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A Consensus Study Report of

The National Academies of

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Environmental Chemicals, the Human Microbiome, and Health Risk: A Research Strategy

Committee on Advancing Understanding of the Implications of Environmental-Chemical Interactions with the Human Microbiome

Board on Environmental Studies and Toxicology

Board on Life Sciences

Division on Earth and Life Studies

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Summary

A great number of diverse microorganisms inhabit the human body and are collectively referred to as the human microbiome. Until recently, the role of the human microbiome in maintaining human health was not fully appreciated. Today, however, research is beginning to elucidate associations between perturbations in the human microbiome and human disease and the factors that might be responsible for the perturbations. Studies have indicated that the human microbiome could be affected by environmental chemicals or could modulate exposure to environmental chemicals. Given those findings, some fear that we might be missing or mischaracterizing health effects of exposure to environmental chemicals and have therefore argued that chemical–microbiome interactions should be considered in assessing human health risk associated with environmental-chemical exposure. Such considerations would add substantial complexity to an already complex analysis. Given the complexity and resource constraints, the US Environmental Protection Agency (EPA) and the National Institute of Environmental Health Sciences (NIEHS) asked the National Academies of Sciences, Engineering, and Medicine to develop a research strategy to improve our understanding of the interactions between environmental chemicals and the human microbiome and the implications of those interactions for human health risk. They also asked the National Academies to identify barriers to such research and opportunities for collaboration.[1] As a result of the request, the National Academies convened the Committee on Advancing Understanding of the Implications of Environmental-Chemical Interactions with the Human Microbiome, which prepared this report.

Here, the committee highlights key aspects of the human microbiome and its relation to health, describes potential interactions between environmental chemicals and the human microbiome, reviews the risk-assessment framework and reasons for incorporating chemical–microbiome interactions, and outlines its research strategy. The committee emphasizes that this report is not a comprehensive review of all microbiome research. The research strategy presented here focuses on addressing questions about the interactions of environmental chemicals with the human microbiome and the implications for human health risk. It is not a research strategy for directly investigating associations between the human microbiome and various diseases.

THE HUMAN MICROBIOME

The *human microbiome* is an all-encompassing term that refers to all microorganisms on or in the human body, their genes, and surrounding environmental conditions. Because of the vast diversity and sheer amount of microbial life that colonizes the human body, human beings are now regarded as ecosystems that are comprised of distinct ecologic niches or habitats, each housing a discrete collection of coevolved microorganisms that interact extensively with each other and with the human host. Coevolution has led to interdependence: the human microbiome contributes a vast array of essential functions to the human host and influences a variety of physiologic, immunologic, and metabolic processes. Perturbations of the composition and function of niche-specific microbial communities have been implicated in an array of neurologic, gastrointestinal, metabolic, oncologic, hepatic, cardiovascular, psychologic, respiratory, and autoimmune disorders or diseases.

[1]The full statement of task is in Chapter 1 of this report.

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One key aspect of the human microbiome is the variation in its composition and function observed among populations, over the human life span, and between body sites. The variation between body sites is particularly noteworthy. Each body site is associated with the presence of a relatively conserved microbial community (a microbiome) that has adapted to the environmental conditions of the site. The site-specific differences in microbial composition yield differences in metabolic capacity and in the aggregate function of the human microbiome. Multiple factors also play roles in the variation observed among individual body sites. For example, age and diet play primary roles in the variation observed in the gut microbiome, and local ecologic conditions, particularly water and nutrient availability, drive the site-specific community states of the skin microbiome. Numerous physiologic and anatomic factors play roles in determining the composition and regional variation in the respiratory microbiome; research suggests that important factors include differences in oxygen tension, airway luminal temperature, mucociliary clearance mechanisms, and other innate defenses. All those factors and others—such as genetics, sex, socioeconomic status, disease state, geography, pregnancy status, diet, and environmental exposures—appear to play roles in shaping the composition and function of microbial communities.

As discussed throughout the present report, animal models provide valuable experimental platforms for studying microbiome structure and function, but it is important to note that the human microbiome differs from the microbiomes of other species in which microorganisms are present, in the relative abundance of dominant microorganisms, and in how the microbial community responds to a given perturbation. The degree to which microbiome composition differs between species (and between humans) depends partially on the taxonomic level at which microbiomes are characterized—whether at the strain, species, genus, family, order, class, or phylum level—and possibly on technical differences among study protocols, which can vary substantially. Although most studies have not compared functional attributes of the microbiomes, such comparison might indicate greater similarity than simply comparing microbial composition. However, given the differences between humans and animals, observations made in animal models, although informative and foundational, might not capture the full breadth of microbial interactions that occur in humans. The strengths and weaknesses of animal models for research into chemical–microbiome interactions are discussed further below.

