UNIVERSITI PUTRA MALAYSIA

LESSER WAX WORM LARVAE, Achroia grisella AS AN ANIMAL MODEL FOR Staphylococcus aureus INFECTION

NORALIA BINTI ABDUL RAZAK

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ABSTRACT

Lesser Wax Worm Larvae, *Achroia Grisella* as An Animal Model for *Staphylococcus Aureus* Infection

Noralia Abdul Razak[a], Johari Jalinas[b], Mohd Nasir Mohd Desa[a], Zulkefley Othman[a]

[a]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University Putra Malaysia

[b]Department of Biology, Faculty of Science and Technology, University Kebangsaan Malaysia

Introduction: *Staphylococcus aureus* Infection is quite prevalent in animals and humans. Numerous animal models for bacterial infection have been developed, but there is always a lack of a thorough description of the infection mechanism. The non-mammalian model organism: *Galleria mellonella* larvae have been widely used as an alternative model for microbial infection study but not *Achroia grisella*. The lesser wax moth, *A. grisella* shares the same life history as the greater wax moth, *G. mellonella*. Consequently, they hold benefits such as reasonable price, readily available, requiring no special laboratory equipment, and short life-cycle which are great for large-scale research. Plus, the innate immune response of the larvae is highly similar to that of vertebrates. **Objective:** This study aims to assess the activity of *A. grisella* larvae upon *Methicillin-resistant Staphylococcus aureus* (MRSA) *and Methicillin-susceptible Staphylococcus aureus* (MSSA) infections. **Methodology:** In the study, the last instar larvae (3-4 weeks of age) were used. For the infection route, intra haemocoelic injection through the skin was applied. The larvae were evaluated by the health index scoring system within five days after infection, which measures the health status of the larvae by assigning scores according to four key observations: mobility of the larvae, formation of cocoons, melanization and survival. Each waxworm's overall health index score was computed by assigning a numerical value to each observation and averaging the scores. Using the Kaplan–Meier method, the survival data was plotted, and the log-rank test was then used to compare the groups. **Results:** The survival percentage for PBS-injected larvae is 40% after five days post-infection. In contrast, MRSA- and MSSA-infected wax worms have higher survival percentages ranging from 70% to 80% at doses above 1.0×10^9. The surviving larvae transform to complete cocoon and moth stages throughout an additional one-week observation. **Discussion:** The death of PBS-uninfected,

MRSA- and MSSA-infected larvae might be due to two reasons: the physical trauma experienced during the administration of the inoculum and melanization which occurred at the injection site of the larvae body (<3 spots on a beige wax worm). As the survival rate of both MRSA- and MSSA-infected larvae are quite similar, the potency of both bacteria might be the same. **Conclusion:** Hence, to appropriately vindicate the concept of MRSA and MSSA pathogenesis, we suggest more in-depth research towards the influence of experimental design on the wax worm larvae.

Keywords: Staphylococcus aureus, *Achroia grisella,* MRSA, MSSA, infection model, wax worm

ABSTRAK

Larva Ulat Lilin Kecil, *Achroia Grisella* Sebagai Model Haiwan Untuk Jangkitan *Staphylococcus Aureus*

Noralia Abdul Razak[a], Johari Jalinas[b], Mohd Nasir Mohd Desa[a], Zulkefley Othman[a]

[a]Jabatan Sains Bioperubatan, Fakulti Perubatan dan Sains Kesihatan, Universiti Putra Malaysia

[b]Jabatan Biologi, Fakulti Sains dan Teknologi, Universiti Kebangsaan Malaysia

Pengenalan: *Staphylococcus aureus* kerap menjangkiti haiwan dan juga manusia. Walaupun terdapat banyak kajian terhadap model haiwan untuk jangkitan bakteria, penerangan menyeluruh mengenai mekanisme jangkitan masih lagi kurang. Organisme model bukan mamalia: larva ulat lilin besar, *Galleria mellonella* telah digunakan secara meluas sebagai model alternatif untuk kajian jangkitan mikrob tetapi tidak *Achroia grisella*. Larva ulat lilin kecil, *A. grisella* mempunyai sejarah kehidupan yang sama dengan larva *G. mellonella*. Jadi, ianya juga mempunyai faedah yang sama seperti harga yang berpatutan, tersedia didapati, tidak memerlukan peralatan makmal khas dan kitaran hayat pendek yang bagus untuk penyelidikan berskala besar. Tambahan pula, larva ini mempunyai tindak balas imun semula jadi yang agak serupa dengan vertebrata. **Objektif:** Kajian ini bertujuan untuk menilai aktiviti larva *A. grisella* setelah dijangkiti dengan dua strain *S. aureus* iaitu *Methicillin-resistant Staphylococcus aureus* (MRSA) *dan Methicillin-susceptible Staphylococcus aureus* (MSSA). **Metodologi:** Dalam kajian ini, larva instar terakhir yang berusia 3 hingga 4 minggu telah digunakan. Suntikan intrahemocoelic melalui kulit larva digunakan sebagai laluan jangkitan bakteria tersebut. Larva dinilai melalui sistem pemarkahan indeks kesihatan selama lima hari selepas jangkitan, yang mengukur status kesihatan larva dengan memberikan skor menurut empat pemerhatian utama iaitu mobiliti larva, pembentukan kepompong, melanisasi dan kelangsungan hidup. Selepas itu, purata skor larva untuk setiap kumpulan telah dihitung. Dengan menggunakan kaedah Kaplan-Meier, data kelangsungan hidup diplot dan uji log pangkat digunakan untuk membandingkan lengkung kemandirian lima kumpulan larva. **Hasil Kajian:** Peratus kelangsungan hidup untuk larva yang disuntikkan dengan PBS adalah 40% selepas lima hari. Sebaliknya, larva yang dijangkiti dengan MRSA dan MSSA mempunyai peratusan kelangsungan hidup yang lebih tinggi iaitu di antara 70% hingga 80% pada dos

melebihi 1.0 x 10^9. Larva-larva yang masih hidup telah bertukar menjadi kepompong dan kupu-kupu sepanjang pemerhatian tambahan selama satu minggu. **Perbincangan:** Kematian larva yang disuntik PBS, dan dijangkiti MRSA dan MSSA mungkin disebabkan oleh dua perkara iaitu trauma fizikal yang dialami semasa pemberian inokulum atau melanisasi hanya berlaku di tempat suntikan pada badan larva (<3 bintik hitam pada badan larva yang berwarna kuning pastel). Oleh sebab kadar kelangsungan hidup larva yang dijangkiti MRSA dan MSSA agak serupa, potensi kedua-dua bakteria berkemungkinan sama. **Kesimpulan:** Oleh itu, untuk mengkaji konsep patogenesis MRSA dan MSSA dengan tepat, kami mencadangkan penyelidikan yang lebih mendalam terhadap pengaruh reka bentuk eksperimen pada larva *A. grisella* dijalankan.

Kata kunci: Staphylococcus aureus, *Achroia grisella,* MRSA, MSSA, model jangkitan, ulat lilin

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LIST OF ABBREVIATIONS

%	Percentage
=	Equal
×	Multiplication
÷	Division
≥	More than or equal
<	Less than
>	More than
β	Beta
^{o}C	Degree Celsius
µL	Microliter
ATCC	American Type Culture Collection
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
G	Gauge
g	Gram
h	hours
LB	Luria-Bertani broth
min	Minutes
ml	Millilitre
mm	Millimetre
MRSA	*Methicillin-resistant Staphylococcus aureus*
MSSA	*Methicillin-susceptible Staphylococcus aureus*
N	Sample size

NA	Nutrient agar
n.d.	No date or year of publication
nm	Nanometre
no.	Number
OD	Optical density
PBS	Phosphate-buffered saline
rpm	Revolutions per minute
UKM	University Kebangsaan Malaysia
UPM	University Putra Malaysia
v	Version

CHAPTER 1

INTRODUCTION

1.1 Background

Known as the endothermic flora, *Staphylococcus aureus* colonises the nose of adults and grows on their skin, where it can cause infections. In healthy individuals, *S. aureus* is rarely responsible for fatal infections. However, breach or damage to the skin may allow the germs to enter and cause infection in internal organs such as the heart (endocarditis), where abscesses can form (Tenover and Gorwitz, 2006). Abscesses around foreign objects and bacteremia caused by staphylococcal infections are common among individuals with a weaker immune system, including those who have intravenous catheters or a prosthetic heart valve, those who have just had surgery, and those who use steroids. Additionally, certain S. aureus strains create various compounds that are harmful to animal cells and result in toxinoses, including toxic shock syndrome (TSS), exfoliatin toxins (scalded skin sickness), and enterotoxins (food poisoning). A further reason for increased interest in *S. aureus* is that the bacteria promptly develop antimicrobial resistance, making it harder to treat with conventional antibiotics (Hiramatsu et al., 2001). Therefore, it is critical to understand the bacteria better since *S. aureus* strains are frequently responsible for infections, especially in hospitalised patients.

Infection with *S. aureus* is quite prevalent in both animals and humans. Numerous animal models for bacterial infection have been developed, but there is always a lack of a thorough description of the infection mechanism. This is because, in the past decade, we have seen the appearance of novel *S. aureus* clones that have spread swiftly across continents, causing widespread skin and soft tissue infections as well as some very severe disorders (Liu, 2009). Although this is true, several well-established murine and primate models of *S. aureus* infection have proven to be essential tools for understanding the processes involved in *S. aureus* infection and the dynamics between pathogen and host. However, due to the increasing expenses and ethical problems associated with mammalian infection models, the use of non-mammalian models of infection is beginning to regain popularity. The nematode *Caenorhabditis elegans* and the common fruit fly *Drosophila melanogaster* are two of the most well-known non-mammalian model organisms used in biological research. Both have numerous advantages compared to higher-order animal models, including that they are easier to procure and utilise, are reasonable price, and are more tolerable from an ethical standpoint (Glavis et al., 2012). In addition, death can also be employed as an endpoint in experiments, which is forbidden for higher-order animal models in most countries, as well as Malaysia, because of the ethical implications (Loh et al., 2013).

