Galleria mellonella as a consolidated in vivo model hosts: New developments in antibacterial strategies and novel drug testing

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ABSTRACT
A greater ethical conscience, new global rules and a modified perception of ethical consciousness entail a more rigorous control on utilizations of vertebrates for in vivo studies. To cope with this new scenario, numerous alternatives to rodents have been proposed. Among these, the greater wax moth Galleria mellonella had a preponderant role, especially in the microbiological field, as demonstrated by the growing number of recent scientific publications. The reasons for its success must be sought in its peculiar characteristics such as the innate immune response mechanisms and the ability to grow at a temperature of 37°C. This review aims to describe the most relevant features of G. mellonella in microbiology, highlighting the most recent and relevant research on antibacterial strategies, novel drug tests and toxicological studies. Although solutions for some limitations are required, G. mellonella has all the necessary host features to be a consolidated in vivo model host.

Introduction
The use of animal models for scientific purposes dates back to ancient Greece, but since the beginning of the 1900s vertebrate preclinical models have represented the gold standard for in vivo tests, as they have provided useful human-like predictions for obtaining mechanistic, efficacious and toxicological information [1]. The fields of drug testing and antimicrobial activity evaluation have been no exception. Here, murine models have often been adopted for infection studies, due to their relatively high similarity to humans about metabolism, body temperature and innate immune response. Nevertheless, these models are expensive and laborious. Moreover, a greater ethical conscience and new global rules and stricter controls mean that it is very time-consuming to obtain authorization for mammalian studies [2,3]. Also, protocols necessitate suitable hosts for the experimental study of in vivo infections. Therefore, the selection of alternative models is fundamental for microbiological research, especially when discrepancies in antimicrobial activity are often observed between in vitro and in vivo testing [4].

Alternatives to rodents have been proposed; the nematode Caenorhabditis elegans (C. elegans) was the first invertebrate model, followed by adult fruit larvae and flies, namely Drosophila melanogaster (D. melanogaster) [5], and, more recently, larvae of the greater wax moth Galleria mellonella (G. mellonella) [6,7]. This latter organism is widely proposed for the study of pathogenesis, virulence mechanisms, immune response and for the evaluation of the potential of antimicrobial compounds. The use of this mini-host offers economic and ethical advantages compared to mammals and its short lifespan makes it suitable for high throughput studies [8,9].

It may seem futile to expect to obtain clinically useful information from species such as insects and nematodes, but some biological mechanisms, which are very well conserved even in evolutionarily divergent species, share the same evolutionary origins as humans [10]. Indeed, from an evolutionary perspective, insects and vertebrates diverged about 500 million years ago, but many aspects of their physiology remain comparable. Although insects do not possess an adaptive immune response, they own innate immune response mechanisms (at epithelial, cellular and humoral levels) that are surprisingly well preserved and which share part of the evolutionary scale to mammals [11–13]. The lack of adaptive immunity is not a disadvantage, rather the insect models permit the study of host-parasite interactions and related innate-immunity mechanisms without the interference of adaptive responses [14].
A review study conducted by Freires et al. in 2016, showed how the use of alternative models for research purposes had increased dramatically from 1990 to 2015. The most frequently used alternative animal models were: D. melanogaster (fruit fly) (41.89%), Danio rerio (zebrafish) (29.74%), C. elegans (roundworm) (26.53%), Galleria mellonella (greater wax moth) (1.14%) and Artemia salina (brine shrimp) (0.70%) [15]. A bibliographic research conducted in March 2019 on PubMed (MEDLINE database), the interest of the scientific community about G. mellonella as in vivo host model in microbiology has greatly increased, from 2016 to 2018 the scientific articles that have as keywords “Galleria mellonella” and “microbiology” are more than 42% (292) compared to those published in the last 10 years (691).

Although its physiological characteristics make the larvae of G. mellonella an ideal host for the study of fungi, especially those of a dimorphic nature [3,16–23], there has been a growth in interest within the scientific community in using the larvae for the study of pathogenic bacteria, which has been particularly marked over the last three years. From 2016 to 2018 thirty-seven research papers about new therapeutic strategies for fourteen different bacterial genera were published (Table 1). Moreover, more than thirteen new molecules and four toxicological studies were assessed using the G. mellonella model (Table 2).

As a result, this review aims to highlight the most relevant and most recent research, published from 1 January 2016 to 31 December 2018, on antibacterial strategies, novel drugs testing and toxicological studies conducted with G. mellonella as an in vivo model.

**In vivo model**

The G. mellonella insect is a member of the Galleriinae subfamily within the Pyralidae family of the Lepidopteran order that naturally infests beehives. The greater wax moth develops through four distinct life stages: egg, larva, pupa, and adult. Galleria larvae are opaque and white in colour, are about 3 cm long, weigh from 0.3 to 0.5g and undergo a metamorphosis to become grey moths. Temperature is a crucial factor for the development of the insect; the optimum averages are from 29 to 33°C; furthermore, larvae can survive at mammalian physiological temperature (37°C) [24,25]. The possibility of breeding larvae at a suitable temperature allows experiments to be carried out in conditions that imitate the mammalian body temperature. Indeed several pathogen temperature-dependent virulence factors can be studied using this model [26]. Moreover, temperature plays a key role in pathogen-host interaction, an increase in temperature after bacterial inoculation reduces larval survival [27]. Compared to other invertebrate models, widely used in microbiological research, such as C. elegans and D. melanogaster, G. mellonella has numerous advantages (Table 3) [28–32]

### Immune system

The principal key factor that makes G. mellonella a helpful preclinical in vivo model is its innate immune response that shares several strategies with the mammalian innate immune system. As mammalians, the innate insect immunity consists of cellular and humoral response and is more advanced than other invertebrates such as nematodes [33]. Of particular interest is the cellular immune response mediated by hemocytes located within the hemolymph. Hemocytes are involved in phagocytosis, nodulation and encapsulation.

The principal mechanisms of pathogen recognition are mediated by:

- hemocytes [6,12,34],
  - recognize pathogenic microorganisms through the direct interaction of their pathogen recognition proteins (PRRs) with pathogen-associated molecular patterns (PAMPs);
  - also indirectly activated by recognition of humoral immune effectors; the Drosophila Toll (like in mammals), and the IL-1 receptor are known to both signal through the NF-KB pathway;

| Characteristics                  | G. mellonella | C. elegans | D. melanogaster | Advantage                                      |
|---------------------------------|---------------|------------|-----------------|-----------------------------------------------|
| Grown temperature [28]          | 37°C          | From 15 to 25°C | From 16 to 29°C | Mammalian temperature allows temperature-dependent virulence factors studies |
| Life span [29]                  | Short (30)    | Long (31)  | Long (32)       | Facilitates experimentation in the laboratory setting |
| Size (length) [29]              | 3 to 30mm     | 1 mm       | 3 mm            | Convenient for handling                        |
| Tissue recovery [28]            | Possible      | Impossible | Impossible      | Tissue studies can be performed                |
| Phagocytosis [28]               | Present       | Absent     | Absent          | Information about host–pathogen interactions. |
Table 2. Use of G. mellonella in antimicrobial drugs evaluation against pathogens.

| Microorganism | Strategy | Reference |
|---------------|----------|-----------|
| Acinetobacter baumannii | Drug combinations | [43], [44], [45], [46] |
| Bacteriophages | ● co-trimoxazole/colistin | [43] |
| | ● levofloxacin/colistin | [44] |
| | ● polymyxin B/netropsin | [45] |
| | ● vancomycin/colistin | [46] |
| Drug repurposing | ● mitomycin C | [47] |
| Burkholderia spp | Drug combinations | [4] |
| | ● avibactam/cefazidime | [50] |
| Bacteriophages | ● Burkholderia phage AP3 | [51] |
| Nutrients from food | ● fish oils | [52] |
| Drug/vitamin combination | ● vitamin E/norfloxacin | [53] |
| Clostridium difficile | Bacteriophages/drug combination | [4] |
| | ● Enterobacter cloacae. | [55] |
| Enterobacter cloacae. | Drug combinations | [55] |
| | ● imipenem/colistin | [55] |
| Bacteriophages | ● Escherichia phage ECP311, Klebsiella phage KPP235, and Enterobacter phage ELPI40 | [56] |
| Escherichia coli | Bacteriophages | [56] |
| | ● Escherichia phage ECP311, Klebsiella phage KPP235, and Enterobacter phage ELPI40 | [56] |
| Helicobacter pylori | Drug repurposing | [57] |
| | ● nicosamide | [57] |
| Klebsiella pneumoniae | Delivery drug system | [58] |
| | ● gentamicin-loaded nanoparticles | [58] |
| Bacteriophages | ● Escherichia phage ECP311, Klebsiella phage KPP235, and Enterobacter phage ELPI40 | [56] |
| | ● capsule depolymerases produced by the Klebsiella phage KP32 | [59] |
| | ● capsule depolymerases produced by the Klebsiella phage KP36 | [60] |
| | ● phage φBO1E | [61] |
| Porphyromonas gingivalis | Plant extract | [62] |
| | ● Punica granatum L. | [62] |
| Pseudomonas aeruginosa | Antimicrobial peptide and drug association | [63] |
| | ● Cecropin A2/Tetracycline | [63] |
| | ● mammalian proline-rich peptide SP-E | [64] |
| | Quorum sensing targeting | [65] |
| | ● clofotol | [65] |
| Bacteriophages | ● cocktail of six P. aeruginosa phages (PY02, DEV, E215, E217, PAK, P1, and PAK P4) | [66] |
| | ● Proteins isolated from bacteriophages | [66] |
| | ● O-specific polysaccharide lyase from the phage LAK1 | [67] |
| | Plant extract and drug combination | [67] |

