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The *Drosophila* model for microbiome research

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Abstract

The gut microbiome is increasingly recognized to play an important role in shaping the health and fitness of animals, including humans. *Drosophila* is emerging as a valuable model for microbiome research, combining genetic and genomic resources with simple protocols to manipulate the microbiome, such that microbiologically sterile flies and flies bearing a standardized microbiota can readily be produced in large numbers. Studying *Drosophila* has the potential to increase our understanding of how the microbiome influences host traits, and allows opportunities for hypothesis testing of microbial impacts on human health. *Drosophila* is being used to investigate aspects of host-microbe interactions, including the metabolism, the immune system and behavior. *Drosophila* offers a valuable alternative to rodent and other mammalian models of microbiome research for fundamental discovery of microbiome function, enabling improved research cost effectiveness and benefits for animal welfare.

For a century, research on *Drosophila melanogaster* (henceforth *Drosophila*) has led to fundamental discoveries in genetics and molecular biology, from elucidation of the chromosomal basis of heredity to the molecular basis of embryogenesis, learning, immunity, aging, the circadian rhythm and more[1]. Over many years, various researchers have investigated how *Drosophila* interacts with microorganisms that are not pathogenic[2], laying the foundations for the recent upsurge of research interest in *Drosophila* as a model for microbiome science.

The microbiology of healthy *Drosophila* can be classified conveniently by the location of the microorganisms, with the two main categories being the gut microbiota, i.e. the community of microorganisms inhabiting the lumen of the gut, and the endosymbionts, which are localized to the internal tissues (body cavity and cells) of the insect. The external surface of *Drosophila* also bears microorganisms, generally of a composition similar to the gut microbiota but at a ten-fold lower abundance[3]. The focus of this article is the gut microorganisms, which are present in all *Drosophila*. Some *Drosophila* in both laboratory culture and natural populations additionally bear endosymbiotic bacteria, notably *Wolbachia* and *Spiroplasma*[4–6], and research on these endosymbionts is providing insights into many

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biological processes, including microbial impacts on chromosomal organization[7], metabolism[8] and immunity[9–14].

The use of *Drosophila* for gut microbiome research is aided by a wealth of genetic and genomic resources and tools available for this model organism. It is also powered by the intrinsic tractability of this system for microbiome study[2,15,16]. In particular, microbiologically sterile *Drosophila* (also known as axenic or germ-free *Drosophila*) can be generated and maintained, with standard protocols that are not technically demanding or costly[2,3,15] (Fig. 1). Although axenic *Drosophila* are phenotypically distinct from their conventional counterparts (i.e. *Drosophila* with an unmodified microbiota) in various ways, e.g. development time, fecundity, lifespan[17–20], they can be maintained as vigorous cultures over multiple generations, if desired (Fig. 1). Furthermore, the axenic insects can be re-conventionalized or administered a standardized microbiota, by supplementing the food with *Drosophila* feces or defined microbial inoculum, respectively. The insects bearing a standardized microbiota are commonly referred to as gnotobiotic.

This article provides an overview of the current state of the art for the use of *Drosophila* in laboratory culture for gut microbiome research. The main topics addressed in this review are: the composition of the microbiota, including the value of research on *Drosophila* with a standardized microbiota; the impact of the microbiota on the nutrition and metabolism of *Drosophila*; and the ways in which the genetic and genomic resources for *Drosophila* are being leveraged to elucidate the molecular basis of *Drosophila*-microbe interactions. To provide context for these sections, the review starts with an overview of the *Drosophila* gut as a habitat for microorganisms. This article closes by considering how *Drosophila* can be used (and, in some instances, is already starting to be used) to investigate the effects of perturbing the microbiota on a range of biological traits of the animal host.

Drosophila gut structure

The gut microbiota of *Drosophila* is extracellular, i.e, microbial cells are not generally internalized into the gut epithelial cells. As is general for insects[21], the *Drosophila* gut has three distinct regions: the proximal foregut which includes the oral cavity, esophagus and, in the adult *Drosophila*, the crop; the midgut, which has four proximal ceca in the larva, but not the adult; and the distal hindgut (Fig. 2). Microbial cells occur in all three regions, including the adult crop and larval ceca, encountering different conditions according to location. This variation relates, in large part, to the functional differentiation of the gut in nutrient acquisition. The foregut mediates physical and chemical processing of ingested food, including initial degradation by enzymes released with the saliva; the midgut is the principal site of enzymatic digestion and assimilation, with a total of 349 putative digestive enzymes including proteases, carbohydrases and lipases identified[22]; and the hindgut mediates the selective assimilation of water, ions and nutrients, especially compounds released from the Malpighian tubules (the insect equivalent of the vertebrate kidney) localized at the midgut-hindgut boundary (Fig. 2).

