Simple animal models for microbiome research

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Abstract | The health and fitness of animals, including humans, are influenced by the presence and composition of resident microbial communities. The development of rational microbial therapies to alleviate chronic immunological, metabolic and neurobiological diseases requires an understanding of the processes underlying microbial community assembly and the mechanisms by which microorganisms influence host traits. For fundamental discovery, simple animal models (that is, lower vertebrate and invertebrate species with low diversity microbiomes) are more cost-effective and time-efficient than mammal models, especially for complex experimental designs and sophisticated genetic screens. Recent research on these simple models demonstrates how microbiome composition is shaped by the interplay between host controls, mediated largely via immune effectors, inter-microorganism competition, and neutral processes of passive dispersal and ecological drift. Parallel research on microbiome-dependent host traits has identified how specific metabolites and proteins released from microorganisms can shape host immune responsiveness, ameliorate metabolic dysfunction and influence behavioural traits. In this Review, the opportunity for microbiome research on the traditional biomedical models zebrafish, *Drosophila melanogaster* and *Caenorhabditis elegans*, which command superb research resources and tools, is discussed. Other systems, for example, hydra, squid and the honeybee, are valuable alternative models to address specific questions.

The study of animal microbiomes is widely perceived as a young discipline, made possible by the advent of sequencing technologies to determine the taxonomic identity and functional traits of microorganisms without cultivation. The meteoric rise of microbiome science over the past 10–15 years has been driven largely by the promise of microbial therapies for chronic human diseases, especially metabolic, immunological and mental health disorders. Consequently, microbiome science is commonly viewed as a biomedical discipline that focuses on the microbiology of humans, assisted by experimental research on the laboratory mouse[1,2].

Nevertheless, important contributions to our understanding of animal microbiomes are being made by research on simple animal models, that is, a select choice of invertebrate and lower vertebrates associated with microbiomes of lower taxonomic diversity than in mammals. Relative to mammals, these simple systems variously support straightforward protocols to manipulate the microbiota and assign function to individual microbial taxa, conduct cost-effective experiments over short timescales, enable complex experimental designs and, especially for invertebrates, bypass important animal welfare issues raised by research on mammals. Three traditional biomedical models — the fruit fly *Drosophila melanogaster* (hereafter *Drosophila*), the zebrafish *Danio rerio* and the nematode worm *Caenorhabditis elegans* (FIG. 1a–c) — are attracting increasing attention because microbiome research is a straightforward extension of the successful use of these systems to investigate the fundamental animal processes of development, neurobiology, immune function, etc. (BOX 1). Microbiome studies are conducted on a wide range of other animals, and some of these systems have yielded novel insights that have subsequently been found to be general, including to human microbiomes. Among these many alternative systems, three associations that have supported sustained research over many years and are yielding unique insights into microbiome–host interactions are considered: the gut microbiome of the honeybee *Apis mellifera*; the bacterial communities associated with the body surface of the freshwater polyp *Hydra vulgaris* (hereafter hydra); and the relationship between the Hawaiian bobtailed squid *Euprymna scolopes* and a single bacterium, *Vibrio fischeri*, which despite its taxonomic simplicity, has made major contributions to our understanding of animal interactions with beneficial microorganisms (FIG. 1d–f; BOX 1). Readers may also be interested to refer to other systems, including the wax moth *Galleria mellonella*[3], crustacean species belonging to the genus

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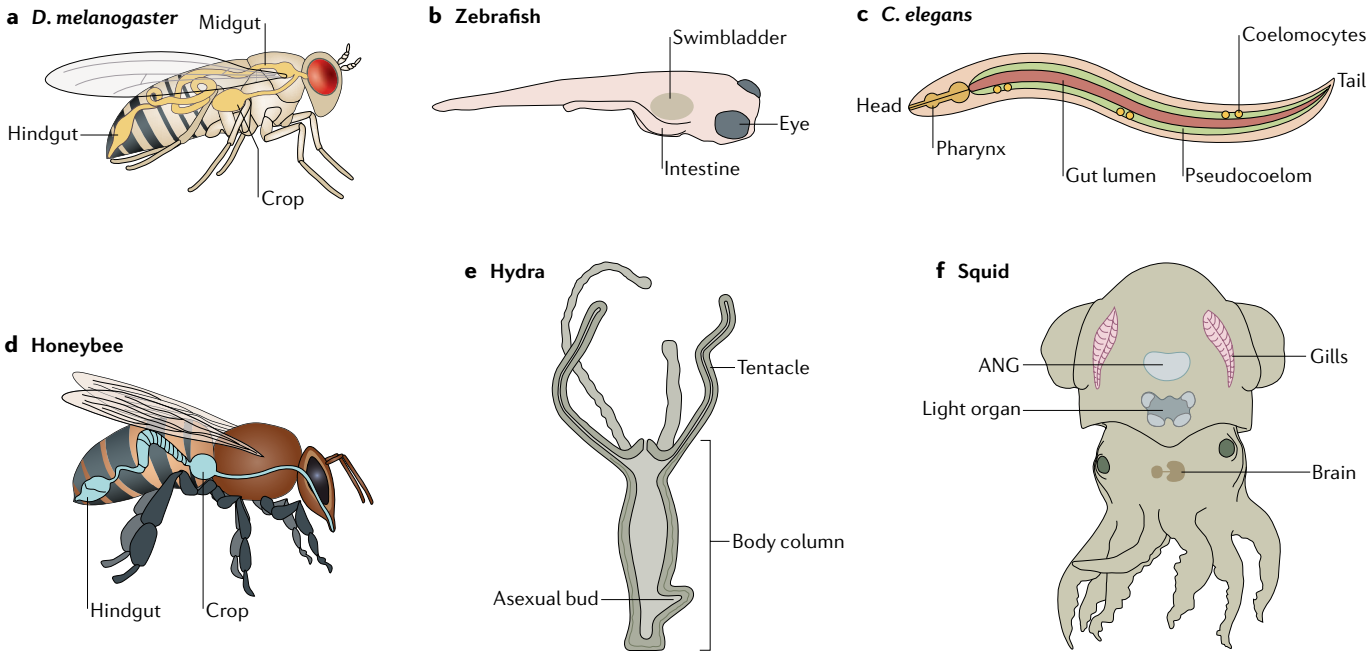