(Continued)

Table 2. (Continued).

| Microorganism | Strategy | Reference |
|---------------|----------|-----------|
| Gram-negative | ● steroidal alkaloids and conessine from Holarrhena antidysenterica/levofloxacin | [68] |
| | ● Plant made bacteriocins | [69] |
| | ● six pyocins produced by P. aeruginosa made using a plant-based transient expression system | [69] |
| | ● Nutrients from food | [52] |
| | ● fish oils | [52] |
| | ● Drug/vitamin combination | [53] |
| | ● vitamin E/norfloxacin | [53] |
| | ● Phytocerm | [70] |
| Shigella sonnei | Drug combinations | [71] |
| ● Cannabis sativa L. essential oil | [72] |
| ● trans-cinnamaldehyde, carvacrol, and thymol | [73] |
| Mycobacterium abscessus | Drug combinations | [74] |
| ● avibactam/piperacillin | [74] |
| Staphylococcus aureus | Drug combinations | [75] |
| ● pleurotomolins (valnemulin, tiamulin and retapamulin) alone, and in combination with tetracycline or ciprofloxacin | [75] |
| Phytochemicals | ● myricetin | [76] |
| ● cinnamaldehyde | [77] |
| Antibacterial peptide | ● temporins | [78] |
| | ● Antimicrobial compound | [79] |
| | ● raf-kinase inhibitor GW5074 | [79] |

○ once phagocytosed, pathogens are killed by the NADPH oxidase, pathways capable of generating superoxide.
○ opsonins [6,35].
○ Apolipoporphorin-III, peptidoglycan recognition proteins, similar to mammalian opsonins;
○ Hemolin and GmCP8 can recognize and bind to the cell wall of different pathogens including fungi and bacteria and stimulate phagocytosis.

As regards hemocytes, Gongqing et al. in 2016 recognized and characterized four different hemocytes in the hemolymph of the G. mellonella, which are known as the plasmatocytes, the granular cells, the spherule cells and the oenocytoïds. Plasmatocytes, oenocytoïds and above all, granulocytes, have been shown to have the ability to phagocytose bacteria. In addition, a variation in the percentage of hemocytes was observed in the different states of development of the larva. There was also a reduction in the granulocyte count and an
Table 3. *G. mellonella* in novel drugs testing and toxicity screening.

| Compound | Antimicrobial target/drug evaluation | Reference |
|----------|------------------------------------|-----------|
| 2-aminimidazole containing urea | Acinetobacter baumanii | [82] |
| 1,2,4-triazolidine-3-thiones derivatives | Acinetobacter baumanii | [83] |
| Aminoglycoside 6′-N-acetyltransferase type Ib (AAC(6′)-Ib) | Klebsiella pneumoniae | [84] |
| 1,2-benziselenazol-3(2H)-one derivatives | Enterobacter cloacae | [85] |
| Steroid-Au(I)-NHC Complexes | Escherichia coli | [86] |
| bis-2-aminimidazole derivative and azithromycin association | Pseudomonas aeruginosa | [87] |
| Silver(I) complex | Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Candida albicans | [88] |
| Tobramycin–lysine conjugates | Pseudomonas aeruginosa | [89] |
| Hybrid antibiotic tobramycin –moxifloxacin hybrid core structure | Pseudomonas aeruginosa | [90] |
| Marine sponge-derived Streptomyces sp. SB7348 extract | Staphylococcus aureus | [91] |
| 1-(4-chlorophenyl)-4,4,4-trifluoro-3-hydroxy-2-buten-1-one | Staphylococcus aureus | [92] |
| N-phenyl-1H-pyrazole-4-carboxamide derivatives | Staphylococcus aureus | [93] |
| 1,2,3-Triazole/Sulfonate Analogue | Toxicity screening | [94] |
| Quinoline thiourea compound | Toxicity screening | [95] |
| Extract from the pulp of Eugenia brasiliensis Lam. (Myrtaceae) | Toxicity screening | [96] |
| Thiazolylhydrazone derivatives | Toxicity screening | [97] |

An increase in the count of oenocytoids during the *G. mellonella* 7th larval instar [36]. These findings led to an important observation: since the variation in the percentage of hemocytes cells changes significantly during the different stages of development of the larvae, it is essential for reproducibility that experiments are carried out in the same stage of the larval life cycle. The humoral response of *G. mellonella* includes melanisation catalyzed by phenoloxidase and synthesis of several anti-microbial peptides which play a crucial role in the last line of defence against pathogens [37].

**Antimicrobial peptides (AMPs) and immune-relevant proteins**

The humoral response of *G. mellonella* includes a wide range of AMPs: cecropin (active against Gram-positive and Gram-negative bacteria), gallerimycin (active against filamentous fungi and yeast but no antibacterial activity), *Galleria* defensin gallerimycin (active against entomopathogenic fungi but not active against yeast), golverin and moricins (particularly active against filamentous fungi but also, against yeast, Gram-positive and Gram-negative bacteria) [38].

Together with AMPs, several immune-relevant peptides involved in the immune response of *G. mellonella* have been identified: Gm proline-rich peptides, Gm anionic peptide, inducible serine protease inhibitor Heliocin-like peptide, lysozyme, moricin-like peptides and x-tox [37–39]. These molecules play a key role in the host defence system, their expression is induced in response to an infection, and it has been observed that their activity is selective towards different pathogenic species. Isolation and characterization of these peptides with antimicrobial activity could be a prospect for the development of novel antimicrobials [34]. Among all the peptides with antimicrobial action, *G. mellonella* produces an insect metalloproteinase inhibitor (IMPI) in response to infection. In 2018, Eisenhardt et al. tested the ability of the fusion protein IMPI-GST (glutathione-S-transferase), produced by fermentation in *Escherichia coli*, to inhibit the proteolytic activity of the M4 metalloproteinases thermolysin and *Pseudomonas* elastase. Results indicated that IMPI is a promising drug candidate for the treatment of *P. aeruginosa* infections [40].

**Influence of diet on larval and immune health**

A key factor, often neglected, in experimental infection models is diet. In nature, *G. mellonella* caterpillars feed on honey or other nutrients from the hive (pupa skins, pollen and beeswax) [41] however, when they are raised in the laboratory, a wrong or nutrient-poor diet can cause developmental problems, increased susceptibility to infections or even death [30,42].

Banville et al. have shown the effects of food deficiency on the immune system, a lower density of hemocytes, reduced expression of a range of antimicrobial peptides) and immune proteins have been observed, thus showing a greater susceptibility to *C. albicans* infection [42].

So, to better evaluate a diet suitable for experimental infection models, in addition to the life cycle parameters (duration of the larval stage, weight and percentage of pupae), the efficiency of the immune system must also be taken into consideration. For this purpose, Jorjão et al. tested the influence of three different feeding regime on both the larval cycle parameters, together with the volume of the hemolymph and the...
concentration of hemocytes. The authors showed that a diet that promotes a short larval phase, a weight increase with an enhanced hemolymph volume and a high concentration of hemocytes, also extend larval survival to bacterial (S. aureus and E. coli) and fungal (C. albicans) infections [30].

G. mellonella as a tool for studying antimicrobial drugs against pathogens

Different therapeutic strategies have been proposed using G. mellonella as an in vivo model. To overcome antimicrobial resistance and decrease the dosage of individual drugs, a combination of antibacterials has been proposed: drug association, drugs/adjuvant and also drug/antimicrobial peptides. Other effective approaches are the repurposing of drugs that are already in use or even bacteriophage therapy. Moreover, hybrid antibiotics, antimicrobial peptides, delivery drug systems, nutrients from food and vitamins and substances obtained from plants have been evaluated (Table 1).

Gram-negative

Eleven bacterial species (Acinetobacter baumannii, Burkholderia cenocepacia, Burkholderia multivorans, Clostridium difficile, Enterobacter cloacae, Escherichia coli, Helicobacter pylori, Klebsiella pneumoniae, Porphyromonas gingivalis, Pseudomonas aeruginosa and Shigella sonnei) belonging to ten different genera are reported in Table 1.