The *Drosophila* gut wall comprises a single layer of epithelial cells, underlain by muscle, and includes two types of differentiated cells, the enterocytes and the enteroendocrine cells,

as well as the stem cells from which the differentiated cells are derived[22,23]. In the healthy insect, the transfer of microbial cells from the gut lumen to the body cavity is prevented by two barriers. The first barrier is provided by the very close apposition between the epithelial cells, mediated by septate junctions, which are functionally equivalent to the tight junction in the mammalian gut epithelium[24,25]. The second barrier is an acellular chitinous layer secreted from the apical surface of the epithelium, i.e. lying between the microbiota and the gut epithelium. In the foregut and hindgut, this layer is continuous with the exoskeleton that bounds the external surface of the insect. The chitinous layer in the midgut is less stable. This structure is called the peritrophic envelope (also known as the peritrophic matrix or peritrophic membrane) and comprises chitin fibrils held together by various chitin-binding proteins[26]. Some of these proteins are extensively glycosylated and structurally similar to mucins in vertebrate mucus[27], raising the possibility that, as for some mucus-associated bacteria in the mammalian gut[28], the *Drosophila* microbiota may interact directly with these proteins. However, the functional properties of most proteins associated with the *Drosophila* peritrophic envelope are unknown. Exceptionally, the structural protein Drosocrystallin has a major protective role as demonstrated by the penetration of orally administered toxins and bacteria through the peritrophic envelope in flies with null *drosocrystallin* mutations, resulting in elevated fly mortality[29]. The peritrophic envelope of *Drosophila* is likely synthesized in the same way as for other dipteran insects[21]: by proximal midgut cells in the larva, with the leading edge of the envelope driven distal-ward as synthesis proceeds, and over the full length of the midgut with “patching together” of the fragments to form a coherent sheet in the adult. How these patterns of synthesis may influence the populations of microorganisms in the gut remains to be investigated in *Drosophila*, although this topic has been addressed in other insects[30].

Physiological traits of the *Drosophila* gut

Studies of the suitability of the *Drosophila* gut as a habitat for microorganisms have focused largely on the midgut. This region of the gut bears an arsenal of antimicrobial effectors, three of which are particularly important in shaping the abundance and composition of the gut microbiota. The first is the acidic region of the midgut, which supports a luminal pH <3[31] and is important for the metabolic health of the insect[32]. Genetic ablation of the cells of the acid region (also known as copper cells) or the *Drosophila* V-ATPase, which mediates H^+ pumping and acidification of this region, results in increased luminal pH and a correlated increase in the abundance of the gut microbiota[33,34], suggesting that the acidic region may lyse or suppress the proliferation of microbial cells in the gut. The second set of effectors influencing the composition of the gut microbiota is the anti-microbial peptides (AMPs), whose production in the midgut is regulated principally by the Immune Deficiency (IMD) signaling pathway[35]. Genetic perturbation of the AMP profile results in changes in the composition of the microbiota that, in one study, resulted in dramatically increased populations of an opportunistic pathogen and high insect mortality[36]. Finally, *Drosophila* have a dual oxidase, Duox, borne on the apical membrane of enterocytes. Duox generates microbicidal hydrogen peroxide, hypochlorite and associated reactive oxygen species via NADPH oxidase and myeloperoxidase activity[37]. The Duox enzyme is activated by a poorly understood cellular pathway in response to the pyrimidine uracil[38,39]. Interestingly, uracil is

released from many bacterial pathogens, but few members of the gut microbiota, with the consequence that the gut Duox has low activity unless the fly is challenged by pathogens[39].

A further feature of the gut that is critically important in shaping the abundance, composition and activities of the microbiota is the redox potential of the gut lumen. Unlike the mammalian gut, the *Drosophila* gut is predicted to lack extensive anoxic regions. The *Drosophila* gut epithelium has aerobic metabolism, with oxygen supplied by an extensive network of tracheae (tracheae are "air tubes": insects differ from vertebrates in that they lack a closed vascular system for oxygen distribution). Because the gut lumen of *Drosophila* has a small diameter, oxygen diffusion from the epithelium is predicted to result in a predominantly oxic/hypoxic gut lumen, suitable for microorganisms with aerobic or aerotolerant metabolism, but unfavorable for obligate anaerobes. Although anoxic conditions have been reported in parts of the adult crop[40], no obligate anaerobic microorganisms are known to associate with *Drosophila*[2,18,41].

The *Drosophila* gut is a highly dynamic organ that can respond adaptively to dietary or microbial challenges by both functional and morphological changes[23]. Underlying these alterations are coordinated changes in gene expression and epithelial cell dynamics, including rates of stem cell proliferation and differentiation. In particular, elimination of the microbiota is associated with 30–40% increase in the length of the midgut of adult *Drosophila*[20,42]. This difference is associated with reduced rates of stem cell proliferation and epithelial turnover of the gut epithelium, together with elevated abundance of enteroendocrine cells in axenic flies[20]. Whether and how this difference in gut morphology between axenic and conventional *Drosophila* influences the observed effects of eliminating the gut microorganisms on the efficiency of nutrient digestion and assimilation[43] remains to be investigated.