INTERACTIONS BETWEEEN ENVIRONMENTAL CHEMICALS AND THE HUMAN MICROBIOME

Scientific research is beginning to elucidate the various ways in which environmental chemicals might interact with the human microbiome. Studies suggest that exposure to environmental chemicals can alter the composition and potentially affect the function of the human microbiome. Other studies indicate that the human microbiome can modulate environmental-chemical exposure. For example, evidence of involvement of the gut microbiome in the metabolic transformation of environmental chemicals in broad chemical classes is compelling.

Many molecular mechanisms likely underlie microbiome interactions. However, research suggests that the human microbiome might modulate the exposure–response relationships of environmental chemicals by a few general mechanisms, as described below.

- *Direct effect of a chemical on the human microbiome*. Distinct microbial compositions can have specific effects on host biology. If exposure to an environmental chemical (or any other factor) causes a perturbation in the microbiome, that perturbation might have distinct effects on the host. It is also conceivable that changes induced by environmental-chemical exposures can result in an altered capacity of the microbiome to metabolize chemicals.
- *Altered epithelial-barrier functions*. Epithelial barriers form the interface between many host tissues and the external environment. Increasing evidence suggests that there are intimate bidirectional interactions between the microbiota and epithelial cells, wherein the composition and activity of the gut microbiota, for example, modulates the structure and function of the intestinal epithelium and vice versa.

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The ability to regulate epithelial permeability and integrity has important implications for the absorption, transport, and excretion of environmental chemicals.

- *Direct chemical transformation.* As noted, the gut microbiome has been shown to metabolize broad classes of environmental chemicals. Microbial metabolic transformations have been generally categorized into reduction and hydrolysis reactions and have been classified further into five major enzymatic families—azoreductases, nitroreductases, β-glucuronidases, sulfatases, and β-lyases.
- *Transformation of host-generated metabolites.* In some cases, detoxification and elimination of environmental chemicals by host liver enzymes might be reversed by microbial hydrolases in the gut. For example, deconjugation reactions by gut β-glucuronidases promote reabsorption of some drug metabolites, which potentially alters their pharmacokinetic profiles, toxicity, or efficacy. Because a wide array of environmental chemicals might be subject to elimination via β-glucuronidation, this mechanism might be more common than is now appreciated.
- *Altered expression of host-tissue metabolic enzymes and pathways.* Recent studies have demonstrated that the gut microbiota can regulate host genes involved in chemical metabolism, although more research is needed to understand the mechanisms by which the gut microbiome and its products interact with host nuclear receptors and whether similar processes can alter expression of other types of host-gene pathways that are involved in toxicity.

Although research has provided important clues regarding microbial transformation of environmental chemicals and vice versa, there are substantial gaps in the understanding of how chemical exposure changes activity or function of a microbiome and the breadth of potential pathways for metabolism of environmental chemicals represented in a given microbiome. Furthermore, it is important to note that each interaction can conceptually increase or decrease chemical exposure, and that the role of the interactions in modifying human susceptibility to toxicity at environmentally relevant exposures remains largely uncertain.

RISK ASSESSMENT: INCORPORATING CHEMICAL–MICROBIOME INTERACTIONS

Research indicates the important role that the human microbiome plays in human health and raises the question of whether some consideration needs to be incorporated into risk assessment. Risk assessment is a process that can be used to estimate the human health risk associated with exposure to an environmental chemical. Although risk assessment used in regulatory programs in the United States and globally has been reformed and advanced over the years, the core elements established in the 1980s—hazard identification, dose–response assessment, exposure assessment, and risk characterization—have remained the same (see Figure S-1). EPA has developed numerous guidelines for the conduct of risk assessment; the guidelines describe the optimal evaluation and use of data that often are inconsistent, and they indicate proper treatment of uncertainty in extrapolating results from animal or human studies of limited scope to policies designed to protect the general public.

