**Galleria mellonella as an experimental model for studying periodontopathogens**

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**Abstract:**

In the present study, *Galleria mellonella* was evaluated as a potential infection model for periodontal bacteria, more specifically, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomyctecemcomitans*. All the bacteria evaluated were pathogenic to *G. mellonella*, causing their death in a concentration-dependent manner, and a decrease in their hemocyte count. Moreover, it was possible to recover the bacteria from the larvae hemolymph and determine the colony-forming units per larvae. *G. mellonella* is an effective model that may help to better understand the host-microbe interactions in periodontics.

**Key words:**

*Aggregatibacter actinomyctecemcomitans*, *Fusobacterium nucleatum*, *Galleria mellonella*, periodontal diseases, *Porphyromonas gingivalis*

**INTRODUCTION**

Periodontitis is a multifactorial inflammatory disease associated with dysbiotic biofilms and caused mainly by Gram-negative, anaerobic, and proteolytic bacteria, such as *Aggregatibacter actinomyctecemcomitans*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*. 

In *vivo* studies are extremely important not only for the analysis of periodontal disease pathogenesis but also for the development of innovative treatments. However, due to ethical issues, reducing the number of animals used in studies by employing alternative techniques and refined experimental protocols should be considered. In this context, invertebrate animal models are one alternative for screening host-pathogen interaction that could reduce the use of vertebrates and add information to the *in vitro* studies. *Galleria mellonella* is an insect frequently used as an experimental model to evaluate microbial virulence, compound toxicity, and antimicrobial effectiveness. These larvae exhibit an immune response analogous to the vertebrates innate immune response. Similar to mammalian blood, the larvae hemolymph contains hemocytes, which are immune cells compared in terms of function to mammalian neutrophils. Through a similar mechanism via superoxide production used by human neutrophils, hemocytes have the ability to phagocytize and kill bacterial cells, in addition to having homologous proteins essential for the production of superoxide.

It is possible to evaluate the host response to the pathogen through hemocyte density. The pathogenicity of the microorganism correlates inversely with the number of hemocytes in the larvae; the higher the pathogenicity of the microorganism, the lower was the number of hemocytes. Few reports describe the use of *G. mellonella* as an infection model for periodontal pathogens.

In this study, *G. mellonella* was evaluated as an animal model for periodontopathogens infection [Figure 1]. For this purpose, determination of survival curves and hemocyte concentration, and colony-forming units (CFU) recovery from the larvae hemolymph were performed after infection with *P. gingivalis* (ATCC 33277), *F. nucleatum* (ATCC 25586), and *A. actinomyctecemcomitans* (ATCC 29522).

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**How to cite this article:** Santos TA, Scorzoni L, Santos AD, Junqueira JC, Anbinder AL. *Galleria mellonella* as an experimental model for studying periodontopathogens. J Indian Soc Periodontol 2020;24:593-6.
The bacteria were grown on Brucella agar supplemented with 5% defibrinated sheep blood and 1% solution of hemin and menadione for 5 days in an anaerobic chamber at 37°C. For the assays, suspensions were prepared at concentrations of $10^7$, $10^8$, and $10^9$ cells/mL, using a spectrophotometer at 660 nm, 550 nm, and 600 nm for P. gingivalis, F. nucleatum, and A. actinomycetemcomitans, respectively.

Light-colored G. mellonella larvae (Lepidoptera: Pyralidae), with no spots on their cuticles and weighting 190–230 mg, were selected for the study. The survival curves were obtained by inoculating 10 µL of periodontopathogens in the larvae last left proleg at the concentrations of $10^7$, $10^8$, and $10^9$ cells/mL, and phosphate-buffered saline for the control group, using micro syringes. Twelve larvae were used for each periodontopathogen concentration evaluation. After inoculation, the animals were incubated at 37°C in the dark, without food. The survival was evaluated daily for 7 days. Sub-lethal concentrations of P. gingivalis ($10^7$ cells/mL), F. nucleatum ($10^8$ cells/mL), and A. actinomycetemcomitans ($10^9$ cells/mL) were used for the evaluation of the hemocyte concentration and CFUs recovery from the hemolymph. After infection, the larvae were incubated at 37°C for 3 h. For hemocyte concentration determination, a hemolymph sample of 3 larvae was collected and diluted with an anticoagulant solution (2% NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, and 10 mM EDTA). The hemocyte concentration was estimated using a Neubauer chamber. For each treatment, four groups of 3 larvae each were analyzed (a total of 12 animals per treatment). For the determination of periodontopathogens CFUs recovered from the larvae, the collected hemolymph was diluted on Brucella blood agar supplemented with 1% hemin and menadione and incubated under anaerobic conditions (37°C for 5 days). The statistical analysis was performed by the log-rank method (Mantel-Cox), ANOVA, or Student’s t-test ($\alpha = 5%$; GraphPad Prism 6, La Jolla, California, USA).

