The Impacts of Microbiota on Animal Development and Physiology

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Abstract Animals evolved in a world dominated by microbes. While pathogenic microbes have long been appreciated as the cause of infectious diseases, only more recently have we understood that diseases can be caused by a lack of beneficial microbes. Microbial genomic sequencing can provide insights into the vast diversity of microbiomes associated with human health and disease, but experimental animal models are required to test hypotheses about the beneficial or detrimental effects of these microbes and their molecular products. Studies in gnotobiotic animal model systems reveal the aspects of animal biology shaped by our microbial associates and shed light on new possible mechanisms underlying human diseases. Here, we survey insights from the widely used animal model systems in microbiome research. We explore emerging shared themes across these diverse animal hosts about the interconnected impacts of microbiota on immune system maturation, intestinal epithelial homeostasis, nervous system development, endocrine signaling, and metabolic regulation. Research in animal models can provide both the basis for uncovering microbial influences on human health and disease, and also the starting point for developing treatment strategies to correct dysregulation of animal-microbe interactions in disease.

Keywords Microbiome · Gnotobiology · *Drosophila* · Zebrafish · Mouse · Immune system · Metabolism · Nervous system

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1 Introduction

Not only oxygen and water but also microorganisms have been a fixture of the world in which animals evolved. Microorganisms inhabited the earth for some three billion years prior to the emergence of the first simple animals, shaping the earth's atmosphere and generating the oxygenated environment that would allow for the existence of aerobically respiring multicellular organisms [1]. The coexistence of animals and microbes is evident in the genomes of single-celled eukaryotes such as amoebae, which encode extensive repertoires of genes with signatures of innate immune sensing and antimicrobial defenses [2]. The biological properties of extant animals, including humans, are shaped by both their evolutionary history with microbes and by their lifelong associations with microbes in and on their bodies [3]. Understanding the normal functioning of animal-microbial interactions is critical for diagnosing diseases in which these associations go awry.

In this chapter, we consider the experimental frameworks necessary for establishing causative relationships in host-microbe systems. We discuss the nature of the molecules produced by microbes and perceived by animal cells and tissues to modulate developmental decisions and physiological programs. We describe several prominent animal models that have been instrumental in revealing the molecular nature of these relationships. We then discuss lessons learned from these animal models about the roles resident microbes play in the development and function of different animal tissues and systems. Studies in these model animal systems reveal the many facets of animal biology that are shaped by our microbial world and highlight new possible mechanisms that can underlie human diseases.

2 Establishing Causation in Host-Microbe Systems

The traditional focus of medical microbiology has been on diseases caused by the presence of a single specific microbe, which we term a pathogen. Studying the action of pathogens in order to develop strategies to treat and prevent infection requires animal models of those infectious diseases. In the late nineteenth century, Robert Koch developed a rigorous experimental framework for using laboratory animals to test the causative role of specific microbes in infectious diseases. Fulfillment of Koch's postulates in an animal model has become accepted as proof that a specific pathogen is both necessary and sufficient to cause a specific disease in humans. In the late twentieth century, with the advent of microbial molecular genetics, this framework was updated in what was termed "molecular Koch's postulates" [4] to establish the causal role of a specific pathogen toxin or effector molecule in a specific disease.

More elusive has been the understanding of diseases caused not by a single microbe but by disturbances in normal associations with microbes. The concept of disease predisposition due to the absence rather than the presence of specific

microbes was first introduced by David Strachan in 1989 as the "hygiene hypothesis," which posited that allergic diseases could be due to an insufficient stimulation of the immune system in the absence of childhood exposure to infections [5]. This idea was further refined by Martin Blaser and Stanley Falkow as the "disappearing microbiota hypothesis" that proposed the loss of certain ancient members of human-associated microbial communities, or microbiota, as the basis for increased incidence of diseases of immune dysregulation in high income countries [6].

The technological capacity to test these ideas about missing microbes and modern diseases followed in subsequent decades with advances in high throughput sequencing that enabled the comprehensive cataloguing of microbial community genomic sequences, or microbiomes, from human tissues. At first the complexity of these communities was overwhelming, with inter-individual differences dwarfing anticipated signatures of health and disease. As cataloguing efforts have become more comprehensive, with studies of people across the globe and longitudinal studies of individuals over time, patterns have emerged that corroborate the hypotheses that lifestyles of high income countries are associated with reduced human-associated microbial diversity. In addition, signatures have started to emerge of microbiomes associated with different diseases. The term "dysbiosis" was coined to describe disease-associated microbial communities that deviate from normal patterns.

