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Galleria Mellonella as a Model Host to Study Gut Microbe Homeostasis and Brain Infection by the Human Pathogen *Listeria Monocytogenes*

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Abstract The gastrointestinal tract in both mammals and insects is associated with microbes (collectively the microbiota), which are controlled by the intestinal immune system. These microbes regulate pathogens that can infect gut epithelial cells, and there is increasing evidence for a reciprocal relationship between beneficial and pathogenic bacteria in the gut and the intestinal immune system. Deciphering these complex interactions between the microbiota and intestinal immune system in mammals requires surrogate model systems, such as larvae of the greater wax moth *Galleria mellonella*. The exposure of *G. mellonella* microbiota to antibiotics induces immunity and stress-related genes in the intestine. The model can also provide insight into the virulence mechanisms of pathogens such as *Listeria monocytogenes* in the human gut and brain. We also discuss the current uses of *G. mellonella* as a model to develop therapeutic strategies against listeriosis.

Keywords Blood–brain barrier · Galleria mellonella · Gut-microbe homeostasis · Listeria monocytogenes

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Abbreviations

CNS Central nervous system
AMP Antimicrobial peptide

Contents

1 Introduction

Prokaryotic microorganisms have existed for more than 3 billion years and have adapted to diverse environments, including the colonization of multicellular eukaryotes. The gastrointestinal tract in mammals contains complex microbial communities that contribute to health and well-being. The complex interplay between gut microbiota and the intestinal immune system has coevolved and confers mutual benefits on both the microbiota and host; thus, its disruption can increase the risk of immune-related diseases [1, 2]. Growing evidence indicates a reciprocal relationship between microbiota in the human gut and intestinal immunity, involving the innate and adaptive immune responses [1, 2]. This relationship provides an example of homeostatic regulation, which improves metabolism and prevents the growth of pathogens. It is valuable to study this relationship using appropriate models.

Insects can be used as model hosts to study interactions between human pathogens and microbiota that prevent infections in the gut [3, 4]. The advantages of insect models over mammals include their convenience, low cost, and ethical acceptability. The insect innate immune system resembles that of humans. The counterpart of mammalian Toll-like receptors was found initially in the fruit fly

Drosophila melanogaster, which can simulate infections by human pathogens and support gut microbial homeostasis [4, 5].

Although *D. melanogaster* is genetically tractable, the greater wax moth *Galleria mellonella* has several other advantages, including its ability to support interactions with human pathogens at 37 °C (the physiological temperature in mammals), which is important because microbial pathogenesis depends on the temperature-sensitive expression of virulence factors. Similar temperature-regulated virulence factors are involved when pathogens infect *G. mellonella* and humans. Another advantage is that larval diets can be supplemented with defined microbial inoculums, allowing the quantitative analysis of immune responses and intestinal homeostasis [6, 7].

G. mellonella has been established as model host for many human pathogens, including the Gram-positive bacterium *Listeria monocytogenes,* which infects the gut and central nervous system (CNS) of humans [8–10]. The role of gut microbiota in the control of gastroenteritis and the mechanisms underlying brain infection in mammals are far from clear. Here, we discuss the recent developments using *G. mellonella* as a model host to study complex microbial interactions within the intestine and CNS.

2 Gut Microbial Homeostasis in Vertebrates and Invertebrates

The interaction between microbiota and the intestinal immune system begins at birth. Neonates share microbial identity with their mother, indicating transgenerational microbial transference. Similarly, insects such as the firebug *Pyrrhocoris apterus* can vertically transmit symbionts to their offspring via an unknown mechanism [11]. Tailoring the intestinal immune response is necessary to promote such reciprocal interactions with the microbiota, which are beneficial because they improve metabolism and immunity. The emergence of adaptive immunity in humans involved diverse antigen-recognition receptors on T and B lymphocytes, which allow specific responses to antigens as well as prolonged immune memory. The adaptive immune system may help to maintain the gut microbiota while eliminating harmful pathogens by tempering innate immune responses programmed for the nonspecific elimination of microbes. In invertebrates, where gut microbiota are solely maintained by innate immunity, it has been suggested that microbial diversity may be responsible for specific immune phenotypes, and the evolution of gut parasites may be driven by interactions with different microbial species as well as host genotypes [12]. A comprehensive understanding of the innate immunity that co-evolves with the microbiota therefore requires further investigation in insect models to eliminate cross-talk with the adaptive immune responses of mammals.