Acinetobacter baumannii (A. baumannii)

Several drug combinations to treat A. baumannii infection have been proposed. A combination of colistin and cotrimoxazole against carbapenem-resistant A. baumannii revealed a greater in vitro bactericidal activity and in vivo activity than the two drugs alone [43]. Similar results have been obtained with levofloxacin and colistin [44], polymyxin B and netropsin [45], vancomycin and colistin [46] against multidrug-resistant strains. For the treatment of persistent infections, Cruz-Muñiz et al. repurposed the anticancer drug mitomycin C that was able to increase the survival of the insect larvae [47].

Regenstein et al. evaluated the use of bacteriophages as a possible therapeutic strategy and succeeded in attenuating the virulence of a strain of A. baumannii pre-exposed to the bacteriophage AB-Army1. In addition to the G. mellonella model, other authors have demonstrated the therapeutic efficacy of the mouse full-thickness dorsal infected wound model, confirming the same results [48].

Betts et al. tested two natural polyphenols, theaflavin–epicatechin, alone or in combination against six clinical isolates of multidrug-resistant A. baumannii. In vivo results in G. mellonella model demonstrated a significantly reduced melanisation score in larvae treated with the theaflavin–epicatechin combination compared with monotherapy [49].

Burkholderia spp

Van den Driessche et al. observed discrepancies in antimicrobial activity between in vitro and in vivo when tobramycin, econazole and miconazole were tested both in combination and individually. Although in vitro tests had demonstrated a synergic effect of the drug combinations, in vivo tests demonstrated that neither treatment with tobramycin, miconazole or econazole individually, nor in combination could protect the larvae against B. cenocepacia infection. The same results were obtained in a mouse pulmonary infection model, corroborating the reliability of G. mellonella as a model of infection [4]. Moreover, ceftazidime-avibactam was shown to improve the survival of larvae infected with extremely drug-resistant B. multivorans isolated from cystic fibrosis patients [50]. Besides antimicrobial drug combinations, the treatment of larvae infected with B. cenocepacia isolated from cystic fibrosis patients with Burkholderia phage AP3 revealed a significant increase in larvae survival in comparison to AP3-untreated infected larvae [51].

The antibacterial activity of nutrients from food has also been investigated. Mil-Homens et al. demonstrated that the survival of larvae infected with B. cenocepacia and Pseudomonas aeruginosa is greater when they are treated with fish oils containing a larger quantity of omega-3 fatty acids. Furthermore, fish oil has been shown to have a prophylactic effect when given 12 days before bacterial infection [52].

Combinations of drugs and vitamins have also been evaluated using G. mellonella as an in vivo model. Using D-tocopheryl polyethylene glycol 1000 succinate (TPGS), a water-soluble vitamin E derivative in combination with the bactericidal quinolone norfloxacin, Naguib et al. obtained a significantly increased larval survival rate upon infection. Furthermore, they demonstrated that increased mortality was due to the vitamin E derivative interference with lipocalin binding. Moreover, similar
results have been obtained with the same combination against *P. aeruginosa* PAO1 infection [53].

**Clostridium difficile (C. difficile)**

Nale et al. assessed *in vivo* colonization of six *C. difficile* clinical strains using single and multiple doses of a filtered *C. difficile* bacteriophages lysates cocktail, both alone and together with vancomycin. The phage cocktail (CDHM1, 2, 5, and 6) was effective as prophylactic therapy and could potentially serve as an intervention therapy to supplement vancomycin and prevent a relapse of the disease [54].

**Enterobacter cloacae (E. cloacae)**

A combination of imipenem and colistin against two clinical isolates of *E. cloacae*, led to significantly increased survival of *larvae* when compared to monotherapy alone. The dosages chosen for *in vivo* virulence assay were comparable to those used to treat human infections both for monotherapy and for the two drug combinations [55].

**Escherichia coli (E. coli)**

The therapeutic potential of bacteriophages against three multi-drug resistant Gram-negative bacteria was assessed by Manohar et al. The antibacterial proprieties of three different phages, *Escherichia* phage ECP311, *Klebsiella* phage KPP235, and *Enterobacter* phage ELP140, against *E. coli* ec311, *K. pneumoniae* kp235 and *E. cloacae* e1410, were evaluated in an infection *in vivo* model. Infected *larvae* treated with multiple phage cocktail doses (6h interval) showed a survival rate superimposable to the untreated group (100% survival) with a significant reduction in the count of bacteria in the hemolymph [56].

**Helicobacter pylori (H. pylori)**

Tharmalingam et al. repurposed the anthelmintic drug niclosamide and significantly, used it to treat larvae with an *H. pylori* (ATCC 49503) infection and recorded a survival rate of up to 70% compared to the no treatment group [57].

**Klebsiella pneumoniae (K. pneumoniae)**

Recently, drug delivery systems as therapeutic strategies have proved to be very promising. Jiang et al. developed and evaluated a poly lactide-co-glycolide /gentamicin formulation capable of successfully treating intracellular *K. pneumoniae* infections. These nanoparticles loaded with gentamicin, have improved larval survival and provided extended prophylactic protection against *K. pneumoniae* [58]. In 2018 Majkowska et al. investigated two capsule depolymerases (KP32gp37 and KP32gp38) produced by the *Klebsiella* phage KP32 with high specificity for the capsular serotypes K3 and K21, that considerably increased the lifespan of *larvae* infected with *K. pneumoniae* in a time (and strain) dependent manner. Previously, in 2016 the same authors had identified another depolymerase enzyme encoded by the *Klebsiella* phage KP36 (depoKP36) with similar results [59,60]. The activity of the lytic phage qBO1E against two carbapenemase-producing *K. pneumoniae* strains (KKBO-1 and KP04C6224) revealed an overall protective capacity for *larvae* from lethal, strain-dependent infection. KKBO-1 was more susceptible to multiple infections than KP04C6224 [61].

**Porphyromonas gingivalis (P. gingivalis)**

The glycolic extract of the pomegranate (*Punica granatum* L.) has been assessed for its antimicrobial action. Concentrations that proved to be non-lethal for the *larvae* showed a high inhibition of the *P. gingivalis* type strain ATCC 33277 inoculated in the *G. mellonella*, in a dose dependent manner. This *in vivo* antibacterial activity may be due to the high presence of gallic tannins and alkaloids in pomegranate extracts [62].

**Pseudomonas aeruginosa (P. aeruginosa)**

Antimicrobial peptides, either alone or in combination with conventional antibiotics, have been used successfully to treat *P. aeruginosa* infections. Zheng et al. evaluated the efficacy of cecropin A2; a 36-residue α-helical cationic peptide derived from *Aedes aegypti* cecropin A, alone and in combination with tetracycline, against *P. aeruginosa*. The administration of cecropin A2 alone protected the *larvae* and prolonged their survival in a dose-dependent manner. Administration of peptide/drug combinations ensured the survival of all *larvae* tested for more than 96h, demonstrating synergistic protection *in vivo* [63]. An evaluation of the therapeutic activity of proline-rich antimicrobial peptide SP-E, isolated from pig saliva, used against *P. aeruginosa* ATCC 9027 led to a significant increase in the survival of *larvae* in comparison to the untreated group at 6 days post-infection. At this point, 14 of the 16 *larvae* in the control group were dead, whereas eight of the peptide-treated group were still alive [64].
The quorum sensing system, which controls virulence factor production and biofilm formation in diverse human pathogens, is another ideal target for antibacterial therapy. D’Angelo et al. tested the anti-virulence activity of clofoctol, an antibacterial compound that inhibits the expression of the transcriptional regulator PqsR, which control virulence traits in *P. aeruginosa*. Experiments conducted on *P. aeruginosa* PAO1 showed the non-toxicity of the drug at the concentrations used (5mM). Furthermore, the treatment with clofoctol led to a larvae survival percentage similar to that observed with the ApqSRI mutant. Overall, these data demonstrate that clofoctol attenuates *P. aeruginosa* PAO1 lethality in *G. mellonella* [65].

A cocktail of six *P. aeruginosa* phages (PYO2, DEV, E215, E217, PAK_P1, and PAK_P4) isolated from strains of different origins has also been tested against *P. aeruginosa* PAK-lumi both in *G. mellonella* and female BALB/c mice obtaining comparable results. In mice, the cocktail produced a rapid reduction of the bacterial load. In contrast, using the systemic *G. mellonella* infection model, a significant reduction in the time taken for the infected larvae to die (20h) compared to those that were untreated was observed upon phage injection. Moreover, the phage cocktail was able to prevent infection [66].