Testing causal relationships between a particular microbial community and a specific disease required new experimental frameworks using animal models that are amendable to microbiota manipulations. For this, researchers turned to the field of gnotobiology in which eukaryotic organisms are grown in the absence of any microbial associations ("germ-free" or "axenic") and then following this sterile derivation, associated with single microbes or defined microbial consortia [7]. Using gnotobiotic animals, researchers could test whether a dysbiotic microbiota was necessary and sufficient for a disease phenotype, using a framework termed "ecological Koch's postulates." [8] Starting with an animal model of a disease with a suspected microbiota etiology, they could test whether deriving the animals germ-free would eliminate the disease symptoms. Conversely, they could test whether a microbial community, harvested from a diseased donor animal or human subject, and transferred to a healthy germ-free recipient animal would confer the disease phenotype in this new host. Such microbiota transplantation experiments have become the standard in the field for establishing the causal relationships between dysbiosis and disease [9].

Evidence that a perturbed microbiota causes a disease does not immediately provide an explanation for how the disease arises. Investigating the mechanistic basis for microbiota-associated disease requires understanding normal microbiota-host interactions in the healthy state. Here gnotobiotic animal models have proved to be invaluable. By studying the properties of animals reared in the absence of their microbial associates, researchers can infer the normal functions these microbes play in animal development and physiology. The same experimental manipulations described in the frameworks of ecological Koch's postulates and molecular Koch's postulates can be employed to test the role of specific microbial communities, microbes, and microbial products in animal development and health.

3 Microbiota-Derived Molecules Perceived by Animals

A major question in the field of microbe-host interactions is the nature of the molecules that modulate animal developmental and physiological programs. The answers now emerging from different experimental models, examples of which are listed in Table 1, provide fundamental insights into animal biology and also suggest new molecular approaches for treatment of human diseases with microbial etiologies. From the studies of bacterial pathogens, using the framework of molecular Koch's postulates, we know of a diversity of microbial molecules that impact animal cells and induce the pathologies of infectious diseases. On one end of the spectrum are generic microbial molecules such as lipopolysaccharide (LPS), the cell wall component of all Gram-negative bacteria that was first discovered as endotoxin based on its capacity to induce many symptoms of infections [10]. On the other end of the spectrum are toxins produced by specific bacterial species or strains that determine their infectious disease pathology, such as the flaccid paralysis caused by Botulinum toxin that cleaves SNARE proteins and inhibits neurotransmitter release [11]. Studies of bioactive molecules from microbiota reveal a similar spectrum of effectors and allow us to understand the nature of the informational exchange between animals and their microbes [3]. On one hand, microbial effectors can be classified as molecular cues, produced for other purposes and perceived by animals to inform them about their microbial residents. On the other hand, these molecules may function as signals specifically produced to communicate with animal cells and elicit responses that are beneficial to the microbial producer.

Clear examples of microbial cues are the generic, microbial-specific molecules like LPS that Charles Janeway and colleagues classified as "Pathogen Associated Molecular Patterns" or PAMPs [12]. Their cognate receptors, such as the LPS binding Toll-Like Receptor 4 (TLR4), were termed "Pattern Recognition Receptors," or PRRs, to describe the innate immune receptors that recognize common microbial molecules. PRRs are ancient and widespread across eukaryotes [2], in contrast to the receptors of the adaptive immune system that are exclusive to the vertebrate lineage of animals. Although these concepts of conserved microbial

Table 1 Different classes of microbial molecules affect host cells

Bioactive microbial molecules Classes and examples	
Microbial molecule	Animal cell receptor or target
Generic microbial associated molecular patterns (MAMPs)	
Lipopolysaccharide (LPS)	TLR4
Generic microbial metabolites	
Short-chain fatty acids (SCFAs)	G protein-coupled receptors (GPCRs)
Species-specific microbial toxins or molecules	
Botulinum toxin	SNARE proteins
Polysaccharide A (PSA)	TRL2/1 heterodimer and Dectin-1

Examples from the classes of microbial molecules and the corresponding host cell receptor or target

detection were transformative, the term PAMPs was a misnomer because molecules such as LPS are not exclusive to pathogens but rather define basic features of microbial cell biology. Identification of bacterial cell wall molecules functioning in beneficial symbioses prompted a rename of these molecules as "Microbial Associated Molecular Patterns" or MAMPs [13]. As discussed below, innate immune reception of such molecules plays important roles in host responses to resident microbiota.

