
# Animal Models for Microbiome Research Advancing Basic and Translational Science: Proceedings of a Workshop



## DETAILS

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# Animal Models for Microbiome Research

## Advancing Basic and Translational Science

PROCEEDINGS OF A WORKSHOP

Joe Alper, Lida Anestidou, and Jenna Ogilvie, *Rapporteurs*

Roundtable on Science and Welfare in Laboratory Animal Use

Institute for Laboratory Animal Research

Division on Earth and Life Studies

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The Roundtable on Science and Welfare in Laboratory Animal Use wishes to express gratitude to the members of the planning committee for their work in developing a far-reaching workshop agenda and the expert speakers who took part in the workshop's discussions.

# Reviewers

This Proceedings of a Workshop was reviewed in draft form by individuals chosen for their diverse perspectives and technical expertise. The purpose of this independent review is to provide candid and critical comments that will assist the National Academies of Sciences, Engineering, and Medicine in making each published proceedings as sound as possible and to ensure that it meets the institutional standards for quality, objectivity, evidence, and responsiveness to the charge. The review comments and draft manuscript remain confidential to protect the integrity of the process.

We thank the following individuals for their review of this proceedings:

Angela Douglas, Cornell University
Robert Dysko, University of Michigan Medical School
Alton G. Swennes, Baylor College of Medicine
Tamara Tal, U.S. Environmental Protection Agency

Although the reviewers listed above provided many constructive comments and suggestions, they were not asked to endorse the content of the proceedings nor did they see the final draft before its release. The review of this proceedings was overseen by Jeffrey Everitt, Duke University School of Medicine. He was responsible for making certain that an independent examination of this proceedings was carried out in accordance with standards of the National Academies and that all review comments were carefully considered. Responsibility for the final content rests entirely with the rapporteurs and the National Academies.

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## BOX, FIGURES, AND TABLE

### BOX



### FIGURES



### TABLE

# Acronyms and Abbreviations

| | |
|---|---|
| BSL-2 | biosafety level 2 |
| CCFA | Crohn's & Colitis Foundation of America |
| DGRP | Drosophila Genetic Resource Panel |
| EEN | exclusive enteral nutrition |
| IACUC | Institutional Animal Care and Use Committee |
| IBD | inflammatory bowel disease |
| LPS | lipopolysaccharide |
| NAPE-PLD | N-acyl phosphatidylethanolamine phospholipase D |
| NIH | National Institutes of Health |
| NKT | natural killer T cells |
| NOD | non-obese diabetic |
| sDMDMm2 | Stable Defined Moderately Diverse Mouse Microbiota 2 |
| SOP | standard operating procedure |
| SPF | specific pathogen-free |
| TMAO | trimethylamine-N-oxide |
| USDA | U.S. Department of Agriculture |

# 1

# Introduction

The surface of the human body and its mucous membranes are heavily colonized by microorganisms. Our understanding of the contributions that complex microbial communities, such as Archaea, bacteria, and eukaryotes, and their viruses (Hugon et al., 2017; Parker, 2016), make to health and disease is advancing rapidly (Cho and Blaser, 2012; Eloe-Fadrosh and Rasko, 2013; Hooper et al., 2012; Lloyd-Price et al., 2016; Lynch and Pedersen, 2016). These projects have produced experimental and computational resources that are enabling investigators to analyze microbial community functions and begin to understand the role that microbial and viral genomes play in normal and disease biology. To capitalize on these new resources and to aggressively explore the relationship between microbiomes and their hosts, including humans, the White House launched the National Microbiome Initiative[1] in May 2016 to "foster the integrated study of microbiomes across different ecosystems" by pulling together federal agencies, academic institutions, and private entities.

Most microbiome research to date has focused on the mouse as a model organism for delineating the mechanisms that shape the assembly and dynamic operations of microbial communities. Mouse microbiome models have also been the primary choice for performing preclinical proof-of-concept tests of causal relationships between given community configurations/memberships and host physiological, metabolic, immune, and neurologic phenotypes and for developing methods to repair or prevent functional abnormalities in these communities that contribute to disease pathogenesis. The mouse, however, is not a perfect surrogate for studying different aspects of the microbiome and how it responds to various environmental and host stimuli. As a result, researchers have been conducting microbiome studies in other animals as well, for instance, zebrafish, piglets, and *Drosophila*.

To examine the different animal models researchers employ in microbiome studies and to better understand the strengths and weaknesses of each of these model organisms as they relate to human and nonhuman health and disease, the Roundtable on Science and Welfare in Laboratory Animal Use of the

[1]See https://obamawhitehouse.archives.gov/the-press-office/2016/05/12/fact-sheet-announcing-national-microbiome-initiative (accessed February 28, 2017).

National Academies of Sciences, Engineering, and Medicine convened a workshop on December 19-20, 2016, in Washington, DC, to discuss animal models of microbiome research. An ad hoc committee (see page v for the committee roster and Appendix B for their biographies) planned this workshop to (1) explore how to improve the depth and breadth of analysis of microbial communities using various model organisms; (2) address the challenges of standardization and biological variability that are inherent in gnotobiotic animal-based research; (3) examine the predictability and translatability of preclinical studies to humans; and (4) discuss strategies for expanding the infrastructure and tools for conducting studies in these types of models (see Box 1-1 for the full Statement of Task). Invited speakers and stakeholders discussed gaps, challenges, and opportunities in this rapidly expanding field, paying particular attention to the care, use, and welfare of the gnotobiotic animals.

This Proceedings of a Workshop was prepared by the rapporteurs as a factual summary of what occurred at the workshop. The planning committee's role was limited to planning and convening the workshop (see Appendix A for the agenda and Appendix B for the biographies of the planning committee members). The views contained in the publication are those of individual workshop participants and do not necessarily represent the views of all workshop participants, the planning committee, or the National Academies of Sciences, Engineering, and Medicine.

**BOX 1-1** Workshop Statement of Task

An ad hoc committee will plan and conduct a public workshop that will provide an overview of the state of the art of microbiome research using animal models. Joshua Lederberg explained that the "microbiome signifies the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease." Most of the animal-based, microbiome-related research depends on murine models to improve our understanding of the physiology, pathology, and relationship to health and disease at both the animal and the translational levels. The workshop would seek to identify and discuss gaps, challenges, and opportunities in this rapidly expanding field. Participants will also discuss husbandry, animal care, and welfare for animals used for such studies. Particular attention will be paid to the care, use, and welfare of the gnotobiotic (germ-free) mice.

The ad hoc committee will develop the workshop agenda, select and invite speakers and discussants, and moderate the discussions. A summary of the presentations and discussions at the workshop will be prepared by a designated rapporteur in accordance with institutional guidelines.

# 2

# A Trans-Kingdom Perspective on Animal Models and Microbiome Research

Herbert Virgin, the Edward Mallinckrodt Professor and chair of the pathology and immunology department at the Washington University School of Medicine in St. Louis, began his presentation by explicitly defining the term *microbiome* as the collection of all the organisms in or on a host, including viruses, bacteria, Archaea, fungi, and protists. All of these organisms interact with each other and the host in a variety of complex and meaningful ways.

The virome—Virgin's area of academic expertise—is a permanent and dynamic contributor to the human metagenome. Every human hosts a number of active, living viruses at any given moment (collectively called "the virome"), most of which are novel and uncharacterized while perpetually shedding virus particles with few overt health consequences (Virgin, 2014).

Every human has a unique virome, whose components constantly interact with each other, with other organisms in the microbiome, and with the host, and influence both the host genotype and phenotype. Virgin hypothesized that these viruses may even be defining how our immune systems react to challenge—"if we're chronically infected but apparently healthy, that may define our immunophenotype." In fact, he and his colleagues have shown that mice with a latent herpesvirus infection are protected from challenge with a very high dose of *Listeria monocytogenes* due to increased expression of gamma interferon (IFN-γ). The same protection is observed in genetically modified mice that lack the HOIL-1 gene, which produces a severely immunocompromised phenotype that is also seen in humans (Boisson et al., 2012; MacDuff et al., 2015).

## THE IMPORTANCE OF TRANS-KINGDOM INTERACTIONS

The complex nature of the interactions between microbiome and host—Virgin calls them trans-kingdom interactions—has important implications for understanding the relationship between disease genotype and phenotype. Virgin related our current understanding of the microbiome to that of the solar system, which only made sense when scientists understood the central position of the

sun rather than the Earth. Relatedly, Virgin argues, researchers now view the microbiome almost exclusively in relation to the host, when the interactions among the components of the microbiome are just as critical.

As an example of this trans-kingdom complexity, Virgin returned to the herpesvirus experiment to note that infection of these mice with a helminth induces interleukin 4 (IL-4), which in turn triggers an active viral infection, that is, reversal of the herpesvirus latency (Reese et al., 2014). The virus contains separate promoters that cause a different response to each of the two cytokines (IFN-γ and IL-4); that is, "this is not about the host controlling the virus. This is about the virus evolving promoters to leverage what the host is doing," noted Virgin.

In a second example of a trans-kingdom interaction, Virgin discussed a series of experiments showing that bacteria can control chronic norovirus infections. Some members of the norovirus family cause severe gastrointestinal distress, while others produce an asymptomatic persistent infection of the spleen, lymph nodes, and other tissues (Nice et al., 2013). Virgin and his collaborators have shown that chronic norovirus infection in a mouse model can increase susceptibility to inflammatory bowel disease (IBD) (Cadwell et al., 2008, 2010). "You can prevent the virus-triggered pathology by giving antibiotics, indicating that the virus is interacting with bacteria," said Virgin, noting that pre-treating the animals with antibiotics protected them from persistent infection, indicating the reliance of the virus on the bacteria to ensure persistence (Baldridge et al., 2015). Subsequent experiments showed that introduction of interferon lambda (IFN-λ) produced the same results against norovirus as exposing the mice to antibiotics (Nice et al., 2015). These were surprising findings, said Virgin, due to the implication that the innate immune system can clear an infection, which goes against the dogma that the innate immune system simply holds an infection in check until the adaptive immune system responds. He noted that other investigators have demonstrated a similar phenomenon with rotaviruses (Uchiyama et al., 2014; Zhang et al., 2014).

Researchers are uncertain whether such trans-kingdom interactions occur in humans, but Virgin presented evidence suggesting that they do. Examination of the bacterial microbiomes and viromes of three patient cohorts with IBD showed decreased microbiome complexity but increased virome complexibity in the IBD patients versus healthy controls due to elevated numbers of *Caudiovirales* (Norman et al., 2015) (see Figure 2-1). Further analysis showed that the viromes associated with Crohn's disease differed from the viromes found in ulcerative colitis patients (both diseases are classified as IBD). Based on these results, Virgin and his colleagues have hypothesized that these diseases develop when viruses are killing beneficial bacteria, rather than in the absence of beneficial or the presence of pathogenic bacteria.

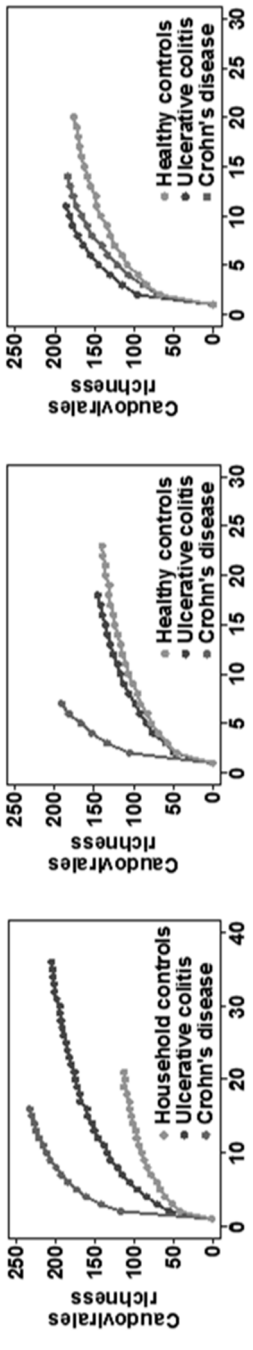


**FIGURE 2-1** The enteric virome becomes more complex in patients with inflammatory bowel disease. NOTE: Samples collected from Cambridge, UK; Chicago, USA; and Boston, USA, respectively. SOURCES: Virgin slide 40 (Norman et al., 2015).

Virgin described another set of experiments which demonstrated that sequential infection of mice with a variety of viruses and one worm altered the animals' response to yellow fever vaccination (Reese et al., 2016). These experiments, designed to mimic the early exposure to infections in humans and the subsequent changes to the immune system's phenotype, showed that early exposure in mice versus humans causes very different phenotypes. This is an important observation regarding animal models because it could explain why mouse models do not predict human vaccine response: "Maybe mice are very representative, but we have cleaned them up to the point where we have made them nonresponsive," said Virgin.

Virgin re-emphasized that the components of the microbiome—viruses, bacteria, worms, and other organisms—do not act in isolation. Their trans-kingdom interactions have substantial physiological effects on the host. In order to elucidate the effect of these interactions on health and disease, the field needs to conduct many more carefully controlled clinical and animal studies. In doing so, Virgin called for researchers to fully and transparently report the experimental conditions and methods used. He further highlighted the need for massive improvements in the scope and annotation quality of genome databases and called for the creation of kingdom-specific bioinformatics tools. In his opinion, the field must evolve from an associational science to one that manipulates the different -omes and tests mechanistic hypotheses, and to do so it needs to improve the quality and reproducibility of sequencing.

Concluding his presentation, Virgin called for researchers to train more students and break down the silos that keep virologists, bacteriologists, mycologists, and others from talking to one another. Integrating disciplines is essential for developing a more complete understanding of the role that the microbiome plays in health and disease.

# 3

# Non-Rodent Models for Microbiome Research

The microbiome research community has focused most of its efforts on mouse models, but as with other branches of preclinical research, exploring the microbiomes of other species could complement mouse studies and generate new knowledge relevant to humans. The four speakers in the workshop's first panel session provided perspectives on the benefits and limitations of animal models beyond mice. The four speakers were Buck Samuel, assistant professor in the Alkek Center for Metagenomics and Microbiome Research and the Department of Molecular Virology and Microbiology at Baylor College of Medicine; Angela Douglas, the Daljit S. and Elaine Sarkaria Professor of insect physiology and toxicology at Cornell University; Karen Guillemin, the Alec and Kay Keith Professor in the Department of Biology and the Institute of Molecular Biology at the University of Oregon; and Jeff Gordon, the Dr. Robert J. Glaser Distinguished University Professor and Director of the Center for Genome Sciences and Systems Biology at Washington University in St. Louis. In a second session, three speakers discussed in vitro systems for studying microbiomes. The three speakers on the second panel were Robert Britton, professor in the Department of Molecular Virology and Microbiology at Baylor College of Medicine; Vincent Young, associate professor in the Department of Internal Medicine/Division of Infectious Diseases and Department of Microbiology and Immunology at the University of Michigan; and Donald Ingber, founding director of the Wyss Institute for Biologically Inspired Engineering at Harvard University and Judah Folkman Professor of Vascular Biology at Harvard Medical School.

## EUKARYOTIC MODELS

### *Caenorhabditis elegans (C. elegans)*

The strength of *C. elegans* as a model organism for microbiome research lies in the ability to conduct high-throughput experiments with a gnotobiotic organism and explore the complex cause-or-effect relationship between the presence or absence of a microbial species and a specific state of health or disease. For Samuel, his goal is to identify the pathways that are open to microbial influence and the molecules mediating that influence.

In the laboratory, *C. elegans* will grow for multiple generations on a nutrient-rich, chemically defined, organism-free medium (Szewczyk et al., 2003). Under these conditions, *C. elegans* grows 35 times slower and lives twice as long as in the wild, modeling how it and other organisms grow under starvation conditions. Beyond that observation, said Samuel, researchers know little about the artificial germ-free state in *C. elegans*, though it is clear that peptide uptake and intestinal metabolism are impaired in the germ-free state, while uptake of complex lipids is not. Despite being able to take up lipids, germ-free *C. elegans* are devoid of fat according to unpublished work from Samuel's group.

The *C. elegans* microbiome is relatively simple, comprising 5-15 microbial strains that support approximately 10,000 colony-forming units per healthy animal (Berg et al., 2016; Dirksen et al., 2016; Samuel et al., 2016), with most of the colonization occurring early in adulthood. So far, Samuel and his colleagues have cultivated 564 different organisms from natural *C. elegans* populations and can now recapitulate communities representing 80 percent of the core operational taxonomic units and 75 percent of the microbial abundance (see Figure 3-1). "We still have some missing taxa that we are interested in, but we definitely have all of the big ones," said Samuel. In one set of knockdown experiments, Samuel and his collaborators identified new signaling pathways that *C. elegans* uses to regulate microbiome form and function. Approximately 40 of the actors involved have direct human orthologs, raising the possibility that this simple system will provide new insights into the basic mechanisms that hosts use to regulate their microbiomes.

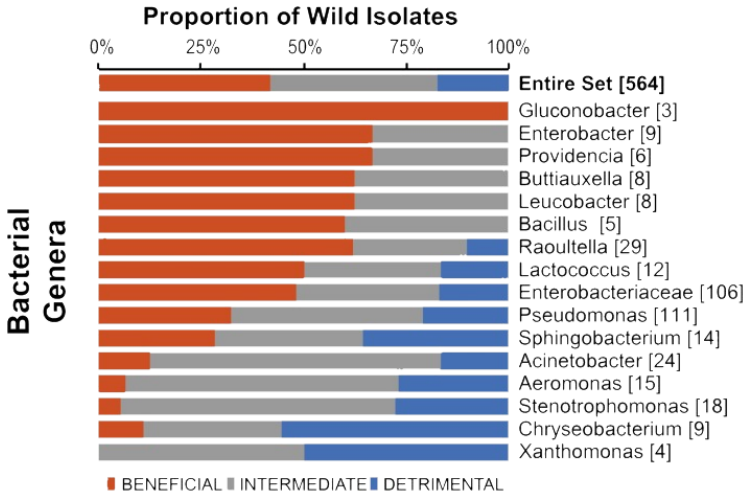


**FIGURE 3-1** Natural microbiome of *C. elegans.* SOURCES: Samuel slide 19 (Samuel et al., 2016).

### *Drosophila melanogaster*

Like *C. elegans*, *Drosophila* is experimentally amenable to manipulation and easy to maintain in a germ-free state. The drosophila gut is not anoxic, explained Douglas, and the organisms that live there—predominantly bacteria—are tolerant of oxygen and readily cultured. Recolonizing axenic, or germ-free, flies involves adding a bacterium or collection of microorganisms to the food on which the larvae or adults feed.

The natural drosophila microbiome is an order of magnitude less diverse than that of mammals, said Douglas, with Acetobacteraceae, Lactobacillales, and γ-Protobacteria being the dominating strains. However, the drosophila microbiome, like that of mice and humans, is inconstant—identical lines of flies grown under the same conditions in two different laboratories will have different microbiome compositions (Chaston et al., 2015; Wong et al., 2013). Characterization of the microbiota in drosophila reared in her laboratory and aggregated across different developmental stages identified five major isolates (see Figure 3-2) that can recapitulate the conventional drosophila phenotype (Wong et al., 2011).

Douglas described an elaborate set of feeding experiments with axenic drosophila as an example of how axenic insects can provide insights on nutritional interactions in the host digestive system. These experiments showed that the gut microbiota, and specifically the lactobacilli, spares drosophila's dietary requirements for B vitamins, especially riboflavin (Wong et al., 2014). Other experiments found that axenic drosophila are inordinately fat, hyperglycemic, and hyperlipidemic (Ridley et al., 2012). Further investigation showed that one genus in the Acetobacteraceae family, *Komagataeibacter*, was present in normal drosophila and not in the obese flies, and that these bacteria protected against hyperlipidemia by competing with the host for dietary sugars (Huang and Douglas, 2015). In other recent unpublished work, Douglas and her colleagues have identified a few bacterial taxa and microbial communities that fail to protect against hyperglycemia.

One advantage of working with *Drosophila* is the ability to harness the wealth of genomic and genetic resources available. "We have tremendous panels of mutants, RNAi lines and so on, readily available from stock centers, and we can make use of the UAS-GAL4 system to exert very precise spatiotemporal control over gene expression," said Douglas. In addition, she noted, CRISPR tools for genetic manipulation are becoming increasingly sophisticated. Two specific resources her laboratory uses are the Drosophila Genetic Resource Panel (DGRP) of 200 inbred lines with sequenced genomes (Huang et al., 2014; Mackay et al., 2012) and the Drosophila Global Diversity Panel of 84 inbred lines from five continents with sequenced genomes (Grenier et al., 2015). Using DGRP lines, she and her colleagues have found that eliminating the microorganisms in these flies causes some genotypes to become obese, others slightly overweight, and still others become even leaner than normal (Dobson et al., 2015). "We see this genetic variation as an opportunity, not as a problem," said

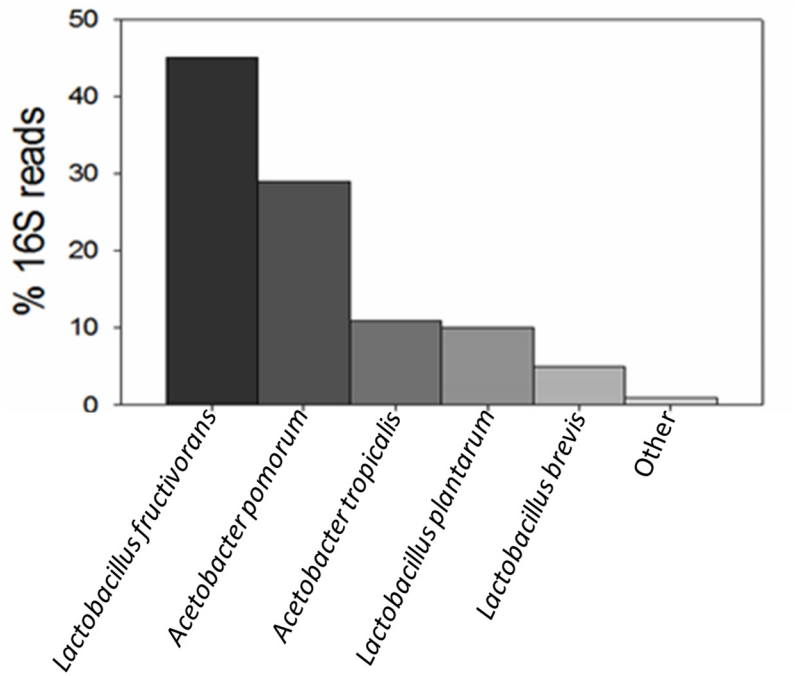


**FIGURE 3-2** The five major bacterial isolates from laboratory-grown *Drosophila*. SOURCE: Douglas slide 7.

Douglas. "It indicates the importance of host genotype as a determinant of microbiota-dependent traits and enables us to apply genome-wide association studies to identify candidate genetic determinants and then, because we have mutants readily available for many of these genes, to validate those genes." Many of the candidate genes her group identified are expressed in the gut or in neurons; have homologs across the animal kingdom, including in humans and other mammals; and belong to pathways that are highly conserved across the animal kingdom. "I think this reflects the fact that the foundation of the microbiome in animals is very ancient," said Douglas.

