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REVIEW

Lepidopteran insects: emerging model organisms to study infection by enteropathogens

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Abstract

The in vivo analysis of a pathogen is a critical step in gaining greater knowledge of pathogen biology and host–pathogen interactions. In the last two decades, there has been a notable rise in the number of studies on developing insects as a model for studying pathogens, which provides various benefits, such as ethical acceptability, relatively short life cycle, and cost-effective care and maintenance relative to routinely used rodent infection models. Furthermore, lepidopteran insects provide many advantages, such as easy handling and tissue extraction due to their large size relative to other invertebrate models, like *Caenorhabditis elegans*. Additionally, insects have an innate immune system that is highly analogous to vertebrates. In the present review, we discuss the components of the insect's larval immune system, which strengthens its usage as an alternative host, and present an updated overview of the research findings involving lepidopteran insects (*Galleria mellonella*, *Manduca sexta*, *Bombyx mori*, and *Helicoverpa armigera*) as infection models to study the virulence by enteropathogens due to the homology between insect and vertebrate gut.

Keywords *Galleria mellonella* · *Helicoverpa armigera* · *Manduca sexta* · *Bombyx mori* · Enteropathogen · Alternative host

Introduction

Even after almost 80 years of the successful use of the first antibiotic (penicillin) against microbes, microbial pathogens still pose a considerable risk to human health. A global rise in resistance to antibiotics and infection-prone aging population (due to chemotherapy, AIDS, indwelling medical devices, and surgical procedures) requires a much better understanding of the infection processes that can be achieved via in vivo modeling (Kemp and Massey 2007). Presently, most research comprising human pathogens uses mammalian hosts (Scully and Bidochka 2006), which have several drawbacks. Apart from the substantial ethical debate over mammalian suffering, they are too costly, and studies utilizing these hosts need a considerable investment of time (Kemp and Massey 2007; Scully and Bidochka 2006; Wang et al. 2013). Thus, there is an urgent requirement for simple, easy-to-handle, rapid, economical, and ethically acceptable in vivo models for assessing the virulence of microbial pathogens (Ahlawat et al. 2021). From the past few years, many researchers have started to utilize insects as model hosts for the study of human pathogens, instead of mammals, because insects are not liable to regulatory control and ethical concerns (Scully and Bidochka 2006). Furthermore, there are insect models that can be easily and economically propagated and produce quick results (Kemp and Massey 2007) (Fig. 1A).

The identification of human pathogens expressing virulence factors is the main focus of disease research (Kemp and Massey 2007). Emerging data suggest that the virulence of many human pathogens is analogous in mammals and insects, and identical virulence factors are utilized by pathogens to infect both the hosts (Wang et al. 2013). Conclusively, both insect and mammalian hosts are susceptible to pathogens and have the same mechanism for the establishment of infection by the pathogens (i.e., adhesion, invasion, systemic spread, and evasion of the immune response). Furthermore, in response to infection, they have developed many mechanisms to safeguard themselves; out of which,

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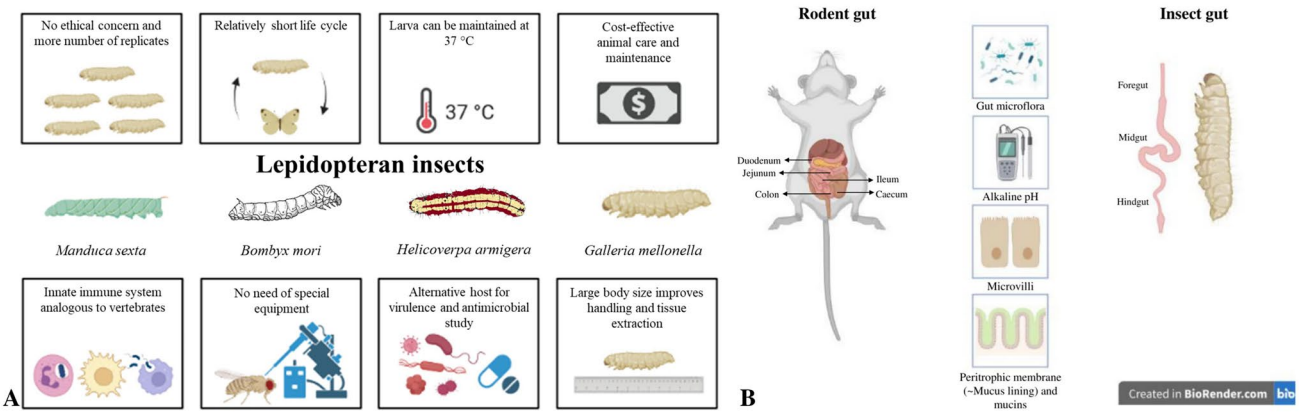

Fig. 1 **A** Various aspects support the use of lepidopteran insects in in vivo experiments: ethical acceptability allows the use of more replicates that increases the statistical support of the study, large size of larva improves handling and extraction of tissues well as it allows direct inoculation of microbes in hemocoel, larva can be reared in the laboratory without the need of special equipment, and they can, to some extent, replace experiments with vertebrates due to similarity in their innate immune system, shorter life cycle (20–40 days), and maintenance at 37 °C. **B** Representation of similarities between the gut of an insect and a rodent. The insect gut carries microorganisms, have alkaline pH, lined by short hair–like projections known as microvilli on the apical side of columnar cells, covered by peritrophic membrane (similar to the mucous lining of vertebrates), and have mucin-like proteins, similar to rodents. These characteristics together allow the use of lepidopteran insects as alternative infection models to study enteropathogens (Image: BioRender.com)

few (as an adaptive immune system) are limited to higher-order metazoans, but others (like physical barriers and innate immune system) are common to both hosts and display high functional homology (Kemp and Massey 2007). These similarities validate the use of insects as model hosts for studying the human pathogens (Scully and Bidochka 2006).

Drosophila melanogaster (Diptera: Drosophilidae) or fruit fly has been used as a model organism by Charles W. Woodworth to study physiology, genetics, and microbial pathogenesis (Wang et al. 2013). He was the first to cultivate *D. melanogaster* in laboratory conditions and suggest that it could be useful in genetic research (Holden 2015). It provides numerous advantages, such as ease of handling and breeding, well-defined published genome, susceptibility to forward and reverse genetics, and commercial availability of genetic tools, such as transgenic cell lines and mutants (Wand et al. 2013). However, it has some disadvantages also, such as small size and hemolymph volume (Kemp and Massey 2007), and needs considerable handling experience and special laboratory equipment (such as microinjectors) (Wand et al. 2013). Furthermore, it cannot be propagated at 37 °C; therefore, over the years, *Galleria mellonella* (Lepidoptera: Pyralidae) or honeycomb moth or greater wax moth has been developed as a major alternative model system to study the microbial infections (Tsai et al. 2016) (Fig. 1A). However, *Manduca sexta* is usually incubated up to 26 °C, and *Bombyx mori* and *Helicoverpa armigera* at 25–28 °C. Thus, the present review discusses lepidopteran insects (*G. mellonella*, *M. sexta*, *B. mori*, and *H. armigera*) as an alternative infection hosts to study infection by the enteropathogens.

