Trends in Microbiology

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Review

Axenic and gnotobiotic insect technologies in research on host–microbiota interactions

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Insects are one of the most important animal life forms on earth. Symbiotic microbes are closely related to the growth and development of the host insects and can affect pathogen transmission. For decades, various axenic insect-rearing systems have been developed, allowing further manipulation of symbiotic microbiota composition. Here we review the historical development of axenic rearing systems and the latest progress in using axenic and gnotobiotic approaches to study insect–microbe interactions. We also discuss the challenges of these emerging technologies, possible solutions to address these challenges, and future research directions that can contribute to a more comprehensive understanding of insect–microbe interactions.

Highlights

Comparable analyses between axenic or gnotobiotic and conventionally reared insects represent a powerful approach to reveal the interplay between the commensal microbiomes and their host insects.

Successful axenic rearing of model and nonmodel insects has helped uncover the microbiome's profound effects on many insect traits, including behavior, development, immune responses, and resistance to xenobiotics.

The classic laboratory model organism *Drosophila melanogaster* was the first invertebrate to be successfully bred axenically. Similar axenic rearing approaches have since been extended to other medically or agriculturally important insects, such as the honey bee, wasp, mosquito, and silkworm.

Establishment of axenic or gnotobiotic insect systems relies on two key aspects: effective sterilization methods and the provision of a sterile diet with adequate nutrition to support insects for generations.

As more insect symbionts are being successfully cultured, the opportunities to study the interaction mechanisms between pathogens, symbionts, and host insects through gnotobiotic approaches will continue to expand.

Insect–microbe multipartner interactions

Over 480 million years of evolutionary history, insects have formed highly diverse symbiotic relationships with microbes (Figure 1), including bacteria, fungi, and viruses [1]. Generally, symbiotic microbes can be classified as **obligate symbionts** (see Glossary) or **facultative symbionts** based on the host's dependency on them for survival [1–3]. Symbionts can exert a powerful influence on host physiology, such as promoting host growth and development, synthesizing essential nutrients, and defending against pathogens. These symbiotic interactions help the host insects to better adapt to their environment.

Many symbionts provide specialized nutritional services to the hosts. These include syntheses of essential amino acids, vitamins, and other nutrients unavailable from the diet and unable to be synthesized by the insect host [4,5]. For example, the obligate symbionts *Buchnera aphidicola* and *Wigglesworthia glossinidia* can synthesize vitamins as supplementary nutrition for their insect hosts, pea aphids and tsetse flies, respectively [6,7]. Apart from directly synthesizing nutrients, symbionts can also break down substances that are indigestible to insects. Examples include the gut microbiota in wood-feeding termites that can efficiently degrade lignocellulose [8] and the gut bacteria (*Gilliamella* spp.) of social bees that digest pollen by secreting pectin-degrading enzymes [9].

Symbionts can also manipulate the behavior of the host. For instance, ***Wolbachia pipientis*** may influence the speciation of *Drosophila paulistorum* by manipulating mating behavior [10]. The gut microbiota in social insects, such as ants, can affect **nestmate recognition** and induce social aggression [11]. Microbes have been found to play a role in the aggregation behavior of insects [12,13], and microbial-based attractants can be used to attract pests for pest management [14]. An improved understanding of symbionts' impact on host biology could lead to potential technological applications. Furthermore, the artificial introduction of *W. pipientis* can activate the host immune system and reduce the **vector competence** of mosquitoes by preventing dengue virus replication in mosquitoes [15,16]. Insecticide-degrading *Burkholderia* strains can establish symbiotic relationships with the bean bug *Riptortus pedestris* and modulate host

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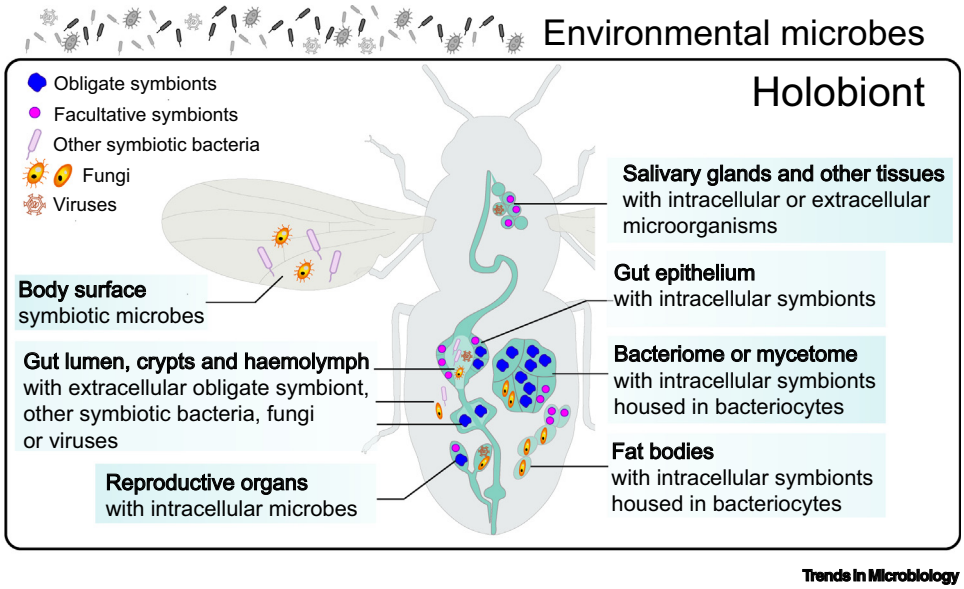

Figure 1. Insect tissues as habitats for symbiotic microbes. The silhouette is a representative image that includes organs from different species of insects and is therefore not anatomically accurate for any given arthropod species, and neither is it to scale to enable visualization of all organs.

detoxification metabolism, suggesting microbes as targets to manage insecticide resistance [17]. Considering experimental tractability, insects are excellent models for studying host–microbe interactions. But challenges still exist in attributing causative effects to specific symbionts from a diverse community of host-associated microbes. Therefore, **axenic rearing approaches** and **gnotobiotic rearing approaches** allow deconstructing and reconstructing insect–microbe multipartner interactions to identify the functions of specific symbionts to the hosts.

About axenic insects

A brief history of axenic insect rearing

At the end of the 19th century, Pasteur proposed that the elimination of intestinal bacteria would lead to the death of vertebrates [18], and to test this assertion, a series of axenic feeding experiments on vertebrates were carried out. Contrary to Pasteur's conjecture, the feasibility of aseptic techniques was confirmed when a variety of axenic vertebrates were successfully raised after refining the experimental methods. The emergence of aseptic organisms filled a major gap in the field of host–microbe interactions. At the same time, a large number of axenic invertebrates, especially **axenic insects**, were successively cultured. To distinguish from sterile insects (non-organic life forms free from living organisms) or germ-free insects (organic life not carrying microbes, especially pathogens), the term axenic insects (organic life that is free from all other demonstrable organisms) was introduced by Baker and colleagues in 1942 [19]. After that, Reyniers *et al*. coined the term **gnotobiotics** to describe the established association of axenic organisms with other fully known microbe species only [20].