Comparing the transcriptome of axenic and gnotobiotic flies from 17 *Drosophila* lines, Douglas and her colleagues found that transcriptome-wide co-expression is significantly weaker in the axenic flies than in gnotobiotic flies (Dobson et al., 2016). They also observed the co-expression of pairs of genes differed significantly between axenic and gnotobiotic flies, leading Douglas to conclude that the microbiome promotes co-expression of specific transcriptional modules. "This work is very recent and there is more about this that we do not understand than that we do," said Douglas. Questions she posed included whether this is a general effect across species and if reduced co-expression is associated with microbiomes that fail to support health.

Douglas said that using invertebrate models such as *Drosophila* and *C. elegans* could substantially reduce the need to conduct experiments using rodents while enhancing their scientific quality, given that researchers can use *Drosophila*, *C. elegans*, and other non-vertebrate systems to more quickly understand fundamental principles of animal microbial associations and then use mammalian models and human data to verify the relevance to humans. Toward that end, Douglas recommended strengthening the framework and infrastructure for integrating model systems with biomedical and clinical science.

## Zebrafish

Zebrafish studies benefit from an abundance of genetic and genomic tools and high-throughput functional assays. As vertebrates, zebrafish offer additional complexity in terms of the kinds of microbial communities they harbor, and their optical transparency provides some unique opportunities to observe dynamic microbial communities in the gut of a living animal. There are challenges to working with zebrafish, including the difficulty of rearing germ-free animals to adulthood, the lack of standardized methods of screening for pathogens, and the lack of knowledge about the nutritional requirements of juvenile and adult animals. Maintaining water quality is an issue, too, because microbes in the natural ecosystem normally play a large part in detoxifying urea and other waste products. Guillemin explained that, while her group and others have accumulated large, curated collections of bacterial isolates and a few species of fungi isolated from zebrafish, there is no defined inoculum for them as there is for mice.

Zebrafish harbor hundreds of different bacterial species and other microorganisms at a level of complexity similar to mammals. Because it is rather straightforward to derive them under germ-free conditions, researchers can build complex microbial communities starting with mono- or di-associations. In one set of experiments, Guillemin and her collaborators found that wild-type and immune-deficient fish raised in isolation had vastly different microbiomes, but the microbial communities converged when the two types of fish were raised in the same tank or when each type of fish was raised in the same tank with others of its genotype. Guillemin said these results suggest that co-housing hosts selects for bacterial members that are transmissible, while raising a host in isolation leads to extinction of the bacterial lineages that have the ecological strategy of moving between hosts. “This has large implications for thinking about how one designs experiments profiling microbiota,” said Guillemin. “The housing conditions can have very profound effects.”

In another study, Guillemin and her colleagues found that *Aeromonas* introduced into the gut of an axenic zebrafish forms clumps that are readily visible in the transparent fish. When a member of the genus *Vibrio* is then added to the gut, this highly motile bacterium rapidly displaces the *Aeromonas* (Wiles et al., 2016). However, in a mutant zebrafish with defective gut peristalsis, the *Aeromonas* population persisted when *Vibrio* was introduced. “This is telling us

that the host environment, in this case peristalsis, contributes to the bacteria-bacteria competition," said Guillemin.

Guillemin has also determined how the microbiome alters zebrafish development (Hill et al., 2016). When zebrafish hatch at three days, they are supported by a single functional islet of pancreatic beta cells. These beta cells double over the next three days, concurrently with the colonization of the patent gut tube by bacteria. In germ-free fish, this doubling does not occur, and consequently, these fish have higher circulating glucose levels relative to the conventionally reared fish. Subsequent experiments identified several *Aeromonas* strains and a *Shewenalla* strain that could reverse this effect and that a specific secreted protein—beta cell expansion factor A, or BefA—produced by the bacteria was responsible for stimulating beta cell production. Treating axenic fish with this protein, which has homologs in human-associated bacteria, triggered expansion of beta cells. Guillemin noted in closing that beta cell expansion in humans occurs during the first year of life, concurrent with the establishment of an infant's gut microbiota.

## Piglets

Studying a suspected link between human malnutrition and human gut microbes, Gordon and his collaborators found evidence that immature microbiota are causally related to undernutrition (Blanton et al., 2016; Subramanian et al., 2014, 2015). They also found that Malawian mothers with severely stunted 6-month-old infants had lower levels of sialylated milk oligosaccharides. When tested in mice, these molecules interact with gut microbes to influence growth phenotypes, including lean body mass gain, bone biology, and metabolism. To determine whether these observations in mice apply to a second species whose physiological and metabolic properties are more similar to those of humans, Gordon and his colleagues spent 2.5 years developing a protocol for birthing germ-free piglets and repeating those experiments in this piglet model.

Birthing germ-free piglets requires delivering the piglet directly from the uterus through a sterile plastic tube. Before they take their first breath, the piglets are submersed in a sterile 2 percent chlorhexidine bath and then placed into a sterile, flexible-film nursery isolator, where they are revived and kept on a heated pad until the remaining piglets in the litter are delivered. Within 24 hours, all of the piglets are transferred from these nursery isolators to larger gnotobiotic isolator tubs, with three to four piglets per isolator, in a room that can be thoroughly disinfected prior to the initiation of any experiments. Initially, the piglets are bottle-fed irradiated sow's milk replacement, and starting at day 4 or 5, they transition to a pelleted diet. The piglets are provided with environmental enrichment, and if they are to be colonized with microbes, that occurs four days after birth by suspending an inoculum in the irradiated sow's milk replacement.

Using the same sialylated milk oligosaccharide-supplemented diet and the same microbial culture they administered to mice, Gordon and his colleagues were able to replicate in the piglets the growth and metabolic effects they ob-

served in mice (Charbonneau et al., 2016). Gordon noted that the germ-free piglet model provides the opportunity to characterize postnatal assembly of the microbiota during the suckling and weaning transition of a mammal with a rapid growth phenotype. The piglet model also allows for studying microbial ecology and microbiota development relative to features such as biogeography that are challenging to characterize in smaller vertebrate or invertebrate models. "We think this model provides opportunities to develop technologies that are relevant for studying human communities, as well as interactions between microbiota and hosts," said Gordon. As examples of such technologies, he listed autonomously functioning devices for remote sampling of communities along the length of the gut and implantable devices for measuring metabolism.

There are challenges of working with such a technically demanding, expensive, and time-consuming model. Experiments are limited to the first 30 days after birth, after which time the animals become too large to manage in a controlled environment; the transition to the weaned state requires careful monitoring and husbandry; and the fact that gut function is compromised during weaning in piglets limits studies with communities containing pathogens. There are also fewer analytic reagents available for the pig. Nonetheless, Gordon said he is enthusiastic about this model for select purposes, and particularly as a second species in a translational medicine pipeline for both proof of concept and mechanistic studies.

## IN VITRO SYSTEMS FOR CHARACTERIZING MICROBIAL CONSORTIA

As a means of studying a microbiome's complex microbial community at both the structural and the functional level, investigators are developing a variety of in vitro systems, including the miniature bioreactor arrays that Britton and his colleagues have created to study how microbial communities resist invasion by pathogens without the need to use mice. Other investigators have developed complex three-vessel bioreactor systems (Freeman et al., 2003; Macfarlane et al., 1998), five-vessel systems such as the Simulator of the Human Intestinal Microbial Ecosystem (Molly et al., 1993), and the ROBOGUT system, used to produce defined microbial communities to treat people with recurrent *Clostridium difficile* infection (Petrof et al., 2013).

Britton's miniaturized bioreactor system uses reaction chambers crafted from commercially available plastic blocks that can be autoclaved, are small enough to fit in an anaerobic chamber, and can be combined in an array of up to 96 bioreactors. Peristaltic pumps feed media into and force waste out of the continuously stirred chambers. Tests using fecal matter from three donors showed that this apparatus could produce stable, distinct microbial communities within three to seven days (Auchtung et al., 2015). The relative proportions of the dominant phyla in the bioreactor-produced communities were similar to those of the original fecal samples. While certain phyla significantly recede and others be-

come more abundant than in fecal samples, Britton believes this system captures approximately half of the species that initially go into the bioreactors.

To test if this system could model what happens in the human gut, Britton and his collaborators conducted an experiment in which they treated some of the reactors with water (controls) and others with clindamycin, an antibiotic clinically associated with *C. difficile* infection in hospitals, followed by inoculation with *C. difficile*. In the water-treated reactors *C. difficile* could not compete and was washed away. It was able, however, to establish a stable invasion in the clindamycin-treated bioreactors, and by day 14 this pathogen was producing toxins and spores. Subsequent experiments showed that as few as 150 cells of *C. difficile* would produce a stable invasion. Britton noted that treatment with clindamycin does not affect the total mass of bacteria growing in the reactors, only the community composition. His group is now trying to determine if they can introduce specific bacteria or bacterial communities to reverse the *C. difficile* invasion.

Other uses for the bioreactor array include studying microbiota-driven drug metabolism, microbiota production of beneficial and detrimental metabolites, and how defined microbial consortia form from purified strains of bacteria. Britton and his collaborators are also using the bioreactor array to establish microbial communities from body sites other than the gut and to grow hard-to-cultivate microbes. Going forward, he plans to develop an interface between this device and human enteroids, grown from autopsy tissue, and organoids, produced from induced human pluripotent stem cells or embryonic stem cells, as an approach to introducing a host component into the system and to explore ways of establishing niches inside the bioreactors.

## Organoids

Young and others are using human intestinal organoids to study the relationship between pathogen and host. Intestinal organoids grown from either human induced pluripotent or embryonic stem cells have both mesenchyme and epithelium (Wells and Spence, 2014), explained Young, whereas enteroids produced from autopsy tissue only have epithelium (Sato et al., 2011). Intestinal organoids have a brush border, microvilli, endocrine cells, lysozyme-producing cells, cells that resemble Paneth cells, and goblet cells that produce mucus, all in a stable matrigel environment (Spence et al., 2011). These organoids are sterile, and they have a functional epithelial barrier.

Using organoids produced from human embryonic stem cells, Young and his colleagues have shown that the *C. difficile* toxin disrupts the endothelial barrier within six to eight hours after injection into the interior of the organoid by disrupting the cytoskeleton of the endothelial cells (Leslie et al., 2015). Repeating this experiment with *C. difficile* itself produced the same results over 12 hours, whereas introducing a strain that does not produce toxin had no effect on barrier function. What was surprising about these experiments, said Young, was that *C. difficile*, an anaerobe, was able to grow in what he assumed was an aero-

bic environment, and further examination uncovered the reason. It turned out that there was an *E. coli* contaminant in one of the pieces of equipment, and *E. coli* reduced the percentage of oxygen in the organoid from 21 percent to approximately 8 percent, low enough to allow *C. difficile* to grow. Additional experiments with *E. coli* alone showed that it induced increases in mucus expression and changes in epithelial cell gene expression corresponding to changes in the types of complex carbohydrates these cells produce (Finkbeiner et al., 2015), suggesting that these organoids can be used to study the molecular details of host-microbe interactions. Young noted that the enhanced mucus production is similar to what happens when a human fetal small intestine is first exposed to bacteria.

Young and his collaborators are now looking at ways of monitoring and manipulating the oxygen level in intestinal organoids to facilitate the study of other anaerobic bacteria that may be even more sensitive than *C. difficile* to oxygen. He believes that, while most of the research conducted so far by his group and others has focused on pathogens, such as *Helicobacter pylori* (Huang et al., 2015; Sigal et al., 2015), *Salmonella* (Forbester et al., 2015; Höner zu Bentrup et al., 2006), and rotavirus (Finkbeiner et al., 2012; Yin et al., 2015), organoids offer the opportunity to examine how mutualistic organisms interact with intestinal tissues.

Other avenues of future research will include introducing increasing complexity to the system. Young and his collaborators, for example, have run some experiments in which they observed immune cells homing in on organoids with bacteria but not to those in the same system that have not been colonized. Recent papers from other groups have reported success at triggering development of an enteric nervous system as part of the organoids (Schlieve and Grikscheit, 2017; Workman et al., 2017), though Young questioned how much additional complexity will prove to be too much. “At which point are we trying to build a mouse or a person?” he asked. “What we need to figure out with these organoid model systems is where they actually fit.”

### Human Organs on Microfluidic Chips

A major issue affecting the drug development enterprise, said Ingber, is that most animal studies do not predict results in human clinical trials, at least in part because animal models lack the human microbiome. To address this problem, he and his colleagues at the Wyss Institute are engineering microchips containing living human cells that reconstitute organ-level functions to accelerate drug development and replace animal testing.

Manufacturing microchips using well-developed photolithographic etching allows control of various features in biocompatible materials at the size scale of living cells (Chen et al., 1997; Singhvi et al., 1994). Ingber and his collaborators’ first major success with this approach involved using a functional alveolus on a microchip to observe the human inflammatory response to bacteria at high resolution (Huh et al., 2010) and study pulmonary edema and drug toxicity. They have

since built a small airway on a chip, complete with differentiated bronchiolar epithelial cells and beating cilia (Benam et al., 2016a,b), and are using it to study influenza virus infection, chronic obstructive pulmonary disease using cells from affected patients, and the effect of cigarette smoke on lung tissues.

Ingber's team has also created what he calls a peristaltic human gut-on-a-chip that re-creates the human intestine, complete with fully developed intestinal villi with mucus-producing cells, endocrine cells, Paneth cells, and cytochrome P450-based drug metabolism (Kim and Ingber, 2013). They have used this system to culture a probiotic *Lactobacillus* found in human intestines and have confirmed that it improves barrier function. They have also cultured a commercial probiotic formulation containing eight different microbes. Gene microarray data showed that this mixture totally changes the phenotype of the human gut epithelium to resemble that of distal human ileum, the one place in the small intestine where microbes are found (Kim et al., 2016a). In contrast, a pathogenic strain of *E. coli* completely overgrows the villi instead of merely growing in the spaces between the villi, which is what the probiotic species do. In addition, flowing peripheral blood mononuclear cells through the vascular channel underlying the intestinal tissue triggers the type of injury response associated with pathogenic *E. coli* and inflammatory bowel disease (Kim et al., 2016b). These studies have identified four combinatorial therapeutic targets and shown that the commercial probiotic could partially protect against injury induced by invasive *E. coli*.

A new project in Ingber's laboratory uses human, mouse, pig, and *Xenopus* gut-on-a-chip devices to study host tolerance to infection. His group has also developed a method for creating primary human small and large intestines and colons on a chip, as well as microfluidic chip-based models of the skin, liver, heart, kidney, brain, and blood-brain barriers. These organs and chip-based models create integrated human body-on-chips that remain coupled and functioning for up to three weeks.

In closing, Ingber said he believes organs-on-chips have the potential to gradually replace animal testing in drug development. He noted that the U.S. Food and Drug Administration, which has provided substantial funding for this work, has said it will accept data from these systems as long as Ingber and his collaborators can demonstrate that the data are as good or better than the data from animal models. His group has already demonstrated the robustness of the organs-on-chips, and therefore Ingber's next step would be to obtain primary and induced pluripotent stem cells and microbiome samples from individual patients as a means of creating personalized medicine approaches to treating disease.

# 4

# Modeling Human Microbiota in Animal Systems

Animal models provide opportunities to define the contributions of members of the microbiota to community function and the mechanisms through which they affect various aspects of host biology. Six speakers addressed current approaches they are using in this regard and addressed how these approaches may promote further basic and translational research in this field. The six speakers were Federico Rey, assistant professor of bacteriology at the University of Wisconsin–Madison; Patrice Cani, a researcher from the Belgian Fund for Scientific Research and a group leader at the Université de Louvain Drug Research Institute; Wendy Garrett, professor of immunology and infectious diseases at the Harvard T.H. Chan School of Public Health; Richard Blumberg, professor of medicine at Harvard Medical School and co-director of the Harvard Digestive Diseases Center; Nancy Moran, the Leslie Surginer Endowed Professor in the Department of Integrative Biology at The University of Texas; and Tracy Bale, professor of neuroscience in the School of Veterinary Medicine and Department of Psychiatry at the Perelman School of Medicine, University of Pennsylvania.

## CONNECTING MICROBES TO METABOLISM USING GNOTOBIOTIC MODELS

Microbes in the gut produce thousands of metabolites that affect mammalian physiology through interactions with host receptors and microbial community dynamics (Krishnan et al., 2015). As an example, Rey noted how the human digestive system cannot absorb the beneficial polyphenols and flavonoids in red wine until gut microbes first metabolize these compounds. At the same time, the choline and carnitine in a steak are not only essential nutrients for humans but also substrates for microbes that ferment them and produce chemicals, such as trimethylamine, that are associated with cardiovascular disease (Romano et al., 2015).

The large interpersonal differences in microbiota composition likely mean that nutrient metabolism and absorption from food will vary from one person to the next, which Rey believes may have differential effects on individual health. Understanding this phenomenon, he said, requires knowing what each of the

myriad species in the gut are doing and the effects they are having on each other and on the individual. His approach to untangling this complexity is to colonize germ-free mice with species that are representative of the native community's phylogeny and function.

Bacterial metabolism in the human gut converts choline into trimethylamine, which the liver then converts into trimethylamine-N-oxide (TMAO). In 2011, researchers showed that high plasma levels of TMAO were a good predictor of cardiovascular disease and that gut microbial metabolism was involved in producing this compound (Wang et al., 2011). Subsequently, a number of groups have found associations between plasma TMAO levels and other diseases, including adipose tissue inflammation, heart failure (Tang et al., 2014), renal failure (Tang et al., 2015), diabetes (Dambrova et al., 2016), colorectal cancer (Bae et al., 2014), and non-alcoholic fatty liver disease (Chen et al., 2016). Experiments in mice have shown that TMAO is causally associated with the development of atherosclerosis through its inhibition of reverse cholesterol transport and platelet activation (Warrier et al., 2015).

Starting from those observations, Rey and his colleagues screened some 100 sequenced human gut isolates representing 91 different species in 37 genera for their capacity to convert choline to trimethylamine. Of these 100 isolates, only 8 consumed any choline at all, and each produced trimethylamine (Romano et al., 2015). Fortunately, said Rey, the human microbiome project identified one strain of *E. coli* that uses the same pathway most of these eight organisms use to convert choline to trimethylamine and a mutant version of this strain that lacks the key enzyme involved in this conversion. This enabled his group to create a community of five gut organisms plus either the wild-type or mutant *E. coli*. Measuring TMAO levels in mice colonized with one of the two communities showed that TMAO was present only in the blood of the mice colonized with wild-type *E. coli*, whose serum levels of choline were lower as was the amount of DNA methylation observed in multiple tissues from these animals.

In other experiments, Rey and his colleagues examined the effect these two communities had on metabolic disease in mice fed a high-fat diet, which is known to increase the body's need for methyl donors. The mice with the wild-type, choline-consuming *E. coli* accumulated more fat and higher levels of triglycerides in their livers compared to mice colonized with the community that cannot metabolize choline. Additional experiments with pregnant mice showed that methylation levels in the brains of the pups were higher in those whose mothers were colonized by the mutant strain of *E. coli*. In addition, when the pups grew to young adulthood, those who were born of mothers colonized by the mutant strain of *E. coli* displayed lower levels of anxiety and obsessive-compulsive behavior than did mice born of mothers colonized with wild-type *E. coli*. One conclusion from these studies, said Rey, is that the microbial choline utilization pathway may limit choline availability during pregnancy and affect fetal brain development. "This is something to think about because current dietary guidelines do not consider interpersonal difference in choline-consuming bacteria in the gut," said Rey.

Like Rey, Cani is interested in finding the mechanistic links between gut microbial communities and human disease. Building off the observation Jeff Gordon and his colleagues made that axenic mice fed a high-fat diet are more resistant to bodyweight gain and fat mass development compared to mice that were not germ-free (Backhed et al., 2007), Cani and his collaborators are using gnotobiotic mice to study how gut microbial communities affect the development of obesity and type 2 diabetes. Several groups have shown that transferring gut microbes from genetically obese or diabetic mice into germ-free recipient mice transfers at least part of the phenotype to the recipient mice (Everard et al., 2014; Geurts et al., 2015; Vijay-Kumar et al., 2010). Similarly, gut microbiota transferred from mice with gastric bypass reduced the weight and fat mass in recipient animals fed a high-fat diet (Liou et al., 2013). Gordon and his colleagues have shown that germ-free mice gain weight when they receive gut microbes transplanted from an obese identical twin human, but not when they received gut microbes from the non-obese identical twin (Ridaura et al., 2013). Some of these studies, said Cani, suggest that cross talk between microbes and hosts may involve short-chain fatty acids binding to specific G-protein-coupled receptors.

Cani and his colleagues have been investigating this link through the lens of low-grade inflammation caused by the administration of bacterial lipopolysaccharides (LPSs) (Cani and Delzenne, 2009). Plasma LPS levels are increased across different strains of mice fed obesity-inducing diets or that were genetically obese (Cani et al., 2007). In these animals, blocking the LPS receptor prevented serum LPS levels from increasing and subsequent inflammation from occurring. Additional experiments showed that intestinal LPS triggered metabolic endotoxemia, insulin resistance, and macrophage infiltration (Cani et al., 2008). The deletion of the enzyme N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) from adipocytes causes mice to develop spontaneous obesity (Geurts et al., 2015); thus germ-free mice that received microbes from the genetically modified obese mice gained both weight and fat mass, while their adipose tissue displayed metabolic changes similar to those observed in the donor mice. The mechanism linking adipose tissue to the microbiome remains unknown, said Cani.

His collaborators have also been studying the inverse correlation between the presence of the bacterium *Akkermansia muciniphila* in the gut microbiome and weight gain. Numerous experiments have shown that this bacterium, which accounts for 1 to 5 percent of the human gut microbiome, can reduce the metabolic endotoxemia and insulin resistance normally associated with a high-fat diet (Everard et al., 2013), perhaps by increasing the thickness of the intestinal mucous layer. He noted that, in humans with gastric bypass, as well as in type 2 diabetics on the drug metformin, the proportion of *A. muciniphila* in the gut microbiome increases significantly. Moreover, individuals on a low-calorie diet who lost weight and whose cholesterol levels, inflammatory tone, and insulin sensitivity improved also had a higher proportion of *A. muciniphila*, as well as 16 other metagenomic species, in their gut microbiota (Dao et al., 2016).