Recently, there has been a resurgence of interest in the larvae of the wax moth, *Galleria mellonella* (greater wax worm), and several groups have explored

the larvae's potential in the modelling of bacterial and fungal infections (Desbois and Coote, 2011). The capacity of the larvae model to live at 37°C is the primary advantage over the *C. elegans* and *D. melanogaster* models (Lionakis, 2011; Hoffman, 1995) since *S. aureus* grows best at temperatures ranging from 37°C to 40°C (Medveďová and Valík, 2012). Besides, insects split from vertebrates roughly during the Cambrian period. Even though vertebrates have evolved an acquired immunity, their natural immunity nevertheless bears striking resemblances to the immunological response observed in insects (Browne et al., 2013). Hence, *G. mellonella* has been increasingly popular as an infection model for studying fungal and bacterial infections and evaluating the efficiency of new antimicrobic medicines in recent years (Tsai et al., 2016). However, Gulati and Kaushik (2004) reported that *G. mellonella* is less spread in Malaysia, but not *Achroia grisella*, the lesser wax worm. *G. mellonella* has a life cycle similar to that of the *A. grisella,* but is larger in size. Since *A. grisella* is more suitable for Malaysia's climate, it is chosen as the animal model of this study (Egelie et al., 2015). Plus, until now (2021), *A. grisella* has never been used as an animal model in *S. aureus* infection study, clearly showing this research's novelty. Therefore, this study generally aims to assess the activity of *A. grisella* larvae upon *S. aureus* infection based on four significant morphological changes: larval mobility, cocoon formation, melanization, and survival.

Serial dilution was performed with exponential phase cultures to determine the pathogenicity of *Methicillin-resistant Staphylococcus aureus* (MRSA ATCC

700699) and *Methicillin-susceptible Staphylococcus aureus* (MSSA ATCC 25923) in *A. grisella*. With each dose of MRSA (ATCC 700699), MSSA (ATCC 25923), or PBS (control), 5 groups consisting of 5-10 wax worm larvae were injected at the bottom-left prolegs. Then, these infected and uninfected wax worms were incubated for five days at room temperature (28-33°C). A total of four key observations were monitored for each wax worm to detect indistinct changes in the larval's health status; larval's activity, cocoon's development, melanization, and survival. The overall health index score of every wax worm was recorded by assigning numerical values during observation over 5 days long and averaging these scores. Using the Kaplan–Meier method, the survival data was plotted, and the log-rank (Mantel-Cox) test was accessed to collate the groups via GraphPad Prism v 9.2.0.

1.2 Objective

1.2.1 General Objective

The general objective of this study is to observe *Achroia Grisella* as an alternative animal model for the *Staphylococcus aureus* bacterial infection study.

1.2.2 Specific Objective

The specific objective of this study is to observe the morphological changes on *Achroia Grisella* upon infection of *Methicillin-resistant Staphylococcus aureus* (MRSA) and *Methicillin-susceptible Staphylococcus aureus* (MSSA).

1.3 Hypothesis

It is hypothesized that the infected lesser wax worm larvae with *Staphylococcus aureus* will most likely manifest change in a pathological manner compared to uninfected lesser wax worm larvae.

CHAPTER 2

LITERATURE REVIEW

2.1 Achroia Grisella

2.1.1 Background

The lesser wax moth, *Achroia grisella*, is a pest of unemployed honey bees, hives, and stored hive supplies (Egelie et al., 2015). On the other hand, wax worms are beneficial insects as they can consume combs leftover when bees colonies die or abandon their nest since they may leave behind microorganisms or pesticide residues in these abandoned combs. Thus, it lowers the future exposure risk toward the unoccupied hive. The life cycle of lesser wax moths is analogous to the greater wax moths, *Galleria mellonella*. *However, A. grisella* has a wide range of distribution and can be found almost anyplace honey bees are kept. It thrives in tropical and subtropical settings but is unable to tolerate lengthy periods of frigid temperatures. Gulati and Kaushik (2004) reported that *G. mellonella* is less spread in Malaysia than *A. grisella* plus it also can live at higher latitudes and lower temperatures than *G. mellonella*.

2.1.2 Life Cycle of Achroia Grisella

The life cycle of *A. grisella* consists of four stages: egg, larvae, pupae, and adult moth, whereby the period within each stage varies depending on temperature and condition (Egelie et al., 2015).

Egg: Mature females usually produce eggs near a food supply in sheltered crevices. The eggs appeared round and creamy white. The time required for an egg to hatch varies depending on the temperature, with higher temperatures resulting in faster development throughout all stages of life. Hatching an egg generally takes about five to eight days on average.

Larvae: Larvae have a white body accompanied by a brown head and pronotal shield. At a temperature of between 29°C to 32°C, larval development takes about one to five months, with an average of six to seven weeks. The larvae moult seven times. The last two instars are when the larvae grow the most, and mature larvae grow about 20 mm in length. Larvae burrow into beeswax combs, spinning silk tunnels, and they cover with frass. The larval is the only stage that consumes food such as honeycombs and hive materials. Brood and pollen comb are preferred by larvae over virgin and honeycomb. In regions where *A. grisella* and *G. mellonella* coexist, *A. grisella* larvae are commonly found eating on the hive floor because *G. mellonella* larvae overpower them for the desired brood comb.

Pupae: Adult larvae pupate in the hive and weave thick silk cocoons surrounding themselves. Pupae appear yellow-tan and measure 11 mm in length. Cocoons appear white and are held in place by webbing. Cocoons can be challenging to spot because they are typically buried with faeces and debris. The maturation of

pupae takes two months, but the usual period until adult emergence is a month and seven days.

Adults: Adults *A. grisella* moths are around half inches long and have thin bodies. Their wingspan is about half inches wide. Males moths are often less in size than females. They have a conspicuous golden head and colouring that varies from silver-grey to beige. Adults moths have a one-week lifespan and are nocturnal. Males use ultrasonic signals to attract females to mating places, which takes place in beehives. Females produce their eggs at nighttime as well. Adults hide in shrubs and trees around hives in the daytime.

2.2 Animal model

The discovery of secure and reliable vaccines and medicines is dependent on a thorough understanding of the benefits and drawbacks of the various animal models relevant to the research problem (Swearengen, 2018). In addition, in some circumstances, like in novels or developing illnesses for which no human data is available, the animal model is essential in understanding the disease's aetiology before researchers can develop vaccines or therapies. A well-designed animal model offers a strong foundation for scientific data and efficient utilisation of human and animal resources. Animal models are particularly significant in infectious diseases research since the resulting condition is often life-threatening or permanently debilitating, making human subjects research difficult. Animal models are critical for scientific progression in human healthcare. Still, if they are not thoroughly defined and understood, incorrect interpretations may be formed, stifling scientific progress and wasting animal lives. Hence, a well-devised animal model involves a detailed grasp of the commonalities and contrasts in human and animal physiology and the incorporation of such knowledge into the study's objectives.

2.3 Invertebrates

2.3.1 Background

Invertebrates, unlike vertebrates, are cold-blooded creatures with no vertebral column (Britannica, 2021). Invertebrates can be soft-bodied animals with no stiff internal skeleton for muscle attachment, but some have a hard exterior skeleton as body protection. Nevertheless, invertebrates make up more than 90% of all extant animal species, either marine or terrestrial, such as crustaceans, molluscs, and arthropods. Invertebrates have a critical role in agriculture as a pest, parasite, and carrier of parasitic illnesses infecting a host. In addition, invertebrates provide nutrients, are crucial components of food chains that feed aves, fish, and various vertebrates, and aid in plant pollination. Despite their importance to the environment, invertebrates are frequently overlooked in research, with substantial vertebrate studies taking precedence. Furthermore, numerous invertebrate taxa are regarded purely as pests, and by the early twenty-first century, widespread pesticide use had resulted in significant population losses of terrestrial insects, bees and wasps. Instead of treating invertebrates such as insects as a pest, it will do good for the environment if the insects or other invertebrates are incorporated as an animal model. In addition, the increase in expenses and ethical problems associated with vertebrate models makes invertebrate models regain popularity. The nematode *Caenorhabditis elegans* and the common fruit fly *Drosophila melanogaster* are two of the most well-known invertebrates models used in biological research.

2.3.2 *Caenorhabditis elegans*

Caenorhabditis elegans is a nematode belonging to the Nematoda phylum. It is a tiny creature, only reaching a length of roughly 1 mm. *C. elegans* is typically found in decaying fruits and stems, and it appears to eat a range of microorganisms, both beneficial and harmful (Balla & Troemel, 2013). Félix and Duveau (2012) stated that because of the growing isolation of wild-caught nematodes and their accompanying microorganisms, the ecology of *C. elegans* in the wild has gotten considerable attention in recent years. *C. elegans* is the most rudimentary organism known, but it bears many essential biological traits that are important in human biology concerns (Edgley, 2015). Hence, *C. elegans* is a valuable model organism for studying microorganisms infection.

C. elegans die in a manner associated with germs buildup within the alimentary tract of nematode throughout several days of feeding with 18 different *S. aureus* isolates (A002, A003, A091, etc.) (Sifri et al., 2020). Approximately three-quarters (70%) of the nematodes were killed during a routine experiment (post-5 days feeding). However, when the nematodes were nourished with heat- or antibiotic-killed *S. aureus*, no death was seen, implying that mortality required the existence of live bacteria. As a result, *C. elegans* was valuable for identifying novel staphylococcal genes and researching mammalian pathophysiology and host innate defense systems.