The development of axenic insect-rearing technologies

The rearing of axenic insects has been attempted since the beginning of the 20th century, tracing back to the bluebottle fly *Calliphora vonzitoria* grown axenically in 1908. However, success is ambiguous due to the lack of detailed and credible experimental reports [21]. In the following 50 years, significant progress was made in the axenic rearing of various insects that can be

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developed into adults and can be subcultured [22,23] (Figure 2). Considering the presence of vertically inherited microorganisms on the egg surface, various chemical reagents (e.g., Zephiran, Lysol, etc.) were used to eliminate egg-surface microorganisms [24]. From the beginning, several chemicals were applied together, and later researchers started to select the one that could achieve the best sterilization effect in the shortest time. Antibiotics have also been used to inhibit the growth of microbes, but experimental results suggested that the use of antibiotics may interfere with insect growth and reproduction. Therefore, antibiotics should only be used as an adjunct to, but not a substitute for, axenic techniques [24,25]. Using chemically defined media, scientists can determine the nutritional requirements of insects by adding or removing specific nutrients, which also facilitated the development of aseptic rearing techniques. Chemically defined media could be used to provide the necessary nutrients for the cultivation of insects from eggs to adults while providing an axenic and specific environment for survival [26]. Among these reports, the successful axenic rearing of *Drosophila melanogaster* was first proposed in 1946 by Schultz *et al*. [27], an important milestone in this field. However, Schultz *et al*. overlooked that newly hatched *Drosophila* larvae generally obtained bacteria by consuming the chorion of the eggs naturally seeded with bacteria by the mothers, as confirmed by methyl blue dye. Marion Bakula refined the axenic technology in 1969, using bleach to obtain **dechorionated embryos** and obtained axenic flies [28]. This approach of dechorionation remains widely adopted today. However, it can be difficult to establish recipes of all the nutrients required for insect development. Therefore, semidefined substrates using natural food ingredients, such as yeasts and liver powder [29], have also been used to raise axenic insects. Over the past decades, many axenic insect models have emerged and facilitated discoveries of insect–microbe interactions.

Technologies for rearing axenic and gnotobiotic insects

Generally, the successful establishment of an axenic insect system relies on three key aspects: rearing of the insects for many generations under sterile laboratory conditions or outdoor controlled conditions, effective sterilization methods, and diets with adequate nutrition [30]. To date, several insects have been reared axenically for research, as highlighted in Figure 3.

Choosing the sterilization methods

The availability of commercialized and standardized sterilization reagents has greatly increased the possibility of obtaining axenic larvae or pupae. Understanding the dynamics of microbiota during the insect's life cycle helps to develop optimal axenic culture methods. Many insects are oviparous and symbionts' transmission is mediated by the material deposition of microbiota on the egg surface ('egg smearing'). Therefore, the successful rearing of axenic insects often depends largely on the effective removal of the egg-surface microbiota. Ethanol and bleach solutions are commonly used for eliminating live microbes on egg surfaces [31] (Figure 3). The concentration of chemical reagents and application time will affect the hatching rate of eggs and also the effectiveness of egg sterilization.

In the case of honey bees, the process of obtaining axenic individuals started at a later life stage [32]. During the pupal stage, bees go through a 'molting' phase when the lining of the gut sheds; this eliminates all the bacteria in the midgut. Therefore, researchers could obtain axenic adult bees by cleansing the pupae and placing them in a sterile rearing environment [33,34].

Making nutritionally adequate sterile food

After generating axenic embryos, the next critical step is to provide sterile food with sufficient nutrients. Natural food or chemical ingredients are mixed in certain proportions and sterilized by autoclaving, irradiation, or filtration [35–37]. Sterile syringe filter and disposable vacuum filtration systems are common tools used to sterilize food. Certain insect-rearing diets also take into

Glossary

Axenic insects: insects that are completely free from any microbes.
Axenic rearing approaches: a method for removing all microbes from special organisms.
Dechorionated embryos: the embryo generated by removing the chorion on the surface of insect eggs without affecting the normal development of the embryos.
Facultative symbionts: symbionts that are generally not essential for host survival, but can exert effects on the host – such as protection against enemies or stress – and can manipulate reproductive systems.
Genome-wide association studies (GWAS): an approach that is used to detect genetic variations of many individuals in a population and associated variants with a phenotype of interest.
Gnotobiotic rearing approaches: describe the artificial introduction of one or more given components.
Gnotobiotics: describes the association of axenic organisms with one or more known species.
Innate immune deficiency (IMD): an important signaling pathway that regulates innate immunity in *Drosophila*; it controls the expression effector antimicrobial peptides.
KDM5 gene family: genes that encode the histone demethylases involved in the epigenetic regulation of genes.
Nestmate recognition: the process whereby social insects recognize whether individuals belong to their own colony to maintain colony-level integrity.
Obligate symbionts: symbionts that are essential for host survival.
Vector competence: the ability of vectors to transmit the pathogen.
***Wolbachia pipientis*:** a widespread intracellular endosymbiotic bacterium in nematodes and arthropods which has a significant impact on several physiological characteristics of the host, especially the reproductive system.

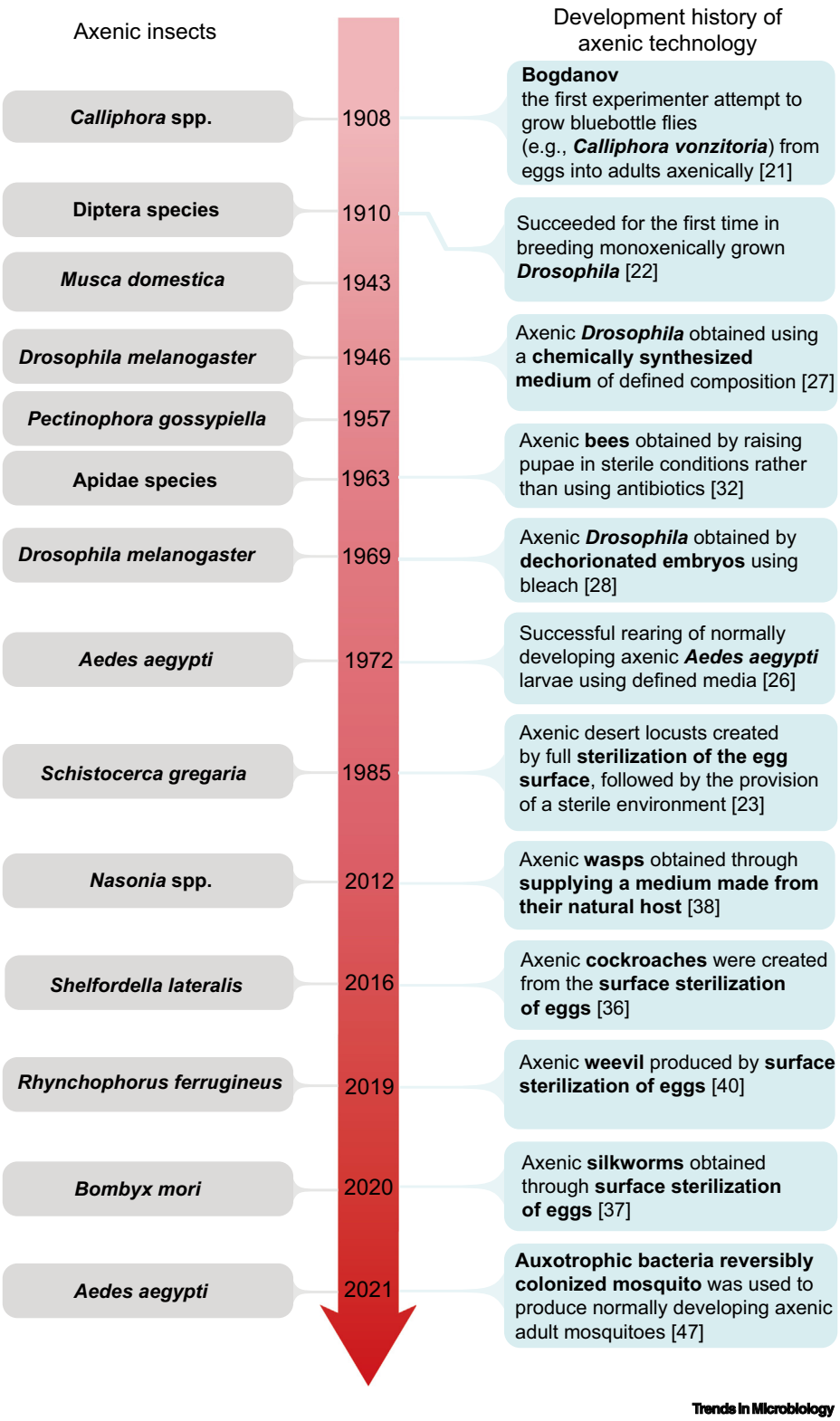

Figure 2. Milestones for axenic techniques of insects and discoveries based on this method.

