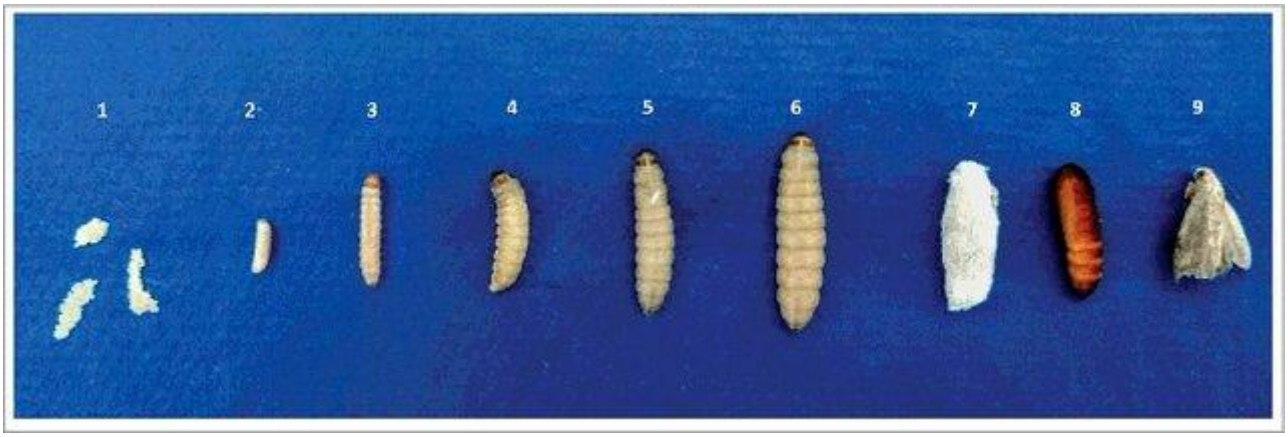

Figure 1. Illustrates the various developmental stages of Galleria mellonella. The stages include eggs (1), a caterpillar that is around 10 days old (2), a caterpillar approximately 20 days old (3), caterpillars aged between 25 to 35 days (4 and 5), a caterpillar in its final larval stage at about 40 days old (6), as well as the pre-pupae and pupae stages (7 and 8), culminating in the adult moths (9) (31).

Methods

Bacterial Strains and Growth Conditions

Larvae of the Greater Wax Moth, G. mellonella (GM) were obtained from Live Foods Ltd (Rooks Bridge, UK). Larvae were stored in the dark at 15˚C and used within 10 to 21 days of receipt. The larvae of the Greater Wax Moth, Galleria mellonella (GM), were acquired from Live Foods Ltd located in Rooks Bridge, UK. They were kept in a dark environment at a temperature of 15˚C and utilized within a timeframe of 10 to 21 days after their arrival.

For the experiments, the selected groups were required to meet certain specifications: a length of 2 to 2.5 cm, a weight ranging from 0.25 to 0.35 g, a cream hue with minimal speckling, and an absence of grey markings, which are considered signs of compromised food health. Groups of ten larvae, all receiving the same treatment, were placed in a 100 mm petri dish and incubated at 37°C following infection. The bacterial inocula underwent washing before the infection to limit the introduction of virulence factors that could have been produced during the microorganism's growth in vitro. In G. mellonella, infection can occur via two methods: either through intra-haemocoelic injection administered in the last left proleg or by direct application through the skin [16].

Bacterial strains

Enterohaemorrhagic E. coli (EHEC) O157:H7 85-170 NalR is a spontaneous nalidixic acid-resistant stx1- and stx2-lacking derivative of strains 84-289 [17]. This strain and its Δqse mutants were gifted by Professor Mark Stevens of the Institute of Animal Health, Compton, UK. The qseC and qseE genes were mutated by λ Red recombinase-mediated integration of linear PCR products [18]. The S. Typhimurium isolate 4/74 and strains were cultured in Luria-Bertani (LB) medium or Standard American Petroleum Institute (serum-SAPI) medium supplemented where appropriate with antibiotics at the following concentrations: nalidixic acid (Nal, 20 µg ml-1), ampicillin (Amp, 100 µg ml-1) and kanamycin (Kan, 50 µg ml-1). The S. Typhimurium qseC and qseE genes were similarly mutated by λ Red recombinase-mediated integration [18].

