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Investigating bacterial infections in *Galleria mellonella* larvae: Insights into pathogen dissemination and behavior

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ABSTRACT

The insect *Galleria mellonella* is an alternative animal model widely used for studying bacterial infections. It presents a wide range of advantages, including its low cost, easy maintenance and lack of ethical constraints. Among other features, their innate immune system is very similar to that of mammals. In this study, we dissected several larvae infected with important human pathogens: *Mycobacterium abscessus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* By observing the fat body, gut, trachea, and hemolymph under the microscope, we were able to describe where bacteria tend to disseminate. We also quantified the number of bacteria in the hemolymph throughout the infection course and found significant differences between the different pathogens. With this work, we aimed to better understand the behavior and dissemination of bacteria in the infected larvae.

1. Introduction

Bacterial infections are becoming more threatening every day. Antimicrobial resistance is one of the major public health concerns of this century and one of the leading causes of death globally (Murray et al., 2022). *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Mycobacterium abscessus* are three opportunistic pathogens that can produce important infections that are also challenging to treat due to the acquisition of resistance to multiple drugs (Guo et al., 2020; Hirsch and Tam, 2010; Petrini, 2006).

Galleria mellonella, an insect from the order *Lepidoptera*, has become a popular animal model for the study of mainly bacterial and fungal infections. Unlike other models, it has a convenient size for manipulation and can be maintained at 37 °C. Their breeding is inexpensive and does not require extra laboratory facilities. Moreover, being an invertebrate, it does not present ethical restrictions. Their innate immune system shares multiple similarities with ours, as they have both cellular and humoral defenses. Therefore, precise doses of pathogens and antimicrobial agents can be injected to test their efficacy and toxicity. These properties make this insect a complete and attractive alternative to study infections (Champion et al., 2016; Ménard et al., 2021; Tsai et al., 2016; Wojda, 2017).

Mycobacterium abscessus colonizes mainly compromised lungs in patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). However, it can cause many types of infections in the skin, soft tissue, bones, lymph nodes and even disseminated infections (Abdelaal et al., 2022). In *G. mellonella*, infected larvae showed similar structures to the granulomas seen in human infections (Meir et al., 2018). *Staphylococcus aureus* is a Gram-positive pathogen, very prevalent and with multiple drug-resistant strains. It has been widely studied in *G. mellonella* (Fábia Pereira et al., 2020). The induction of immune peptides and the formation of nodules have been previously reported in this insect, resembling some human infections (Sheehan et al., 2019). The main colonization site in humans is the anterior nares, although other tissues of interest are the skin, throat, perineum, vagina and gastrointestinal tract. Therefore, it can cause a wide range of different infections (Brown et al., 2013). *Pseudomonas aeruginosa* is also very difficult to treat due to the rapid acquisition of antibiotic resistance. It causes acute and chronic infections in patients with CF, COPD and burns, among others (Qin et al., 2022). *G. mellonella* has been used to study the virulence of this pathogen, which has been correlated with mice infections (Jander et al., 2000).

In this work, we have dissected several *G. mellonella* larvae infected with different significant pathogens. We aimed to study how these bacteria disseminate, and which tissues are the most colonized in this animal model. The search for common patterns and differences can

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contribute to investigating new antimicrobial targets and a better understanding of bacteria dissemination in this excellent model.

2. Material and methods

2.1. Bacteria culture conditions

In this study, we used three bacterial strains that constitutively express green fluorescent protein (GFP). *Pseudomonas aeruginosa* (MK171 - PAO1::eGFP) (Moya-Andérico et al., 2020) was cultured on Luria-Bertani medium (LB; Scharlab, S.L., Barcelona, Spain), while *Staphylococcus aureus* (8325-4) transformed with the plasmid pHRG (Catalan-Moreno et al., 2021) and *Mycobacteroides abscessus* (ATCC 19977) with the plasmid pFPV27 (Campo-Pérez et al., 2021), were both cultured on tryptic soy broth (TSB; Sharlab, S.L.).

P. aeruginosa and *S. aureus* were cultured overnight at 37 °C with agitation. The bacterial broth was centrifuged at 2500*g* (Labnet Spectrafuge™ 6C) for 10 min to prepare the injection inoculum. The supernatant was discarded, and the cell pellets were resuspended in 5 mL of 1 × PBS (Fisher Scientific, Madrid, Spain) for three washes. The optical density (OD_{590}) was measured using a 1/10 dilution with 1 × PBS and equalized to a final OD_{590} of 1. *M. abscessus* was grown on a plate for a week at 37 °C. A loopful of isolated colonies was mixed with 1 × PBS, and the OD_{590} was measured and equalized to 0.35. Afterwards, 10-fold serialized dilutions of all equalized cultures were made with 1 × PBS for larvae injection.