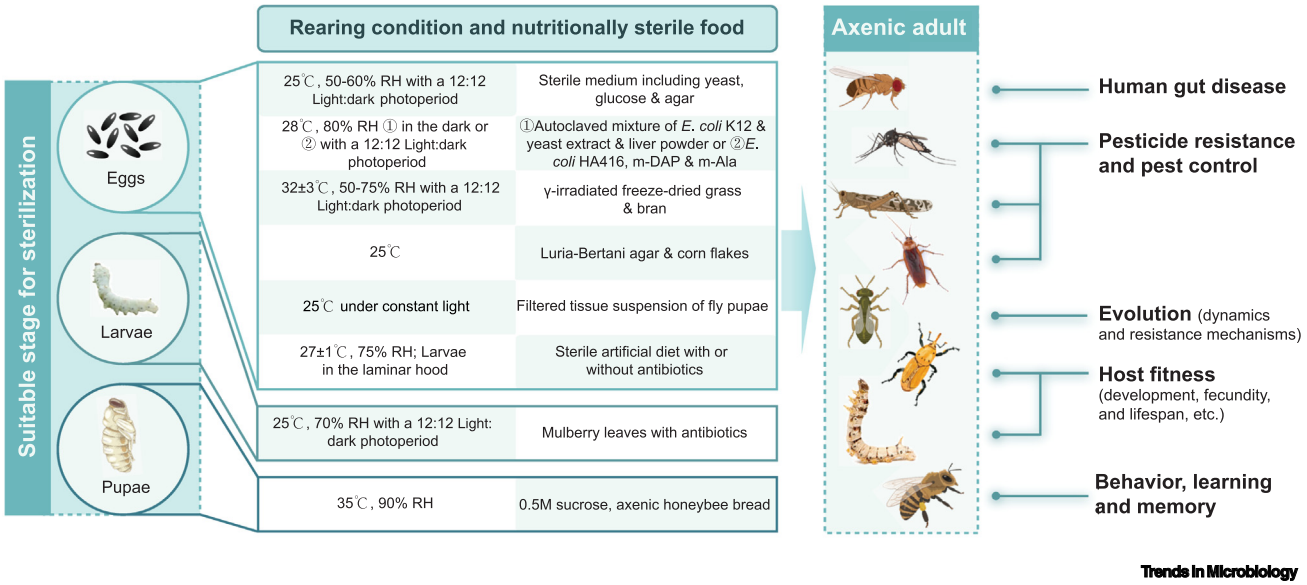

Figure 3. Comparison of key steps in the rearing of important axenic insects and their applications. The rearing of axenic insects often starts from a special period when axenic individuals are easily obtained. Then, providing them with a sterile environment and essential nutrients enables them to grow and develop. Axenic strains, *Drosophila*, mosquito, locust, cockroach, and red palm weevil, are obtained from sterilized eggs. Antibiotic-treated 2nd larvae are often used to rear axenic silkworms. And for honey bees and wasps, pupae are fed sterile food and grow into axenic adults. RH refers to relative humidity. Figure created with BioRender.

account the parasitic characteristics of insects by supplying a sterile medium consisting of their natural hosts, which can be broadly applied to the axenic rearing of insect parasitoids [38,39].

Given that the mechanism of microbial action on insect development was unclear, and in the absence of the ability to determine the complete nutrient composition required by insects, the gnotobiotic insect-breeding technology has developed (Figure 4). Colonization with microbes can improve the development of axenic larvae [40], and even help axenic larvae successfully develop into adults [31]. Usually, the target microbes were added to the larval growth environment, such as food, or injected directly into the insect. Axenic larvae can be exposed to a single bacterial colony [41] or combinations of several microbes such as core microbiota originally present in the host or less compatible bacteria [42,43]. In addition, methods have been creatively expanded to transplant the whole microbiota from donor mosquitoes into axenic larvae [44].

In most cases, the food needs to be supplemented with other nutrients after sterilization to support the development of axenic insects. The current challenge is how to find the most suitable nutrients to make sterile food, which can be a time-consuming and labor-intensive process. Considering the potential role of microbially produced metabolites in insect development, making sterile food from large amounts of autoclaved bacteria is an innovative approach [45]. This led to the discovery that microbes do provide specialized metabolites that need to be kept away from light to prevent degradation [46]. But it is worth thinking about how to choose suitable bacteria, and whether the complex composition of metabolites produced by dead bacteria will be harmful to the insect.

Conversely, the transient colonization technique is a fine-tuned gnotobiotic technique that aims at the stage-specific colonization of auxotrophic live bacteria to produce the final axenic adults while meeting the nutritional requirements of the insects [47]. This method greatly promotes the survival

