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Evaluation of Rifampicin Toxicity and Antimicrobial Efficacy Against Methicillin-Resistant *Staphylococcus aureus* (MRSA 14/0440) Using the *Galleria mellonella* Infection Model

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Abstract

In this study, we injected the Galleria mellonella larvae with rifampicin in a dose-dependent manner to assess the toxicity of the antibiotic in both 1-day and 6-day-old larvae. In the toxicity assay, we monitored larvae survival, health index score, and percentage of melanization to assess the potential toxic effect of rifampicin (antibiotic). Rifampicin at a 40 mg/kg dose percentage of melanization was lower in 1-day-old larvae; the same dose of rifampicin showed a high level of melanization in 6-day-old larvae. In the HISS and survival rate, both 20 and 40 mg/kg rifampicin doses showed less toxicity in Galleria mellonella larvae at 37°C in both 1-day and 6-day-old larvae (age group). In this study, we observed that rifampicin in lower doses (5 mg/kg) has a high level of toxicity. In the efficacy assay, Galleria mellonella larvae were infected with 7.03×10^5 CFU/L MRSA 14/0440 and treated with rifampicin (100, 40, and 5 mg/kg). Here, we observed that among all doses of rifampicin, the 40 mg/kg dose showed a significant level of protection to larvae infected with MRSA 14/0440 (EMRSA-15) in both 1-day-old and 6-day-old larvae and showed a 90% survival rate in 1-day-old and 100% survival in 6-day-old larvae. In hemolymph, rifampicin somehow improves hemocyte density in larvae after 48h post-treatment. In both 1-day and 6-day-old larvae, the larvae treated with rifampicin at 24 h post-infection had a spike in hemocyte density. Especially in 40 mg/kg dose-treated larvae, hemocyte density at 24 h post-treatment was 6.42E+06 cells/ml; after 48 h post-treatment, hemocyte density spiked to 1.48E+07 cells/ml in 1-day-old larvae. Even in 6-day-old larvae, at 24 h post-treatment, hemocyte density was 6.07E+06 cells/ml; after 48 h post-treatment, it spiked to 1.72E+07 cells/ml. The larvae that were infected with MRSA 14/0440 (7.03×10^5 CFU/L) and treated with a 40 mg/kg rifampicin dose showed a spike in hemocyte density. At 24h post-treatment, hemocyte density dropped to 4.50E+06 cells/ml, but after 48h post-treatment, hemocyte density spiked to 1.48E+07 cells/ml in 1-day-old larvae. In 6-day-old larvae, hemocyte density dropped to 5.44E+06 cells/ml at 24h post-treatment; at 48h post-treatment, hemocyte density was spiked to 6.07E+06 cells/ml but not as high as we observed in 1-day-old larvae. Taken together, results of this study suggest that a 40 mg/kg rifampicin dose has low toxicity, a 95% survival rate against MRSA 14/0440 infection, and helps in improving hemocyte density in larvae.

Keywords: *Galleria mellonella, Rifampicin, MRSA, Antimicrobial efficacy, Toxicity, Haemocyte density*

1. Introduction

The clinical investigations and in vivo experiments employing rodent models (rats and mice) were the gold standards in the early phases of research for a better understanding of the physiopathology of infectious illnesses (Scully *et al*., 2006). Because they have similar metabolisms, body temperatures, and immunological responses, vertebrates like mouse models are used as conventional hosts in studies of human infections. According to a 2019 estimate by Bismuth et al., about 75 to 100 million vertebrates get used for scientific research each year, mainly rodent models. In recent years, there has been a growing voice to safeguard animals from being used in research (Pereira *et al*., 2018). As a result, laws have gotten stricter, and obtaining clearance from ethical rule authorizations has taken significantly longer. Furthermore, working on invertebrate models necessitated specific training, legislation, and equipment; animal adaptation time; and significant cost, and the use of many animals for research can be challenging for financial, technical, ethical, and unpredictable data reasons (Menard *et al*., 2021).

The 3Rs (Replacement, Reduction, and Refinement) are widely recognized as a framework for carrying out high-quality scientific studies in the academic and industrial sectors, with a greater emphasis on developing alternatives that do not entail the use of animals (Graham *et al*., 2015). Because of this, it is now possible to provide mechanistic, informative, and toxicological data for drug testing and antimicrobial evaluation using alternative invertebrate models that provide realistic human-like predictions (Cutuli *et al*., 2019). Since the beginning of 1990, especially in the most recent years, there has been much research on these invertebrate models. The earliest invertebrate models were the worm *Caenorhabditis elegans* (*C. elegans*), mature fruit flies, and larvae, namely *Drosophila melanogaster* (*D. melanogaster*). More recently, models have been made from the larvae of the greater wax moth *Galleria mellonella* (*G. mellonella*) (Cutuli *et al*., 2019).

In studies on pathogenesis, virulence mechanisms, immunological responses, and the potential of antibiotic compounds, this latter organism is frequently brought up. From an economic and ethical point of view, utilizing this mini host is preferable to using mammals, and due to its short lifespan, it is suitable for high-throughput research studies. The regulations of ethics have not yet been applied to these invertebrate models (insects and nematodes) or home office legislation (UK) (Hardin-Pouzet et al., 2019). A review study by Freires et al. (2016) showed how the use of alternative research models has significantly increased between 1990 and 2015. *D. melanogaster* (fruit fly) (41.89%), *Danio rerio* (zebrafish) (29.74%), *C. elegans* (roundworm) (26.53%), *G. mellonella* (greater wax moth) (1.14%), and *Artemia salina* (brine shrimp) (0.70%) were the alternative animal models that were employed the most (Freires *et al*., 2017). However, *Galleria mellonella* has recently gained a lot of attention as an invertebrate model, as seen by the more than 3100 published scientific articles to date (search "*Galleria mellonella"* on PubMed). Figure 1.1 shows a definite growth in research that was held on *G. mellonella* from 2016 to 2022.

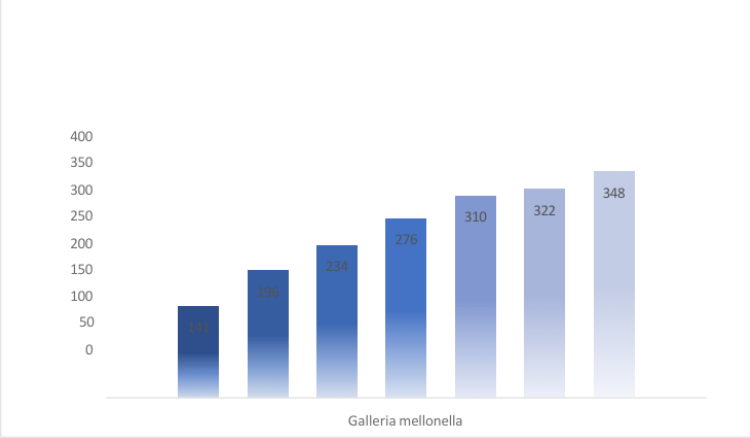

Figure 1.1: The bar chart provides clear information about the number of publications on *G. mellonella* over the period of 2016-2022. Adapted from PubMed.

Many scientists who study the interactions between hosts and pathogens were interested in the findings of recent research that suggest invertebrates have very efficient immune responses to protect them from infectious diseases. The immune system's cellular and humoral reactions work together to promptly recognize, neutralize, or immobilize the pathogen (Sheehan *et al*., 2020). Hemocytes, which are phagocytes that either destroy or immobilize infections, are responsible for mediating the cellular immune response. Melanin and antimicrobial peptides produced by hemocytes are both part of the humoral immune response (Sheehan *et al*., 2020). The various antimicrobial peptides (AMPs) that insects generate can be effective antibacterial and antifungal agents. Although lacking an adaptive immune system, these invertebrates share a similar innate immune system with vertebrates; for example, insect hemocytes show structural and functional similarities to mammalian phagocytic cells (Browne *et al*., 2013).

One of these alternate invertebrate models is *Galleria mellonella*. Recent research/review publications on *G. mellonella* list all the pros and drawbacks of this microhost model in addition to describing some of its drawbacks. The innate immune system of *G. mellonella*, which is controlled by hemocytes, can deal with a wide range of pathogens. Fast and high reproductive rates, low costs, and easy laboratory upkeep without the need for expensive equipment are further intriguing traits. *G. mellonella* can maintain itself in a wide range of temperatures (18°C to 37°C) compared to other invertebrate models (Lionakis *et al*., 2011; Durieux *et al*., 2021), which aids in the research of human infections. The whole genome of *G. mellonella* was sequenced in 2018 (Lange *et al*., 2018), enabling the creation of a biobank with a database like that of other invertebrate models. In addition, it is not possible to perform genetic manipulation. The main disadvantage of this model, according to Eisenman *et al.* (2015), Amorim-Vaz *et al.* (2015), and more recently, Champion *et al.* (2018), is the reproducibility of results when compared with mouse models. The primary causes include the larvae's origin, storage temperature, feeding, genetics, and age. There are no standardized procedures, especially in the obtaining and storage of the larvae.