These results, said Cani, suggest that *A. muciniphila* might be a therapeutic candidate for treating obesity, type 2 diabetes, and other associated disorders. While the bacterium itself is too sensitive to oxygen to be contained within a pill, a protein from the bacterium's outer membrane produces the same effect in obese and diabetic mice as the live bacterium (Plovier et al., 2017). Preliminary experiments have shown that humans can safely take pasteurized *A. muciniphila*, and Cani and his colleagues are planning further clinical studies in humans.

## REVISITING KOCH'S POSTULATES[1] FROM A MICROBIAL COMMUNITY PERSPECTIVE

When Garrett first began exploring how microbes contribute to colon cancer, she and her colleagues found that the bacterium *Fusobacterium nucleatum*, an anaerobic gram-negative macrobacterium, was enriched in human colorectal tumors and stools (Kostic et al., 2012). Feeding a human isolate of *F. nucleatum* to a strain of mice predisposed to develop intestinal adenomas increased the rate at which these mice developed tumors (Kostic et al., 2013). Additional experiments showed that specific strains of *F. nucleatum* greatly expanded the number of multiple types of myeloid immune cells at the earliest stages of colorectal tumor development. To determine if these results had any bearing on what was happening in human colorectal cancer, Garrett and her collaborators examined RNAseq data generated as part of The Cancer Genome Atlas program and found the same signatures in humans that they saw in mice. The researchers also found that the presence of certain strains of *F. nucleatum* correlated with different T cell subsets in human colorectal cancer patients and that the presence of these strains correlated with a poorer prognosis (Mima et al., 2015, 2016).

Screening of a library of *F. nucleatum* strain 23726 revealed two clones that did not impair natural killer (NK) cell–induced cytotoxicity, and detailed molecular studies conducted with collaborators at the Hebrew University of Jerusalem identified the protein Fap2, an adhesin, as the protein that impairs NK cytotoxicity. Experiments using human cell lines then identified TIGIT, an immune checkpoint inhibitor, as the binding partner on NK cells for Fap2 and showed that Fap2-TIGIT binding protected tumors from immune cell attack (Gur et al., 2015).

Meanwhile, Garrett and her collaborators found that a different binding region of Fap2 interacted with the tumor-expressed sugar galactose-N-acetyl galactose, and this interaction mediated the enrichment of *F. nucleatum* in colo-

[1]Koch's postulates are a set of four criteria for judging whether a given microbe is the cause of a given disease: (1) the bacteria must be present in every case of the disease, (2) the bacteria must be isolated from the host with the disease and grown in pure culture, (3) the specific disease must be reproduced when a pure culture of the bacteria is inoculated into a healthy susceptible host, and (4) the bacteria must be recoverable from the experimentally infected host (see https://www.medicinenet.com/script/main/art.asp?articlekey=7105 [accessed March 2, 2018]).

rectal tumors (Abed et al., 2016). They also found that other bacteria were capable of producing immune system changes to ensure their own survival while promoting cancer growth and spread. Experiments in mice identified a chemokine called CCL2 that enriches myeloid-derived suppressor cells in colon tumors. Taken together, said Garrett, these results suggest that, in the human mouth, *F. nucleatum* is an innocuous symbiont, but occasionally it will colonize the gut and create an immune microenvironment that is permissive for microbes and tumors.

With regard to animal welfare, Garrett said, animal protocols and monitoring are often constructed to catch signs of distress secondary to procedures; therefore simple complementary workflows that minimize labor but help maintain the animals' health are crucial. She also believes simple methods for recognizing signs of suffering and for taking action are needed, such as using paper-based bedding for housing an animal developing prolapse from a tumor. Also, her group is developing noninvasive monitoring methods, such as luminescence-based in vivo imaging, to replace invasive monitoring procedures, such as colonoscopy, and refine experimental procedures.

In addition to obesity and cancer, avenues of research have linked a lack of early exposure of the human microbiome to microbes in the environment to a set of conditions known as atopic diseases, a set of allergic hypersensitivities that include food allergy, atopic eczema, allergic rhinitis, and asthma (Bach, 2002; Carpenter et al., 1989; Ege et al., 2011). Research has also suggested that inflammatory bowel disease (IBD), which is not thought of as an atopic disease but shares immunological characteristics and pathways, may also result from a lack of early life exposures to environmental microbes (Benchimol et al., 2009; Shaw et al., 2010).

Blumberg's approach to testing the hypothesis that early exposure to specific microbes affects immune function and susceptibility to atopic diseases, IBD, and other ailments has been to study the effect that early exposure has on natural killer T (NKT) cells. Doing so, he explained, requires exposing germ-free animals to microbes early in life and looking for the development of a phenotype that does not appear if germ-free animals are exposed to the same microbes later in life. So-called invariant NKT cells recognize host and microbial lipid antigens presented by the molecule CD1d and play a critical role in the early immune response as orchestrators of downstream events. Invariant NKT cells, said Blumberg, are important regulators of bacterial commensalism, whether it involves a pathogen, such as *Pseudomonas aeruginosa*, or a commensal organism, such as *E. coli* or *Lactobacillus gasseri*, a normal inhabitant of the lower reproductive tract in healthy women (Nieuwenhuis et al., 2002, 2009).

Research in multiple laboratories, on both mice and humans, supports the involvement of CD1d and invariant NKT cells in the pathogenesis of IBD (Boirivant et al., 1998; Fuss et al., 2004; Heller et al., 2002; Iijima et al., 2004; Jostins et al., 2012; Liao et al., 2012). Blumberg and his colleagues have shown that exposure to microbial colonization in the early stages of life protects germ-free mice from high invariant NKT cell infiltration in the colon and lung (Olszak et al., 2012). Furthermore, they showed that germ-free mice are highly susceptible to oxazolone-induced colitis associated with triggers that activate invariant NKT cells, and that this susceptibility is eliminated if the mice are exposed to what he called microbial programming during the first couple of weeks of their lives.

Invariant NKT cells are also involved in the development of asthma (Akbari et al., 2003; Albacker et al., 2013; Iwamura and Nakayama, 2010), and Blumberg and his colleagues have shown that early life exposure to microbes protects germ-free mice from allergic asthma. "So two different diseases, one an atopic disease, the other a complex disease, could only be normalized in terms of their sensitization to later triggers of those diseases, if they received the microbial education during the early part of life," said Blumberg. The mechanistic connection, he explained, may be the chemokine ligand CXCL16. This ligand is important for NKT cell recruitment, and germ-free mice exposed to microbes early in life have low levels of CXCL16, whereas germ-free mice not exposed to microbes have high levels of this ligand in serum, the colon, and the lung (Lexmond et al., 2014). Similarly, when the offspring of antibiotic-treated pregnant mice are given antibiotics during the first two weeks of life, they become quite susceptible to oxazolone-induced colitis in a CD1d-dependent and invariant NKT-dependent manner, said Blumberg.

Other experiments may have identified at least one symbiotic organism—*Bacteroides fragilis*—and one specific molecule—a glycosphingolipid called GSL-Ff717—that can normalize invariant NKT levels in the colon of germ-free mice (An et al., 2014) through a pathway that is CKCL16 independent. This molecule, said Blumberg, represents a new class of microbial immunomodulatory molecules. Though early bacterial colonization normalizes invariant NKT cell levels in the lung, other organisms must be involved, he noted, because *B. fragilis* was not able to normalize NKT cell levels in that tissue.

Since publishing the results of these studies, other investigators have found a similar effect from early microbial colonization in germ-free mice on other immune system components, including IgE (Cahenzli et al., 2013) and regulatory T cells in the skin and lungs (Gollwitzer et al., 2014; Scharschmidt et al., 2015). Taken together, said Blumberg, these studies support the hypothesis that atopic disorders and numerous complex diseases, including IBD, originate from inappropriate microbial exposure during early life through pathways that he believes are developmentally regulated. He explained that his current hypothesis suggests that NKT cell infiltration into the colon is a developmentally regulated process influenced by microbes. As a result, it is likely there exist age-dependent pathways linked to later life sensitivity to environmental events. Iden-

tifying and understanding these pathways in humans are challenging, but perhaps *Drosophila* or *C. elegans* could serve as appropriate models for their study.

## THE INTERFACE BETWEEN MICROBES AND NEUROSCIENCE: TWO CASE STUDIES

### Bee Microbiome

When Moran began working with *Apis mellifera*, the Western or European honeybee, about 6 years ago, her goal was to use this species to study different aspects of how its distinct microbiome comes together, how the microbes within that microbiome interact, and how those interactions affect the behavior of bees. The honeybee gut microbiota comprises nine bacterial species that form dense, spatially organized communities in the hindguts of adult workers (Kwong and Moran, 2016) (see Figure 4-1). Any honeybee in the world will have these nine species, said Moran, and only one of these species, an Acetobacteriaceae, is found outside of the bee in nectar. In many ways, this is similar to what occurs in the mammalian gut. "The things that live in our gut for the most part only live in the gut. We do not find them in our food or in the environment," said Moran. A major difference between the mammalian and honeybee gut microbiomes is that the mammalian microbiome comprises hundreds of species in contrast to the nine species that make up more than 95 percent of the honeybee microbiome.

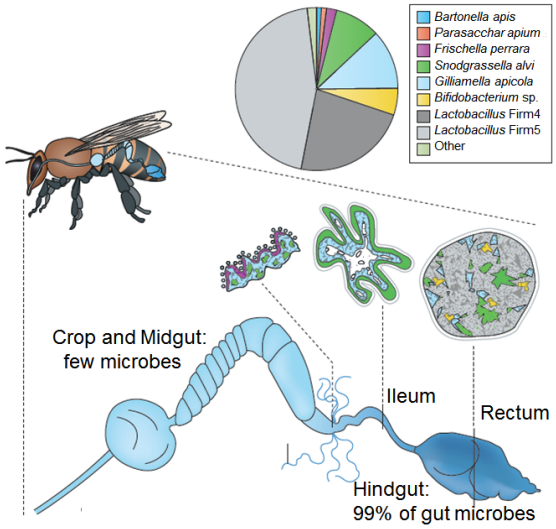


**FIGURE 4-1** The honeybee gut microbiome. SOURCES: Moran slide 4 (Kwong and Moran, 2016).

Honeybee and mammalian gut microbiota are similar, though, in that they are both transmitted socially early in life, mostly within family groups, and both host immune systems that can modulate community composition. High levels of strain diversity within symbiont species exist in honeybee and mammalian microbiota. For example, there can be up to 100 strains of *Gilliamella apicola* within an individual bee. Both honeybee and mammalian gut microbiomes include mixtures of gram-negative and gram-positive bacteria living in the oxygen- and nutrient-poor distal gut, and both can utilize complex plant polymers. Stress, age, antibiotics, and pathogens, such as Enterobacteriaceae, viruses, and trypanosomatids, can disrupt both honeybee and mammalian microbiomes.

When a new adult bee emerges from a capped hexagonal cell 21 days from when the queen lays an egg, its gut has few bacteria, but over the next 5 days a stable community of approximately one billion organisms establishes itself. Moran explained that, when the bee larva turns into a pupa, it sheds its entire gut lining so that when it emerges as an adult it is essentially germ-free. This enables Moran to take the pupae out of the hive when they are still in the capped pupal cells and allow them to emerge in the lab as germ-free individuals that she and her colleagues can manipulate as an experimental system. She also noted that her group developed methods to grow the nine species of bacteria in the honeybee gut microbiome as pure cultures in the laboratory and to introduce fluorescence or luminescence genes that enable them to monitor microbiome composition.

Inoculating the bees is simply a matter of feeding a sucrose solution or pollen laced with one or more of the bacterial species. Imaging of naturally and experimentally colonized honeybee gut tissue showed that experimentally introduced bacteria colonize the same niches in the gut as do the naturally colonized species. In addition, experimentally introduced microbiota can be passaged and replicated by co-housed bees. These experiments also revealed that colonization appears to occur in a particular sequence. For example, *Snodgrassella* needs to colonize the gut before *Gilliamella* can establish itself.

Genomic and metabolomic studies (Engel et al., 2012, 2014; Kwong et al., 2014; Powell et al., 2016) have shown that the members of the honeybee microbiome cross-feed and are interdependent on each other. They have also revealed that many of the interactions of the different species are antagonistic, involving toxins and bacteria type VI secretion systems. These studies have shown that some *G. apicola* strains can degrade pollen cell wall components, such as pectin, and use the resulting sugars as an energy source. This bacterium and others in the bee microbiome can also metabolize sugars that would otherwise be toxic to the bees (Zheng et al., 2016). Moran noted that, just as in the human gut, different bacterial strains break down different plant polysaccharides, a phenomenon that may be important nutritionally for the host given that the bee itself is incapable of metabolizing many of these polysaccharides.

Turning to the subject of bee behavior, Moran noted that there is an extensive literature on bee behaviors, and researchers have developed a number of assays for learning, motility, aggression, sociality, gustatory response, buzzing re-

sponse, and other behaviors (Fahrbach and Robinson, 1995; Zayed and Robinson, 2012). In one learning experiment, Moran and her colleagues, using the plasmid system they created to introduce genes into honeybee microbiome species, studied the effect on bee behavior of adding L-dOPA, a precursor to dopamine, into bee gut bacteria. Other investigators had shown previously that bees fed dopamine learn to associate a color with punishment, as measured by a sting extension response, faster than control bees, and that feeding them a dopamine antagonist diminishes that learned response (Agarwal et al., 2011). This experiment found that bees inoculated with the L-dOPA-producing bacteria learned faster and demonstrated better memory than bees inoculated with bacteria engineered to produce green fluorescent protein.

Though this result was not surprising, Moran said it serves as proof of the principle that gut bacteria can produce chemicals that alter their host's behavior and that this process can be studied in germ-free honeybees. Ongoing studies in her laboratory include examining how insulin produced by the bee microbiome affects hunger, as measured by proboscis extension, and the effect of isopentyl acetate, an alarm pheromone, on aggression, stinging, and cohort alert.

With regard to animal welfare issues, Moran said that Institutional Animal Care and Use Committee policies do not cover bees. Nonetheless, she and her collaborators strive to avoid procedures that might result in prolonged suffering of the bees. At the end of an experiment, the bees are killed by freezing, a common way for them to die in nature. In addition, because bees are an agriculturally important species and the subject of many types of studies, there are established protocols for using bees in research.

In closing, Moran discussed some of the challenges her group has encountered working with bees as a model organism. Given that honeybees have complex social lives in large colonies, studying them in the laboratory environment outside of the context of a colony and without a queen being present is highly artificial, she said, and nobody has been able to establish a germ-free colony. Bee behavior also varies genetically and with age, which requires controlling for numerous sources of variability. In addition, despite homologies in endocrine systems, immune systems, and nervous systems, many aspects of human biology do not apply to bees. Still, she said, one motivation for studying bees is the bees themselves. "They are important and a lot of them are dying so we are hopeful that some of what we find out will actually be helpful in improving the health of bees as pollinators," said Moran. She added that the bee microbiome does protect against pathogens to some extent, though the mechanism is not known.

### Maternal Microbiome

Bale's interest in the microbiome stems from her work on how events that occur during pregnancy affect brain development. In particular, Bale was curious as to how maternal stress might affect the mother's vaginal microbiome, and

hence the initial inoculum that seeds the infant's microbiome, and whether changes in the maternal vaginal microbiome had different effects on neurodevelopment in female and male newborns. She noted that neurodevelopmental disorders occur more frequently in males than females, and that research has identified many factors that influence male vulnerability during the neonatal window compared to females (Bale, 2015).

Over the past 14 years, Bale and her collaborators have cataloged a host of effects from maternal stress during early pregnancy that pass through at least two generations of offspring. These effects include changes in the behavioral stress response, the hypothalamic–pituitary–adrenal axis response to stress, activity of stress regulatory genes, cognitive deficits, and reduced post-pubertal weight gain (Howerton and Bale, 2014; Howerton et al., 2013; Morgan and Bale, 2011; Mueller and Bale, 2008). The effects occur in male but not female offspring developing in the same uterus. "Could there be an aspect by which the stress that is influencing Mom changes the contents or the composition of the vaginal microbiome such that when the babies are born they are getting a different inoculant than they would otherwise, which is influencing brain development?" asked Bale.

To answer that question, Bale and her colleagues looked at whether maternal stress during early pregnancy in mice changed the vaginal microbiome in a manner that persisted until the time of birth. In fact, early stress changes both the bacterial and the viral composition of the vaginal microbiome that persists through at least two days after birth (Jasarevic et al., 2015b) (see Figure 4-2). Proteomic analysis revealed significant changes in the vaginal tissues after exposure to stress, particularly among proteins involved in the immune response. Next, they showed that the mother passes these changes in the maternal microbiome to her offspring's microbiome and that these changes result in metabolic reprogramming in the offspring's gut and brain (Jasarevic et al., 2015a). In particular, said Bale, there is a dramatic drop in *Lactobacillus* levels in the maternal vagina, though not in maternal feces, and a corresponding drop in the gut microbiome of both male and female offspring.

Then came some surprising results: by day 4 after birth, these differences went away, but then at day 28, when puberty begins in mice, dramatic changes appeared in the male gut, while only slight changes occurred in the female gut. These changes in the male gut microbiota were associated with many-fold increases in mitochondrial, carbohydrate, and energy metabolism, which together could be affecting the availability of nutrients in the brain. Going back to the postnatal day 2 offspring, Bale and her colleagues found that amino acid transport into the paraventricular nucleus at day 2 was markedly different in males than in females. The paraventricular nucleus is the part of the brain that regulates stress reactivity, and it plays a role in the brain's homeostatic response to feedback from the periphery, Bale explained.

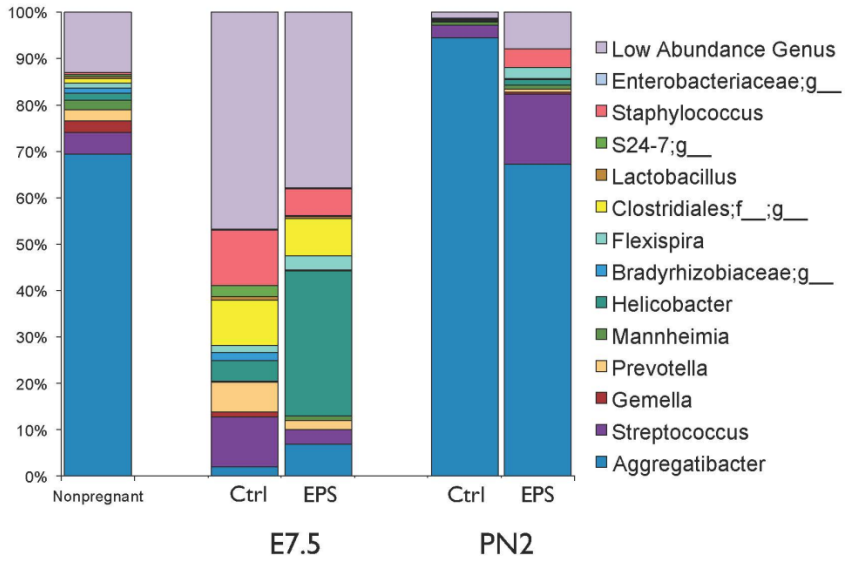


**FIGURE 4-2** Early pregnancy stress changes the mouse vaginal microbiome when comparing embryonic day 7.5 with postnatal day 2. SOURCES: Bale slide 7 (Jasarevic et al., 2015b).

These associations are interesting, she noted, but the real goal was to demonstrate causality. To get at causality, she and her colleagues performed a difficult set of experiments in which they delivered the pups via cesarean section and inoculated them with vaginal lavages from stressed and control mothers, a procedure that appears to reproduce the bacterial load and diversity of the vaginal microbiome in the gut of the offspring. It also reproduces the elevated stress response in post-pubertal males delivered vaginally from mothers exposed to stress. However, males from stressed mothers delivered by cesarean section who were then inoculated with a vaginal lavage from a non-stressed mother still show an elevated stress response. Males from control mothers who received a vaginal lavage from stressed mothers showed a slightly elevated stress response.

One factor Bale had not considered was that prenatal maternal stress could be affecting the development of the gut during fetal development in a sex-specific manner and that any such differences could be interacting with the maternal inoculant. In fact, when she and her colleagues conducted a proteomic analysis of the male and female gut at embryonic day 18.5, they found huge sex-related differences. "So right before birth, there are huge differences in the development of the gut that likely are interacting with the mother's microbiome as they pass through the same vagina," said Bale. Further analysis showed that immune system genes in the male gut were activated in a manner similar to that seen in a response to *Leishmania* infection, though there was no infection by this parasite. "This was surprising to us because they have not been inoculated yet

and you are already seeing differences in priming of the gut for these animals," said Bale. These differences, present at birth, then interact with the initial vaginal inoculant.

These differences, though not by themselves, manifesting as disease or a behavioral disorder, are establishing how the male and female gut and brain respond to the environment later in life. For example, exposing these animals to a week of chronic stress lowers gut permeability in the males but not females born of mothers who experienced stress during early pregnancy. Bale noted, too, that changes in the microbiota of these animals are associated with changes in plasma levels of various metabolites and may affect their transport into the brain.

In closing, she pointed to the common practice among neuroscientists of shipping pregnant mice for use in research studies. Doing so, she said, exposes the pregnant mice to a variety of stressors and different environments. Given the results she and her colleagues have obtained, she wondered how the effects of early pregnancy stress might be affecting the results of neuroscience and behavioral studies involving those pregnant mothers and their offspring. She also commented on the possibility that these types of differences could be related to the increased vulnerability of human males during the prenatal and neonatal period that manifests in lower survival rates and increased rates of developmental disorders, such as autism, which occurs four to five times more frequently in males than females.

# 5

# Experimental Reproducibility Using Gnotobiotic Animal Models

The second day of the workshop focused on challenges stemming from working with gnotobiotic animal models, both with regard to experimental reproducibility and in building and operating facilities supporting the use of gnotobiotic animals. The speakers who addressed experimental reproducibility were Andrew Macpherson, professor of medicine and director of gastroenterology at the University Hospital of Bern; Craig Franklin, professor of veterinary pathology and director of the Mutant Mouse Resource and Research Center at the University of Missouri; Aldons Lusis, professor of microbiology, human genetics, and medicine at the University of California, Los Angeles; Jeremiah Faith, assistant professor at the Immunology Institute and the Institute for Genomics and Multiscale Biology at the Icahn School of Medicine at Mount Sinai; Gary Wu, the Ferdinand G. Weisbrod professor of medicine at the University of Pennsylvania's Perelman School of Medicine; and Alexander Chervonsky, professor of pathology and chair of the committee on immunology at The University of Chicago.

## CREATING STABILIZED MICROBIOMES IN LABORATORY ANIMALS

One of the most difficult aspects of animal husbandry, said Macpherson, is controlling or standardizing the animal microbiome, in part because it is still not well understood. Consequently, it is difficult for researchers to "make robust measurements, to look at the underlying biology, to design suitable controls in our experiments, and to reduce the number of animals [used]," Macpherson stated. Given the reduced genetic variance of inbred strains, environmental microbiota and experimental manipulations predominantly influence the phenotypic variance. Macpherson's main question, and the focus of his talk, was how much further this variance could be reduced through the application of gnotobiology.