Fig. 1 | **Simple animal models for microbiome research.** Traditional biomedical models and alternative systems are being used to study microbiome–host interactions. **a** | In *Drosophila melanogaster* (vinegar fly), bacteria and yeasts colonize the gut lumen of adults (shown) and larvae. Microorganisms localize to the midgut and hindgut of adults and larvae and adult crop (diverticulum of foregut) (*D. melanogaster* larvae lack crop). **b** | In zebrafish (*Danio rerio*), bacteria colonize the gut lumen of larvae and are retained throughout the life of the fish. Most microbiome research projects use transparent larvae (5 days post-fertilization larva is shown). **c** | In *Caenorhabditis elegans* (nematode), bacteria colonize the gut lumen of juvenile and adult worms. **d** | In the honeybee (*Apis mellifera*), bacteria and yeasts colonize the gut lumen, especially crop and the hindgut of adult workers (shown). The gut microbiota of larval honeybees is poorly defined, and the gut microbiome of male (drone) and reproductive female (queen) honeybees is little studied. **e** | Bacteria colonize the external surface of the hydra (*Hydra vulgaris*) body column and tentacles, and are transferred to the asexually produced buds (shown) and surface of sexually produced eggs (not shown). **f** | A single bacterial species (*Vibrio fischeri*) derived from the water column colonizes the light organs of juvenile Hawaiian bob-tailed squid (*Euprymna scolopes*). This inoculum is retained throughout the squid lifespan. Various different bacteria colonize the accessory nidamental gland (ANG).

Daphnia[4], the medicinal leech *Hirudo medicinalis*[5] and the sea anemone *Nematostella vectensis*[6].

In this Review, an overview of simple animal models for microbiome research, including their value and limitations as models, is first provided. Then, the contribution of these models to the two major topics of microbiome research is examined, including the factors that shape the taxonomic composition of the microbiome and how the microbiome, and individual microbial taxa, influences host traits, focusing particularly on the metabolic and immunological status of the host and host behaviour. The Review concludes by considering the most effective strategies to use simple animal models in animal microbiome research.

Overview of simple animal models

Traditional simple animal models. Microbiome research on *Drosophila*, *C. elegans* and zebrafish (FIG. 1a–c) is facilitated by the wealth of resources, including standardized laboratory protocols and many tools for genetic manipulation, already developed by the wider research community (TABLE 1). In addition, microbiome-related discoveries can be connected to prior information about the immunity, nutrition, behaviour, etc. of the animal. Most important of all, these traditional models are amenable to the core procedures for experimental analysis of animal–microbiome interactions: to obtain axenic hosts (that is, without the microbiota) and resynthesize associations with standardized microorganisms to generate gnotobiotic animals (TABLE 1).

Large numbers of axenic hosts can be generated by surface-sterilizing eggs, commonly using bleach, and raising the resultant animals in sterile dishes or tubes[7–9]. To obtain gnotobiotic animals, the culture medium is supplemented with the desired microorganisms derived from gut homogenates, faecal pellets or cultured microbial strains[7,10]. Axenic *Drosophila* can be maintained through multiple generations, and likely indefinitely, on nutrient-rich media. By contrast, most experiments involving axenic *C. elegans* and zebrafish are restricted to young larvae. *C. elegans* needs bacteria for sustained growth and development, although this requirement can be alleviated somewhat by supplementing the medium with artificial liposome nanoparticles[11]. Axenic zebrafish can be reared to adulthood, but the procedure is costly and labour intensive. The problem relates to feeding the axenic zebrafish after the yolk sac is depleted (~8 days post-fertilization): sterilized commercial fish food is toxic for axenic zebrafish, and live food, for example, sterile members of the genus *Tetrahymena*[9],

is time-consuming to produce and administer. Long-term experiments on axenic zebrafish also require rigorous procedures to exclude microorganisms, ideally using isolators equipped with filtered air under positive pressure[12].

Zebrafish larvae and all life stages of *C. elegans* have the important advantage that they are transparent, enabling ready visualization of fluorescently labelled microorganisms in the gut. Tracking the spatiotemporal distribution of gut bacteria in real-time is further facilitated in the zebrafish system by the application of light-sheet microscopy, which allows for imaging over large fields of view and focal depth with minimal phototoxicity to the fish[13].

Special consideration needs to be given to the design and interpretation of microbiome experiments for *Drosophila* and *C. elegans* because these hosts are microbivores, that is, many of the microorganisms taken in the gut are digested as food items, making a quantitatively important contribution to host nutrition. The natural diet of *Drosophila* and *C. elegans* is dominated by microorganisms that mediate the decomposition of fruits and other decaying plant material[14,15]. By contrast, zebrafish are omnivores, feeding on aquatic insects, crustaceans and plant material[16]. The standard protocols for laboratory culture of *Drosophila* and *C. elegans* administer microorganisms as food. Dead yeast is an important component of most laboratory *Drosophila* diets, and *C. elegans* is maintained routinely on an *Escherichia coli* strain that is killed and digested in the worm gut[17]. Nevertheless, viable microorganisms can be isolated routinely from the gut of both species[17–19] and some bacteria persist, and often proliferate, for extended periods in the gut of *Drosophila*[20–22]. For the design of many microbiome experiments in *Drosophila* and *C. elegans*, it is important to consider how the host interacts with living and food microorganisms.

Alternative simple animal models for microbiome research. Some alternative animal models are better suited than the traditional models to address certain questions. The hydra and squid models provide the opportunity to study interactions between bacteria and animal epithelia that are more accessible than the gut epithelium of most animals[23,24]. The honeybee *A. mellifera* is ideally suited to investigate the effects

Box 1 | **Choosing simple animal models for microbiome research**

Drosophila melanogaster (hereafter *Drosophila*), *Caenorhabditis elegans* and the zebrafish (*Danio rerio*) were developed primarily as genetic models, for example, to study development, neurobiology and behaviour, and genetic diseases, including cancer, founded on their ease of laboratory maintenance, short generation time and high fecundity (see the table). Being a vertebrate, the zebrafish has particular value as a biomedical model, even though its generation time is considerably longer than for *Drosophila* and *C. elegans*.