Culture preservation

Stock cultures of each species of bacteria were maintained in Luria broth by adding 25 % (v/v) sterile glycerol, dividing cultures into 1 ml aliquots, and freezing at -80°C. An aliquot of this stock was thawed, and to ensure the validity of the strains, reactivated, cultured on plates once every 24 hours for three days before use in the experiments.

Evaluation of LD50 in Galleria mellonella Larval infections

Observation findings were recorded if larvae' colour changed from their normal pale cream coloration to brown or black, indicative of melanisation. The pathogenicity of E. coli and Salmonella Wild Type (WT) and their QseC/E mutants was compared. To establish the 50% lethal dose (LD50). Larvae were subjected to injections of different doses, including 103, 104, 105, 106, and 107 Colony Forming Units (CFU) of both the wild type and its mutant strains. Larvae that received an inoculation of 106 colony-forming units of WT/larvae exhibited the highest mortality rates, resulting in the death of 50% of the larvae within 24 hours

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(Fig. 2, Fig. 3). In culture dilutions lower than 106, only one or two out of ten worms died when exposed to wild-type E. coli, Salmonella, and their respective mutants. However, at dilutions exceeding 10^6, there were no recorded fatalities among the larvae injected with Buffered Phosphate Saline (BPS). Thus, the subsequent experimental assay, set at 106 CFU per larva, was carried out to compare the virulence of the bacterial strains. Overnight growth of the bacterial strains was conducted in Luria broth at 37°C, followed by collection via centrifugation at 4000xg for 10 minutes. The cells were then washed twice with 1 mL of BPS, and the resulting cell suspensions were standardized to an optical density (OD600) of 1.0. The colony-forming units (CFU/mL) were confirmed through a viable count assay. To limit the introduction of virulence factors released during in vitro growth, bacterial inocula were washed before the infection process. The most frequently utilized route for infection is through intrahemocoelic injection [19] which is performed either via the last left pro-leg or through the skin [16].

For each strain's serial dilution (103,104, 105, 106, and 107 CFU/10 µl), a minimum of three biological replicates were prepared, with 10 larvae placed in 100 mm Petri dishes. These were injected using a Hamilton syringe (26-gauge, 10 µl capacity) within a 96-well flat plate. For control, 10 larvae were administered with PBS. The larvae were subsequently incubated at a temperature of 37°C in a dark environment, and the lethal dose that resulted in the death of 50% of the larvae (LD50) for each replicate was assessed after 24 hours. Each experiment involved the use of 10 larvae from each strain being tested. Bacterial cell counts were determined by performing serial dilutions of the inoculum and plating them onto Luria agar.

Regular evaluations of individual larvae were conducted during the experiment to monitor signs of melanization and viability, based on their reflex actions in response to contact. The results are expressed as survival percentages and reflect the outcomes of at least three separate experiments.

Statistical Analysis

Statistical analysis was performed as needed, utilizing One-way ANOVA (Analysis of Variance) or two-way ANOVA through the GraphPad Prism software. A P value of less than 0.05 denotes statistical significance.

Results

Observational data were noted when the larvae transitioned from their usual pale cream hue to brown or black, which indicates melanization and the level of infection severity.

In Figure 2, Galleria mellonella larvae were inoculated with Salmonella wild-type and its QseC, QseE, and QseEC mutants for a duration of 24 hours, resulting in discoloration caused by melanization. A minimum of three biological replicates, each comprising 10 larvae in 100 mm Petri dishes, were subjected to injections with various concentrations (103,104, 105, 106, 107 CFU/10 µl) and incubated at 37°C in the absence of light. The LD50, indicating the dilution that led to 50% larval mortality, was calculated for each replicate after 24 hours. The findings are reported as percentage survival and are representative of at least three independent experiments.