1.1 Galleria mellonella model:

Galleria mellonella, known also as the wax moth. The phrase "wax moth" describes a variety of moth species that prey on, invade, and harm honeybee colonies and hive productivity. The Lepidopteran order's *Pyralidae* family, which includes *Galleria mellonella*, is divided into *Galleriinae* subfamilies (Kwadha *et al*., 2017). Kwadha *et al*. provided a detailed description of *Galleria mellonella's* whole morphology, characteristics, and life cycle. Wax moth larvae are around 1-3 mm long and 0.12–0.15 mm in diameter when they first hatch; later instar larvae are 25–30 mm long and 5-7 mm wide and weigh approximately 300 mg (Kwadha *et al*., 2017; Firacative *et al*., 2020). The wax moth larvae have eight prolegs on the third to sixth abdominal segments, six legs on the thorax, an anal proleg, a neural system, a silk gland, a dorsal vessel, a Malpighian tubule, a crop, and a digestive system (Figure 1.2). The *Galleria mellonella life* cycle can last anywhere from a few weeks to months, and both biotic factors like food competition, cannibalism, parasiticides, honeybees, and tiny hive beetles as well as abiotic ones like temperature and humidity have an impact. Food composition, particularly when grown in a lab, may influence the duration of the larval stage, the volume of the hemocyte, and the density of the hemocyte. Additionally, other study teams reported varying nutritional conditions for the test larvae, including starving for a week prior to infection vs no starvation and providing food after vaccination vs not providing food after vaccination (Firacative *et al*., 2020). Even hemocyte density and volume will affect susceptibility infection.

Recent studies have found that *Galleria mellonella* larvae provide a quick and reliable way to evaluate toxicity, with the results demonstrating an important correlation to cell lines such as 3T3, human keratinocytes, and those from the mouse model. It's notable to note that a few researchers think that *G. mellonella* can deliver better results for toxicity tests than cell lines. Low-toxicity substances from being classified as harmful may be avoided by using *G. mellonella* instead of cell lines to evaluate toxicity (Serrano *et al*., 2023). *G. mellonella*, for instance, was used to investigate the toxicity of many substances, including chemicals (Dolan *et al*., 2016; Allegra *et al*., 2018), solvents (Suay-Garcia *et al*., 2019), food preservatives (Maguire *et al*., 2016), nanoparticles (Moya-Anderico *et al*., 2021), and ionic liquids (Megaw *et al*., 2015).

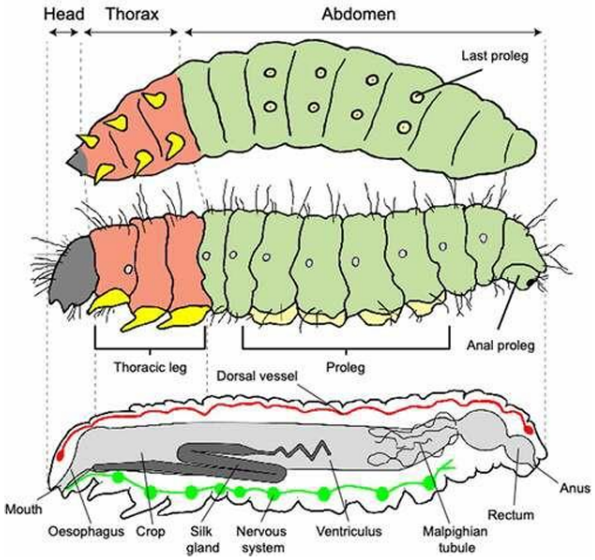

Figure 1.2: Anatomy of larvae of *Galleria mellonella*, taken from Durieux *et al*., 2021.

1.2 MRSA (Methicillin-resistant *Staphylococcus aureus*)

Staphylococcus aureus is one of the most encountered causes of infections in hospitals and the population. The bacteria S. aureus causes anything from minor skin and soft tissue infections (SSTIs) to serious disorders such as endocarditis, osteomyelitis, bacteremia, meningitis, and pneumonia (Lade *et al*., 2023). Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most serious pathogenic forms of Staphylococcus aureus. In comparison to other strains such as methicillin-susceptible *S. aureus* (MSSA), vancomycin-intermediate *S. aureus* (VISA), and vancomycin-resistant *S. aureus* (VRSA). Several antibiotics are used to treat *Staphylococcus aureus*, targeting different bacterial processes like cell wall synthesis, translation, transcription, and DNA synthesis (Vestergaard *et al*., 2019). MRSA is the most challenging kind of *S. aureus* to treat due to its high virulence and resistance to specific antibiotics (Otarigho *et al*., 2023), further complicated due to the emerging strains that are resistant to the oxazolidinone drug, linezolid, through mutation in 23S rRNA and the ribosomal methyltransferase-encoding cfr gene (Haaber *et al*., 2017). Several dynamic genetic factors contribute to the emergence of antibiotic resistance. Antibiotic resistance emerges through multiple kinds of processes, including changed drug targets, enzymatic drug inactivation, enhanced efflux of antimicrobial chemicals, and altered drug accessibility. While resistance has been noted for all substances, specific strains resistant to every medication have not surfaced.

MRSA is responsible for 20–80% of nosocomial *S. aureus* infections and is becoming an increasing concern for medical professionals (Álvarez *et al*., 2019), and 0.9–1.5% of the healthy population are continuously colonized by MRSA (Zhao *et al*., 2023; Turner *et al*., 2019). Diseases caused by MRSA strains are typically challenging to treat since the bacteria develop resistance to all antimicrobials put into clinical use, including last-resort medicines like daptomycin, vancomycin, and linezolid. As a result, the emergence of *S. aureus* in hospital settings has emerged as one of the most important public health problems due to the restricted treatment choices (Witek, K., et al., 2023). Despite advancements in medical care, estimates show that the death rate from multidrug-resistant *S. aureus* bacteremia persists around 15 – 50% (Turner *et al*., 2019). In the 1960s, MRSA initially appeared in hospitals. In the 1980s, it left both the community and hospitals, spreading globally and establishing reservoirs in both environments. Hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) were formerly thought to be epidemiologically distinct clones because of the differences in their acquisition mechanism and pattern of antibiotic resistance (Loewen *et al*., 2017; Abrudan *et al*., 2023). Furthermore, CA-MRSA caused major disease or even death to healthy people, whereas HA-MRSA mostly afflicted specific at-risk populations in hospitals. Since CA-MRSA has grown more invasive, transmissible, and challenging to distinguish from HA-MRSA in recent years, the situation has altered.

In 1961, the United Kingdom detected the first MRSA strain and observed a rapid increase in MRSA infection cases all around the world. The global epidemic MRSA population is unstable clonal structures. CC1, CC5, CC8, CC22, CC30, and CC45 are most frequently reported MRSA clonal structures throughout the world. Among them, CC5 and CC8 are most common (Monecke *et al*., 2023). The ST22 (CC22) MRSA lineage was first identified in the United Kingdom as EMRSA-15. It's the most rapidly transmitted hospital-acquired MRSA clone in Europe. The main concern of this strain (EMRSA-15), which contains the lukS/F gene that encodes Panton-Valentine Leukocidin (PVL), is the increased frequency of severe infection (Witte *et al*., 2007). This PVL toxin is a bicomponent pore-forming toxin that is highly selective for human leukocytes. It is associated with severe, chronic, or recurrent skin and soft tissue infections, as well as rare but extremely deadly necrotizing pneumonia. Due to their location on prophages, the PVL genes (lukF-PV and lukS-PV) can be horizontally transmitted between *S. aureus* strains and lineages (Monecke *et al*., 2023). This ST22-MRSA (EMRSA-15) has a strong possibility to become an epidemic MRSA clone (Witte *et al*., 2008).

1.3 Rifampicin:

Rifampicin (also known as rifampin), a semi-synthetic derivative of rifamycin, was first synthesized in 1965. Rifamycin was initially found in 1957 from the bacterium Nocardia mediterranei. Rifampicin was initially used in clinical treatment in 1968 (Abulfathi *et al*., 2019), and because of its sterilizing properties and capacity to expedite therapy, it is still a key medication for the treatment of TB illness caused by *susceptible bacilli* (Grobbelar *et al*., 2019). Rifampicin exhibits a broad spectrum of antibacterial action against a wide range of pathogens, including mycobacteria, Gram-positive cocci, *Clostridium difficile*, and selective Gram-negative pathogens, including *Neisseria meningitides*, *N. gonorrhoeae*, and *Hemophilus influenza*. Rifampicin is not effective against most Gram-negative infections (Rothstein *et al*., 2016). Additionally, it exhibits a potent bactericidal effect against non-replicable intra-macrophage and drug-tolerant microorganisms (Rinaldi *et al*., 2021).

Rifampicin, an antibiotic drug used in combination therapy, inhibits transcription by binding to the rpoB gene-encoded β-subunit of the bacterial DNA-dependent RNA polymerase (Wang *et al*., 2019). Rifampicin is highly efficient against intracellular infections because it easily diffuses into tissues, live cells, and bacteria (Campbell *et al*., 2001). The 2D structure of rifampicin was observed in Figure 1.3.