Reducing microbiome-associated phenotypic variability is challenging for two reasons, said Macpherson. First, while the gut and skin microbiomes of a typical human remain relatively constant over a period of weeks, across individuals they vary tremendously. Second, experiments in germ-free animals have

shown that manipulating a host's microbiome creates a plethora of strong effects throughout the body. Therefore, a single microbiome cannot serve as the exemplar for a species.

Different experimental models (see Table 5-1) are useful for characterizing and ultimately minimizing microbiome-associated phenotypic variability, said Macpherson. His research has largely focused on bottom-up models, using axenic animals or those with simple microbiotas, but noted that these models illuminate only portions of the biology of the complex microbiomes studied using top-down models (i.e., models with complex microbiotas).

An important limitation of top-down models is their inability to precisely define microbial consortia and the resulting ambiguity in assigning phenotypic effects to particular species within those consortia. However, Macpherson feels that the biggest disadvantage of these models is the lack of reproducible results.

The reduced phenotypic variability of inbred animals allows experiments to reach statistical significance using smaller numbers of animals than when using outbred strains. Using different inbred strains in an appropriately powered factorial design can compensate for the loss of genetic traits. Standardization, however, extends beyond genetics given that the microbiota also varies greatly from facility to facility. Breeding littermate controls is one approach to help clarify whether the microbiome or host genetics are contributing to phenotypic traits (Stappenbeck and Virgin, 2016), and can be applied in all animal facilities, Macpherson said. He added that researchers should be aware that raising mice in vivaria protected from natural environmental pathogens would change the immunological maturity of the mice.

**TABLE 5-1** Different Types of Experimental Models for Understanding Microbiome-Associated Phenotypic Variability

| | Bottom-Up Models | Convergence | Top-Down Studies |
|---|---|---|---|
| **Definition** | Studying axenic models or those with very simple microbiotas, e.g., germ-free or monocolonizations | Studying defined components of complex microbiotas (e.g., IgA-bound bacteria) in a gnotobiotic system | Studies of complex and natural microbiotas (e.g., human samples, SPF) |
| **Disadvantages** | 1. Limited scope<br>2. May omit microbial metabolic pathways and metabolite exchanges between bacteria in complex microbiotas<br>3. Mostly mouse models | | 1. Imprecise definitions of microbial consortia<br>2. Ambiguity in assigning effects to species<br>3. Reproducibility issues<br>4. Ethical issues limit human experimentation |
| **Advantage** | Molecular mechanisms and interactions between microbes or their metabolites and the immune system can be defined | Defined and reproducible system with a microbiota that aims to be representative of a natural situation and is amenable to experimentation | Representation of a natural situation that models or directly shows the human condition |

Attempts to create inbred mouse strains with standardized microbiota have not been successful (Pang et al., 2012). One approach is to transfer isogenic embryos into a second isogenic strain with the required microbiota so that the pups produced will have acquired the defined microbiota of the second strain. Another approach is to gavage an isogenic strain with organisms from a pure culture or a fecal sample, or even add a colonized animal to a cage. A reversible approach would be to gavage a mouse with bacteria that cannot survive within its digestive system so that—in time—it becomes germ-free (Hapfelmeier et al., 2010).

The best-known diverse, standardized consortium is the altered Schaedler flora, which consists of eight microorganisms derived from mice (Wymore Brand et al., 2015). Strains are first inoculated, and then a microbial consortium that complements the model's pathways and metabolomics must be carefully chosen. Macpherson posited that isobiotic strains should, ideally, "be stable over generations on open source diets" to ensure reproducibility. The criteria for creating an isobiotic strain are that its microbiome is stable across multiple generations, that the members of the consortium can be cultured and have published genomic sequences, and that the original germ-free mouse comes from an open-source stock so that other laboratories can regenerate the microbiota. Such criteria would enable transfer of the microbiome to mice of different genetic backgrounds and to different institutional animal facilities.

Macpherson elaborated on the ideal consortium: it should be regenerated from pure cultures, have known microbial metabolic pathways, and not cause abnormalities in clinical chemistry, hematology, histology, body composition, development, or fecundity. Moreover, it should express representative metabolic and immunological profiles, induce pathogen resistance and inflammatory response, and be relatively stable under aseptic husbandry conditions in individually ventilated cages. He and his team have designed the Stable Defined Moderately Diverse Mouse Microbiota 2 (sDMDMm2) consortium, which has been successfully transferred to other institutions and remains reasonably stable over time if the host animals are consistently fed a standardized diet. If the diet is changed, both representation and transcriptomic signatures of the consortium members change significantly.

In conclusion, Macpherson stated that "one microbiota is insufficient for a scientific community," but he believes that significant progress can be achieved by analyzing a microbiota that is standardized across multiple institutions. Since it is possible to maintain defined microbial consortia and isobiotic strains with reasonable stability over time, experiments can be designed with small sample sizes and/or involving multiple institutions. He stressed that no one isobiotic model should be considered exclusive. Instead, researchers should view using isobiotic models in a manner that matches particular situations to particular models. This targeted use, he believes, is statistically more powerful and also

contributes to the Three Rs,[1] as it reduces the number of animals necessary to perform an experiment.

## COMPLEX GNOTOBIOLOGY: AN EMERGING PARADIGM IN THE ERA OF NEXT-GENERATION SEQUENCING

The Mutant Mouse Resource and Research Center that Franklin directs collects the mutant mice that other investigators generate. These mice come into the center with a particular microbiome, but their microbiomes are likely to be different when the center distributes them to other investigators. "Is that problematic for the phenotypes of these models that we have collected and are distributing? Intuitively, we thought that it could be," said Franklin.

The problem with trying to answer that question rests with the fact that the animals that come into the facility are not gnotobiotic and have complex communities of microbes. Next-generation sequencing and advances in bioinformatics provide the opportunity to better characterize these complex communities, though it is not possible yet to take all of the sequence data down to the level of the operational taxonomic unit, said Franklin. In addition, sequence data, even with the help of new predictive statistical tools, cannot provide information on functionality and overall phenotype. He added that, while studies with classical gnotobiotic animals with defined communities provide important insights about function, it might be important to take what these studies show and see if the results hold true in animals with a complex microbial community with other bacteria, viruses, fungi, and organisms.

Franklin and his colleagues began exploring the issue of complex microbiota by looking at whether the gut microbial communities vary in contemporary rodent colonies. "We knew that animals produced at Jackson Labs and Taconic were probably different, but we wanted to look beyond those," said Franklin. Principal component analysis comparing the microbiota of animals from Jackson Laboratories and Harlan Sprague Dawley, another supplier, showed differences in certain families of bacteria (Ericsson et al., 2015b) that were small between animals from the same vendor versus between animals obtained from the two different vendors.

He and his colleagues also looked at microbiota during the first weeks of a mouse's life and found that diversity is low at week 1, increases dramatically in week 2, and by week 3 it is similar to that of an adult animal. It would be useful, said Franklin, to know the physiological changes during this period and how these may impact conditions later in life.

[1]The Three Rs [3Rs] refer to the concepts of replacement, refinement, and reduction. Russell, W. M. S., and R. L. Burch. 1959. *The Principles of Humane Experimental Technique*. London: Methuen and Co.

In another study, he and his colleagues examined the effect of diet, bedding, and housing on the mouse microbiome. While there was some variation among most of the combinations, when the mice were housed in static microisolators with Aspen bedding there was a marked difference in cecal, but not fecal, microbiota. "We rely on feces, but there is a lot going on upstream that we may not be detecting by only focusing on feces," said Franklin.

Diet, bedding, and housing are just three of the many variables that can modulate microbiota, but the important question, Franklin said, is whether those shifts matter, and he believes they do. An initial proof of concept experiment examined the effect of introducing microbiota from mice obtained from three commercial suppliers into a knockout mouse model of IBD. The results showed that different microbiota present in rodent communities might be modulating disease phenotypes (Ericsson et al., 2015a). Experiments with a rat model of colon cancer produced similar variations in disease phenotype depending on which of three commercially available rats served as the microbiota source. These experiments, Franklin noted, are identifying targets to be explored in gnotobiotic studies at some point in the future, just as banking feces could potentially allow the reconstitution of phenotypes that have disappeared over time. When investigators move from one laboratory to another, he explained, they might lose the phenotype they were studying, so they request soiled bedding from their former animal facility, add it to the cages housing their animals at their new facility, and reconstitute the phenotype. "This is indirect evidence that the microbiota is playing a role," said Franklin.

His team tested whether banked feces can reconstitute a gut microbiome and found that feces from a low-diversity donor did not reconstitute the original microbiome when transplanted into a high-diversity recipient treated with a cocktail of four antibiotics (Ericsson et al., 2017). The results were the same, he added, regardless of whether they used fresh or frozen feces or a cecal transplant. Going from a high-diversity donor into a low-diversity recipient treated with the same antibiotic cocktail is partially successful. He also noted that, when two animals with different microbiota live in the same cage, their microbiomes hybridize, something that may be important depending on the phenotype in question. As to whether mouse gut microbiomes are translatable to the study of human immune responses, he noted that a number of investigators are exploring this issue and finding that many organisms other than bacteria can trigger responses that would be useful for studying human diseases (Baxter et al., 2014; Beura et al., 2016; Chudnovskiy et al., 2016; Reese et al., 2016; Tan et al., 2016; Weldon et al., 2015; Wu et al., 2010; Zackular et al., 2016).

As a final comment, Franklin said that improving the definition of gut microbiota could help minimize variability and reduce the number of animals needed to properly power studies. He also raised the possibility that the microbial content of feces could serve as a biomarker for non-terminal experimental end points. "Can we start looking at the feces and what is happening in some of our disease models and say it is time to shut down?" he asked. As to whether the field should move toward using a standardized microbiota in future experiments,

he believes the answer is no, that perhaps it would be preferable to have a collection of complex microbiomes that can be used as tools.

## THE ROLE OF HOST GENETICS

To get some insights into factors that lead to microbiota variability across members of a species, including humans, Lusis and his colleagues have been studying a set of some 100 commercially available inbred strains of mice selected for their genetic diversity. The genome of the members of the Hybrid Mouse Diversity Panel has been sequenced, or at least densely genotyped (Bennett et al., 2010). The diversity in their microbiomes is similar to the diversity observed in human populations (see Figure 5-1). The variability within a particular mouse strain is small compared to the variability between strains, said Lusis, which suggests the influence of a host genetic component on the variance in microbiota. The consistency within a strain could, for example, result from maternal seeding, since all of the mice in a strain were derived initially from the same mother.

To address that question, Lusis and his colleagues analyzed the genome sequences of the strains in the Hybrid Mouse Diversity Panel to map how they are related to one another given that they were all derived from a pool of pet mice around 100 years ago. Based on the sharing of particular microbes they calculated a measure of heritability, that is, the relationship between genetic and phenotypic similarity (Org et al., 2015). The results of these calculations were surprising. "Heritability is high, unexpectedly high," said Lusis, which means that the genetics of the mouse strain, given a common environment, accounts for about 50 percent of the variability of the microbiome in that strain. A similar study of some 1,200 monozygotic and dizygotic twins in the United Kingdom performed the same analysis and calculation and found for a number of microbial genera that heritabilities were 20 to 30 percent (Goodrich et al., 2016), which Lusis said is consistent with his results in mice. In comparison, the heritability of heart disease and type 1 diabetes are approximately 40 percent and 70 percent, respectively.

Given the presentations during the first day of the workshop, it should not be surprising that host genetics play an important role in microbiome composition, said Lusis. "We have talked about how we have adapted to the microbiome over millions of years, and that there will be factors we produce that affect the microbiome," he said. "If those factors vary in the population, then the microbiota will vary in the population." As an example, he and his colleagues performed a small experiment in which they compared the microbiomes of gonadectomized male and female mice to controls and found that the microbiomes shifted in composition between the matched groups. Testosterone replacement in the gonadectomized males shifted their microbiomes back to match those of the sham-treated animals.

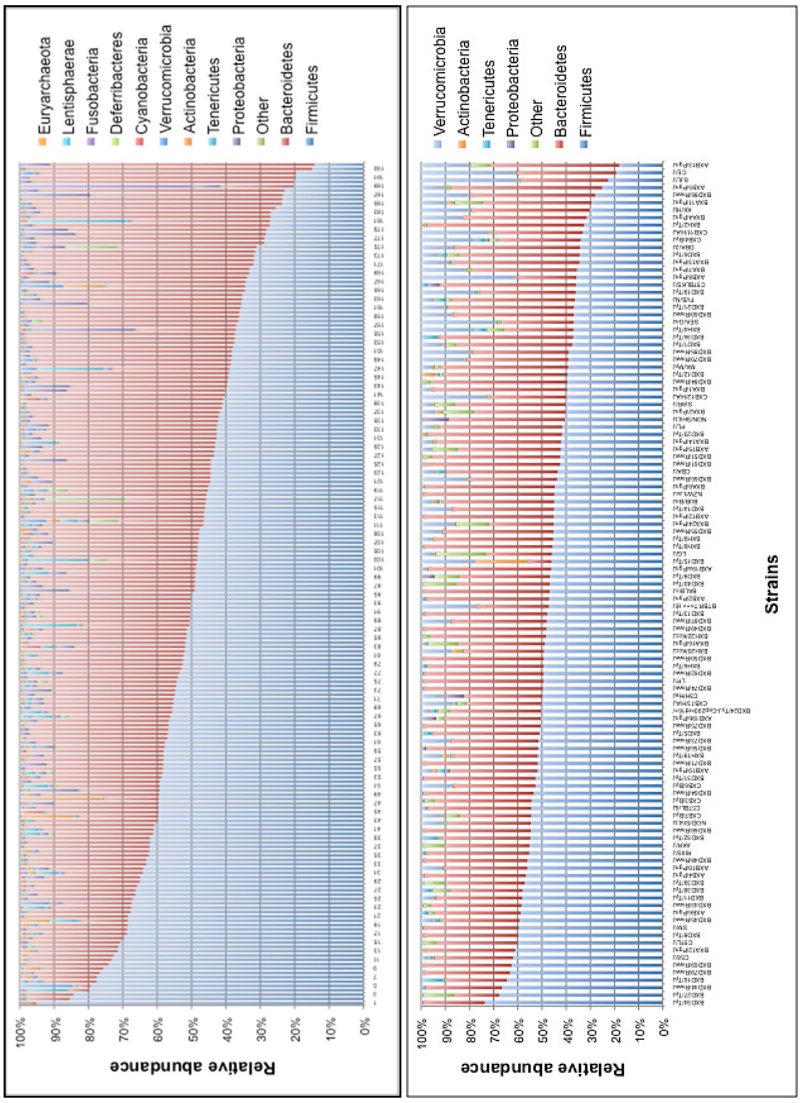


**FIGURE 5-1** Microbial diversity in a population of 200 Finnish men ages 50 to 70 years (top) and across 113 mouse strains in the Hybrid Mouse Diversity Panel (bottom). SOURCE: Lusis slide 6.

While genetics contributes to microbiome composition, Lusis said that diet and environment will trump genetics. In fact, an experiment looking at the effects of gene-by-diet interactions found substantial changes in microbiota within the same strains depending on whether they were fed standard mouse chow, a high-fat and high-sucrose diet, or a high-fat and high-cholesterol diet. The changes, though, are not additive or linear and are still a function of genetics, he added. "The genetics determines whether an individual, or a mouse in this case, responds by changing a lot or not at all," said Lusis.

Lusis also discussed how he and his colleagues have been using host genetic variation to study host-gut microbe interactions. "If there is this big genetic component to the composition of gut microbiota, we should be able to apply genetic mapping to identify the genes responsible," he said. A genome-wide association study of the mice fed a high-fat and high-sucrose diet identified one or two loci that correlated with the abundance of a number of microbiota genera (Org et al., 2015). He and his colleagues are now working to identify the genes controlling gut microbial content.

It is also possible, said Lusis, to look for correlations between gut microbiota and host traits to identify candidate bacteria that influence host physiology or disease. His research group measured fat gain in response to a high-fat, high-sucrose diet and found that some strains respond greatly and others very little. The strains that had high proportions of *Akkermansia muciniphila*, for example, tended to not gain weight in response to this diet (Parks et al., 2013). "This is not causality, just correlation, but it allows you to formulate a hypothesis that you can then test." In fact, when they gavaged *A. muciniphila* into obesity-prone mice—the mice were not germ-free or treated with antibiotics—and later started them on the high-fat, high-sucrose diet, the increase in body fat, plasma lipids, and glucose metabolism was less than when the mice were gavaged with heat-killed *A. muciniphila*. As Patrice Cani noted in his presentation, he and his colleagues have purified a membrane protein from this bacterium that improves metabolism in obese and diabetic mice (Plovier et al., 2017).

One practical application of these findings, said Lusis, relates to how his group treats mice they purchase from their mouse supplier. "If we get mice from Jackson Laboratories, they are very different from the mice in our place," he said. Instead of using them in experiments immediately, his research group first breeds the mice in their vivarium for a few generations.

In closing, he said that embracing diversity presents an opportunity to unravel the incredible complexity of microbial interaction with the host. "Simplifying things is the classic way that scientists have operated to dissect mechanisms, but at the same time, in terms of microbiota, I think looking at diversity is important as well," said Lusis. He also said that it is important to look at both human and mouse populations to identify similarities and differences.

## THE ROLE OF IMMUNOLOGICAL VARIATION

In 2009, researchers published what Faith considers the key paper concerning the interactions between the microbiome and the immune system (Ivanov et al., 2009). This paper showed that colonization of the mouse intestine by segmented filamentous bacteria originally absent from that particular murine strain would trigger a robust immune response that was protective against infection by an intestinal pathogen. These experimental results were important, he said, because they showed that a specific microbe could change the immune system on top of a complex background.

Faith noted that mice from Jackson Laboratory and Taconic are frequently used in immunological studies of the microbiome which have repeatedly demonstrated that differences in the microbiome affect an animal's immune response. For example, investigators studying the anticancer effect of the protein PD-L1 found that Jackson mice treated with this molecule produced a vigorous antitumor response, whereas Taconic mice given the same treatment produced a much smaller response (Sivan et al., 2015).

Faith and his coworkers have investigated the relative versus absolute abundance of different microbiome components. Measuring relative abundance, he said, yields an incomplete picture of how the microbiota responds to various challenges. For example, dosing a Jackson mouse with the antibiotic vancomycin eliminates the animal's microbes, and plotting the relative abundance of different bacteria as the microbiome recovers would suggest that a particular bacterial phylum, the Firmicutes, expands rapidly in the intestines by day 7. A plot of absolute abundance shows that, in fact, the microbial community remains decimated for at least another week. However, when they repeated this experiment using Jackson mice ordered on a different day, the results were markedly different—vancomycin had little effect on the total microbial biomass, though the absolute response showed there had been a bloom of vancomycin-resistant *Akkermansia.* From these results, Faith concluded that variation of microbiota across animal facilities, between rooms, and across time is confounding results.

Faith's solution to the non-standardized microbiota of a supposedly reference murine strain is to work with gnotobiotic mice and to take advantage of the fact that gnotobiotic mice housed in the same cage will maintain their defined microbiome communities, assuming the animal facility staff are well trained and follow strict handling procedures. From a practical perspective this implies that, when students leave his laboratory, they can re-create their animals in a new facility merely by taking a sample of the microbiota used to create the gnotobiotic animals and innoculating axenic animals housed at their new location. He noted that his research group has developed a robotic microbiota "manufacturing" system that can reliably assemble identical standardized microbial communities. Using this system, he and his colleagues have produced defined microbiomes from more than 50 human fecal samples (Faith et al., 2013; Goodman et al., 2011).

Using defined colonies derived from fecal samples from humans with or without IBD, Faith and his colleagues have demonstrated that human gut microbes transfer colitis to susceptible mice by measuring weight loss in the recipient mice. The response of the mice to different human microbiota was variable—the microbiota from some healthy humans produced symptoms of colitis in the recipient mouse, for example. This variability, said Faith, points to the need to examine samples from multiple individuals and look at the resulting distribution of immune profiles in the recipient mice. "I think the most frequent question at microbiome talks is, 'What is normal?'" said Faith. In fact, when his group assessed the immune-stimulating properties of different human microbiota in an unchallenged mouse model, the responses fell along a spectrum.

Because every human microbiota triggers an increase in regulatory T cell production in the recipient mice, Faith and his colleagues set out to determine which bacteria were responsible for this response (Faith et al., 2014). They identified seven different bacteria that could increase regulatory T cell numbers relative to germ-free animals. Using a microbial community derived from an individual with Crohn's disease, his group identified an immunomodulatory effector strain of Enterobacteriaceae as measured by its ability to increase Th17 cells (Ahern et al., 2014). They were able to replicate this response by adding this specific bacterium to a commercially available seven-member model community that by itself did not trigger an increase in Th17 cells.

Going forward, Faith said that these types of immunological experiments would benefit from developing standard operating procedures designed to keep animals free from known pathogens and enable keeping microbiota reasonably constant for several generations. A goal for the research community, he added, should be to create a set number of microbiotas with different properties that a laboratory could order from a vendor to create a defined human microbiota in a mouse. "Logistically, this is complicated, but I think more important than anything would be to change SOPs [standard operating procedures] and training of animal staff to be able to handle this," said Faith.

He said he would also like to see the development of an effector strain collection of individual or groups of microbes with known function and known ability to engraft on defined microbiotas. "If we all had the same baseline communities, we would know how good each effector strain is at invading a certain number of communities," said Faith. It would be good to know how robust a newly discovered virus is across the standard defined communities, for example, to understand how well that virus can manipulate a particular immune cell population. To make these resources available, the field needs to develop a system of government suppliers, commercial vendors, and internal standard operating procedures to standardize microbiotas worldwide.

## STANDARDIZING AND CHARACTERIZING DIETS

The effects of diet on the human microbiome are difficult to analyze: humans adhere poorly to standardized dietary regimens, diet can have profound

impacts on host biology independent of the gut microbiota, and intensive controlled feeding experiments and large outpatient cohort studies are expensive and challenging to complete. Animal models can address each of these challenges, said Wu. Animal studies, for example, enable tight control over defined diets for long periods, while germ-free animals allow researchers to examine the effect of diet independently of the gut microbe.