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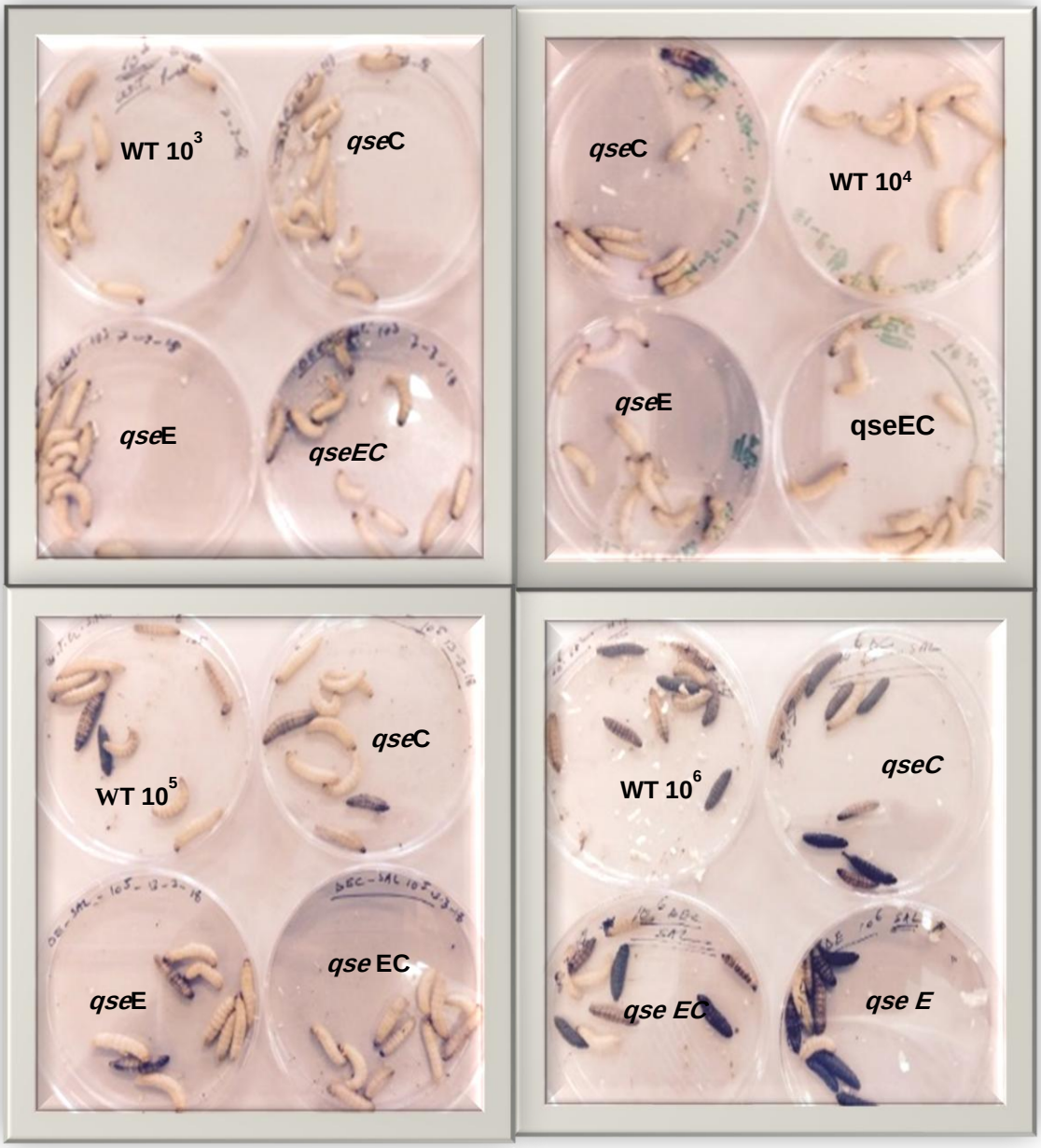

Figure 2. Inculcation of Galleria mellonella with Salmonella wild-type

In Figure 3, Galleria mellonella larvae were inoculated with E. coli wild type, as well as QseC, QseE, and QseEC mutants, and observed after 24 hours. The observed discoloration is attributed to melanization. A minimum of three biological replicates, each consisting of 10 larvae in 100 mm Petri dishes, were injected with varying concentrations (103,104, 105, 106 and 107 CFU/10 µl), followed by incubation at 37°C in darkness. The lethal dose required to kill 50% of the larvae (LD50) for each replicate was assessed after 24 hours. Data are presented as percentage survival and represent the results of at least three independent experiments.

Figure 4 shows that G. mellonella larvae were observed 24 hours post-inoculation with PBS. A minimum of three biological replicates, each comprising 10 larvae in a 100 mm Petri dish, received injections of BPS at concentrations of (103,104, 105, and 106 CFU/10 µl),, followed by incubation at 37°C.