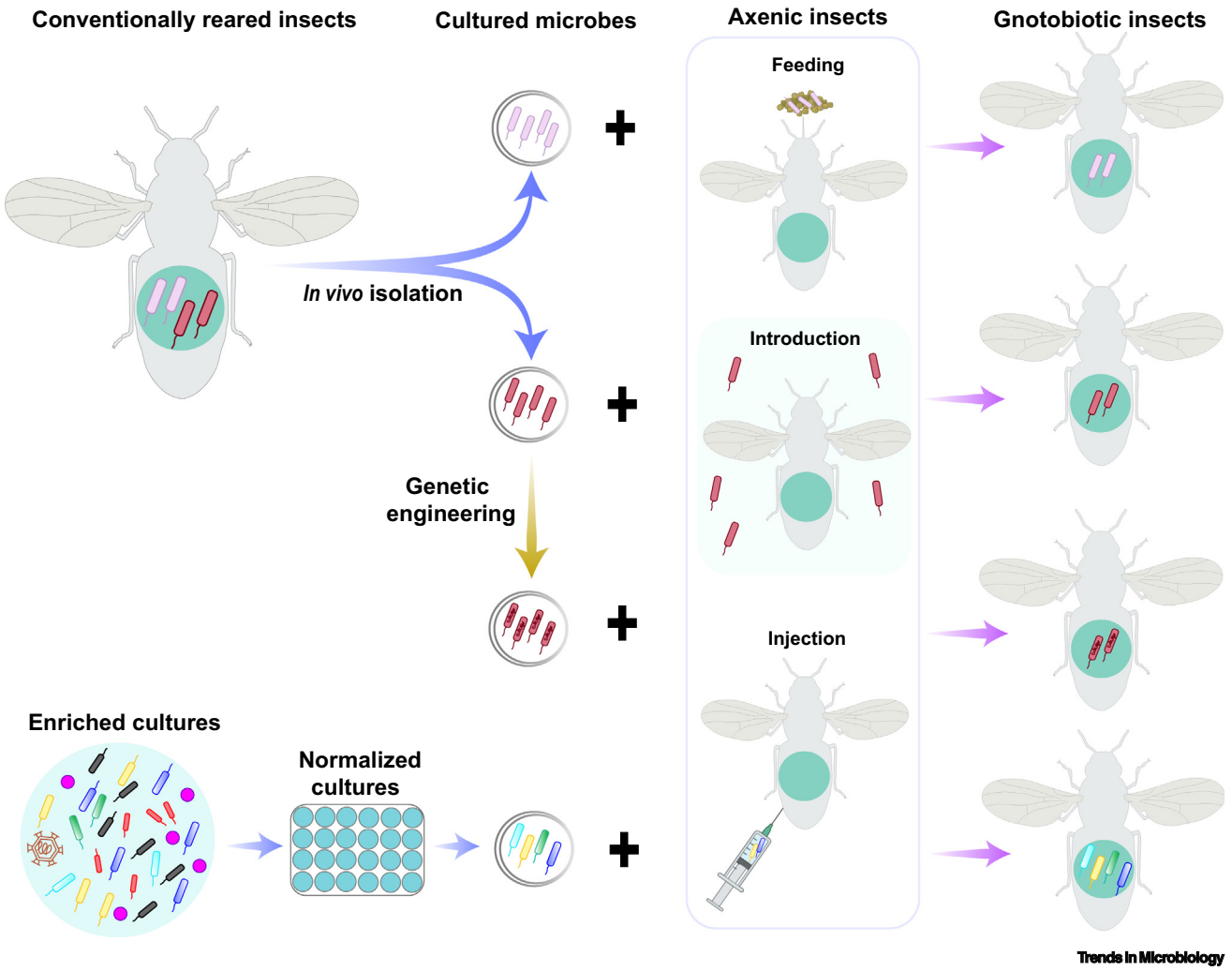

Figure 4. Studying insect–microbe interactions in conjunction with gnotobiotic rearing systems. Gnotobiotic insect rearing systems are established by artificially introducing a single bacterial isolate or complex communities into the axenic host. Figure created with BioRender.

rate of the axenic insects and shortens their development time, and is expected to be used for batch rearing of axenic insects. However, considering that exposure to different microbes during larval development may have carryover effects on adult traits [41], this method needs to be applied with caution to the study of host–microbe interaction mechanisms.

Verifying the axenic status of insects

When establishing an axenic rearing system, it is necessary to check the axenic status of the insects. The commonly used detection methods include: (i) 16S rRNA gene PCR to detect the presence of bacteria in the samples [45]; (ii) plating insect homogenates on nutrient medium or broth [35,36]. The culture method is straightforward and quick to apply, it can detect the presence of live bacteria, but there is a limit to the microbes that can be cultured. PCR tests can detect all microbes, including dead ones. However, nucleic acid contamination can cause false positives, and amplification thresholds do not completely exclude false negatives. Considering their advantages and disadvantages, both methods are often used to verify the axenic status of insects.

Major discoveries using axenic and gnotobiotic insects

The establishment of axenic and gnotobiotic rearing methods allows for precise manipulation of the insect microbiome, leading to advances in understanding the impact of symbionts on many host traits, such as metabolism, insecticide resistance, immunity, and behavior, as listed in Figure 3. Examples are as follows.

Microbiota on insect metabolism

Using the axenic approach, the gut microbes have been shown to affect host weight and gene expression of the insulin/insulin-like signaling pathway in honey bees [48]. Axenic bee pupa inoculated with different bacteria showed differences in metabolomic profiles, revealing that the gut microbiota can influence the host's ability to metabolize polysaccharides [49].

Adult lipid metabolism is profoundly affected when the axenic larvae of mosquitoes are colonized with different bacteria at the juvenile stage, manifesting as differences in the resistance of adult mosquitoes to starvation [50].

Symbiotic microbes can directly affect insect development by providing essential nutrients. The reintroduction of gut microbes in axenic red palm weevil *Rhynchophorus ferrugineus* can significantly increase the levels of nutrients in the hemolymph and promote development [40]. Especially, intestinal hypoxia caused by bacterial aerobic respiration may serve as a signal to promote insect developmental processes [51,52]. After the artificial severance of these connections by the axenic insect system, the metabolic process of the host was disturbed [53], and the host developmental defects appeared under the lighted rearing environment. For example, mosquito larval development arrest was observed at the first-instar stage when they are reared axenically [31]. However, the growth of axenic mosquito larvae can be rescued by colonization with live bacteria [47] or drastically changing the growth conditions (rearing in complete darkness), and supplement of specific nutrients, such as folic acid supplementation at the L3 stage of axenic mosquito [46].

In addition, the symbiotic gut bacterium *Lactobacillus plantarum*, colonized in axenic larvae of *Drosophila* under a low-nutrient environment, will undergo adaptive changes after multiple generations of culture; this, in turn, causes changes in the bacterial metabolic processes. Recolonization of evolved strains in independent axenic larvae significantly promoted larval growth [54], which is also helpful for a further extensive understanding of the evolution of host–microbe symbiosis.

Considering that most insects harbor specialized gut microbiota, the metagenomic sequencing technology will contribute to find microbes of interest and create gnotobiotic insect. Combined with metabolomic analysis, the gnotobiotic system becomes a powerful tool for demonstrating the metabolic function of specific microbes. But it should be noted that insects raised in different environments may have different microbiota, and the metabolic function of the given single bacterial isolates in the axenic host may be weakened or replaced in insects living in other environments, which may lead to differences in experimental results. In addition, the nutrient content of sterile food can significantly affect the survival status or physiological indicators of axenic insects, so axenic insect rearing systems need to be kept consistent in their entirety.

Symbiont-mediated insecticide resistance

Chemical insecticides are widely used for controlling insect pests, but insecticide resistance has become an increasing problem worldwide. Axenic or gnotobiotic insects have demonstrated that symbiotic microbes can confer host resistance to chemical insecticides in a variety of indirect or direct ways.

Removal of microbes from insects or re-establishment of association with microbes significantly affects host insecticide resistance [55,56]. Microbes could provide nutrients to enhance the adaptation of insect hosts to insecticide. For example, specific gut bacteria recolonizing axenic silkworms, *Bombyx mori*, were shown to enhance host resistance to organophosphate pesticides by providing essential amino acids [37]. And the microbiota of *Drosophila* is primarily responsible for the nitro-reductive metabolism of imidacloprid (IMI), thus exposure of axenic *Drosophila* larvae to IMI resulted in a reduction of nitro-reduced metabolites, but not to zero [57]. After the removal of *Wolbachia* using specific antibiotics, the brown planthopper, *Nilaparvata lugens*, became more sensitive to pesticides and gene expression analysis supported *Wolbachia* as a key symbiont regulating host detoxification metabolism [58]. Meanwhile, the insect microbiota has also been shown to confer host resistance to xenobiotics by exposure-induced adaptive changes. In the wasp, *Nasonia vitripennis,* atrazine exposure induced adaptive changes within the microbiota structure and function [59]. Especially, the rare gut bacteria *Serratia marcescens* NVIT01 and *Pseudomonas protegens* NVIT02 were shown to mediate pesticide resistance by metabolizing atrazine [60]. Adaptive changes in the gut microbiota conferred host resistance, as evidenced by the loss of resistance in the axenic progeny of resistant wasps [59]. The survival of axenic *N. vitripennis* was greatly improved by optimizing the sterilization method and producing natural media, making it valuable for future symbiont-related research [39].