Comparison of the immune system of mammalian and insect hosts

The conserved innate immune system in both the hosts, i.e., mammalian and insect, perceives external invaders in the same way and utilizes similar pathways to activate the immune responses (Pereira et al. 2020). In both the hosts, innate immune systems have pattern recognition receptors (PRRs) that identify the pathogen-associated molecular patterns (PAMPs) and elicit suitable immune responses (Scully and Bidochka 2006). For instance, in *D. melanogaster*, two protein families, i.e., Gram-negative binding proteins (GNBPs) and peptidoglycan recognition proteins (PGRPs), activate pathways related to immune response. Binding of PRRs to PAMPs triggers a cascade of serine proteases. Gram-negative bacteria stimulate the Imd pathway in insects that is homologous to the TNF pathway in mammals, whereas Gram-positive bacteria and fungi stimulate the Toll pathway in insects that is homologous to the TLR pathway in mammals (Müller et al. 2008). Therefore, even though insects do not have adaptive immune response, their innate immune response shows notable similarities with innate immune response in vertebrates (Tsai et al. 2016). In insect, the immune responses in the body cavity involve the function of hemocytes and fat body, while in the gut, immune responses are different from immune responses in the body cavity, which are complicated by peritrophic membrane and resident gut microbiota (Wu et al. 2016).

Comparison of systemic immunity

Deprived of adaptive immune components, insects have cellular (cell-mediated) and humoral innate immune components. In cellular response, hemocytes (similar to human phagocytes) in hemolymph (similar to mammalian blood) phagocytose or nodulate the invading microbes or encapsulate the foreign bodies that are too large to be phagocytosed (Müller et al. 2008), while the humoral response includes melanin synthesis and soluble effector molecules, such as complement-like proteins (opsonins) and antimicrobial peptides (AMPs) that are secreted from the fat body (equivalent to the human liver) to immobilize or kill the pathogens (Tsai et al. 2016; Pereira et al. 2020; Ali Mohammadie Kojour et al. 2020). In insects, encapsulation starts with the attachment of a foreign target (larger microbes) to the granular cells that triggers the release of plasmatocyte spreading peptides that results in a smooth capsule due to the attachment of many layers of plasmatocytes around the foreign target (Tsai et al. 2016). Encapsulation is also found in mammals along with granuloma formation that is analogous to nodule formation in invertebrates (Müller et al. 2008).

Cell-mediated innate immunity

A broad variety of hemocytes exist in different species of insects that differ in morphology and function. For instance, *D. melanogaster* has phagocytic and nodule-forming plasmatocytes, encapsulating lamellocytes and granulocytes, and phenoloxidase (PO)-producing crystal cells (Müller et al. 2008), while lepidopteran insects have plasmatocytes and granulocytes that are involved in cellular defense, oenocytoids that produce enzymes of melanization cascade, and spherulocytes whose immune function is still not known (Feng et al. 2021). The concentration of hemocytes varies in response to pathogens (Pereira et al. 2020). On the contrary, in mammals, various cell types, such as phagocytic cells (macrophages, neutrophils, and dendritic cells) and granule-containing cells (basophils, eosinophils, mast cells, and natural killer (NK) cells), support the innate immune response against the microbial invasion. In addition, they have primitive lymphocytes (T cells and B cells). Macrophages have a role in antigen presentation to induce an appropriate adaptive immune system. They produce factors of the innate immune system (chemokines and complement factors), proinflammatory cytokines, and effector molecules, like reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) (Müller et al. 2008). Reactive oxygen species (ROS) production has also been found in hemocytes, specifically superoxide anion species ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2, dismutation product of $O_2^{\bullet-}$) has been reported in plasmatocytes of *G. mellonella* and *D. melanogaster* (Bergin et al. 2005). Furthermore, phagocytic cells in both insects and mammals have similar receptors, such as calreticulin on their surface, and produce extracellular traps (NETs) containing proteins and nucleic acids to immobilize and kill pathogens (Browne et al. 2013). Thus, insect hemocytes work in a similar way as human phagocytic cells (Bergin et al. 2005).

Humoral innate immunity

Both insects and mammals respond to infections by strongly synthesizing AMPs (Müller et al. 2008). For instance, infection of *H. armigera* larvae with *Klebsiella pneumoniae* induced the expression of various AMP-related genes, like *HaAtt* (attacin); *HaCec-1*, *HaCec-2*, and *HaCec-3* (cecropin-1, cecropin-2, and cecropin-3); *HaCob* (cobatoxin-like); *HaGall* (gallerimycin-like); *HaGlo* (gloverin-like); *HaGali* (galiomicin-like); *HaIip* (immune inducible protein); *HaLys* (lysozyme); and *HaMor* (moricin-like) (Wang et al. 2010). Similarly, in mammals, the production of AMPs seems to be induced by Th17 cells, which produce IL-22 that acts on non-immune cells to induce the production of AMPs (Müller et al. 2008).

Similar to PRRs found in mammals, opsonins recognize and bind microbial components, like lipopolysaccharide (LPS) (Pereira et al. 2020). The participation of opsonins, i.e., complement factors and antibodies, in the immunity of vertebrates is well known, where opsonization makes the engulfment of pathogens by phagocytes a much easier task. The phagocytosis of invading microorganisms is carried out by several mammalian cell types, including neutrophils, whereas insect hemolymph contains hemocytes that have a role similar to the neutrophils (Kemp and Massey 2007). As discussed earlier, phagocytes, i.e., hemocytes, in insects and neutrophils in mammals have evolved conserved intracellular killing mechanism involving ROS production (Müller et al. 2008). For instance, as observed in neutrophils, the hemocytes in *G. mellonella* produce $O_2^{\bullet-}$ via the induction of NADPH oxidase (NOX) against the phagocytosed bacteria (Bergin et al. 2005). On the other hand, in insects, opsonins, i.e., complement-like proteins, C-type lectins, and C-reactive proteins, compensate for the absence of antibody-mediated features, improve encapsulation, and activate the pro-phenoloxidase (ProPO) system, thereby synchronizing humoral and cellular effector systems (Müller et al. 2008). According to the conventional views about immunity in insects, hemocyte-stored ProPO are released upon the recognition of foreign bodies (Whitten and Coates 2017). In insects, the coupling of PRRs to target molecules triggers the serine protease cascade, which results in the cleavage of inactive zymogen ProPO to PO that catalyzes the oxidation of phenols to reactive quinones, which polymerize to synthesize melanin around the invading pathogens. However, the activation of PO is tightly regulated by protease inhibitors as its overproduction leads to the production of cell-damaging

ROS (Ahlawat et al. 2020). Thus, melanization is the synthesis and accumulation of melanin to enclose pathogens at the wound site, accompanied by hemolymph coagulation and opsonization (Tsai et al. 2016). Besides innate immunity, melanins are synthesized in insects for many purposes, like clot formation, cuticle sclerotization, and organogenesis. Interestingly, PO activity and melanin production are not limited to hemolymph or cuticle, but recent evidence points towards the role of melanin in gut homeostasis (Whitten and Coates 2017).