The composition of the *Drosophila* microbiome

The microbial taxa.

Research on the composition of the gut microbiota in laboratory cultures of *Drosophila* has focused principally on bacteria. Multiple studies using 16S rRNA gene amplicon sequencing yield 1–70 OTUs (operational taxonomic units, specifically defined as a cluster of sequence reads with 97% sequence identity[44–49]). The microbiota of *Drosophila* reared on diets containing complex polysaccharides, such as cornmeal or soy flour, tends to be composed of a high abundance of *Lactobacillus* species (Firmicutes of the order Lactobacillales), while *Drosophila* fed sugar-rich diets have microbiomes commonly dominated by Acetobacteraceae (α-Proteobacteria), especially *Acetobacter* and *Gluconobacter* species. In some *Drosophila* cultures, either γ-Proteobacteria, including members of the Enterobacteriaceae and Xanthomonadaceae, or *Enterococcus*, including *E. faecalis* (related to *Lactobacillus* in the order Lactobacillales) are very abundant, often to the exclusion or near-exclusion of the Acetobacteraceae and *Lactobacillus*[50,51]. The Acetobacteraceae, Lactobacillales and γ-Proteobacteria also dominate the gut microbiota of natural populations of *Drosophila*[45,52–55], but the microbiota in wild flies is more diverse at the OTU level, comprising 100–200 16S OTUs in samples that pool multiple individuals, and up to 80 OTUs per individual fly[52,55]. There are many taxonomic differences between the microbiota

in laboratory and wild *Drosophila*. In particular, *Lactobacillus* is low abundance or undetectable in some natural populations, but different representatives of the Lactobacillales, including *Leuconostoc, Enterococcus* and *Weissella*, are often abundant. For the Acetobacteraceae, laboratory and wild *Drosophila* are not strongly differentiated by 16S taxonomy[56].

Yeasts are regularly detected in the guts of wild *Drosophila* populations and in laboratory *Drosophila* fed on rotting fruits, and the dominant taxa include *Hanseniaspora, Pichia* and *Candida* species[57–59]. However, there has been little investigation of the incidence of these yeasts in laboratory cultures of *Drosophila* reared on artificial diets. The yeast *Saccharomyces cerevisiae* has been used in various studies on *Drosophila*-microbe interactions, but the interpretation of these studies has been called into question because this species is not known to be an associate of wild *Drosophila*[60].

The taxonomic variability of the *Drosophila* microbiome.

A robust feature of the gut microbiome of *Drosophila*, reported by many researchers, is its variability. The composition of the bacterial community in a single *Drosophila* strain maintained on the same diet under strictly uniform conditions can vary over time within a single laboratory and between laboratories following the same fly husbandry protocols[44,45]. There is a growing consensus that much of this variability is stochastic, i.e. driven by chance events of gain and loss from the gut. This interpretation is supported by population modeling analyses[40]. These dynamics appear not to be a laboratory artefact because stochastic processes have also been inferred to have a role in shaping the gut microbiome composition in wild *Drosophila* populations[55]. The variability in composition of the microbiome can be explained, in broad terms, by the fact that the *Drosophila* gut is an open system and microbial cells are both ingested with food and shed in feces. Some ingested microorganisms are lysed in the gut or pass through the gut with the bulk flow of food, while others persist in the gut for variable lengths of time[61,62]. *Drosophila* can also influence the composition of the microorganisms depending on the substrate on which they feed[62,63]. Although the fate of ingested cells can vary with *Drosophila* genotype and the strain and species of the microorganisms, there is a growing consensus that passive dispersal of the microorganisms between flies and chance loss of taxa from individual flies play an important role in shaping the gut microbiota[40,47,55,61,63]. Interestingly, laboratory rodent models display similar variation, often known as “the cage effect”, in gut microbiome composition[64–66].

One important consequence of the inconstancy of the composition of the *Drosophila* gut microbiome is that conventional *Drosophila* can yield variable results for microbiota-dependent traits. For most experimental purposes, therefore, it is crucial to work with *Drosophila* that harbor a standardized microbiota, i.e. gnotobiotic *Drosophila* (Fig. 1). A detailed step-by-step protocol has been published[15]. Briefly, the strategy is to eliminate the naturally occurring microorganisms, generating axenic (also known as “germ-free”) *Drosophila*, and then administer the microorganisms of choice, which are ingested readily by both larval and adult *Drosophila*. Because antibiotic treatments do not, generally, eliminate all gut-associated microorganisms and can have nonspecific deleterious effects on

Drosophila[67], the preferred method to generate axenic *Drosophila* is to rinse eggs in hypochlorite solution, which removes all surface microorganisms[68], and then to add the microbial taxa of choice either immediately, providing for colonization of the *Drosophila* hatchlings, or at any desired developmental stage of the insect[19,43]. This methodology has two limitations. First, microorganisms that are transmitted from the mother into the egg cytoplasm are not eliminated, meaning that axenic cultivation can be obtained only using *Drosophila* lines that are free of *Wolbachia* and other vertically transmitted endosymbionts. (These endosymbionts can be eliminated by antibiotic treatment[9], although care is required to avoid direct deleterious effects on the *Drosophila*[69].) Second, this method precludes experiments involving microbiota manipulations in adults that are derived from conventional larvae because gut symbionts are transmitted through metamorphosis from the larval insect to the adult[68,70].