The causal relationship between microbes and host resistance is intricate and needs to be studied in depth. Exposure to pesticides leads to changes in the microbiome, and the genetic background of the microbiome itself may also lead to differences in susceptibility to pesticides. Current reports suggest that insect symbionts play a key role in metabolic resistance. Studies using axenic and gnotobiotic insects have demonstrated that the exposure-induced adaptive changes of insects to pesticides may be driven by their symbiotic microbes. Besides, transcriptome analysis revealed that the expression of detoxification genes is associated with microbe abundance. The changes in the 'insecticide-resistant microbiome' in the host possibly affect the host susceptibility to insecticides, and the spread of these microbes may contribute to the resistance level of the host population [61]. Axenic and gnotobiotic models could pave the way for resistance mechanistic studies, provide a reference on existing pesticide applications, and aid in the development of eco-friendly resistance-management approaches.

Microbiota on host insect immunity and susceptibility to infection

By unraveling and reconstructing host–symbiont interactions, the axenic insect systems provide effective tools for explaining the relationship between microbes and host immunity and susceptibility to infection. In the case of axenic *D. melanogaster*, several gut physiological and immune processes are modulated by the microbiota, for example, gut microbes can promote intestinal epithelial cell renewal, activate immune pathways, defend against pathogen infection, and maintain intestinal homeostasis [62–64]. Using gene-editing technology to induce wing cell necrosis in flies containing normal microbial community would induce an over-activation of the immune system and result in a systemic inflammatory response, as opposed to this, the over-activation of immune signaling pathways in axenic flies was suppressed, showing that host–microbe interactions affect host immune responses [65].

Colonization of the core intestinal bacteria *Acetobacter* and *Lactobacillus* in gnotobiotic *Drosophila* via feeding shows that bacterial recognition protein responds differently to commensal microbes as compared to pathogens [66]. Immune regulators and immune recognition proteins can also interact to defend against microbial invasion while building immune tolerance to the gut microbiota [67]. In addition, it has been reported that the microbial metabolite acetate of

gnotobiotic *Drosophila* can replace the role of gut microbes to activate the **innate immune deficiency** (**IMD**) signaling pathway and restore host metabolic homeostasis [68].

Because of the global burden of mosquito-borne diseases, such as malaria and dengue, and a lack of effective vaccines for these diseases, researchers are eager to explore new approaches to control mosquito populations and reduce mosquito vector competence. Symbiont-based control strategies, such as the introduction of *Wolbachia* or manipulation of gut microbial communities to block pathogens, are being actively researched [69,70]. Axenic mosquitoes can be applied to study how specific microbes may attenuate vector competence. The symbiotic gut bacterium *Serratia ureilytica* YN1 was shown to inhibit the development of *Plasmodium* in gnotobiotic mosquitoes by secreting antimalarial lipase [71]. Follow-up studies revealed that this bacterium can be transmitted among mosquito populations in the laboratory, demonstrating its potential to combat malaria transmission. Another valuable application of gnotobiotic mosquitoes is to perform experiments related to the *Plasmodium* infection of humans. Gnotobiotic mosquitoes infected with *Plasmodium falciparum* are used to bite volunteers and can prevent the introduction of other pathogens that can harm the volunteers [72]. This approach is safer and has a higher infection rate, and is also used to produce spores free of other microbes for making vaccines.

Frontier research is inseparable from the discussion of microbe-related host health and disease, as well as microbial intervention therapy. The axenic system can prove whether the microbiota interacts with the host's immune and metabolic systems to maintain host homeostasis. Given the conserved phenotypes of immune pathways, well-established axenic models such as *Drosophila* are expected to advance the study of host immune mechanisms in depth. More importantly, the development of microbiome transplantation technology helps to reveal the dynamic change mechanism of microbial acquisition and community composition, such as the first established cross-generic transplantation of entire microbial communities of mosquitoes, as mentioned earlier. It helps to reveal the dynamic change mechanism of microbial acquisition and community composition. Whereas considering the influence of environmental factors and other factors on transplantation efficiency, the results of transplantation microbiota are not always positive, so how to completely change the recipient microbiota is a problem worth solving.

Effects of symbionts on host behavior and the gut–brain axis

Emerging evidence has highlighted the effects of microbiota on host behavior and the gut–brain axis. The use of axenic and gnotobiotic insects has helped to advance the field of gut–brain axis research.

Honey bees (e.g., *Apis mellifera* and *Apis cerana*) are social insects, which have not only high economic value to humans but also great ecological contributions as pollinators. Recently, axenic honey bee rearing has emerged as an experimental approach to explore microbial effects on complex behavior, including learning and memory. Compared with bees colonized with normal gut microbiota, axenic bees did not learn stimulus odors and link them to rewarding, exhibiting unsuccessful memory and behaviors [73]. Gnotobiotic bees colonized with *Lactobacillus* strains showed improved learning and memory behaviors.

Metabolomic analysis demonstrated that gut microbes can influence the gut–brain axis by modulating tryptophan metabolism. In addition, the gut physiological characteristics of *Drosophila* and humans share some similarities [74,75], and axenic *Drosophila* provides a tractable model to study the relationship between the gut microbiota and the gut–brain axis. Genetically modified gut bacteria may be applied to treating autism and intellectual disabilities of humans by altering the patient's gut microbiome. For example, in axenic *Drosophila*, mutations in genes encoding histone methylesterases, in members of the **KDM5 gene family**, alter the composition of gut

microbes, while colonization by *L. plantarum* may partly rescue social behavior in *Drosophila* [76]. Axenic and monoxenic flies showed different foraging preferences for media containing different bacteria, with axenic flies showing a less clear preference while monoxenic flies preferred the bacterium they are associated with. These observations raise the possibility that gut bacteria may influence the gut–brain axis, such as through the releases of metabolites [77]. Similarly, by modulating the expression of a specific gut neuropeptide, changes in the gut microbiome can affect amino acid levels in the host, which may in turn affect compensatory appetite for essential amino acids [78,79]. In addition, axenic male *Drosophila* showed significantly lower aggression, while after recolonization with microbes, they exhibited higher aggressive behavior owing to the expression of octopamine in males [80]. The axenic approach has also been extended to the agricultural pest *Drosophila suzukii*. Axenic females of *D. suzukii* were shown to be less active in foraging despite having a higher level of starvation-induced locomotion compared with conventionally reared female flies. Interestingly, the effect was not observed in axenic male *D. suzukii*, pointing to sex differences in microbiome effects on behavior [81].

The swarm behavior of pests is a key aspect of pest management, and the correlation between microbes and swarm behavior has also been proved. The aggregation behavior of the German cockroach, *Blattella germanica*, was shown to be elicited by volatile carboxylic acids (VCAs) from the feces of normal and gut bacteria-inoculated cockroaches. Feces from axenic cockroaches lack these VCAs and thus fail to induce a robust aggregation response [13]. Microbes play an important role in communication between the gut and brain. The unusual behaviors exhibited by germ-free insects after the removal of the microbiome have repeatedly demonstrated the powerful potential of the axenic insect model as a blank tool. But how to establish the link between microbes and host behavior is debatable. In addition, how to standardize and scientifically standardize the sterile breeding conditions of axenic models is a problem that needs attention. Different breeding conditions may lead to differences in experimental results. In particular, the behavior of insects is affected by many factors, and external interference factors should be reduced as much as possible.

Limitations of axenic and gnotobiotic technologies in research

Although a variety of axenic insects have been established in recent decades, these systems retain some drawbacks and limitations. It is worth thinking about how to introduce microbes into the axenic system and establish stable microbial community. And whether the microbes colonized in the laboratory population will be compatible with and play a role in the natural population. These are all problems that need to be solved urgently [82]. Developmental stunting in axenic insects is a common phenomenon under laboratory conditions [45]. Many social insects, such as termites, are difficult to establish in axenic models due to their social habits and the presence of obligate symbionts. Termites have so far failed to be raised axenically because of their complete dependence on obligate gut microbes for lignin digestion. And termites are internally differentiated and hierarchical. There is a high degree of cooperation among nestmates, and newly hatched larvae cannot live independently and need nourishment from nestmates until they are capable of foraging on their own [83].