Another example of microbial cues is metabolites that are the products of specific microbial physiologies. These molecules are less generic than MAMPs, and thus metabolite perception can confer information about the identity of the producing microbes, although some metabolites are made by phylogenetically unrelated microbial lineages and through different enzymatic processes. The best studied of these types of molecules are the short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate that are the byproducts of the fermentation reactions of many anaerobic bacteria [14]. The absolute and relative abundances of SCFAs can modulate properties ranging from intestinal barrier function to nervous system activity. These molecules are perceived by G protein-coupled receptors (GPCRs) expressed on many cell types throughout the body. More generally, animal genomes encode large numbers of orphan GPCRs, hormone receptors, and other receptors that are likely involved in detecting microbial metabolites [15].

Fewer examples exist in the microbiome literature that resemble the specificity of bacterial toxins which target particular host receptors or signaling pathways. One such effector molecule, the *Bacteroides fragilis* polysaccharide A (PSA), has potent immunomodulatory activity that can correct immune system immaturity in germ-free mice [16] and behavioral abnormalities in a maternal immune activation mouse model of autism spectrum disorder [17]. Although potent and specific in its effects, PSA is a cue rather than a signal that is produced by *B. fragilis* as a component of its protective capsule and is perceived by receptors of the innate immune system: TLR2/1 heterodimers signaling in parallel with the C-type lectin carbohydrate receptor Dectin-1 [18]. The challenge of identifying microbiota-derived signals, similar to pathogen toxins, may come from the complexity of animal-associated microbial communities. Another possibility is that our perception of bacterial toxins' specificity for animals has been warped by a focus on human infections without considering the ecology of the producing bacteria [19]. For example, the fitness benefit of Botulinum toxin production conferred on the soil bacterium *Clostridium botulinum* is unknown, but more plausibly involves competition with other soil bacteria than intoxication of animals. Similar selective pressures that drive *C. botulinum* to produce its toxin may induce members of animal-associated microbiota to produce specific molecules that happen to have potent and specific collateral effects on their hosts.

4 Gnotobiotic Animal Models

Exploring the impacts of resident microbes and their associated molecules on animal biology requires experimentally tractable gnotobiotic models. Here we provide brief descriptions of several of the prominent gnotobiotic animal systems that have advanced our understanding of the impacts of microbiota (Fig. 1). Each system has its unique strengths. Studies of different animal models complement each other and advance our understanding of the common ways in which microbiota shape animal tissues and the common mechanisms through which animals perceive and respond to their microbial inhabitants.

4.1 The Bobtail Squid Model

The bobtail squid, *Euprymna scolopes*, forms an exclusive symbiosis with the luminescent marine bacterium *Vibrio fischeri*. The squid, a night-active predator, harbors an active culture of light-producing bacteria in a specialized tissue called the light organ, which allows it to evade detection while moonlit hunting. Pioneered as a

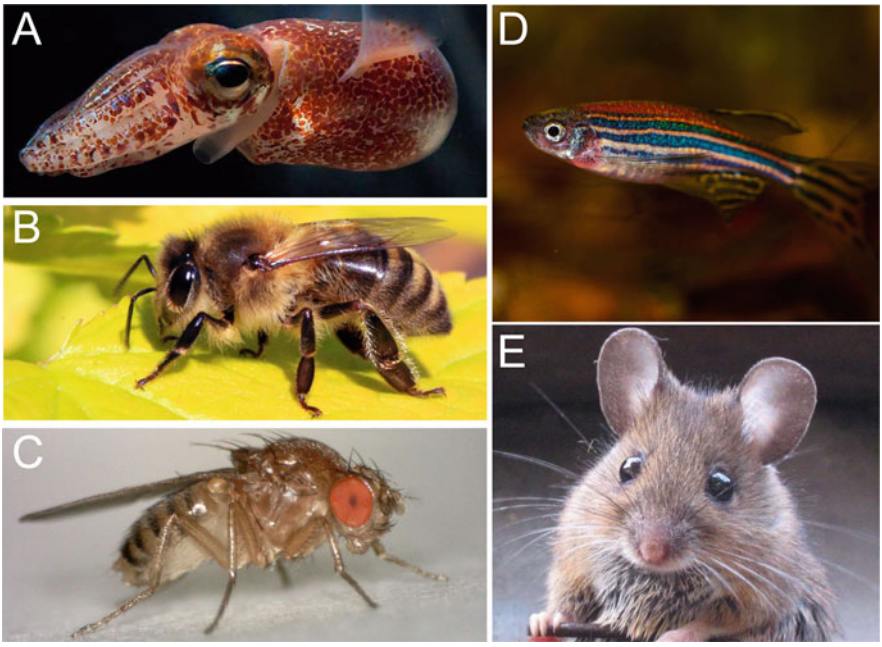

Fig. 1 Gnotobiotic animal model systems. Animal models used in research of host and microbiota interactions include (**a**) *Euprymna scolopes*, the Hawaiian bobtail squid; (**b**) *Drosophila melanogaster*, the fruit fly; (**c**) *Apis mellifera*, the Western honey bee; (**d**) *Danio rerio*, the zebrafish; and (**e**) *Mus musculus*, the laboratory mouse

model system by Margaret McFall-Ngai, Edward Ruby, and colleagues, the squid-*Vibrio fischeri* symbiosis features a simple binary association between an animal host and a bacterium [20, 21]. Importantly, the two partners can be grown in isolation from each other, making the system tractable to experimental manipulations of the symbiosis.