Drosophila is very amenable to experiments involving the resynthesis of the gut microbiome. *Drosophila* has been colonized with success by single bacterial taxa from multiple phyla, co-cultures of 2–12 taxa, and undefined communities in feces or dissected guts from conventional flies[19,36,40–43,71]. it is good practice to administer known numbers of microorganisms because the starting inoculum size can influence the density of microorganisms in gnotobiotic flies over short timescales (e.g. 3 days) after administration[40], although not over timescales of >10 days[19]. However, the numbers of *Drosophila* in the vial can influence colonization patterns of multitaxa inocula, with evidence that some taxa can be lost in *Drosophila* cultures maintained at low density[63].

Host-microbe interactions and *Drosophila* nutrition

Drosophila is an excellent model system to study the effects of gut microbiota on host nutrition and metabolism. In particular, it is amenable to the two key methodologies in nutritional physiology: the impact of diet composition on animal performance and indices of metabolic function, e.g. metabolite or nutrient content, rates of biosynthesis or degradation of specific compounds. Informative experimental designs test how the animal performance and metabolic indices are influenced by the presence and composition of the microbiota (Fig. 3). Research to date has focused on three classes of nutrients: B vitamins, energy storage molecules (especially lipids), and protein nutrition.

B vitamin nutrition.

Early dietary studies demonstrated that axenic *Drosophila* have extended larval development times and high mortality when individual B vitamins were omitted from the diet[72]. Evidence that the gut microbiota contributes to the B vitamin requirements of *Drosophila*, especially for riboflavin (vitamin B_2) and folate (vitamin B_9), comes from recent experiments that demonstrate the recovery of *Drosophila* performance, either by providing the fly-conditioned food (which contains the gut microorganisms) or B vitamins to the axenic *Drosophila*[73–75]. These experiments used yeast-depleted or chemically defined diets because B vitamin provisioning by the gut microbiota is not required for sustained *Drosophila* performance on most standard laboratory diets which contain B vitamin-rich dried yeast or yeast extract. It remains to be established whether the B vitamins are released from living microbial cells

(either in the food or in the *Drosophila* gut) or made available by digestion of the microbial cells in the insect gut.

Energy storage.

The role of the gut microbiota in *Drosophila* energy storage has been investigated by analysis of both the major macromolecular energy stores, lipid and glycogen, and also two free sugars, glucose and trehalose. (Trehalose is a non-reducing glucose disaccharide and the principal sugar in insect hemolymph, which is the insect equivalent of vertebrate blood[76].) The levels of all these indices are elevated in axenic flies, relative to conventional flies reared on the same diet[19,42,70]. These results are the reverse phenotype of the germ-free mouse, which displays a significantly leaner phenotype (i.e. lower fat content in the body)[77]. The likely reason for this difference is that the microbiota in the mammalian hindgut (colon and, for mouse, the cecum) ferment plant polysaccharides to short chain fatty acids (SCFAs), which are then assimilated by the host as a source of calories. Although many of the microbial taxa associated with *Drosophila* produce SCFAs and simüar fermentation products[78] that can be utilized by the insect host, this interaction appears not to be a quantitatively important source of calories for *Drosophila* under the laboratory conditions tested[79].

Although axenic *Drosophila* do not reproduce the energy storage phenotype of axenic mice, their phenotype is reminiscent of the pro-obesogenic effects of microbiome perturbation caused by antibiotic treatment in the mouse and possibly also humans[80]. Consequently, the interaction between the gut microbiota and energy storage in *Drosophila* has potential relevance to public health. Two processes have been identified in the *Drosophila* system. First, some members of the *Drosophila* microbiota, notably Acetobacteraceae, use dietary sugars at sufficiently high rates to deplete the sugar availability to the host[41,43]. For flies on high sugar diets, this is quantitatively sufficient to account for the difference in lipid content between flies containing and lacking a gut microbiota[43]. In mammals, including humans, the principal carbon compounds utilized by the microbiota in the small intestine are low molecular weight metabolites, such as sugars,[81] raising the possibility that, as in *Drosophila*, this microbiota promotes a lean host phenotype by competing for calories.