2.2. G. mellonella maintenance and infection

G. mellonella larvae were fed an artificial diet (15% corn flour, 15% wheat flour, 15% infant cereal, 11% powdered milk, 6% brewer's yeast, 25% honey, and 13% glycerol) and reared at 34 °C in darkness (Moya-Andérico et al., 2021). Groups of 8 larvae (approximately 200 mg) were injected with different dilutions through the top right proleg using a 26-gauge microsyringe (Hamilton, Reno, NV, USA). The different inoculum doses were plated onto agar plates to determine bacterial counts. Larvae were monitored at 37 °C during the infection course, and survival curves were plotted with GraphPad Prism 9.0 software to define inoculum doses.

2.3. G. mellonella dissection

Infected larvae with defined doses were killed by freezing at −20 °C for at least 24 h. Then, under a stereoscopic microscope (VWR) with the VisiLight CL-150 (VWR), larvae were cut with a sterile surgical blade (size 23, Paramount Surgimed, New Delhi, India) and selected tissues of interest (fat, gut and trachea) were removed with dissection forceps and placed on top of microscope coverslips (thickness 1.5). Images of *G. mellonella* dissections were performed with a mobile phone camera of 48 mpx (Oppo A74).

2.4. Tissue imaging by confocal microscopy

Tissue samples from *G. mellonella* larvae were dyed with FM™ 4–64 (Invitrogen) at a concentration of 5 μg/mL. Then, they were visualized under an LSM 800 confocal laser scanning microscope (Zeiss, Aalen, Germany) with a 63×/1.4 oil objective. The images obtained were analyzed using ZEN Microscopy Software (version 2.6) and ImageJ FIJI (version 1.52p).

2.5. Hemolymph extraction and analysis

Infected larvae were anesthetized on ice and their tails were cut off using a sterile surgical blade (size 23). The hemolymph was collected into Eppendorf tubes, and to prevent melanization, the hemolymph was kept on ice during the whole process. Hemolymph was pooled from 8 larvae group and observed with an inverted fluorescence microscope ECLIPSE Ti − S/L100 (Nikon, Tokyo, Japan) coupled with a DS-Qi2 camera (Nikon, Tokyo, Japan) using a 100×/1.30 oil objective with the GFP filter for the bacteria green fluorescence.

After hemolymph collection, serial dilutions were made, and the number of colony-forming units (CFUs) was determined by plating on agar at different time points after infection. In addition, entire larvae were disrupted and homogenized with a 20G syringe. The samples were then placed in an ultrasonic bath for 5 min and vortexed for 30 s. Serial dilutions were made from the homogenized samples and plated onto agar to determine the number of CFUs.

3. Results and discussion

3.1. Bacterial infection of G. mellonella larvae

Various groups of larvae were infected with different concentrations of each bacterial strain (*M. abscessus, S. aureus* and *P. aeruginosa*) to determine the optimal doses and incubation time for *G. mellonella* tissue collection (Fig. 1). The figure shows that all three pathogens exhibit diverse virulence patterns in *G. mellonella* larvae survival. Consequently, different doses of bacteria inoculation were employed based on the type of strain used (see Table 1). All doses were selected based on the survival curves (Fig. 1). We were interested in a dose and an incubation time that would enable us to have a substantial number of live bacteria during the infection course.

3.2. Larvae dissection and microscope observation of infected G. mellonella tissues

After the incubation (see Material and Methods and Table I), the larvae were frozen at −20 °C. This freezing step is crucial for dissecting the larvae, as it makes them more rigid and easier to cut, allowing the isolation of individual tissues. A general anatomical diagram of *G. mellonella* was created to facilitate the location of the main organs and tissues (Fig. 2A).

Once under the stereoscopic microscope and after making a sagittal incision (from the head to the last part of the abdomen, see Fig. 2B), very different melanization patterns were observed (Fig. 3, see circles) depending on the bacteria used for infection. Melanization is an immune response that can occur in insects due to injury or microbial infection. As in mammals, infectious pathogens are identified as foreign entities by the immune system. Pattern recognition receptors (PRRs) bind to molecules such as lipopolysaccharides (LPS) and peptidoglycan, which are components found in bacterial cell walls (Pereira et al., 2018). This triggers in insects the activation of the prophenoloxidase cascade, which leads to the production of melanin that deposits around the foreign target (in our case, bacteria) or damaged tissue. The aim is to restrain the dissemination of the infection to other tissues. This process is easily identified by melanin pigmentation (grey or black spots) (González-Santoyo and Córdoba-Aguilar, 2012; Nakhleh et al., 2017).

We observed specific black nodules or a disseminated dark pigmentation covering the larvae's entire body, as in the case of *P. aeruginosa* (Fig. 3). This tends to occur when the larvae are close to death, or a very high concentration of bacteria is injected into their system. The fat (and fat body) was the first tissue to be localized after the incision, as it is found just under the larvae cuticle. When part of the fat body (indicated as F in Fig. 3) was removed, the gut (G) was easily recognized for its yellowish color. The gut was also removed for analysis using the dissection forceps. The trachea (T) was distributed along the body and particularly attached to the gut. Carefully, some pieces of it were also extracted for microscopy. During the dissection, images of all infected larvae and one uninfected control were taken (see Fig. 3).