It is fundamentally important, he said, to understand that diet not only shapes the composition of the microbiome but also serves as a substrate for the microbiota to produce molecules that can circulate widely throughout the body and affect distant organs (Holmes et al., 2012). For example, consumption of a particular milk fat delivers more sulfated bioacids to the gut microbiota, triggering a bloom of the sulfate-reducing bacterium *Bilophila wadsworthia*, which in turn stimulates the immune response that exacerbates colitis in a mouse model of IBD (Sartor, 2012). Similarly, a high-fat diet provides choline, which is metabolized to trimethylamine. As Federico Rey previously noted, the liver then converts trimethylamine into trimethylamine-N-oxide, a molecule that accelerates coronary vascular disease (Wang et al., 2011).

One human disease that diet affects is IBD and its many manifestations, and in fact, dietary modification is a first-line therapy in Europe, Japan, Israel, and some U.S. and Canadian centers for Crohn's disease. More people would use dietary therapy, said Wu, except that these diets are monotonous, often unpalatable, and require delivery by nasal gastric tube. In addition, said Wu, "Despite their efficacy, we really do not understand how they actually work." In that regard, his goal is to answer two fundamental questions:

1. Does this exclusive enteral nutrition (EEN) provide something "good" for patients with IBD that is not abundant in the regular diet?
2. Does the consumption of EEN exclude something that is "bad" for patients with IBD in the regular diet?

Various animal models have shown that substances present in the human diet today, such as artificial sweeteners and emulsifiers that were not present several decades ago, alter the microbiota in a way that favors inflammation said Wu (Chassaing et al., 2015; Suez et al., 2014). While he does not claim that artificial sweeteners and dietary emulsifiers cause IBD or any type of disease, this idea is worth exploring. Work from Jeff Gordon's group has shown in both humans and mice that diet does not have to alter microbiota composition to produce a physiological response (Faith et al., 2011; McNulty et al., 2011). For example, humans and gnotobiotic mice fed a fermented milk product with live microbes experienced no significant change in the composition of their microbiota, but there was a specific and reproducible transcriptomic signature, related to polysaccharide metabolism, seen in both humans and mice. This type of experiment, said Wu, shows that researchers can use gnotobiotic mice to mimic and understand the human response to a particular dietary intervention.

Computational biologists, Wu noted, have begun to mine clinical metadata, including microbiome data, to predict how someone will respond to diet. One group of investigators, for example, used genomic flux modeling to predict how certain microbes would respond to diet and how that response would lead to changes in serum amino acid levels (Shoaie et al., 2015). Another group devised a machine-learning algorithm that integrates various physiological parameters, dietary habits, physical activity, and data from gut microbiota to predict personalized postprandial glycemic response to meals (Zeevi et al., 2015).

Numerous research groups, said Wu, have shown that the gut microbiome can have a significant effect on the metabolome of the host animal (Wikoff et al., 2009; Xie et al., 2013; Zheng et al., 2011). Researchers have for the most part conducted these studies using germ-free and colonized mice, and studies have yet to confirm if these results hold true in humans, he added. "I personally believe that a lot of the input, at least in the metabolome in terms of diet, is independent of the gut microbiota," said Wu.

As Lusis noted earlier, though, diet certainly has a strong effect on mouse microbiota, and Wu and his collaborators have shown the same effect in humans in a study of 15 vegans and 16 omnivores. They have also demonstrated that the plasma and urinary metabolomes of omnivores and vegans differ to such an extreme that a computational analysis of an individual's plasma metabolome can predict with 94 percent accuracy whether a person is a vegan or omnivore (Wu et al., 2016). Despite these huge differences, the gut microbiota composition of vegans and omnivores differed only modestly, as did the diversity of the microbiomes. One explanation could be that organisms other than bacteria—fungi, viruses, or bacteriophages, for example—could be responsible for the observed metabolomic shifts, and this is something Wu plans to examine in a future study.

These results, said Wu, seem to contradict those of a large number of studies, including one he conducted showing that a change in diet rapidly and reproducibly alters the human gut microbiome (David et al., 2014; O'Keefe et al., 2015; Wu et al., 2011). These studies, however, all involved relatively extreme dietary changes and were of relatively short duration—the vegans in his study had been so for at least six months. In addition, Wu learned from speaking with these investigators that the variability between subjects was far greater than the variability in one individual.

With regard to the challenges of studying how diet affects the mouse microbiome and translating those results to humans, Wu said the issues include the fact that mice will eat their own feces and the mouse chow diet is monotonous relative to the variability of the human diet. In addition, mouse digestive physiology and its response to diet is different from that of humans—mice, for example, are hindgut fermenters and have a large cecum, whereas humans, who are not, have a small cecum—and the response of the endogenous mouse gut microbiota to diet differs in magnitude and consistency relative to that in humans. As far as the studies themselves go, the lack of a standardized mouse chow and whether it is sterilized or not can be a cause of variance.

## THE ROLE OF BIOLOGICAL SEX

Research has shown that males and females have different sensitivities to infectious disease (Úbeda and Jansen, 2016) and responses to vaccination (Voysey et al., 2016). There are also differences in the incidence of various cancers and autoimmune diseases between the two sexes (Klein and Flanagan, 2016) (see Figure 5-2). Most studies that aim to explain these differences have focused on differences in sex hormones, overexpression of X-linked genes, and even the expression of mitochondrial genes. What these studies often overlook, Chervonsky said, is that the microbiota could be responding to one of the strongest biological stressors, sex; that there exist sex-specific microbiota; and as several speakers have noted, that the microbiota can have a marked effect on its host's immune system.

Studies looking at the effect of sex on autoimmune disorders, such as type 1 diabetes, have largely used non-obese diabetic (NOD) mice, a strain of NOD mice in which the incidence of diabetes is 1.3 to 4.4 times higher in females than males. However, Chervonsky and his colleagues found that this gender bias disappeared in germ-free NOD mice (Yurkovetskiy et al., 2013). Their analysis of the microbiota in post-pubertal male and female mice showed that there were marked sex differences, but these differences normalized when the males were castrated, which Chervonsky said confirmed that androgens influence gut microbiota composition. They then looked at gnotobiotic males and females colonized with the same microbiota and again found clear differences after puberty. Further analysis found that, while there are always microbiome differences between males and females, the differences change, showing that there is no male-specific microbiota signature. One conclusion, said Chervonsky, is that gender bias seen with type 1 diabetes does not depend on the specific microbial lineage. It is also possible, he noted, that the expansion of specific microbial lineages is also irrelevant to the gender bias of disease, but experiments with individual bacterial lineages showed that not all bacteria can influence a gender bias and that bacteria of very different families can affect gender bias.

One possible hypothesis to explain a connection between microbiota, autoimmunity, and sex predicts that microbes can affect the levels of hormones that reduce autoimmunity. It also infers that male microbiota should affect disease development in females. To test this hypothesis, Chervonsky and his colleagues tested the effect of colonizing mice with different bacteria and found that some bacteria induce a rise in testosterone levels, whereas others do not. They then did an experiment using NOD mice and found that mice with a wide range of testosterone levels, including very low levels, and a protective microbiota did not score high on a marker for diabetes. However, mice without a protective microbiome did develop the signs of diabetes. Moreover, protective microbiota from males transferred to female NOD mice had no protective effect in females, indicating the protective bacteria require male hormones to produce that effect. Taken together, he said, these results mean it is likely that this hypothesis is incomplete.

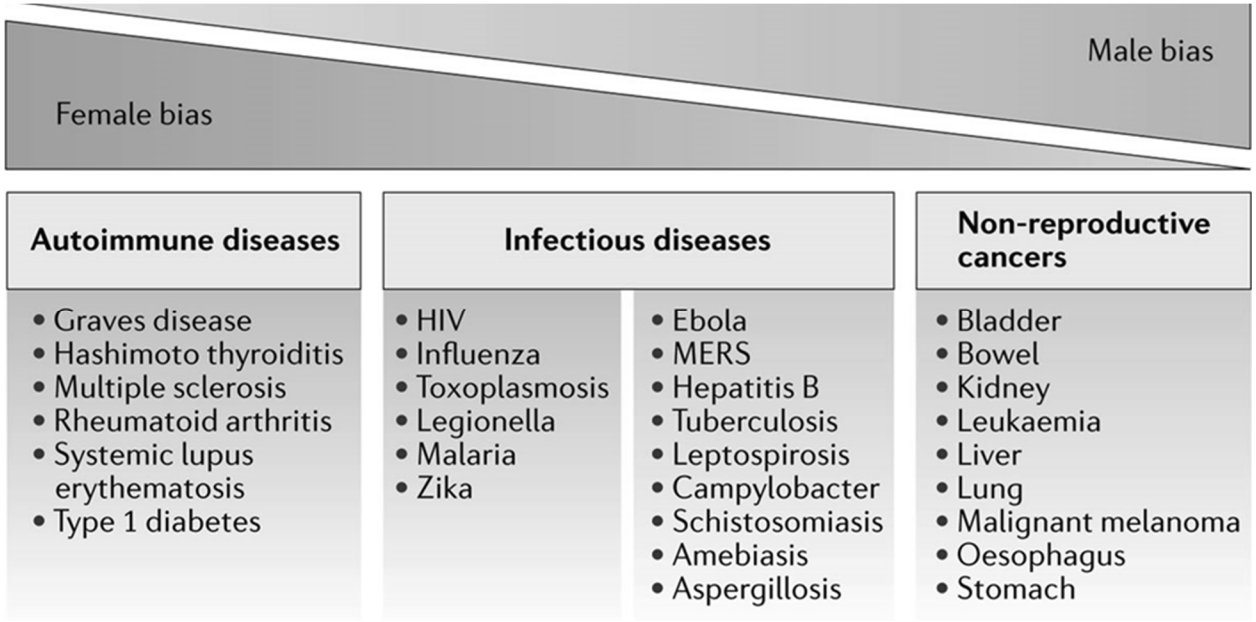


**FIGURE 5-2** Female and male differences in disease susceptibility related to immunological differences. NOTE: MERS, Middle East Respiratory Syndrome. SOURCES: Chervonsky slide 4 (Klein and Flanagan, 2016).

A so-called dual-signal model, in which both androgens and microbes work in concert to reduce type 1 diabetes in males, is more likely to explain gender differences, said Chervonsky. In this model, some hormones might amplify some microbes and some microbes might amplify hormone levels. "These two signals do not have to be applied simultaneously, but can be differential effectors during development," he said. He noted that he and his colleagues are testing this model and have some early evidence to support it.

# 6

# Establishing and Evolving Gnotobiotic Facilities

The workshop's final session focused on how to establish and maintain the facilities and infrastructure needed to house gnotobiotic animals. It also presented ways with which investigators have received support from institutional leaders to ensure that the appropriate resources are available to conduct microbiome-focused research.

## BUILDING AND MAINTAINING A GNOTOBIOTIC FACILITY

Timothy Hand, assistant professor of pediatrics and immunology and chair of the committee on gnotobiotics at the University of Pittsburgh, is in the process of establishing a gnotobiotic facility, the first ever at his institution. He identified three critical steps to this process: (1) education and training in his institution, (2) choosing the layout of the facility and the appropriate equipment, and (3) building a gnotobiotic infrastructure.

Regarding education, Hand noted that educating the senior leadership at his institution was critical for obtaining funding to support the facility. The University of Pittsburgh did not have space that could be easily converted into a gnotobiotic facility, so Hand needed to convince senior administration to provide significant funding needed to renovate a space, as well as pay for the trained staff that is essential to the successful operation of a gnotobiotic facility. Part of Hand's proposal was to demonstrate how such a facility would benefit the university faculty by controlling the costs of conducting gnotobiotic research. Hand explained that having investigators purchase individual gnotobiotic animals from large breeders was not fiscally sustainable, citing an average $500 cost for a single axenic mouse. Breeding mice in house would allow for less expensive long-term research. R. Balfour Sartor, distinguished professor of medicine, microbiology, and immunology and director of the University of North Carolina's Multidisciplinary Inflammatory Bowel Disease Center, also noted that it is critical to ensure that the facility's mission and cost structure matches those of the users, and especially that an institution would truly benefit from a gnotobiotic facility. He also underscored the importance of developing sustained, broad-based funding. His 31-year-old facility at the University of North

Carolina, for example, is funded by the National Institutes of Health (NIH) as a national resource facility, similar to the national primate centers, and as a resource center for investigators funded by the Crohn's & Colitis Foundation of America (CCFA). In addition to securing this stable and external funding, Sartor has also positioned his facility as a resource for local, regional, national, and international investigators who want to explore hypotheses related to the effect of resident microbiota on animal physiology. Chriss Vowles, co-manager of the germ-free research laboratory at the University of Michigan, also stressed the importance of a funding source other than user fees to support ongoing operations of a gnotobiotic facility, particularly in terms of retaining highly trained staff. He noted that the labor alone for preparing materials for the facility often has to begin a full month ahead of the actual experimentation schedule. Increased labor costs, because everything needs to be sterilized, mean that "it is extremely unrealistic for the core to support itself on user fees alone."

Hand also informed the senior administration that development of an in-house gnotobiotic facility would facilitate the experiments. He noted that collaborating with investigators at other universities or contracting all germ-free work was not sustainable, as such collaborations or contract arrangements would create additional legal and administrative hurdles given the need to establish material transfer agreements for every experiment.

Sartor added that there are additional reasons beyond ease of experimentation that should incline administrators to want an on-campus gnotobiotic facility. Having such a facility at an institution increases faculty competitiveness for NIH and foundation grant applications, and the facility serves as a tool for recruiting faculty who will generate more grant support and will increase an institution's national visibility and reputation. Betty Theriault, associate professor of surgery and clinical veterinarian in the Animal Resources Center at The University of Chicago, noted that having a gnotobiotic facility creates an environment for collaborative studies with investigators from other institutions who bring ideas and funding to her home institution. The one downside of becoming a center for collaboration, she said, is that it requires developing more memoranda of understanding, material transfer agreements, and training investigators from those outside institutions. While many investigators have some understanding of sterile techniques, for example, working in a gnotobiotic facility requires a level of sterile handling that most researchers are not familiar with and may find challenging.

Hand has found it important to educate the university's research community about the particular requirements of gnotobiotic research, how gnotobiotic animals are produced, the advantages and limitations of gnotobiotic research, and what can and cannot be done with gnotobiotic animals. Given the level of interest from his colleagues to examine the role microbiota might play in transplant rejection, he has had to explain how difficult such experiments would be. "Without discussing the nuts and bolts and the technical issues associated with a gnotobiotic isolator, you cannot really explain to someone who is a transplant

immunologist what is going to be difficult about transplanting hearts onto the aorta of germ-free mice," Hand explained.

He added that this educational effort served a purpose beyond informing his colleagues—it also became an ad hoc recruitment effort to utilize the facility. Even though Hand needed to obtain funding from his university to maintain the facility, he had to ensure that his colleagues were actually going to use the facility. To help with recruitment, he and his colleagues at the Center for Medicine and the Microbiome held a full-day symposium in spring 2016 and a "Microbiome Boot Camp" in early 2017.

These activities provided Hand with the opportunity to learn what his colleagues' interests in gnotobiotic research were. Somewhat to his surprise, significant interest was focused on the pulmonary microbiome, the relationship of the microbiome to cancer therapies, and the metagenomics of the human microbiome. This knowledge changed how Hand equipped and staffed his gnotobiotic facility, because these research interests required long-term rather than short-term housing, which affected the type of isolators to purchase. Hand recognized Alexander Chervonsky and his team at The University of Chicago for helping with the design of the University of Pittsburgh's facility, and segued into his second key step to establishing a gnotobiotic facility—facility layout and equipment. His planned facility will be composed of two independent facilities, each operating autonomously with no crossover between the adjacent spaces. "This provides redundancy," said Hand. "If one facility gets contaminated, we will still have germ-free mice on the other side that we can repopulate our facility with." Other notable features of the facility include placing the autoclaves in separate spaces away from the isolators to reduce noise and stress for the animals, security features to prevent unauthorized and untrained personnel from entering, and a heating and ventilation system that will vent positive to the hallway, ensuring that no outside microbes are brought in via air circulation. Sartor noted that, if possible, one should plan a new facility with expansion in mind, so that the facility can be upgraded as technology develops, the needs of users evolve, and the science advances.

Hand explained his rationale for choosing one type of isolator over another, as an example of the many decisions to be made. The first one was a vinyl isolator of the type used at the Centers for Disease Control and Prevention that can house mice for many months, including during breeding. While this isolator has well-established standard operating procedures (SOPs), it is large and not amenable to conducting experiments involving 30 or more microbiomes.

Hand also considered a hermetically sealed, independently ventilated caging system that can house 34 experiments simultaneously in a small space. Turnover time with this type of isolator is fast, but this system cannot be used for breeding because changing the cages is extremely laborious. Vowles reviewed three types of housing used in his facility. The first type is a soft-sided bubble isolator, a double barrier system, that requires very basic personal protective equipment (plastic gowns, gloves). These units can house large experimental populations, but only one microbe or group of microbes may be exam-

ined at a time because cross contamination is unavoidable. The second type is a biosafety level 2 (BSL-2) cabinet that provides easy access and good dexterity, while the animals can be housed in basic, static caging. However, this housing type limits available space and community size significantly, only allowing up to five groups plus controls in each. The third type is the same hermetically sealed system Hand described. Vowles said that decontamination of this system requires extremely toxic chemicals, which are expensive and risk damaging the equipment. It also takes Vowles and his team a great deal of time to prepare the materials needed to work with these systems. Vowles has found that a combination of isolators and rack systems is the safest way to house an axenic colony. "It is easier to rear the mice in the isolators and then transfer them to the individual ventilated units in the rack system," he explained.

Sartor noted that his facility has developed a customized surgical isolator, complete with warming blankets, stereotactic microscope, and surgical gloves that are only slightly thicker than normal surgical gloves—so not all additions to the design and construction of a facility need to be purchased. When considering how to incorporate various pieces of test equipment into an isolator, Theriault recalls the answer Philip Trexler, the inventor of the first flexible-film isolator, gave when asked that question: instead of getting the equipment into an isolator, wrap the isolator bubble around the test equipment and the animals. She briefly described several approaches to maintaining sterility when transferring animals for imaging or surgery, including the use of a sterilized biological safety cabinet in the gnotobiotic animal facility and training investigators to use this setup (Theriault et al., 2015).

Regarding sourcing mice for the facility, Hand said that the decision to obtain genetically modified strains of gnotobiotic mice from an external source or re-derive them in house would depend on the availability of experienced investigators able to perform these experiments.

In addition to the actual facility, supportive infrastructure is necessary. A germ-free facility, for instance, will not be very effective without the ability to grow organisms to place in the animals. At the University of Pittsburgh, the only anaerobic growth chamber is in Hand's laboratory, at which he is growing communities for eventual use in the facility. The university has invested heavily in sequencing capabilities and bioinformatics expertise, including hiring two investigators with expertise in assembling full bacterial genomes using shotgun sequences of microbiomes. Hand relies heavily on lessons learned at other facilities. Following SOPs developed in other facilities also helps ensure the welfare and well-being of the mice in his facility. "We are not changing anything from what we did at NIH," said Hand. "We really are trying to reproduce that facility."

## Maintaining and Operating a Gnotobiotic Facility

Once a facility is operational, additional considerations are necessary to foster sustained success. Sartor, whose facility was established in 1985, said that

stable scientific and technical leadership, as well as staff continuity, is essential, a point with which Hand agreed.

Perhaps the most important aspect of establishing a gnotobiotic facility, said Hand, is hiring dedicated, detail-oriented staff who understand and observe SOPs and who can be trained in an existing gnotobiotic facility, something Theriault and Vowles also stressed. Working in a gnotobiotic facility can be very physically demanding, as most operations need to be done manually. Therefore, staff should possess a commitment to cleanliness and attention to details, said Theriault. All interested parties should be aware of this issue. Vowles similarly noted the intensity and high level of fatigue. "You cannot make any mistakes. You could lose months and months of work contaminating an isolator by just touching something that might be dirty." Vowles noted that training could take up to six months before new staff could work unsupervised. A gnotobiotic technician is a highly skilled staff member deserving a higher salary than a standard animal facility technician, which Sartor also emphasized.

Another element of success is having a committed core user base. Sartor's facility, for example, has a core group of 15 or so investigators and another 20 to 25 additional users each year. Given that all 40 researchers cannot use the facility simultaneously, it is important to have transparent and equitable prioritization, he said. His facility utilizes a web-based scoring system that takes into account NIH or CCFA funding, being a local center member or young investigator, how long someone has been on the waiting list, and if someone has a grant or manuscript pending. The higher an investigator's score, the sooner that investigator will be able to use the facility.

Theriault noted that prior to 2005 very few people engaged in gnotobiotic research and very few germ-free facilities existed. Between 2005 and 2010, several institutions, including hers, began to develop such facilities, and today it appears that everyone would like to develop some type of gnotobiotics program. She cautioned that "nothing with this technology is as easy as it appears."

Vowles believes that a lack of national standards for gnotobiotic facilities increases the challenges of operating such facilities. "I think as a gnotobiotic community, we should come together, have an open dialog and create some of these standards in the next few years," said Vowles. He also advocated for Institutional Animal Care and Use Committee (IACUC) standards for gnotobiotic facilities and animals.

Typically, most rodents coming into Theriault's facility are from approved vendors with well-characterized specific pathogen-free (SPF) status. Animals from non-approved sources are quarantined to keep the general animal facility free of specified pathogens. Vowles commented that veterinarians and technicians must also be aware of potential contamination to the facility even while treating the animals. Medications and associated materials brought into the facility must be sterile. Furthermore, noted Theriault, veterinarians need to be cognizant not only of the health of the animals within the facility but also of the potential for contamination when the veterinarian interacts with the animals.

The University of Chicago first built its gnotobiotic animal facility in a remodeled old storage building. Increased demand led at first to an expansion into a neighboring storage closet and then to an entirely new, dedicated facility. Referring to Sartor's prior comments, Theriault repeated that this technology is ergonomically challenging, labor intensive, and expensive. The current standard approach to monitoring for adventitious pathogens in SPF colonies is labor intensive and requires euthanizing animals. However, polymerase chain reaction–based assays to evaluate the microbial status of the colonies are in development, which would help reduce the number of animals to be euthanized for monitoring purposes.

Housing these animals and keeping them germ-free is only a means to the end of conducting research with them. Protecting these animals from pathogens and other microbes starts, she said, with education, training, and communication, both within and outside of her university. "We have to educate new members of our research community," said Theriault. "We have to educate our IACUCs, our biosafety committees and program visitors, or maybe even our USDA [U.S. Department of Agriculture] inspectors."

Maintaining the health of germ-free animals entails more than just protecting them from infection by adventitious pathogens, said Vowles. As they age, for example, germ-free animals develop an abnormally large cecum, which occasionally causes volvulus. Providing proper nutrition is challenging as well, because key nutrients, such as thiamine and vitamin K, degrade during the chow autoclaving process. Even irradiating food or using supplements can be unreliable.