These traditional models are increasingly being adopted for microbiome research as the parallels in patterns and processes of host–microbiome interactions across the animal kingdom are becoming apparent[27,28]. Some alternative models of microbiome research have a similar historical basis. For example, hydra was initially adopted as a model to study regeneration and morphogenesis[127], but its anatomical simplicity makes this system ideal for research on host–microbiome interactions at the cellular level (rather than the organ level)[24]. Alternative models can also offer unique opportunities to address specific topics in microbiome research, despite the disadvantages of relatively long generation times (see the table) and constraints on laboratory culture (TABLE 1). Notably, the association between the sepiolid squid *Euprymna scolopes* and the bacterium *Vibrio fischeri* has been developed specifically to investigate host interactions with beneficial microorganisms. The key strength of this system is its exquisite specificity for a single bacterium, *V. fischeri*, which has the readily detectable functional trait of luminescence[23], facilitating research on the molecular processes underlying bacterial colonization of animal epithelia. Economically important animals can also yield fundamental insights into host–microbiome interactions. For example, the honeybee commands a wealth of scientific and technical information linked to its importance for honey production and pollination services, and is emerging as a valuable model for the study of microbiome interactions with pesticides and other xenobiotics, and microbiome correlates of complex behavioural traits.

Model	Time to adulthood (pre-adult stages) under standard laboratory conditions	Reproductive output per adult
Drosophila (dipteran fly)	12–16 days (eggs, three larval stadia and pupa with complete metamorphosis to adult fly)	150–900
C. elegans (nematode worm)	3–4 days (egg and four larval stages (L1–L4); under harsh conditions, L1/L2 transform into dauer (diapause), which can survive for up to 4 months	100–300
Zebrafish (cyprinid fish)	90–100 days (egg, larva and pre-reproductive juvenile)	Up to 200 eggs per week, with reproductive lifespan of up to 3.5 years
Honeybee (social hymenopteran insect)	21 days for worker (egg, five larval stadia and pupa with complete metamorphosis to adult)	Queen (one per colony) and 200,000 eggs over 2–5 years
Hydra (hydrozoan coelenterate)	Not applicable	0.1–0.15 asexual buds produced per day
Squid (cephalopod mollusc)	80–90 days (egg and juvenile)	100–200

Table 1 | **Strengths and limitations of simple animal models for microbiome research**

Animal host[a]	Dominant microbial partners	Strengths[b]	Limitations
Drosophila melanogaster (*Drosophila*, also known as vinegar fly; ancestrally tropical Africa, now cosmopolitan as a human commensal)	Acetobacteraceae, Lactobacillales and Enterobacteriaceae; ascomycete yeasts: Saccharomycetales[7,14,129]	• Conventional hosts that are readily maintained through the life cycle in the laboratory • Axenic *D. melanogaster* can be generated in large numbers and maintained through multiple generations on nutrient-rich food • Microbial partners are culturable in vitro • Superb resources for genetic manipulation of host and representative members of the genus *Acetobacter* and the genus *Lactobacillus* symbionts amenable to genetic manipulation	Methods for genetic transformation for some microbial partners have not been developed
Danio rerio (zebrafish; shallow streams in the Indian subcontinent)	γ-Proteobacteria (e.g. Aeromonadaceae, Enterobacteriaceae, Shewanellaceae and Vibrionaceae), β-proteobacteria (e.g. Comamonadaceae), α-proteobacteria (e.g. Rhizobiaceae and Rhodobacteraceae) and Firmicutes (e.g. Staphylococcaceae and Streptococcaceae)[9,12,16,130,131]	• Conventional hosts readily maintained through the life cycle in the laboratory • Axenic eggs can be produced in large numbers for experiments on non-feeding larvae over 7–9 days • Microbial partners are culturable in vitro • Host and bacterial partners amenable to genetic manipulations	Time-consuming and costly to raise axenic zebrafish to adulthood
Caenorhabditis elegans (*C. elegans*; cosmopolitan but most abundant in temperate regions)	α-Proteobacteria (e.g. Acetobacteraceae), γ-proteobacteria (e.g. Lactobacillales, Pseudomonadaceae and Enterobacteriaceae) and Firmicutes (e.g. Lactobacillaceae)[8,10,15,85]	• Conventional hosts readily maintained through the life cycle in the laboratory • Axenic *C. elegans* can be generated in large numbers • Bacterial partners are culturable in vitro • *C. elegans* amenable to genetic manipulation	*C. elegans* require bacteria for sustained growth and development. Methods for genetic transformation of native bacterial partners have not been developed. Protocols developed for bacteria isolated from *D. melanogaster* are likely suitable (perhaps requiring some optimization) for the taxonomically similar bacteria in *C. elegans*
Apis mellifera (honeybee; domesticated in Middle East 4,000 years ago and maintained by humans worldwide)	Various, including *Lactobacillus*, *Bifidobacterium*, β-proteobacteria and γ-proteobacteria[18,37,132]	• Bacterial partners are culturable and amenable to genetic manipulation • Newly emerged adult workers are naturally microorganism-free (or nearly so) and are readily colonized by bacterial partners, facilitating short-term experiments on axenic and gnotobiotic bees	• Facilities and beekeeping expertise are required to maintain honeybee hives, although micro-colonies of queen-free workers can be maintained in the laboratory for limited periods • Long-term cultivation of axenic honeybees is unrealistic, although axenic adult workers can be produced for short-term experiments • The honeybee is not amenable to genetic manipulation, although RNA interference can be used to investigate responses to expression knockdown of specific genes
Hydra vulgaris (hydra; freshwater ponds, cosmopolitan)	Burkholderiales, including members of the genus *Curvibacter* and the genus *Duganella*[24]	• Conventional hosts can be maintained indefinitely in the laboratory, with asexual budding and sexual reproduction • Hydra is amenable to genetic manipulation via sexual eggs • Bacterial partners are readily culturable in vitro	• It is time-consuming and technically demanding to feed axenic hydra aseptically, and so most experiments on axenic hydra use starved animals • Protocols for genetic manipulation of the bacterial associates of hydra have not been developed
Euprymna scolopes (Hawaiian bob-tailed squid; shallow coastal waters in Hawaii)	Luminescent *Vibrio fischeri*[23]	• Adult squid can be collected from natural populations and maintained for several months, producing many eggs that hatch into juveniles lacking *V. fischeri* • *V. fischeri* is readily culturable and amenable to genetic manipulation	• It is time-consuming and technically demanding to raise the squid through the life cycle in the laboratory • Methods for genetic manipulation of the host have not been developed

[a]The commonly used name and natural distribution of the animal are indicated in parentheses. [b]Strengths are indicated where standardized, cost-effective protocols are available.

of environmental stressors (pesticides, antimicrobials, etc.) on the microbiome because the health of honeybee populations is already the focus of intensive research[25]. Additionally, it provides unique opportunities to investigate interactions between the microbiome and complex behaviour[26].