Considering that insect–microbe and microbe–microbe interactions are complex and diverse, gnotobiotic insects are not representative of the complex microbiome [42]. Axenic and gnotobiotic approaches allow researchers to have precise controls of microbiome configurations, but microbiome effects can vary by genotype. Thus, additional technical tools may be required to integrate with axenic/gnotobiotic approaches to study the complex insect–microbe interactions.

As an important component of the gut–brain axis, microbes influence host behavior primarily through their involvement in metabolic processes. In combination with behavioral devices, axenic

insects can help to reveal the effects of specific metabolites on host behavior, which may help to develop new strategies for pest control. With the growing evidence supporting the role of the microbiome in the gut–brain axis, another potential caveat is that axenic insects may exhibit abnormal behaviors compared to those conventionally reared. These phenomena may be confounding factors in studying other microbiome effects, for example, on metabolism and physiology. For example, axenic *Drosophila* exhibits hyperactive behaviors, including a significant increase in walking speeds [84]. Recolonization of axenic cockroaches with a fraction of gut microbiota also failed to rescue development, and it seems that gnotobiotic insects bear greater energy stress than axenic insects [85]. Therefore, there is a need to consider the effect of nutritional stress on host behavior.

Concluding remarks and future perspectives

The construction of axenic and gnotobiotic insects provides effective means to study the functions of microbiota in hosts (see Outstanding questions). Some emerging and future themes involving axenic and gnotobiotic technologies include the following.

Fine-tuning of axenic and gnotobiotic technologies

On the one hand, the production of fully nutritious media will further improve the establishment of axenic insect systems when combined with techniques such as metabolomics to delve into the nutritional requirements of insects and solve physiological defects such as developmental delay. Combining techniques such as untargeted metabolomics and gas chromatography–mass spectrometry, changes in metabolites of developmentally defective insects can be identified. It offers the possibility to determine the nutrients provided by microbes and, based on the results, allows targeted supplementation during the development of axenic insects. But considering that many metabolites probably cannot be characterized, how to determine the structure of unknown metabolites is also a major difficulty.

On the other hand, the optimization of gnotobiotic technology can focus on the composition and timing of introducing microbes, etc. The colonization of the host's native microbiota or foreign microbiota (such as artificially selected and assembled complex microbial communities) can advance the study of microbe–host–microbe interactions [86]. And the colonization efficiency of different strains of the same bacteria varies in the same host [87], and the amount of injected bacteria can affect the chances of successful colonization [88]. Meanwhile, given the difficulty of permanent and stable colonization of microbes, reversible colonization has also become a powerful research tool. Transient colonization can be used to assess the life stage- or age-specific effects of the microbiota or the carryover effect on adult traits. In addition, the composition and abundance of microbes are different in the juvenile, pupal, and adult stages of insects, so a staged introduction can fully demonstrate the stage effect of microbes.

Axenic insects, combined with genetic modifications [89,90] and other molecular techniques, will aid in the discovery of the fundamental mechanisms governing host–microbe symbiosis and improve our understanding of how heritable microbes influence host phenotypes and genotypes. For example, knocking out specific genes in bacteria and then introducing axenic insects with the genetically modified bacteria could help to identify microbial genes and effectors underlying colonization and host phenotypic effects [91,92]. Considering that the instability of genetically engineered bacteria and the host's selection pressure on microbes will affect the colonization efficiency of the introduced microbes, engineering the dominant bacterial strains in the host may achieve persistent colonization.

Outstanding questions

How can we determine the roles of a large number of symbiont microbes that cannot be cultivated *in vitro* combining omics and *in situ* experimental methods?

Besides the axenic insects that are currently established, what other insects are worth further exploring for axenic rearing techniques?

Is it possible to correspond symbiotic microbes to host genes, one by one, and precisely study how host genes manipulate microbial composition?

What kind of microbial community composition is best suited to colonize axenic insects?

Can we achieve permanent colonization of foreign microbes by modifying the dominant strain in the host?

Can we identify any potential probiotics as supplementation for current agriculture, food, and human/animal health based on axenic or gnotobiotic rearing systems?

Combining new technologies to study the mechanisms of host–microbe interactions

Host–microbe interactions are multiscale and constitute a complex network structure. It is possible to infer general microbiota colonization patterns and forecast changes in host microbiota at large scales for extension and application to a wider range of fields by establishing the causal relationship between the two at various scales. Integrating the construction of gene networks, quantitative trait locus (QTL), and **genome-wide association studies** (**GWAS**), we can capture complex host–microbe relationships at multiple scales, link genes to phenotypic traits, and analyze microbial community dynamics and genetic influences [92]. The host's genetic background can significantly affect the colonization of alien microbes. GWAS analysis can be used to better understand how the host regulates the dynamic composition of the microbial community *in vivo*. In particular, this technique can help to reveal differences in microbiota–genome associations and changes in the genetic background of the host or microbe after multiple generations of axenic or gnotobiotic passage. In addition, a novel clustered regularly interspaced short palindromic repeats (CRISPR)-based real-time recording technology can be used to record the transcriptional dynamics of gut bacteria and identify active genes [93]. After the engineered *Escherichia coli* cells are injected into the host, transcript information can be stored in a CRISPR array, and subsequent sampling of the host fecal matter can help to reconstruct mRNA information. How gut bacteria adapt and compete with each other in the face of hosts in different states can be recorded. This innovative technique could capture the status of gut microbes and the expression information of microbial genes in the living host, and is expected to be applied to research to determine whether the metabolites of microbes can be used by the host or how to help the host digest food to obtain nutrients.

Insights into agricultural and human/animal health issues caused by insects

The relationship between insects and humans is multifaceted. On the one hand, humans actively find ways to eliminate insects that are prone to transmit human/plant diseases. On the other hand, we need to protect beneficial insects such as pollinators that provide significant ecosystem and agricultural services. An in-depth understanding of insects can help to provide timely insight into agricultural and human/animal health problems caused by insects, and there is great scope for the development of biological control technologies based on axenic and gnotobiotic insect systems in the future. For example, pest resistance to insecticides or natural toxins could be attributed to specific bacteria strains or species that colonized specific tissues of the gnotobiotic insect. By revealing the mechanisms of host resistance and key microbes involved in the process, together with microbial control technology and genetic engineering, targeting the microbes of highly resistant pests may provide an environment-friendly pest control strategy without affecting nontarget organisms. In addition, by identifying microbes that support insect development and growth, detoxifying chemicals, and fighting diseases, it may also be possible to engineer probiotics for natural enemies of pests and pollinator insects.

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Declaration of interests

There are no interests to declare.