Animal toxicology studies have traditionally provided the data for hazard identification and dose–response assessment, but epidemiology (human) studies have provided the primary evidence on a few chemicals, such as arsenic and formaldehyde. In vitro assays and computational approaches are also being developed in light of scientific and technologic advances in biology and related fields and substantial increases in computational power. The hope is that the new approaches can predict toxicity on the basis of an understanding of the biologic processes that lead to adverse effects. Exposure science has also undergone remarkable advances in the last few decades; technologies for developing rapid and comprehensive exposure profiles, from the use of remote and personal sensors to identification and sampling of key biomarkers, are contributing copious new data for risk assessment. Regardless of the approaches used to provide data for various risk-assessment elements, none has explicitly considered or incorporated the human microbiome. Therefore, risk assessments might mischaracterize the nature of a hazard associated

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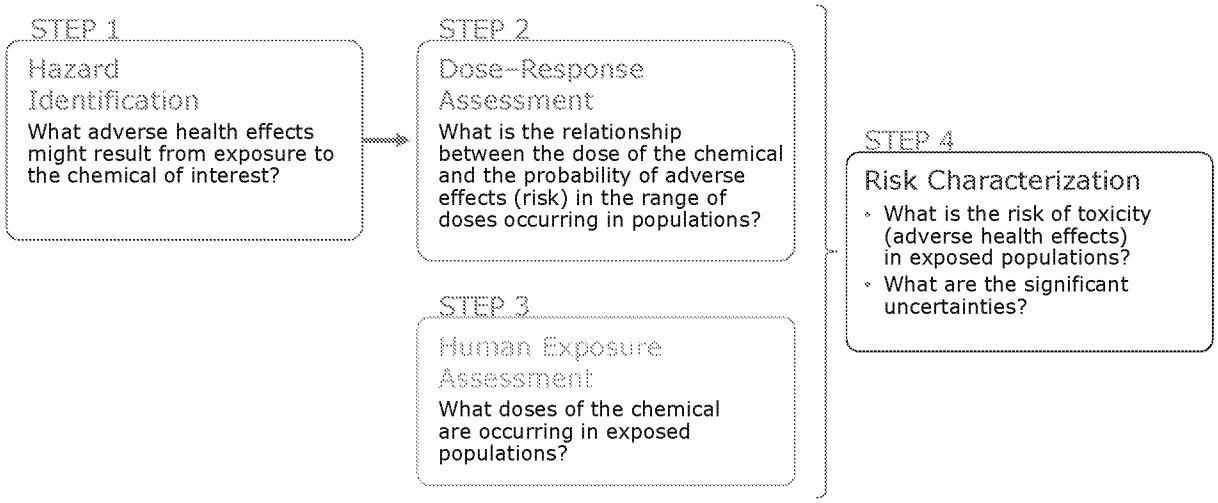

FIGURE S-1 Standard four-step framework for risk assessment.

with an exposure or overestimate or underestimate the risk associated with the exposure, particularly when the results from studies in animals or in a specific population are used to characterize risk to another species or population that has a microbiome different from that of the studied population.

Studies on chemical–microbiome interactions and their consequences suggest that further research could substantially advance understanding of human health risk posed by exposure to environmental chemicals. Specifically, research might explain differences between animal toxicology studies and human responses, provide greater confidence in extrapolating findings of animal studies to humans, and identify unrecognized health consequences of environmental exposures. Furthermore, differences in responses to chemical exposure reported in epidemiology studies conducted on different populations might be explained by the population variation in microbiome composition and function. Given the recent research on the human microbiome, it is reasonable to hypothesize that its adequate consideration in risk assessment could improve the understanding of health risks posed by exposures to environmental chemicals.

RESEARCH STRATEGY

Development of a research strategy to understand the interactions between environmental chemicals and the human microbiome and the implications of those interactions for human health risk is a complex task. One reason is that our understanding of how perturbations of the human microbiome might cause or contribute to the development of various diseases is in its infancy, so the task of understanding how environmental chemicals fit into the picture is even more difficult than it might otherwise be. Initially, the committee envisioned a research strategy that was similar to a flowchart or decision tree in which the results of one or more experiments would lead naturally to a next set of experiments. However, such a straightforward approach is not feasible given the state of the science. Thus, the committee determined that the research strategy should focus broadly on the three general topics: the effects of environmental chemicals on the human microbiome, the role of the human microbiome in modulating environmental-chemical exposure, and the importance of variation in the human microbiome in modulating chemical–microbiome interactions. The discussion below provides the primary goals of the research, identifies some possible barriers, and highlights the need for collaboration. A more detailed discussion of experimental approaches and barriers related to each topic can be found in Chapter 6 of the committee's report with criteria for selecting chemicals for experimental approaches. It is important to note that the committee is not recommending that all the research described in this report be undertaken at once. Discoveries

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made in trying to understand the relationships between microbiome perturbations and disease will influence the course of the committee's proposed research strategy, and various agencies and organizations will have different priorities and interests in pursuing various research topics described here. The committee hopes that the near-term research will help to elucidate whether the microbiome is an important contributor to human health risks associated with exposure to environmental chemicals and the need for and direction of research in this area.