2.1 The Composition of the Gut Microbiota in Vertebrates and Invertebrates

The gastrointestinal tract in most insects contains many nonpathogenic microbes, specifically reflecting their diverse habitats, lifestyles, and nutritional sources. For example, the honeybee (*Apis melifera*) contains a particularly diverse microbial community, providing functional capabilities linked to host interaction, biofilm formation, and carbohydrate degradation [13, 14]. Metagenomic sequencing has identified genes encoding pectin-degrading enzymes in γ-proteobacterial species, possibly conferring upon bees the ability to break down pollen grain walls [15]. The microbiota in social bees resembles that of the human gut, albeit with substantially reduced complexity.

Regardless of differences in size, physiology, and diet, the same types of bacteria dominate the distal guts of both humans and mice. These include Firmicutes, Bacteroidetes, and Actinobacteria, whereas Proteobacteria, Verrucomicrobia, Fusobacteria, and Cyanobacteria are present as minor constituents [16]. Comparative analysis of the human and mouse microbiomes indicates only 15 % identity. It is estimated that the human microbiome contains as many as 10^{14} bacteria, 10-fold more than the number of cells present in the human body, whereas *D. melanogaster* contains approximately 10^5 microbial cells [17–19]. More than 500 bacterial species are found along the epithelial barrier of the human gut, compared to 5–20 different species in *D. melanogaster* [18, 20, 21].

Compared with *D. melanogaster*, the gut microbiota of other insects can be extremely diverse and complex. For example, the firebug has a microbiome similar in complexity to humans, with 454 sequencing indicating the dominance of Actinobacteria (*Coriobacterium glomerans* and *Gordonibacter* spp.), Firmicutes (*Clostridium* spp. and *Lactococcus lactis*), and Proteobacteria (*Klebsiella* spp. and Rickettsiales bacterium) [11]. Abundant bacteria are also found in the lepidopteran species *Spodoptera littoralis* and *Helicoverpa armigera*. Based on 16S rRNA sequencing and microarray analysis, the microbiota in these insects include Enterococci, Lactobacilli, and Clostridia among the Firmicutes, which are also prevalent in the human gut [21]. Microbiota are also present in the midgut of the lepidopteran model host *G. mellonella,* but the details remain unclear. Although bacteria have been studied in detail, the roles of viruses, archaea, and unicellular eukaryotes in the gastrointestinal tract are poorly understood.

2.2 Contribution of the Gut Microbiota to Infection, Immunity, and Metabolism

It is unclear how the microbiota in the human gut influence pathogens and improve immune responses, so this remains a subject of intense research. Probiotic prophylaxis exploits the antagonistic activity of beneficial bacteria against invading pathogens [22]. Typically, beneficial gut microbes form a physical barrier to

prevent pathogens infecting host epithelial cells; they also occupy pathogen attachment sites, consume nutrients required by pathogens, and induce host antimicrobial responses. Gram-positive bacteria of the genius *Lactobacillus*, which are found in the human gut, can prevent infections with the pathogens *L. monocytogenes* and *Escherichia coli* by modulating epithelial immunity and secreting compounds that inhibit colonization, respectively [23, 24]. The gut microbes in the insect model *Anopheles gambiae* help to prevent infection by the malaria pathogen *Plasmodium falciparum*, and pyrosequencing has confirmed that the microbiota proliferates following infection [3]. This indicates that the microbiota protects the mosquito against infection with *P. falciparum*, although further evidence is required to understand the molecular mechanism of homeostasis. Understanding these mechanisms may facilitate the development of new targets for the treatment of chronic gastroenteritis and associated human infections [25].

The host diet is another key factor that can regulate the gut microbiota, particularly diets that promote beneficial bacteria thus improving health and well-being. Phenolic compounds can reduce or reverse the development of colitogenic changes in the intestinal mucosa, offering prophylaxis against colorectal carcinomas [26]. The gut microbiota maximizes the caloric availability of ingested nutrients by extracting additional sugars from indigestible carbohydrates and also modulates nutrient absorption and utilization by the intestinal epithelium, thus improving metabolism. The human intestinal microbiota also modulates the uptake and absorption of lipids [27] and increases glucose uptake by upregulating the Na^+/glucose cotransporter at the intestinal epithelium [28]. The gut microbiota also helps to metabolize microbial toxins and xenobiotic compounds such as drugs, influencing toxicological studies in the pharmaceutical industry and the development of personalized medicines. These processes provide energy not only for the host but also to maintain the microbial population [26].