G. mellonella larvae are examined after being inoculated with Salmonella and E. coli strains, including the wild type and the QseC, QseE, and QseEC mutants, at a dose of 107 CFU over a 24-hour period. The discoloration noted is attributed to melanization, and full melanization, represented by black larvae, is linked to the death of the larvae (Figure 5).

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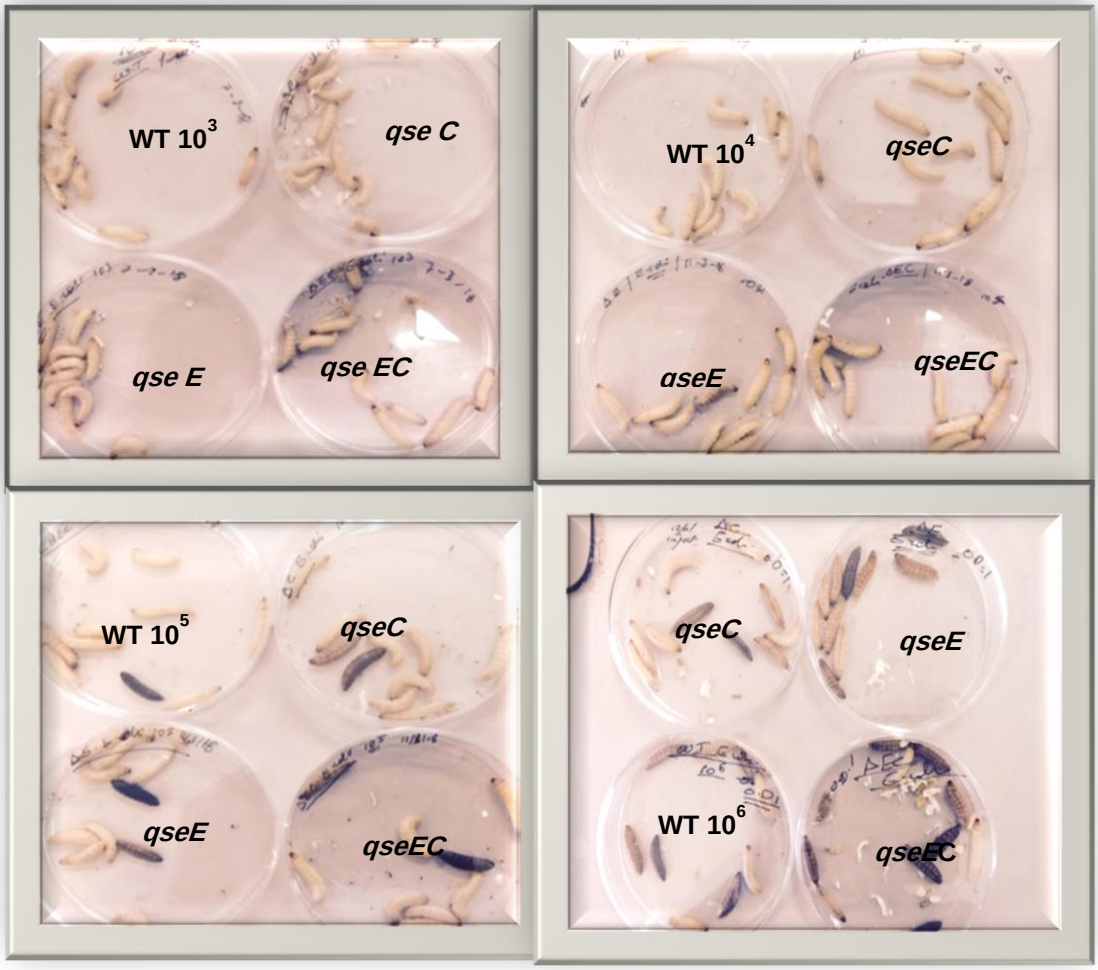

Figure 3. Inculcation of Galleria mellonella with E. coli wild-type

Figure 4. G. mellonella larvae 24 hours post-inoculation

As shown in figure 6, a minimum of three biological replicates, each with 10 larvae in a 100 mm Petri dish, were injected with concentrations of (103,104, 105, 106 and 107 CFU/10 µl), The samples were incubated at 37°C in a dark environment, and the dilution that resulted in 50% larval mortality (LD50) was evaluated after 24 hours. The findings are shown as percentage survival and are representative of at least three independent experiments.