Interest in members of the genus of the freshwater polyp hydra as a model arises from its basal position in the animal kingdom. Hydra are members of the phylum Cnidaria (also including jellyfish and corals) and comprise just two epithelial layers with a blind-ended gut and nerve net, but no defined organs. Research on these basal animals should provide unique insights because their limited morphological (and likely molecular) divergence from ancestral animals predicts similar interactions with microorganisms to those displayed by ancestral animals, and these basal interactions may be conserved across the animal kingdom[27,28]. The hydra microbiota is localized on the extracellular glycocalyx on the external surface of the polyp and can be cultured readily[29]. Axenic hydra can be generated by antibiotic treatment for 2–3 weeks, and bacteria from long-term cultures or homogenates of conventional hydra readily colonize the external surface of axenic individuals, generating gnotobiota hydra[30]. Similar to zebrafish (see above), long-term maintenance of axenic hydra is constrained by difficulties in providing sterile food, and most experiments involving axenic hydra use animals that have not been fed for up to 3–4 weeks[24]. In the squid model *Euprymna scolopes*, the biological context of the interactions between bacteria and a non-gut animal epithelium is morphologically complex and functionally specialized, comprising a pair of light organs containing luminescent bacteria[23]. The epithelium in each light organ comprises three blind-ended crypts that harbour the luminescent bacteria and are connected via a common duct to the surface. The bacteria in the crypts are a single species, *V. fischeri*, which is readily culturable and amenable to genetic manipulation. The light organ of newly hatched squid is bacteria free, and is colonized by *V. fischeri* from the water column. For experimental studies, adults are collected from shallow waters and cultured in aquaria with running sea water in the laboratory, where they produce thousands of juveniles. Experimental studies focus on adults bearing a natural *V. fischeri* inoculum and the colonization of juveniles by cultured *V. fischeri*, including fluorescently labelled strains suitable for study by confocal microscopy[31]. However, culture of the squid through its life cycle is technically difficult on a routine basis[32]. The recent sequencing of the squid genome[33] and anticipated development of genome editing, including gene knockouts using CRISPR–Cas technologies, will further extend the tractability of this model.

The honeybee offers a cost-effective route to address the effects of anthropogenic factors on the relationship between microbiome status and the host health. There is global concern about honeybee health, with drastic population declines associated with pesticides, antimicrobials and various disease agents[25,34]. Impaired bee health has been linked with perturbations to metabolism, immune function, behaviour and the microbiome[35], analogous to the putative links between microbiota loss and chronic metabolic, immunological and neurological diseases in humans[1,36]. However, honeybees are not a straightforward laboratory system[26]; they are best maintained as outdoor hives in habitats with flowering plants. Although not costly to maintain, beekeeping skills are essential. Axenic adults can be generated by isolating pupae (which are naturally sterile) under aseptic conditions; once they reach adulthood, the bees can be fed on bacteria and either studied in the laboratory or marked (for example, with paint) and returned to the hive. Isolates of gut bacteria are available in long-term culture, and several are amenable to genetic manipulation[37]. Genetic transformation technologies are not practical for the honeybee because each hive supports a single reproductive queen, but protocols for RNA interference-mediated gene expression knockdown are available.

Determinants of microbiome composition

The taxonomic composition of the microbiome associated with the gut or external surface of most animals is variable. Despite reproducible differences in microbiota composition among hosts of different developmental age or reared on different diets, the basis for much of the variation between host individuals or within one host over time is not well understood.

Extensive research on associations involving single microbial taxa has identified a predominant role of host control over the identity of its microbial partners, mediated largely by immune effectors and other bioactive molecules[38]. The exceptional tolerance of *V. fischeri* for high nitric oxide levels in the ducts leading to the squid light organ contributes to the high specificity of this association[39] and variation in the low diversity bacterial communities associated with *Hydra* species is attributable largely to species-specific profiles of the arminin family of antimicrobial peptides[30]. Mutant studies of other simple animal models reinforce the central importance of immune-mediated host control, although host-derived nutrients and mechanical factors may also contribute[40,41]. Gut-residing *Aeromonas veronii*, which supports the healthy development of wild-type zebrafish larvae, proliferates rapidly and kills mutant larvae that lack a key immune cell — the neutrophil[42]. Similarly, genetic ablation of transforming growth factor-β/bone morphogenetic protein-dependent immunological signalling in *C. elegans* leads to overgrowth of *Enterobacter* species that are beneficial for wild-type worms[43] and transcriptional knockdown of the gut transcription factor *caudal* in *Drosophila* perturbs gut antimicrobial peptide production, resulting in excessive proliferation of the gut bacterium *Gluconobacter morbifer* and early host death[44].

There is a growing recognition, however, that the animal host has weaker control over the composition of taxonomically diverse microbiota than in systems involving one or a few taxa (FIG. 2). Many animal hosts are relatively permissive, somewhat akin to an environmental filter, and the microbiota composition in any individual animal at a specific time may be shaped by processes that are independent of, or weakly influenced by, host factors[40,45,46]. Simple animal models, particularly

Blind-ended gut
The gut has a single opening to the exterior, through which the food is ingested and, following digestion and absorption, waste is egested.

Nerve net
Two-dimensional lattice of neurons connected by synapses that includes sensory, motor and integrative elements, and transmits impulses in all directions.

Glycocalyx
Extracellular matrix of glycoprotein and glycolipid bounding the external surface of many cells.