3.2.1. Fat body

The fat body is a distinct and unique organ in insects, encompassing

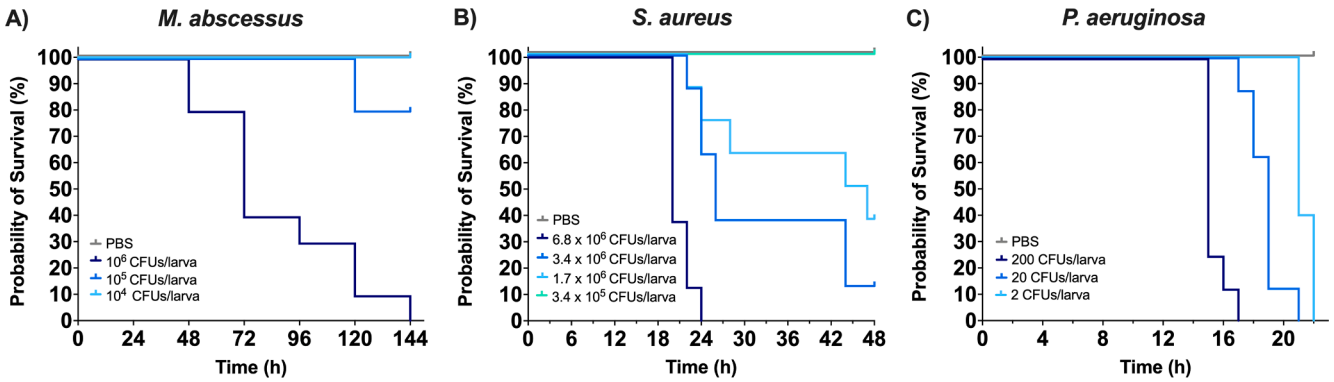

Fig. 1. Kaplan-Meier survival curves of *G. mellonella* after infection with *M. abscessus* **(A)**, *S. aureus* **(B)** and *P. aeruginosa* **(C)**. Graphs were plotted with GraphPad Prism 9.0 software.

Table 1
Optimized bacterial infection doses and incubation time were used for subsequent experiments.

	M. abscessus	*S. aureus*	*P. aeruginosa*
Bacterial injection	2 x10^5 CFUs/larva	2x10^6 CFUs/larva	10 CFUs/larva
Incubation time at 37 °C	48 h	20 h	16 h

functions beyond what is commonly known as fat or adipose tissue. It serves as a site for the synthesis and storage of energy reserves such as fat and glycogen. Additionally, it plays a role in innate immunity, participating in the synthesis of hemolymph proteins and antimicrobial peptides. The fat body is also involved in metabolism and insect development, among other functions (Arrese and Soulages, 2010; Li et al., 2019).

One common feature of all three bacterial species studied is that they were widely found and disseminated in the fat. This can be observed in Fig. 3, in the cases of *M. abscessus* and specifically *S. aureus*, where plenty of melanized nodules are found in the fat (see circles in the images). Locating those specific melanized areas in *P. aeruginosa*-infected larvae is challenging as melanization tends to occur more suddenly and throughout the body. However, by looking at all the samples under the confocal microscope, we could confirm that all three pathogens widely colonized the fat tissue preferentially (Fig. 4A). This is unsurprising, as after injecting the bacteria into the hemocoel, the fat body is the first organ bacteria would reach. Under the cuticle, larvae have a broad layer of fat (fat body) that surrounds and protects the organs found in the abdominal, thorax, and head areas. Being one of the most critical tissues in insects for its multiple functions, it is arranged in thin lobes maintaining direct contact with the hemolymph and integrating the signals that come from all the body (Skowronek et al., 2021).

Previously, *M. abscessus* has been found in the fat body of *G. mellonella* larvae, displaying a similar structural pattern to that observed in human granulomas. Bacteria grew inside the larvae and killed them in a reproducible manner. As a result, *G. mellonella* was proposed as a potential model for studying host-pathogen interactions (Meir et al., 2018). Moreover, a recent study demonstrated the accumulation and growth of *M. abscessus* in the fat bodies of silkworms, another species of Lepidoptera (Matsumoto et al., 2023).