The Effects of Environmental Chemicals on the Human Microbiome

The question for this research to answer is whether environmental-chemical exposures or doses that are in the range of known or anticipated human exposures can induce microbiome alterations that modulate adverse health effects. As noted, recent evidence indicates that exposures to some environmental chemicals can alter the microbiome, but there is little evidence that the alterations have adverse effects on health status. To address the question posed, the research program should focus on defining toxicity end points for the microbiome, on identifying environmental chemicals that can perturb the microbiome structurally and functionally, and on using animal and epidemiology studies to demonstrate that microbiome perturbations by environmental chemicals cause or modulate a change in health. Although individual microbial physiology can be detailed robustly, no end points for microbiome toxicity have been established. Thus, defining quantifiable end points that reflect toxicity to the microbiome are of paramount importance, and comprehensive approaches will be needed to capture all aspects of microbiome responses to a given toxicant. Establishing toxicity end points for the microbiome will enable the development of high-throughput bioreactors that can screen environmental chemicals in a uniform manner for their ability to perturb microbiomes. Once chemicals that perturb microbiomes have been identified, they can be investigated in animal models and in epidemiology studies.

Epidemiology studies constitute a considerable undertaking, so it is important to note that existing epidemiology and population studies could be leveraged for this research. For example, one could identify a human population in which a chemical exposure of interest has been tracked and collect new samples appropriate for microbiome analyses, one could generate new microbiome-relevant data from stored samples from such a cohort, or one could add measurements of environmental-chemical exposures to a human population that is being followed for other purposes, including microbiome measurements. Simple measures of microbiome structure might be sufficient to identify cases in which a perturbation occurs in tandem with or after chemical exposure and manifestation of adverse health outcomes; the microbiome changes would then need to be investigated in more detail to characterize their functional or clinical consequences, if any. In such cases, it will also be crucial to separate health effects mediated by microbial activity from those induced directly by chemical exposures of the host.

The Role of the Human Microbiome in Modulating Environmental-Chemical Exposure

The question for this research to answer is, What is the role of the human microbiome in modulating absorption, distribution, metabolism (activation or inactivation), and elimination (ADME) of environmental chemicals? The research program would focus on generating pharmacokinetic–pharmacodynamic data from animal and in vitro experiments. The animal experiments would assess the effects of the microbiome on ADME processes in vivo and the magnitude of the effects. The in vitro experiments would be used to define functional traits for a microbial community that transforms an environmental chemical, to identify microorganisms and microbial interactions implicated in chemical transformations, to identify microorganism-modified metabolites, and to obtain microorganism-specific chemical transformation rates. The data generated from the experiments could be used to develop a microbiome component for physiologically based pharmacokinetic or pharmacodynamic models that would permit better assessment of human responses to chemical exposures.

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Another aspect of the research program would be identification of specific microorganisms and their enzymes that mediate chemical transformation processes by using new chemical probes and chemical screening technologies. Ultimately, linking the specific microorganisms, genes, and enzymes to particular chemical transformation processes is essential if substantive progress is to be made in addressing individual susceptibility and interspecies extrapolation at a mechanistic level and in understanding the degree of functional redundancy that exists within a microbiome.

The Importance of Microbiome Variation

Two aspects of microbiome variation need to be investigated. The first is the microbiome variation in the human population; the question is whether knowledge of population variation in the human microbiome improves understanding of individual health risks and susceptibility to effects of environmental chemicals. The research goals are to understand the importance of human microbiome variation at any given life stage or among specific populations and ultimately to ensure that studies consider such variation adequately and appropriately when assessing the human health risks posed by exposure to environmental chemicals. Variation will be best understood by conducting comparative studies that assess functional similarities and differences of the factors known or hypothesized to affect microbiome diversity. The studies should emphasize populations that represent key windows of potential vulnerability—such as pregnant women, infants, adolescents, and geriatric populations—and resilience, such as healthy adults. As discussed above, existing epidemiology and population studies could be leveraged for this research to obtain results in the near term.

The second aspect of variation that needs to be explored is that between species. One question is whether the differences are so great that effects are being missed or mischaracterized by using animal models to predict human health risk associated with environmental-chemical exposure. Another question is whether the intraspecies uncertainty factors that are used to extrapolate effects in animals to humans account adequately for the microbiome variation. The research program would focus on comparative studies that ultimately could reveal the functional capacity encoded by the human microbiome so that animal species and study designs that are most appropriate for extrapolating to humans could be identified. Specifically, near-term research could focus on identifying functional pathways that are uniquely encoded by microbiomes of select model organisms and humans, on understanding differences and similarities between model-organism and human-host responses to environmental-chemical exposures, and on assessing the redundancy in the microbiomes of various model organisms and humans.