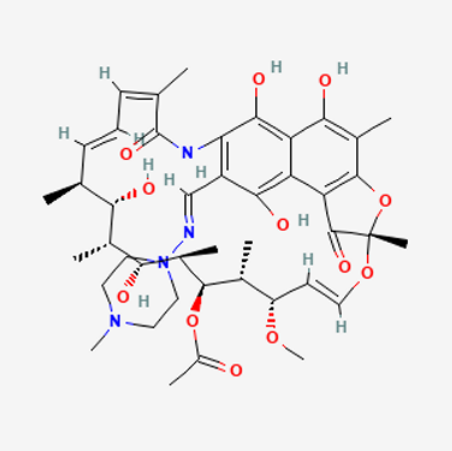

Figure 1.3: 2D structure of Rifampicin, adapted from https://pubchem.ncbi.nlm.nih.gov/compound/Rifampicin

2. Materials and Methods

2.1 Reagents, microorganisms, and insects

Fisher bioreagents, Aldrich chemicals, and Sigma-Aldrich (Merck Life Sciences, UK) were the suppliers of the growth media and reagents. The supplier of *Galleria mellonella* larvae is UK Waxworms Ltd., Sheffield, UK. One of our Ph.D. students provided a clinical isolate of MRSA 14/0440 bacteria. Fisher Scientific was the supplier of rifampicin antibiotics (Fisher Scientific, Leicestershire, UK).

2.2 Bacterial strain

In this work, the MRSA (EMRSA15) strain 14/0440, a clinical isolate that exhibits prominent levels of mupirocin resistance, was used. Mueller Hinton agar was used to subculture the strain, and it was incubated at 37 °C. Following the bacterial growth, one colony from the subculture was inoculated into 10 ml of Mueller Hinton (MH) broth, and it was then incubated overnight at 37 °C at 200 rpm (shaken incubator, aerobic condition). Before infecting the larvae, the overnight culture's OD read at 600 nm (JENWAY 6300 Spectrophotometer) was taken, and the cells were back re-suspended to an OD600 of 0.05 concentration into 50 ml of Mueller Hinton broth for two hours at 37°C and 200 rpm. The cells were then centrifuged for 10 minutes at 4°C at 4200 rpm, and the medium was removed by twice washing them in phosphate-buffered saline (PBS), diluted to a concentration of 10^8 CFU/ml.

2.3 Galleria mellonella larvae

Galleria mellonella larvae are purchased in the final instar stage from UK Waxworms Ltd. On the day of delivery, larvae were sorted by removing larvae with melanization spots, darkening of the cuticle, and small size (below 1 cm); the remaining larvae were weighed on a weighing scale to calculate the average weight of the larvae. Once it is done, half of the larvae are used 1 day after delivery, and the remaining half are used after 6 days. For experiment purposes, larvae are considered 1-day and 6-day old from the time of delivery. Always, larvae weighed between 240 and 280 mg each on average. Half of the larvae were divided into six groups, with 10 larvae in each group placed on sterile petri dishes lined with Whatman paper. The remaining larvae are used 6 days from the time of delivery and are stored in the dark, at room temperature in sawdust.

2.4 Antibiotic preparation

The antibiotic used in this experiment was rifampicin. Rifampicin was dissolved in 100% DMSO. This experiment used varying concentrations of rifampicin (100 mg/kg, 80 mg/kg, 40 mg/kg, 20 mg/kg, and 5 mg/kg). The average weight of the larvae—which varies depending on the experiment—is used for determining these concentrations. To get the final concentration of 142.50 mg/ml, 142.50 mg of rifampicin was diluted in 3.25 ml of DMSO to make the primary stock. Rifampicin's remaining concentrations were made by diluting the primary stock with 100% DMSO.

By using basic formula $C_{1}V_{1} = C_{2}V_{2}$ concentration calculation:

Dose per larva = 1/average weight of larvae. Dose/larvae = Dose per kg / Dose per larva.

Concentration = Dose per larva × 50 (times concentrated)

2.5 Determination of the infection dose of MRSA 14/0440

The spread plate technique was utilized to ascertain the infectious dose of MRSA. After centrifuging the pellet at 4200 rpm for 10 minutes at 4°C, it was diluted into 1 ml of phosphate-buffered saline (PBS) that makes up a concentration of 1.25×10^8 CFU/ml. The 1 ml of culture was subsequently re-suspended into 2 ml using the formula $C_1V_1 = C_2V_2$, in which the larvae were infected. Prior to infection, 100 µl was used in a 10-fold serial dilution up to 10^6 using the 2 ml. Spread plates with dilution factors 10^5 and 10^6 were plated and incubated at 37°C for 24h to determine the infection dose. In these experiments, the infection dose was 7.03×10^5 CFU/L in 20 µl.

2.6 Rifampicin toxicity test procedure

Six groups, each group with 10 larvae (240-280 mg each), were injected with a 27-gauge syringe (Sigma-Aldrich, St. Louis, USA). Each group of larvae was inoculated with different concentrations of rifampicin in 1% DMSO (100 mg/kg, 80 mg/kg, 40

2.7 Galleria mellonella larvae infection

G. mellonella larvae weighed 240-280 mg each and were used in all experiments. The larvae are sorted as described in 2.3. Once sorted into groups, larvae are incubated at 37°C for 2h before inoculation. During that time, MRSA 14/0440 culture concentrations of 10^7 CFU ml^{-1} and 10^8 CFU ml^{-1} were prepared. After 2h pre-incubation, each treatment group larva was inoculated with 20 µl of 10^7 CFU ml^{-1} (n=1) and 10^8 CFU ml^{-1} (n=2) through the top right proleg using an insulin syringe, and a control group larva was inoculated with 20 µl PBS. Larvae are incubated for 2h post-infection at 37°C.

2.8 Rifampicin efficacy test procedure

The rifampicin toxicity test gave a clear idea to use 100 mg/kg, 40 mg/kg, and 5 mg/kg concentrations for the rifampicin efficacy test. After 2 h of post-infection, each treatment group's larvae were inoculated with 20 µl of 100 mg/kg, 40 mg/kg, and 5 mg/kg concentrations through the last left proleg using an insulin syringe, and control group larvae were inoculated with 20 µl of 1% DMSO. Larvae are incubated in the dark at 37°C throughout the experiment. The same process was used in both 1-day-old and 6-day-old larvae from the time of delivery. Each experiment was repeated two times (n=2) using larvae from different batches each time. To assess the dose-dependent effect of rifampicin against MRSA 14/0440 (methicillin-resistant *Staphylococcus aureus*).

2.9 Monitoring of *Galleria mellonella* larvae

Larvae that had been inoculated or infected were examined individually every day for seven days. to assess each larvae's activity, melanization, cocoon development, and survival separately. Every day, the following items were checked to assess the larvae's overall health index. The appropriate scale for measuring *G. mellonella* larvae is the health index score. To determine the overall health index score for each treatment group, a distinct score was assigned to each category, which was then added up. It provides an understandable overview of every substance used in a dose-dependent manner. Typically, healthy, uninfected, and uninoculated larvae receive a total score of 9, while dead or black larvae receive a score of 0 (Figure 2.1).

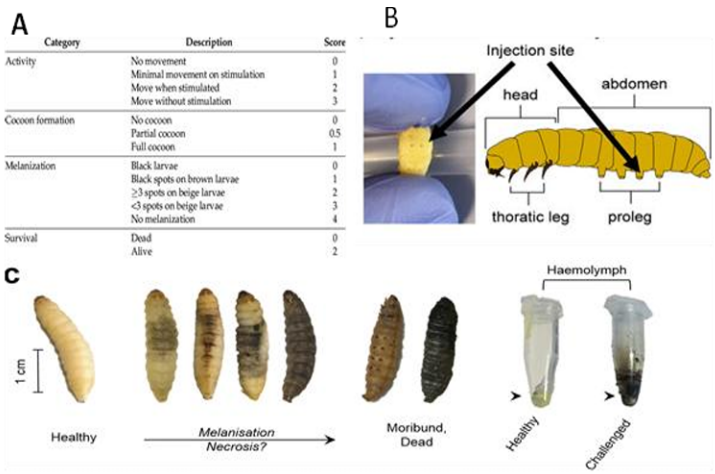

Figure 2.1: G. mellonella larvae health index score system. A: scoring system for larvae post-inoculation/infection. B: injection site of larvae. C: Larval melanization: uninfected larvae show no melanization (4), <3 melanization spots (3), ≥ 3 melanization spots (2), black spots on brown larvae (1), or black larvae (0). Table (A) and figure (B) are adapted from a Google image. Figure (C) is taken from Coates *et al*. (2019).

2.10 Determination of hemocyte density

To measure hemocyte density, the hemolymph was extracted from three *G. mellonella* larvae by bleeding them into a microcentrifuge, which is coated with N-phenylthiourea to prevent melanization. After that, 10 µl of hemolymph was used in a 10-fold dilution in 0.37% (v/v) of 2-mercaptoethanol-supplemented PBS, and the density of hemolymph was determined by using a hemocytometer. Hemocyte density was expressed in the form of hemocyte density/ml. Experiments were conducted two times (n=2), and the means ± SD were determined.