In the case of *P. aeruginosa,* bacteria invaded and caused destruction of the insect *Oncopeltus fasciatus'* fat body (Dorn, 1977). During infection, it is well-known that bacteria can undergo changes in their metabolic pathways, altering their overall metabolisms to optimize the utilization of host nutrients available for growth. Therefore, genes involved in the beta-oxidation and the glyoxylate shunt pathways have been reported to be upregulated in a *P. aeruginosa* infection (Crousilles et al., 2015). Similar results were described in *in vitro* biofilms of *M. abscessus*, with increased mycolic acid biosynthesis (Dokic et al., 2021). In *S. aureus, fadABDE* genes involved in the beta-oxidation pathway were also upregulated in a mouse liver infection (Hamamoto

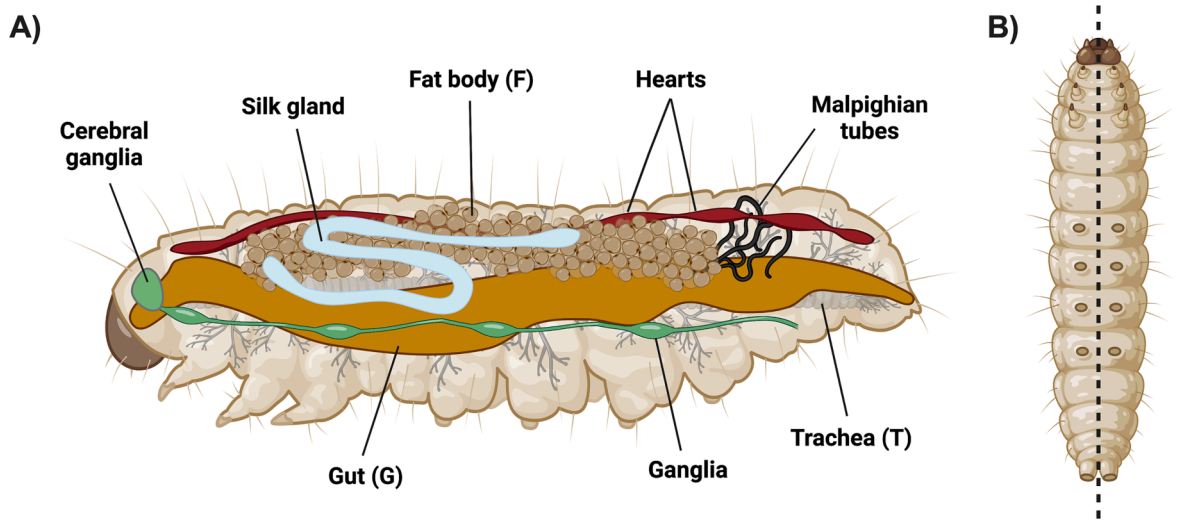

Fig. 2. General anatomy of *G. mellonella* larvae. **A)** Most important tissues and organs of *G. mellonella* larvae. **B)** Sagittal incision for dissecting *G. mellonella* larvae. Created with www.Biorender.com (accessed on the 3rd of March 2023).