The squid-*Vibrio fischeri* symbiosis has been an especially powerful model for understanding the impact of microbes on animal development [22]. In the absence of their luminescent symbiont, juvenile squid will fail to undergo a normal developmental transformation of the epithelial tissue surrounding their symbiont-harboring light organ. Elegant studies exposing aposymbiotic squid to bacterial products showed that the normal developmental events of tissue remodeling around this organ could be triggered by a combination of LPS and a specific fragment of the bacterial cell wall polymer peptidoglycan [13]. The model has also been powerful for understanding how animals maintain long-term associations with resident microbes. Within the squid, *Vibrio fischeri* undergoes diurnal cycles of growth and expulsion, which maximizes light production for the squid during its nightly hunting. Transcriptional and metabolomic profiling reveals dramatic daily cycles of changes in the genetic regulation and chemical environment of the partnership, reminiscent of circadian cycling in humans [23, 24].

4.2 The Fruit Fly Model

The fruit fly *Drosophila melanogaster* has a long history as a model organism, and research with this simple animal has resulted in six Nobel Prizes to date. It was first studied in depth by Thomas Hunt Morgan and colleagues in the early twentieth century to elucidate basic principles of genetic inheritance. The genetic tools developed by these studies enabled the whole genome, forward genetic screens of Christiane Nüsslein-Volhard, Eric Wieschaus, and colleagues that uncovered the genetic basis for patterning of the basic animal body plan during development. These foundation screens uncovered many genes whose protein products are important in signal transduction pathways employed across the animal kingdom. One example is the Toll receptor, discovered in a subsequent screen by Kathryn Anderson as being required for dorsal-ventral patterning, and subsequently found by Jules Hoffmann and colleagues to be one of a family of innate immune sensors critical for protection against infectious diseases [25, 26].

More recently, *Drosophila* has emerged as a model for studying animal interactions with their resident microbes [27]. The foundational knowledge of developmental biology and innate immunity, the sophisticated genetics tools, and the ease of fruit fly husbandry have fueled this field. In addition, a strong history of *Drosophila* population genetics and ecology research propelled analyses of the microbes associated with fruit flies in the wild. In comparison to vertebrate animals, the gut microbiomes of both wild caught and laboratory fruit flies proved to be relatively simple in composition, typically consisting of less than a dozen distinct strains.

Drosophila melanogaster gut microbiomes are similar to the communities found associated with rotting fruit, dominated by bacteria belonging to the genera *Lactobacillus*, *Acetobacter*, *Gluconobacter*, and *Enterococcus*, and yeasts belonging to the genera *Kloeckera*, *Pichia*, and *Saccharomycodes* [28].

The phenotypes of germ-free *Drosophila* reveal that the microbiota is required for aspects of normal growth and metabolism, intestinal development, insulin signaling, and behavior. The low cost and ease of rearing *Drosophila*, which enabled the large screens described above, have advanced the field of microbiome research by allowing unbiased discovery of host and bacterial factors that determine these traits. Researchers have conducted genome-wide association studies to identify host genes that modulate microbiota composition [29] and host metabolic responses to microbiota [30]. Additionally, the ability to screen large populations of hosts has allowed studies in which *Drosophila* are mono-associated with individual isolates from collections of bacterial mutants to identify bacterial determinants of host-microbe interactions, for example, with *Acetobacter pomorum* [31] and *Lactobacillus plantarum* [32]. Large population sizes and experimental tractability have also enabled comprehensive studies of the interactions between host diet, microbiota, and physiology.

4.3 The Honey Bee Model

The Western honey bee, *Apis mellifera*, is a newer insect gnotobiotic model [33]. Developed independently by Nancy Moran and Irene Newton to study microbiota assembly and function, the model builds on extensive research about honey bee social behavior and ecology as a pollinator for economically important crops. Similar to fruit flies, honey bees have relatively low complexity microbiomes. Although the model lacks as extensive a history of genetic manipulations, recent innovations in genome engineering enable transgenesis and disruption of honey bee genes [34]. In addition, members of the honey bee microbiota can be genetically manipulated [35], which has been used to study the microbiota-host association and to engineer beneficial microbiota-dependent properties such as increased immune activation and protection against pathogens [36].