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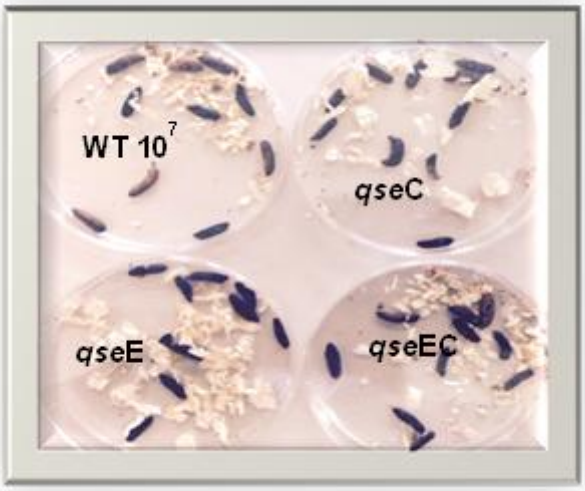

Figure 5. G. mellonella larvae after being inoculated with Salmonella and E. coli strains

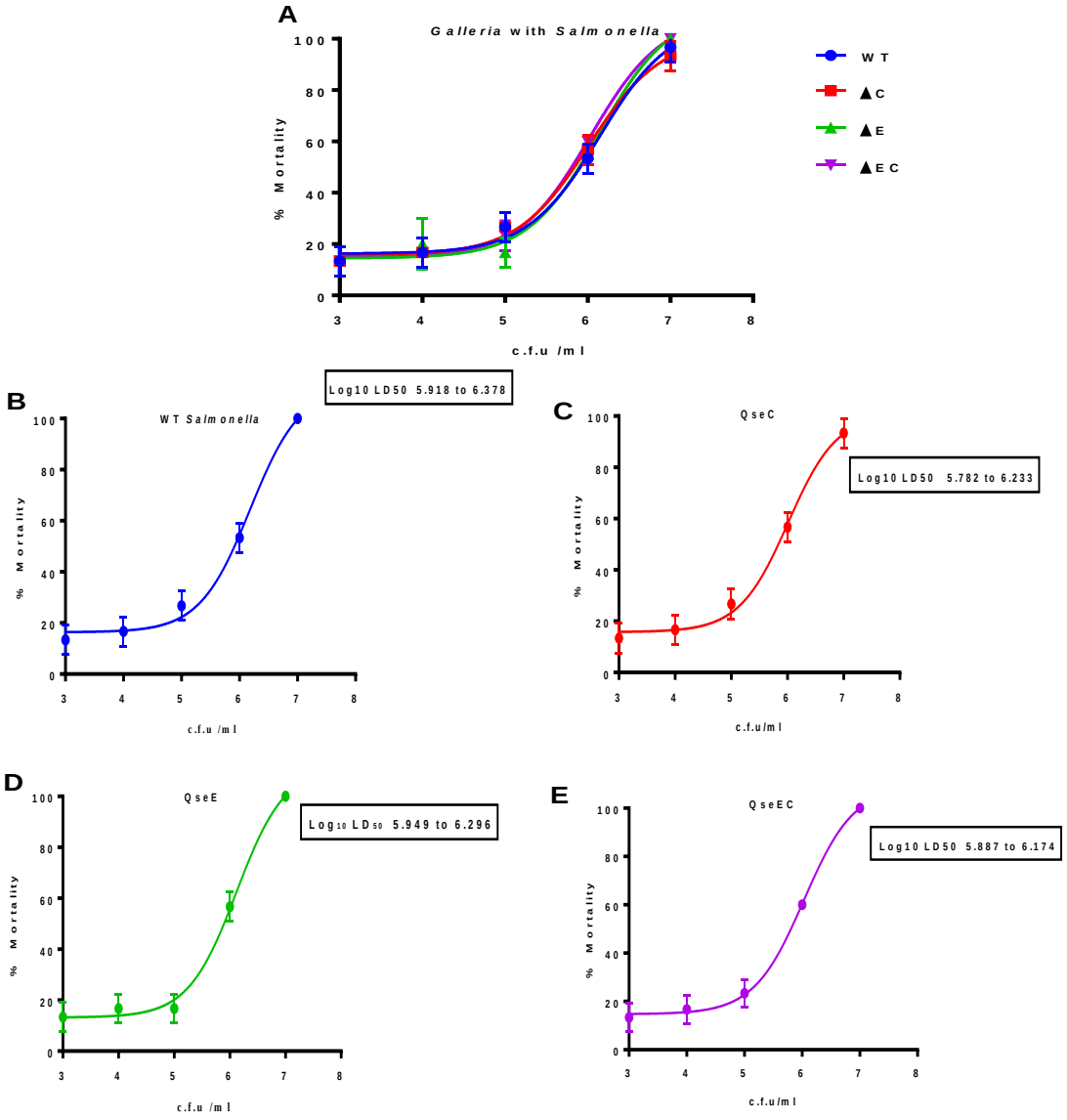

Figure 6. A comparison of the virulence (LD50) between wild-type Salmonella and its qse C/E mutants.

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As shown in figure 7, a minimum of three biological replicates, each consisting of 10 larvae placed in 100 mm Petri dishes, were injected with varying concentrations (103,104, 105, 106 and 107 CFU/10 µl), The larvae were then incubated at 37°C in darkness, and the dilution that resulted in the death of 50% of the larvae (LD50) for each replicate was assessed after 24 hours. The data are presented as percentage survival and represent the outcomes of at least three independent experiments.