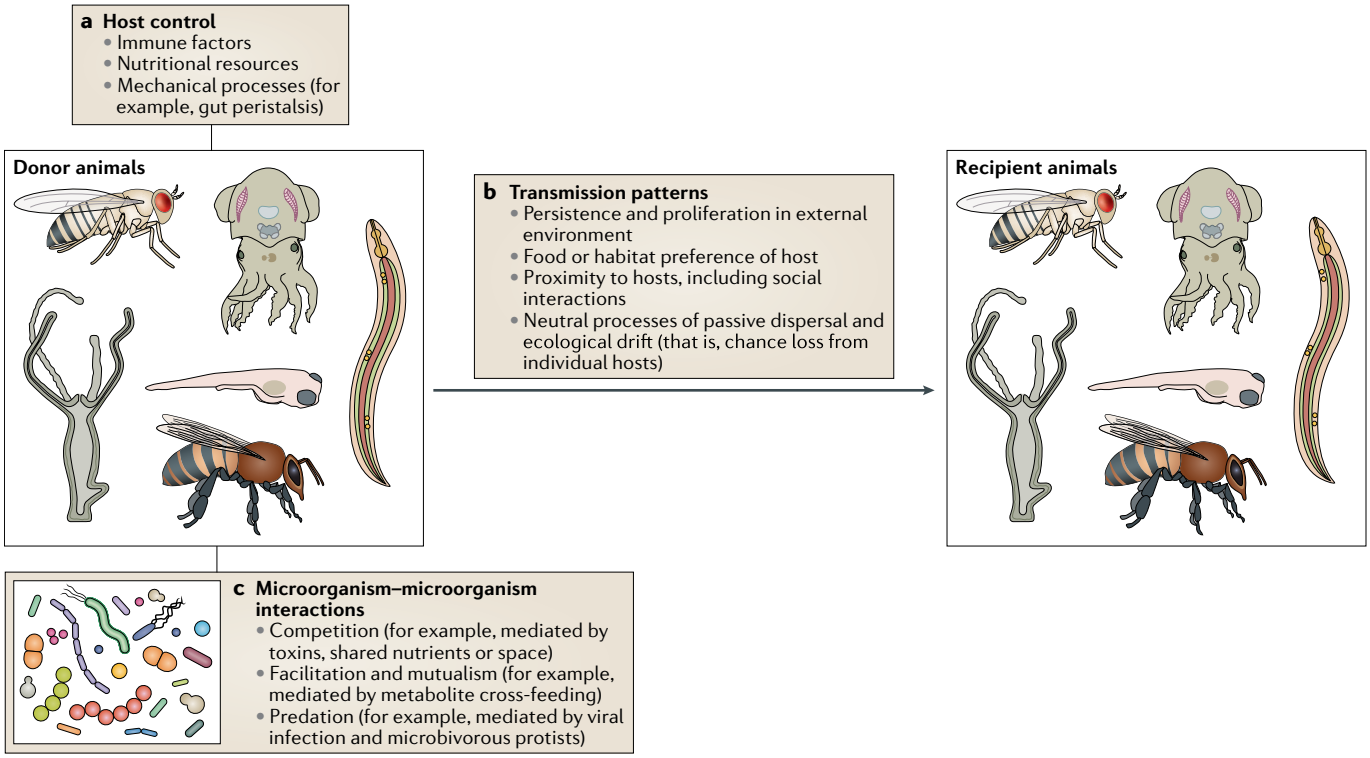

Fig. 2 | **Determinants of microbiota composition in animal hosts.** Three broad categories have been identified: host control (part **a**), transmission patterns (part **b**) and microorganism–microorganism interactions (part **c**). Host controls and microorganism–microorganism interactions (competition, mutualism, etc.) operate at the level of the individual host. Host controls are diverse and vary between host taxa; they include immune factors, the availability of nutrients as influenced, for example, by diet choice and host metabolism, and mechanical factors (for example, gut peristalsis favours the loss of microbial cells that are planktonic or associated with food particles). Transmission patterns confer population-level and community-level influences on the microbiota composition of individual hosts because the availability of microorganisms to an individual host is influenced by their distribution and abundance in the external environment and the behaviour of the host (including proximity to other host individuals who are shedding microorganisms). In many host populations, the abundance and prevalence of many microorganisms conform to the predictions of neutral assembly dictated by ecological processes that are independent of the traits of the microorganism.

Drosophila, are making an important contribution to this area of research because associations with different combinations of taxa are readily constructed and analysed.

There is now overwhelming evidence that the abundance of bacterial taxa in the *Drosophila* gut is influenced by inter-microorganism interactions, particularly between *Acetobacter* and *Lactobacillus* species. In experimental associations with two taxa, the abundance of *Acetobacter* species in the flies is consistently promoted by co-association with members of the genus *Lactobacillus*[47]. This effect is abolished in members of the genus *Acetobacter* with mutated gene *ppdk*, encoding pyruvate phosphate dikinase, which mediates the first step in gluconeogenesis from pyruvate, suggesting that *Lactobacillus* species fermentation products may be cross-fed to *Acetobacter* species[48]. In a larger experimental design involving five *Acetobacter* and *Lactobacillus* species associated in all 32 possible combinations (from mono-associations to the full five-taxa association), negative effects of co-association on the abundance of individual bacterial species predominated, especially in associations involving multiple taxa[49], consistent with the prediction that competitive interactions become more prevalent in communities of increasing diversity[41]. This conclusion is supported by studies of the microbiome composition of conventional *Drosophila* in laboratory cultures, with evidence that individual taxa of bacteria co-occur less frequently than expected by chance[50].

In *Drosophila* and other simple animal models, the effects of host control and inter-microorganism interactions are increasingly recognized as insufficient to explain the substantial between-host variation in microbiota composition. Much of the observed variation appears to be stochastic[22,51–54]. In other words, the processes determining microbiome composition can be largely independent of the traits of the microbiota or host, and comprise passive dispersal (between hosts and from environment to host) and the chance loss of a microbial taxon from the host. The significance of passive dispersal is reinforced by experiments on zebrafish larvae, in which the difference between the microbiota of wild-type and an immune-deficient mutant (*Myd88*$^{-}$) strain was abolished when the two fish strains were co-housed, enabling bacterial transmission between the fish[55].

These studies on simple animal models suggest that the determinants of microbial community assembly include between-host transmission dynamics and

microbial population processes in the external environment (FIG. 2). These results are fully congruent with evidence from mammal studies that microbiome composition can be predicted from social interactions, for example, in free-ranging baboons, chimpanzees and humans[56–58]. Sustained research on simple animal models has great potential to elucidate these processes, especially in the context of social behaviour of variable complexity, including shoaling in zebrafish[16], aggregative feeding in *Drosophila*[59] and highly structured inter-individual interactions in the honeybee[25].

The microbiome and animal traits

General principles. Simple animal models are superbly suited to investigate the processes underlying correlations between the presence or composition of the microbiome and host traits of interest. Causality can be inferred from comparisons of host traits across conventional animals, axenic animals and gnotobiotic animals bearing different microbial taxa. Furthermore, the amenability of these models to complex experimental designs and sophisticated genetic screens facilitates mechanistic analyses, and the opportunity for experimental evolution studies enables analysis of the proximate and ultimate processes underlying host–microbiome interactions[60–62]. Complementing these approaches, the availability of mutant libraries for various bacterial taxa has facilitated identification of microbial products mediating microbiome-dependent host traits[48,63–67].