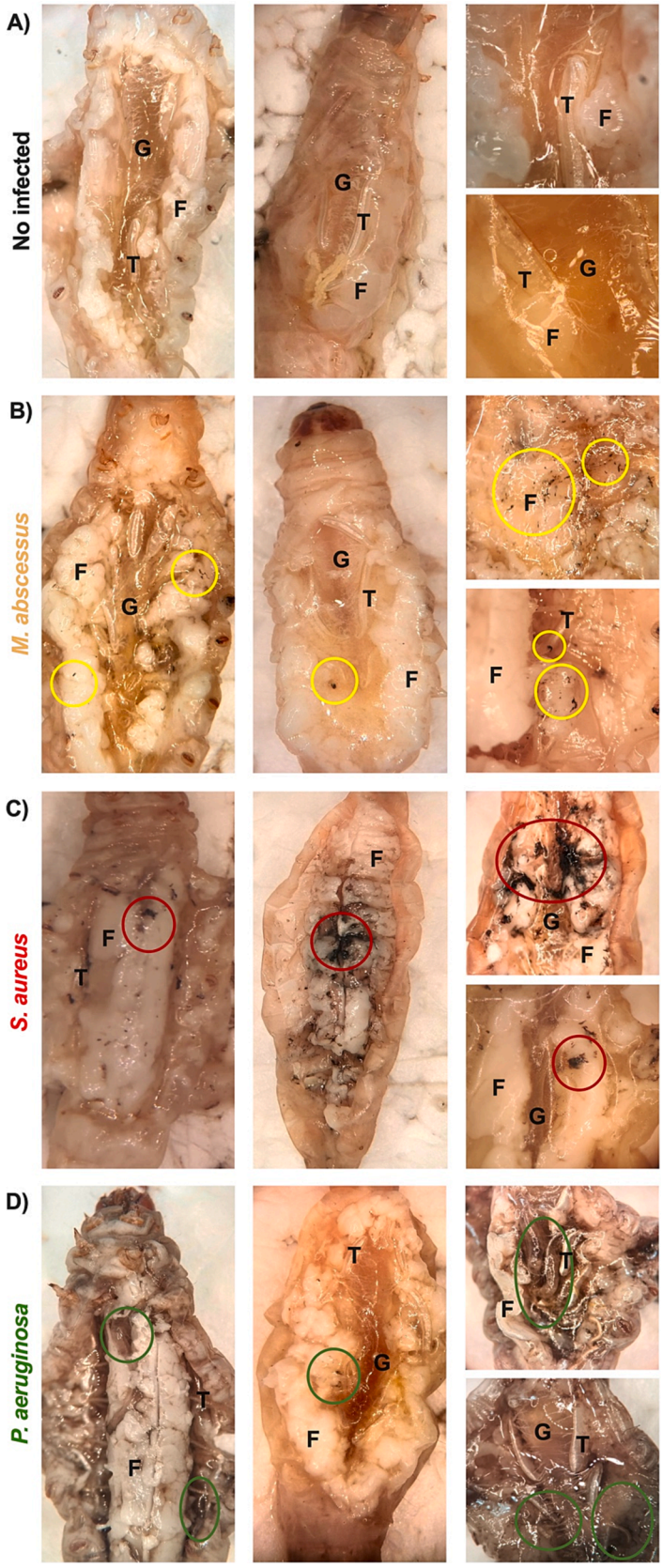

Fig. 3. *G. mellonella* dissection images under the stereoscopic microscope. **A)** Uninfected larvae. **B)** Larvae infected with *M. abscessus* at 48 h. **C)** Larvae infected with *S. aureus* at 20 h. **D)** Larvae infected with *P. aeruginosa* at 16 h. Tissues are marked as follows: Fat body (F), Gut (G), and Trachea (T). Circles indicate melanized areas.

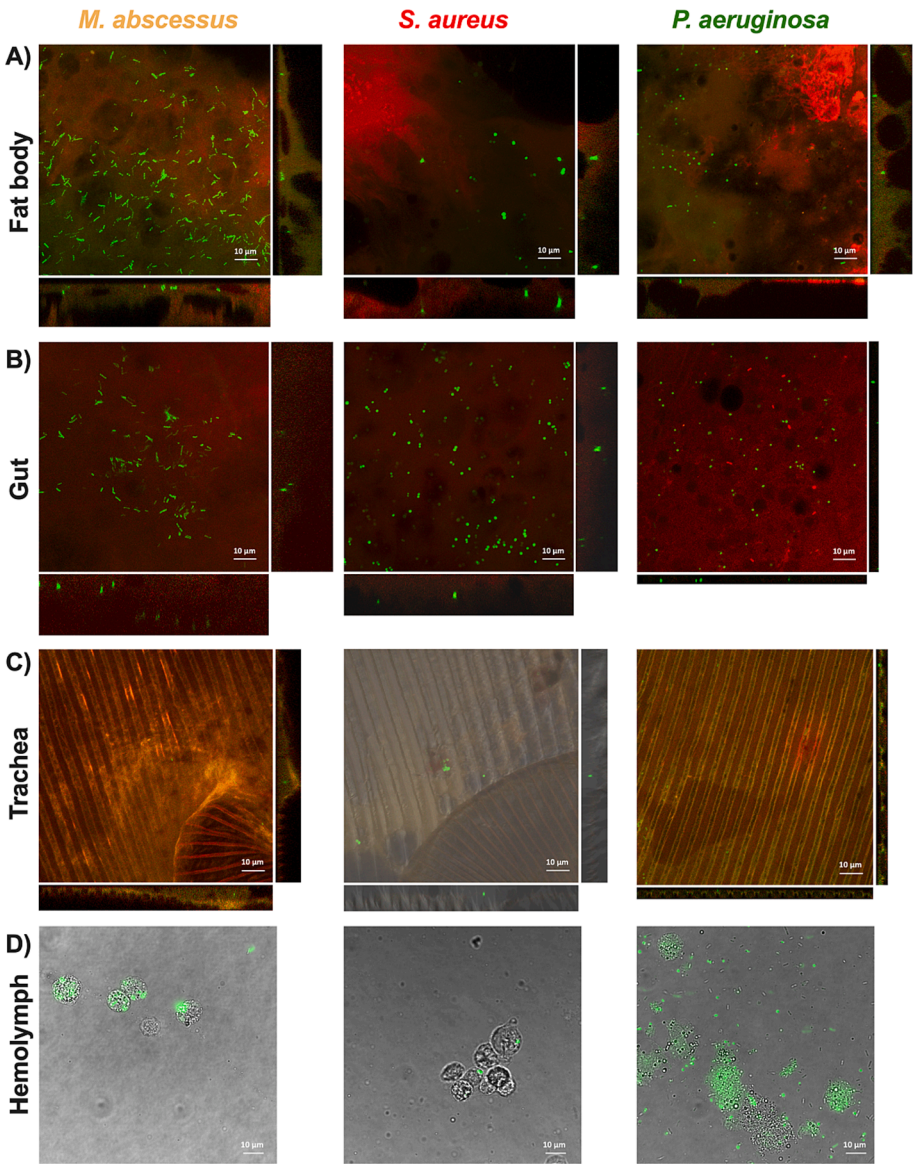

Fig. 4. Microscopic images of different dissected tissues: **A)** fat body, **B)** gut, **C)** trachea and **D)** hemolymph, from *G. mellonella* larvae infected with *M. abscessus* (48 h), *S. aureus* (20 h) and *P. aeruginosa* (16 h) respectively. Images **(A-C)** were taken with confocal microscopy, with a magnification of 63 ×. **D)** Phase contrast images merged with green fluorescence taken at a magnification of 100 × in a fluorescence microscope. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2022). Therefore, there is no doubt that the fat body plays a very significant role during all bacterial infections, as we have also observed, in this work, the broad distribution of pathogens in this specific tissue (Fig. 3, Fig. 4).