The complex social structure of the honey bee hive contributes to the assembly of distinct intestinal microbiomes of workers and queens [37]. The queens are the most important individuals in the hives, and as such they are protected from pathogens both by being isolated from the foraging workers who are at greatest risk of acquiring infections and by being provisioned with microbiota members that prevent infection [38]. The properties of honey bee microbiota members that confer protection against different pathogens are beginning to emerge [38, 39], and this knowledge will have immediate applications for protecting honey bee populations used in agriculture.

Studies of germ-free honey bees have revealed important roles for the microbiota in growth and metabolism. Similar to germ-free fruit flies, honey bees derived

without their microbiota exhibit decreased growth [40]. In addition, expression of key genes involved in the insulin pathway are decreased in expression in germ-free honey bees, demonstrating critical roles for the honey bee microbiota in regulating endocrine signaling.

4.4 The Zebrafish Model

The zebrafish, *Danio rerio*, was established as a model system by George Streisinger at the University of Oregon in the late 1970s with the hope of applying powerful forward genetic screening approaches to a vertebrate animal [41]. The model had many important attributes that would attract prominent researchers from other systems, including Christiane Nüsslein-Volhard from *Drosophila*, and propel the zebrafish to become a major platform for biomedical research. Its high fecundity and relative ease of husbandry enables forward genetic screens and other experiments with large population sizes. The embryos' optical transparency and rapid, ex-utero development have shed new light onto processes of vertebrate embryogenesis. In the last few decades, an explosion of genetic engineering approaches has opened up new avenues of investigation with zebrafish. These include transgenic expression of fluorescent reporters of cell types and processes and CRISPR/Cas9 mediated site-specific mutagenesis.

All of these attributes make zebrafish a powerful model for microbiome studies [42]. Foundational work for this model profiled the gut microbiome composition of lab reared and wild-caught zebrafish [43] and surveyed gut microbiomes across development [44]. This work revealed high complexity microbial communities, with hundreds of bacterial species belonging to similar bacterial domains as represented in the mammalian intestine, but with different proportional representation and species membership. The larval stages, when the animals are first colonized after hatching, are dominated by facultative aerobes of the *Gammaproteobacteria*, similar to the early neonatal stages of human microbiome assembly. Cultivation and genomic engineering of representative gut bacteria from larval zebrafish [45] has generated a collection of fluorescent protein expressing strains, allowing visualization of processes of bacterial colonization dynamics in living animals [46]. This work reveals how bacterial behaviors, such as swimming motility versus biofilm formation, influence the biogeography of the microbiota [47] and host immune responses to resident bacteria [48].

4.5 The Mouse Model

The laboratory mouse, *Mus musculus*, has a long history of use in gnotobiology, dating back to the 1940s [49]. These small mammals are well-accepted animals for preclinical studies modeling human diseases and responses to therapeutics. Decades

of mouse research have yielded sophisticated genetic resources such as large collections of mutants and tools for cell type specific gene manipulations. The field of immunology is built on mouse research, which has generated deep molecular insights into innate and adaptive immune responses to microbes. All of these tools have been invaluable for characterizing host-microbiota interactions in the mouse.

Of the standard laboratory models for microbiome research, the mouse harbors a gut microbial community that is most similar in composition to that of humans, dominated by the same phyla of *Bacteroidetes* and *Firmicutes*. The mouse is also the only standard gnotobiotic animal model that can be efficiently transplanted with human microbiota samples, creating "humanized" microbiome mice [50]. Transplantation of human microbiomes has become a standard approach for evaluating the potential functional properties of human microbiota samples. For example, fecal samples from twins discordant for obesity were shown to differentially impact the metabolism of transplanted germ-free mice, with the murine recipients of microbiota from an obese twin gaining more weight than those that received microbiota from the corresponding lean twin [51]. However, others have argued that the conclusions drawn from such humanized mouse experiments are subject to investigator bias and over-interpretation [52].

5 Impacts of Resident Microbes and Lessons for Human Health

Collectively, these major gnotobiotic animal models are teaching us about the impacts of resident microbes on various animal tissues and organ systems (Fig. 2). Comparisons across animal systems reveal generalities about the nature of microbial factors and activities that influence animal biology.