References

1. Douglas, A.E. (2015) Multiorganismal insects: diversity and function of resident microorganisms. *Annu. Rev. Entomol.* 60, 17–34
2. Fisher, R.M. *et al.* (2017) The evolution of host–symbiont dependence. *Nat. Commun.* 8, 15973

3. Kucuk, R. (2020) Gut bacteria in the Holometabola: a review of obligate and facultative symbionts. *J. Insect Sci.* 20, 22
4. Whittle, M. *et al.* (2021) Insect-host control of obligate, intracellular symbiont density. *Proc. R. Soc. B Biol. Sci.* 288, 20211993
5. Moya, A. *et al.* (2008) Learning how to live together: genomic insights into prokaryote–animal symbioses. *Nat. Rev. Genet.* 9, 218–229
6. Shigenobu, S. *et al.* (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* 407, 81–86
7. Michalkova, V. *et al.* (2014) Vitamin B6 generated by obligate symbionts is critical for maintaining proline homeostasis and fecundity in tsetse flies. *Appl. Environ. Microbiol.* 80, 5844–5853
8. Brune, A. and Dietrich, C. (2015) The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. *Annu. Rev. Microbiol.* 69, 145–166
9. Zheng, H. *et al.* (2016) Metabolism of toxic sugars by strains of the bee gut symbiont *Gilliamella apicola*. *mBio* 7, e01326–01316
10. Miller, W.J. *et al.* (2010) Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. *PLoS Pathog.* 6, e1001214
11. Teseo, S. *et al.* (2019) The scent of symbiosis: gut bacteria may affect social interactions in leaf-cutting ants. *Anim. Behav.* 150, 239–254
12. Dillon, R. *et al.* (2002) A note: gut bacteria produce components of a locust cohesion pheromone. *J. Appl. Microbiol.* 92, 759–763
13. Wada-Katsumata, A. *et al.* (2015) Gut bacteria mediate aggregation in the German cockroach. *Proc. Natl. Acad. Sci. U. S. A.* 112, 15678–15683
14. Ishii, Y. *et al.* (2015) Effective trapping of fruit flies with cultures of metabolically modified acetic acid bacteria. *Appl. Environ. Microbiol.* 81, 2265–2273
15. Moreira, L.A. *et al.* (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell* 139, 1268–1278
16. Zug, R. and Hammerstein, P. (2015) *Wolbachia* and the insect immune system: what reactive oxygen species can tell us about the mechanisms of *Wolbachia*–host interactions. *Front. Microbiol.* 6, 1201
17. Kikuchi, Y. *et al.* (2012) Symbiont-mediated insecticide resistance. *Proc. Natl. Acad. Sci. U. S. A.* 109, 8618–8622
18. Pasteur, L. (1885) Méthode pour prévenir la rage après morsure. *Compt Rend. Acd. Sc.* 101, 765–773
19. Baker, J.A. *et al.* (1942) Growth of platyfish (*Platypoecilus maculatus*) free from bacteria and other microorganisms. *Proc. Soc. Exp. Biol. Med.* 51, 116–119
20. Reyniers, J.A. *et al.* (1949) The need for a unified terminology in germfree life studies. *Lobund Reports* 2, 151–162
21. Dougherty, E.C. (1959) Introduction to axenic culture of invertebrate metazoa: a goal. *Ann. N.Y. Acad. Sci.* 77, 27–54
22. Delcourt, A. and Guyenot, E. (1910) The possibility of studying certain Diptera in a defined environment. *CR Hebd. Acad. Sci.* 151, 255–257
23. Charnley, A. *et al.* (1985) The germ-free culture of desert locusts, *Schistocerca gregaria*. *J. Insect Physiol.* 31, 477–485
24. Greenberg, B. (1970) Sterilizing procedures and agents, antibiotics and inhibitors in mass rearing of insects. *Bull. ESA* 16, 31–36
25. Boorman, J. (1967) Aseptic rearing of *Aedes aegypti* Linn. *Nature* 213, 197–198
26. Lang, C.A. *et al.* (1972) Growth, composition and longevity of the axenic mosquito. *J. Nutr.* 102, 1057–1066
27. Schultz, J. *et al.* (1946) A chemically defined medium for the growth of *Drosophila melanogaster*. *Anat. Rec.* 96, 540
28. Bakula, M. (1969) The persistence of a microbial flora during postembryogenesis of *Drosophila melanogaster*. *J. Invertebr. Pathol.* 14, 365–374
29. Trager, W. (1935) The culture of mosquito larvae free from living microorganisms. *Am. J. Epidemiol.* 22, 18–25
30. Trager, W. (1941) The nutrition of invertebrates. *Physiol. Rev.* 21, 1–35
31. Coon, K.L. *et al.* (2014) Mosquitoes rely on their gut microbiota for development. *Mol. Ecol.* 23, 2727–2739
32. Jay, S.C. (1963) The development of honeybees in their cells. *J. Apic. Res.* 2, 117–134
33. Zheng, H. *et al.* (2018) Honey bees as models for gut microbiota research. *Lab. Anim.* 47, 317–325
34. Powell, J.E. *et al.* (2014) Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*. *Appl. Environ. Microbiol.* 80, 7378–7387
35. Koyle, M. *et al.* (2016) Rearing the fruit fly *Drosophila melanogaster* under axenic and gnotobiotic conditions. *J. Vis. Exp.* 113, e54219
36. Tegtmeier, D. *et al.* (2016) Oxygen affects gut bacterial colonization and metabolic activities in a gnotobiotic cockroach model. *Appl. Environ. Microbiol.* 82, 1080–1089
37. Chen, B.S. *et al.* (2020) Gut bacteria of the silkworm *Bombyx mori* facilitate host resistance against the toxic effects of organophosphate insecticides. *Environ. Int.* 143, 105886
38. Brucker, R.M. and Bordenstein, S.R. (2012) *In vitro* cultivation of the hymenoptera genetic model, *Nasonia*. *PLoS ONE* 7, e51269
39. Wang, G.H. and Brucker, R.M. (2022) An optimized method for *Nasonia* germ-free rearing. *Sci. Rep.* 12, 219
40. Habineza, P. *et al.* (2019) The promoting effect of gut microbiota on growth and development of red palm weevil, *Rhynchophorus ferrugineus* (Olivier)(Coleoptera: Dryophthoridae) by modulating its nutritional metabolism. *Front. Microbiol.* 10, 1212
41. Dickson, L.B. *et al.* (2017) Carryover effects of larval exposure to different environmental bacteria drive adult trait variation in a mosquito vector. *Sci. Adv.* 3, e1700585
42. Gould, A.L. *et al.* (2018) Microbiome interactions shape host fitness. *Proc. Natl. Acad. Sci. U. S. A.* 115, E11951–E11960
43. Kozlova, E.V. *et al.* (2021) Microbial interactions in the mosquito gut determine *Serratia* colonization and blood-feeding propensity. *ISME J.* 15, 93–108
44. Coon, K.L. *et al.* (2022) Interspecies microbiome transplantation recapitulates microbial acquisition in mosquitoes. *Microbiome* 10, 58
45. Correa, M.A. *et al.* (2018) Generation of axenic *Aedes aegypti* demonstrate live bacteria are not required for mosquito development. *Nat. Commun.* 9, 4464
46. Wang, Y. *et al.* (2021) Riboflavin instability is a key factor underlying the requirement of a gut microbiota for mosquito development. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2101080118
47. Romoli, O. *et al.* (2021) Production of germ-free mosquitoes via transient colonisation allows stage-specific investigation of host–microbiota interactions. *Nat. Commun.* 12, 942
48. Zheng, H. *et al.* (2017) Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. *Proc. Natl. Acad. Sci. U. S. A.* 114, 4775–4780
49. Zheng, H. *et al.* (2019) Division of labor in honey bee gut microbiota for plant polysaccharide digestion. *Proc. Natl. Acad. Sci. U. S. A.* 116, 25909–25916
50. Giraud, É. *et al.* (2022) Mosquito–bacteria interactions during larval development trigger metabolic changes with carry-over effects on adult fitness. *Mol. Ecol.* 31, 1444–1460
51. Coon, K.L. *et al.* (2017) Bacteria-mediated hypoxia functions as a signal for mosquito development. *Proc. Natl. Acad. Sci. U. S. A.* 114, E5362–E5369
52. Valzania, L. *et al.* (2018) Hypoxia-induced transcription factor signaling is essential for larval growth of the mosquito *Aedes aegypti*. *Proc. Natl. Acad. Sci. U. S. A.* 115, 457–465
53. Vogel, K.J. *et al.* (2017) Transcriptome sequencing reveals large-scale changes in axenic *Aedes aegypti* larvae. *PLoS Negl. Trop. Dis.* 11, e0005273
54. Martino, M.E. *et al.* (2018) Bacterial adaptation to the host's diet is a key evolutionary force shaping *Drosophila-Lactobacillus* symbiosis. *Cell Host Microbe* 24, 109–119
55. Barnard, K. *et al.* (2019) The contribution of gut bacteria to insecticide resistance and the life histories of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). *Sci. Rep.* 9, 9117
56. Daisley, B.A. *et al.* (2018) Microbiota-mediated modulation of organophosphate insecticide toxicity by species-dependent interactions with Lactobacilli in a *Drosophila melanogaster* insect model. *Appl. Environ. Microbiol.* 84, e02820–17
57. Fusetto, R. *et al.* (2017) Partitioning the roles of CYP6G1 and gut microbes in the metabolism of the insecticide imidacloprid in *Drosophila melanogaster*. *Sci. Rep.* 7, 11339
58. Zhang, Y.H. *et al.* (2021) Decline in symbiont-dependent host detoxification metabolism contributes to increased insecticide susceptibility of insects under high temperature. *ISME J.* 15, 3693–3703