2.3.3 *Drosophila melanogaster*

Drosophila melanogaster, the fruit fly, is the most widely utilized and well-studied model organism. Fruit flies are about 3 mm long. It is frequently found among rotten and immature fruit (Scott, 2021). Based on Needham et al. (2004), *D. melanogaster* was infected with over 30 strains of *S. aureus* (SH1000, SJF1385, MHK10A, etc.), which develops a systemic infection and eventually dies. All of the flies perished about 16 hours after being infected. For instance, flies were killed by S. aureus SH1000 cells at doses ranging from 1.9×10^7 to 1.9×10^9 CFU/mL, whereas those injected with autoclaved S. aureus SH1000 (4.6×10^9 CFU/mL) and sterile PBS stayed alive. This confirms that the existence of live bacteria, rather than shock, causes the mortality of infected *D. melanogaster* same result as *C. elegans*. The mortality rate was correlated to the number of bacteria administered, with 100% killing post-23 hours with a 1.9×10^8 CFU/mL inoculum. As a result, D. melanogaster serves as a useful *S. aureus* infection model with a predetermined result.

2.5 Galleria mellonella

2.5.1 Background

Galleria mellonella, the greater wax moth ('ulat lilin besar'), belongs to the *Lepidoptera* order and the *Pyralida*e family of insects (Scoble, 1995). It can destroy combs and beehive materials during the larval stage, especially a weakened colony's comb, without harming the adult bees. In addition, when the larval is prepared to undergo metamorphosis, they often gnaw out an area of the beehive to weave their silk, causing damage to that place (McGregor, 2017). Technically, they used beehives (food) as fuel to enter the next stage of life. *G. mellonella* has just been established as a new model for studying pathogenic infestations. Over a thousand articles concerning the *G. mellonella* infection model have been uploaded on PubMed, with over 268 of them published only in 2020–2021, illustrating the infection model's growing popularity. Many microbial genome studies have been completed in the previous decade, resulting in many "hypothetical proteins" in the database. The genome annotations were found through open reading frames or bioinformatics analysis, however, without an experimentally proven function. Nevertheless, most newfound proteins were thought to be virulent, so identifying their roles will help us better understand disease pathogenesis and, in turn, pave the way for the development of new therapeutics (Tsai et al., 2016). Thus, *G. mellonella* larvae could be one of the infection models that would be fully utilised in testing the efficiency of new antimicrobial medications, in current years.

2.5.2 Benefits of *Galleria mellonella* as Animal Model

G. mellonella larvae are less expensive to establish and maintain than standard mammal model hosts since they require no specialised instrumentation (Ramarao et al., 2012). These larvae are great for high-throughput investigations because they require no ethical approval and have a short life cycle (Tsai et al., 2016) but it depends on two factors: temperature (29-33°C) and humidity level (29-33%) (Kwadha et al., 2017). Besides, based on the research conducted in Kansas, *G. mellonella* larvae could also live normally at 37°C (Warren & Huddleston, 1962) allowing researchers to investigate temperature-dependent microbial virulence factors compared to other infection models like *C. elegans* and *D. melanogaster* (Konkel & Tilly, 2000). Despite the fact that vertebrates possess an evolve acquired immunity, their first line defense seems comparable to that of insects (Browne et al., 2013). For example, arthropod hemocytes and mammal neutrocytes functioned in capturing and eliminating infections, and both types of cells synthesize superoxide through p47 and p67 proteins that are remarkably similar (Kavanagh & Reeves, 2007).

2.5.3 The Melanization of *Galleria mellonella*

In this study, melanization is used as a parameter to observe the morphological changes in *A. grisella* larvae upon infection, hoping they can exhibit the same response as *G. mellonella* larvae. Melanization is classified as the humoral response of the *Insecta* through the phenoloxidase pathway. First, larval would

produce melanin as a reaction toward pathogen invasion as it aids in the trapping and killing of pathogenic organisms at the injury site. Then, the coagulation and opsonization of larval hemolymph were followed, and these processes are reported to be similar to the formation of abscesses in mammal infections (Tang, 2009). Finally, the melanin could be seen by the colour changes of the larval body surface from beige to brown or black. In the end, larvae would die following complete melanization as their body is overwhelmed with host-pathogen interactions (Loh et al., 2013).

2.5.4 *Galleria mellonella* Infection Model

Many Gram-positive bacteria, such as *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Enterococcus faecalis* have been studied using the *G. mellonella* infection model (Tsai et al., 2016).

Streptococcus pneumoniae: Serotypes 2, 4, 14, 19A, 19F were used to detect the differences in virulence. Evans and Rozen (2012) reported that the existence or lack of known virulence factors correspond to the virulence differences in *S. pneumoniae* serotypes. In addition, they found that LD_{50} values between 1.36 x 10^5 CFU and 1.56 x 10^6 CFU were greater than those seen in the mouse infection model.

Staphylococcus aureus*:* The infected *G. mellonella* larvae mortality is depended on the infection dose (Desbois and Coote, 2011). For example, post-one day 1 x 10^7 CFU infection, all infected larvae died while post-five-day 1 x 10^5 CFU infection, only 20% of the infected larvae died. In addition, when evaluated at 25ºC, 30ºC, and 37ºC, the virulence depended on the temperature, with higher mortality rates at higher temperatures.

Enterococcus faecalis*: G. mellonella* larvae were infected with 5 x 10^5 CFU *E. faecalis* into their hemocoel, leading to severe melanization post-5 minutes and mortality post-30 minutes. In *G. mellonella* infections, extracellular gelatinase (GelE) has been identified as a key of virulence factor (Parck et al., 2007).

To summarize, the virulence of a particular bacteria depending on the dose and temperature in showing larvae mortality and survivability.

2.6 Staphylococcus aureus

Staphylococcus aureus is classified as a Gram-positive facultative anaerobic bacterium that infects around 30% of the world's population across various body sites. *S. aureus* can be identified morphologically as golden medium size colonies on growth agar such as nutritional agar and is capable of causing β-hemolysis on sheep blood agar (Ryan and Ray, 2004). *S. aureus* is the most frequently seen pathogen associated with biofilms or medical devices, most likely due to its commensal nature (Otto, 2008). The nasal, epidermis and digestive tract are the most frequently colonized locations by *S. aureus* (Brown et al., 2014). *S. aureus* is an infectious agent that can infect people when the host's defense system is weakened. They are typically infected by the commensal *S. aureus* carried by them (Kluytmans et al., 1997, William et al., 1959). While most cases of *S. aureus* colonization are asymptomatic, it is capable of producing a wide variety of illnesses, ranging from furuncles and boils to severe infections like pneumonia, toxic shock syndrome, and endocarditis (Tong et al., 2015). Currently, community- and hospital-acquired *S. aureus* infections have grown, resulting in the emergence of antibiotic-resistant isolates, which increased concern in combating it (Rağbetli, 2016). In this study, *Methicillin-resistant Staphylococcus aureus* (MRSA ATCC 700699) and *Methicillin-susceptible Staphylococcus aureus* (MSSA ATCC 25923) were used to assess the morphological changes that happened in *A. grisella* larvae after being infected.

CHAPTER 3

METHODOLOGY

3.1 Culture Media Preparation

3.1.1 Nutrient Agar (NA)

NA agar (GranuCult™) was made by dissolving 14 g of NA agar powder in 500 ml of distilled water. A hot stir plate was used to completely dissolve the media for one minute at a high temperature. After that, the mixture was autoclaved at 121°C for 15 minutes. The agar was then cooled to 45°C-55°C before being poured onto a 4 mm Petri dish on a flat surface to ensure consistent depth. The agar was left at room temperature until fully solidified and stored in the chiller (4-8°C).

3.1.2 Luria-Bertani broth (LB)

LB broth (Difco™) was made by dissolving 6.25 g of LB powder in 250 ml of distilled water. A hot stir plate was used to completely dissolve the media for one minute at a high temperature. After that, the mixture was autoclaved at 121°C for 15 minutes. After autoclaving, the broth was cooled at room temperature before being used or stored in the chiller.

3.2 Bacterial Strains and Cultures

3.2.1 Bacterial Culture on The Nutrient Agar

MRSA and MSSA bacteria colonies were taken from the stock using sterile loops and streaked on nutrient agar (the stationary phase of bacterial growth). Then, the cultured agars were incubated inside an incubator (SASTEC) with a temperature of 37^{o} C for 16 hours without shaking. The following day, the growth of bacteria was observed before proceeding to bacterial subculture into Luria-Bertani broth.

3.2.2 Subculture of Bacteria into Luria-Bertani Broth

After subculturing bacteria on NA agar, three to five colonies were taken from the cultured nutrient agar and were subcultured into the LB broth. Then, the cultured broths were incubated inside an incubator with a temperature of 37°C for 16 hours without shaking (the exponential phase of bacterial growth). On the following day, the growth of bacteria was observed before proceeding with 1/10 dilution of the bacterial suspension.

3.2.3 Bacterial Suspension and OD600 Measurement

The bacterial suspensions (100 µL) were taken from the previously cultured LB broth and pipetted into test tubes that contained 9900 µL of LB broth to make up for 1/10 dilution of the bacterial suspension. Then, the suspension was incubated at 37°C without shaking until it reached OD of 600 = 0.4-0.6. The optical density

at 600 nm of the bacterial suspensions for MRSA and MSSA was measured using a spectrophotometer. The OD600 is used to regulate the development of cells, bacteria, or yeast in liquid culture media. The growth stage is often determined when measuring the optical density (OD600) of bacterial culture in ensuring that cells are collected at an optimal point that corresponds to an acceptable density of living cells (Implen, 2021). The time for bacterial incubation was controlled carefully to achieve the desired optical density which takes 5.5 to 6.0 hours for MRSA and MSSA to reach OD600 = 0.4-0.6 (**Table 3.1**).

3.2.4 Centrifugation

A volume of 1.5 ml of bacteria suspension was taken and placed into the Eppendorf tube after the OD reached 0.4-0.6. The bacterial suspensions were centrifuged (Eppendorf Centrifuge Model 5424R) at 11000 rpm for 2 minutes. Then, the supernatant was discarded, and the pellet was rinsed with phosphate-buffered saline (PBS) twice. Afterwards, 1 ml of PBS was added, and the pellet was resuspended before continuing with the 100X serial dilution.