Various recent studies have demonstrated the PO activity in the lumen of the gut of insects, like cricket (Joseph 2014), silkworm (Shao et al. 2012), and cotton bollworm (Whitten and Coates 2017), to protect the insect gut microbiota, to protect the insect gut against the microbial overpopulation, and to prevent insect toxic shock-like responses to overgrowth. Interestingly, certain gut bacteria and stress conditions in the gut also trigger the melanization. For instance, oral infection with *Pseudomonas entomophila* in *Drosophila* and occurrence of *Frischella perrara* in European bee species are linked to melanization in larval midgut/foregut junction and specifically located darkened scabs, respectively (Vodovar et al. 2005; Emery et al. 2017). Interestingly, the above studies suggest the regional localization of melanization to the foregut and hindgut. The limited involvement of the midgut is may be due to its suboptimal conditions, like very high pH, which could inhibit the PO cascade, or it may be due to different embryonic origins of the gut regions, as the midgut is derived from endodermal cells and the foregut and hindgut develop from the ectoderm. Furthermore, in most insects, the midgut is lined by a secreted peritrophic matrix, and the foregut and hindgut are lined with a chitinous exoskeleton. Also, there is a need to protect symbionts in the midgut that precludes the excessive PO activity in the region (Whitten and Coates 2017).

Melanogenesis occurs mainly in the midgut epithelium, cuticular structures, or hemolymph with a role in darkening and hardening the cuticle and immune defense, like non-self-recognition and encapsulation of invading pathogens. Thus, dark or melanic morphs of insects possess high concentration of melanin, and there exists a positive correlation among melanism, PO activity, and resistance to microbial pathogens. In a past study, dark (melanic) morphs of *G. mellonella* were studied for their heightened resistance to infection with an entomopathogenic fungus, i.e., *Beauveria bassiana*, where these morphs have thickened cuticle, higher numbers of circulating hemocytes, upregulated cuticular PO activity, higher expression of stress management genes and immunity-related genes, and an increased ability to encapsulate the fungus. In response to fungal infection, the net effect is decreased cuticular fungal penetration, lower propensity to develop hemolymph infections, and increased larval survival times. However, in the absence of infection, heavy defense investments result in lower biomass or lesser size, decreased longevity, and lower fecundity. The presence of melanin in the insect cuticle not only suppresses the growth of certain fungus (by acting like a physical barrier) but also limits the synthesis of cuticle-degrading enzymes, thereby impeding the cuticle penetration by the entomopathogenic fungi (Dubovskiy et al. 2013). On the other hand, to colonize an insect host, the entomopathogenic fungi must first attach to and penetrate the cuticle layers of integument. In a study, cuticle of the melanic morphs of *G. mellonella* was shown to have melanin accumulation, higher L-di-hydroxy-phenylalanine (DOPA) decarboxylase activity, and fewer hydrocarbons that lead to the decreased attachment and germination of conidia of *Metarhizium brunneum* and increased expression of stress-linked genes. The lack of conidia adherence to the cuticle demonstrated the decreased ability of the fungus to overcome the host preformed defenses, which negatively impacted the host mortality (Grizanova et al. 2019). Lastly, according to the study by Tsai and co-workers, melanization begins with black spots on the surface of larval cuticle and larva becomes completely melanized as the infection progresses, which correlates with its death soon after (Tsai et al. 2016; Pereira et al. 2020).

Thus, insects and mammals remove pathogens by both similar and distinct mechanisms. The innate immune system of both hosts shares common features like discrimination between non-self and self, identification of pathogens through PRRs, opsonization of pathogens, uptake of pathogens by phagocytosis, secretion of reactive effector molecules to kill microorganisms, and control of microorganisms via granuloma or nodule formation. But other features, like alternative splicing of PRRs have only evolved in insects, and this may partially balance the lack of a highly specific adaptive immune system among them. Furthermore, insects encapsulate the pathogens that they cannot eliminate, whereas mammals use adaptive immune system when the innate immune system fails. Insects lack immune memory of mammals but may utilize other approaches leading to specific immune priming that allows for stronger phagocytic responses upon reinfection with the same pathogen (Müller et al. 2008).

Comparison of gut immunity

Altogether, anatomical barriers, physiological barriers, and phagocytic barriers are the three main components of the innate immune system in mammals, where anatomical barriers consist of the epidermis that produces a physical barrier and secretions, such as mucus for the clearance of unattached microbes. In insects, the cuticle acts in a protective manner similar to the mammalian epidermis, where cells of insect's reproductive and digestive tract produce protective secretions (Kemp and Massey 2007). Also, glycoconjugate receptors

for microbe-derived toxins are found on the microvillar surfaces of both the insect midgut and the mammalian intestine (Scully and Bidochka 2006). The insect gut can be divided into the foregut, midgut, and hindgut (Marzban et al. 2013), where the midgut with an alkaline pH is the longest part of an insect gut with a role in almost all digestive and absorptive functions (Marzban et al. 2013; Pauchet et al. 2008). It consists of columnar cells, basal regenerative cells, and goblet cells with an apical end of columnar cells lined by short hair–like projections called microvilli as an absorptive lining of an insect gut lumen (Marzban et al. 2013). The epithelial arrangement of the smooth septate junctions and columnar cells is highly analogous to the tight junctions and controls permeability (Emery et al. 2019). Furthermore, the insect digestive tract is covered by an invertebrate-specific structure known as the peritrophic membrane (Zhang and Guo 2011) that is a hollow mesh work of the chitinous fibers cross-linked by proteins (Pauchet et al. 2008). It is crucial for insect survival with a role in numerous physiological functions, including protection from microbial infections (Zhang and Guo 2011), prevention of oxidation of biomolecules, and provides home for toxin binding and digestive enzymes (Campbell et al. 2008). Therefore, it serves as the first line of defense (Zhang and Guo 2011) and is highly similar to the mucous lining of a vertebrate gut (Campbell et al. 2008). Furthermore, the vertebrate mucus layer has been reported to have associated mucins that are large glycoproteins with high proline, serine, and threonine content. Likewise, the peritrophic membrane of an insect has shown to possess a mucin-like protein, i.e., HaIIM86, which is similar to the vertebrate mucin, having threonine and O-glycosylation (Zhang and Guo 2011) (Fig. 1B). Moreover, the complement system, interferon, and lysozyme form the part of physiological barriers in mammals. Similarly, in insects, soluble factors, such as AMPs, are produced (Kemp and Massey 2007).