2.10.1 Determination of rifampicin activity of hemocytes

Larvae of *G. mellonella* are divided into three groups, each having eight larvae. Subsequently, the larvae in each treatment group were injected into the final right proleg with 20 µl of rifampicin (100 mg/kg, 40 mg/kg, and 5 mg/kg) and incubated for 48 hours at 37°C. Three larvae from each concentration were extracted for hemolymph every 24 hours to measure the hemocyte density. The means ± standard deviation were calculated after the experiments were run twice (n = 2).

2.10.2 Determination of rifampicin activity on hemocytes with MRSA 14/0440 load

G. mellonella larvae are infected with 7.03 × 10^5 CFU/L MRSA 14/0440 in the last right proleg and incubated at 37°C for 2 h. After 2 h, each group of larvae was injected with 20 µl of rifampicin (100 mg/kg, 40 mg/kg, and 5 mg/kg) into the last left proleg and incubated for 48 h at 37°C. After every 24h, hemolymph was extracted from three larvae from each concentration group for assessing the hemocyte density.

3. Results

3.1 Dose-dependent effect of Rifampicin on G. mellonella larvae.

Evaluated the percentage of melanization (<3 spots) in the larvae of *G. mellonella* to examine the dose-dependent effect of rifampicin. The melanization graphs of the 1-and 6-day-old larvae (from the time of delivery) were analyzed, and the results showed no discernible variations between the rifampicin doses and the 1% DMSO control. The melanization graph of 1-day-old larvae revealed that (Figure 3.1, A), although 40 mg/kg and 100 mg/kg were compared with 1% DMSO, The ANOVA general linear model comparison test (Tukey pairwise comparison), p = 1.000, showed the same level of melanization in the larvae at 20 mg/kg (rif) and 1% DMSO (control). The General Linear Model comparison test (Tukey pairwise comparison) indicates a marginal shift in the percentage of melanization (40 mg/kg, p = 0.438; 100 mg/kg, p = 0.380). However, there is not a significant distinction between 100 mg/kg (rif) and 40 mg/kg (rif) (P = 1.00). The percentage of melanization in the 40 mg/kg (rif) larvae, which were 6 days old (measured from the time of delivery) (Figure 3.1, B), showed a slight variation in P value when compared to the 1% DMSO (p = 0.508), 5 mg/kg (p = 0.414), 20 mg/kg (p = 0.140), 80 mg/kg (p = 0.589), and 100 mg/kg (p = 0.082). When compared to the remaining doses and 1% DMSO (control), the overall observation reveals that in 1-day-old larvae, 100 mg/kg rifampicin exhibits a lower percentage of melanization and 1% DMSO (control) shows high levels, and in 6-day-old larvae, 40 mg/kg rifampicin exhibits a higher percentage of melanization compared to the remaining doses.

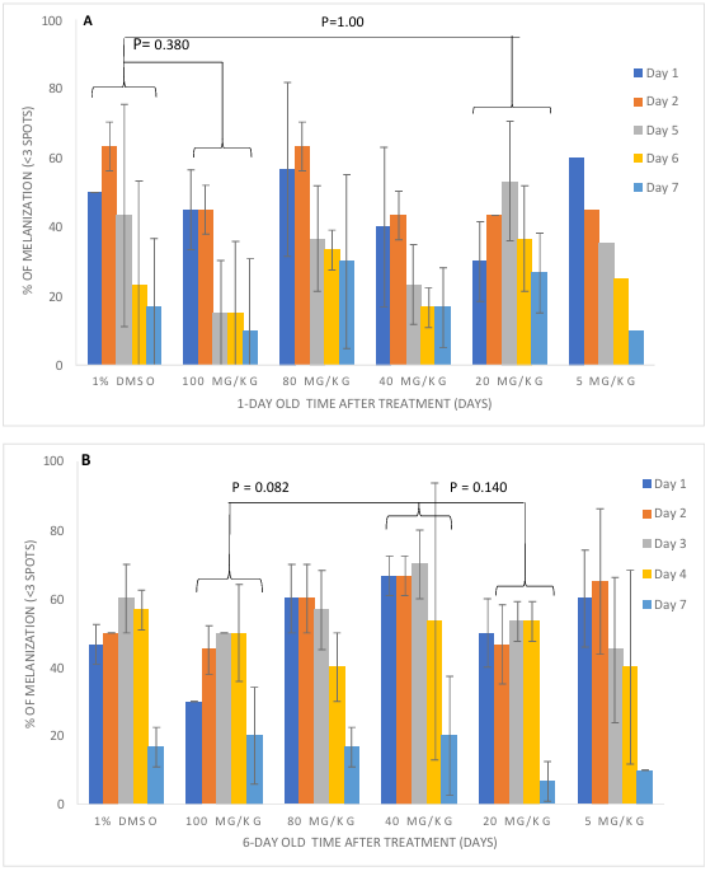

Figure 3.1: Dose- and time-dependent effect of rifampicin on *G. mellonella* larvae melanization status 1 day and 6 days from time of delivery. Larvae were injected with 20 µl of rifampicin and the control with 20 µl of 1% DMSO and subsequently incubated at 37°C. Larvae were scored based on melanization (< 3 spots). Results and mean ± SD of three independent tests. **A:** Melanization score of 1-day-old larvae (from time of delivery); **B:** Melanization score of 6-day-old larvae (from time of delivery). The statistical difference is as compared (Tukey pairwise comparison) to 1% DMSO (control) and different doses of rifampicin (ns, no significant differences (two-way ANOVA, general linear model)).

3.2 Toxicity of Rifampicin on *G. mellonella* is dose- and time-dependent and the HISS.

By comparing the mean survival rate and larval health using the HISS (Health Index Scoring System), it was possible to determine if *G. mellonella* larvae could be used to evaluate the toxicity of rifampicin. After analyzing the HIS (Health Index Score) graph (Figure 3.2) of *G. mellonella* larvae that were one day and six days old (as of the time of delivery), results revealed a substantial difference. Figure 3.2A comparison was made between larvae injected with a dose of 20 mg/kg and those injected with 1% DMSO (control) ($p = 0.001$), as well as 40 mg/kg and 1% DMSO (control) ($p < 0.05$). In addition, there were no discernible differences between the remaining doses (100, 80, and 5 mg/kg) and their health index scores. The Health Index Score (HIS) graph (figure 3.2B) of 6-day-old larvae revealed no significant difference in the rifampicin and 1% DMSO (control) treatments. On the other hand, the level of health score was similar for 40 mg/kg of rifampicin and 1% DMSO (control) ($p = 1.00$); no discernible variations in the p-value were noted. Overall, Figures 3.2A and 3.2B demonstrate that both 1-day and 6-day-old larvae exhibit significant levels of toxicity when exposed to 5 mg/kg rifampicin and 1% DMSO. In contrast, compared to the remaining rifampicin dosages, 20 mg/kg showed modest levels of toxicity. In survival graphs of 1-day-old (Figure 3.3.A) and 6-day-old (Figure 3.3.B) larvae, there was no mortality in 20 mg/kg and 40 mg/kg and in 1-day-old larvae and 20 mg/kg, 40 mg/kg, and 80 mg/kg of rifampicin in 6-day-old larvae. In both age groups, the mortality rate was less than 20%. While significant differences in mortality rates were observed between 20 mg/kg rifampicin and 1% DMSO (control) ($p = 0.001$) and 40 mg/kg rifampicin and 1% DMSO (control) ($p = 0.001$), no significant differences were observed in the mortality rate of 5, 80, and 100 mg/kg rifampicin with 1% DMSO (control) in Figure 3.3A. In the 6-day-old larvae survival graph (figure 3.3.B), no significant differences were observed between the mean mortality rate of 20 mg/kg ($p = 0.098$), 40 mg/kg ($p = 0.098$), 80 mg/kg ($p = 0.098$), and 1% DMSO. No significant difference was observed between 20 mg/kg – 5 mg/kg ($p = 0.061$, pairwise comparison) and 40 mg/kg and 80 mg/kg rifampicin with 5 mg/kg ($p = 0.061$, pairwise comparison). An overall observation of Figures 3.3A & 3.3B shows that 80 mg/kg showed a high death rate when compared to other doses of rifampicin in 1-day-old larvae. In contrast, the same concentration of rifampicin, 80 mg/kg, showed a zero-death rate in 6-day-old larvae (figure 3.3B), while 5 mg/kg showed a high death rate.