3.2.5 Serial Dilution in 96-well Plate

Bacterial suspensions (1 µL) were taken from the mixture and placed into the first well filled with 99 µL PBS to make up for the 100X serial dilution to dilute the bacterial suspension until the desired concentration is achieved. Then, 10 µL bacterial suspensions from the first well were added into the second well and

continued for the remaining four wells. The second to the seventh wells were filled with 90 µL PBS.

3.2.6 Viable Count

The bacterial suspension (10 µL) from 10^{-6} and 10^{-7} concentrations of MRSA and MSSA were added onto the nutrient agar for the viable count, colony-forming unit (CFU). The bacteria suspension was spread using a hockey stick and was incubated at 37°C without shaking. Plate counting is a technique used to approximate the number of live cells present in a given sample (Boundless Microbiology, n.d.). The following day, the growth of bacteria was observed and bacterial colony-forming units per millilitre (CFU/mL) was calculated (**Table 3.2**) prior to administration of the bacteria into larvae.

Colony-Forming Units (CFU/mL) = no. of colonies ÷ (total dilution × volume of the culture plate

Optical Density at 600 nm (OD600)									
Incubation Period (min)	**Blank**	**MRSA**				**MSSA**			
		Reading 1	Reading 2	Reading 3	Average	Reading 1	Reading 2	Reading 3	Average
240	0.000	0.255	0.253	0.251	**0.253**	0.314	0.314	0.312	**0.313**

Table 3.1. The optical density of MRSA and MSSA at 600 nm (OD600).

Note: One-hour additional incubation period was added to reach OD600 = 0.4-0.6 (data not shown).

Viable Cell Counting				
Group	**Volume of Culture Plated (mL)**	**Dilution Factor**	**Colony Count**	**CFU/mL**
MRSA	0.01	10^{-6}	8	8.0×10^{8}
MRSA	0.01	10^{-7}	1	1.0×10^{9}
MSSA	0.01	10^{-6}	3	3.0×10^{8}
MSSA	0.01	10^{-7}	1	1.0×10^{9}

Table 3.2. The viable cell counting of MRSA and MSSA.

3.3 Infection of *Achroia grisella* Larvae

A. grisella larvae (Faculty of Science and Technology, UKM) was injected with 10 µL of inoculum at the lower-left prolegs using a 32 G insulin syringe. The last instar larvae (at age of 3 - 4 weeks) with a weight between 0.025 and 0.033 g was used. There are 2 groups of subjects: the uninfected or negative control group (injected with PBS alone) to account for the possibility that physical trauma will have an impact on mortality and the infected group (injected with four different concentrations: of MRSA and MSSA). For each group, 10 larvae were used with a total of 45 larvae (N=45) per batch. The larvae were kept in the Petri dish in the dark at room temperature for up to 120 hours post-infection and were monitored for survival every 24 hours; larvae were declared dead if they did not move when the Petri dish was shaken. The dead larvae were put inside an Eppendorf tube filled with 70% alcohol for DNA extraction and polymerase chain reaction on the presence of bacterial DNA to confirm that the death of larvae was solely due to MRSA and MSSA.

3.4 Monitorization of *Achroia grisella* Larvae

The larvae were observed by measuring the larvae's health status (assigning scores) based on four major morphological changes: larvae mobility, cocoon formation, melanization, and survival. The results of each observation were given a score, and the total health index score for each wax worm was computed. The average score for every group was calculated (**Table 4.1**) in order to generate a

bar graph of the mean health index score of *A. grisella* larvae upon MRSA and MSSA infection in a period of five days post-infection.

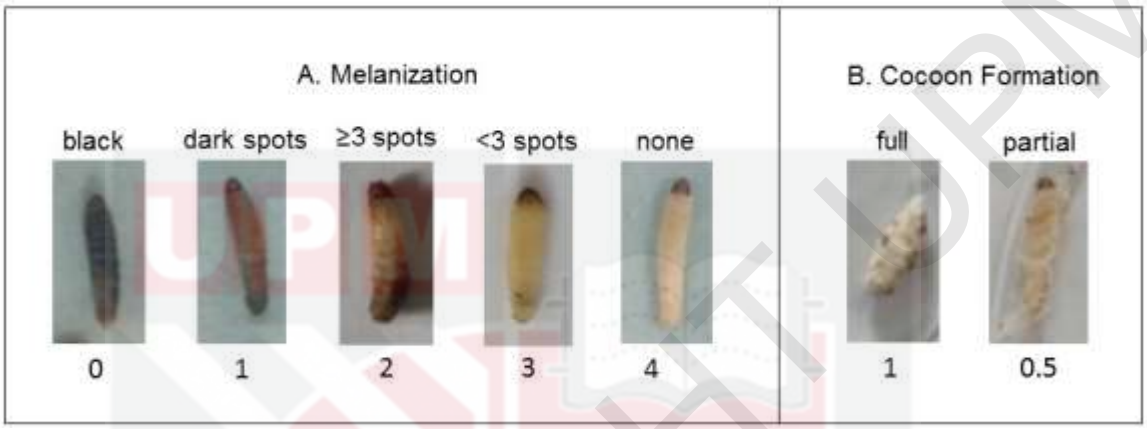

Figure 3.1. Images of *A. grisella* larvae at various stages of the disease. **A:** Melanization, which begins with the production and accumulation of melanin to capture microorganisms at the injured area (the fourth picture from left), is generally preceded by hemolymph coagulation and opsonization. Full melanisation (black larvae, left picture) is shortly after the larvae died. **B:** Reduction of cocoon development in *A. grisella* larvae can be utilised as a biomarker for the species' illness (right image). Additionally, the numbers below also indicate the larval health index scores (refer to **Table 3.3**).

Category	Description	Score
Larvae mobility	No activity	0
	Minimal activity on stimulation	1
	Active when stimulated	2
	Active without stimulation	3

Cocoon formation	No cocoon	0
	Partial cocoon	0.5
	Full cocoon	1
Melanization	Complete melanization (black)	0
	Dark spots on brown wax worm	1
	≥3 spots on beige wax worm	2
	<3 spots on beige wax worm	3
	No melanization	4
Survival	Dead	0
	Alive	2

Table 3.3. The Health Scoring System of *A. grisella* (Loh et al., 2013).

3.5 Statistical Analysis

The Kaplan–Meier method was used to plot the survival data, and the log-rank test (Mantel-Cox) was used to differentiate the survival curves between infected and uninfected groups (negative control) through Graph Pad Prism v 9.2.0. If p-value ≤ 0.05, all of the survival curve for every group was regarded as significantly different from each other.

CHAPTER 4

RESULT

4.1 The Survivability of *A. grisella* Larvae Upon MRSA and MSSA Infection

Negative control larvae were injected with PBS alone to account for the possibility that physical trauma will have an impact on mortality (Velikova et al., 2016). Larvae were also inoculated with two different concentrations of MRSA inoculum (8.0×10^8 and 1.0×10^9 CFU/mL) or MSSA inoculum (3.0×10^8 and 1.0×10^9 CFU/mL). Then, they were left in the dark box at room temperature (25-33°C) and larvae survivability was assessed over five days. PBS-injected larvae survivability reduced to 80+16.92-59.62% and 40+35.28-34.80% as one larva died after 24 h and two larvae died after 48 h (**Fig. 4.1.A**). The MRSA inoculum of 8.0×10^8 CFU/mL resulted in changes of larval survivability over five days, with three larvae died (**Fig. 4.1.A:** 2 larvae died after 24 h and 1 died after 96 h). The larvae viability for 8.0×10^8 CFU/mL MRSA inoculum reduced to 80+14.59-39.13% and 70+19.20-37.12%. The MRSA inoculum of 1.0×10^9 CFU/mL also resulted in the same changes of larval survivability, with three mortalities after 24 h, 48 h and 96 h (**Fig. 4.1.A**). The larval survivability for 1.0×10^9 CFU/mL MRSA inoculum was reduced to 90+8.53-42.70%, 80+14.59-39.13%, and 70+19.20-37.13% respectively. The inoculum of 3.0×10^8 CFU/mL MSSA resulted in changes of larval survivability over five days, with three larvae died (**Fig. 4.1.A:** 1 each after 24 h, 48 h and 120 h). The larvae viability for 3.0×10^8 CFU/mL MSSA inoculum shared the same survival percentage as 1.0×10^9 CFU/mL MRSA inoculum: 90+8.53-42.70%, 80+14.59-

39.13% and 70+19.20-37.13% respectively. The inoculum of 1.0×10^9 CFU/mL MSSA only has one mortality after 24 h (**Fig. 4.1.A**) resulting in the small reduction of larval survivability: 80+16.92-59.62%. Meanwhile, the surviving larvae had turned to a complete cocoon and moth stage throughout an additional one-week observation (data not shown). According to the log-rank (Mantel-Cox) test, p-value > 0.05 (p=0.6776), indicating that there is no significant difference between the survival curves of *A.grisella* infected and uninfected larvae.