In parallel, acetic acid, a product of the periplasmic respiratory chain in the Acetobacteraceae, has been identified as a signaling molecule that modulates insulin signaling in *Drosophila*, resulting in reduced lipid storage[42]. The relative importance of the microbiome as a determinant of the nutritional inputs to *Drosophila* versus a source of signaling molecules that modulate *Drosophila* regulation of metabolic function is an important area for future research This research has important general significance, in the context of the evidence that acetic acid can also influence lipid storage in the mammalian liver[82].

Drosophila protein nutrition.

Various bacteria and yeasts in the gut microbiota promote the protein nutrition of *Drosophila*, especially females and on low protein diets. This interaction contributes to extended lifespan[71,74,83] and also sustained egg production of *Drosophila* reared on diets

deficient in specific essential amino acids (i.e. amino acids that animals, including *Drosophila*, cannot synthesize)[84]. One microbial process contributing to this effect is amino acid harvesting. Specifically, the yeast *Issatchenkia orientalis* has been demonstrated to accumulate amino acids as it grows on the fly food, such that *Drosophila* acquire limiting amino acids at a higher rate when feeding on yeast-inoculated food than on sterile food. The significance of amino acid harvesting for *Drosophila* can be very pronounced, doubling the lifespan of the flies on low-protein diets[84]. It is not known whether other yeast or bacterial associates of *Drosophila* also mediate amino acid harvesting. In principle, the gut microbiota could also enhance *Drosophila* protein nutrition by provisioning amino acids that are limiting *Drosophila* protein synthesis[71] and by microbial recycling of *Drosophila* nitrogenous waste into amino acids or other limiting nitrogenous compounds[56]. Further research is required to investigate the incidence and significance of these interactions.

Drosophila sterol nutrition.

As for arthropods generally, *Drosophila* has a dietary requirement for sterols, which is provided by yeast (or yeast extract) in most laboratory diet formulations and by or purified cholesterol in chemically defined diets[85,86]. *Drosophila* provided with yeasts as the sole source of dietary sterols are generally assumed to obtain their sterol requirement by the digestion of ingested yeast cells, but this has not been investigated experimentally.

Leverage from *Drosophila* genetics and genomics for microbiome research

A major goal in microbiome science is to understand microbiota-dependent effects on host phenotype in molecular terms. Which genes influence the magnitude of microbiota-dependent host traits, and how is this effect related to the sequence and expression pattern of the genes? Two complementary approaches founded on the superb genomic resources available for *Drosophila* have been very productive: transcriptome analysis, especially RNA-Seq, and genome-wide association (GWA) studies.

Impacts of the microbiome on *Drosophila* gene expression.

Transcriptome analyses have focused principally on the effect of eliminating the gut microbiota on gene expression, either of the gut epithelium or the whole animal. *Drosophila* raised in a sterile environment display altered gene expression patterns across the entire body, relative to conventional flies, but the greatest effects are on the gut transcriptome. Comparisons across multiple studies[20,52,87,88] suggest that: first, the gut transcriptome is responsive to elimination of the microbiota, especially with respect to metabolism-related and immune-related genes; and second, the transcriptional response is not strongly affected by the composition of the gut microbiota (including different bacteria taxa and also yeasts). Additional insights into the microbial effects on *Drosophila* gene expression will require detailed analysis of transcriptional patterns in flies associated with microbial partners of systematically-varied composition. System-level expression responses to microbiota elimination have also been studied, revealing that the strength of gene co-expression among a subset of genes is significantly greater in gnotobiotic flies than in axenic flies[80]. These data suggest that the microbiota influence not only the expression levels of individual genes, but also the overall structure of the transcriptional network[89].

Genome-wide association studies of *Drosophila*.

Drosophila microbiome science has made use of the wealth of genome sequence data available for this species. In particular, the genomes of more than 200 inbred lines derived from a single natural population have been sequenced, and this Drosophila Genetic Reference Panel (DGRP)[90,91] has been used extensively to correlate genetic variants, especially SNPs, with phenotypic traits. Analyses of microbiota-dependent traits have, to date, focused on the effect of the microbiota on nutritional traits and the population size of the microbiota[50,92]. Extensive genetic variation in both sets of traits was detected. For example, although axenic cultivation results in increased lipid content of most *Drosophila* genotypes, the magnitude of this effect varies and some strains show the reverse phenotypic response, i.e. are leaner in the absence of the microbiota, indicating that the impact of the microbiota on host phenotype can be strongly genotype-dependent. Many of the genetic variants could be assigned to genes with functions in cell signaling, including the insulin/TOR nutritional signaling pathways, and that have homologs across the animal kingdom, including to humans and other mammals. The broad relevance of these studies is reinforced by comparative analysis of genetic associations with microbiome-related traits that identified parallels between GWAS results obtained for *Drosophila* and other animal systems[93].

Future directions

Drosophila as a model to study the fundamentals of microbiome-animal interactions.