The larvae survival rates inversely correlate with microorganism pathogenicity. All bacteria evaluated were able to kill G. mellonella in a concentration-dependent manner. The infection with P. gingivalis at $10^7$ cells/mL resulted in 50% of larvae survival after 7 days, but when infected with the concentrations of $10^8$ and $10^9$ cells/mL of P. gingivalis, all larvae were dead within 24 h [Figure 2a]. When the larvae were infected with $10^7$, $10^8$, and $10^9$ cells/mL of F. nucleatum, survival rates of 66.67%, 13.33%, and 6.67%, respectively, were observed after 7 days [Figure 2b]. Infection with A. actinomycetemcomitans at $10^9$ cells/mL caused 100% caterpillar mortality within 48 h of inoculation; this was significantly higher than the

![Figure 1](image1.png)

**Figure 1:** Galleria mellonella experimental model: (a) Inoculation of periodontopathogen in the larvae last left proleg; (b) Alive larvae after 1 h of PBS inoculation; (c) Melanized alive larvae after 1 h of infection with Aggregatibacter actinomycetemcomitans; (d) Melanized dead larvae after 24 h of infection with Aggregatibacter actinomycetemcomitans

![Figure 2](image2.png)

**Figure 2:** Survival curve of Galleria mellonella after infection with different concentrations of periodontopathogens; (a) Porphyromonas gingivalis. There was a statistical difference between the concentrations of $10^7$ and $10^8$ cells/mL ($P=0.0001$) and $10^7$ and $10^9$ cells/mL ($P=0.0001$); (b) Fusobacterium nucleatum. There was a statistical difference between the concentrations of $10^7$ and $10^8$ cells/mL ($P=0.0022$), and $10^7$ and $10^9$ cells/mL ($P=0.0003$); (c) Aggregatibacter actinomycetemcomitans. There was a statistical difference between the concentrations of $10^7$ and $10^8$ cells/mL ($P=0.0023$), and $10^8$ and $10^9$ cells/mL ($P=0.0362$). Same letters over the lines indicate the absence of statistical difference after Log-Rank test (Mantel Cox)
mortality observed at other concentrations. Infections with $10^7$ and $10^8$ cells/mL led to similar results ($P = 0.0645$) after 7 days of infection [Figure 2c]. P. gingivalis was the most pathogenic bacteria for G. mellonella, causing 100% mortality within 24 h at lower concentrations than the other bacteria.

Regarding hemocyte concentration after 3 h of infection, a reduction in the hemocyte count was observed with all evaluated bacteria [Figure 3]. Infection with P. gingivalis led to a reduction of 63.88% in the number of hemocytes, while F. nucleatum and A. actinomycetemcomitans led to a decrease of 72.34% and 60.11%, respectively.

The amount of P. gingivalis recovered from the larvae hemolymph 3 h after infection was lower ($2.9 \times 10^4$ cells/mL) than the initially inoculated concentration ($10^7$ cells/mL), in contrast to the other bacteria tested. In the larvae infected with $10^8$ cells/mL of F. nucleatum and A. actinomycetemcomitans, the microbial recovery was found to be $2.295 \times 10^8$ cells/mL and $3.65 \times 10^8$ cells/mL, respectively, equivalent to the initial inoculated amount. Oxygen contact may have influenced the recovery of strictly anaerobic microorganisms such as P. gingivalis. Although we have evaluated the bacterial burden only in the hemolymph, bacteria can also invade other tissues of the larvae, such as the gut and fat body. Furthermore, in addition to the hemocytes, the immune response of the larvae is also mediated by proteins, antimicrobial peptides, and melanin, which possibly act against P. gingivalis. Despite the significantly reduced amount of P. gingivalis recovered, the mortality rate was still higher. This could be explained by P. gingivalis virulence factors such as capsule, fimbriae, and lipopolysaccharides, and by the activation of the immune system, which requires a high-energy cost and may not be sustained if the bacterial stimulus is intense, leading to larvae septic death. The specific role of P. gingivalis virulence factors in mortality rates should be investigated further.

This study demonstrated that G. mellonella could be used as a model for infection by the facultative anaerobic bacterium A. actinomycetemcomitans, as well as by strictly anaerobic bacteria P. gingivalis and F. nucleatum. Although it cannot replace vertebrate models of periodontitis induction, this simple model effectively allows the study of the interaction of periodontopathogens and the host. It was possible to evaluate the virulence of the microorganisms by evaluating G. mellonella survival curves, hemocyte counts, and microbial recovery. Different branches of research in periodontics, such as the investigation of alternative treatments and evaluation of the virulence of clinical strains, may benefit from the use of G. mellonella as a model of infection for periodontopathogens.

**Financial support and sponsorship**

This project was funded by São Paulo Research Foundation FAPESP (2016/06946-1 and 2017/05439-1) and by Coordination of Improvement of Higher Education Personnel CAPES.

**Conflicts of interest**

There are no conflicts of interest.

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