4.2 The Mean Health Index Scores of *A. grisella* Larvae Upon MRSA and MSSA Infection

Each wax worm's overall health index score was computed by assigning a numerical value to every four observations and averaging the score per group (**Table 4.1**). The mean health index scores pattern of *A. grisella* wax worms injected with PBS was decreased from 6.70 (Day 1) to 5.25 (Day 2) but increased to 7.50 (Day 3) and gradually decreased to 6.75 (Day 4) and 3.00 (Day 5). However, the mean health index scores pattern of *A. grisella* wax worms infected with MRSA (8.0×10^8 CFU/mL) has an opposite trend whereby the mean value increased from 6.15 (Day 1) to 7.50 (Day 2), decreased to 5.19 (Day 3) and 3.81 (Day 4) but slightly increased to 4.57 (Day 5). Meanwhile, the mean health index scores pattern of *A. grisella* wax worms infected with MRSA (1.0×10^9 CFU/mL) has quite the same trend as the PBS-injected larvae at the beginning whereby it slightly decreased from 7.50 (Day 1) to 7.06 (Day 2) but increased to 7.81 (Day

3), rapidly decreased to 3.00 (Day 4) and remained the same at the final day of observation (Day 5). As for the mean health index scores pattern of *A. grisella* wax worms infected with MSSA (3.0×10^8 CFU/mL), it produced the same trend with the larvae infected with MSSA (1.0×10^9 CFU/mL) whereby the mean value increased from Day 1 (6.80, 6.60) to Day 3 (7.38, 8.00) and gradually decreased at Day 5 (3.44, 4.25). (**Fig. 4.1.B**)

Achroia grisella Larvae's Health Score						
Day	**Group**	**Larvae mobility**	**Cocoon formation**	**Melanization**	**Survival**	**Mean**
Day 0	Control (PBS)	5(3)	5(0)	5(4)	5(2)	9.00
	MRSA (8.0×10^8)	10(3)	10(0)	10(4)	10(2)	9.00
	MRSA (1.0×10^9)	10(3)	10(0)	10(4)	10(2)	9.00
	MSSA (3.0×10^8)	10(3)	10(0)	10(4)	10(2)	9.00
	MSSA (1.0×10^9)	5(3)	5(0)	5(4)	5(2)	9.00
Day 1	Control (PBS)	2(2), 2(1), 1(0)	1(0.5), 4(0)	4(4), 1(3)	4(2), 1(0)	6.70
	MRSA (8.0×10^8)	5(3), 1(2), 2(1), 2(0)	3(0.5), 7(0)	1(4), 7(3), 2(-)	8(2), 2(0)	6.15
	MRSA (1.0×10^9)	9(3), 1(0)	2(0.5), 8(0)	2(4), 7(3), 1(-)	9(2), 1(0)	7.50
	MSSA (3.0×10^8)	8(2), 1(1), 1(0)	4(0.5), 6(0)	9(3), 1(4)	9(2), 1(0)	6.80
	MSSA (1.0×10^9)	1(3), 3(2), 1(0)	5(0)	4(3), 1(4)	4(2), 1(0)	6.60
Day 2	Control (PBS)	2(2), 2(0)	2(0.5), 2(0)	4(3)	2(2), 2(0)	5.25
	MRSA (8.0×10^8)	8(2)	8(0.5)	8(3)	8(2)	7.50

	MRSA (1.0 x 10^9)	5(3), 2(2), 2(0)	1(1), 7(0.5), 1(0)	8(3), 1(-)	8(2), 1(0)	7.06
	MSSA (3.0 x 10^8)	8(2), 1(0)	8(0.5), 1(0)	8(3), 1(2)	8(2), 1(0)	6.89
	MSSA (1.0 x 10^9)	4(2)	4(0.5)	4(3)	4(2)	7.50
Day 3	Control (PBS)	2(2)	2(0.5)	2(3)	2(2)	7.50
	MRSA (8.0 x 10^8)	8(2)	5(1), 3(0.5)	8(3)	8(2)	5.19
	MRSA (1.0 x 10^9)	4(3), 1(2), 3(1)	2(1), 1(0.5), 5(0)	8(3)	8(2)	7.81
	MSSA (3.0 x 10^8)	2(3), 2(2), 4(1)	4(1), 2(0.5), 2(0)	8(3)	8(2)	7.38
	MSSA (1.0 x 10^9)	3(3), 1(2)	2(0.5), 2(0)	4(3)	4(2)	8.00
Day 4	Control (PBS)	1(2), 1(0)	1(1), 1(0.5)	2(3)	2(2)	6.75
	MRSA (8.0 x 10^8)	1(3), 1(2), 6(0)	5(1), 1(0.5), 2(0)	2(3), 6(-)	7(2), 1(0)	3.81
	MRSA (1.0 x 10^9)	8(0)	7(1), 1(0)	1(3), 7(-)	7(2), 1(0)	3.00
	MSSA (3.0 x 10^8)	3(2), 5(0)	5(1), 1(0.5), 2(0)	5(4), 3(3)	8(2)	7.06
	MSSA (1.0 x 10^9)	1(3), 1(1), 2(0)	3(1), 1(0)	1(3), 3(-)	4(2)	4.50
Day 5	Control (PBS)	2(0)	2(1)	2(-)	2(2)	3.00

	MRSA (8.0 x 10^8)	1(3), 1(2), 5(0)	5(1), 2(0.5)	1(4), 1(3), 5(-)	7(2)	4.57
	MRSA (1.0 x 10^9)	7(0)	7(1)	7(-)	7(2)	3.00
	MSSA (3.0 x 10^8)	1(2), 7(0)	6(1), 1(0.5), 1(0)	1(2), 1(3), 6(-)	7(2), 1(0)	3.44
	MSSA (1.0 x 10^9)	1(3), 3(0)	3(1), 1(0)	1(3), 3(-)	4(2)	4.25

Table 4.1. The health score of the uninfected and infected group at room temperature (25-33°C) for 5 days.

Note: no. of larvae(score); (-) = uncounted/no value

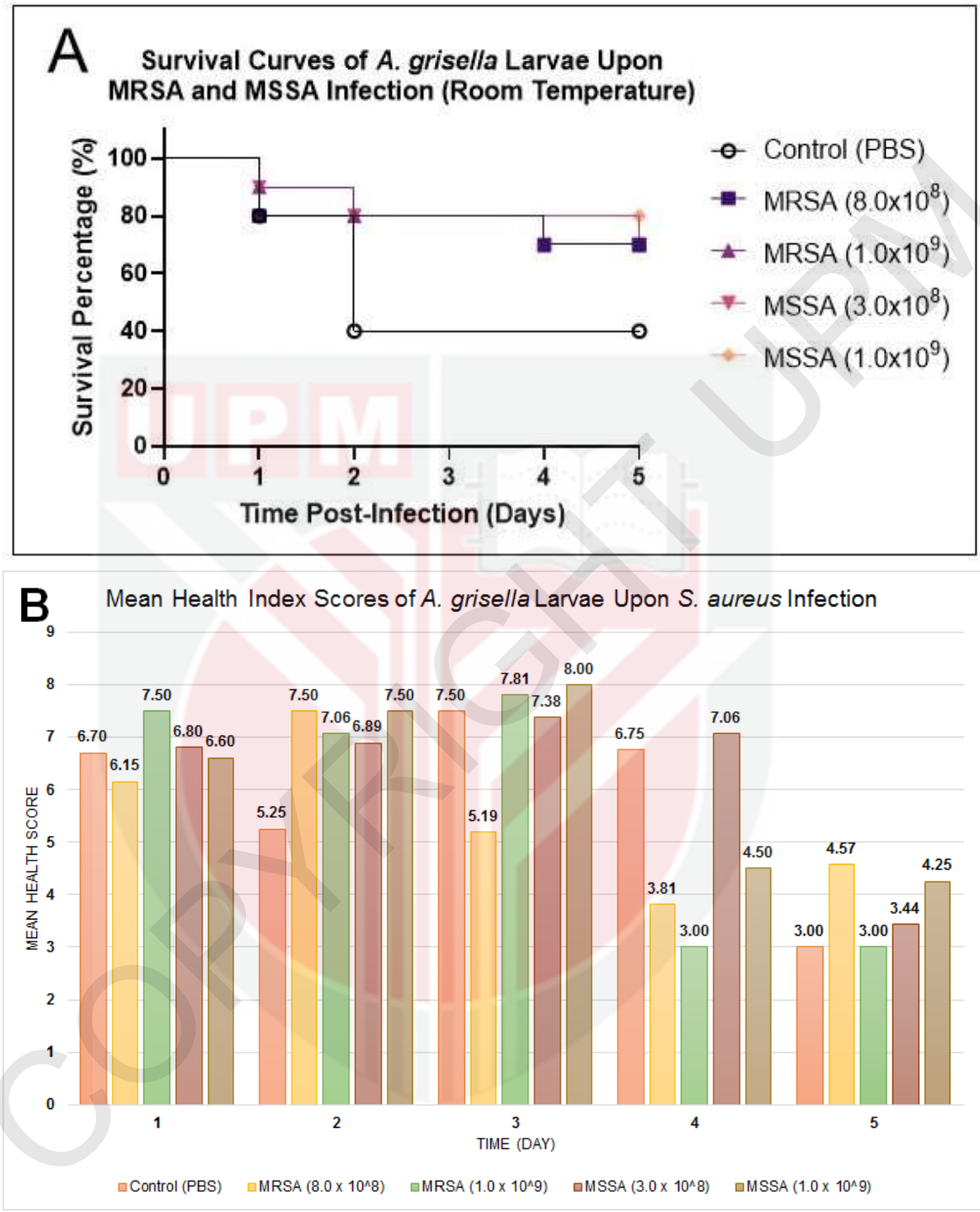

Figure 4.1. A: Kaplan-Meier survival curves of *A. grisella* wax worms infected with four different concentrations of the exponential phase of MRSA and MSSA. *p > 0.05 (log-rank). **B:** Mean health index scores of *A. grisella* wax worms infected with four different concentrations of the exponential phase of MRSA and MSSA.

CHAPTER 5

DISCUSSION

5.1 The Possibility Death Causes of *A. grisella* Larvae

The survival percentage for PBS-injected larvae is 40% after five days post-infection. In contrast, MRSA- and MSSA-infected wax worms have higher survival percentages ranging from 70% to 80% at doses above 1.0 x 10^9 CFU/mL. The death of PBS-uninfected, MRSA- and MSSA-infected larvae might be due to two possible reasons: the physical trauma experienced during the administration of the inoculum (Velikova et al., 2016) or the melanization that occurred at the injection site of the larvae body (<3 spots on a beige wax worm). According to the Kaplan-Meier survival curves of *A. grisella* wax worms (**Fig. 4.1.A**), the survival rate of both MRSA- and MSSA-infected larvae are pretty similar, and the potency of both bacteria might be the same as there is no significant difference between the survival curves of *A. grisella* infected and uninfected larvae ($p>0.05$). However, the sample size for a population batch is only 45 larvae (N=45), which is too low to give a reliable survival curve that represents a population. Therefore, in the future, the sample size needs to be increased in order to generate reliable results.