3.2.2. Gut

We also isolated and collected gut samples (denoted as a G in Fig. 3) to analyze the presence of bacteria in this organ. Its main role is nutrient absorption and digestion, but it also defends the system from ingested pathogens. The peritrophic membrane is a semipermeable chitin and glycoprotein layer that defines the insect midgut and has a similar role to the mucous secretions of vertebrate digestive tract (Rosales, 2011; Wu et al., 2016). This membrane was found severely damaged in silkworms infected with *S. aureus*, showing signs of bacteria dissemination into the midgut (Pan et al., 2023).

In our case, although there was no sign of gut melanization and the bacteria inoculums were injected into the hemocoel, all three types of bacteria were found and visualized in the gut when observed under the confocal microscope (Fig. 4B). As we can see in Fig. 3, the gut is covered by a large fat layer, so on occasion, it might be difficult to separate both tissues perfectly. Probably because of this fat tissue proximity, bacteria can also disseminate easily into the gut. Furthermore, *S. aureus* has already been reported as a common colonizer and resident of the human gut (Acton et al., 2009). This pathogen can evade the innate immune system and can migrate and disseminate through the body inside phagocytic cells (Raineri et al., 2022).

Regarding *P. aeruginosa*, it has been described a rise of intestinal colonization, especially in hospitalized patients who have cancer, immunosuppression, or surgery which are also related to changes in the general gut microbiome (Krezalek et al., 2016). This bacterium presents a wide range of virulence factors (toxins and different enzymes) that can damage epithelial cells while disrupting the epithelial barrier and modifying its permeability (Markou and Apidianakis, 2014). On the other hand, there is no evidence of *M. abscessus* colonizing the human intestine. A histological study revealed that *M. abscessus* multiplied in the body cavity and specifically in the abdomen of *Drosophila melanogaster* while causing serious tissue damage (Oh et al., 2013). To our knowledge no *G. mellonella* studies have analyzed the gut as a possible target for a *M. abscessus* infection, although in our work we found this bacterium in this specific organ.

Recently, label-free multimodal imaging was performed for the first time in *G. mellonella* larvae infected with *Enterococcus faecalis*. This type of methodology allowed the observation of gut deterioration and the loss of the microvilli brush border (Quansah et al., 2022). One limitation of

confocal microscopy is that we cannot obtain this kind of detailed and valuable information for a better understanding of bacteria pathogenicity.

3.2.3. Trachea

We also collected samples of the larvae trachea. The insect trachea (denoted T in Fig. 3) is a complex system of tubes and small ramifications (Fig. 2), called tracheoles, that exchange oxygen and carbon dioxide with the different tissues and organs. It can be found very close to the cuticle and the gut (Fig. 3), but it is easily recognizable due to its segmentation pattern (Harrison, 2009). As observed under confocal microscopy (Fig. 4C), almost no bacteria were seen in this tissue, and the presence of a few bacteria could be easily explained by sample contamination. As described before, the trachea can be attached to other tissues containing bacteria. The insect trachea has a chitin padding that protects and hardens the structure. The low humidity and lack of nutrients make the trachea a hostile environment for colonization (Wojda, 2017).

All studied pathogens in this work are opportunistic and known for causing significant lung infections in immunocompromised patients. Unfortunately, this type of pulmonary disease cannot be reproduced in this animal model due to the lack of a comparable respiratory system to vertebrates. The *G. mellonella* model offers other possibilities, such as studying host-pathogen interactions or the morphological and transcriptional changes generated after infection (Sheehan et al., 2018). Larvae have an innate immune system very similar to mammals, and this response is the first line of defense in our lungs (Cools et al., 2019).

3.2.4. Hemolymph

Insects have an open circulatory system composed of fluid (hemolymph), the body cavity (hemocoel), and several muscular pumps. The main driver of hemolymph is the dorsal vessel, a cylindrical tube that goes along the insect's length. This system delivers nutrients and hormones to the tissues while removing waste and maintaining the body's homeostasis (Hillyer and Pass, 2020). Regarding hemolymph samples (Fig. 4D), larvae infected with *S. aureus* were the only condition with fewer bacteria. Moreover, interestingly, not a single *S. aureus* cell was found freely circulating in the hemolymph, as they had all been phagocytosed by the hemocytes (Fig. 4D). On the other hand, samples related to the infection of *P. aeruginosa* presented the highest number of bacteria, where a good number of them were free in the hemolymph. In the case of *M. abscessus,* a significant percentage had already been phagocytosed, but a few were still outside the hemocytes.