In what ways can research on *Drosophila* continue to contribute most effectively to the discipline of microbiome science? Let us start with what *Drosophila* microbiome is not: a miniaturized version of the human gut microbiome. Despite some similarities between the human and *Drosophila* gut, e.g. both are divided into three regions (the foregut, the digestive midgut and the post-digestive hindgut) and include a highly acidic proximal midgut region, there are also many differences. For example, the microbiota in *Drosophila* is separated from the gut epithelium by a chitinous layer, not mucus; the *Drosophila* hindgut is not a fermentation chamber, equivalent to the mammalian colon; and the *Drosophila* gut is widely considered to be an unsuitable environment for the obligate anaerobic microorganisms that dominate the gut microbiota of mammals. Consequently, *Drosophila* cannot be humanized, i.e. colonized with most gut microbes from humans. Bearing in mind recently expressed doubts about the biological relevance of research on rodent models with a humanized microbiota[94,95], it can very easily be argued that a humanized fly (even if possible) would not be informative because of the major differences between the organization of the human and *Drosophila* gut.

The very real value and potential of *Drosophila* for microbiome research should not be decided by the extent to which it matches the human condition, but by its long track record as a successful model for discovery of fundamental biological mechanisms[1]. As summarized in this article, *Drosophila* is already proving itself as a system for understanding how the microbiome influence host nutrition and metabolism. Furthermore, various recent studies are signposting the opportunities for the use of *Drosophila* beyond metabolic effects, especially in relation to microbiome effects on immunity and behavior.

The microbiome and immune system function.—Most research to date on the interaction between the *Drosophila* immune system and the gut microbiota has focused on how immune effectors, especially antimicrobial peptides, influence the composition and abundance of the gut microbiota, e.g.[20,36,96,97]. However, research on other systems suggests that the interaction between the host immune system and the microbiota is bi-directional, involving microbial effects on immunological function as well as the reverse[98,99]. Indications that the impact of the gut microbiota on *Drosophila* immune function is a fruitful topic for fixture study come especially from two recent studies. The first study[100] shows that the gut microbiota is an important regulator of *Drosophila* crystal cells, a class of hemocytes (immune cells) that mediates melanin synthesis in the encapsulation of pathogens and wound healing. The second study[101] reveals that expression of the growth factor Pvf2 (the homolog of the mammalian platelet-derived growth factor (PDGF)/vascular endothelial growth factor (VEGF) in the gut epithelium is enhanced by gut microbe-induction of the IMD signaling pathway, and that this interaction plays a necessary role in the activation of antiviral immunity in the *Drosophila* gut. More generally, there is growing evidence for parallels between *Drosophila* and the mouse in relation to aging-related changes in the gut microbiota, correlated with gut inflammation and the breakdown of gut compartmentalization[34,102,103].

The microbiome and behavior.—The effect of the gut microbiota on various aspects of host behavior, including appetite, learning, locomotory activity and social interactions, is becoming an active area of research, especially on rodent models[104,105]. Because various behavioral traits of *Drosophila* have been dissected in great detail from both neurobiological and genetic perspectives, *Drosophila* is an attractive system to study the molecular mechanisms underlying microbiome effects on behavior. There is now growing evidence that key behavioral traits of directed movement, oviposition choice and food choice are influenced by microbial volatiles[78,79,106], including acetic acid and acetoin. The microbiota has also been reported to influence *Drosophila* mate choice[107]. Although this effect is not observed in all *Drosophila* strains or laboratories[108,109], further research is certainly warranted to investigate whether and how the complex chemosensory basis of courtship and mate choice is influenced by microbial volatiles and microbial effects on *Drosophila* secondary metabolism. A further area of great potential opportunity is the role of the microbiota in stabilization of biological rhythms in the animal host. In the light of the widespread interest in the possible bi-directional link between perturbation of the microbiota and disruption of the circadian rhythm in people[110,111], research on microbiota effects on the circadian pattern of *Drosophila* behavior (including the pattern of movement, feeding and sleep) offers great opportunity for fundamental discovery.

Integrating *Drosophila* with other model systems for microbiome research.

As the studies summarized in this article illustrate, *Drosophila* is a superb system for gut microbiome research because it commands a wealth of genetic and genomic resources and is exceptionally amenable to the experimental manipulations of the microbiome. Furthermore, *Drosophila* should not be considered entirely as an alternative to other model systems. For many purposes, especially relating to biomedical research, *Drosophila* can be used in conjunction with rodent and other mammalian models: *Drosophila* to identify the scale and

pattern of microbiota effects on the host phenotype of interest and the underlying molecular mechanisms, and the mammalian model to assess how the *Drosophila* results translate to a mammalian system. This approach has already been adopted with great success, for example in the study of the impact of probiotic *Lactobacillus* on gut epithelial cell proliferation in *Drosophila* and the mouse[112]. As well as achieving research outputs in a more timely and cost-effective way, the use of *Drosophila* (and other invertebrate models) has the great benefit of reducing the number of vertebrate animals required for microbiome research.