Furthermore, the composition of the gut microbiota of mouse has been reviewed elsewhere in detail with *Actinobacteria*, *Anaerotruncus*, *Bacteroidetes*, *Candidatus arthromitus*, *Deferribacteres*, *Firmicutes*, *Pseudoflavonifractor*, *Proteobacteria*, *Turicibacter*, *Tenericutes*, *Mucispirillum*, *Verrucomicrobia*, *Lactobacillus*, *Coprobacillus*, *Marvinbryantia*, *Roseburia*, *Bifidobacterium*, *Dialister*, *Alistipes*, and *Faecalibacterium* as the major dominants (Hugenholtz and de Vos 2018; Clavel et al. 2016; Nguyen et al. 2015). Similar to a vertebrate intestine, an insect gut also carries microorganisms. The insect gut is sterile initially, and the microbiota it carries is determined by the food habits of insect (Krishnan et al. 2014). For instance, *B. mori* are predominant in *Arcobacter* and *Bacillus* when fed on mulberry leaves and in *Bacteroides* and *Acinetobacter* when fed on lettuce leaves in a bio-regenerative life support system (Liang et al. 2014). In an earlier study, 11 bacterial isolates, i.e., *Bacillus circulans*, *Aeromonas* sp., *Citrobacter freundii*, *Enterobacter* sp., *Escherichia coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, *P. fluorescens*, *Proteus vulgaris*, *Erwinia* sp., and *Serratia liquefaciens*, were obtained from the gut of mulberry leaf-fed *B. mori* (Anand et al. 2010). However, the gut microbiota of *H. armigera* majorly consists of clostridia, enterococci, and lactobacilli (Tang et al. 2012), and different parts of the *H. armigera* gut have been reported to carry different bacterial and yeast genera/species; for instance, the foregut is dominant in *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus pumilus*, *Proteus myxofaciens*, *Klebsiella* sp., *Saccharomyces kluyveri*, and *Rhodotorula graminis*; the midgut is prevalent in *Bacillus alvei*, *Serratia marcescens*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Staphylococcus* sp., and *Salmonella* sp., whereas the hindgut is pre-dominated by *Bacillus subtilis*, *Bacillus alvei*, *B. pumilus*, *Bacillus firmus*, *B. megaterium*, *P. vulgaris*, *Enterococcus faecalis*, *Pseudomonas stutzeri*, and *E. coli* (Mishra and Tandon 2003). The gut microbiome of *G. mellonella* laboratory lines is mainly composed of Firmicutes, Proteobacteria (Barrionuevo et al. 2022), *Enterococcus* (Polenogova et al. 2019) (*E. gallinarum/saccharolyticus* (Allonsius et al. 2019), *E. faecalis*, and *E. mundtii* (Ignasiak and Maxwell 2018)), *Enterobacter*, *Pseudomonas*, and *Bacillus* (Dubovskiy et al. 2016; Lou et al. 2020). Similarly, the gut bacterial community in *M. sexta* involves various Gram-positive cocci and coryneforms, like *Bacillus*, *Curtobacterium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Pediococcus*, *Kocuria*, and *Staphylococcus* (Van Der Hoeven et al. 2008) (Fig. 2). Thus, overall, Firmicutes, Actinobacteria, and Proteobacteria are the major bacterial communities found in the insect gut (Shinde et al. 2019). Altogether, the insect midgut microbiota differs from species to species, and commensal and symbiotic bacteria provide protection as in some lepidopteran insects, the depletion of midgut microbiota enhances the susceptibility to infection, and increased immune activity enhances the midgut microbial load (Wu et al. 2016). In a previous study, anexic (germ-free) *B. mori* larvae were found to be more susceptible to infection by baculovirus and *Serratia piscatorum* (Rajagopal 2009). Lastly, just like the vertebrate gut microbiota, the gut microbiota of insects has a crucial role in functions, like digestion, metabolism, pesticide degradation, detoxification of plant materials, pheromone production (Shinde et al. 2019), defense against parasites and pathogens, and production of nutrients to supplement poor diet (Wu et al. 2016).

The insect midgut is the initial site of contact with pathogens and is shown to produce various key immunity proteins needed for protection, such as immune-related Hdd 13, cyclophilin A, cyclophilin in *B. mori* (Zhang et al. 2011) and GNBP (Wu et al. 2016; Pauchet et al. 2008). Depending on the bacterial target, i.e., symbiont or pathogenic agent, insects produce various antimicrobial compounds both in the hemocoel and intestinal tract (Müller et al. 2008). To support the first line of immune

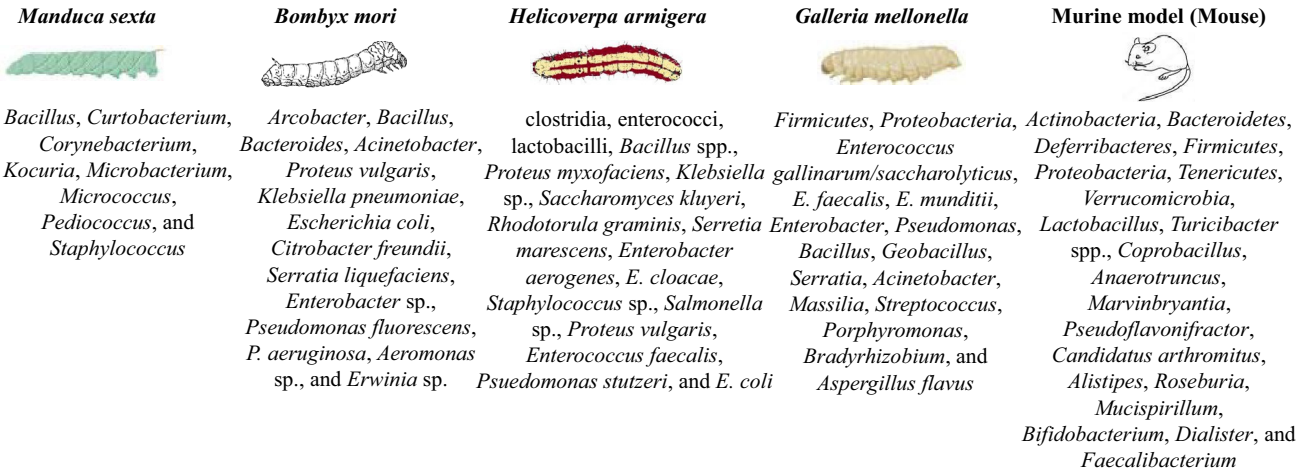

Fig. 2 Comparison between the major gut colonizing microbiota of each of the selected insect models (*Manduca sexta*, *Bombyx mori*, *Helicoverpa armigera*, and *Galleria mellonella*) and a rodent (Image: BioRender.com)