To assess how the bacteria were distributed during the infection course, in both the hemolymph and the larva, samples were plated to count bacterial numbers (CFUs) (Fig. 5). The results obtained correlate with what we had observed earlier in the confocal microscope analysis (Fig. 4D). For *P. aeruginosa,* the bacterium load increases five orders of magnitude in the hemolymph during the infection course, especially from 8 h post-infection. Thus, for *P. aeruginosa*, most bacteria must be in the hemolymph as the amount in the entire larvae is similar to the hemolymph counts. In the case of *M. abscessus*, the bacteria load in the hemolymph decreases two orders of magnitude in the first 24 h of infection, whereas it is almost maintained in the whole larvae. This indicates an internal redistribution of bacteria but not a reduction of the infection, which makes sense for the expected slow growth of *Mycobacteria* species. For our *S. aureus* strain, the number of bacteria clearly decreased over time in both the larvae and the hemolymph. At eight hours post-infection, three orders of magnitude were reduced in the hemolymph and two orders of magnitude in the entire larvae. After twenty hours of infection, the bacteria load in the hemolymph decreased by one order more in magnitude. Our results show a clearing of the infection by the larvae immune system, which lowers the number of bacteria and is fighting the infection through mechanisms such as phagocytosis, as we observed (Fig. 4D). Nevertheless, the reduction of the pathogen load is not enough to revert the infection, as larvae ended up dying in a few hours. Our results clearly indicate a different response of the larvae to the infection caused by different bacteria, in our case for three different types of microorganisms (*P. aeruginosa, S. aureus* and *M. abscessus*).

Back in the seventies, it was already observed that *S. aureus* was able to escape nodulation in a *G. mellonella* infection. The infected larvae were dead within 24 h, presenting massive tissue invasion and necrosis (Ratcliffe and Gagen, 1976). Nodulation refers to the formation of hemocytes aggregates that entrap large numbers of bacteria. When these aggregates grow bigger, we refer to them as nodules, which become covered by layers of flattened hemocytes. Nodulation is an impressively effective response to clear the hemolymph from bacteria. Gagen and Ratcliffe showed that one hour after infection, heat-killed *Bacillus cereus* was removed from the larvae's hemolymph circulation. Surprisingly, the major reduction occurred just within the first five minutes. On the other hand, one hour after infection, they observed that heat-killed *S. aureus* behaved completely different and remained in the hemolymph (Gagen and Ratcliffe, 1976; Rosales, 2011).

Today, it is well established that *S. aureus* is a complete master evading the human immune system. It possesses a wide range of strategies to avoid being eliminated by neutrophils, including the release of toxins and the use of mechanisms that destroy phagocytes. Another example is the inhibition of complement factors. *S. aureus* leaves our bloodstream to invade organs and tissues where it forms encapsulated abscesses. In cases of systemic murine infections, *S. aureus* reaches soon the organs. This not only requires immune evasion, but also the ability to adhere to the tissues. Once an abscess is established, bacterial proliferation results in the infiltration of multiple leukocytes (Cheung et al.,

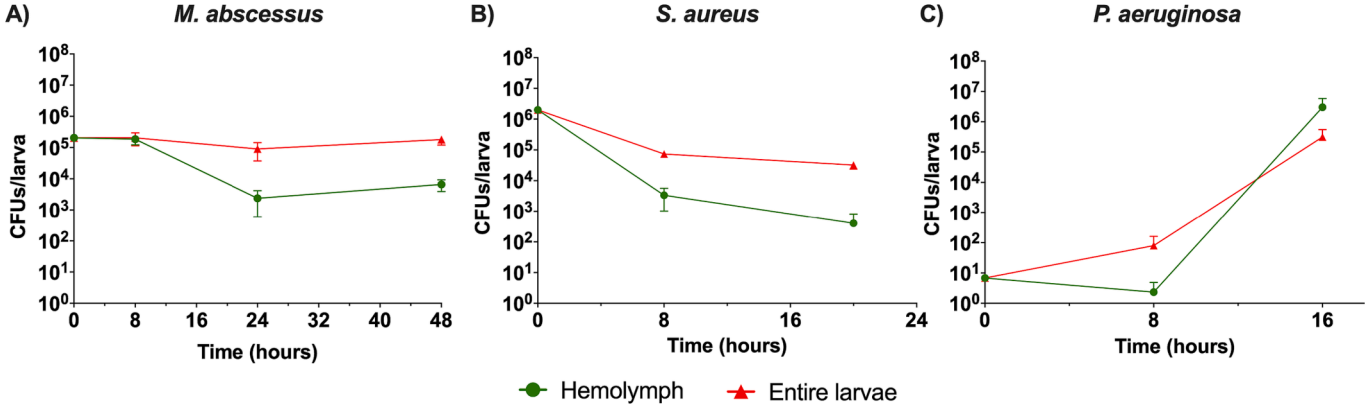

Fig. 5. Distribution of pathogens in *G. mellonella* larvae. **A)** *M. abscessus,* **B)** *S. aureus*, **C)** *P. aeruginosa*. The number of bacteria is expressed in CFUs/larva, from *G. mellonella* injection (0 h) and through the infection course of both the hemolymph and the entire larvae. Graphs were plotted with GraphPad Prism 9.0 software.