3 The Model Host *Galleria mellonella* Complements the Study of Gut Microbe Homeostasis in Humans

G. mellonella is increasingly favored as a preclinical research model for the investigation of bacterial and fungal pathogens that infect humans and innate immune system dysfunction following infection, correlating with data obtained from mammalian models [29]. The insect and mammalian gastrointestinal tracts share similar tissues, anatomy, and physiological functions [30, 31]. The microvilli of the *G. mellonella* midgut contain microbes that resemble those found in the intestinal microvilli of mammals. Because gut microbe homeostasis in *G. mellonella* can be studied at the physiological temperature of mammals, it is possible to identify orthologs of human genes that contribute to such reciprocal interactions.

Antimicrobial peptides are an evolutionarily-conserved component of the innate immune systems of vertebrates and invertebrates that can help to maintain or eliminate microbial associations. In mammals, antimicrobial peptides (AMPs)

produced in the gut, such as defensins, cathelicidins, and C-type lectins, disrupt the cell membranes of both commensals and pathogens [32, 33]. These AMPs are induced both by gut microbes and the compounds they produce; however, the hyperactivation of innate immunity can reduce the abundance of gut microbiota, so there must be a balance to maintain a productive coexistence. The innate immune response to gut microbiota is limited by the physical separation of bacteria and host cells and by the modulation of localized immunity to achieve tolerance. Furthermore, homeostatic interaction downregulates host pro-inflammatory responses to facilitate microbial colonization of the gut [34].

G. mellonella has a diverse repertoire of AMPs, including candidates with structures and functions that are not found in mammals such as insect metalloprotease inhibitor [35], gallerimycin displaying exclusive activity against mycelia-forming fungi [36], and cobatoxin (scorpion toxin-like) [37]. The *G. mellonella* genes encoding AMPs are induced during tissue remodeling [38, 39], hunger stress, and infection with pathogens, suggesting they may play a role in the specific microbial selection process that occurs in the insect midgut. Modulating the composition of the microbiota with antibiotics can disrupt this homeostatic balance, promoting the proliferation of pathogens [40–47]. The long-lasting effects of antibiotic treatment even after discontinuation can interfere with the activity of the gut and the maintenance of microbial populations [48–52].

For example, plating midgut extracts from *G. mellonella* larvae on nutrient medium results in the growth of bacterial colonies, but the levels of AMPs such as gallerimycin, as well as markers of cellular and humoral stress, remain at normal levels (Fig. 1). However, when larvae are fed an antibiotic cocktail, gallerimycin is downregulated after initial induction (Fig. 1a, b), increasing the number of bacterial colonies (data not shown) and also inhibiting a number of stress markers involved in cellular immunity, metabolism, and anti-inflammatory responses (Fig. 1, Table 1). This result clearly indicates a coordinated response that stabilizes the immune system and maintains microbial growth in the midgut region, as reported in mammals. Force-feeding *G. mellonella* larvae with either pathogenic or nonpathogenic bacteria can also provide insight into microbial dysbiosis (loss of balance between protective and harmful microbes) and its involvement in foodborne infections by human pathogens such as *L. monocytogenes* (Fig. 2).

4 The Prospective Role of Gut Microbiota in the Control of Brain Infections by the Foodborne Pathogen *L. monocytogenes* in *G. mellonella*

Microbial dysbiosis in the human gut often correlates with the progression of infections and diseases such as gastroenteritis, metabolic imbalance, inflammatory bowel disease and colorectal cancer [53]. Dysbiosis can be investigated using the *G. mellonella* model by the oral delivery of specific microbial populations or