defense, insects use ROS and AMPs to clean the gut pathogens and protect the commensals. Ingested bacteria translocate from the midgut to hemocoel. Thus, immunity responses are not limited to the midgut after oral infection; rather, both hemocoel immunity and metabolism are also altered. Most viruses enter the hemocoel via the gut, while most bacteria invade the hemocoel via wounds. In contrast, fungi penetrate the integument and further infect the hemocoel. In response, lepidopteran insects initiate immunity responses against the pathogenic agents by increasing the expression of important immunity genes (Wu et al. 2016). For instance, when silkworm larvae were fed with food carrying *E. coli* and *Staphylococcus aureus*, transcription of lysozyme, gloverin, and ceropin A genes was upregulated (Wu et al. 2010). Injection of soluble peptidoglycan into *B. mori* larvae increased the transcription of cecropin A and B in the larval midgut (Yamano et al. 1994). Furthermore, increased expression of dual oxidase (DUOX) was observed in *B. mori* larval midguts when the larvae were orally fed with *E. coli* and nucleopolyhedrovirus (NPV). DUOX produces ROS under the control of the p38 pathway (He et al. 2013), whereas BmPrx5 protects the larva against the oxidative stress by degrading the increased levels of H_2O_2 (Zhang and Lu 2015). In *G. mellonella* larvae, the activities of lysozyme, PO, and other antibacterial proteins were induced upon feeding of the pathogenic and non-pathogenic bacteria (Freitak et al. 2014).

Lepidopteran insects as host for enteropathogens

From the past two decades, *G. mellonella* has gathered huge attention as a "model host" among scientific researchers due to an increase in the number of reports utilizing it as an alternative host (Junqueira et al. 2021) to study virulence factors and pathogenesis of numerous human pathogens (Cook and McArthur 2013). In addition, recently, its entire genome was sequenced, further raising the understandings and prospects for the future investigations (Junqueira et al. 2021). Most importantly, in contrast to other invertebrate models, like *Caenorhabditis elegans*, using *G. mellonella*, analysis can be accomplished at 37 °C, i.e., the optimal temperature for most of human pathogens. Furthermore, injection of bacteria into larval hemolymph provides an advantage by allowing the application of a defined dose of bacteria (Bender et al. 2013). *G. mellonella* larvae may be easily and precisely inoculated by force-feeding or by rolling of a layer of spores or via intra-hemocoel injection, and various parameters including mortality, change in microbial load, hemocyte density and/or population composition, movement, formation of pupa, alteration in gene expression, extent of melanization, and variations in proteome may be applied to analyze their response to infections (Piatek et al. 2020). In addition to *G. mellonella*, *B. mori* has an extensive history in the area of host–pathogen interaction. In last few years, silkworms have gained attention as a model for studying the innate immunity, and genome-wide transcriptional responses of silkworm to various pathogens have been examined based on the International Silkworm Genome Consortium (containing 14,623 protein-coding gene dataset and a 22,987 oligo-nucleotide probe microarray) (Cheng et al. 2016). With the establishment of its genomic and protein database, *B. mori* has emerged as a valuable model in the scientific research (Meng et al. 2017).

A recent label-free proteomic study suggested the utilization of *H. armigera* as an in vivo model to study the

enteropathogenic infection by the pathogenic *Yersinia enterocolitica* strain 8081 biovar 1B. On performing proteomics, a two-component system, secretory systems (such as T3SS and T6SS), and putative hemolysin appeared as major pathogenic proteins. In turn, *Yersinia*-added diet-fed insect larvae manifested altered cytoskeleton due to increased melanization and ROS production. Overall, this study suggested that the mechanism of *Y. enterocolitica* infection and host (*H. armigera*) response mimics *Yersinia*-mammalian gut interactions (Ahlawat et al. 2021). T3SS is a central element of virulence of various enteropathogens, like enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Shigella* spp. (Kang et al. 2018), and *Yersinia* spp. (Ahlawat et al. 2021), where T3SS effector proteins manipulate the host defenses and cellular processes, which support the bacteria to colonize, multiply, and cause disease (Kang et al. 2018). Besides, AggR, a transcriptional regulator of enteroaggregative *E. coli* (EAEC), regulates the expression of various putative virulence factors, like dispersin, dispersin translocator Aat, Aai T6SS, and aggregative adherence fimbriae (AAF) (Morin et al. 2013). In a study, *Salmonella* Typhimurium strain NCTC 12023 virulence determinants, i.e., PhoPQ two-component signal transduction system and LPS O-antigen (OAg), were identified as key factors for the colonization of *G. mellonella* (Bender et al. 2013), where LPS composed of lipid A, oligosaccharide core, and OAg is crucial for the virulence of *Salmonella* Typhimurium as a recognized defense against the host complement system, in mammals also (Murray et al. 2005). Besides, *H. armigera* and *Y. enterocolitica* are orally toxic to *M. sexta* (Bresolin et al. 2006). Thus, numerous original studies focusing on the pathogenic mechanisms of different human enteropathogens, like *E. coli*, *Campylobacter jejuni*, *Shigella* sp., *Vibrio* sp., *Salmonella* sp., and *Yersinia* sp., as well as their interaction with the lepidopteran insect's immune system are presented in Table 1.