Theriault explained that many experiments might require animals to be anesthetized, immobilized, X-rayed, and even subjected to radiotherapy—on top of routine administration of test agents and sample collection—all of which require special procedures in the germ-free and gnotobiotic context. This should not be surprising given that the composition and diversity of the microbiome significantly influence homeostatic and metabolic processes. These differences, she added, can create additional challenges regarding protocol review, because many institutions' guidelines apply to conventional animals. As a result, she often has to educate IACUCs about the possibility of straying outside of these guidelines. In fact, she advocates that researchers include pilot trials to assess anesthetic regimens in germ-free and gnotobiotic animals as part of their study design.

Given the challenges of operating these types of facilities, and the fact that an increasing number of institutions want to establish microbiome research programs, Theriault wondered if it was time to consider establishing regional gnotobiotic and microbiome centers of expertise and excellence.

## ALTERNATIVES TO GNOTOBIOTICS: NORMALIZING THE ENVIRONMENT

Stephen Jameson, professor of laboratory medicine and pathology at the University of Minnesota, investigates whether the characteristics of a mouse's

immune system would change if the mice had a more natural, physiological, and immunological experience. His concern with raising mice in a gnotobiotic facility is that animals are separated from natural threats in the environment that enable their immune system to develop as it might in the wild. To explore how a broader physiological infectious history would alter the immune system and immunological responses in inbred mice, Jameson and his collaborators are generating and working with what they call "dirty mice." They house wild mice or mice from pet stores with genetically modified ones to produce dirty mice. "We know that these wild and pet store animals have been exposed to many pathogens, some of which we can define, and have their own particular blend of commensal microbes," said Jameson. "Some of these pathogens and commensals will be acquired by the co-housed mice, but we do not try to control this." He believes the dirty mice are a more authentic reflection of the human microbial experience and suggested that these models may better reflect a human immunological response. "[Dirty mice] are essentially the opposite of gnotobiotic mice."

There are many logistical challenges to this approach, said Jameson. Dirty mice carry pathogens excluded from most animal facilities, and perhaps others that are equally dangerous, so working with them requires installing an isolation barrier. His group houses its animals under BSL-3[1] conditions, at great expense, even though none of the pathogens involved are above BSL-2 status. He noted that deliberate sequential infection is a valuable alternative to working with wild-caught or pet store mice because it allows controlling an animal's infection history.

Jameson commented on the tremendous frustration among mouse immunologists that predictions regarding the human immune system have not always proved to be true (Mestas and Hughes, 2004; Payne and Crooks, 2007; Rivera and Tessarollo, 2008; von Herrath and Nepom, 2005). Data show, for example, that immune cell populations from SPF mice correspond to those found in human umbilical cord blood (naïve CD8 T cells in both cases), which he said is fine if the goal is to model a newborn, but not if the goal is to model the adult human immune system.

Investigators use mice maintained in barrier facilities under SPF conditions to normalize the immune system prior to study. Humans, however, are exposed to a wide range of pathogens and commensals that shape the immune

[1]"Animal Biosafety Level 3 involves practices suitable for work with laboratory animals infected with indigenous or exotic agents … and requires that: 1) access to the animal facility is restricted; 2) personnel must have specific training in animal facility procedures… 3) personnel must be supervised by individuals with adequate knowledge of potential hazards… and 4) procedures involving the manipulation of infectious materials, or where aerosols or splashes may be created, must be conducted in BSC's (biological safety cabinets) or by use of other physical containment equipment." See https://www.cdc.gov/biosafety/publications/bmbl5/bmbl5_sect_v.pdf (accessed March 2, 2018).

system, which is the reason his research group began a collaboration with Dave Masopust's group at the University of Minnesota to explore what happens in dirty mice. Large populations of CD8 T cells from wild mice had an effective memory phenotype, whereas the vast majority of CD8 T cells from SPF mice had a naïve phenotype (Beura et al., 2016). The problem with wild-caught mice is that there are many variables, such as their pre-capture diet and the season in which they are caught, that could affect the characteristics of these animals.

Mice from pet stores are easier to obtain and work with, plus they also present with the phenotypic conversion of CD8 T cells seen in wild-caught mice. Their main limitation is that they are not inbred. One solution is to convert inbred SPF mice to the pet store mouse phenotype by housing the SPF mice with pet store mice for two months. Jameson noted that crossover is close to 100 percent except for pinworms and mites, and that in some cases pathogen transfer can be fatal for the previously unexposed mice. However, after two months of co-habitation, the CD8 T cell phenotype of the SPF mice stabilizes, and activated T cells and other characteristics of a maturing immune system appear. Though these studies are in early stages, the investigators have found that the microbiota of co-housed mice becomes more similar to that of pet store mice; that is, it is changing. Jameson noted that gene expression analysis showed that co-housing pet store animals with standard SPF mice produced a profile that compared well with that seen in adult human blood, including the activation of type 1 interferon-inducing genes.

Regarding animal welfare, 20 percent mortality among B6 mice following exposure to pet store animals is a concern, though the mortality was much higher in BALB/c mice. Jameson noted that SPF-designated pathogens are the most likely cause of death, based on data from necropsies and pathology. Using contaminated bedding instead of co-housing leads to some reduction in mortality. Another way to avoid this problem, Jameson added, would be to establish sequential infection models to recapitulate the dirty mouse phenotype. Doing so could eliminate the need for costly BSL-3 equipment.

# 7

# Reflections on the Workshop

Microbes—bacteria, Archaea, eukaryotes, and viruses—are everywhere, including in every animal model of disease, said Vincent Young in his reflections on the important lessons of the workshop. "Therefore, if you use an animal model of any human disease or physiological process, you have a microbio-animal model," said Young. When considering the role microbes play, researchers may be forced to reevaluate what they understand about their model. In the end, he said, the microbiota may not matter, but doing the experiments to determine whether it does or does not is necessary.

Young discussed some of the issues raised by the speakers: *scale*, both in terms of cost but also with regard to which interactions are important to study in a given set of experiments. "How much are you going to focus on various aspects of the host, how much on the various aspects of the indigenous microbiota, and how much on pathogens?" asked Young. *Complexity* is another element both in terms of technical issues and with respect to what can be controlled in any set of experiments. *Translatability to human conditions* is yet another consideration, as are *relevance* and *variation*. How much variation should one allow in an experiment given that variability is part of what defines being human?

The choice of which animal model to use and how to evaluate it stems from the scientific question that drives a research project, said Young. In some cases, it will be important to define the exact microbial community, while at other times using "dirty mice" may be appropriate. "It all depends on what you are asking," said Young. It is important, he added, "to control what you can and be ready, willing, and able to measure what you cannot." Ultimately, he said, when there are more questions than answers, reach out to the rest of the community. "We talked about *having things we can share*—reagents, mice, methods, technologies, strains. Perhaps that is the right way to do science."

Joseph Newsome, associate professor of pathology and clinical director of the Division of Laboratory Animal Resources at the University of Pittsburgh, reminded the workshop audience that the idea behind specific pathogen-free animal facilities and models arose in the 1950s and 1960s as a means of standardizing animal models in order to reduce variability and improve translation of results to humans. Today, researchers have developed a host of new models for investigating the microbiome, relying on the work of the field's pioneers. At the

same time, many of the technologies currently used have been minimally changed over time. Newsome suggested that the research community at large, and the laboratory animal community specifically, begin to challenge dieticians, cage manufacturers, and animal vendors to examine the issues discussed at this workshop to improve the translatability of animal models.

Newsome, like Young, commented on the need for *more collaboration* given the complexity of microbiome research. "This probably requires us to think and ask for support in ways that do not currently exist," said Newsome. "Can we streamline the interactions and sharing of data, create repositories, and get animals from one place to the other [faster]?" he asked.

Newsome mentioned the need for *training and infrastructure development*, and he referred to Betty Theriault's suggestion that the nation might need centers of excellence to train investigators and share resources. He also referred to Vowles's suggestion that the field would benefit from *standards and regulations for gnotobiotic facilities*. His final comment was the need to *educate IACUCs* on the specific requirements for working with gnotobiotic animals.

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# Appendix A

# Workshop Agenda

**ANIMAL MODELS FOR MICROBIOME RESEARCH:
ADVANCING BASIC AND TRANSLATIONAL SCIENCE**

A WORKSHOP OF THE ROUNDTABLE ON SCIENCE
AND WELFARE IN LABORATORY ANIMAL USE

December 19-20, 2016

500 Fifth Street NW, Washington DC 20001
National Academies of Sciences, Engineering, and Medicine
Keck Center, Room 100

**MONDAY, DECEMBER 19**

**(Gnotobiotic) Model Organisms and Microbiome Research:
Choices, Challenges, and Proposed Solutions**

| | |
|---|---|
| 7:30–9:00 am | **Registration** |
| 9:00 | **Animal Models and Microbiome Research: A Trans-Kingdom Perspective**<br>Herbert "Skip" Virgin, Washington University |
| 9:45 | **Coffee Break** |
| 10:00 | **Session 1-1. Non-Rodent Animal Models for Microbiome Research**<br>*Much of current microbiome research has focused on mouse models. As with other branches of preclinical research, exploring the microbiome in other species complements and advances knowledge gleaned from mice. This session will provide perspectives on the benefits and limitations of some of these animal models.*<br><br>***C. Elegans* -** Buck Samuel, Baylor College of Medicine<br>***Drosophila* -** Angela Douglas, Cornell University<br>**Zebrafish -** Karen Guillemin, University of Oregon<br>**Piglets -** Jeff Gordon, Washington University (Planning Committee Member) (remotely) |

12:00 pm **Lunch** (Will not be provided. There is a cafeteria on the third floor of the Keck Center.)

1:00 **Session 1-2. In Vitro Systems for Characterizing the Properties/Dynamic Operations of Microbial Consortia**
*One of the benefits stemming from advances in in vitro systems is the opportunity to reduce the number of animals needed to develop and test hypotheses, and in some cases replace their use entirely. Speakers in this session will present three non-animal systems for microbiome research, including thoughts on their ability to complement animal use now and in the future.*

**Bioreactors -** Robert Britton, Baylor College of Medicine
**Organoids -** Vincent Young, University of Michigan (Planning Committee Member)
**Microfluidics: Human organs on chips -** Donald Ingber, Wyss Institute at Harvard University (remotely)

2:30 **Coffee Break**

2:45 **Session 1-3. Modeling Human Microbiota in Animal Systems**
*Animal models provide opportunities to define the contributions of members of the microbiota to community function and the mechanisms through which they affect various aspects of host biology. This session illustrates current approaches that are being used and how these approaches may be advanced to promote further basic and translational research in this field.*

**A. Connecting Microbes to Metabolism Using Gnotobiotic Models**
- Biologically significant metabolites produced by the gut microbiota: their origins and functions - Federico Rey, University of Wisconsin–Madison
- Mechanistic studies of how the gut microbiota influences host metabolism - Patrice Cani, Université Catholique de Louvain, Belgium

**B. Revisiting Koch's Postulates from a Microbial Community Perspective**
- *Fusobacterium nucleatum* and colorectal carcinogenesis - Wendy Garrett, Harvard T.H. Chan School of Public Health (Planning Committee Member) (remotely)
- Microbes and atopic disorders - Richard S. Blumberg, Harvard Medical School

**C. The Interface Between Microbes and Neuroscience**
- The effects of the microbiome on the behavior of bees - Nancy Moran, The University of Texas at Austin
- Maternal stress and the microbiome: Programming of offspring neurodevelopment - Tracy Bale, University of Pennsylvania

5:45 pm **Adjourn**

**TUESDAY, DECEMBER 20**

**Methodological Challenges in Characterizing Gnotobiotic Animal Models**

| | |
|---|---|
| 9:00 am | **Session 2-1. Reproducibility: Within and Across Experiments** |
| | **Creating Stabilized and Defined Microbiomes in Laboratory Animals**<br>Andrew Macpherson, University Hospital, Bern, Switzerland |
| 9:45 | **Coffee Break** |
| 10:00 | **The role of host genetics -** Aldons J. Lusis, University of California, Los Angeles |
| | **The role of immunological variation -** Jeremiah Faith, Icahn School of Medicine at Mount Sinai |
| | **The role of diets: Standardization and characterization -** Gary Wu, University of Pennsylvania |
| | **The role of gender -** Alexander Chervonsky, The University of Chicago |
| 12:00 pm | **Lunch** (Will not be provided. There is a cafeteria on the third floor of the Keck Center.) |
| 1:00 | **Session 2-2. Establishing and Evolving Gnotobiotic Facilities and Their Technologies: Examining the Present and Looking to the Future** *Establishing the necessary infrastructure for microbiome research is challenging. How can a successful gnotobiotic facility be planned? What are some of the attributes that can ensure sustainability, community sharing, and support of such a facility (including rederivation and "archiving" of animals as germ-free)? The speakers will share their experiences regarding challenges and solutions encountered. They will also focus on advances in the support systems and facility operations that enable animal care personnel to provide for the improved well-being of the specialized animals used in microbiome research.* |
| | **Establishing a new gnotobiotic facility: Education, missions, and accommodating success -** Timothy Hand, University of Pittsburgh |
| | **Evolving an established gnotobiotic facility -** R. Balfour Sartor, University of North Carolina at Chapel Hill |
| | **Complex gnotobiology: An emerging paradigm in the era of next-generation sequencing -** Craig Franklin, University of Missouri |

**Alternatives to gnotobiotics: Normalizing the environment -** Stephen Jameson, University of Minnesota

**Veterinary management challenges and future directions, including technical considerations for imaging and surgery in gnotobiotic animals -** Betty Theriault, The University of Chicago

**Unique challenges and future directions related to managing mouse gnotobiotic husbandry facilities -** Chriss J. Vowles, University of Michigan

4:30 **Overview of Workshop**
Joseph Newsome and Vincent Young

5:00 pm **Adjourn**

# Appendix B

# Biographical Sketches of Planning Committee

**James G. Fox** (*Co-Chair*) is a professor and director of the Division of Comparative Medicine and a professor in the Department of Biological Engineering at the Massachusetts Institute of Technology. He is also an adjunct professor at the Tufts University School of Veterinary Medicine and the University of Pennsylvania School of Veterinary Medicine. He is a diplomate and a past president of the American College of Laboratory Animal Medicine, past president of the Massachusetts Society of Medical Research, past chairman of the Association for Assessment and Accreditation of Laboratory Animal Care Council, past chairman of the National Center for Research Resources, National Institutes of Health (NIH/NCRR) Comparative Medicine Study Section and past president of the American Association of Veterinary Medical Colleges. He has served on the editorial board of several journals, is a past member of the NIH/NCRR Scientific Advisory Council, and the Institute for Laboratory Animal Research Council of the National Academy of Sciences. He currently serves on the National Academies of Sciences, Engineering, and Medicine's Board on Global Health and an advisory committee to the NIH directors concerned with the physician-scientist workforce. Dr. Fox was elected to the National Academy of Medicine in 2004. Dr. Fox is considered an international authority on the epidemiology and pathogenesis of gastric and enterohepatic helicobacters in humans and animals. He is largely responsible for identifying, naming, and describing many of the diseases attributed to various helicobacter species; most notably their association with hepatitis, liver tumors, inflammatory bowel disease, and colon cancer. His past and current research has been funded by NIH and the National Cancer Institute, as well as by private industrial sources, for the past 35 years. He has been the principal investigator of an NIH postdoctoral training grant for veterinarians for the past 25 years and has trained 60 veterinarians for careers in biomedical research. He also has an NIH training grant for veterinary students and has introduced more than 100 veterinary students to careers in biomedical research.

**Joseph T. Newsome** (*Co-Chair*) is currently an associate professor in the Department of Pathology and the clinical director of the Division of Laboratory Animal Resources at the University of Pittsburgh. He received a BSc in microbiology in 1980, a master's degree in pathobiology in 1982, working with the research team

of Dr. Richard Olson that developed the first vaccine for feline leukemia, and obtained a doctorate of veterinary medicine in 1986 from The Ohio State University College of Veterinary Medicine. For the next 10 years he wore multiple hats at Georgetown University in Washington, DC, including as facility manager, assistant professor of surgery and pathology, and clinical veterinarian overseeing surgery and radiological research support. In 1996 he became a diplomat of the American College of Laboratory Animal Medicine and concurrently completed a postdoctoral training program in experimental pathobiology, assisting in the development of the animal models for the team that eventually led to the current human papillomavirus vaccine. From 2000 to 2012 he was the University of Pittsburgh's attending veterinarian. He is the author or co-author of more than 65 articles and book chapters. During his career he has been the principal investigator (4) or co-investigator (6) on multiple National Center for Research Resources, Office of Pharmaceutical Industry Research, National Institutes of Health–funded grants focused on renovations or new construction projects related to vivaria in multiple institutions. He is involved in national and industry level organizations such as American College of Laboratory Animal Medicine (ACLAM), Association of Primate Veterinarians, American Association for Laboratory Animal Science (AALAS), and American Veterinary Medical Association with leadership roles such as being a subcommittee chair for the ACLAM foundation since 2010 and was vice chair of the Policies & Procedures Coordinating Committee of AALAS. His current focus and expertise are in management, biosecurity, biocontainment, facility design and operations, and cancer modeling, immunology, and virology.

**Wendy S. Garrett** is a physician-scientist and her basic science laboratory is located at the Harvard T.H. Chan School of Public Health. Dr. Garrett is a physician at the Dana-Farber Cancer Institute and Brigham and Women's Hospital. Her laboratory is focused on defining the dynamic interactions between the mucosal immune system and gut microbiota. The Garrett laboratory's experimental questions are grounded in understanding how interactions between intestinal microbial communities and the immune system contribute to the development of inflammatory bowel disease and colorectal cancer. Dr. Garrett has recently received the following awards for her research: a Damon Runyon Foundation Fellowship, a Burroughs Wellcome Career in Medical Sciences Award, a V Foundation Scholar Grant, a Cancer Research Institute Investigator Award, and a Searle Scholars Award.

**Jeffrey I. Gordon** is the Dr. Robert J. Glaser Distinguished University Professor at Washington University in St. Louis. He received his AB from Oberlin College and his MD from The University of Chicago. He joined the Washington University faculty after completing his clinical training in internal medicine and gastroenterology and doing postdoctoral research at NIH. He was head of the Department of Molecular Biology and Pharmacology before becoming the founding Director of a university-wide, interdisciplinary Center for Genome Sciences and

Systems Biology. His group has developed new experimental and computational approaches to characterize the assembly and dynamic operations of human gut microbial communities; this work has involved studies of novel gnotobiotic animal models, twins concordant or discordant for physiologic phenotypes, and children and adults representing diverse geographic, cultural and socioeconomic conditions. A central question he and his students are pursuing is how our gut microbiomes contribute to obesity and childhood undernutrition. Gordon has been the research mentor to more than 120 PhD and MD/PhD students and postdoctoral fellows since he established his lab. He is a member of the National Academy of Sciences, the American Academy of Arts and Sciences, the National Academy of Medicine, and the American Philosophical Society.

**Vincent B. Young** is an associate professor in the Department of Internal Medicine/Infectious Diseases and the Department of Microbiology & Immunology at the University of Michigan Medical School. He received his undergraduate degree from the Massachusetts Institute of Technology and received his MD and PhD from Stanford University. He completed his clinical training in internal medicine and infectious diseases at Massachusetts General Hospital. He was previously on the faculty at Michigan State University prior to joining the University of Michigan in 2007. Dr. Young has a long-standing interest in understanding the pathogenesis of bacterial infections of the gastrointestinal tract and the role of the normal microbiota in human health and disease. Dr. Young led a Human Microbiome Project on the role of the microbiome in inflammatory bowel disease. He is also involved in projects that look at microbial communities in the lungs of patients with HIV infection and cystic fibrosis. Current research in the Young lab includes a "team science" effort to understand the pathogenesis *Clostridium difficile* infection by an integrated approach that combines clinical research, bacterial genomics, microbial ecology, and immunology/host response projects. He is also leading a group of investigators that is developing the use of stem cell–derived intestinal organoids as a novel alternative model system for the study of enteric disease agents.

# Appendix C

# Biographical Sketches of Workshop Speakers and Moderators

**Tracy L. Bale** is a professor of neuroscience in the School of Veterinary Medicine and in the Department of Psychiatry of the Perelman School of Medicine. She obtained her PhD from the University of Washington in the Department of Pharmacology and the Program in Neurobiology. She completed her postdoctoral training with Dr. Wylie Vale and the Salk Institute in La Jolla, California. Her research focuses on understanding the role of stress dysregulation in neurodevelopmental and neuropsychiatric diseases, and the sex differences that underlie disease vulnerability using mice as the model organism. Mechanistic examination includes studies on the contributions of the placenta, germ cells, and microbiome in epigenetic programming of the brain. Dr. Bale is the co-director of the Penn Center for the Study of Sex and Gender in Behavioral Health, which is funded by a National Institute of Mental Health (NIMH) and Office of Research on Women's Health (ORWH) Specialized Centers of Interdisciplinary Research (SCOR) P50 grant, and is the director of research for the Building Interdisciplinary Research Careers in Women's Health (BIRCWH) Faculty Scholars. She serves on many internal and external advisory committees, panels, and boards and is currently a reviewing editor at the *Journal of Neuroscience* and serves as chair of the Neuroendocrinology, Neuroimmunology, Rhythms and Sleep, Center Scientific Review (NNRS CSR) study section. She has been the recipient of several awards for her research in this area, including the career development award for early career achievement and promise by the Society for Neuroscience, the Richard E. Weitzman Memorial award as exceptionally promising investigator award by the Endocrine Society, the Medtronic Award from the Society for Women's Health Research for outstanding research that has led to the improvement of women's health, and most recently, the Daniel H. Efron Research Award from the American College of Neuropsychopharmacology.

**Richard S. Blumberg** trained in internal medicine (The New York Hospital, 1982), infectious diseases (Massachusetts General Hospital, 1986), and gastroenterology and hepatology (Brigham and Women's Hospital, 1989). He is currently Senior Physician in Medicine and Gastroenterology at Brigham and Women's Hospital where he holds the position of division chief of gastroenter-

ology, hepatology and endoscopy, is a professor of medicine at Harvard Medical School, co-director of the Harvard Digestive Diseases Center, and immediate past-director of the Brigham Research Institute. In addition, Dr. Blumberg serves on the Executive Advisory Committee of the Department of Medicine. He also currently serves on the Gastrointestinal Pathobiology Study Section at the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and has served as a member of the Immunology Sciences Study Section of the National Institute of Allergy and Infectious Diseases (NIAID), a member on the National Commission of Digestive Diseases of the NIDDK, scientific consultant to the Human Microbiome Project of the National Human Genome Research Institute (NHGRI), a member of the Vaccine Branch External Advisory Board of the National Cancer Institute (NCI), and a member of the Board of Scientific Councilors. Dr. Blumberg has served as the chair of the National Scientific Advisory Committee of the Crohn's & Colitis of America (2002-2005) and was former president of the Society for Mucosal Immunology (2007-2009). Dr. Blumberg is an elected member of the American Association of Clinical Investigation and the Association of American Physicians and the recipient of a Merit award from the National Institutes of Health (NIH) (2005), the William Beaumont Prize from the American Gastroenterological Association (2012), the Distinguished Scientific Achievement Award from the Crohn's and Colitis Foundation of America (2012), and Lifetime Achievement Award from the Society of Mucosal Immunology (2015). He has been an NIH-funded investigator since 1989 whose long-standing research programs focus on mucosal immunology and specifically directed the role of CD1d-NKT cells, the unfolded protein response, CEACAM1, and FcRn in immunobiology. Dr. Blumberg was the scientific founder of Syntonix Pharmaceuticals that developed long-acting therapeutic agents, which were recently approved by the U.S. Food and Drug Administration and European Medicines Agency for the treatment of hemophilia, and is the scientific founder of Syntimmune, Inc., which is developing inhibitors of FcRn that are currently being tested in phase 1 studies.