The effect of a tail spike protein (LKA1gp49) encoded by *Pseudomonas* phage LKA1 on *P. aeruginosa* PAO1 strain pathogenicity has also been evaluated in the *G. mellonella* model. Whether by administering the protein after larval infection or by incubating it with the bacterium an hour before infection, a greater survival rate than the untreated groups was observed during the 72h infection period [67].

Siriyog et al. investigated the efficacy of combinations of steroidal alkaloids and conessine obtained from the Thai medicinal plant Holarrhena antidysenterica with levofloxacin against *P. aeruginosa* strains overexpressing either the MexAB-OprM or MexEF-OprN efflux pumps. Appropriate non-toxic concentrations were determined by injecting larvae with triple doses (of the steroidal alkaloids or conessine) for 96h. Combination therapies of conessine or steroidal alkaloids with levofloxacin restored antibiotic efficacy in vivo. The enhanced efficacy of the combination treatments was most pronounced with conessine and correlated with a reduced larval burden for those infected with *P. aeruginosa* [68].

Using a plant-based transient expression system, Paslikačvičius et al., were able to produce, isolate and purify six different antibacterial proteins, pyocins, produced by *P. aeruginosa* (S5, PaEM, L1, L2, L3 and a new one, PaEM4). Their antibacterial proprieties against *P. aeruginosa* strains PAO1 and A19 were evaluated with the *G. mellonella* infection model. The survival rates of larvae treated with pyocins, especially when mixed together, were significantly higher than those of the untreated group, although the authors observed differences between the two tested strains of *P. aeruginosa*, due to the virulence of different strains [69].

**Shigella sonnei**

Geraniol, a natural substance present in the essential oils of plants such as rose and lemongrass, significantly improved larval survival when compared to those that were untreated. Moreover, in a 5-day cytotoxicity experiment, the tolerance level was at least 10-fold higher than the therapeutic level required to control *S. sonnei* infection [70].

**Gram-positive**

Five bacterial species (*Enterococcus faecalis, Enterococcus faecium, Listeria monocytogenes, Mycobacterium abscessus and Staphylococcus aureus*) belonging to four different genera are reported in Table 1.

**Enterococcus spp**

The effects of different antibacterial combinations (rifampicin, tigecycline, linezolid or vancomycin) against five *Enterococcus faecalis* and three *Enterococcus faecium* isolates using an *in vitro* approach and an *in vivo* *G. mellonella* infection model has been assessed. Antibacterial combinations that displayed synergy in *in vitro* tests were selected for an *in vivo* infection model. Combination treatment demonstrated higher protection of larvae post-infection in comparison with antibiotic monotherapy. In particular, rifampicin in combination with tigecycline or vancomycin significantly enhanced survival [71].

**Listeria monocytogenes (L. monocytogenes)**

The ability of a sublethal concentration (256 μg/mL) of *Cannabis sativa* *L.* essential oil extracted from the French monoeocious variety Futura 75 to attenuate the virulence of eleven *L. monocytogenes* strains isolated from patients diagnosed with invasive listeriosis has been evaluated by a survival experiment. After 6 days of infection survival rates of infected groups compared with those who were not infected increased remarkably (from 50 to 90%) [72].
The efficacy of three phytochemicals (trans-cinnamaldehyde, carvacrol and thymol), alone and in combination, in reducing the virulence of three strains of *L. monocytogenes*, and their effects on the transcription of antimicrobial peptide genes in *G. mellonella* (responsible for host defence) has also been investigated. In a 5-day infection experiment, all phytochemicals enhanced survival rates. In particular, a combination of carvacrol and thymol was found to be the most effective treatment. Moreover, all phytochemicals were found to upregulate the expression of antimicrobial peptide genes in larvae challenged with *L. monocytogenes*. However, the expression of antimicrobial peptide genes was not significantly affected in uninfected larvae treated with phytochemicals [73].

**Mycobacterium abscessus (M. abscessus)**

The effect of drug combinations used against the non-tuberculous mycobacterium *M. abscessus* has been evaluated by an *in vivo* experiment. Infection was carried out using the luminescent strain *M. abscessus* mDB158, meropenem, piperacillin and ampicillin alone or with the addition of avibactam in two daily doses during a 72h infection period. Larvae treated with either meropenem or piperacillin/avibactam had a significantly lower infection burden compared to the untreated control groups. Piperacillin and avibactam alone had no significant inhibitory effect, similar results were obtained in a single dose experiment [74].

**Staphylococcus aureus (S.aureus)**

The *in vivo* antibacterial propriety of three different pleuromutins (valnemulin, tiamulin and retapamulin) alone, and in combination with tetracycline or ciprofloxacin against two standard *S. aureus* strains (MSSA ATCC 29213 and MRSA ATCC 43300) and two *S. aureus* clinical strains (MSSA N54 and MRSA N9) has been assessed. Administration of pleuromutins alone was found to lead to increased larval survival, although all combinations with tetracycline provided better results. Among all combinations, tetracycline and valnemulin was the best combination. The combinations with ciprofloxacin did not improve the larvae survival rates as compared with monotherapy [75].

The potential protective effect of flavonoid myricetin against *S. aureus* Newman and ATCC 6538 infection in *G. mellonella* has also been investigated. Myricetin was shown to exhibit anti-virulence effects without modulating bacterial growth; indeed, after infection, the treated larvae demonstrated increased survival, and larval bacterial counts of the treated group remained similar to those in the untreated control group (PBS-treated) [76].

Another phytochemical, namely cinnamaldehyde, the predominant active compound found in cinnamon oil from the stem bark of the *Cinnamomum cassia*, was found not to induce any toxicity and enhanced the survival rate of larvae when they when infected by *S. aureus* ATCC 25923. Moreover, cinnamaldehyde significantly reduced the number of bacteria in larvae hemolymph in comparison with that in those in the untreated groups [77].

Mishra et al. demonstrated the efficacy of the antimicrobial peptide Temporin-10La (T10La), isolated from skinsecretions of the *Rana okaloo sae* against the methicillin-resistant *S. aureus* USA300. They found that the deaths of larvae were reduced with a single dose of treatment [78].

Johnston et al. tested the ability of a commercially available Raf-kinase inhibitor, namely GW5074 (benzyllidene-1H-indol-2-one), to protect *G. mellonella* and *C. elegans* from the methicillin-resistant *S. aureus* MW2 infection. The administration of a single dose of the compound showed an increase in the survival of species. Moreover, after 5 days of infection about 42% of the *G. mellonella* larvae had survived, demonstrating the long-term protection capacity of GW5074 [79].

**Novel drugs**

The excessive and uncontrolled use of antibiotics leads to the spread of bacterial resistance. Several nosocomial and community microbial agents have become resistant to most antibiotics, drastically complicating therapy [80,81]. Therefore, the research for new therapeutic strategies is becoming a pressing issue. As described previously, the *G. mellonella* model represents an important tool for the preliminary screening of antimicrobial compounds. It can be used as an *in vivo* model for a rapid and reliable evaluation of the activity and toxicity of novel antimicrobial drugs and thus should reduce the number of experiments needed using mammalian models.

**Antimicrobial testing**

Minrovic et al., carried out a toxicity and antibacterial assay using the *G. mellonella* model to test, *in vivo*, five colistin adjuvants based on a new urea-containing class of 2-aminoimidazole compounds against a highly virulent isolate of *Acinetobacter baumannii* AB5075. During the 24h observation period, an increase in the survival of infected larvae treated with five different
combinations of colistin and adjuvant compounds was observed [82].

The antibacterial activity of several compounds with 1,2,4-triazolidine-3-thione scaffold against MDR A. baumannii AB5075 was assessed by Huggins et al. Although many derivatives had good in vitro test results, single dose in vivo tests showed conflicting results. When the two most active lead compounds in vitro were tested in the G. mellonella infection model with AB5075, one drug did not confirm any activity while the other showed only modest activity, partially protecting the larvae against infection [83].

Using mixture-based combinatorial libraries, the scaffold ranking approach, and the positional scanning strategy, Tran et al. were able to identify three new aminoglycoside 6'-N-acetyltransferase type Ib (AAC (6')-Ib) inhibitors, a type of enzyme that induces resistance to aminoglycosides, including amikacin. These molecules, in combination with amikacin, showed good in vitro activity (Checkerboard Assay), but further in vivo tests demonstrated that only one combination could protect the larvae from infection by the resistant strains A. baumannii A155 and K. pneumoniae JHCK1 [84].

Evaluation of a newly synthesized molecule, derived from the scaffold of Ebslen (an NDM-1 inhibitor) as a meropenem adjuvant was assessed by Jin et al. After estimating the non-toxic adjuvant dosage, the larvae were inoculated with a lethal dose of carbapenem-resistant Enterobacter cloacae and treated with a combination of meropenem and a new compound or the two alone. Results showed a significantly reduced mortality rate when larvae were treated with the drug/adjuvant combination, demonstrating the synergistic action of the two compounds [85].