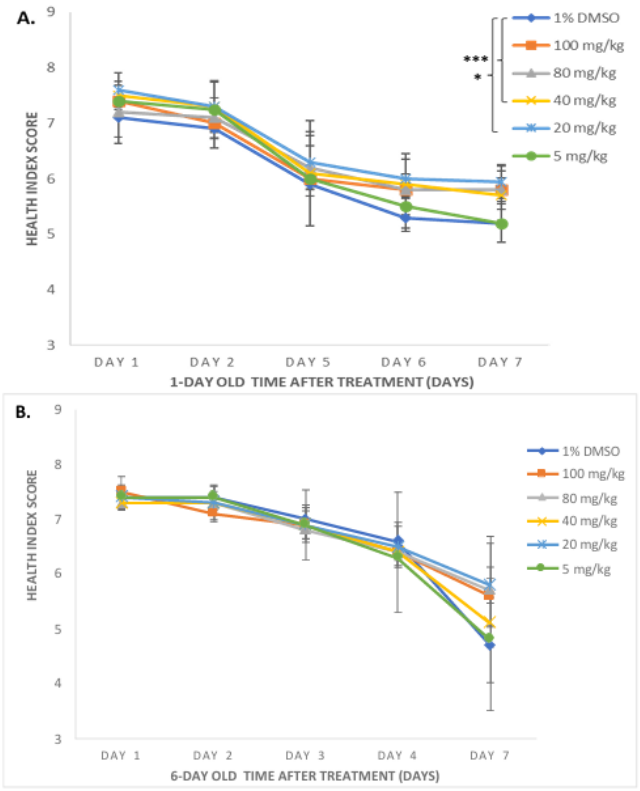

Figure 3.2: Dose-dependent effect of rifampicin on *G. mellonella* larvae. Larvae were injected with a dose of 100 mg/kg, 80 mg/kg, 40 mg/kg, 20 mg/kg, and 5 mg/kg and a control with 20 µl of 1% DMSO and subsequently incubated at 37°C. Larvae health was scored based on activity, cocoon formation, melanization, and survival. Results are mean ± SD of three independent tests. Statistical differences were compared to 1% DMSO (control). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, no significant differences (two-way ANOVA) (general linear model). **A:** Health index score of 1-day-old larvae (from time of delivery); **B:** Health index score of 6-day-old larvae (from time of delivery).

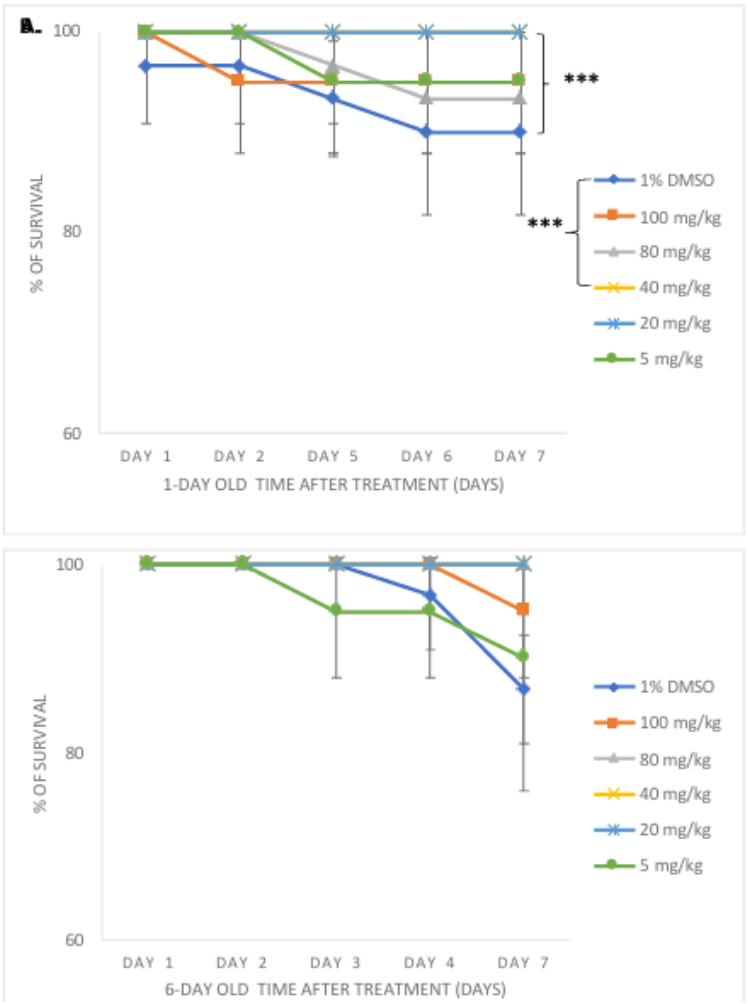

Figure 3.3: Survival of *G. mellonella* larvae injected with different doses of rifampicin. Larvae were injected with a dose of 100 mg/kg, 80 mg/kg, 40 mg/kg, 20 mg/kg, and 5 mg/kg and a control with 20 µl of 1% DMSO and subsequently incubated at 37°C. Results are mean ± SD of three independent tests. Statistical differences were compared to 1% DMSO (control). ***P < 0.001; ****P < 0.0001; ns, no significant differences (two-way ANOVA, general linear model) (Tukey pairwise comparison). **A:** percentage of survival, 1-day-old larvae (from time of delivery) were injected with different doses of rifampicin; **B:** percentage of survival, 6-day-old larvae (from time of delivery) were injected with different doses of rifampicin.

3.3 MRSA 14/0440 (Methicillin-resistance *Staphylococcus aureus*) virulence in *Galleria mellonella* larvae.

The results showed MRSA 14/0440 has a time- and dose-dependent virulence in *Galleria mellonella* larvae, as the effect of the two doses on larvae survival varies at each time point and throughout the course of observation. For instance, Figure 3.3A 7.03E+05 CFU shows a gradual decrease in mortality till day 5; after that, the mortality rate stabilized at 40 percent by day 7. At the same time point, 2.8E+05 CFU treatment was almost 80 percent; larvae survival was observed. In contrast, 2.8E+05 CFU MRSA 14/0440 shows only 40 percent larval survival in 6-day-old larvae (Figure 3.4B). In both 1-day- and 6-day-old larvae, a 7.03E+05 CFU infection in *Galleria mellonella* larvae showed a gradual decrease in survival of larvae till day 5; after that, stability of larvae survival was observed at 40%. Additionally, 2.8E+05 shows a steady survival rate of larvae till day 5 at 90 percent, but on day 6 and day 7, the survival rate dropped to 40 percent in 6-day-old larvae (Figure 3.4B) and another 10 percent in 1-day-old larvae (Figure 3.4A). In the graph (figure 3.4A), there is an observed significant difference between 7.03E+05 CFU and 2.8E+05 CFU (ANOVA, General Linear Model, ****: $p < 0.0001$) and 7.03E+05 CFU and controls (1% DMSO & PBS + 1% DMSO) (ANOVA, General Linear Model, ****: $p < 0.0001$), but no significant variations between 2.8E+05 CFU and controls (1% DMSO & PBS + 1% DMSO).

In contrast, in 6-day-old larvae (figure 3.4B), a significant difference exists between all the 7.03E+05 and 2.8E+05 and controls (1% DMSO & PBS + 1% DMSO) (ANOVA, General Linear Model, pair-wise comparison, **: $p < 0.01$, ****: $p < 0.001$). The significant difference between 7.03E+05 CFU and 2.8E+05 CFU in 6-day-old larvae ($p = 0.018$) (from the time of delivery) differences were very low when compared to a significant difference in 1-day-old larvae ($p = 0.000$). It provides a clear view that the age of the larvae plays an important role in the virulence of bacteria cultures.

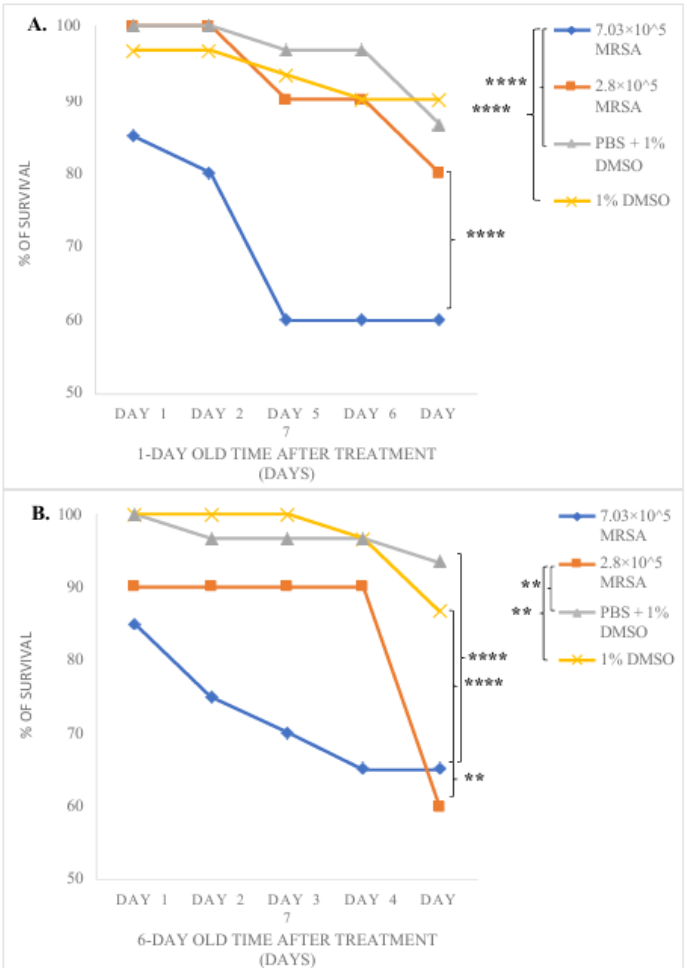

Figure 3.4: *Galleria mellonella* larvae susceptibility to MRSA 14/0440 (clinical isolate, CC22) infection is dose dependent. Bacteria cultures were grown in MH broth for 2 hours, washed twice, and resuspended in PBS. Larvae were inoculated with a dose of 7.03×10^5 CFU and 2.8×10^5 CFU, and controls with 20 µl of PBS and 1% DMSO were incubated at 37°C. Results are mean ± SD of two independent tests. Statistical comparisons are done by using ANOVA, the general linear model, and pairwise comparison. **A:** percentage of survival of 1-day-old larvae. No significant difference was observed between controls and 2.8×10^5 CFU (TWO-ANOVA, General Linear Model, ***: $p < 0.0001$). **B:** percentage of survival in 6-day-old larvae (TWO-ANOVA, General Linear Model, **: $p < 0.01$, ****: $p < 0.0001$).