There is the strongest expectation and, to date, evidence that host–microbiota interactions are biochemical, generally mediated by the release of bioactive molecules (metabolites, proteins, lipids and small RNAs) from microbial cells, although we cannot exclude the possibility that biomechanical forces (for example, forces exerted by microbial biofilms) and electrical effects[68] may also play a role. Some microbial products contribute directly to host metabolism and nutrition. For example, microbiota-derived B vitamins are required for *Drosophila* survival on nutrient-poor diets[69,70], microbiota-derived riboflavin (vitamin B2) promotes healthy feeding and growth in *C. elegans*[71], and honeybees derive supplementary carbon from fermentation products released from hindgut bacteria that degrade complex cell wall polysaccharides of ingested pollen grains[72]. Microbial products can also function as info-chemicals that modulate the host regulatory circuits controlling host traits. For example, normal proliferation of insulin-producing β-cells in the developing pancreas of larval zebrafish is dependent on a specific protein (BefA) derived from bacteria of the genus *Aeromonas* in the zebrafish gut[73]. Homologues of BefA are encoded in the genomes of various bacteria, including members of the human gut microbiota[73]. In the squid model, peptidoglycan cell wall fragments released from proliferating *V. fischeri* cells in the light organ crypts are key info-chemicals leading to the developmental maturation of the light organ[74].

The production of some microbial products may be influenced by interactions with other taxa in the microbial community. In these circumstances, microbiome-dependent host traits are not obtained by associations with any single microbial taxon because the microbial determinant is the product of metabolite cross-feeding or other interactions between microorganisms. This is illustrated elegantly by two different microbiome-dependent traits of hydra. Both the regular pattern of body wall contractions in conventional hydra and the resistance of conventional hydra to a fungal pathogen *Fusarium* sp. can be recapitulated by colonizing axenic hydra with a mixture of bacteria representative of the natural microbiome, but not by any individual bacterial taxon[75,76]. In some associations, the inter-microorganism interactions underlying microbiome-dependent host traits are characterized. For example, the metabolite acetoin (a ketone, also known as 3-hydroxybutanone), which stimulates aggregation of *Drosophila* larvae, is produced by cross-feeding of sugar fermentation products between yeasts, lactobacilli and Acetobacteraceae, the three major taxonomic groups contributing to the gut microbiota[77,78]. However, the scale and significance of metabolic cross-feeding are not necessarily extensive in microbiomes, as illustrated by the honeybee, where the metabolic function of the hindgut microbiome comprises the sum of traits obtained with mono-associations with the various bacterial taxa[72].

The burgeoning literature for microbiome effects on animal traits demonstrates that these interactions can be pervasive and often strongly context dependent. Simple animal models are ideally suited to analysis of these complexities because they are amenable to controlled and sophisticated experimental designs. Extensive studies, especially using *C. elegans* and *Drosophila*, have revealed that the presence and composition of the microbiota can have substantial effects on host growth, reproduction and lifespan, varying with host genotype and physiological condition, diet, temperature and other factors[17,49,79–81]. Increasingly, these studies of microbiome effects on host fitness traits are being complemented by research on the underlying physiological processes. In the following sections, some of these studies are reviewed from the perspective of three host physiological traits: the immune system, metabolism, and neurobiology and behaviour.

The immune system. The evidence for microbiome effects on animal immune function comes from two approaches[27]. First, axenic animals generally display reduced expression of immune effector genes and have a heightened susceptibility to microbial pathogens, relative to conventional animals. Second, the immune responsiveness of the animal host varies with the composition of the microbiota; certain microbial taxa or communities may promote inflammation and disease, whereas others promote immune tolerance and host health[82,83]. There is currently intense interest to discover novel bioactive molecules that interact with the host immune system, either to promote immune responses against pathogens or to reduce inflammation. Simple animal models are valuable to identify microbial immunomodulatory molecules and to investigate the underlying mechanisms of action, especially where associations are constructed with single microbial taxa. However, findings cannot be applied precisely to mammals because invertebrates lack

Shoaling
Staying together as a group while swimming, for example, in fish.

Aggregative feeding
Feeding in a group.