Velle et al. designed an Au(I)-N-heterocyclic carbene complex bound with either ethynyl oestradiol or ethisterone. Both carbene precursors and complexes containing the oestriadiol had antibacterial activity in vitro against E.coli and S. aureus, but in vivo tests with G. mellonella showed conflicting results. All the complexes tested showed no toxicity and the larvae infected with E. coli and treated with the compound containing oestradiol demonstrated increased survival, while the carbene precursors did not show any significant increase in larval survival even if in vitro studies demonstrated their antibacterial activity [86].

Another molecule which is potentially active against metal-beta-lactamase is zinchophore [S,S]-ethylenediamine-N,N0-disuccinic acid (EDDS), which is produced by several bacteria. This zinc chelator has been tested alone and in combination with imipenem against NMD-I producing K. pneumoniae. Treatment with the drugs individually did not result in a significantly higher survival rate for larvae when compared to those left untreated. However, the combination of EDDS and imipenem was proven to be very effective by counteracting the resistance mechanisms of the bacterium [87].

A new therapeutic strategy against P. aeruginosa infections conceived by Hubble et al. involved the association of a macrolide (azithromycin) with a new synthetic adjuvant (a bis-2-aminoimidazole derivative) against a highly resistant macrolide, P. aeruginosa laboratory strain PAO1. Authors performed both toxicity and infection studies, demonstrating that the adjuvant is not toxic at the tested concentration and that it increased the survival of larvae when administered in a single dose in combination with azithromycin. This is equivalent to the treatment of infected worms with clavulanic acid/penicillin, one of the few clinically approved antibiotic/adjuvant combinations [88].

Jakobsen et al. synthesized and characterized the in vitro and in vivo anti germmic proprieties of tetrameric-iodine and polymeric silver complexes of the omeprazole scaffold. The tetrameric iodide complex did not show significant antimicrobial action in vitro while the polymeric complex showed activity comparable to AgNO3. The in vivo performance of the in vitro active polymeric compound showed less toxicity than AgNO3. Moreover, it was able to both significantly stimulate the immune system (increase in blood cell density), and increase the larvae survival rate after Candida albicans, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa infections [89].

A study performed by Lyu et al. reported on the antibacterial properties of new amphiphilic tobramycin-lsine conjugates alone and in association with minocycline and rifampicin. After in vitro studies, tests with the G. mellonella model were conducted in order to evaluate toxicity and antibacterial action. The larvae were infected with XDR P. aeruginosa P262, and treated with different non-toxic doses of the drugs, individually or in combination. In the groups treated with minocycline and rifampicin a mortality rate of 100% was recorded, while the combination of these two antibiotics with amphiphilic tobramycin-lysine protected larvae by reducing mortality to 77%. Surprisingly, a lower dose of rifampicin with amphiphilic tobramycin-lysine showed an improved antibacterial action in vivo (about 40% survival). This outcome does not reflect
the evidence obtained with the in vitro tests where the adjuvated minocycline had a higher bacteriostatic activity [90].

Gorityala et al. developed a novel antipseudomonal agent, a tobramycin–moxifloxacin hybrid core structure that protects G. mellonella larvae from the lethal effects of MDR P. aeruginosa 104354 (resistant to all classes of antipseudomonal agent except colistin). In this compound, moxifloxacin is linked via a C12-tether to the C-5 position of tobramycin. No toxic effects were reported, and efficacy studies of single dose treatment resulted in a 100% survival rate after 24 h, with enhanced long-term survival effects [91].

Balasubramanian et al., found a new bioactive compound (SKC3) isolated from Streptomyces marine sponges derived from Streptomyces sp. SBT348. At the concentrations tested the compound showed no larvae toxicity in 24h. The antibacterial action observed in vitro against S. epidermidis RP62A was not confirmed in the in vivo model G. mellonella. Indeed SKC3 had not protected the larvae from an infection of S. aureus USA300 Lac (community-acquired MRSA isolated from a wrist cancer) [92].

A novel protonophore 1-(4-chlorophenyl)-4,4,4-trifluoro-3-hydroxy-2-buten-1-one active versus methicillin-resistant Staphylococcus aureus MW2 (MRSA) was tested in C. elegans and in G. mellonella. The molecule had an antibacterial effect comparable to that of vancomycin in the two models tested, both before and after inoculation of MRSA. The good correlation between the results obtained with two different unconventional models is interesting [93].

The ability of several N-phenyl-1H-pyrazole-4-carboxamide derivatives and other pyrazoles, to improve the survival of wax moth larva infected with S. aureus ATCC 29213 was investigated by Cascioferro et al. After a preliminary in vitro screening, the most active compound showed good results after 24h of infection, proving there is a good protective effect. Low toxicity was also demonstrated since there was a slightly lower survival percentage difference between uninfected larvae and those treated [94].

**In vivo toxicity screening**

The in vivo toxicity assay using G. mellonella was performed on two triazole analogues (one derived from carvacrol and the other derived from 2-hydroxy-1,4-naphthoquinone) bearing carboxylic acid, which had previously been tested for their antibacterial activity in vitro [95]. Results showed nontoxic behaviour towards the larvae viability. Moreover, the hemocyte density of larvae treated with tested compounds was not significantly affected if compared to untreated ones [95].

A series of new thiourea-containing compounds, with in vitro bacteriostatic activity towards different gram-positive and gram-negative strains, were injected into larvae at different doses to determine whether treatment could interfere with normal larval development. The compounds tested were non-toxic because a 100% survival rate was observed in most cases and the number of pupated larvae was comparable to the untreated group [96].

Lazarini et al., tested on G. mellonella, an extract from the pulp of Eugenia brasiliensis Lam. (Myrtaceae) with a high total phenolic content (389.88 ± 3.48 mg GAE/g). Although this extract showed in vitro antibacterial activity (MIC and MBC) against Staphylococcus aureus ATCC 25923 (MSSA), Staphylococcus aureus ATCC 33591 (MRSA), Pseudomonas aeruginosa ATCC 27853, Streptococcus mutans ATCC 700690, Escherichia coli ATCC 43895 and Lactobacillus acidophilus ATCC 4356 together with an in vitro effect on mature biofilm survival, it did not exert toxic effects on the larvae when administered at antibiofilm concentrations (10 × MIC –625 µg/mL, which corresponds to doses of 0.025 g/kg of extract). Moreover, the authors did not find the lethal doses able to kill 50% (LD50) of the larvae [97].

Cruz et al. evaluated the toxicity of thiazolylhydrazone derivatives in G. mellonella. Although these compounds showed antifungal activity, the authors observed an LD50 < 10mg/kg. This low toxicity was confirmed by a hemolysis assay performed using human erythrocytes [98].

**Limitation**

A limitation in the use of G. mellonella could be due to its feeding in nature. As reported by Betts et al., the high tolerance of larvae to phenolic compounds such as theaflavins and epicatechins could be due to natural adaptation, since bee honeycomb is naturally rich in natural phenolic compounds [49]. These findings are supported by Lazarini et al. testing a plant extract (Eugenia brasiliensis Lam.) with a high phenol content. The authors failed to find an LD50 using G. mellonella as in vivo model [97].

Moreover, unlike C. elegans and D. melanogaster, the G. mellonella genome (GenBank NTHM00000000) is still a shotgun project that has not been fully analysed [99]. Also, few microarrays [100,101], libraries of RNA interference and mutant strains are available [6,102]. Recently, the analysis of host-pathogenic interactions at the molecular level using the G. mellonella model has
shown many advances. Indeed, genome sequencing and studies on the immune response at the proteomic, epigenetic and transcriptomic levels have opened up important new areas of research \([100,101,103,104]\).

As already highlighted by Champion et al. in 2018 \([18]\), the major problem using \textit{G. mellonella} as a non-mammalian infection model is the lack of standardized procedures. Although these authors examined the problem in the field of fungal pathogens, their conclusions can also be extended to bacterial infections. Usually, in most experiments using larvae of \textit{G. mellonella}, infection is induced by an intra hemocoelic injection of a titrated bacterial \textit{inoculum} through the last left pro-leg. The response to infection can be assessed by different parameters: mortality, melanization, \textit{larvae} mobility, cocoon formation, quantification of hemoocytes and microorganisms in the hemolymph, alteration in gene expression and variations in the proteome \([105–107]\). Although the infection method and the scoring parameters used in microbial infection studies are very similar, there are numerous experimental variations: preparation or quantity of inoculum injected, source and management of larvae, experimental conditions or subjective interpretation of scoring parameters (morbidity or mortality). These diversities can lead to variability in results, which does not allow a direct comparison of different published studies.