Another pathogen, *Helicobacter pylori*, colonizes the human digestive tract and causes various conditions from peptic ulcers to gastric carcinomas. In a study, 25 mg/kg niclosamide protected *G. mellonella* larvae by improving the larval survival rates (up to 70%) after 5 days (Piatek et al. 2020). Another study reported the susceptibility of *G. mellonella* larvae to infection by the enterohepatic species of the genus *Helicobacter* (EHH), which are emerging pathogens linked to hepatobiliary and gastrointestinal (GI) diseases in humans (Ochoa et al. 2021). Moreover, in a recent study by Consentino and co-workers (2021), the expression of *B. cereus* genes involved in iron homeostasis, virulence, and oxidative stress in the gut of germ-free *G. mellonella* was analyzed, where *B. cereus* is a Gram-positive opportunistic pathogen involved in intestinal infections and iron is crucial for the growth and virulence of the pathogens during infection. To perform the analyses, a technique, i.e., laser-capture microdissection (LCM), was utilized for specific in situ gene expression analysis of bacteria in *G. mellonella*. The results demonstrated that iron homeostasis has a crucial role in colonization of the *G. mellonella* intestine by *B. cereus* (Consentino et al. 2021). Furthermore, a study by Scalfaro and co-workers evaluated the protective activity of probiotic bacteria against GI bacterial pathogens using *G. mellonella* larvae as an in vivo model. Before challenging with the pathogens (*Listeria monocytogenes*, EPEC, or *Salmonella enterica* Typhimurium), the insect larvae were pre-inoculated with either *Clostridium butyricum* Miyairi 588 or *Lactobacillus rhamnosus* GG. The survival rates and hemocyte density increased in the probiotic pretreated larvae in comparison to control larvae inoculated with pathogenic bacteria only. Overall, the results suggest *G. mellonella* larvae as a potentially useful in vivo model for pre-screening of the candidate probiotic bacteria (Scalfaro et al. 2017). In another study, the treatment of *G. mellonella* larvae with bovine herpes simplex virus-1 (BHSV-1) has stimulated eicosanoid-mediated nodulation response and PO activation. The viral challenge has provoked nodulation in a manner that increased with increasing viral load and incubation time. However, nodulation was severely impaired in a dose-dependent way in larvae that were treated with an eicosanoid biosynthesis inhibitor, i.e., indomethacin, before inducing the viral infection, thereby suggesting that hemocytic nodule formation in *G. mellonella* larvae in response to viral infection and antiviral nodulation reaction is mediated by eicosanoids (Büyükgüzel et al. 2007).

G. mellonella has also been used as a model system to test the efficacy of antifungal, antibiotic (Tsai et al. 2016; Pereira et al. 2020), or phage treatments (Scalfaro et al. 2017; Abbasifar et al. 2014). *K. pneumonia*, among the routinely found pathogens in the nosocomial infections, has developed resistance to the last resource antibiotics. Thus, the multi-drug-resistant (MDR) *K. pneumoniae* producing OXA-48-like or KPC carbapenemases have been perceived as a major health threat globally. In a recent study, the virulence potential of OXA-48(+) and KPC(+) isolates was tested using the *G. mellonella* model. On average, KPC(+) was reported to be more virulent than OXA-48(+). Furthermore, a synthetic polycationic oligomer, i.e., L-OEI-h, exerted significant bactericidal activity; thus, it was suggested as a promising therapeutic approach for treating the MDR *K. pneumoniae* infections (Mil-Homens et al. 2021). Earlier, *G. mellonella* was used as a host to conceptually approximate the *K. pneumoniae*-triggered pneumonia. *G. mellonella* distinguished between the pathogenic and non-pathogenic strains of *Klebsiella*. Virulence factors needed in the mouse model were also indicated in the *G. mellonella* model; in turn, *K. pneumoniae* infection of *G. mellonella* larvae showed some of the known features of the *Klebsiella*-induced pneumonia (Insua et al. 2013).

Table 1 List of studies utilizing lepidopteran insects as alternative hosts for the study of enteropathogens (* ↑ represents increased and ↓ represents decreased)

S. no.	Enteropathogen	Route of administration	Temperature	Virulence factors	Host response	Reference
Galleria mellonella (honeycomb moth or greater wax moth) (Lepidoptera: Pyralidae)						
1	Enteropathogenic *Escherichia coli* (Migula) (EPEC)	Intrahemocoelically	30 °C	Type III secretion system (T3SS)	Melanization, hemolymph coagulation, AMP production, nodulation, and phagocytosis; dose-dependent ↑ mortality, ↓ survival time, delayed pupation, ↓ pupal mass, ↑ pupal duration, and hemocytopenia	(Chen and Keddie 2021)
2	*Salmonella* Typhimurium strain NCTC 12023	Injected into the hindmost left proleg	37 °C	PhoPQ two-component signal transduction system and lipopolysaccharide (LPS) O-antigen (OAg)	Dose-dependent melanin production	(Bender et al. 2013)
3	*Campylobacter jejuni* strain 11168H, *C. jejuni* strain G1, and *C. jejuni* strain 81–176	Injected in the right foreleg	37 °C	O-Methyl phosphoramidate (MeOPN) capsule modification	Dose-dependent killing of larvae	(Champion et al. 2010)
4	Enterohemorrhagic *E. coli* (EHEC)	Injected into the larval hemolymph via 4th posterior proleg	37 °C	T3SS (*tir* and *espA*), *E. coli* common pilus (*ecpA*), and motility (*fliC*) genes; TolA protein	Despite ↑ *espA*, *tir*, and *ecpA*, *tolA*-deleted EHEC strains show ↓ virulence and adherence to cultured HT-29 colonic epithelial cells	(Morgan et al. 2014)
5	EHEC	Injected into the larval hemolymph via last left proleg	37 °C	Sensor histidine kinase CpxA and lon protease	*cpxA* mutant ↓ larvae killing; *cpxA lon* mutant unable to kill larvae, suggests T3SS-independent virulence in *G. mellonella*	(De la Cruz et al. 2016)
6	Enteroaggregative *E. coli* (EAEC)	Injected into the hemocoel	37 °C	AggR regulon	Typical (having AggR regulon) and atypical strains have the same virulence; EAEC 042 *aggR* mutant strain show ↓ mortality	(Guerrieri et al. 2019)
7	*Enterobacter cloacae* ATCC 13047, *Salmonella* Typhimurium ATCC 14028, and *Enterobacter bugandensis* (EB-247)	Injected	37 °C	–	EB-247 led to rapid larval death within 6 h compared to 9 h for *E. cloacae* and 5 h for *S.* Typhimurium	(Pati et al. 2018)
8	*Campylobacter jejuni*	Injected into the hemocoel via the right foreleg	37 °C	–	Bacteria in hemocoel and gut, bacteria caused midgut damage, and pigmented nodules in infected larvae; isolates belonging to ST257 were most virulent and those belonging to ST21 were least virulent in *G. mellonella*	(Senior et al. 2011)
9	*Vibrio parahaemolyticus*	Injected into the right foremost proleg	37 °C	*mutT* encoded nudix hydrolase	*mutT* mutant unable to kill larvae	(Wagley et al. 2018)

Table 1 (continued)