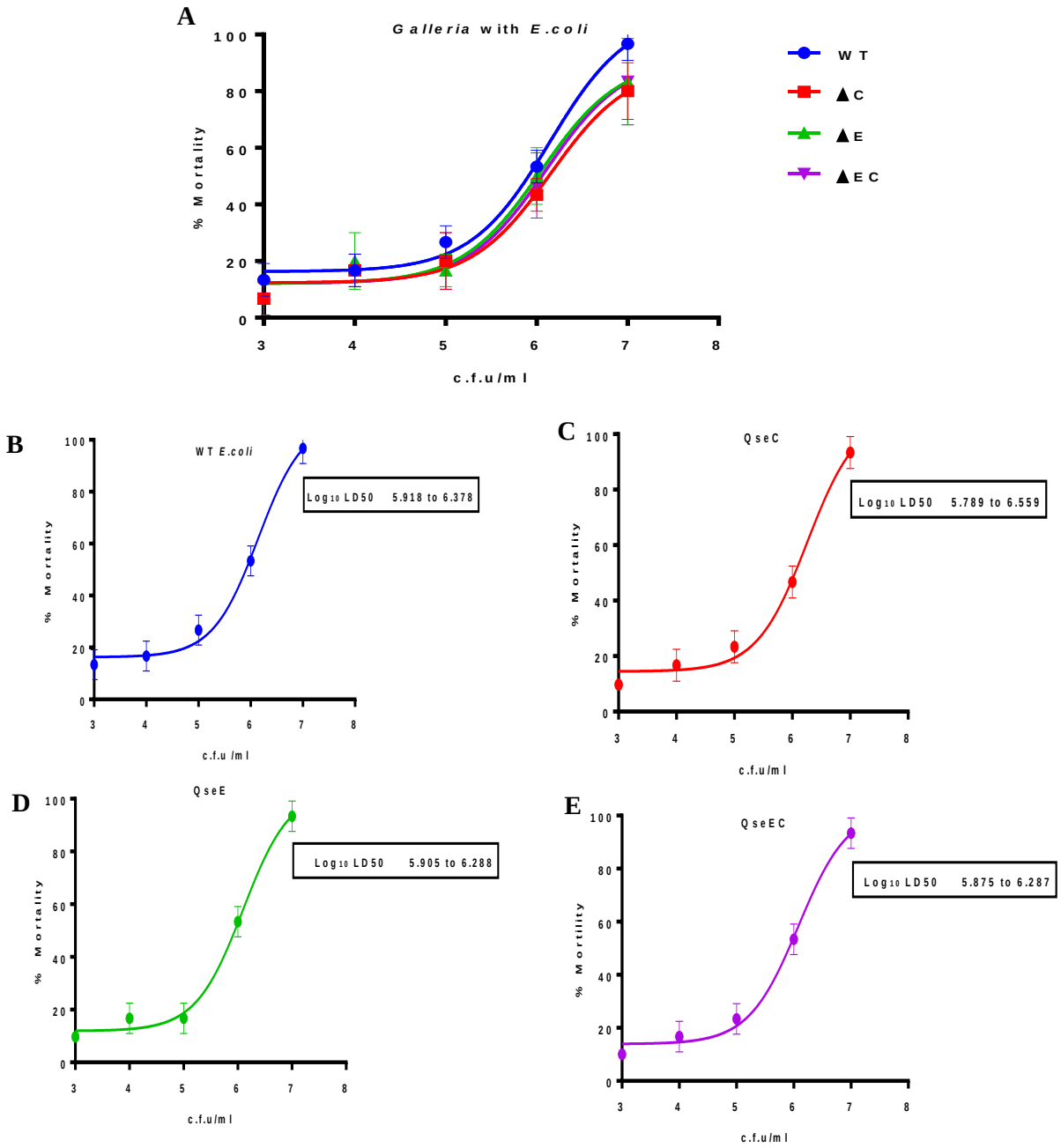

Figure 7. The virulence (LD50) of wild-type E. coli and QseC/E mutants in Galleria mellonella larvae.

Discussion

The findings of our study suggest that G. mellonella could be an effective alternative model for examining the virulence of bacterial infections, including their mutants, namely Salmonella enterica serovar Typhimurium strain ST4/74 and Enterohaemorrhagic E. coli (EHEC) O157:H7. In vivo experiments, using laboratory animals, are an essential element for determining the degree of pathogenicity of bacterial strains and understanding interactions between the host and the pathogen. Despite using of rodent models (as an *in vivo* experiment) is widely in studies concerning the pathogenesis of bacterial infections, these types of studies have many disadvantages, such as time time-consuming, requiring an expensive experimental setup, and being associated with significant ethical issues. The use of invertebrate models can participate in reduction of research costs and minimize ethical concerns. A variety of research studies have indicated

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and validated that the Wax moth G. mellonella serves as an effective model for examining certain aspects of bacterial strain pathogenicity. [20, 21]. Our study's results demonstrate that the virulence of Salmonella and E. coli, evaluated using a G. mellonella infection model, is dependent on the concentration of the inoculum administered.

At high concentrations of 107 CFU/larva was enough to induce death of the worm after 24 hours (figure 5). However, those which injected with different dilutions of PBS buffer for control purposes were not affected, and they were alive (Figure 4). At 106 (figure 6 & figure 7), these levels of bacteria killed half of the worms and were considered as lethal dose LD50 [22]. The melanization response refers to the process of synthesizing and depositing melanin to encase pathogens at the site of injury, which is subsequently accompanied by hemolymph coagulation and opsonization. This response is comparable to the formation of abscesses in infections observed in mammals [23, 24]. The process of melanization is viewed as an integral aspect of the innate immune response of arthropods in response to parasitic challenges, making it a useful tool for evaluating the health of insects. Furthermore, the synthesis of melanin was found to be induced in a dose-dependent manner, culminating in the emergence of 100% dark-colored larvae at 24 hours following inoculation with 1 × 107 bacteria. The larvae injected with PBS exhibited no indications of melanization (Figure 4) [25-27]. They displayed no symptoms, and the survival rate for the control larvae was 100% [24, 28, 29].