Lack of standardization does not only concern the procedures but also the larvae of \textit{G. mellonella} used for scientific purposes. Larvae can easily be purchased from different commercial sources, as food for reptiles and birds in captivity or as fishing bait and also can be created and standardized directly in research labs \([30]\). Factors such as age, weight, nutrition and breeding conditions must be known to be able to use these larvae as a model of infection, as well as the presence of antibiotics and hormones that can alter their metabolism, generating inconsistent experimental responses \([18]\). For over a decade, standardized \textit{G. mellonella} larvae have been available on the market, although the cost is higher than that of common larvae, these ones offer greater consistency and reproducibility for experiments with pathogenic bacteria \([108,109]\).

**Conclusions**

There is undoubtedly a current and increasing interest within the scientific community in using the \textit{G. mellonella} as an \textit{in vivo} pre-clinical model. Although used as a pre-screening procedure, \textit{in vitro} tests alone are often inconclusive, especially in the microbiological field, where new therapeutic strategies or novel antibacterial drugs are sought. Discrepancies in antimicrobial activity between \textit{in vitro} and \textit{in vivo} experiments are often observed. For example, associations of already known drugs \([4]\), probiotic function in humans \([110]\), and even novel synthetic molecules with good \textit{in vitro} activity can have the same or a reduced level of effectiveness or they may not be effective at all when tested with \textit{G. mellonella in vivo} models \([83,84,86,90,92]\). This may be because common \textit{in vitro} models for susceptibility testing (MIC determination methods and checkerboard assay) involve contact between the bacterium and the drug in a suitable medium, lacking numerous factors present in the \textit{in vivo} models such as the cellular and humoral immunity of the host.

Besides the numerous advantages previously described, \textit{G. mellonella} proves to be very reliable even when compared to other invertebrate models \([28,29]\), as demonstrated in two studies of \textit{S. aureus} methicillin resistance, where the survival rate of the larger wax \textit{larvae} was comparable to that of the nematode \textit{C. elegans} \([79,93]\). Furthermore, despite the large evolutionary differences between insects and mammals, the results obtained by \textit{G. mellonella} are comparable to those obtained with mice \([4,48,66,111,112]\).

The use of this larval model has provided several advantages over the use of the murine model, including the ability to test many bacterial strains in a limited period at low cost with easy insect management. These characteristics make \textit{G. mellonella} an ideal model for the \textit{in vivo} evaluation of new therapeutic strategies, the efficacy of novel drugs and the characterization of host-pathogen interactions. We strongly believe that, although the use of this insect as an \textit{in vivo} pre-clinical model is now a well-established part of the laboratory routine, the full potential of \textit{G. mellonella} has not yet been developed. Indeed, overcoming some limitations such as the need for full genomic characterization and the formulation of standardized methods are necessary for the progress of scientific research.

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References

[1] Clark RE, Squire LR. An animal model of recognition memory and medial temporal lobe amnesia: history and current issues. Neuropsychologia. 2010;48(8):2234–2244, ISSN 0028-3932.

[2] O’Callaghan D, Vergunst A. Non-mammalian animal models to study infectious disease: worms or fly fishing? Curr Opin Microbiol. 2010;13:79–85.

[3] Fuchs BB, Mylonakis E. Using non-mammalian hosts to study fungal virulence and host defense. Curr Opin Microbiol. 2006;9:346–351.

[4] Van den Driessche F, Vanhoutte B, Brackman G, et al. Evaluation of combination therapy for Burkholderia cenocepacia lung infection in different in vitro and in vivo models. PLoS One. 2017;12:e0172723.

[5] Pham LN, Dionne MS, Shirasu-Hiza M, et al. A specific primed immune response in Drosophila is dependent on phagocytes. PLoS Pathog. 2007;3:e26.

[6] Tsai CJ-Y, Loh JMS, Prof T. Galleria mellonella infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence. 2016;7:214–229.

[7] Panayidou S, Ioannidou E, Apidianakis Y. Human pathogenic bacteria, fungi, and viruses in Drosophila: disease modeling, lessons, and shortcomings. Virulence. 2014;5:253–269.

[8] Brunke S, Quintin J, Kasper L, et al. Of mice, flies—and men? Comparing fungal infection models for large-scale screening efforts. Dis Model Mech. 2015;8:473–486.

[9] Conery AL, Larkins-Ford J, Ausubel FM, et al. High-throughput screening for novel anti-infectives using a C. elegans pathogenesis model. Curr Protoc Chem Biol. 2014;6:25–37.

[10] Holt WV. Exploitation of non-mammalian model organisms in epigenetic research. In: Fazeli A, Holt WV, editors. Periconception in physiology and medicine. Advances in experimental medicine and biology. Vol. 1014. Cham: Springer; 2017. p. 155–173.

[11] Kavanagh K, Reeves EP. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. FEMS Microbiol Rev. 2004;28:101–112.

[12] Browne N, Heelan M, Kavanagh K. An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes. Virulence. 2013;4:597–603.

[13] Irazoqui JE, Urbach JM, Ausubel FM. Evolution of host innate defence: insights from Caenorhabditis elegans and primitive invertebrates. Nat Rev Immunol. 2010;10:47.

[14] Kavanagh K, Sheehan G. The use of galleria mellonella larvae to identify novel antimicrobial agents against fungal species of medical interest. J Fungi. 2018;4:113.

[15] Freires IA, Sardi JDCO, de Castro RD, et al. Alternative animal and non-animal models for drug discovery and development: bonus or burden? Pharm Res. 2017;34:681–686.

[16] Trevijano-Contador N, Zaragoza O. Immune response of galleria mellonella against human fungal pathogens. J Fungi. 2018;5:3.

[17] Singulani JL, Scorzoni L, De Oliveira HC, et al. Applications of invertebrate animal models to dimorphic fungal infections. J Fungi. 2018;4:118.

[18] Champion O, Titball R, Bates S. Standardization of G. mellonella larvae to provide reliable and reproducible results in the study of fungal pathogens. J Fungi. 2018;4:108.

[19] Arvanitis M, Glavis-Bloom J, Mylonakis E. Invertebrate models of fungal infection. Biochim Biophys Acta (BBA) - Mol Basis Dis. 2013;1832:1378–1383.

[20] Lionakis MS. Drosophila and Galleria insect model hosts: new tools for the study of fungal virulence, pharmacology and immunology. Virulence. 2011;2:521–527.

[21] Junqueira JC. Models hosts for the study of oral candidiasis. In: Mylonakis E, Ausubel F, Gilmore M, Casadevall A, editors. Recent advances on model hosts. Advances in experimental medicine and biology. Vol. 710. New York (NY): Springer; 2012. p. 95–105.

[22] Mylonakis E. Galleria mellonella and the study of fungal pathogenesis: making the case for another genetically tractable model host. Mycopathologia. 2008;165:1–3.

[23] London R, Orozco BS, Mylonakis E. The pursuit of cryptococcal pathogenesis: heterologous hosts and the study of cryptococcal host–pathogen interactions. FEMS Yeast Res. 2006;6:567–573.

[24] Kwadha CA, Ong’amo GO, Ndewga PN, et al. The biology and control of the greater wax moth, Galleria mellonella. Insects. 2017;8:61.

[25] Nathan S. New to Galleria mellonella: modeling an ExPEC infection. Virulence. 2014;5:371–374.

[26] Fleming ID, Krezalek MA, Belogortseva N, et al. Modeling Acinetobacter baumannii wound infections: the critical role of iron. J Trauma Acute Care Surg. 2017;82:557.

[27] Yang H-F, Pan A-J, Hu L-F, et al. Galleria mellonella as an in vivo model for assessing the efficacy of antimicrobial agents against Enterobacter cloacae infection. J Microbiol Immunol Infect. 2017;50:55–61.

[28] Desalermos A, Fuchs BB, Mylonakis E. Selecting an invertebrate model host for the study of fungal pathogenesis. PLoS Pathog. 2012;8:e1002451.

[29] Andrea A, Krogfelt KA, Jenssen H. Methods and challenges of using the greater wax moth (Galleria mellonella) as a model organism in antimicrobial compound discovery. Microorganisms. 2019;7:85.

[30] Jorjão AL, Oliveira LD, Scorzoni L, et al. From moths to caterpillars: ideal conditions for Galleria mellonella rearing for in vivo microbiological studies. Virulence. 2018;9:383–389.

[31] Klass MR. Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. Mech Ageing Dev. 1977;6:413–429.

[32] Economos A, Lints F. Growth rate and life span in Drosophila. I. Methods and mechanisms of variation of growth rate. Mech Ageing Dev. 1984;27:1–13.
[33] Lemaitre B, Hoffmann J. The host defense of Drosophila melanogaster. Annu Rev Immunol. 2007;25:697–743.

[34] Pereira T, de Barros P, Fugisaki L, et al. Recent advances in the use of galleria mellonella model to study immune responses against human pathogens. J Fungi. 2018;4:128.