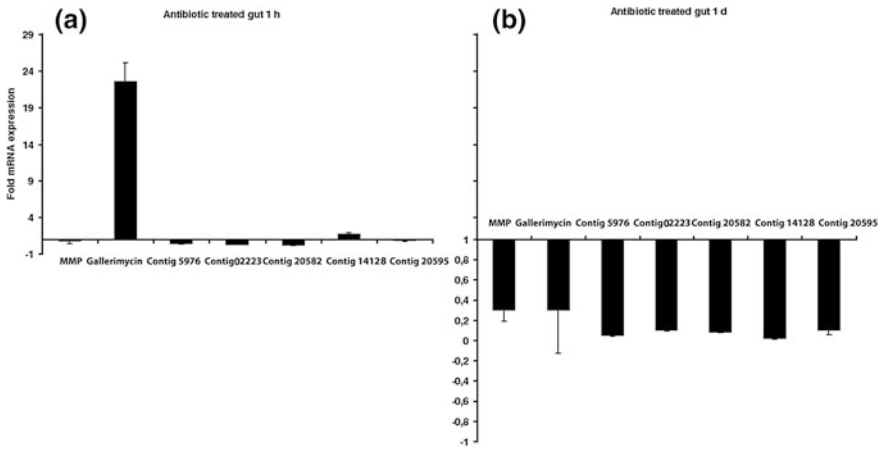

Fig. 1 Transcriptional activation of matrix metalloproteinase, gallerimycin, and potential stress markers in the midgut of *G. mellonella* following the ingestion of an antibiotic cocktail. Larvae were fed (10 μl/larva) with an antibiotic cocktail containing erythromycin, kanamycin, ampicillin and gentamicin. Expression levels in the midgut were determined by quantitative real-time reverse-transcription polymerase chain reaction and are shown relative to the midgut of untreated larvae after 1 h and 1 day of feeding. The selected expressed sequence tags include Contig 5976.0, Contig 02223_1.f1.exp, Contig 20582_1.exp, Contig 14128_1.exp and Contig 20595_1.exp. Values were normalized against the expression of the housekeeping gene 18*S* rRNA. The experiment was carried out three times with similar results

Table 1 Selected *Galleria mellonella* stress markers used for real-time reverse-transcription polymerase chain reaction analysis and their assignment to biological processes based on gene ontology categories

Biological process	GenBank accession no. of expressed sequence tag
Antiapoptosis, GTP biosynthetic process, CTP biosynthetic process, purine base metabolic process, pyrimidine base metabolic process	Contig 5976.0
Cell cycle arrest, ubiquitin-dependent protein catabolic process, negative regulation of cell proliferation, induction of apoptosis by intracellular signals, ubiquitin cycle, G1/S transition of mitotic cell cycle, calcium ion transport, signal transduction	Contig 02223_1.f1.exp
Inflammatory response, L-phenylalanine metabolic process, tyrosine metabolic process, signal transduction	Contig 20582_1.exp
Defense response to Gram-positive bacterium, innate immune response, xenobiotic metabolic process, antifungal humoral response, transport	Contig 14128_1.exp
G-protein coupled receptor protein signaling pathway, response to stress	Contig 20595_1.exp

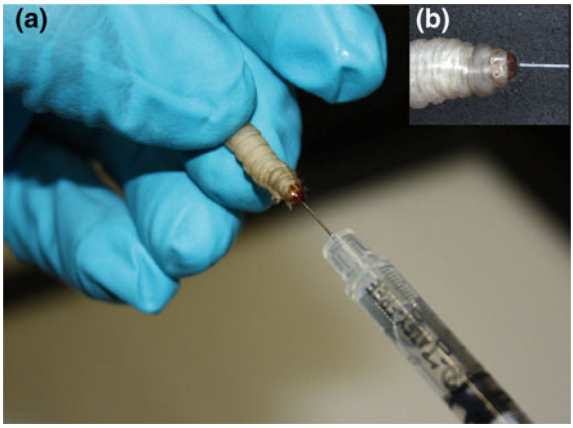

Fig. 2 Force-feeding method used in *G. mellonella* larvae

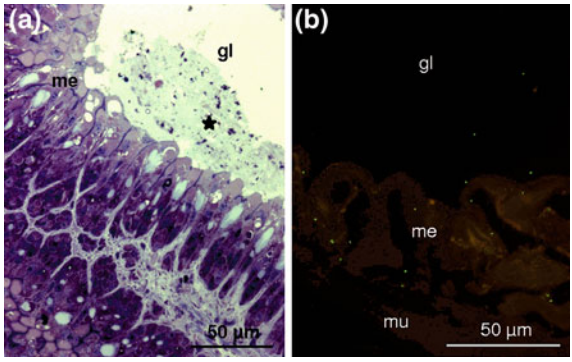

Fig. 3 Midgut epithelium of *G. mellonella*. **a** Bacteria-contaminated diet in the gut lumen (*asterisks*); semi-thin section (1 µm), toluidine *blue* staining. **b** Cryosection (10 µm) of specimens fed with Texas *Red*-labeled bacterial probes in the final larval instar. *gl* gut lumen, *me* midgut epithelium, *mu* muscle

antibiotics. Furthermore, if *G. mellonella* larvae are fed a microbe-contaminated diet, the bacteria survive and colonize the midgut (Fig. 3).