5.2 MRSA and MSSA Virulence

Besides, we do acknowledge that MRSA is more virulent than MSSA (Nozin, 2019). This is because the lowest concentration of MRSA (1.0×10^9 CFU/mL) showed the same mortality rate as the highest concentration of MRSA (8.0×10^8 CFU/mL), whereby they both held the same amount of dead larvae compared to the highest and lowest concentration of MSSA. It means that even at a lower dose, MRSA can cause a high mortality rate. In contrast, the highest concentration of MSSA (3.0×10^8 CFU/mL) showed a higher mortality rate than the lowest concentration of MSSA (1.0×10^9 CFU/mL), proving its potency. Although it was challenging to collate the strains due to varying doses, it was apparent that the wax worm model exhibited a wide range of reactions when infected with MRSA and MSSA (**Table 4.1**). Bacterial potency can also be determined by enumerating their growth within the larvae upon infection via plating larval extracts onto agar surface (Wand et al., 2013) or by utilising light-producing bacteria to pinpoint pathogen load through biophotonic imaging (La Rosa et al., 2013).

5.3 Factors Influence The Rise and Fall of Larval Mean Health Index Scores

The mean health index scores of *A. grisella* wax worms infected with four different concentrations of the exponential phase of MRSA and MSSA have shown fluctuated values (**Fig. 4.1.B**). This is due to significant differences between the individual score of each larva in a group. The factors that influenced the rise and

fall of larval mean health index scores are larval activity, survivability, cocoon formation and melanization as discussed below.

5.3.1 The Association Between Larval Activity and Survivability

Both activity and survivability of a larva were assessed by a nudge with forceps or shaking the Petri dish to determine whether the larvae are still alive or dead. The ability for the larvae to move indicates the larvae is alive. If the larvae are immobile or with no activity, it signifies that they are dead, as Loh et al. (2013) reported that higher activity is equivalent to a healthier larva. Previously, it was predicted that a healthy and uninfected larva score is in between 9-10, while an infected and dead larva scores 0. However, it is not usually the case. This study found that a healthy and uninfected or infected larva scored only up to 3 obtained from their ability to form a complete cocoon (score 1) and being alive (score 2) inside the cocoon. Therefore, the larvae were considered healthy even though they had no movement because the larvae have metamorphosed into pupae, and their movement was restrained inside the cocoons (Jorjão et al., 2018).

5.3.2 The Association Between Cocoon Formation and Larval Activity

Larval cocoon formation was assessed by observing a white silken thread that has been woven across the body of a larva, as shown in **Figure 4.1.B.** This study discovered that the cocoon formation had influenced the movement of the larvae

depending on whether the larvae are inside a complete, partial or none cocoon. Most larvae, either infected or uninfected, were immobilized whenever they reached the complete cocoon stage. This condition has questioned the larval survivability and contradicted with what has been reported by Loh et al. (2013). They stated that a wax worm that is more active and produces many cocoons is healthier. The cocoon acts as a protective shield for the last instar larvae as they used their own body (or food) as fuel to develop into moths (Britannica, 2021). However, in this study, the larvae were considered alive even though they were immobilized because they had developed into the pre-pupae and focusing on entering the moth stage, which took about 5-21 days (Mahgoub et al., 2015). Besides, the larvae were considered dead if they could not develop to the next stage within 5-21 days. This is what has been highlighted by Loh et al. (2013) which showed a decrease in cocoon development could also be utilized as a marker for illness in the larvae, which might be why all of the dead larvae were unable to proceed to the next stage of their life. However, insect life cycle studies have confirmed that the larval stage's lifetime is dependent on external variables such as weather (Ellis et al., 2013) and demand for food availability (Mohammadi et al., 2010). Hence, the larvae tend to have no activity when they are inside a complete cocoon, but the larvae immobility did not necessarily correspond to their death.

5.3.3 The Association Between Melanization and Cocoon Formation

Larval melanization was assessed by observing the production of melanin (black spot) on the body of a larva, as shown in **Figure 3.1.A.** The production of melanin by the wax worm happened due to their immune reaction towards MRSA and MSSA infection, as melanin aids in the trapping and killing of pathogenic organisms. It is common for the wax worm to die shortly after achieving complete melanization, which indicates an overwhelmed immune response (Loh et al., 2013). In this study, most of the larvae showed one black dot of melanization only at the site of injection. Furthermore, this study also discovered that the cocoon formation influenced the larval melanization depending on whether they are inside a complete, partial or none cocoon. For some larvae, either infected or uninfected, melanization could not be appropriately assessed whenever they reached the complete cocoon stage, as the observation could not be conducted as the larvae are covered by the cocoon. Jorjão et al. (2018) stated that the larvae had turned into dark reddish-brown colour as they proceed into the next stage of life (pupae). These larvae were assigned as blank (-), and it was unaccounted for since there is no suitable score in the health scoring system (**Table 3.3**), which affected the mean health score tremendously. Thus, it is suggested to add this new feature (dark reddish-brown colour) into the health scoring system.

5.3.4 The Association Between Melanization and Decomposition

Besides, the melanization for almost all dead larvae cannot be assessed because of the loss of their posterior part, which only left approximately at least 0.5 cm length of their body, probably due to the decomposition process post-death. The decaying process is a common phenomenon that happened after the death of a living organism. The dead larvae that appear good physically do not escape this process as the bacteria might have been feasting on the gut's insides but late enough to digest the whole body. They were submerged in 70% ethanol on time compared to dead larvae that lost their posterior. Alcohol is the most often used fluid preservative to sustain and protect the dead larvae against deterioration (Kwapis, 2021). Dead larvae lost their posterior part because the bacteria have broken free from the intestine and begin eating the interior organs surrounding them. Additionally, the larval digestive enzymes generally found in the colon had circulated their body, aiding in its decomposition. Minutely, when a cell dies, enzymes are released and begin to degrade the cell's structure and connections to other cells (The Australian Museum, 2020). Hence, it is suggested to monitor the survivability of the larvae often to avoid the decaying process.

CHAPTER 6

CONCLUSION

6.1 General conclusion

Throughout the last decade, the need for invertebrates organisms like *C. elegans, D. melanogaster*, and *G. mellonella* to understand microorganisms disease and host response had made a significant contribution to biomedical research. *A. grisella* can now be added to that list as an alternative animal model, as demonstrated in this study. There is relatively little information on the actual reason for the *S. aureus* outbreak, as a focal of invasion is undetected in approximately 25% of cases. Even though this study only assesses the morphological changes on *A. grisella* upon infection of *S. aureus*, it still serves its purpose as a starting point in discovering the usefulness of the *A. grisella* larval infection model. Interestingly, this study shows significant morphological changes on *A. grisella* larvae upon infection of two strains of *S. aureus*, MRSA and MSSA. The survival rate of both MRSA- and MSSA-infected larvae are pretty similar, and the potency of both bacteria might be the same. However, the outcomes are partially unconvincing due to many limitations and challenges faced throughout the study. Hence, we suggest more in-depth research towards the influence of experimental design on the wax worm larvae has been carried out to vindicate the concept of MRSA and MSSA pathogenesis appropriately.

6.2 Challenges Faced Throughout This Study

Throughout this study, many challenges were faced due to the implementation of movement control order (MCO) and technical problems, but we managed to overcome them.

6.2.1 The Effect of Movement Control Order (MCO)

During MCO, the time for the laboratory to operate was only four hours, leading to the inability to incubate bacteria according to the desired time. However, both bacteria were stored inside a chiller, and then incubation was continued the following day. Besides, lab assistants could not come to the laboratory since the distance between their residence-to-faculty is more than 10 km, but they eventually received permission from UPM. A concrete reason must convince UPM authorities before permission was given. Furthermore, we were unable to retrieve *A. grisella* larvae from a supervisor, and it was solved by using Lalamove, an application that allows customers to deliver items.

6.2.2 Technical Problems

It was difficult to determine the correct incubation time for MRSA and MSSA to reach an OD of 600 nm = 0.4-0.6 as it took 3-4 weeks to discover the correct incubation period. Besides, the spectrophotometer that we used in MPG 8 malfunctioned, and luckily, we were permitted to use a spectrophotometer from Lab Microbe, Level 2, with the help of a PhD student. Moreover, there were some

changes and difficulties encountered in part of the methodology. First, the dilution factor was changed from 10X to 100X because the viable count for both bacteria was too numerous to count (TNTC). Next, it was difficult to infect the *A. grisella* larvae because of their small size, which needed total concentration, and it took quite some time to inject 45 larvae per batch. Lastly, an unsuitable incubator was used, resulting in all larvae died, including the control group, after one-day incubation at 37 ^{o}C. The death of larvae might be due to suffocation for air, or they require a high humidity environment to survive. Unfortunately, we cannot try alternative incubators from other laboratories because of the difficulty of gaining access due to the MCO constraint. In the end, we just incubate them at room temperature in College Seventeenth's Dorm of UPM, ranging from 25-33 ^{o}C.

6.3 Limitation of This Study

Even though *A. grisella gives* a fast and cost-effective way to gather preliminary data; it may not completely replace the mammals model. This is because the *A. grisella* infection model is very new and not well recognized compared to other invertebrates models (Cook & McArthur, 2013). Besides, *A. grisella*'s genomic sequences have not been completely mapped, and no accessibility to microarrays or RNA interference libraries has been established (Koseva et al., 2019). Moreover, there seem to be no stock centres for grisella, as there are for Drosophila, in which the research team could obtain a particular genotype reared under specified conditions. Once the larvae are obtained, handling aspects, such

as husbandry, lighting, nutrition, mating condition, and management, may differ among research centres. These variations might produce a different outcome of larval survivability after being infected by a selected microbe. Therefore, these issues could be resolved by establishing stock centres that provide the best standard population of *A. grisella*.

6.4 Future Recommendations

For future studies involving the *A. grisella* larvae infection model, the sample size for a population batch should be 10-20 larvae per group to generate a reliable survival curve that represents a population. Besides, a beginner researcher tends to misinterpret the condition of larvae during observation, especially when the larvae are metamorphosing into pupae. Therefore, it is suggested to add a new feature dark reddish-brown colour of larvae's body into the health scoring system not to influence the larval mean health index greatly. Lastly, it is suggested to monitor the survivability of the larvae frequently to avoid the decaying process as it can destroy the body of a larva.