59. Wang, G.H. *et al.* (2021) Coadaptation between host genome and microbiome under long-term xenobiotic-induced selection. *Sci. Adv.* 7, eabd4473
60. Wang, G.H. *et al.* (2020) Changes in microbiome confer multigenerational host resistance after sub-toxic pesticide exposure. *Cell Host Microbe* 27, 213–224.e7
61. Zhang, Y. *et al.* (2023) Microbiome variation correlates with the insecticide susceptibility in different geographic strains of a significant agricultural pest, *Nilaparvata lugens*. *NPJ Biofilms Microbiomes* 9, 2
62. Buchon, N. *et al.* (2009) Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. *Genes Dev.* 23, 2333–2344
63. Buchon, N. *et al.* (2010) *Drosophila* EGFR pathway coordinates stem cell proliferation and gut remodeling following infection. *BMC Biol.* 8, 152
64. Buchon, N. *et al.* (2009) *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation. *Cell Host Microbe* 5, 200–211
65. Kosakamoto, H. *et al.* (2020) Local necrotic cells trigger systemic immune activation via gut microbiome dysbiosis in *Drosophila*. *Cell Rep.* 32, 107938
66. Bosco-Drayon, V. *et al.* (2012) Peptidoglycan sensing by the receptor PGRP-LE in the *Drosophila* gut induces immune responses to infectious bacteria and tolerance to microbiota. *Cell Host Microbe* 12, 153–165
67. Lhocine, N. *et al.* (2008) PIMS modulates immune tolerance by negatively regulating *Drosophila* innate immune signaling. *Cell Host Microbe* 4, 147–158
68. Kamareddine, L. *et al.* (2018) The *Drosophila* immune deficiency pathway modulates enteroendocrine function and host metabolism. *Cell Metab.* 28, 449–462
69. McMeniman, C.J. *et al.* (2009) Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* 323, 141–144
70. Dong, Y. *et al.* (2009) Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog.* 5, e1000423
71. Gao, H. *et al.* (2021) A natural symbiotic bacterium drives mosquito refractoriness to *Plasmodium* infection via secretion of an antimalarial lipase. *Nat. Microbiol.* 6, 806–817
72. Lyke, K.E. *et al.* (2010) *Plasmodium falciparum* malaria challenge by the bite of aseptic *Anopheles stephensi* mosquitoes: results of a randomized infectivity trial. *PLoS ONE* 5, e13490
73. Zhang, Z. *et al.* (2022) Honeybee gut *Lactobacillus* modulates host learning and memory behaviors via regulating tryptophan metabolism. *Nat. Commun.* 13, 2037
74. Wong, A.C. *et al.* (2016) The interplay between intestinal bacteria and host metabolism in health and disease: lessons from *Drosophila melanogaster*. *Dis. Model. Mech.* 9, 271–281
75. Wu, S.C. *et al.* (2017) Intestinal microbial dysbiosis aggravates the progression of Alzheimer's disease in *Drosophila*. *Nat. Commun.* 8, 24
76. Chen, K. *et al.* (2019) *Drosophila* histone demethylase KDM5 regulates social behavior through immune control and gut microbiota maintenance. *Cell Host Microbe* 25, 537–552.e8
77. Wong, A.C. *et al.* (2017) Gut microbiota modifies olfactory-guided microbial preferences and foraging decisions in *Drosophila*. *Curr. Biol.* 27, 2397–2404
78. Kim, B. *et al.* (2021) Response of the microbiome–gut–brain axis in *Drosophila* to amino acid deficit. *Nature* 593, 570–574
79. Leitão-Gonçalves, R. *et al.* (2017) Commensal bacteria and essential amino acids control food choice behavior and reproduction. *PLoS Biol.* 15, e2000862
80. Jia, Y.C. *et al.* (2021) Gut microbiome modulates *Drosophila* aggression through octopamine signaling. *Nat. Commun.* 12, 2698
81. Shu, R. *et al.* (2021) Sex-dependent effects of the microbiome on foraging and locomotion in *Drosophila suzukii*. *Front. Microbiol.* 12, 656406
82. Al-Asmakh, M. and Zadjali, F. (2015) Use of germ-free animal models in microbiota-related research. *J. Microbiol. Biotechnol.* 25, 1583–1588
83. Mikaelyan, A. *et al.* (2016) Deterministic assembly of complex bacterial communities in guts of germ-free cockroaches. *Appl. Environ. Microbiol.* 82, 1256–1263
84. Schretter, C.E. *et al.* (2018) A gut microbial factor modulates locomotor behaviour in *Drosophila*. *Nature* 563, 402–406
85. Vera-Ponce de León, A. *et al.* (2021) Microbiota perturbation or elimination can inhibit normal development and elicit a starvation-like response in an omnivorous model invertebrate. *mSystems* 6, e00802–00821
86. Cheng, A.G. *et al.* (2022) Design, construction, and in vivo augmentation of a complex gut microbiome. *Cell* 185, 3617–3636 e3619
87. Zhou, W. *et al.* (2019) Selective colonization ability of human fecal microbes in different mouse gut environments. *ISME J.* 13, 805–823
88. Obadia, B. *et al.* (2017) Probabilistic invasion underlies natural gut microbiome stability. *Curr. Biol.* 27, 1999–2006.e8
89. Heu, K. *et al.* (2021) The effect of secondary metabolites produced by *Serratia marcescens* on *Aedes aegypti* and its microbiota. *Front. Microbiol.* 12, 645701
90. Wang, S. *et al.* (2017) Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. *Science* 357, 1399–1402
91. Gallo, M. *et al.* (2022) Beneficial commensal bacteria promote *Drosophila* growth by downregulating the expression of peptidoglycan recognition proteins. *iScience* 25, 104357
92. Powell, J.E. *et al.* (2016) Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. *Proc. Natl. Acad. Sci. U. S. A.* 113, 13887–13892
93. Schmidt, F. *et al.* (2022) Noninvasive assessment of gut function using transcriptional recording sentinel cells. *Science* 376, eabm6038