2021).

Injection of *S. aureus* into the veins of mice or guinea pigs has also led into a rapid clearance of the bacterium from the blood (Edwards and Massey, 2011). We have seen the exact same pattern in infected *G. mellonella* larvae, mimicking the vertebrate infection. Neutrophils can create an environment that enhances intracellular growth of *S. aureus* and contributes to the spread of infection. Rather than destroying macrophages, *S. aureus* preserves their integrity and uses them as an intracellular niche. Thus, *S. aureus* can survive within human macrophages for several days until at a certain point, bacteria escape the intracellular confinement, proliferate in the surrounding environment and cause cell death (Kubica et al., 2008). According to this, our results at 20 h post-infection could suggest that part of the hemocytes with intracellular *S. aureus* have already established colonization in the other tissues as the hemolymph sample does not show a substantial presence of either hemocytes or bacteria (see Fig. 4D).

In the case of *M. abscessus*, natural killer (NK) cells can eliminate infected macrophages, but they are unable to kill the intracellular mycobacteria (Touré et al., 2023). Furthermore, the transition from smooth to rough morphotypes increases bacterial virulence and promotes survival inside macrophages (Kim et al., 2019). Related to this, the reduction of CFUs observed in our findings (Fig. 5A and 5B) may suggest apart from an actuation of the immune system, that a proportion of *S. aureus* and *M. abscessus* could have remained hidden intracellularly. Despite our efforts to maximize the recovery of bacteria through sonication and vortex, it is possible that some larvae cells remained intact, leading into a potential underestimation of the bacterial load.

While bacteria are in the hemolymph, bacteria secrete virulence factors like lipases, exotoxins or proteases that can digest immune system proteins or the different larvae tissues, perpetuating the infection. Exotoxin A (*exo*A) is one of the main virulence factors secreted by *P. aeruginosa.* It inhibits protein synthesis, disrupting cell protein homeostasis and leading cells to death. It has been demonstrated that ExoA modifies the concentration of hemocytes and suppresses host immune response in insects (Iwánski et al., 2023; Iwánski and Andrejko, 2022) thus it is not surprising to find this bacterium inside the different *Galleria* larvae organs, as we see in our experiments.

Besides the cellular component of the innate response that includes several types of hemocytes, larvae also possess humoral defenses. Molecules such as antimicrobial peptides, reactive oxygen species, and complement-like proteins play important roles in clearing an infection. Enzymatic cascades involved in melanization and clotting also contribute to the immune response. Overall, cellular and humoral branches are mutually connected and regulated to fight against bacterial infections in *G. mellonella* (Pereira et al., 2018; Strand, 2008; Wojda, 2017).

4. Conclusions

This study provides valuable insights into the dissemination of the bacterial strains studied within the main tissues and organs of *G. mellonella* larvae, thereby validating its utility as a significant animal model for studying tissue invasion and host-pathogen interactions. We have demonstrated both the differences and similarities in the pathogenicity of three different bacteria (*Mycobacteria, Staphylococcus* and *Pseudomonas*). We have also identified the fat body as a primary site for bacteria colonization during infection. This interesting organ not only serves as a nutrient source for the bacteria but also represents an easy anatomical site to reach after entering the hemolymph. Additionally, the gut is also an attractive location, while the trachea remains free of infection. The hemolymph serves as a gateway into *G. mellonella* tissues and is particularly associated with the larvae's immune system, playing a significant role in infection restraint. The pattern observed in the hemolymph differs among the three strains examined. *P. aeruginosa* shows a significant increase in bacterial growth over time, while the amount of *M. abscessus* slightly diminishes, and the infection of *S. aureus* has a clearing tendency in both the hemolymph and the entire body within the first 20 h of infection. As seen in literature, some of our findings align with those observed in other animal models, suggesting that bacterial behavior across species shares fundamental characteristics that could also correlate with human pathology.

Overall, our research can contribute to a better understanding of the infections caused by *M. abscessus*, *S. aureus,* and *P. aeruginosa* in this alternative model organism. While we acknowledge the limitations of our work, we hope that our findings provide insights to unravel new targets involved in pathogen dissemination and aid in combating these spotlight bacteria.

Author Contributions

JA and ET write the original draft and review and edit the final manuscript. JA performed data curation, formal analysis investigation and methodology. ET supervised, validated and supervisised the research. All authors have approved the final version of the manuscript.

Declaration of Competing Interest

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

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