REFERENCES

Balla, K. M., & Troemel, E. R. (2013). *Caenorhabditis elegans* as a Model for Intracellular Pathogen Infection. *Cellular Microbiology*. *15*(8), 1313–1322. https://doi.org/10.1111/cmi.12152

Boundless Microbiology. (n.d.). Counting Bacteria. Boundless Microbiology. Lumen Candela.https://courses.lumenlearning.com/boundless-microbiology/chapter/counting-bacteria/

Britannica, T. (2021). Invertebrate. Encyclopedia Britannica. https://www.britannica.com/animal/invertebrate

Britannica, T. (2021). Larva. Encyclopedia Britannica. https://www.britannica.com/science/larva

Brown, A. F., Leech, J. M., Rogers, T. R., & McLoughlin, R. M. (2014). *Staphylococcus Aureus* Colonization: Modulation of Host Immune Response and Impact on Human Vaccine Design. *Frontiers in immunology*, 4, 507. https://doi.org/10.3389/fimmu.2013.00507

Browne, N., Heelan, M., & Kavanagh, K. (2013). An Analysis of The Structural and Functional Similarities of Insect Hemocytes and Mammalian Phagocytes. Virulence. 4:597-603; http://dx.doi.org/10.4161/viru.25906

Cook, S. M., & McArthur, J. D. (2013). Developing *Galleria mellonella* as a Model Host for Human Pathogens. *Virulence*. 1;4(5): 350-3. DOI: 10.4161/viru.25240.

Desbois, A. P., & Coote, P. J. (2011) Wax Moth Larva (*Galleria mellonella*): An In Vivo Model for Assessing the Efficacy of Anti-*Staphylococcal* Agents. 66:1785-90; http://dx.doi. org/10.1093/jac/dkr198

Edgley, M. (2015). What is *C. elegans?. College of Biological Sciences*. University of Minnesota. https://cbs.umn.edu/cgc/what-c-elegans

Egelie, A. A., Mortensen, A. N., Barber, L., Sullivan, J., & Ellis, J. D. (2015). Lesser Wax Moth Scientific Name: *Achroia grisella Fabricius* (Insecta: *Lepidoptera*: *Pyralidae*). University of Florida. https://entnemdept.ufl.edu/creatures/MISC/BEES/Achroia_grisella.htm

Ellis, J. D., Graham, J. R., & Mortensen, A. (2013). Standard Methods for Wax Moth Research*. Journal of Apicultural Research*, 52(1), 1-17. https://doi.org/10.3896/IBRA.1.52.1.10

Evans, B. A., Rozen, D. E. (2012). A *Streptococcus Pneumoniae* Infection Model in Larvae of The Wax Moth *Galleria mellonella. Eur J Clin Microbiol Infect*. 31:2653-60. http://dx.doi.org/10.1007/s10096- 012-1609-7

Félix, M. A., Duveau, F. (2012). Population Dynamics and Habitat Sharing of Natural Populations of *Caenorhabditis elegans* and *C. briggsae*. *BMC Biology.* https://doi.org/10.1186/1741-7007-10-59

Glavis-Bloom, J., Muhammed, M., & Mylonakis, E. (2012) Of Model Hosts and Man: Using *Caenorhabditis elegans*, *Drosophila melanogaster* and *Galleria mellonella* As Model Hosts for Infectious Disease Research. *Adv Exp Med Biol*. 710:11-7. http://dx.doi.org/10.1007/978-1-4419-5638-5_2

Hiramatsu, K., Cui, L., Kuroda, M., & Ito, T. (2001). The Emergence and Evolution of Methicillin-Resistant Staphylococcus aureus. Trends Microbiol. 9, 486–493. DOI: 10.1016/s0966-842x(01)02175-8

Hoffmann, J. A. (1995). Innate Immunity of Insects. *Curr Opin Immunol*. 7:4-10; http://dx.doi.org/10.1016/0952-7915(95)80022-0

Implen. (2021). The OD600 Basics. Best OD600 Tool to Generate Microbial Growth Curves. Implen NanoPhotometers. The Best in Spectrophotometers. https://www.implen.de/od600-diluphotometer/od600/

Jorjão, A. L., Oliveira, L. D., Scorzoni, L., Lívia, M. A., Figueiredo-Godoi, M., Prata, C. A., Olavo, A., Jorge, C., Junqueira, J. C., Jorjão, A. A. L., & Figueiredo-Godoi, M. A. (2018). From Moths to Caterpillars: Ideal Conditions for *Galleria mellonella* Rearing For In Vivo Microbiological Studies. https://doi.org/10.1080/21505594.2017.1397871

Kluytmans, J., Van, B. A., & Verbrugh, H. (1997). Nasal Carriage of *Staphylococcus Aureus*: Epidemiology Underlying Mechanisms Associated Risks. *Clinical Microbiology Reviews* 10: 505-520. DOI: 10.1128/CMR.10.3.505

Konkel, M. E., & Tilly, K. (2000). Temperature-Regulated Expression of Bacterial Virulence Genes. *Microbes Infect*. 2:157-66; http://dx.doi.org/ 10.1016/S1286-4579(00)00272-0

Koseva, B. S., Hackett, J. L., Zhou, Y., Harris, B. R., Kelly, J. K., Greenfield, M. D., Gleason, J. M., & Macdonald, S. J. (2019). *Quantitative Genetic Mapping and Genome Assembly in the Lesser Wax Moth Achroia grisella*. https://doi.org/10.1534/g3.119.400090

Kurokawa, K., Kaito, C., & Sekimizu, K. (2007). Two-Component Signaling in The Virulence of *Staphylococcus aureus*: A Silkworm Larvae-Pathogenic Agent Infection Model of Virulence. In *Methods in enzymology* (Vol. 422, pp. 233-244). Academic Press. DOI: 10.1016/S0076-6879(06)22011-1

Kwadha, Charles, A., Ong'amo., George, O., Ndegwa., Paul, N., Raina., Suresh, K., Fombong, & Ayuka, T. (2017). *The Biology and Control of the Greater Wax Moth, Galleria mellonella*. *Insects*. 8 (2):61. DOI:10.3390/insects8020061.

Kwapis, M. A. (2021). *Wet Specimens - A General Guide*. Mickey Alice Kwapis. https://mickeyalicekwapis.com/blog/2015/9/10/wet-specimens-a-general-guide

La Rosa, S. L., Casey, P. G., Hill, C., Diep, D. B., Nes, I. F., & Brede, D. A. (2013). In Vivo Assessment of Growth and Virulence Gene Expression During Commensal and Pathogenic Lifestyles of Lux-ABCDE-Tagged *Enterococcus faecalis* Strains on Murine Gastrointestinal and Intravenous Infection Models. *Appl Environ Microbiol*. 79:3986-97. http://dx.doi.org/10.1128/AEM.00831-13

Lionakis, M. S. (2011). *Drosophila* and *Galleria* Insect Model Hosts: New Tools for The Study of Fungal Virulence, Pharmacology and Immunology. *Virulence*. 2:521-7; http://dx.doi.org/10.4161/ viru.2.6.18520

Liu, G. Y. (2009). Molecular Pathogenesis of *Staphylococcus aureus* Infection. *Pediatric research*, *65*(5 Pt 2), 71R–77R. https://doi.org/10.1203/PDR.0b013e31819dc44d

Loh, J. M., Adenwalla, N., Wiles, S., & Proft, T. (2013). *Galleria Mellonella* Larvae as An Infection Model for Group A *Streptococcus*. *Virulence*, 4(5), 419-428. https://doi.org/10.4161/viru.24930

Mahgoub, M. O., Lau, W. H., & Omar, D. (2015). Observations on the Biology and Larval Instars Discrimination of Wax Moth *Achroia grisella F*. (*Pyralidae*: *Lepidoptera*). *Journal of Entomology* 12 (1): 1-11, DOI: 10.3923/je.2015.1.11

McGregor, S. E. (2017). *Beekeeping - Disease and pest control.* Encyclopedia Britannica. https://www.britannica.com/topic/beekeeping

Medveďová, A., & Valík, L. (2012). *Staphylococcus aureus*: Characterization and Quantitative Growth Description in Milk and Artisanal Raw Milk Cheese Production. DOI: 10.5772/48175

Needham, A. J., Kibart, M., Crossley, H., Ingham, P. W., & Foster, S. J. (2004). *Drosophila melanogaster* as a Model Host for *Staphylococcus aureus* Infection. *Microbiology*, *150*(7), 2347–2355. https://doi.org/10.1099/mic.0.27116-0

Otto, M. (2008). *Staphylococcal* Biofilms. *Bacterial Biofilms*, 207-228. https://link.springer.com/chapter/10.1007/978-3-540-75418-3_10

Park, S. Y., Kim, K. M., Lee, J. H., Seo, S. J., Lee, I. H. (2007). Extracellular Gelatinase of *Enterococcus faecalis* Destroys a Defense System in Insect Hemolymph and Human Serum. *Infect Immun*. 75:1861-9. http://dx.doi.org/10.1128/IAI.01473-06

Peleg, A. Y., Monga, D., Pillai, S., Mylonakis, E., Moellering, R. C., Jr., Eliopoulos, G. M. (2009). Reduced Susceptibility to Vancomycin Influences Pathogenicity in *Staphylococcus aureus* Infection. *J Infect*. 199:532-6; http://dx.doi.org/10.1086/596511

Quiblier, C., Seidl, K., Roschitzki, B., Zinkernagel, A. S., Berger-Bachi, B., & Senn, M. M. (2013). Secretome Analysis Defines the Major Role of SecDF in *Staphylococcus aureus* Virulence. *PLoS One.* 8:e63513; http://dx.doi.org/10.1371/journal.pone.0063513

Rağbetli, C., Parlak, M., Bayram, Y., Guducuoglu, H., & Ceylan, N. (2016). Evaluation of Antimicrobial Resistance in *Staphylococcus aureus* Isolates by Years. *Interdisciplinary perspectives on infectious diseases*, *2016*, 9171395. https://doi.org/10.1155/2016/9171395

Ramarao, N., Nielsen-Leroux, C., & Lereclus, D. (2012). The Insect *Galleria mellonella* As a Powerful Infection Model to Investigate Bacterial Pathogenesis. (70):e4392; http://dx.doi.org/ 10.3791/4392.