3.4 Antimicrobial efficacy testing in vivo and the HISS.

To assess the slightest differences in larvae, the Modified Health Index Score System (HISS) was used. The HISS evaluates larvae activity, cocoon formation, and melanization in addition to survival (Figure 2.1A).

In 1-day-old larvae (from the time of delivery) (Figure 3.5A), rifampicin provided a shield to *Galleria mellonella* larvae against MRSA 14/0440 (clinical isolate, CC22, EMRSA-15). For instance, compared to 100 and 5 mg/kg doses, Figure 3.5A demonstrated that a 40 mg/kg rifampicin dosage offers a significant level of protection against the MRSA 14/0440 strain. When it comes to protecting larvae from MRSA infection, the lowest dose of rifampicin—5 mg/kg—showed a significant difference from doses of 40 and 100 mg/kg. One-day-old larvae treated with 100 and 40 mg/kg of rifampicin doses showed comparable immunity to those infected with 7.03×105 CFU/L of MRSA 14/0440, followed by a dosage of 5 mg/kg. Notably, there were no significant differences between the dose of 5 mg/kg and MRSA (7.03×105 CFU/L) + 1% DMSO (positive control).

Each dosage of rifampicin offers a high level of protection to MRSA 14/0440-infected larvae (7.03×10^5 CFU/L) in 6-day-old larvae (figure: 3.5B). A statistically significant variation was noted between the rifampicin dosages and the positive control (MRSA + 1% DMSO) in Figure 3.5B. Day 7 post-infection revealed the same level of health score index for all rifampicin dosages (100, 40, and 5 mg/kg) and the negative control.

Overall observation of both 1-day- and 6-day-old larvae HIS graphs revealed that a 40 mg/kg dose of rifampicin protects larvae from MRSA infection compared to 100 and 5 mg/kg rifampicin doses; even 100 mg/kg also showed the same level of protection. According to HISS, the 5 mg/kg concentration that protects G. mellonella larvae is greater in 6-day-old larvae than in 1-day-old larvae. Set that each dosage of rifampicin gave 6-day-old larvae a consistent shield against MRSA 14/0440 infection for the duration of the experiment, based on the HISS score. On the other hand, rifampicin dosages administered to 1-day-old larvae likewise function as a shield, albeit less successfully than those administered to 6-day-old larvae (Figure 3.5B).

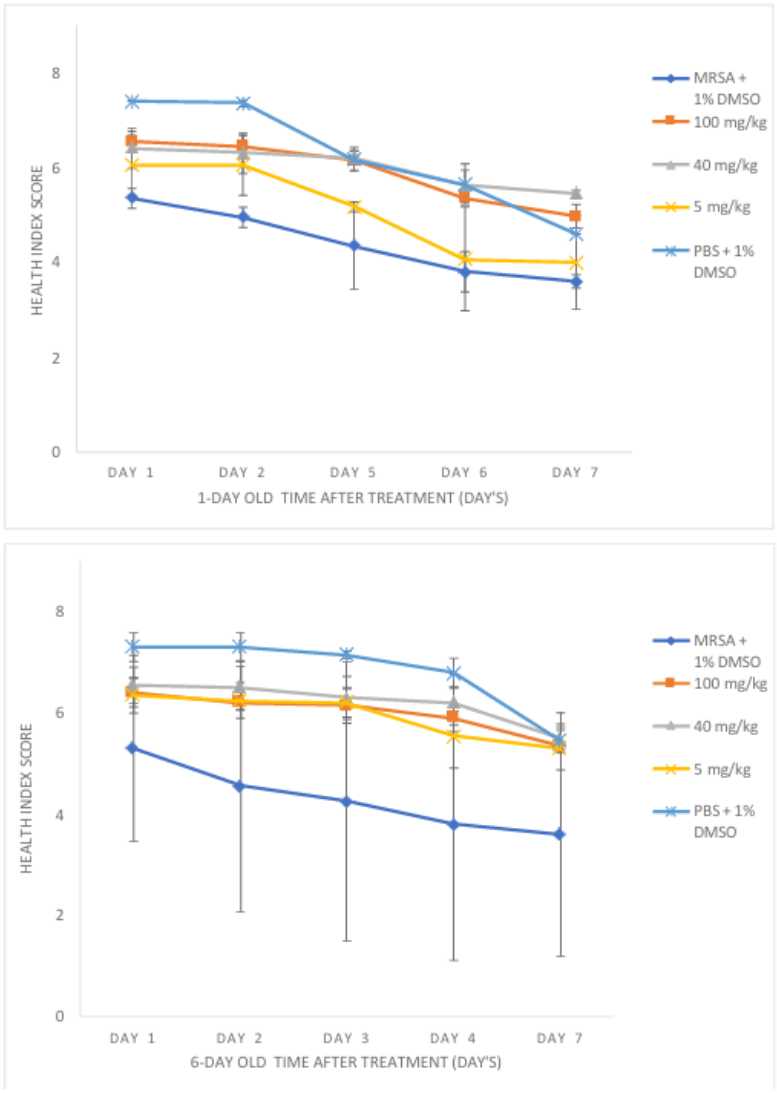

Figure 3.5: Dose-dependent effect of rifampicin on 7.03E+05 CFU MRSA 14/0440 in *Galleria mellonella* larvae's health post-infection. Larvae (n=10) were infected with 7.03E+05 CFU MRSA 14/0440 and incubated for 2 h at 37°C and inoculated with different doses of rifampicin and controls with 20 µl of PBS + 1% DMSO (negative control) and MRSA + 1% DMSO (positive control) and incubated at 37°C. Results are mean ± SD of two independent tests.

3.5 Antimicrobial efficacy testing in vivo and the survival rate.

The effect of a single dose of rifampicin (antibiotic) 2 h post-infection with MRSA 14/0440 (clinical isolate, EMRSA-15, inoculum 7.03E+05 CFU/L) on the survival of *Galleria mellonella* larvae is shown in figure 3.6. One-day-old and 6-day-old larvae (from the time of delivery) that have been infected with the MRSA 14/0440 strain showed dose-dependent increases in larvae survival.

In 1-day-old larvae (figure 3.6A), significant differences were observed between positive (MRSA + 1% DMSO) and negative control (PBS + 1% DMSO), and significant differences within different doses of rifampicin with the positive control (MRSA + 1% DMSO) were 100 mg/kg (rif) and 40 mg/kg (rif) – MRSA + 1% DMSO; the survival rate of larvae was 90%. In 5 mg/kg (rif) – MRSA + 1% DMSO, the survival rate of larvae was 75 ± 21.21%. No significant differences were observed when different doses of antibiotics were compared to PBS + 1% DMSO. In 1-day-old larvae, rifampicin 100 and 40 mg/kg doses showed a similar level of mortality rate throughout the investigation: a 90 percent survival rate. The larvae infected with 7.03E+05 CFU/L MRSA + 1% DMSO (positive control) showed that the larvae survival rate dropped to 60 percent.

In 6-day-old larvae (Figure 3.6B), different doses of rifampicin showed a high level of larvae survival, and both PBS + 1% DMSO (negative control) and 40 mg/kg (rif) showed a 100% survival rate of larvae when compared to larvae infected with MRSA 14/0440 (7.03E+05 CFU/L). Significant differences were observed when comparing 100 mg/kg (rif), 40 mg/kg (rif), and PBS + 1% DMSO (negative control) with MRSA + 1% DMSO (positive control). In 6-day-old larvae, the survival rate dropped to 65 percent in larvae that are infected with MRSA 14/0440 (positive control).