S. no.	Enteropathogen	Route of administration	Temperature	Virulence factors	Host response	Reference
Galleria mellonella (honeycomb moth or greater wax moth) (Lepidoptera: Pyralidae)						
10	*Shigella* spp.	Injected into the hemocoel via the last left proleg	30 °C, 37 °C	IpaB (T3SS effector protein)	Dose, temperature, growth phase, and *Shigella* virulence plasmid-dependent killing of larvae; ↓ mortality in IpaB mutants infected larvae	(Barnoy et al. 2017)
11	EAEC	Injected via the last right proleg	22 °C, 37 °C	AggR, aii island encoded type VI secretion system (T6SS), dispersin, and dispersin transporter	Dose, time, and temperature-dependent killing of larvae, which can differentiate between virulent and non-pathogenic strains	(Jønsson et al. 2017)
12	EPEC	Injected into the hemolymph via the hindmost left proleg	30 °C	T3SS	Bacterial growth and virulence factors are necessary for killing of larvae in a dose-dependent manner	(Leuko and Raivio 2012)
13	*Salmonella* Dublin strains, *Salmonella* Typhi ATCC 19430, *Salmonella* Typhimurium ST313, and *S.* Typhimurium ST19	Injected into the hemocoel via the last right proleg	37 °C	–	Differential killing of larvae	(Vilela et al. 2020)
14	*Yersinia pseudotuberculosis*	Injected into the right foremost leg	37 °C	Superoxide dismutase C (SodC)	↓ larvae killing by *Y. pseudotuberculosis* Δ*sodC*; virulence was restored in complemented *sodC* mutant	(Champion et al. 2009)
15	Symbiotic *Bacteroides vulgatus* and pathobiotic *E. coli*	Oral force-feeding	37 °C	–	Strong immune responses in pathobiont-fed larvae by production of MAMP-binding recognition proteins, induction of ROS and RNS, and secretion of AMPs	(Lange et al. 2018)
16	*Yersinia enterocolitica* strains	Injected	25 °C, 37 °C	Flagella (*flbB*)	*Y. enterocolitica* strain 5303 belonging to phylogroup 1 was most virulent in *G. mellonella*; *flbB* mutant has ↓ virulence that was restored upon complementation with the *flbB* gene	(Alenizi et al. 2016)
17	*Y. enterocolitica*, *Yersinia pestis*, and *Y. pseudotuberculosis*	Injected via the left hindmost proleg	21 °C, 37 °C	–	*Y. enterocolitica* had ↑ virulence than *Y. pestis* and *Y. pseudotuberculosis*	(Erickson et al. 2011)
18	*Salmonella*	Injected in the right foreleg	37 °C	–	Dose-dependent larval killing; severe damage to larval gut; bacterial replication in hemolymph and vacuoles containing bacteria in hemocytes	(Card et al. 2016)

Table 1 (continued)

S. no.	Enteropathogen	Route of administration	Temperature	Virulence factors	Host response	Reference
Galleria mellonella (honeycomb moth or greater wax moth) (Lepidoptera: Pyralidae)						
19	Colostrum and/or *Cordyceps sinensis* before receiving indomethacin or *Campylobacter jejuni*	Force-feeding and injected	30 °C	–	Nutraceutical blend had positive impacts on larval health (weight gain, delayed pupation, and reduced permeability); *C. sinensis* was superior against *C. jejuni*	(Emery et al. 2021b)
20	*Y. enterocolitica* strain W22703, *Y. enterocolitica* strain W22703-*tcaA::Tn5lux*, *Y. enterocolitica* strain 2594 and *Y. enterocolitica* strain 4466	Oral uptake	22 °C	*tc* genes (*tc*-PAI^{Ye})	Highest killing (83%) in *tc*-PAI^{Ye} harboring *Y. enterocolitica* strain 2594	(Fuchs et al. 2008)
21	*Salmonella enterica* subspecies *enterica* serovar *Enteritidis* phage type 4578 (*Salmonella* PT4)	Injected in the right proleg	37 °C	*rpoS*, *arcA*, *arcB*, and *invA*	Virulence genes are under the regulation of quorum sensing system; *Salmonella* PT4 growing in presence of N-dodecanoyl-homoserine lactone (C12-HSL) showed increased expression of virulence genes and promoted larval mortality	(de Freitas et al. 2021)
22	*S. typhimurium* strain 7 Suc LL and LT2	Injection and per os or via mouth	28 °C	–	Mortality; ↑ number of bacteria; ↓ total hemocyte count; impairment of phagocytosis	(Kurstak and Vega 1968)
23	*Y. pseudotuberculosis* YP126, isogenic MAM deletion strain of YP126 i.e., ΔMAM, and ΔMAM + MAM complemented strain	Injected in the last right proleg	37 °C	Multivalent adhesion molecule (MAM)	MAM is needed for bacterial binding and uptake by host phagocytes. Loss of MAM and ↓ bacteria-phagocyte interaction, ↑ bacterial pathogenesis and bacterial clearance. Phagocyte unbound bacteria hyper-activated melanin synthesis, thereby ↑ phagocyte death and host mortality	(Krachler et al. 2021)
24	Okadaic acid (80–400 µg/kg) and *Escherichia coli* K12	Injected in the last left (ventral) proleg or force-fed (gavage)	30 °C	–	↓ larval survival to 47% in comparison to microbial challenge (73%) or toxin (90%) alone, tissue disruption (loss of organ architecture, gross epithelial displacement into lumen, and nuclear aberrations associated with cell death), shift in resident microbiome population	(Emery et al. 2021a)

Table 1 (continued)

Manduca sexta (tobacco hornworm) (Lepidoptera: Sphingidae)						
1	*Y. enterocolitica* strain W22703, *Y. enterocolitica* strain W22703-*tcaA::Tn5lux*, *Y. enterocolitica* strain 2594 and *Y. enterocolitica* strain 4466	Subcutaneous infection	15 °C	–	Similar lethality of *Y. enterocolitica* strain W22703 and *Y. enterocolitica* strain W22703-*tcaA::Tn5lux*	(Fuchs et al. 2008)
2	*Y. enterocolitica* strain W22703 and mutant W22703Δ*tca*A	Added in artificial diet	25 °C	*tc*-PAI^{Ye}	Whole-cell extracts of *Y. enterocolitica* strain W22703 cultivated at 10 °C, but not at 30 °C, led to insect mortality when fed to *M. sexta* larvae, in contrast to an insertional tcaA mutant	(Bresolin et al. 2006)
3	Heat-killed *E. coli* strain XL1-blue	Injected	25 °C	*M. sexta* Toll (MsToll) gene	MsToll is induced in insect hemocytes by *E. coli* with a role in innate immunity against *E. coli* infection	(Ao et al. 2008)
Bombyx mori (silk worm) (Lepidoptera: Bombycidae)						
1	Non-pathogenic *E. coli*	Orally (added in artificial diet)	25 °C	–	↑ *JHBP6* and *7*, trypsin family genes, tubulin genes; ↓ muscle-related genes; modulated Imd and Toll signaling pathways	(Cheng et al. 2016)
2	*E. coli*	Injected	–	–	Imd pathway activated in response to infection	(Liu et al. 2015)
3	*Vibrio cholera* (p1418, Ogawa form), *E. coli* (NIHJ), and *E. coli* (W3110, K12-3)	Injected into the first midleg	Room temperature	–	90% larvae died within 2 days when injected with *V. cholera*; no significant difference in mortality with control larvae when injected with *E. coli*	(Kaito et al. 2002)