[35] Kim CH, Shin YP, Noh MY, et al. An insect multiligand recognition protein functions as an opsonin for the phagocytosis of microorganisms. J Biol Chem. 2010;285:25243–25250.

[36] Wu G, Liu Y, Ding Y, et al. Ultrastructural and functional characterization of circulating hemocytes from Galleria mellonella larva: cell types and their role in the innate immunity. Tissue Cell. 2016;48:297–304.

[37] Mak P, Zdybicka-Barabas A, Cytryńska M. A different repertoire of Galleria mellonella antimicrobial peptides in larvae challenged with bacteria and fungi. Dev Comp Immunol. 2010;34:1129–1136.

[38] Wojda I. Immunity of the greater wax moth Galleria mellonella. Insect Sci. 2017;24:342–357.

[39] Brown SE, Howard A, Kasprzak AB, et al. A peptidomics study reveals the impressive antimicrobial peptide arsenal of the wax moth Galleria mellonella. Insect Biochem Mol Biol. 2009;39:792–800.

[40] Eisenhardt M, Schlupp P, Höfer F, et al. The therapeutic potential of the insect metalloproteinase inhibitor against infections caused by Pseudomonas aeruginosa. J Pharm Pharmacol. 2019;71:316–328.

[41] ANWAr mohAmed A, Ansari MJ, Al-Ghamdi A, et al. Effect of larval nutrition on the development and mortality of Galleria mellonella (Lepidoptera: Pyralidae). Revista Colombiana de Entomologia. 2014;40:49–54.

[42] Banville N, Browne N, Kavanagh K. Effect of nutrient deprivation on the susceptibility of Galleria mellonella larvae to infection. Virulence. 2012;3:497–503.

[43] Khalil MA, Moawad SS, Hefzy EM. In vivo activity of co-trimoxazole combined with colistin against Acinetobacter baumannii producing OXA-23 in a Galleria mellonella model. J Med Microbiol. 2018;68:52–59.

[44] Wei W, Yang H, Hu L, et al. Activity of levofloxacin in combination with colistin against Acinetobacter baumannii: in vitro and in a Galleria mellonella model. J Microbiol Immunol Infect. 2017;50:821–830.

[45] Chung J-H, Bhat A, Kim C-J, et al. Combination therapy with polymyxin B and netropsin against clinical isolates of multidrug-resistant Acinetobacter baumannii. Sci Rep. 2016;6:28168.

[46] Yang H, Lv N, Hu L, et al. In vivo activity of vancomycin combined with colistin against multidrug-resistant strains of Acinetobacter baumannii in a Galleria mellonella model. Infect Dis (Auckl). 2016;48:189–194.

[47] Cruz-Mutín MY, López-Jacome LE, Hernández-Durán M, et al. Repurposing the anticancer drug mitomycin C for the treatment of persistent Acinetobacter baumannii infections. Int J Antimicrob Agents. 2017;49:88–92.

[48] Regimbal JM, Jacobs AC, Corey BW, et al. Personalized therapeutic cocktail of wild environmental phages rescues mice from A. baumannii wound infections. Antimicrob Agents Chemother. 2016 Sep;60(10):5806–5816.

[49] Betts JW, Hornsey M, Wareham DW, et al. In vitro and in vivo activity of theflavin–epicatechin combinations versus multidrug-resistant acinetobacter baumannii. Infect Dis Ther. 2017;6:435–442.

[50] Papp-Walace KM, Becka SA, Zeiser ET, et al. Overcoming an extremely drug resistant (XDR) pathogen: avibactam restores susceptibility to ceftazidime for Burkholderia cepacia complex isolates from cystic fibrosis patients. ACS Infect Dis. 2017;3:502–511.

[51] Rosznioński B, Latka A, Maciejewska B, et al. The temperate Burkholderia phage AP3 of the Pseudovirinae shows efficient antimicrobial activity against B. cenocepacia of the IIIA lineage. Appl Microbiol Biotechnol. 2017;101:1203–1216.

[52] Mil-Homens D, Ferreira-Dias S, Fialho A. Fish oils against Burkholderia and Pseudomonas aeruginosa: in vitro efficacy and their therapeutic and prophylactic effects on infected Galleria mellonella larvae. J Appl Microbiol. 2016;120:1509–1519.

[53] Naguib MM, Valvano MA. Vitamin E increases antimicrobial sensitivity by inhibiting bacterial lipopolysaccharide binding. mSphere. 2018;3e00564–00518.

[54] Nale JY, Chutia M, Carr P, et al. ‘Get in early’; biofilm and wax moth (Galleria mellonella) models reveal new insights into the therapeutic potential of Clostridium difficile bacteriophages. Front Microbiol. 2016;7:1383.

[55] Yang H, Chen G, Hu L, et al. Enhanced efficacy of imipenem-colistin combination therapy against multiple-drug-resistant Enterobacter cloacae: in vitro activity and a Galleria mellonella model. J Microbiol Immunol Infect. 2018;51:70–75.

[56] Manohar P, Nachimuthu R, Lopes BS. The therapeutic potential of bacteriophages targeting gram-negative bacteria using Galleria mellonella infection model. BMC Microbiol. 2018;18:97.

[57] Tharmalingam N, Port J, Castillo D, et al. Repurposing the anthelmintic drug niclosamide to combat Helicobacter pylori. Sci Rep. 2018;8:3701.

[58] Jiang L, Greene MK, Insua IL, et al. Clearance of intracellular Klebsiella pneumoniae infection using gentamicin-loaded nanoparticles. J Control Release. 2018;279:316–325.

[59] Majkowska-Skrobek G, Latka A, Berisio R, et al. Phage-borne depolymerases decrease Klebsiella pneumoniae resistance to innate defense mechanisms. Front Microbiol. 2018;9:2517.

[60] Majkowska-Skrobek G, Latka A, Berisio R, et al. Capsule-targeting depolymerase, derived from Klebsiella KP36 phage, as a tool for the development of anti-virulent strategy. Viruses. 2016;8:324.

[61] D’andreà MM, Marmo P, De Angelis LH, et al. φBO1E, a newly discovered lytic bacteriophage targeting carbanemase-producing Klebsiella pneumoniae of the pandemic Clonal Group 258 clade II lineage. Sci Rep. 2017;7:2614.

[62] Aparecida Procópio Gomes L, Alves Figueiredo LM, Luiza do Rosário Palma A, et al. Punica granatum (L. (Pomegranate) extract: in vivo study of antimicrobial activity against Porphyromonas gingivalis in Galleria
[63] Zheng Z, Tharmalingam N, Liu Q, et al. Synergistic efficacy of aedes aegypti antimicrobial peptide cercopin A2 and tetracycline against pseudomonas aeruginosa. Antimicrob Agents Chemother. 2017;61:e00686–00617.

[64] Ciociola T, Giovati L, Giovannelli A, et al. The activity of a mammalian proline-rich peptide against Gram-negative bacteria, including drug-resistant strains, relies on a nonmembranolytic mode of action. Infect Drug Resist. 2018;11:969.

[65] D’Angelo F, Baldelli V, Halliday N, et al. Identification of FDA-approved drugs as antivirulence agents targeting the pqs quorum-sensing system of Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2018;62:e01296–01218.

[66] Forti F, Roach DR, Cafora M, et al. Design of a broad-range bacteriophage cocktail that reduces Pseudomonas aeruginosa biofilms and treats acute infections in two animal models. Antimicrob Agents Chemother. 2018;62:30251–30267.

[67] Olszak T, Shneider MM, Latka A, et al. The O-specific polysaccharide lyase from the phage LKA1 taspilke reduces Pseudomonas virulence. Sci Rep. 2017;7:16302.

[68] Siriyong T, Voravuthikunchai SP, Coote PJ. Steroidal alkaloids and conessine from the medicinal plant Holarrhena antidysenterica restore antibiotic efficacy in a Galleria mellonella model of multidrug-resistant Pseudomonas aeruginosa infection. BMC Complement Altern Med. 2018;18:285.

[69] Paškevičius Š, Starkevič U, Misūnas A, et al. Plant-expressed pyocins for control of Pseudomonas aeruginosa. PloS One. 2017;12:e0185782.

[70] Mirza ZR, Hasan T, Seidel V, et al. Geranial as a novel antivirulence agent against bacillary dysentery-causing Shigella sonnei. Virulence. 2018;9:450–455.

[71] Skinner K, Sandoe JA, Rajendran R, et al. Efficacy of rifampicin combination therapy for the treatment of enterococal infections assessed in vivo using a Galleria mellonella infection model. Int J Antimicrob Agents. 2017;49:507–511.

[72] Marini E, Magi G, Ferretti G, et al. Attenuation of Listeria monocytogenes virulence by Cannabis sativa L. essential oil. Front Cell Infect Microbiol. 2018 Aug 22;8:293.