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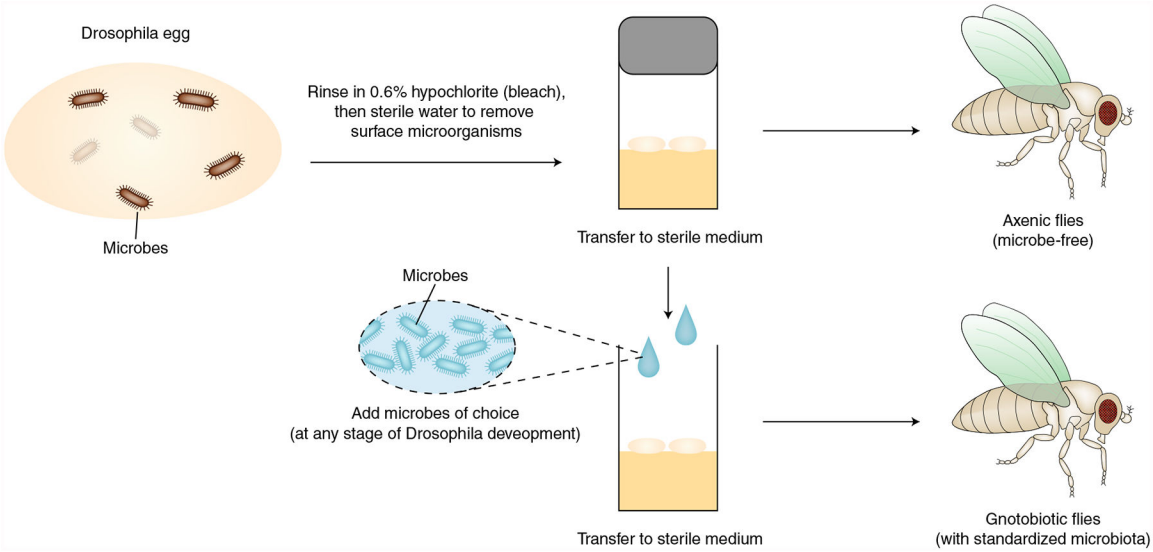

Fig. 1|. The *Drosophila* gut microbiome is amenable to manipulation, to generate axenic flies (i.e. microbiologically sterile, often referred to as "germ-free") and gnotobiotic flies (i.e. with a standardized microbiota).

To produce axenic flies, eggs are rinsed in hypochlorite, which removes all surface microorganisms, and then aseptically transferred to sterile medium, on which the insect develops to adulthood. Microbes of choice can be added to the vials containing axenic insects. Both larvae and adults feed readily on microorganisms, so establishing an association.

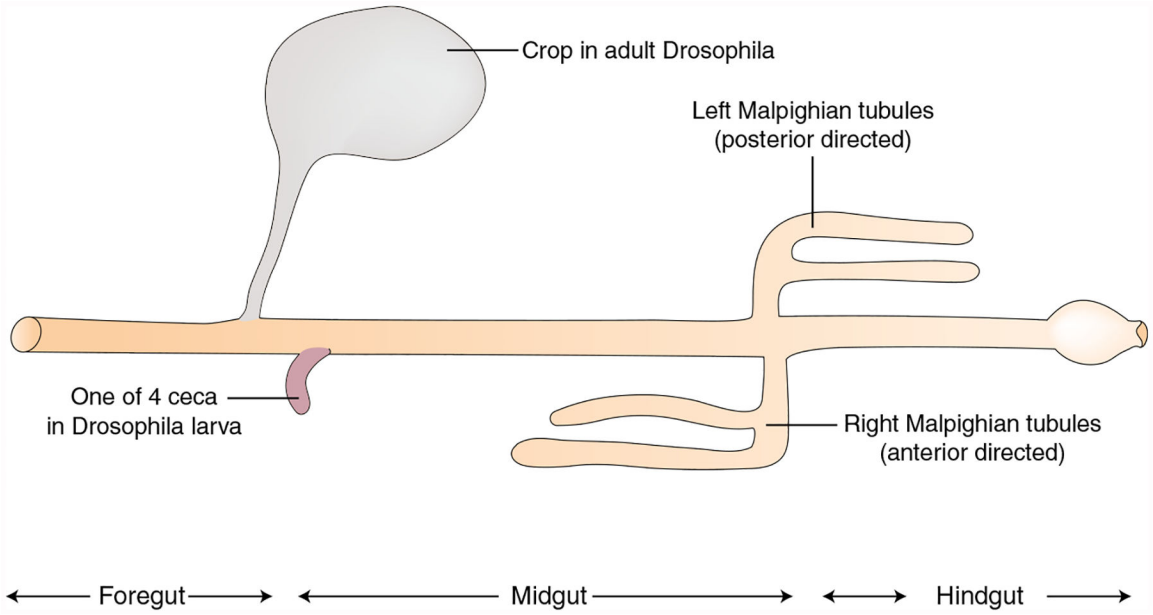

Fig. 2|. The organization of the *Drosophila* gut.