Table 1 (continued)

Helicoverpa armigera (cotton bollworm) (Lepidoptera: Noctuidae)						
1	*Y. enterocolitica* strain 8081 (1B/O: 8)	Orally (added in artificial diet)	27 ± 1 °C	Secretory systems (T3SS, T6SS), two-component system, and hemolysin	↑ melanin synthesis, iron uptake proteins and anti-pathogenic response proteins of lipocalin family; ↓ body weight and ↑ oxidative stress	(Ahlawat et al. 2021)
2	*Enterobacter cloacae* strain (strain no. 1.2022)	Injected	28 ± 1 °C	–	Immunity-related genes were activated in bacterium-challenged larvae (↑ expression of recognition and modulator genes in fat body and signal pathway genes in hemocytes)	(Xiong et al. 2015)

In addition to the use of *G. mellonella* as "mini-hosts" for the study of microbial pathogenicity and virulence factors, they are also used for the screening of xenobiotics or toxins. In a study, it was used to determine the relative toxicity of 0.50–7.5 µg/larvae or 2–30 mg/kg indomethacin (a non-steroidal anti-inflammatory drug (NSAID)) in insect larvae via two inoculation methods (gavage or force-feeding and intrahemocoelic injection). Upon indomethacin treatment, tissue damage (such as epithelial sloughing and cell necrosis) and raised gut leakiness were observed. The degeneration of the midgut was followed by a significant rise in the detoxification-linked activities (like glutathione-S-transferase and superoxide dismutase (SOD)), thereby showing the vast symptoms of the gastric damage analogous to their vertebrate counterparts (Emery et al. 2019). In a study by Coates and co-workers (2019), *G. mellonella* larvae injected with okadaic acid (a polyether toxin that causes diarrheic shellfish poisoning) at a concentration of ≥ 75 ng/larva or ≥ 242 µg/kg were monitored to evaluate the potential adverse effects of the okadaic acid. Upon treatment, larvae showed broad symptoms of immune cytotoxicity and oxidative damage, such as decreased larval survival (> 65%) and circulating hemocytes (> 50%), reduced hemocyte viability and increased PO activity in hemolymph, and increased malondialdehyde level and SOD activity in the midgut. Interestingly, little difference was seen in lethality between either route of administration, i.e., injection and force-feeding in contrast to threefold more requirement of okadaic acid to induce lethality in rodents when force-fed, compared to intraperitoneal injection (Coates et al. 2019). Another study performed to evaluate the effect of okadaic acid (80 µg/kg) intoxication on *E. coli*-led infection in *G. mellonella* larvae showed reduction in larval survival levels (to 47%) in a dose-dependent manner in comparison to bacterial (73%) or toxin (90%) challenge alone, displayed tissue disruptions, like nuclear aberrations linked with cell death, gross epithelial displacement into lumen, and loss of organ architecture, and represented a shift in resident bacterial population with decline in richness (Chao-1) and diversity (Shannon) indices. Thus, okadaic acid–induced disintegration of insect alimentary canal mimics the changes caused by okadaic acid in the human GI tract (Emery et al. 2021a).

Moreover, an entomopathogenic bacterium, i.e., *Bacillus thuringiensis* (Bt), produces a variety of insecticidal proteins, including Cry toxins that have been used as insecticides. After ingestion of toxins, they get activated by cleavage of N and C termini by midgut proteases. Thereafter, they pass through the peritrophic membrane, and according to a pore-formation model, mature toxins interact with cadherin-like receptors on the columnar cells, which results in the formation of oligomers that bind to aminopeptidase N and get inserted into the membrane. Upon insertion, pores are created in the cells, which causes cell death. However, it has

been reported that Bt resistance is due to reduction in active mature toxins because of mutant midgut proteases, increase in attachment of toxins on the peritrophic membrane due to reduction in permeability of mutant peritrophic membrane, or lowered toxins binding to epithelial cells due to mutant receptors on these cells (Mitsuhashi and Miyamoto 2020). In view to this, an earlier work on resistant and susceptible lines of *G. mellonella* suggests that the resistant line exploits multi-factorial adaptations for resistance to Bt, like the occurrence of the more intact midgut in the resistant line. Furthermore, the resistant line secretes antimicrobial factors, which mitigate Bt activity and affect the survival of other resident gut bacteria (Dubovskiy et al. 2016). Furthermore, in an earlier transcriptomic study, RNA sequence expression profiling was done to determine the host (i.e., *B. mori* larvae) response to hemocoel injection of Bt. Among differentially expressed genes (DEGs), genes involved in insecticide resistance or detoxification, pattern recognition, immune melanization, AMPs, cytoskeleton reorganization, and other immune effectors were identified (Wu and Yi 2018).

Besides the success of *G. mellonella* larva as an alternative host, its propagation conditions and diet vary between groups with a critical need for implementation of the standardization procedures, such as availability of reference population and propagation and maintenance of strains under controlled and standardized environmental and experimental conditions (Cook and McArthur 2013). Additionally, several studies found that *B. mori* was highly sensitive to human pathogens, antibiotics, and pesticides; thus, their use as a model organism for studying the human tumor and metabolic and degenerative diseases has become a research focus (Meng et al. 2017). In a recent study, recombinant laccase (from *Y. enterocolitica*) feeding to larva of *H. armigera* induced the significant damage in the midgut and decrease in body weight, thereby suggesting the use of *H. armigera* larva to study the effect of microbial metabolites on the host anatomy, physiology, and survival (Ahlawat et al. 2020). Altogether, due to a great level of similarity between physical, physiological, and functional structure of the gut and innate immune system of insects and mammals, insects can be utilized as an alternative host for the enteric pathogens.

Conclusion

In conclusion, the great similarity of the innate immune system between lepidopteran insects and vertebrates, and relatively short life cycle, easy handling, no ethical constraint, cost-effective maintenance, large body size, and no need for special equipment and hands-on training for insects, makes them an ideal alternative host for the study of virulence by enteropathogens. Furthermore, it also minimizes the mammalian suffering and produces fast results. Thus, this review highlighted the usage of the lepidopteran insects (*G. mellonella*, *M. sexta*, *H. armigera*, and *B. mori*) as the alternative hosts for the study of infection by many enteropathogens that include *E. coli*, *Shigella* sp., *Salmonella* sp., *Yersinia* sp., *C. jejuni*, and *Vibrio* sp. However, there is an urgent need for further standardization of environmental and experimental conditions for maintenance and propagation of insect population to ensure experimental comparability and reproducibility, globally. In addition to it, more knowledge about the innate immune system and genetic background of the insects is needed for their success as alternate infection hosts.

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Declarations

Conflict of interest The authors declare no competing interests.

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