Overall observation of both 1-day-old and 6-day-old larvae survival graphs revealed that both 100 mg/kg and 40 mg/kg rifampicin-treated groups showed high levels of survival rates in both age groups. Figures 3.6A & 6B showed that 5 mg/kg (rif) provided more shield to 6-day-old (95% survival) than 1-day-old larvae (75% survival). The larvae infected with 7.03E+05 MRSA 14/0440 and treated with doses of antibiotic (rif) showed higher levels of survival rate in 6-day-old larvae than in 1-day-old larvae. Significant differences were observed between 1-day and 6-day-old larvae survival graphs. In terms of negative control (PBS + 1% DMSO), the survival rate was dropped to 80 percent in larvae treated with positive control. In contrast, the survival rate of the positive control group in 6-day-old larvae was 100 percent.

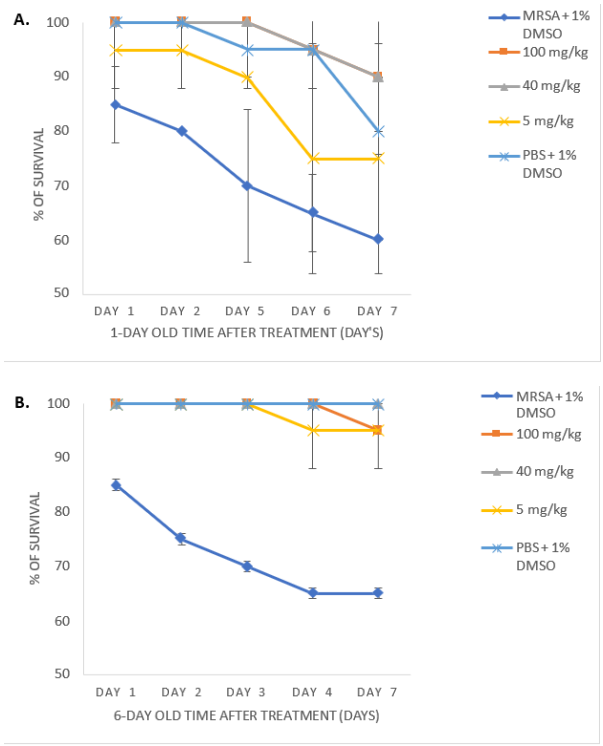

Figure 3.6: Dose-dependent mortality effect of rifampicin on 7.03E+05 CFU MRSA 14/0440 *Galleria mellonella* larvae survival rate. Larvae (n=10) were infected with 7.03E+05 CFU MRSA 14/0440 and incubated for 2 h at 37°C and inoculated with different doses of rifampicin and controls with 20 µl of PBS + 1% DMSO (negative control) and MRSA + 1% DMSO (positive control) and incubated at 37°C. Results are mean ± SD of two independent tests. **A:** survival rate of antimicrobial efficacy tests in 1-day-old larvae. **B:** the survival rate of antimicrobial efficacy tests in 6-day-old G. mellonella larvae.

3.6 Hemocyte density and function in response to doses of rifampicin and MRSA + doses of rifampicin

Inoculation of larvae with rifampicin (100 mg/kg, 40 mg/kg, and 5 mg/kg) resulted in the spike of hemocyte density at 24 h: 100 mg/kg 9.13 × 10^6 cells/ml, 40 mg/kg 6.42 × 10^6 cells/ml, and 5 mg/kg 6.44 × 10^6 cells/ml as compared to the control (no treatment) 6.09 × 10^6 cells/ml in 1-day-old larvae. There was also a spike in hemocyte density of larvae infected with MRSA 14/0440 (7.03 × 10^5 CFU/L) and treated with rifampicin (100 mg/kg, 40 mg/kg, and 5 mg/kg). At 24 h, hemocyte density in MRSA + 100 mg/kg (9.08 × 10^6 cells/ml) and MRSA + 5 mg/kg (6.92 × 10^6 cells/ml). Larvae infected with MRSA14/0440 and treated with 40 mg/kg showed a decrease in hemocyte density (4.50 × 10^6 cells/ml) when compared to larvae with no treatment and larvae inoculated with a 40 mg/kg rifampicin dose. In contrast, at 48h hemocyte density was spiked to 1.48 × 10^7 cells/ml in larvae treated with MRSA + 40 mg/kg, and the same spike was observed in 40 mg/kg rifampicin treatment in 1-day-old larvae. 5 mg/kg rifampicin showed a spike in hemocyte density after 48h. In 6-day-old larvae (Figure 3.7B), a similar kind of spike was observed in 1-day-old larvae. 6-day-old larvae: at h, the hemocyte density was spiked to 10^7 cells/ml in treatments of 100 mg/kg, 40 mg/kg, and 5 mg/kg. In larvae treated with MRSA + doses of rifampicin, significant differences were observed at 24 h compared to the no-treatment group (7.67 × 10^6 cells/ml). At 48 h, a decrease in hemocyte density was observed in MRSA + 100 mg/kg (3.69 × 10^6 cells/ml) and MRSA + 5 mg/kg (4.27 × 10^6 cells/ml), but a small spike was observed in MRSA + 40 mg/kg-treated larvae (6.07 × 10^6 cells/ml) at 24 h, with hemocyte density at 5.44 × 10^6 cells/ml. An overall observation of larvae with different treatments showed that a 40 mg/kg rifampicin dose spiked the larvae's hemocyte density at 24-hour time intervals. In larvae infected with MRSA 14/0440, 40 mg/kg somehow spikes the hemocyte density of larvae and protects larvae from death. In both 1-day-old (Figure 3.7A) and 6-day-old (Figure 3.7B) larvae, it was observed that larvae infected with MRSA and treated with 100 mg/kg and 5 mg/kg decreased in hemocyte density after 48 h, but 40 mg/kg showed a significant increase in hemocyte density in larvae treated with or without MRSA 14/0440 infection. Significant differences were observed in hemocyte density in larvae treated with rifampicin compared to no-treatment larvae (Figure 3.7).

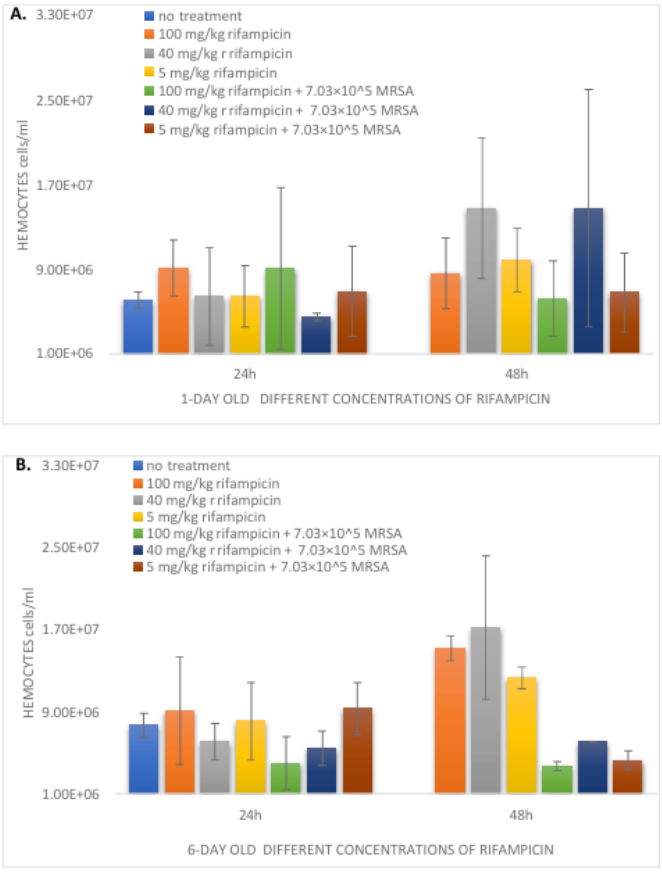

Figure 3.7: Changes in hemocyte density in *G. mellonella* larvae inoculated with the MRSA 14/0440 (7.03E+05 CFU/L) strain and different doses of rifampicin (antibiotic). Larvae (n=10) were infected with 7.03E+05 CFU/L MRSA 14/0440 and incubated for 2 h at 37°C and inoculated with different doses of rifampicin, and larvae were injected with only rifampicin doses and incubated at 37°C. After every 24 h, hemolymph was collected from 3 alive larvae, and haemocyte density was quantified on a hemocytometer. Results are mean ± SD of two independent tests. A: hemocyte density in 1-day-old larvae (from time of delivery). B: hemocyte density in 6-day-old larvae.

Discussion

Galleria mellonella larvae have recently been employed as an infection model for bacterial, fungal, and viral diseases to investigate antibiotic treatment effectiveness, dosage, and toxicity. A large number of studies are also being conducted on *Galleria mellonella* larvae to evaluate the toxicity of various substances such as chemicals (Dolan *et al*., 2016; Allegra *et al*., 2018), solvents (Suay-Garcia *et al*., 2019), food preservatives (Maguire et al., 2016), nanoparticles (Moya-Anderico *et al*., 2021), and ionic liquids (Megaw *et al*., 2015) and the efficacy of antimicrobial agents (Peleg *et al*., 2009; Mylonakis *et al*., 2005; Debois *et al*., 2010; Kaskatepe *et al*., 2023; Jeon *et al*., 2019). Larvae of *Galleria mellonella* are a promising model that provides an ethically free platform for testing various substances and therapies. Moreover, this model has the potential to drastically reduce the number of vertebrate models used in medication and infection research. Furthermore, this model's primary benefit over other invertebrate models is its capacity to sustain human body temperature, which aids in the development of accurate infection and medication dosage calculations. *Galleria mellonella* larvae do, however, have several disadvantages and limitations, the most important of which are associated with the lack of established protocols, particularly with regard to age and nutrition.