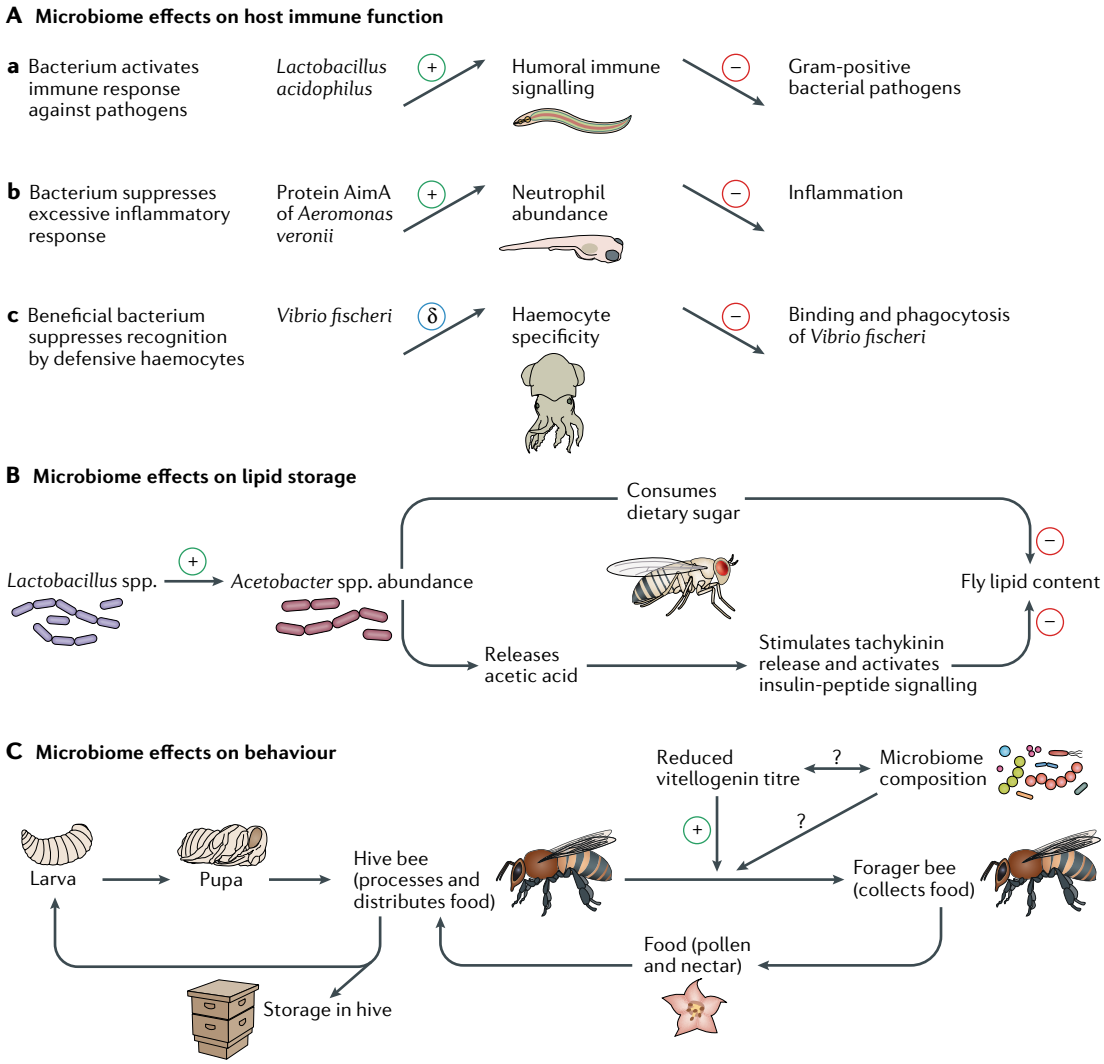

Fig. 3 | **The impact of the microbiome on host traits. A** | The microbiome influences host immune function, for example, in *Caenorhabditis elegans*, *Lactobacillus acidophilus* reduces Gram-positive bacterial pathogens by promoting the humoral immune systems[128] (part **Aa**). In zebrafish, the protein AimA of *Aeromonas veronii* suppresses the abundance of pro-inflammatory immune cells called neutrophils[42] (part **Ab**), and in the Hawaiian bob-tailed squid, the bacterial symbiont *Vibrio fischeri* modifies the specificity of squid haemocytes, such that *V. fischeri* is not targeted by these immune cells[87] (part **Ac**). **B** | Lipid storage in *Drosophila melanogaster* is reduced by some strains of *Acetobacter*, with two processes identified: the consumption of dietary sugar and activation of insulin-like peptide signalling via release of the neuropeptide tachykinin; the *Acetobacter* populations are promoted by some *Lactobacillus* strains[48,66,90,91]. **C** | Behaviour of adult honeybee workers, with particular focus on the behavioural transition from food processing in the hive (hive bee) to food collection from the external environment (forager bee). A shift in gut microbiome composition is correlated with (and potentially contributes to) this behavioural transition[117,118]. +, have positive effect; –, have negative effect; δ, change in effect.

an adaptive immune system, and adaptive immunity of zebrafish is not developed in the larvae used in most microbiome studies.

Protocols are now well established to investigate how the microbiome promotes the immune responses of *C. elegans* against pathogens. In particular, experiments using mutant panels of *C. elegans* indicate that multiple signalling pathways, including p38 MAPK, are required for protection by *Lactobacillus* and *Enterobacter* species against Gram-positive pathogens (FIG. 3Aa), and also by *Pseudomonas mendocina* against *Pseudomonas aeruginosa*[84]. Furthermore, these interactions may be co-evolved: *Enterobacter cloacae* isolates from *C. elegans* and the related nematode *Caenorhabditis briggsae* protect their native host from the pathogen *Enterococcus faecalis*, but are not protective when experimentally associated with the non-native host[85]. Research on the underlying protective mechanisms is facilitated by methods for high throughput screening of the immune function of *C. elegans* colonized with different bacteria, together with the very considerable taxonomic and functional diversity of the gut microbiota in natural *C. elegans* populations[10,85,86].

Interactions between the gut microbiota and the cellular immune system have been studied in the zebrafish and squid models. For zebrafish, the primary focus is one class of innate immune cells, the neutrophils, which mediate a pro-inflammatory response (FIG. 3Ab). Elevated neutrophil counts in the host gut are induced by

a mutant of the bacterium *A. veronii* that lacks a functional type II secretion system (T2SS), suggesting that T2SS-competent *A. veronii* release a protein or proteins with an immunomodulatory function[42]. This observation led to the identification of an *A. veronii* protein (AimA) that is structurally similar to mammalian lipocalin-2 with a known immunomodulatory function. The essential role of AimA was demonstrated using *aimA* deletion mutants: zebrafish colonized with these bacteria displayed increased gut neutrophil counts, pathological inflammation and high mortality[42]. The squid immune cells of interest are phagocytic haemocytes that circulate in the blood and infiltrate the light organ housing the luminescent *V. fischeri* (FIG. 3Ac). Adult squid treated with antibiotics to eliminate the bacteria bind *V. fischeri* avidly, whereas haemocytes from untreated squid are unresponsive[87]. The implication that *V. fischeri* likely releases anti-inflammatory compounds that alter haemocyte function is supported by transcriptomic and proteomic evidence for differences in immune-related gene products between the haemocytes of antibiotic-treated and untreated squid[88,89]. The ready availability of *V. fischeri* mutants will facilitate molecular dissection of this highly specific interaction.

Metabolism. Simple animal models are making an important contribution to understanding the impact of the gut microbiome on metabolic health, especially in relation to metabolic syndrome (obesity, impaired control over glucose and cardiovascular disease) induced by feeding on high lipid or high sugar diets. The gut microbiome of *Drosophila* is protective against obesity, with evidence that gut bacteria of the genus *Acetobacter* reduce the lipid content of flies, especially on sugar-rich diets[47,66]. Two contributory processes have been identified: bacterial consumption of dietary sugar, thereby reducing the calories gained by the flies[90], and bacterial release of the short-chain fatty acid acetic acid, which promotes lipid mobilization via increased production of the peptide hormone tachykinin from gut enteroendocrine cells, leading to increased insulin signalling[66,91]. The value of *Drosophila* to study microbial determinants of metabolic health is enhanced by its well-established use as an obesity model[92,93], and by the immediate relevance of the microbiome effects in *Drosophila* to the known contributions of microbial short-chain fatty acids in reducing weight gain and promoting glucose homeostasis in mice and humans[94]. However, *Drosophila* differs from mammalian systems in one important respect. Elimination of the gut microbiota promotes energy storage in *Drosophila*[47], but germ-free rodent models are lean, even when fed on high-fat diets[95]. An important determinant of the lean phenotype of germ-free mice is impaired digestion and assimilation of dietary lipid in the small intestine, with depletion of lipid reserves in the intestinal epithelium[96]. Axenic zebrafish larvae display an equivalent dysfunction in assimilation of dietary lipid[97], whereas axenic *Drosophila* have the reverse trait of an excessive lipid load in the gut epithelium[91]. Investigation of the factors contributing to these differences should improve our overall understanding of the underlying mechanisms.