Ryan, K. J., Ray, C. G. (2004). Sherris Medical Microbiology: An Introduction to Infectious

Scoble, M. (1995). Classification of the *Lepidoptera*. Oxford University Press,

Scott, S. (2021). *Why use the fly in research?* Your genome. https://www.yourgenome.org/facts/why-use-the-fly-in-research

Sheehan, G., Dixon, A., & Kavanagh, K. (2019). Utilization of *Galleria Mellonella* Larvae to Characterize the Development of *Staphylococcus aureus* Infection. *Microbiology*, 165(8), 863-875. DOI: 10.1099/mic.0.000813

Sifri, C. D., Begun, J., Ausubel, F. M., & Calderwood, S. B. (2003). *Caenorhabditis elegans* as a Model Host for *Staphylococcus aureus* Pathogenesis. *Infection and immunity*, *71*(4), 2208-2217. https://doi.org/10.1128/IAI.71.4.2208-2217.2003

Swearengen, J. R. (2018). Choosing The Right Animal Model for Infectious Disease Research. *Animal Models and Experimental Medicine*, *1*(2), 100–108. https://doi.org/10.1002/ame2.12020

Tang, H. (2009). Regulation and Function of the Melanization Reaction in *Drosophila*. *Fly (Austin)*. 3:105-11. http://dx.doi.org/10.4161/fly.3.1.774

Tenover, F. C., and Gorwitz, R. J. (2006). The Epidemiology of *Staphylococcus* Infections. In ‘‘Gram-Positive Pathogens’’. pp. 526–534. ASM Press, Washington, DC.

The Australian Museum. (2020). *Stages of Decomposition*. https://australian.museum/learn/science/stages-of-decomposition/

Tong, S. Y., Davis, J. S., Eichenberger, E., Holland, T. L., & Fowler Jr, V. G. (2015). *Staphylococcus aureus* Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. *Clinical microbiology* reviews, 28(3), 603-661. https://doi.org/10.1128/CMR.00134-14

Tsai, C. J. Y., Loh, J. M. S., & Proft, T. (2016). *Galleria mellonella* Infection Models for The Study of Bacterial Diseases and for Antimicrobial Drug Testing. *Virulence*, 7(3), 214–229. https://doi.org/10.1080/21505594.2015.1135289

Velikova, N., Kavanagh, K., & Wells, J. M. (2016). Evaluation of *Galleria mellonella* Larvae for Studying the Virulence of *Streptococcus suis*. *BMC Microbiology*, *16*(1), 1–9. https://doi.org/10.1186/s12866-016-0905-2

Wand, M. E., McCowen, J. W., Nugen,t P. G., & Sutton, J. M. (2013). Complex Interactions of *Klebsiella pneumoniae* with the Host Immune System in a *Galleria mellonella* Infection Model. *J Med Microbiol*. 62:1790-8; http://dx.doi.org/10.1099/jmm.0.063032-0

Warren, L. O., Huddleston., & Paul. (1962). Life History of the Greater Wax Moth, *Galleria mellonella L*., in Arkansas. *Journal of the Kansas Entomological Society*. 35(1): 212–216. https://www.jstor.org/stable/25083247

APPENDICES

APPENDIX 1

Individual Score for Every *A. grisella* Larvae Upon Injection of PBS and Infection of MRSA and MSSA

1. Negative uninfected control (5 larvae injected with PBS alone)

Control (PBS)	Day 1					Day 2					Day 3					Day 4					Day 5				
	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score
C1	2	0.5	4	2	8.5	2	0.5	3	2	7.5	2	0.5	3	2	7.5	2	1	3	2	8	0	1	unvisible (inside cocoon)	2	3
C2	2	0	4	2	8	2	0.5	3	2	7.5	2	0.5	3	2	7.5	0	0.5	3	2	5.5	0	1	unvisible (inside cocoon)	2	3
C3	1	0	4	2	7	0	0	3	0	3															
C4	1	0	4	2	7	0	0	3	0	3															
C5	0	0	3	0	3																				
Total Score					6.7	Total Score				5.25	Total Score				7.5	Total Score				6.75	Total Score				3

2. (i) MRSA infected group (10 larvae injected with 8.0 x 10^8 CFU/mL)

MRSA (8.0 x 10^8)	Day 1					Day 2					Day 3					Day 4					Day 5				
	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score
R1	3	0.5	4	2	9.5	2	0.5	3	2	7.5	2	1	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R2	3	0.5	3	2	8.5	2	0.5	3	2	7.5	2	1	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R3	3	0.5	3	2	8.5	2	0.5	3	2	7.5	2	1	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R4	3	0	3	2	8	2	0.5	3	2	7.5	2	1	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R5	3	0	3	2	8	2	0.5	3	2	7.5	2	1	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R6	2	0	3	2	7	2	0.5	3	2	7.5	2	0.5	3	2	7.5	3	0.5	3	2	8.5	3	0.5	4	2	9.5
R7	1	0	3	2	6	2	0.5	3	2	7.5	2	0.5	3	2	7.5	2	0	3	2	7	2	0.5	3	2	7.5
R8	1	0	3	2	6	2	0.5	3	2	7.5	2	0.5	3	2	7.5	0	0	unvisible (crushed butt)	0	0					
R9	0	0	unvisible (crushed butt)	0	0																				
R10	0	0	unvisible (crushed butt)	0	0																				
Total Score					6.15	Total Score				7.5	Total Score				5.19	Total Score				3.81	Total Score				4.57

2. (ii) MRSA infected group (10 larvae injected with 1.0 x 10^9 CFU/mL)

MRSA (1.0 x 10^9)	Day 1					Day 2					Day 3					Day 4					Day 5				
	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score
R1	3	0.5	4	2	9.5	3	1	3	2	9	1	1	3	2	7	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R2	3	0.5	4	2	9.5	3	0.5	3	2	8.5	2	1	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R3	3	0	3	2	8	3	0.5	3	2	8.5	2	0.5	3	2	7.5	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R4	3	0	3	2	8	3	0.5	3	2	8.5	3	0	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R5	3	0	3	2	8	3	0.5	3	2	8.5	3	0	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R6	3	0	3	2	8	2	0.5	3	2	7.5	3	0	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R7	3	0	3	2	8	2	0.5	3	2	7.5	3	0	3	2	8	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
R8	3	0	3	2	8	0	0.5	3	2	5.5	3	0	3	2	8	0	0	3	0	3					
R9	3	0	3	2	8	0	0	unvisible (crushed butt)	0	0															
R10	0	0	unvisible (crushed butt)	0	0																				
Total Score					7.5	Total Score				7.06	Total Score				7.81	Total Score				3	Total Score				3

3. (i) MSSA infected group (10 larvae injected with 3.0 x 10^8 CFU/mL)

MSSA (3.0 x 10^8)	Day 1					Day 2					Day 3					Day 4					Day 5				
	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score
S1	2	0.5	4	2	8.5	2	0.5	3	2	7.5	1	1	3	2	7	0	1	4	2	7	0	1	unvisible (inside cocoon)	2	3
S2	2	0.5	3	2	7.5	2	0.5	3	2	7.5	1	1	3	2	7	0	1	4	2	7	0	1	unvisible (inside cocoon)	2	3
S3	2	0.5	3	2	7.5	2	0.5	3	2	7.5	1	1	3	2	7	0	1	4	2	7	0	1	unvisible (inside cocoon)	2	3
S4	2	0.5	3	2	7.5	2	0.5	3	2	7.5	1	1	3	2	7	0	1	4	2	7	0	1	unvisible (inside cocoon)	2	3
S5	2	0	3	2	7	2	0.5	3	2	7.5	2	0.5	3	2	7.5	0	1	3	2	6	0	1	unvisible (inside cocoon)	2	3
S6	2	0	3	2	7	2	0.5	3	2	7.5	2	0.5	3	2	7.5	2	0.5	3	2	7.5	0	1	unvisible (inside cocoon)	2	3
S7	2	0	3	2	7	2	0.5	3	2	7.5	3	0	3	2	8	2	0	4	2	8	2	0.5	3	2	7.5
S8	2	0	3	2	7	2	0.5	3	2	7.5	3	0	3	2	8	2	0	3	2	7	0	0	2	0	2
S9	1	0	3	2	6	0	0	2	0	2															
S10	0	0	3	0	3																				
Total Score					6.8	Total Score				6.89	Total Score				7.38	Total Score				7.06	Total Score				3.44

3. (ii) MSSA infected group (5 larvae injected with 1.0 x 10^9 CFU/mL)

MSSA (1.0 x 10^9)	Day 1					Day 2					Day 3					Day 4					Day 5				
	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score	Activity	Cocoon	Melanization	Survival	Score
C1	3	0	4	2	9	2	0.5	3	2	7.5	2	0.5	3	2	7.5	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
C2	2	0	3	2	7	2	0.5	3	2	7.5	3	0.5	3	2	8.5	0	1	unvisible (inside cocoon)	2	3	0	1	unvisible (inside cocoon)	2	3
C3	2	0	3	2	7	2	0.5	3	2	7.5	3	0	3	2	8	1	1	unvisible (inside cocoon)	2	4	0	1	unvisible (inside cocoon)	2	3
C4	2	0	3	2	7	2	0.5	3	2	7.5	3	0	3	2	8	3	0	3	2	8	3	0	3	2	8
C5	0	0	3	0	3																				
Total Score					6.6	Total Score				7.5	Total Score				8	Total Score				4.5	Total Score				4.25