This investigation has focused on the virulence of two pathogenic bacterial types, Salmonella and E. coli, including their mutants, employing G. mellonella as an in vivo infection model. G. mellonella is regarded as a suitable model for investigating the virulence factors of bacteria and their impact on the host. It leverages the innate immune response to microorganisms, thereby serving as an initial starting point in research related to human health.[30]. A key feature of G. mellonella is its innate immune system, which exhibits functional structures similar to those found in mammals [31]. The study reveals that both the strains and their mutants possess the same LD50. Additionally, the deletion of the QseC and QseE proteins does not alter the virulence of either the wild-type Salmonella or E. coli strains, nor their mutants. This highlights the potential role of these receptors as one of multiple mechanisms through which bacteria can exhibit virulence. The findings suggest the QseC/E proteins have no role in the virulence activity of the bacteria tested in this infection model. Both types of wild-type and mutant bacteria can infect and kill G. mellonella in a dose and time-dependent manner. In conclusion, the data collected indicate the importance of G. mellonella in the study of bacterial pathogens. This organism serves as an effective model owing to its beneficial experimental characteristics, simplicity in handling, and cost-effectiveness. Additionally, its brief life cycle facilitates efficient testing durations. In addition, its resemblances to the human immune system render it an excellent tool for experimentation. Nevertheless, despite these benefits, there are notable disadvantages to take into account; the primary issue is the absence of regulations governing its application, which hinders the ability to compare experimental results across different laboratories. Despite the restrictions associated with employing larvae as an in vivo model, it continues to serve as a significant resource for investigating bacterial infections, owing to its various benefits. Several studies have examined both virulence and immune responses, along with the impact of antimicrobials. It is widely accepted that, while the application of this insect as a pre-clinical in vivo model has become a standard practice in laboratories, the complete potential of G. mellonella remains to be fully realized. To further scientific research, it is imperative to achieve a complete genomic profile of G. mellonella and to develop standardized procedures.

Conclusion

The findings of this study underscore the potential of Galleria mellonella as a valuable alternative model for assessing bacterial virulence, particularly for pathogens such as Salmonella enterica serovar Typhimurium and enterohemorrhagic E. coli (EHEC) O157:H7. Our results demonstrate that bacterial virulence in this model is dose-dependent, with higher inoculum concentrations leading to increased larval mortality and melanization, a key immune response analogous to mammalian abscess formation. Notably, the study revealed that the deletion of QseC and QseE proteins did not alter the virulence of the tested bacterial strains, suggesting that these receptors may not be critical for pathogenicity in this infection model. This highlights the complexity of bacterial virulence mechanisms and the need for further investigation.

Conflict of interest. Nil

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المستخلص

في هذا البحث، استخدمت يرقات جاليريا ميلونيلا كنموذج حيّ لدراسة ضراوة نوعين من البكتيريا الممرضة، وهما السالمونيلا التيفية الفأرية والإشريكية القولونية، بالإضافة إلى طفرات مستقبلات استشعار النصاب الخاصة بهما، qseC و qseEيُعد الحقن داخل الخصية اليسرى الأخيرة (وهو الأسلوب المستخدم في هذه الدراسة) أكثر طرق التلقيح شيوعًا لجاليريا. ولتحديد الجرعة البكتيرية المثلى، استخدمت مجموعة من اللقاحات (103، 104، 105، 106، و107 وحدة تشكيل مستعمرة/10 ميكرولتر من حجم الحقن)؛ وحُقنت هذه اللقاحات باستخدام محقنة هاميلتون (عيار 26، سعة 10 ميكرولتر). ولأغراض الضبط، تم تلقيح 10 يرقات بمحلول ملحي مُنظم بالفوسفات (PBS) فقط. كانت الجرعة المميتة (LD50) أداة مفيدة للتمييز بين الإمراضية العالية والمنخفضة، ولكنها لم تكن كذلك للإمراضية المتوسطة. حُضنت اليرقات بعد ذلك عند 37 درجة مئوية في بيئة مظلمة، وقُيّم تخفيف المزرعة الذي أدى إلى نفوق 50٪ من اليرقات (LD50) لكل تكرار بعد 24 ساعة. لكل تجربة، استخدم ما لا يقل عن 10 يرقات (موجودة في أطباق بتري 100 مم) لكل سلالة للاختبار. وبالمثل، يمكن أن يعمل التصبغ الجلدي كمعيار لتقييم إمراضية العزلات، ولكن من الضروري ربطه بعوامل أخرى. طوال التجربة، تم تقييم اليرقات الفردية باستمرار بحثًا عن مؤشرات التصبغ الجلدي وقابليتها للبقاء من خلال مراقبة استجاباتها الانعكاسية للتلامس الجسدي. كانت معدلات الوفيات ومنحنيات البقاء على قيد الحياة أدوات مفيدة لتوصيف العزلات غير المسببة للأمراض والعزلات شديدة الإمراض. تم تحديد الأعداد الدقيقة للبكتيريا الملقحة في اليرقات عن طريق وضع تخفيفات متسلسلة من الملقح على أجار لوريا. وتُعبر النتائج عن نسبة البقاء على قيد الحياة، وهي مستمدة من ثلاث تجارب تجريبية مستقلة على الأقل.

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