**Robert Britton** is a professor in the Department of Molecular Virology and Microbiology and is a member of the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine. He currently directs the Therapeutic Microbiology laboratory, which is focused on the use of microbes to prevent and treat human disease. Currently funded research projects in the laboratory range from the study of how traditional probiotic strains can ameliorate osteoporosis to how intestinal microbial communities resist invasion by the diarrheal pathogen *Clostridium difficile*. His laboratory has made several advances in the development of genetic and microbial growth platforms to aid in the understanding of how microbes promote health and disease. These include the development of precision genome engineering technologies for lactic acid bacteria and the development of human fecal minibioreactor arrays to study the function of microbial communities in a high-throughput manner. Dr. Britton received a BS in Biology from the University of Nebraska–Lincoln and a PhD in

cell and molecular biology from Baylor College of Medicine. After performing postdoctoral training at the Massachusetts Institute of Technology he started his own laboratory at Michigan State University. After rising to the rank of professor in 2014 he moved to his current position at the Baylor College of Medicine.

**Patrice D. Cani** is a professor and senior researcher from the Belgian Fund for Scientific Research, and a group leader at the Louvain Drug Research Institute from University College London (UCL), Brussels, Belgium. He is a dietitian, earned an MSc in nutrition, an MSc in health sciences, and a PhD in biomedical sciences (2005). His main research interests are the investigation of interactions between the gut microbiota, the host, and specific biological systems, such as the endocannabinoid system and the innate immune system in the context of obesity, type 2 diabetes, cardiometabolic disorders, and metabolic inflammation. In 2007, he published the discovery of the concept of metabolic endotoxemia. More recently, he discovered the beneficial role of the bacteria *Akkermansia muciniphila* on obesity and cardiometabolic risk factors. Dr. Cani is a Walloon Excellence in Lifesciences and Biotechnology (WELBIO) investigator and the recipient of prestigious grants: European Research Council (ERC) Starting Grant 2013 (ENIGMO), a PoC ERC grant 2016, the prize "Baillet Latour Grant for Medical Research 2015," and the International Prize of Physiology Lucien Dautrebande (2016). He is the author/co-author of more than 195 scientific research publications.

**Alexander Chervonsky** is a professor in the Department of Pathology at The University of Chicago. He is also a chair of the Committee on Immunology at The University of Chicago. Dr. Chervonsky joined The University of Chicago in 2005 from The Jackson Laboratory in Bar Harbor, Maine, where he was a staff scientist. His research interests are in the broad area of immunology with an emphasis on autoimmunity. He has contributed to the understanding of the mechanisms of destruction of the target tissues by the autoimmune response in organ-specific autoimmunity, to the understanding of trafficking of effector T cells to the site of antigen expression, and to the role of innate immunity in regulation of autoimmunity. His later work has been focused on the role of commensal microbes (the microbiota) in the regulation of autoimmunity. He has published a seminal paper describing the connections between the microbiota, innate immunity mechanisms, and the development of autoimmunity. The continuation of this work has revealed the role of the microbiota in the sexual dimorphism of autoimmunity. In addition, Dr. Chervonsky's laboratory has discovered a role of the inducible changes in glycosylation of the mammalian host's tissues under conditions of microbial invasion as a protective mechanism decreasing microbial virulence and increasing the wellness of the host. Dr. Chervonsky holds an MD degree from the 1st Moscow Medical School, Moscow, Russia, and a PhD in immunology from the Cancer Research Center, Moscow, Russia. He completed his postdoctoral training at Yale University, New Haven, Connecticut.

**Angela Douglas** is the Daljit S. and Elaine Sarkaria Professor of Insect Physiology and Toxicology at Cornell University in Ithaca, New York. She joined Cornell University in August 2008 from The University of York, United Kingdom, where she had been a professor (personal chair). Dr. Douglas's research experience concerns beneficial microorganisms in animals, with a particular interest in the nutritional role of microorganisms in insects and their use as a biomedical models for metabolic health. Her works include more than 200 refereed research publications and four academic books on beneficial microbes and animal physiology. In addition to her publications, Dr. Douglas has given many invited lectures and has a strong record of service activities. She has held leadership positions in scientific societies, served on scientific advisory boards, and received awards for her research. She holds a BA in zoology from Oxford University, United Kingdom and a PhD in microbiology from the University of Aberdeen, United Kingdom.

**Jeremiah Faith** received his PhD in bioinformatics and systems biology from Boston University. He did his postdoctoral training in the laboratory of Jeffrey Gordon at Washington University in St. Louis Medical School with a focus on the structure and function of the gut microbiome in healthy individuals. He is currently an assistant professor in the Immunology Institute and the Institute for Genomics and Multiscale Biology in the Icahn School of Medicine at Mount Sinai in New York. His research focuses on modeling the interactions between diet, gut microbes, and host physiology with an emphasis on inflammatory bowel disease. Ongoing research in the lab includes (1) quantifying the influence of diet and the gut microbiota on host health and disease, (2) identifying microbial strains that modulate host phenotypic variation, and (3) the stability of the human gut microbiota.

**James Fox** received his veterinary training at Colorado State University. He was an NIH postdoctoral fellow at Stanford University before accepting a position at the University of Colorado Medical Center as an assistant professor. He was later recruited to Massachusetts Institute of Technology and is currently a professor and the director of the Division of Comparative Medicine. He is also an adjunct professor at the Tufts University School of Veterinary Medicine. Dr. Fox has received numerous scientific awards, and was elected to the National Academy of Medicine in 2004. He has been the principal investigator of an NIH postdoctoral training grant for veterinarians for 28 years and has trained 80 veterinarians, physicians, and PhDs for careers in biomedical research. He also has an NIH training grant for veterinary students and has introduced more than 120 veterinary students to careers in biomedical research. He has been funded by NIH and NCI to study infectious diseases of the gastrointestinal tract for the past 40 years and has focused on the pathogenesis of *Campylobacter* spp. and *Helicobacter* spp. infection in humans and animals. Dr. Fox has a long-standing interest in studying the gastrointestinal microbiome and how it interfaces with

and influences the host's immune response to gastrointestinal pathogens, particularly *Helicobacter* species. These studies are complemented by extensive experience with mouse models, including those maintained under gnotobiotic conditions. His laboratory developed the ferret as a model for both *Campylobacter*- and *Helicobacter*-associated disease as well as the first rodent model to study *Helicobacter*-associated gastric disease, including gastric cancer. Dr. Fox is considered an international authority on the epidemiology and pathogenesis of gastric and enterohepatic helicobacters in humans and animals. He is largely responsible for identifying, naming, and describing many of the diseases attributed to various *Helicobacter* species; most notably their association with hepatitis, liver tumors, inflammatory bowel disease, and colon cancer.

**Craig Franklin** attended the University of Missouri (MU), where he received his DVM and PhD. He is currently a professor of veterinary pathobiology at MU and directs the Mutant Mouse Resource and Research Center, an NIH-funded repository of genetically engineered mutant mice; the Comparative Medicine Program, a post-DVM laboratory animal medicine residency and advanced degree program; and the Veterinary Research Scholars Program, a summer research program for veterinary students. He also co-directs the new MU Metagenomics Laboratory and is a co-investigator for the Rat Resource and Research Center. He has more than 25 years of experience in rodent disease and diagnostics with an emphasis on infectious and intestinal diseases. His current research home, the Comparative Metagenomics Laboratory, studies environmental variables that may modulate rodent microbiota, the impact of differing microbiota on rodent model phenotypes (e.g., inflammatory and neoplastic diseases of the intestine), and methods to practically manipulate and control complex microbiota to optimize model reproducibility. He also performs collaborative studies involving numerous rodent and domestic species models of disease.

**Wendy S. Garrett** is a physician-scientist, and her basic science laboratory is located at the Harvard T.H. Chan School of Public Health. Dr. Garrett is a physician at Dana-Farber Cancer Institute and Brigham and Women's Hospital. Her laboratory is focused on defining the dynamic interactions between the mucosal immune system and gut microbiota. The Garrett laboratory's experimental questions are grounded in understanding how interactions between intestinal microbial communities and the immune system contribute to the development of inflammatory bowel disease and colorectal cancer. Dr. Garrett has recently received the following awards for her research: a Damon Runyon Foundation Fellowship, a Burroughs Wellcome Career in Medical Sciences Award, a V Foundation Scholar, a Cancer Research Institute Investigator Award, and a Searle Scholars Award.

**Jeffrey Gordon** is the Dr. Robert J. Glaser Distinguished University Professor at Washington University in St. Louis. He received his AB from Oberlin College and his MD from The University of Chicago. After completing his clinical training in internal medicine and gastroenterology, and a postdoctoral fellowship at

NIH, he joined the faculty at Washington University where he has spent his entire career, first as a member of the Departments of Medicine and Biological Chemistry, then as the head of the Department of Molecular Biology and Pharmacology, and for the past decade as the founding director of an interdepartmental Center for Genome Sciences and Systems Biology. Students in his lab have created new types of gnotobiotic animal models and developed new experimental and computational approaches for characterizing the assembly, dynamic operations, functional properties, and biological effects of human gut microbial communities. He has combined these models with human studies involving twins as well as members of birth cohorts living in low-, middle-, and high-income countries representing diverse geographic locations and cultural traditions. His group is focused on addressing the global health challenges of obesity and childhood undernutrition through new understanding of the interactions between diets and the gut microbiome and new ways of promoting healthy development of the gut community during the first several years of postnatal life. He has been the research mentor to more than 125 PhD and MD/PhD students and postdoctoral fellows. He is a member of the National Academy of Sciences, the American Academy of Arts and Sciences, the National Academy of Medicine, and the American Philosophical Society.

**Karen Guillemin** is the Alec and Kay Keith Professor in the Department of Biology and the Institute of Molecular Biology at the University of Oregon. She is also the founding director of the Microbial Ecology and Theory of Animals (META) Center for Systems Biology, an NIH-funded National Center for Systems Biology established in 2012. Guillemin received her bachelor's degree in biochemical sciences from Harvard College in 1991 and her PhD from the Department of Biochemistry at Stanford University School of Medicine in 1998, where she worked with Dr. Mark Krasnow studying organ development in the model organism of the fruit fly. She continued her postdoctoral training at Stanford in the Department of Microbiology and Immunology, studying bacterial–host interactions with Dr. Stanley Falkow, studying the bacterial pathogen and carcinogen, *Helicobacter pylori*. In 2001 she joined the faculty of the University of Oregon, where she established an independent research program that combines her interests in animal development and bacterial–host interactions. Her research group has been instrumental in pioneering the use of gnotobiotic zebrafish to study how resident microbial communities assemble and modulate host biology.

**Timothy Hand** is an assistant professor of pediatrics and immunology and the chair of the Committee on Gnotobiotics at the University of Pittsburgh. Dr. Hand's laboratory is within the R.K. Mellon Institute for Pediatric Research at the Children's Hospital of Pittsburgh. Dr. Hand received his PhD from Yale University in immunology and followed these studies with a productive postdoctoral fellowship at NIH with Dr. Yasmine Belkaid. Dr. Hand's research focuses on the interaction between the host immune system and the intestinal microbiota, with a particular focus on how this relationship is contextually shaped by diet

and infection. Current lab focuses include studies directly relevant to children, such as oral vaccination, cystic fibrosis, necrotizing enterocolitis, and inflammatory bowel disease.

**Donald E. Ingber** is the founding director of the Wyss Institute for Biologically Inspired Engineering at Harvard University, the Judah Folkman Professor of Vascular Biology at Harvard Medical School, and the Vascular Biology Program at Boston Children's Hospital, and professor of bioengineering at the Harvard John A. Paulson School of Engineering and Applied Sciences. He received his BA, MA, MPhil, MD, and PhD from Yale University. Dr. Ingber is a pioneer in the field of biologically inspired engineering, and at the Wyss Institute he currently leads a multifaceted effort to develop breakthrough bioinspired technologies to advance health care and to improve sustainability. His work has led to major advances in mechanobiology, tumor angiogenesis, tissue engineering, systems biology, nanobiotechnology, and translational medicine. Through his work, Dr. Ingber has also helped to break down boundaries between science, art, and design. Dr. Ingber has authored more than 400 publications and 125 patents, founded four companies, and been a guest speaker at more than 450 events internationally. He is a member of the National Academy of Medicine, National Academy of Inventors, American Institute for Medical and Biological Engineering, and the American Academy of Arts and Sciences. He was named one of the Top 20 Translational Researchers worldwide in 2012 (*Nature Biotechnology*) and a Leading Global Thinker of 2015 (*Foreign Policy* magazine). He has received numerous other honors in a broad range of disciplines, including the Robert A. Pritzker Award and the Shu Chien Award from the Biomedical Engineering Society, the Rous Whipple Award from the American Society for Investigative Pathology, the Lifetime Achievement Award from the Society of In Vitro Biology, the Leading Edge Award from the Society of Toxicology, and the Department of Defense Breast Cancer Innovator Award. Some of Dr. Ingber's most recently developed technologies include an anticoagulant surface coating for medical devices that replaces the need for dangerous blood-thinning drugs; a dialysis-like sepsis therapeutic device that clears blood of pathogens and inflammatory toxins; a shear stress–activated nanotherapeutic that targets clot-busting drugs to sites of vascular occlusion; and human organs-on-chips created with microchip manufacturing methods and lined by living human cells, which are being used to replace animal testing as a more accurate and affordable in vitro platform for drug development and personalized medicine. In 2015, Dr. Ingber's organs-on-chips technology was named Design of the Year by the London Design Museum and was also acquired by the Museum of Modern Art (MoMA) in New York City for its permanent design collection.

**Stephen Jameson** is in the Center for Immunology and Department of Laboratory Medicine and Pathology at the University of Minnesota. He obtained his PhD in Cambridge, England, and postdoctoral training at Scripps Research

Institute (La Jolla) and University of Washington (Seattle). His research has focused on factors that regulate CD8 T cell development, homeostasis, and function. A major current area of interest is control and maintain of protective memory CD8 T cells, capable of rapid effector responses and efficient control of pathogens and tumors. These studies included application of novel techniques to isolate and characterize memory-like cells from the pre-immune CD8 T cell pool, building from expertise in the use of peptide/MHC tetramers. Recent work involved characterization of highly protective CD8 T cells, which can be generated by modification of vaccination techniques and novel studies involving mice with more natural exposure to normal environmental pathogens ("dirty mice"), which were shown to be a more faithful model of the adult human immune system. Other studies revolve around the KLF2 transcription factor, which was shown to regulate numerous aspects of lymphocyte trafficking, including key roles in directing the production of recirculating versus resident memory CD8+ T cells and the formation of follicular helper CD4+ T cells. Dr. Jameson plays an active role in graduate student and postdoctoral education and training. He has trained/is training 14 graduate students and 14 postdoctoral fellows, in addition to several undergraduate researchers and two research associates. He was the director of Graduate Studies for the Microbiology, Immunology and Cancer Biology (MICaB) PhD graduate program from 2013 to 2016 and is a member of the steering committee for the NIH-funded T32 Cancer Biology Training Grant.

**Aldons (Jake) Lusis** is professor of microbiology, human genetics and medicine at the University of California, Los Angeles (UCLA). He obtained his PhD in biophysics from Oregon State University and did postdoctoral work in molecular genetics and mouse genetics at Roswell Park Memorial Institute prior to joining the faculty of UCLA. Dr. Lusis's lab studies naturally occurring genetic variations in mice and in humans to help understand interactions underlying complex cardiovascular and metabolic disorders. A major focus of the lab over the past decade has been to leverage common genetic variation in populations to integrate clinical traits with "intermediate" phenotypes obtained using high-throughput technologies, such as expression arrays, sequencing, or proteomics, an approach known as "systems genetics." To facilitate this approach, they have developed a reference resource termed the Hybrid Mouse Diversity Panel that can be used to carry out whole genome association mapping.

**Andrew J. Macpherson** is a professor of medicine and the director of gastroenterology at the University Hospital of Bern, Switzerland. He studied biochemistry and medicine at Cambridge University and did his PhD on sugar-proton symport systems in the laboratory of Sir Hans Kornberg and Peter Henderson. His clinical medical studies and clinical specialty training in gastroenterology were in Cambridge and London. The results (of control experiments) during a project in London on immune-mediated damage to intestinal epithelial cells focused his interest on the way in which the mucosal immune system responds to commensal intestinal microbes. In 1997 he moved to work with Rolf Zinkernagel at the Insti-

tute of Experimental Immunology in Zürich. Between 2004 and 2008 he was the Farncombe Professor of Medicine and a Canada Research Chair holder at McMaster University in Hamilton. His work has shown that there are different pathways of induction of immunoglobulin A in the intestinal mucosa by commensal intestinal microbes, with and without help from T cells. He has also shown a compartmentalization between the mucosal and systemic responses to commensals, since mucosal immune responses are driven locally in the mucosal compartment by dendritic cells that have sampled commensals at the epithelial surface. More recently his lab has developed methods of reversible colonization of germ-free mice to allow intestinal colonization with commensals and mucosal immune priming to be experimentally uncoupled, to address mucosal immune memory, and the functional consequences of mucosal immune responses in host–microbial mutualism and the effects of maternal colonization on immune system development in early life.

**Nancy A. Moran** is the Leslie Surginer Endowed Professor at the University of Texas in the Department of Integrative Biology. She obtained a BA from The University of Texas in 1976 and a PhD (zoology) from the University of Michigan in 1982. From 1986 to 2010, she served on the faculty of the University of Arizona and from 2010 to 2013 she was a professor at Yale University. She has mentored more than 30 graduate students and postdoctoral researchers. Dr. Moran has been elected to membership in the U.S. National Academy of Sciences, the American Academy of Arts and Sciences, and the American Academy of Microbiology. She received the 2010 International Prize for Biology from the Japan Society for the Promotion of Science, the 2014 James Tiedje Award for lifetime contribution in microbial ecology, and the 2016 Lifetime Research contribution award in molecular evolution from the Society for Molecular Biology and Evolution. Dr. Moran studies the evolution of bacteria and insects, using genomic approaches, and has focused on the evolution of symbiotic bacteria that affect insect ecology.

**Joseph T. Newsome** is currently an associate professor in the Department of Pathology and the clinical director-Division of Laboratory Animal Resources, University of Pittsburgh. He received a BSc in microbiology in 1980, master's in pathobiology in 1982 working with the research team of Dr. Richard Olson which developed the first vaccine for feline leukemia, and obtained a DVM in 1986 from The Ohio State University College of Veterinary Medicine. For the next 10 years he wore multiple hats at Georgetown University in Washington, DC, including facility manager, assistant professor of surgery and pathology, and clinical veterinarian overseeing surgery and radiological research support. In 1996 he became a diplomat of the American College of Laboratory Animal Medicine and concurrently completed a postdoctoral training program in experimental pathobiology, assisting in the development of the animal models for the team that eventually led to the current human papilloma virus vaccine. From 2000 to 2012 he was the University of Pittsburgh's attending veterinarian. He is

the author or co-author of more than 65 articles and book chapters. During his career he has been the principal investigator (4) or co-investigator (6) on multiple National Center for Research Resources, National Institutes of Health–funded grants focused on renovations or new construction projects related to vivaria in multiple institutions. He is involved in national and industry-level organizations, such as the American College of Laboratory Animal Medicine (ACLAM), American Association for Laboratory Animal Science (AALAS), and American Veterinary Medical Association (AVMA), with leadership roles such as being a subcommittee chair for the ACLAM foundation since 2010, and being the vice chair of the Policies & Procedures Coordinating Committee (PPCC) of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. His current focus and expertise are in management, biosecurity, biocontainment, facility design and operations, and cancer modeling, immunology, and virology.

**Federico Rey** is an assistant professor in bacteriology at the University of Wisconsin–Madison. His research program focuses on the human microbiome, with a special interest in how gut microbial metabolism impacts cardiometabolic disease. He earned his bachelor's and master's degrees in clinical chemistry from Universidad Nacional de Cordoba in Argentina. As an undergraduate he explored how free radicals modulate vascular tone and atherosclerosis. He did his doctoral work with Professor Caroline Harwood (University of Iowa), studying anaerobic microbial metabolism. He engineered photosynthetic bacteria for improved hydrogen production. After obtaining his PhD, he went on to do postdoctoral studies with Professor Jeffrey Gordon (Washington University in St. Louis), where he explored how human gut microbes interact with each other and their host. In this work, he revealed the metabolic niches of several key members of the community, as well as identifying how they contribute to host health and disease. In Madison, he is expanding on this work, looking at how cardiometabolic disease is impacted by the interplay between host-genetics microbial metabolism and diet.

**Buck Samuel** aims to comprehensively define the genetic pathways that mediate the influences of microbes on host health. He is an assistant professor in the Alkek Center for Metagenomics and Microbiome Research and Department of Molecular Virology and Microbiology at Baylor College of Medicine. He earned two bachelor's degrees (magna cum laude with honors) from the University of Idaho and served in the Science Division at the U.S. Department of State in Paris, France. He completed his PhD at Washington University under the mentorship of Jeffrey Gordon, studying the foundations of how microbes shape host metabolism, and performed his postdoctoral developing *Caenorhabditis elegans* as a facile, high-throughput amenable gnotobiotic system under Gary Ruvkun at Harvard Medical School. Dr. Samuel has been recognized as a National Science Foundation (NSF) Graduate Research Fellow, NIH Ruth L. Kirschstein National Research Service Award (NRSA) Postdoctoral Fellow, and

Charles King Trust Postdoctoral Fellow. His interdisciplinary research brings to bear expertise in organismal biology and genomics to high-throughput molecular studies on how microbes and their products impact host physiology.