When treating larvae, several aspects need to be taken into account, such as their age, size, food availability, physical stress from transit, and storage temperature (Champion *et al*., 2018). Different study teams reported testing larvae under different nutritional settings, such as starving them for a week before infection vs not feeding them at all (Firacative *et al*., 2020). The current study, however, confirms that the larvae used immediately after delivery and the larvae starved for six days before infection exhibited significant differences in each experiment. It also found that the larvae stored in a sterile petri dish with Whatman paper a week before infection consumed the paper and formed holes in it. In our study, we repeated the same type of experiment in larvae that were one day and six days old (from the time of delivery) to see how the age of the larvae can affect the outcome of the experiment. The age of the larvae should be regarded as an important component to any kind of experiment performed by using *Galleria mellonella* larvae. Both the toxicity and effectiveness findings for larvae that were one day and six days old showed a significant difference. It demonstrates that the age of the larvae ought to be taken into account in all types of experiments. Though technically the larvae used in the experiment were not 1-day-old or 6-day-old, if we were to produce larvae in a lab and perform the same experiment, we could state with certainty that the age of the larvae can affect the outcome of the experiment.

The toxicity and effectiveness of antimicrobial drugs have previously been assessed using *Galleria mellonella* larvae; however, this work is the first to demonstrate the toxicity of rifampicin, in particular, and the efficacy of a clinically licensed antibiotic against MRSA 14/0440 infection. Fascinatingly, rifampicin showed efficacy in *Galleria mellonella* larvae at a dose of 40 mg/kg, which was comparable to the effective dose of 50 mg/kg vancomycin and daptomycin in MRSA infection (Desbois *et al*., 2011). In the Galleria mellonella model, rifampicin at 40 mg/kg likewise showed minimal toxicity in 1-day-old larvae; however, the same dose demonstrated greater levels of toxicity in 6-day-old larvae compared to doses of 100, 80, and 20 mg/kg. Furthermore, the amount of toxicity in both 100 mg/kg and 5 mg/kg was the same in both 1-day-old and 6-day-old larvae (Figure 3.1). Interestingly, the larvae injected with 100 mg/kg and 80 mg/kg rifampicin excreted approximately 30% of the administered dose in the form of urine and fecal matter; it was clearly observed on Whatman paper due to the strong orange color of the antibiotic (rifampicin). It was almost similar in humans; approximately 30% of the administrated drug was eliminated through urine (Beloor suresh *et al*., 2023). We didn’t observe excretion of antibiotics (rifampicin) in lower doses like 40, 20, and 5 mg/kg. It showed that even by injecting a higher dose of antibiotic into larvae, we couldn’t ensure that the drug was 100% showing its effect against infection in *Galleria mellonella* larvae.

According to Sheehan *et al*. (2020), invertebrates have a very effective immune response to protect them from infectious pathogens; hemocytes are phagocytes that either destroy or immobilize the infection and are responsible for the cellular immune response. Browne *et al*., 2013. State that these invertebrates share a similar innate immune system to vertebrates and show structural and function similarities with mammalian phagocytic cells. In our study, larvae are injected with rifampicin in a dose-dependent manner, resulting in a significant change in hemocyte density in both 1-day and 6-day-old larvae at 48h. After injecting rifampicin in a dose-dependent way, at 24 h only 100 mg/kg showed a significant change in hemocyte density when compared to no treatment (negative control), but 40 and 5 mg/kg showed a similar hemocyte density to no-treatment larvae (negative control). The same experiment was conducted by infecting the larvae with MRSA 14/0440 and treated with rifampicin in a dose-dependent manner to assess the changes in hemocyte density. Infecting the larvae with 7.03×10^5 CFU/L MRSA resulted in a significant increase in hemocyte density in 100 and 5 mg/kg at 24 h in 1-day-old larvae.

In 6-day-old larvae, hemocyte density dropped significantly in both 100 mg/kg and 40 mg/kg, except for 5 mg/kg at 24h. After 48h, larvae infected with 7.03×10^5 CFU/L MRSA and treated with 100 mg/kg (higher concentration) and 5 mg/kg (lowest concentration) showed a significant decrease in hemocyte density in both 1-day and 6-day-old larvae. In both cases, 40 mg/kg treated larvae hemocyte density dropped initially at 24 h, but after 48 h hemocyte density increased. We don't know how rifampicin at 40 mg/kg improves hemocyte density in both with and without MRSA infections. According to Ratcliffe NA, 1985. There is a possibility of an increase in hemocyte density after infection due to the release of hemocytes, which are attached to the linings of internal organs such as body fat. Even if the hemocyte is attached to an internal organ or fat, it goes into or is released into the system right after the infection, but in this study, we collected the hemocytes from 3 larvae at 24 and 48h. In Galleria mellonella larvae, hemocytes can be differentiated into at least five types of cells; the most common types are prohemocytes, granulocytes, and plasmatocytes (İzzetoğlu, S., 2012). We just only observed significant changes in hemocyte density in larvae treated with only rifampicin and larvae infected with MRSA and treated with rifampicin; we don't know which kind of hemocyte was increasing and which type of hemocyte in Galleria mellonella larvae was effectively increased by rifampicin, especially the 40 mg/kg dose. The innate immune system of Galleria mellonella larvae is highly sensitive to external factors like thermal, physical, and chemical stress (Sheehan et al., 2018; Browne *et al*., 2014). Browne et al. highlighted that response to thermal stress leads to transient increase in hemocyte density and production of antimicrobial peptides in anticipation of infection.

The findings that were presented included information on the toxicity of rifampicin, its effectiveness against MRSA 14/0440 infection, and the humoral reaction of larvae to both rifampicin and MRSA 14/0440. According to these findings, rifampicin, at a dose of 40 mg/kg, increased the survival rate of larvae infected with MRSA 14/0440 by 90%. Rifampicin blocks transcription by attaching to the bacterial DNA-dependent RNA polymerase's β-subunit, which is encoded by the rpoB gene (Wang *et al*., 2019). It also prevents cell division in the larvae, but it doesn't eliminate MRSA. When an MRSA strain is treated with a combination therapy, such as rifampicin and vancomycin, the first medication prevents MRSA from proliferating inside the larvae, while the second medication, vancomycin, kills any residual MRSA cells inside the larvae. These findings can also be utilized to investigate the innate immune system (hemocyte) and the dose-dependent interaction of antibiotics with hemocytes.

Conclusion

This study successfully evaluated the toxicity and antimicrobial efficacy of rifampicin against MRSA 14/0440 using the *Galleria mellonella* infection model. The findings demonstrated that *G. mellonella* provides a convenient, cost-effective, and ethically acceptable platform for assessing both drug toxicity and therapeutic efficacy. Rifampicin exhibited dose-dependent effects on larval health, survival, melanization, and hemocyte responses. Among the concentrations tested, 40 mg/kg consistently showed the most favourable balance between safety and efficacy, producing minimal toxicity while providing substantial protection against MRSA infection.

The antimicrobial efficacy experiments revealed that treatment with rifampicin significantly improved larval survival following infection with MRSA 14/0440. Notably, the 40 mg/kg dose achieved survival rates of up to 90–100% and was associated with improved health index scores compared with untreated infected controls. Furthermore, haemocyte density analyses suggested that rifampicin may contribute to enhanced host immune responses, particularly at the 40 mg/kg concentration, where increased haemocyte counts were observed following treatment. Exactly, we don't know how a 40 mg/kg concentration acts as a sweet spot to trigger the innate immune response to improve haemocyte concentration, which helps in reducing the MRSA infection. If we find the exact mechanism and sweet spots, how specific a concentration can trigger an innate immune response without giving high doses of drugs to kill any bacterial infection will improve the entire drug delivery system.

An important outcome of this research was the observation that larval age influenced both toxicity and efficacy results. Significant differences were identified between larvae used immediately after delivery and those stored for six days prior to experimentation. These findings emphasize the need for standardized larval age and handling procedures to improve reproducibility and reliability across *G. mellonella* studies.

Overall, this work supports the use of *Galleria mellonella* as a valuable alternative infection model for antimicrobial research and demonstrates that rifampicin possesses significant activity against MRSA 14/0440 within this system.

Future studies should investigate the molecular mechanisms underlying rifampicin-mediated changes in haemocyte responses, evaluate combination therapies to reduce the risk of resistance development, and further establish standardized experimental protocols for the *G. mellonella* model. Such investigations will strengthen the utility of this model and contribute to the development of effective therapeutic strategies against multidrug-resistant bacterial pathogens.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

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