5.1 Intestinal Epithelial Renewal

The epithelium that lines the intestinal tract is the tissue in the closest contact with the densest and most populous microbial community of the animal body. A conserved response of this epithelium to the presence of resident microbes is an elevated rate of epithelial renewal. Across the model systems of flies, zebrafish, and mice, the absence of a microbiota results in reduced numbers of proliferating cells as compared to conventionally reared counterparts [53]. It is not yet known whether the molecular mechanism that stimulates intestinal epithelial cell proliferation in response to microbiota is conserved across these animal hosts. However, the intestinal epithelium in all of these organisms responds to inflammatory insults and physical injury by upregulating programs of epithelial renewal through conserved pathways such as the Jak/Stat, EGF, and Wnt pathways [54]. Additionally,

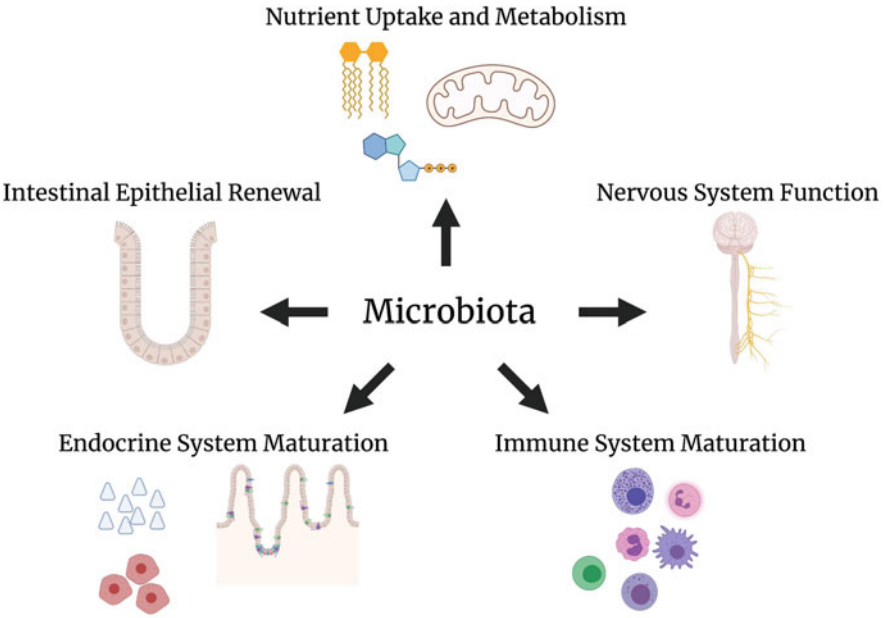

Fig. 2 Summary of the impact of resident microbes on animal tissues and organ systems. Research using gnotobiotic animal models has revealed microbiota influences on diverse host aspects including *(clockwise from top)* nutrient uptake and metabolism functions, such as mitochondria, lipid metabolism, and ATP synthesis; nervous system function and development; maturation of the immune system; endocrine function including insulin signaling (triangles), beta cell development, and intestinal enteroendocrine cell number; and the proliferation rate of intestinal epithelium. See text for details

conserved innate immune signaling pathways are required for sensing and responding to microbiota derived cues that stimulate intestinal epithelial proliferation. For example, zebrafish deficient for the common TLR adaptor protein, Myd88, have low rates of intestinal epithelial proliferation, resembling germ-free rates [55]. Both the presence of microbiota and Myd88 is also required for colonic epithelial proliferation in a mouse model of intestinal injury with the chemical irritant dextran sodium sulfate [56]. Thus, it is plausible that homeostasis of the intestinal epithelium in response to the presence of colonizing gut microbes is a result of a subtle triggering of inflammatory and tissue repair programs by generic microbial stimulants perceived through innate immune pathways.

5.2 Nutrient Uptake and Metabolism

The primary function of the intestine is to absorb nutrients that are subsequently metabolized and disseminated throughout the body. There is a complex interplay

between gut microbiota, diet, and metabolism [57]. Dietary changes have profound impacts on gut microbiota composition. Reciprocally, microbiota influence the processes of nutrient absorption and utilization. A general feature of germ-free animals is that they typically have the metabolic traits of an undernourished state even when given unlimited access to food. Germ-free *Drosophila* are delayed in their development in the transition from their larval to pupal stages, and under nutrient-limited conditions they will fail to pupate and die as larvae. Germ-free zebrafish and mice share conserved programs of microbiota-regulated nutrient acquisition gene expression [58], and both exhibit nutrient acquisition defects such as reduced lipid absorption [54, 59].

Dissecting the complex interactions between microbiota, diet, and metabolism requires not just gnotobiology but also experimental control of nutrient intake, which can be challenging even with laboratory animals. The *Drosophila* field has developed elementally defined diets for fruit flies, allowing them to systematically eliminate macro- and micronutrient components of the diet and study the impact of these dietary manipulations in the context of colonization with different gut bacteria [60]. These studies show that gut bacteria provision certain essential nutrients to their hosts including essential amino acids and trace metals. Similar provisioning, for example, of sphingolipids, also occurs in the mammalian intestine [61].