The foodborne pathogen *L. monocytogenes* causes listeriosis, which is responsible for approximately 30 % of food-related deaths in the United States. *L. monocytogenes* in its most severe form can invade the CNS and cause fatal meningitis, but the role of gut microbial dysbiosis in the progression of the disease is unknown. The bacteriocins produced by *L. salivarius* in the human gut antagonize *L. monocytogenes*, suggesting possible therapeutic approaches based on probiotic bacteria against listeriosis [54]. Probiotic treatment was shown to enhance anti-inflammatory responses following infection, particularly those involving interleukin-10. The injection of heat-killed *L. monocytogenes* into *G. mellonella* larvae induced anti-inflammatory responses against live bacteria, suggesting probiotic approaches could be useful to prevent infections. *G. mellonella* is the only insect model that discriminates between *Listeria* species and serotypes based on their similar pathogenic profile in mammals [55, 56]. The *G. mellonella* model shows that the pathogenicity of *L. monocytogenes* in insects is

regulated by the same six genes involved in human infections [55, 56]. Pathogenic *L. monocytogenes* can colonize *G. mellonella* cells, tissues, and organs, and AMPs can potentially inhibit this process. High-throughput sequencing, transcriptomics, and metabolomics in *G. mellonella* will help to identify the factors that control *L. monocytogenes* infection [57].

5 *G. mellonella* as a Model Host to Study Brain Infections by the Human Pathogen *L. monocytogenes*

The blood–brain barrier is an integral part of the CNS that separates circulating blood from the extracellular fluids in the brain, regulating the transfer of nutrients, proteins, and cells. Certain bacterial, fungal, viral, and protozoan pathogens can breach this barrier and cause life-threatening diseases that are difficult to treat. Bacterial meningitis is one of the most lethal infectious diseases, with up to half of survivors left with permanent neurological sequelae [58]. Human pathogens such as *Mycobacterium tuberculosis*, *Chlamydia pneumoniea,* and *Neisseria meningitidis* are the main causes of brain infections, although *L. monocytogenes* has been recognized as one of the leading causes of community-acquired acute bacterial meningitis in adults [59, 60], leading to meningoencephalitis, brain abscesses, and rhombencephalitis [61]. The bacteria may gain access to the CNS either via internalin–cadherin interactions or translocation by phagocytic cells, although conclusive evidence is yet to be provided [62].

Recently, *G. mellonella* has been established as a surrogate model host to study brain infections by *L. monocytogenes* in order to promote its use in preclinical research [9]. *L. monocytogenes* infects hemocytes during the early stages of infection but later infects the brain tissue and the nerve cords. The colonization strategy used to overcome the protective immune response in the brainstem system is not yet understood. *L. monocytogenes* induces *G. mellonella* larvae to produce melanin, which traps the bacteria and stimulates the expression of neuronal repair genes [9]. However, the trapped bacteria find it easy to evade the bactericidal activity of the larval brain and eventually kill the larvae. The comprehensive *G. mellonella* transcriptomic database has been used to identify genes that are likely to play a role in neuronal repair, based on their induction in larvae infected with *L. monocytogenes* at later infection stages. Furthermore, *G. mellonella* has been used to screen potential anti-inflammatory compounds; for example, treatment with diclofenac increased the survival of larvae followed by the complete disappearance of melodized spots from the larval brain. The *G. mellonella* model can therefore be used to test the efficacy of novel therapeutics indicated for bacterial infections of the CNS [9].

6 Conclusions

Insects such as the greater wax moth *G. mellonella* are valuable model hosts that can be used to investigate the virulence mechanisms of human pathogens. The larva of the greater wax moth *G. mellonella* is a favored model that can be used to decipher the complex interactions between the gut microbiota, pathogenic bacteria, and the immune system of the intestinal tract. This model host can also be used to explore mechanisms that allow human pathogens such as *L. monocytogenes* to penetrate the intestinal epithelia and infect the brain. The availability of such whole-animal high-throughput systems provides an opportunity to develop therapeutic strategies and antibiotics against foodborne diseases.

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