**R. Balfour Sartor**, distinguished professor of medicine, microbiology, and immunology; director of the University of North Carolina Multidisciplinary Inflammatory Bowel Disease Center; and director of the Broad Medical Research Program at the Crohn's & Colitis Foundation of America (CCFA), is a board certified gastroenterologist specializing in inflammatory bowel disease (IBD) and a basic and translational scientist, running a large NIH- and foundation-funded laboratory. Research interests include pathogenesis of Crohn's disease and ulcerative colitis, microbial/genetic/immunological interactions in the intestine, using gnotobiotic mice and rats to explore mechanisms of resident bacterial induction of chronic, immune-mediated inflammation versus homeostatic protective mucosal immune responses, and translating basic science knowledge to improve IBD diagnosis and treatment. Dr. Sartor has published more than 330 research articles and reviews and edited five books. Recent studies have emphasized commensal bacterial induction of regulatory T and B cells, particularly IL-10-mediated protection, and identification of novel resident protective bacterial species. Recent translational research searches for microbial, immunological, genomic, and genetic predictors of post-operative recurrence of Crohn's disease and for biomarkers that predict the natural history of Crohn's disease (aggressive disease requiring biologic therapies with complications leading to Crohn's disease versus Endoline, easily treated course). He directs a large gnotobiotic unit and uses germ-free mice to investigate mechanisms by which resident microbiota initiate, perpetuate, and protect against chronic intestinal inflammation. He currently serves on the American Gastroenterological Association's Microbiome Advisory Committee, and has previously been an American Gastroenterological Association (AGA) council member as chair of the Immunology, Microbiology and IBD Section. He now directs the Broad Medical Research Program at CCFA after previously serving as the CCFA's chief medical advisor, national board member, chairman of the National Scientific Advisory Committee and chairman of the Senior Research and Training Award Review Committees. He has a passion for better understanding immunopathogenic mechanisms and host–microbial interactions of IBD, improving therapies, and training the next generation of scientists and clinicians.

**Betty Theriault** is an associate professor in the Department of Surgery and clinical veterinarian within the Animal Resources Center at The University of Chicago. She is a veterinarian with more than 30 years of experience working with animals in a variety of settings and across a broad spectrum of species. Dr. Theriault joined The University of Chicago in 2005 and in 2006 embarked on developing The University of Chicago's Gnotobiotic Research Animal Facility (GRAF), which has experienced 10 years of steady growth and success. Initially developed to support the research of a few scientists at the university, the gnotobiotic program currently supports the work of 12 principal investiga-

tors directly as well as The University of Chicago's Digestive Disease Research Core Center (DDRCC) Host Microbiome Core Gnotobiotic Mouse Component, for which she is co-director. Her work focuses on assisting researchers with developing animal models of human disease and adapting technologies to unique applications. She has been a primary or co-author of publications spanning a diverse range of models and species, including but not limited to, animal models of transplantation, ovarian cancer metastasis, food allergy, circadian rhythm, and obesity. She is the immediate past president of the Chicago branch of the American Association for Laboratory Animal Science and president elect for both the Association for Gnotobiotics, which is currently being revitalized, and the International Society for Gnotobiology. She has received a Distinguished Leader in Program Development and Distinguished Faculty Award from The University of Chicago, as well as the Bengt E. Gustafsson Award presented by the 17th International Society for Gnotobiology and 34th Society of Microbial Ecology and Disease Joint Congress held in Yokohama, Japan, in 2011. Dr. Theriault has presented lectures on topics of gnotobiology at scientific meetings locally, nationally, and internationally and has led workshops at national meetings held for the laboratory animal community. She received her DVM from the University of California, Davis, completed a small animal medicine and surgery internship at the University of Pennsylvania, and is a diplomate of the American College of Laboratory Animal Medicine.

**Herbert "Skip" Virgin** is the Edward Mallinckrodt Professor and chair of the Department of Pathology and Immunology at the Washington University School of Medicine in Saint Louis, Missouri. He received his AB, MD, and PhD from Harvard University, trained in internal medicine and infectious diseases, and performed postdoctoral studies with Dr. Bernard Fields. He is a member of the American Society for Clinical Investigation, the Association of American Physicians, the American Academy of Microbiology, and the National Academy of Sciences. He serves on the Board of Reviewing Editors of *Science* and the Editorial Board of *Cell*. The Virgin laboratory uses genetic, structural, computational, and sequencing methods to define mechanisms of viral pathogenesis and immunity in vivo, with many studies focusing on mouse models. They have identified the physiological role and molecular mechanisms of several RNA and DNA virus immune evasion molecules and studied immune effector mechanisms, including ISG15, interferon-γ, interferon-λ, cGAS, and autophagy. They discovered the first murine norovirus and developed the first culture system for a norovirus. Studying this virus, they showed that virus-plus-host-gene interactions define disease phenotypes. They have recently focused on "trans-kingdom" interactions within the metagenome and on interactions between microbial and viral components of the metagenome and host immunity. They found that persistent γ-herpesvirus infection can "complement" genetic immunodeficiency, and can symbiotically protect the host against bacterial infection. Recent studies also reveal that enteric helminth infection reactivates murine γ-herpesviruses from latency through host cytokine competition at a viral promoter and that

these cytokine effects are conserved in a human herpesvirus. They found that bacteria control persistent enteric norovirus infection in a manner dependent on interferon-λ, and identified interferon-λ-induced sterilizing innate immune responses to enteric viral infection. They have used metagenomics to identify constituents of the mammalian virome and to show that the enteric virome is altered in humans and macaques with lentivirus infection as well as in humans with inflammatory bowel disease. Together these studies identify trans-kingdom interactions within the metagenome as key contributors to mammalian biology.

**Chriss J. Vowles** co-manages a multi-investigator germ-free research laboratory at the University of Michigan in Ann Arbor. He began his career at the University of Michigan in 2003, working full time as a husbandry technician in the Unit for Laboratory Animal Medicine. In 2006, he discovered gnotobiotic technology. At that time, the Germ Free Laboratory was just starting. Mr. Vowles joined the research group on the ground floor and has been growing with it ever since. He has authored the first practical guide in the field of gnotobiotics titled *Gnotobiotic Mouse Technology: An Illustrated Guide*.

**Gary D. Wu** is the Ferdinand G. Weisbrod Professor of Medicine at the Perelman School of Medicine at the University of Pennsylvania, where he is the associate chief for research in the Division of Gastroenterology, the associate director of the Center for Molecular Studies in Digestive and Liver Disease, and the co-director of the University of Pennsylvania and Children's Hospital of Philadelphia (PennCHOP) Microbiome Program. He was the inaugural director and chair of the Scientific Advisory Board for the American Gastroenterological Association Center for Gut Microbiome Research and Education and is an elected member of both the American Society for Clinical Investigation and the American Association of Physicians. Research programs in the Wu laboratory focus on the mutualistic interactions between the gut microbiota and its host, with a particular emphasis on metabolism, including nitrogen balance, intestinal oxygen regulation, and epithelial intermediary metabolism. Of particular interest is the effect of diet on the gut microbiome and its relationship to therapeutic responses associated with the use of defined formula diets in the treatment of Crohn's disease. Insights gained from these projects will hopefully lead to the development of better diets for patients with IBD.

**Vincent B. Young** is an associate professor in the Department of Internal Medicine/Infectious Diseases Division and the Department of Microbiology and Immunology at the University of Michigan Medical School. He received his undergraduate degree from the Massachusetts Institute of Technology and received his MD and PhD from Stanford University. He completed his clinical training in internal medicine and infectious diseases at the Massachusetts General Hospital. He was previously on the faculty at Michigan State University prior to joining the University of Michigan in 2007. Dr. Young has a long-standing interest in understanding the pathogenesis of bacterial infections of the gastrointestinal

tract and the role of the normal microbiota in human health and disease. Dr. Young led a Human Microbiome Project on the role of the microbiome in inflammatory bowel disease. He is also involved in projects that look at microbial communities in the lungs of patients with HIV infection and cystic fibrosis. Current research in the Young Lab includes a "team science" effort to understand the pathogenesis of *Clostridium difficile* infection by an integrated approach that combines clinical research, bacterial genomics, microbial ecology, and immunology/host response projects. He is also leading a group of investigators that is developing the use of stem cell–derived intestinal organoids as a novel alternative model system for the study of enteric disease agents.

# Appendix D

# Glossary

**Adaptive immune system:** A collective term given to a group of highly specialized, systematic cells and processes that prevent vertebrates from certain death by pathogenic infections. (Alberts B, et al. 2002. *Molecular Biology of the Cell*, 4th Edition. New York: Garland Science.)

**Anoxic:** An absence or deficiency of oxygen reaching the tissues. (The Oxford English Dictionary)

**Archaea:** One of the three main branches of evolutionary descent (Archaea, Eukaryota, and Bacteria), archaea are single-celled organisms once classified as extremophiles (being found in harsh environments such as hot springs and salt lakes), yet recent evidence shows that archaea are widely distributed in nature. (IOM [Institute of Medicine]. 2013. *The Science and Applications of Microbial Genomics: Workshop Summary*. Washington, DC: The National Academies Press.)

**Axenic:** Free of all microorganisms, including those that are typically found in the gut (thus truly germ free). Axenic mice (for example) are produced by hysterectomy rederivation and must be maintained in isolators under very strict handling procedures to keep them germ-free. (The Jackson Laboratory: https://www.jax.org/news-and-insights/jax-blog/2013/may/the-difference-between-germ-free-and-specific-pathogen-free-mice. Accessed March 2, 2018.)

**Bacteria:** Microscopic, single-celled organisms that have some biochemical and structural features different from those of animal and plant cells. (IOM. 2014. *Microbial Ecology in States of Health and Disease: Workshop Summary*. Washington, DC: The National Academies Press.)

**Commensal/commensalism:** Two (or more) species coexist, one deriving benefit from the relationship without harm or obvious benefit to the other. (IOM. 2014. *Microbial Ecology in States of Health and Disease: Workshop Summary*. Washington, DC: The National Academies Press.)

**Commensal organism:** An organism that derives benefits from its association with humans or animals without causing harm (NASEM [National Academies of Sciences, Engineering, and Medicine]. 2017. *Microbiomes of the Built Environment: A Research Agenda for Indoor Microbiology, Human Health, and Buildings*. Washington, DC: The National Academies Press. doi: https://doi.org/10.17226/23647.)

**Conventionalization:** A method in which germ-free animals (particularly mice) are inoculated with gut microbiota to populate the gastrointestinal tract. (Cho I, Blaser MJ. 2012. The human microbiome: At the interface of health and disease. *Nat Rev Genet* 13:260-270.)

**Ecosystem:** A biological community of interacting organisms and their physical environment. (The Oxford English Dictionary)

**Effector strain:** Molecules that either facilitate infection (virulence factors or toxins) or that trigger host defense (avirulence factors or elicitors). (Kamoun S. 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. *Annu Rev Phytopathol* 44:41-60.)

**Endogenous:** Growing or originating from within an organism. (The Oxford English Dictionary)

**Enteroids:** Epithelial structures that contain crypt- and villus-like domains reminiscent of normal gut epithelium. Commonly termed "enteroids" when derived from the small intestine and "colonoids" when derived from the colon, they are different from organoids that also contain mesenchymal tissue. (Mahe MM, et al. 2015. Establishment of human epithelial enteroids and colonoids from whole tissue and biopsy. *J Vis Exp* 97:e52483.)

**Eukaryota:** One of the three domains of life. The two other domains, Bacteria and Archaea, are prokaryotic and lack several features characteristic of eukaryotes (e.g., cells containing a nucleus surrounded by a membrane and with DNA bound together by proteins [histones] into chromosomes). Animals, plants, and fungi are all eukaryotic organisms. (IOM. 2014. *Microbial Ecology in States of Health and Disease: Workshop Summary*. Washington, DC: The National Academies Press.)

**Genome:** The complete set of genetic information in an organism. In bacteria, this includes the chromosome(s) and plasmids (extra-chromosomal DNA molecules that can replicate autonomously within a bacterial cell). (Ibid.)

**Genomics:** The study of genes and their associated functions. (Ibid.)

**Genotype:** In a broad sense, the term *genotype* refers to the genetic makeup of an organism; in other words, it describes an organism's complete set of genes. In a narrower sense, the term can be used to refer to the alleles, or variant forms of a gene, that are carried by an organism. (SciTable by Nature Education: https://www.nature.com/scitable/definition/genotype-234. Accessed March 2, 2018.)

**Gnotobiotic:** An animal that is colonized solely by known strains of bacteria or other microorganisms. The term also describes germ-free animals because the status of their microbial communities is known. (Cho I, Blaser MJ. 2012. The human microbiome: At the interface of health and disease. *Nat Rev Genet* 13:260-270.)

**Gram negative:** Refers to the inability of a microorganism to accept a certain stain. This ability is related to the cell wall composition of the microorganism and has been useful in classifying bacteria. (IOM. 2013. *The Science and Applications of Microbial Genomics: Workshop Summary*. Washington, DC: The National Academies Press.)

**Gram positive:** Refers to the ability of a microorganism to accept a certain stain. This ability is related to the cell wall composition of the microorganism and has been useful in classifying bacteria. (IOM. 2013. *The Science and Applications of Microbial Genomics: Workshop Summary*. Washington, DC: The National Academies Press.)

**Homolog:** One of two or more genes that are similar in sequence as a result of derivation from the same ancestral gene. The term covers both orthologs and paralogs. (IOM. 2009. *Microbial Evolution and Co-Adaptation: A Tribute to the Life and Scientific Legacies of Joshua Lederberg*. Washington, DC: The National Academies Press.)

**Immunophenotype/immunophenotyping:** A process that uses antibodies to identify cells based on the types of antigens or markers on the surface of the cells. Immunophenotyping may also be used to separate cells into different groups based on the markers they have on the surface. (National Cancer Institute Dictionary of Cancer Terms: https://www.cancer.gov/publications/dictionaries/cancer-terms?cdrid=341450. Accessed March 2, 2018.)

**Infection:** The invasion of the body or a part of the body by a pathogenic agent, such as a microorganism or virus. Under favorable conditions, the agent develops or multiplies, with results that may produce injurious effects. (IOM. 2014. *Microbial Ecology in States of Health and Disease: Workshop Summary*. Washington, DC: The National Academies Press.)

**Innate immune system:** Innate (natural) immunity is so named because it is present at birth and does not have to be learned through exposure to an invader. It thus provides an immediate response to foreign invaders. However, its components treat all foreign invaders in the same way. They recognize only a limited number of identifying substances (antigens) on foreign invaders. However, these antigens are present on many different invaders. Innate immunity, unlike acquired immunity, has no memory of the encounters, does not remember specific foreign antigens, and does not provide any ongoing protection against future infection. (Merck Manual: http://www.merckmanuals.com/home/immune-disorders/biology-of-the-immune-system/innate-immunity. Accessed March 2, 2018.)

**Islet:** A portion of tissue structurally distinct from surrounding tissues. (The Oxford English Dictionary)

**Isobiotic:** Colonized with only a defined set of microbes. (Stappenbeck TS, Virgin HW. 2016. Accounting for reciprocal host-microbiome interactions in experimental science. *Nature* 534:191-199.)

**Isogenic:** Organisms having the same or closely similar genotypes. (The Oxford English Dictionary)

**Loci:** The positions of a gene or mutation on a chromosome. (The Oxford English Dictionary)

**Metabolites:** Substances made or used when the body breaks down food, drugs or chemicals, or its own tissue (for example, fat or muscle tissue). This process, called metabolism, makes energy and the materials needed for growth, reproduction, and maintaining health. It also helps get rid of toxic substances. (National Cancer Institute Dictionary of Cancer Terms: https://www.cancer.gov/publications/dictionaries/cancer-terms?cdrid=462687. Accessed March 2, 2018.)

**Metabolome:** The census of all metabolites present in any given tissue, space, or sample. (Adapted from Marchesi JR, Ravel J. 2015. The vocabulary of microbiome research: A proposal. *Microbiome* 3(1):31. Cited in NASEM. 2017. *Microbiomes of the Built Environment: A Research Agenda for Indoor Microbiology, Human Health, and Buildings*. Washington, DC: The National Academies Press. doi: https://doi.org/10.17226/23647.)

**Metabolomics:** Systematic global analysis of nonpeptide small molecules, such as vitamins, sugars, hormones, fatty acids, and other metabolites. It is distinct from traditional analyses that target only individual metabolites or pathways. (NASEM. 2016. *Genetically Engineered Crops: Experiences and Prospects*. Washington, DC: The National Academies Press. doi: 10.17226/23395.)

**Metagenome:** The collection of genomes and genes from the members of a microbiota or microbial community. (Marchesi JR, Ravel J. 2015. The vocabulary of microbiome research: A proposal. *Microbiome* 3(1):31.)

**Metagenomics:** A culture-independent method used for functional and sequence-based analysis of total environmental (community) DNA. (Adapted in part from IOM. 2014. *Microbial Ecology in States of Health and Disease: Workshop Summary*. Washington, DC: The National Academies Press.)

**Metaproteomics:** The large-scale characterization of the entire protein complement of environmental or clinical samples at a given point in time. (Marchesi JR, Ravel J. 2015. The vocabulary of microbiome research: A proposal. *Microbiome* 3(1):31.)

**Microbe:** A microscopic living organism, such as a bacterium, fungus, protozoan, or virus. (IOM. 2014. *Microbial Ecology in States of Health and Disease: Workshop Summary*. Washington, DC: The National Academies Press.)

**Microbial community/microbiota:** A collection of microorganisms existing in the same place at the same time. (Adapted from Ibid.)

**Microbiome:** The collection of all the organisms in or on a host, including viruses, bacteria, archaea, fungi, and protists. These organisms interact with each other and the host in a variety of complex and meaningful ways. (Presentation of HW Virgin)

**Monocolonization:** Inoculation of germ-free animals with one type of microbe. (Wiktionary)

**Nonpathogenic:** Refers to an organism or other agent that does not cause disease. (Adapted from Alberts B, et al. 2002. *Molecular Biology of the Cell*, 4th ed. New York: Garland Science.)

**Operational Taxonomic Unit:** OTUs, defined as clusters of 16S/18S small subunit (*SSU*) rRNA gene similarity, are used as theory-agnostic approximations of microbial taxa. (Schmidt TSB, Matias Rodrigues JF, von Mering C. 2014. Ecological Consistency of SSU rRNA-Based Operational Taxonomic Units at a Global Scale. *PLoS Comput Biol*. https://doi.org/10.1371/journal.pcbi.1003594.)

**Organoid:** An in vitro 3D cellular cluster derived exclusively from primary tissue, embryonic stem cells, or induced pluripotent stem cells, capable of self-renewal and self-organization, and exhibiting similar organ functionality as the tissue of origin. (Fatehullah A, Tan SH, Barker N. 2016. Organoids as an in vitro model of human development and disease. *Nat Cell Biol* 18:246-254.)

**Ortholog:** One of two or more genes that are similar in sequence as a result of derivation from the same ancestral gene. The term covers both orthologs and paralogs. (IOM. 2009. *Microbial Evolution and Co-adaptation: A Tribute to the Life and Scientific Legacies of Joshua Lederberg*. Washington, DC: The National Academies Press.)

**Pathogen/pathogenic:** An organism or other agent that causes disease. (Alberts B, et al. 2002. *Molecular Biology of the Cell*, 4th ed. New York: Garland Science.)

**Phenotype:** The term *phenotype* refers to the observable physical properties of an organism; these include the organism's appearance, development, and behavior. An organism's phenotype is determined by its genotype, which is the set of genes the organism carries, as well as by environmental influences on these genes. (SciTable by Nature Education: https://www.nature.com/scitable/definition/phenotype-phenotypes-35. Accessed March 2, 2018.)

**Proteomic:** The large-scale characterization of the entire protein complement of a cell line, tissue, or organism. A more recent definition combined protein studies with analyses that have a genetic readout, such as mRNA analysis, genomics, and the yeast two-hybrid analysis. (Graves PR, Haystead TAJ. 2002. Molecular biologist's guide to proteomics. *Microbiol Mol Biol* Rev 66(1):39-63.)

**Protista Kingdom:** Eukaryotic organisms that are unicellular and sometimes colonial or less often multicellular and that typically include the protozoans, most algae, and often some fungi (as slime molds). (Merriam-Webster Dictionary) (https://www.merriam-webster.com/dictionary/protist. Accessed March 2, 2018.)

**Shotgun sequencing:** Sequencing of a genome that has been fragmented into small pieces. (IOM. 2014. *Microbial Ecology in States of Health and Disease: Workshop Summary*. Washington, DC: The National Academies Press.)

**Specific pathogen free (SPF):** SPF mice are mice that are demonstrated to be free of a specific list of pathogens by routine testing. (The Jackson Laboratory: https://www.jax.org/news-and-insights/jax-blog/2013/may/the-difference-between-germ-free-and-specific-pathogen-free-mice. Accessed March 2, 2018.)

**Symbiotic:** Involving interaction between two different organisms living in close physical association. (The Oxford English Dictionary)

**Taxa:** A term used to refer to all the organisms that fall under a particular taxonomic criterion (such as kingdom, phylum, class, order, family, genus, species, or subspecies). (NASEM. 2017. *Microbiomes of the Built Environment: A Research Agenda for Indoor Microbiology, Human Health, and Buildings*. Washington, DC: The National Academies Press. https://doi.org/10.17226/23647.)

**Taxonomic/taxonomy:** The systematic classification, identification, and nomenclature of organisms. (Adapted from Baron S, ed. 1996. *Medical Microbiology*, 4th ed. Galveston, TX: University of Texas Medical Branch at Galveston.)

**Transcriptome:** The transcriptome is the full range of messenger RNA, or mRNA, molecules expressed by an organism. The term can also be used to describe the array of mRNA transcripts produced in a particular cell or tissue type. (SciTable by Nature Education: https://www.nature.com/scitable/definition/transcriptome-296. Accessed March 2, 2018.)

**Transmissibility:** The ease with which a microorganism(s) can spread from a source to a host. (NASEM. 2017. *Microbiomes of the Built Environment: A Research Agenda for Indoor Microbiology, Human Health, and Buildings*. Washington, DC: The National Academies Press. https://doi.org/10.17226/23647.)

**Transmission:** The transfer of a microorganism(s) from a source to a host. (Adapted from Baron S, ed. 1996. *Medical Microbiology*, 4th ed. Galveston, TX: University of Texas Medical Branch at Galveston.)

**Virome:** The composition of the human virome includes viruses that infect human cells, ancient virus-derived elements inserted in our chromosomes, and bacteriophages that infect a broad array of bacteria that inhabit us. (Zou S, Caler L, Colombini-Hatch S, Glynn S, Srinivas P. 2016. Research on the human virome: Where are we and what is next. *Microbiome* 4:32.)

**Virus:** A small infectious agent that can replicate only inside the cells of another organism. Viruses are too small to be seen directly with a light microscope. They infect all types of organisms, from animals and plants to bacteria and archaea. (IOM. 2014. *Microbial Ecology in States of Health and Disease: Workshop Summary*. Washington, DC: The National Academies Press.)