The gut comprises three regions, the foregut, midgut and hindgut, each of which contains morphologically, genetically and functionally distinct regions[21,113,114]. The gut epithelium is bounded by a stable chitinous layer in the foregut and hindgut and a dynamic layer, the peritrophic envelope in the midgut; the adult has a single extensible foregut evagination, the crop[115] and the larva has four proximal midgut ceca; and the Malpighian tubules are strictly oriented, with the right pair (dorsal view) extending anteriorly and the left pair extending posteriorly (the tips are commonly attached to the distal end of the hindgut)[116,117].

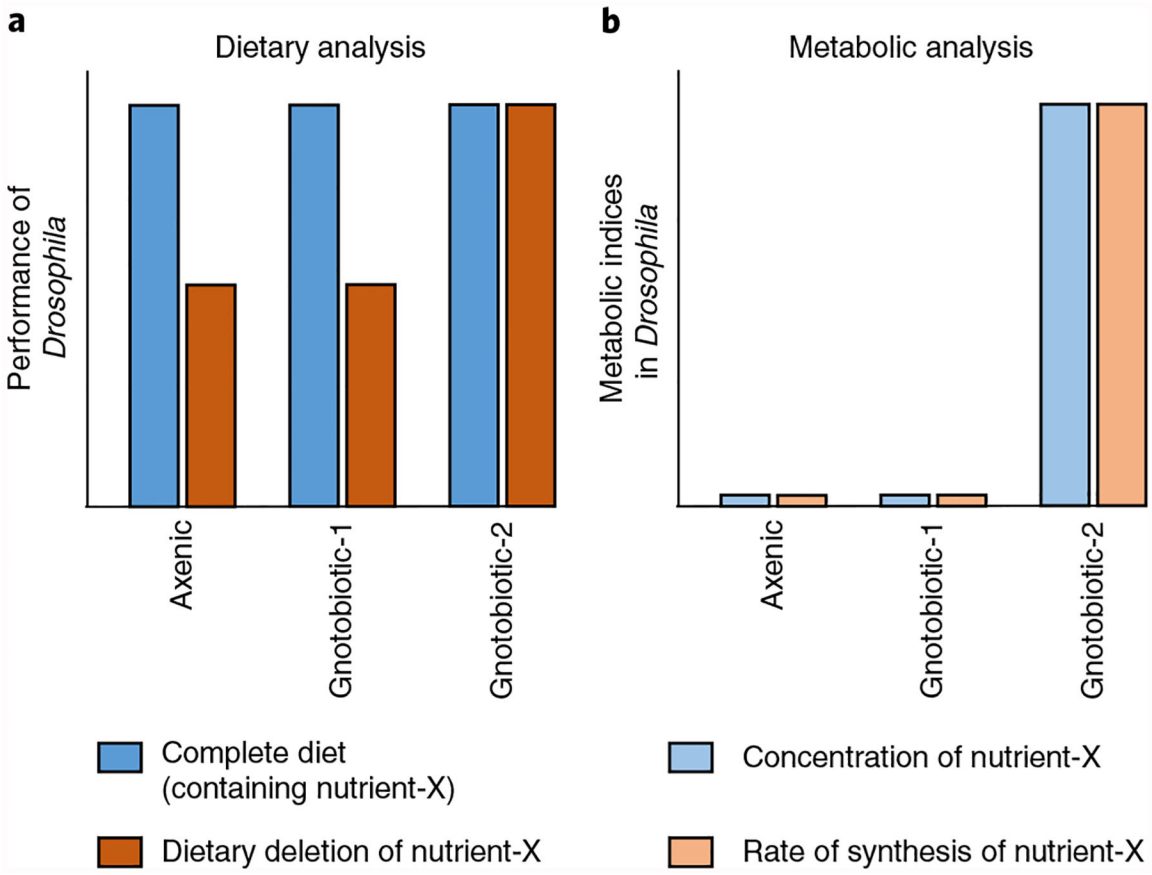

Fig. 3|. Contribution of the gut microbiota to *Dmsophila* nutrition.

The illustrated experimental design comprises axenic *Drosophila* and *Drosophila* with two alternative standardized microbiota (an ineffective microbiota in gnotobiotic-1, i.e., which does not promote insect performance and an effective microbiota in gnotobiotic-2, which does promote insect performance), **a**. The dietary analysis compares the performance that is lifespan, growth rate, fecundity etc on diets containing and lacking the nutrient (X) of interest, **b**. The metabolic analysis compares the concentration of the nutrient X or related metabolite and its biosynthesis in *Drosophila*. The values are for illustration, and the magnitude of differences may vary.