The fact that germ-free animals generally exhibit reduced metabolic rates may reflect a requirement for additional factors normally provisioned by resident microbes. Recent whole body transcriptomic and metabolomic profiling of conventionally reared versus germ-free *Drosophila* reveal that the lack of bacteria causes an overall reduction of host mitochondrial function and ATP production [62]. This deficit could be reversed by supplementation of bacterial riboflavins, which are precursors of the universal mitochondrial co-enzymes FAD and FMN, suggesting that bacteria normally are sources of these molecules. Limited metabolic capacity could then impact developmental programs throughout the body, similar to developmental alterations associated with nutrient deprivation [63]. Indeed, early childhood deprivation of nutrients can impair normal programs of microbiome maturation in humans and result in developmental defects such as growth stunting and neurological deficits reminiscent of developmental defects in germ-free animals [64]. Mechanistic studies in model systems are critical for providing molecular insights into the diversity of human metabolic diseases of both under- and overnutrition, such as environmental enteropathy, diabetes, and cardiovascular disease, which are linked, based on epidemiological studies, to interactions between diet and the gut microbiota.

5.3 Endocrine System Maturation

Critical for nutrient utilization is the regulation of cellular metabolism by endocrine hormones. Across multiple animal models, endocrine signaling is impacted by the

microbiota. This occurs both at the level of signaling regulation and through impacts on the development of endocrine cells and tissues.

The quintessential endocrine signaling pathways is the insulin pathway. Insulin signaling is reduced in germ-free fruit flies [31] and honey bees [40], resulting in their reduced growth in the absence of their microbiota. Forward genetic screening in the fruit fly commensal *Acetobacter pomorum* identified a metabolic pathway involved in acetic acid production as a critical cue for promoting normal insulin signaling [31]. Additionally in fruit flies, the immune deficiency (IMD) innate immune signaling pathway was found to be critical for sensing acetate, the bacterial fermentation SCFA, and regulating insulin signaling [65].

In germ-free zebrafish, insulin levels are reduced, and circulating glucose is elevated because the larvae fail to develop the normal number of insulin-producing beta cells in their pancreas [66]. This defect can be rescued by supplementation with a single commensal bacterial secreted protein, Beta Cell Expansion Factor (BefA), of novel sequence and function [66]. BefA homologues are found in the genomes of human intestinal microbiota members, raising the possibility that lack of this protein during early postnatal development could predispose individuals to the development of type 1 diabetes, a disease of beta cell paucity.

Within the intestinal epithelium, specialized enteroendocrine cells secrete hormones that regulate metabolism and intestinal function. The specification of these cells is dependent on the presence of microbiota and innate immune signaling. Germ-free zebrafish have fewer enteroendocrine cells in their intestines, a trait that is recapitulated in conventionally reared animals lacking the TLR adaptor Myd88 [67]. Germ-free mice have reduced numbers of an enteroendocrine cell type, enterochromaffin cells, which are also reduced in mutants lacking Tlr2 and Myd88 and restored by addition of the commensal bacterium *Clostridium ramosum* [68] or by the enteric parasite *Trichuris muris* [69].

In addition to their impacts on enteroendocrine cell development, microbiota also play key roles in regulating the functions of these cells. For example, serotonin secretion is reduced from germ-free mouse enterochromaffin cells and restored by addition of certain spore-forming bacteria [70]. In zebrafish, signaling from enteroendocrine cells was found to be silenced by high fat diet, but this silencing required the presence of the microbiota or monoassociation with a commensal *Acinetobacter* strain [71]. The ability of enteroendocrine cells to sense both microbial and nutrient information makes them important cells to consider in the etiology of human metabolic disorders with a microbial component.

5.4 Immune System Maturation

Another common feature of germ-free fruit flies, zebrafish, and mice is an immature immune system. In fruit flies, this has been studied as the lack of antimicrobial peptide expression [72], in zebrafish as a lack of neutrophil immune cells recruited to the intestine [73], and in mice as T cell deficiencies [16]. In-depth studies of

immunological deficiencies in each of these models reveal both local and systemic effects of the presence or absence of microbes. For example, the skin microbiota of mice has been shown to modulate the maturation of local innate immune cells, as well as to educate adaptive T cell populations with systemic functions [74]. One emerging theme is that the context of microbial exposure, both as a function of the cell type and the developmental timing, influences host responses. The importance of timing may explain why early childhood experiences, such as infections or repeated courses of antibiotics, have been linked to adult diseases of immune dysregulation. Another important theme is the connection between immunity and metabolism. Immune cells have been found to play key roles in sensing endogenous perturbations in tissue metabolism, such as drops in nutrient availability [75]. This has prompted a new appreciation for the same diseases discussed above, such as environmental enteropathy, diabetes, and cardiovascular disease, as being immunometabolic disorders, with immune and metabolic dysfunction being inextricably linked in the disease pathologies.