Simple animal models can also contribute to understanding the relationship between microbiome composition, diet and metabolic health of the host. As also found in the mouse[96,98], the microbiome composition is altered by high-fat or high-sugar diets in zebrafish[99] and *Drosophila*[100], and the composition of the microbiota can influence lipid deposition in *Drosophila*[47,101] and *C. elegans*[102]. Furthermore, probiotic members of the genus *Lactobacillus* are protective against hyperlipidaemia and hyperglycaemia in these models. To date, different mechanisms have been identified in the various systems. Members of the genus *Lactobacillus* promote the populations of bacteria that reduce host lipid content in *Drosophila*[47,48] (FIG. 3b) and depress the expression of host genes involved in lipid metabolism in zebrafish[103,104].

Neurobiology and behaviour. A major impetus for the study of microbiome effects on animal behaviour is the potential of microbial therapies for the treatment of neurodevelopmental and neurodegenerative diseases and mental illnesses in humans[105,106]. Research has focused largely on the gut microbiome, with the expectation that metabolites released from gut microorganisms may influence the enteric nervous system or alter brain function by affecting the activity of nerves linking the gut and brain or by transfer via the blood system to the brain. Simple animal models can make major contributions to this field because they offer high throughput behavioural assays linked to genetic and biochemical dissection of mechanisms.

Elimination of the gut microbiome causes locomotor hyperactivity in the mouse[107], zebrafish[108,109] and *Drosophila*[110]. In *Drosophila*, hyperactivity is linked to activation of octopaminergic neurons in the brain, and is reduced by the gut bacterium *Lactobacillus brevis*. Surprisingly, the bioactive molecule in *L. brevis* is not a small metabolite, but rather a sugar-converting enzyme, xylose isomerase, that mediates reduced titres of the main insect blood sugar, trehalose[110].

Drosophila is also providing insights into the microbial determinants of social behaviour. Microbial volatiles, especially acetoin and acetic acid, have been implicated in larval aggregation and egg laying by females, respectively[77,78]. The production of these small molecules is likely promoted by cross-feeding of metabolic intermediates between different microbial taxa[77], with the implication that *Drosophila* may be using these products as a cue for a microbial community rather than for a specific microorganism. Gut bacteria have also been implicated in *Drosophila* mate choice, with evidence that flies prefer mating partners that contain the same bacterial taxa, perhaps as a consequence of microbial effects on the hydrocarbon profiles of the fly cuticle[111]. Although these results are not universally reproducible[112,113], the indication that gut microorganisms may influence the composition of cuticular hydrocarbons deserves further investigation because these surface molecules play an important role in social interactions in *Drosophila* (and other insects) and are responsive to both the physiological condition of the insect and environmental factors[114,115].

Octopaminergic neurons
Neurons that release the neurotransmitter octopamine.

Vitellogenin
The major yolk protein in animal eggs, also present in the haemolymph (blood) of the non-reproductive worker caste of the honeybee.

Glyphosate
An organophosphorus compound that inhibits the plant enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase, and widely used as a herbicide under the trade name Roundup.

The honeybee has great potential for the study of microbial impacts on various aspects of social behaviour, including the task allocation of workers to hive activities (food processing, feeding of larvae, etc.) versus foraging for nectar and pollen as well as cognition and communication in foraging bees (FIG. 3c). As yet, evidence is fragmentary and largely correlative. For example, a key determinant mediating the transition of hive bees to foraging is declining titres of the protein vitellogenin[116] and *vitellogenin* gene expression is influenced by microbiome composition[117], which also differs between hive bees and foraging bees of the same age[118]. Another intriguing correlation relates to glyphosate, a widely used herbicide in agricultural habitats. Glyphosate both suppresses the gut microbiome[119] and interferes with navigation by foraging workers[120]. The detailed knowledge of the microbiome, together with protocols to generate axenic and gnotobiotic bees and to study worker behaviour[18,37], offers an excellent basis to investigate the causal basis and mechanism of these correlations.

Outlook

How can simple animal models be used most effectively to promote microbiome research? The greatest strength of these systems is as an engine for fundamental discovery. As described in this Review, these model systems are playing a leading role in demonstrating that the taxonomic composition of animal microbiomes is shaped by the several processes of host control, interactions between microorganisms and neutral processes. Similarly, simple animal models are illuminating the multiple ways in which individual microorganisms and microbial communities influence host immune responses and metabolic health, as well as offering simple assays to investigate microbiome effects on animal behaviour. Discoveries made with these systems can be used to construct precise hypotheses of function in less tractable but important associations. Currently, this research strategy has the greatest application to biomedical science, with the opportunity for subsequent hypothesis testing in mammalian models, followed by clinical trials in humans. The data obtained for simple animal models are equally applicable to other emerging applications of microbiome science, including the management of pests[121], species and habitat restoration[122], and improved domestic animal production[123]. These non-biomedical applications will likely become increasingly important aspects of animal microbiome science in the coming years.

Simple animal models can also be used for the reciprocal purpose to investigate the molecular and cellular mechanisms that underpin patterns of host–microbiome interactions already identified in humans or other relatively intractable animals. However, the results cannot provide reliable explanations of mechanism for the human microbiome; the data provided by model animals should only be used to generate hypotheses of function in humans. The need for caution in interpreting results from lower vertebrate and invertebrate models is reinforced by the growing recognition that even microbiome studies on the mouse often fail to translate reliably to humans[124]. In this regard, a key strength of using multiple models is to identify host–microbiome interactions that are conserved and are thus likely to be relevant to humans. Furthermore, there is increasing opportunity to complement all animal models with alternative approaches, including custom bioreactors to study interactions among human microorganisms[125] and gut organoid cultures to investigate interactions between human tissues and the microbiome[126].

Another important question relating the use of simple animal models is: what is the most appropriate model species to use? Beyond the species considered in this Review (TABLE 1), research on many animal species can make meaningful contributions to our general understanding of animal–microbiome interactions[27,28]. In this respect, the research community and funding agencies have the important responsibility to balance the opportunities for novel insights from a diversity of systems against the opportunity cost of investing time and funds for similar discoveries in multiple systems. It is anticipated that many of the major discoveries on the fundamentals of animal–microbiome interactions in the coming years will come from studies of traditional simple animal models, powered by the superb resources and tools that these species command.

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Competing interests
The author declares no competing interests.