[73] Upadhyay A, Venkatanarayanan K. In vivo efficacy of trans-cinnamaldehyde, carvacrol, and thymol in attenuating Listeria monocytogenes infection in a Galleria mellonella model. J Nat Med. 2016;70:667–672.

[74] Meir M, Bifani P, Barkan D. The addition of avibactam renders piperacillin an effective treatment for Mycobacterium abscessus infection in an in vivo model. Antimicrob Resist Infect Control. 2018;7:151.

[75] Dong C-L, Li L-X, Cui Z-H, et al. Synergistic effect of pleuromutilins with other antimicrobial agents against staphylococcus aureus in vitro and in an experimental galleria mellonella model. Front Pharmacol. 2017;8:553.

[76] Silva L, Hora G, Soares T, et al. Myricetin protects Galleria mellonella against Staphylococcus aureus infection and inhibits multiple virulence factors. Sci Rep. 2017;7:2823.

[77] Ferro TA, Araújo JM, dos Santos Pinto BL, et al. Cinnamaldehyde inhibits staphylococcus aureus virulence factors and protects against infection in a galleria mellonella model. Front Microbiol. 2016;7:2052.

[78] Mishra B, Wang X, Lushnikova T, et al. Antibacterial, antifungal, anticancer activities and structural bioinformatics analysis of six naturally occurring temporins. Peptides. 2018;106:9–20.

[79] Johnston T, Hendricks GL, Shen S, et al. Raf-kinase inhibitor GW5074 shows antibacterial activity against mexitillin-resistant Staphylococcus aureus and potentiates the activity of gentamicin. Future Med Chem. 2016;8:1941–1952.

[80] Normark BH, Normark S. Evolution and spread of antibiotic resistance. J Intern Med. 2002;252:91–106.

[81] Bonev BB, Brown NM. Bacterial resistance to antibiotics: from molecules to man. Hoboken (NJ): John Wiley & Sons; 2019.

[82] Minrovic BM, Jung D, Melander RJ, et al. A new class of adjuvants enables lower dosing of colistin against acinetobacter baumannii. ACS Infect Dis. 2018;4(9):1368–1376.

[83] Huggins WM, Minrovic BM, Corey BW, et al. 1, 2, 4-Triazolidine-3-thiones as narrow spectrum antibiotics against multidrug-resistant Acinetobacter baumannii. ACS Med Chem Lett. 2016;8:27–31.

[84] Tran T, Chiem K, Jani S, et al. Identification of a small molecule inhibitor of the aminoglycoside 6'-N-acetyltransferase type Ib [AAC (6')-Ib] using mixture-based combinatorial libraries. Int J Antimicrob Agents. 2018;51:752–761.

[85] Jin WB, Xu C, Cheng Q, et al. Investigation of synergistic antimicrobial effects of the drug combinations of meropenem and 1, 2-benzisoxazol-3 (2H)-one derivatives on carbapenem-resistant Enterobacteriaceae producing NDM-1. Eur J Med Chem. 2018;155:285–302. DOI:10.1016/j.ejmech.2018.06.007.

[86] Vellé A, Maguire R, Kavanagh K, et al. Steroid–aul–NHC complexes: synthesis and antibacterial activity. ChemMedChem. 2017;12:841–844.

[87] Proschak A, Kramer J, Proschak E, et al. Bacterial zincofphore [S, S]-ethylenediamine-N, N'-disuccinic acid is an effective inhibitor of MBLs. J Antimicrob Chemother. 2017;73:425–430.

[88] Hubble VB, Hubbard BA, Minrovic BM, et al. Using small-molecule adjuvants to repurpose azithromycin for use against pseudomonas aeruginosa. ACS Infect Dis. 2019;5(1):141–151.

[89] Jakobsen V, Viganor L, Blanco-Fernández A, et al. Tetrameric and polymeric silver complexes of the omeprazole scaffold: Synthesis, structure, in vitro and in vivo antimicrobial activities and DNA interaction. J Inorg Biochem. 2018;186:317–328.

[90] Lyu Y, Yang X, Goswami S, et al. Amphiphilic tobramycin–lysine conjugates sensitizes multidrug resistant gram-negative bacteria to rifampicin and minocycline. J Med Chem. 2017;60:3684–3702.

[91] Gorityala BK, Guchhait G, Goswami S, et al. Hybrid antibiotic overcomes resistance in P. aeruginosa by enhancing outer membrane penetration and reducing efflux. J Med Chem. 2016;59:8441–8455.
Balasubramanian S, Skaf J, Holzgrabe U, et al. A new bioactive compound from the marine sponge-derived Streptomyces sp. SBT348 inhibits staphylococcal growth and biofilm formation. Front Microbiol. 2018;9:1473.

Tharmalingam N, Jayamani E, Rajamuthiah R, et al. Activity of a novel protonophore against methicillin-resistant Staphylococcus aureus. Future Med Chem. 2017;9:1401–1411.

Cascoferro S, Maggio B, Raffa D, et al. Synthesis and biofilm formation reduction of pyrazole-4-carboxamide derivatives in some Staphylococcus aureus strains. Eur J Med Chem. 2016;123:58–635.

Aneja B, Azam M, Alam S, et al. Natural product-based 1, 2, 3-triazole/sulfonate analogues as potential chemotherapeutic agents for bacterial infections. ACS Omega. 2018;3:6912–6930.

Dolan N, Gavin DP, Eshwika A, et al. Synthesis, antibacterial and anti-MRSA activity, in vivo toxicity and a structure–activity relationship study of a quinoline thiourea. Bioorg Med Chem Lett. 2016;26:630–635.

Lazarini JG, Sardi JDCO, Franchin M, et al. Bioprospection of Eugenia brasiliensis, a Brazilian native fruit, as a source of anti-inflammatory and antibiofilm compounds. Biomed Pharmacother. 2018;102:132–139.

Cruz L, Lopes L, de Camargo Ribeiro F, et al. Anti-candida albicans activity of thiazolylhydrazone derivatives in invertebrate and murine models. J Fungi. 2018;4:134.

Lange A, Beier S, Huson DH, et al. Genome sequence of Galleria mellonella (greater wax moth). Genome Announc. 2018;6:e01220–01217.

Heitmueller M, Billion A, Dobrindt U, et al. Epigenetic mechanisms regulate innate immunity against uropathogenic and commensal-like Escherichia coli in the surrogate insect model Galleria mellonella. Infect Immun. 2017;Sep:85(10):e00336–e00417.

Mukherjee K, Vilcinskas A. Development and immunity-related microRNAs of the lepidopteran model host Galleria mellonella. BMC Genomics. 2014;15:705.

Lange A, Schäfer A, Bender A, et al. Galleria mellonella: a novel invertebrate model to distinguish intestinal symbionts from pathobionts. Front Immunol. 2018 Sep;9:2114. PubMed PMID: 30283451; PubMed Central PMCID: PMC6156133.

Sheehan G, Kavanagh K. Proteomic analysis of the responses of candida albicans during infection of galleria mellonella larvae. J Fungi. 2019;5:7.

Mukherjee K, Vilcinskas A. The entomopathogenic fungus Metarhizium robertsii communicates with the insect host Galleria mellonella during infection. Virulence. 2018;9:402–413.

Loh JM, Adenwalla N, Wiles S, et al. Galleria mellonella larvae as an infection model for group A streptococcus. Virulence. 2013;4:419–428.

Rossoni RD, Fuchs BB, de Barros PP, et al. Lactobacillus paracasei modulates the immune system of Galleria mellonella and protects against Candida albicans infection. PLoS One. 2017;12:e0173332.

de Melo NR, Abdrahman A, Greig C, et al. Myriocin significantly increases the mortality of a non-mammalian model host during Candida pathogenesis. PLoS One. 2013;8:e78905.

Thelaus J, Lundmark E, Lindgren P, et al. Galleria mellonella reveals niche differences between highly pathogenic and closely related strains of Francisella spp. Front Cell Infect Microbiol. 2018;Jun 5;8:188. PubMed PMID: 29922601; PubMed Central PMCID: PMC5996057.

Wagley S, Borne R, Harrison J, et al. Galleria mellonella as an infection model to investigate virulence of Vibrio parahaemolyticus. Virulence. 2018;9:197–207.

Blandino G, Fazio D, Di Marco R. Probiotics: overview of microbiological and immunological characteristics. Expert Rev Anti Infect Ther. 2008;6:497–508.

Sá NP, Lima CM, Dos Santos A, et al. A phenylthiazole derivative demonstrates efficacy on treatment of the cryptococcosis & candidiasis in animal models. Future Sci OA. 2018;4:FSO305–FSO305.

Rossoni RD, Barbosa JO, Vilela SFG, et al. Competitive interactions between C. albicans, C. glabrata and C. krusei during biofilm formation and development of experimental candidiasis. PLoS One. 2015;10: e0131700.