A limitation of laboratory animals for investigating human immunological diseases is their capacity to model human immune system function. Much of immunological research is based on using "specific pathogen free" (SPF) mice, reared in clean, barrier facilities, as the normal reference against which to compare other treatment groups, such as germ-free mice. However, recent work has called into question the normalcy of the immune development of SPF mice. Analysis of the immune systems of wild caught mice or even pet store mice has shown them to have immune cell populations more similar to adult humans, whereas the SPF mouse immune system resembled that of human neonates [76]. Similar immune system maturation can be induced by "wilding" laboratory mice through co-housing or fecal exposures, demonstrating that the microbiomes of the donor mice are responsible for the immune maturation. Although the procedures for generating "dirty" mice pose experimental challenges that SPF mice were designed to overcome, such as lack of reproducibility and the introduction of new pathogens [77], they demonstrate the extent to which microbial exposures mediate immune system development and function, and provide further experimental evidence for associations between human diseases of immune dysregulation and microbiome dysbiosis.

5.5 Nervous System

Of the many impacts of the microbiota on animal biology, one of the most fascinating is its impacts on the nervous system, with implications for regulation of behavior and cognition. These impacts have been studied at different levels, from behavior to neuroanatomy [78]. Across different model systems, deprivation of microbiota is associated with alterations in behavior. For example, germ-free adult *Drosophila* exhibit increased walking activity, which was normalized by colonization with certain bacterial residents, exposure to a bacterial enzyme xylose isomerase that modules host sugar metabolism, or by modulating octopaminergic neuronal

signaling [79]. Similarly, germ-free zebrafish exhibit hyperactivity, which could be reversed by colonization with certain gut bacteria but not by exposure to heat-killed products [80]. Germ-free mice exhibit a number of aberrant behaviors, which vary across genetic backgrounds and settings, but generally can be categorized as increased baseline exploration behaviors and impairments in social behaviors [81]. Modulations of hypothalamic-pituitary-adrenal responses to stress appear to underlie many of these behaviors [82]. The molecular nature of the microbial cues that impact nervous system function is still being uncovered, but conserved microbial molecules and products of metabolism seem to be critical for mediating many of the responses to complex microbiotas [83]. As with the immune system, microbiota impacts on the nervous system are influenced by location, developmental timing, and metabolism.

The microbiome is emerging as an important feature of many human neurodevelopmental disorders, such as autism spectrum disorder (ASD) and schizophrenia, and of neurodegenerative diseases, such as Parkinson's and Alzheimer's disease [84]. There is an urgent desire to deploy knowledge about microbiome dysbiosis in these diseases for therapeutic purposes, but major challenges remain. One hurdle is the complexity of many of these neurological disorders, which cannot easily be modeled in laboratory animals. For example, the social and communication deficits that define ASD cannot be recapitulated in animals that lack the capacity for complex language acquisition. Better understanding of the cellular and molecular bases of these neurological disorders will be needed to reveal the potential for microbial interventions as therapeutics.

6 Conclusions

When viewed through the lens of any specific human disease, microbiota-host interactions can appear intractably complex. Yet when examined through the lens of the common responses to microbiomes shared across well-studied gnotobiotic animal models, certain themes emerge that help provide context for individual human diseases. The importance of resident microbes as sources of limiting nutrients explains their profound impacts on the metabolic states of tissues and organs, setting rates of tissue homeostasis to match metabolic capacities. Resident microbes are also important immunological stimulants of tissue homeostasis, defense, and repair programs. The molecular cues that trigger these programs are likely to be diverse but highly redundant. Collectively, the metabolic state and immune activation of an organism, as determined by its microbiota, will impact the developmental trajectories of many tissues and organ systems, each of which may have different critical windows of sensitivity. Thus, a productive starting point for understanding microbiome-associated human diseases is to uncover the earliest manifestations of metabolic and immunological dysregulations in the affected tissues. Such metabolic and immunological processes may be amenable to experimental modeling in gnotobiotic animal models, providing a path forward for uncovering molecular

mechanisms of disease and developing effective microbial prophylactic and therapeutic treatment strategies.

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Compliance with Ethical Standards
For this article no studies with human participants or animals have been performed by the author.

Conflict of Interest The authors declare no conflicts of interest.

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