Barriers to Research

To accomplish the research described in the committee's report, tools will need to be developed, and barriers will need to be overcome. Some barriers are specific to the research described, and others are broadly applicable. A few overarching barriers are highlighted below (further details are provided in Chapter 6 of this report).

- *Resources*. Many experiments that the committee describes are likely to require substantial investments of time and resources, are exploratory and thus unlikely to be supported through traditional funding mechanisms, and require multidisciplinary expertise not found within a single laboratory.
- *In vitro model systems*. Despite advances, in vitro model systems that faithfully model, for example, the gut environment have not yet been developed. Current in vitro model systems are unable to incorporate microbial communities that represent naturally occurring microbiomes fully, and researchers do not yet understand how various factors change microbiome gene expression and metabolism and which factors need to be recapitulated in an in vitro system. Furthermore, in vitro systems are not yet able to capture fully all the functional diversity of a microbiome and its interactions with its host.

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- *Standardization*. Lack of standardization in experimental approaches results in an inability to reproduce findings related to chemical–microbiome interactions. Investigators need to control and disclose variables relevant to microbiome assessments, including animal-care procedures and conditions, choices in laboratory reagents, and methods for processing samples and measuring outcomes.
- *Microbial reference communities*. There is no consensus regarding reference strains or microbial communities. Past initiatives have provided data on the composition of microbial communities from healthy adults, but additional microbial reference communities and standardized microbial populations that faithfully recapitulate the variation present in the human microbiome are needed; their development and use will allow comparison of study results among institutions and increase reproducibility of results.
- *Reference information*. The vastness and complexity of the microbiome has resulted in genomic databases that contain scores of unannotated genes about which scientists know almost nothing. Similarly, much in metabolomics databases remains to be annotated and identified, including chemical structure, metabolite source (human vs microbe), and metabolic pathway. Genomic, transcriptomic, and metabolic databases and libraries will need to expand their coverage of relevant strains, genes, enzymes, metabolite identities and function, and associated characteristics of microbiome sources to enable understanding of microbiome dynamics. Large-scale data generation and data-integration efforts will be required to develop computational models that can predict chemical–microbiome interactions and their consequences.

Collaboration

In the United States, several agencies play roles in assessing health risks associated with exposures to environmental pollutants. Similarly, microbiome-related research is being conducted by several agencies and sectors. Progress in fields related to risk assessment and in microbiome research has occurred largely independently, and the segregation of such research programs poses a major barrier to advancing knowledge on interactions between environmental chemicals and the human microbiome and the implications of the interactions for human health risk. Funding mechanisms that promote interdisciplinary research and specifically encourage collaboration are vital for implementing the research strategy detailed in the committee's report.

To support such efforts effectively, agencies and research entities that conduct microbiome and human-health research are encouraged to develop collaborations with their counterparts in risk-assessment fields and vice versa. For example, collaborations between the National Institutes of Health and EPA or state agencies that have a long history of assessing the health risks posed by environmental-chemical exposures are encouraged. That type of interdisciplinary collaboration should be sought out, encouraged, and supported to make the best use of available knowledge and resources in each agency or organization. Likewise, initiatives similar to the Center for Children's Health, the Environment, the Microbiome and Metabolomics at Emory University, jointly funded by EPA and NIEHS, should be considered as vehicles for stimulating and fostering the types of interdisciplinary research needed. The participation of experts in diverse research disciplines during the entire research cycle—planning and designing studies, conducting the experiments, and analyzing the data—is likely to result in studies that are well suited to address the research recommended by the committee. Such interdisciplinary initiatives could also serve as an ideal training environment for the next generation of researchers whose expertise spans several fields.

CONCLUDING REMARKS

Implementation of the committee's proposed research strategy should substantially advance understanding of whether and to what extent the human microbiome affects the nature and magnitude of adverse health effects caused by exposures to environmental chemicals. In the relatively near term (2–4 years), results of the proposed research should allow judgments to be made about whether explicit consideration of microbiome interactions in the study of environmental-chemical toxicity yields information that is not available from traditional studies (ones that do not explicitly consider microbiomes). Within a simi-

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lar time frame, it should also be possible to determine whether new information is gained by studying the effects of chemicals on the human microbiome, the role of the human microbiome in modulating chemical exposures, or both. The research should lead to the type of information needed to assess the importance of the human microbiome as a contributor to human health risks associated with exposures to environmental chemicals and thus permit informed decisions about the need for and nature of continuing research in this field.

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1

Introduction

The human body is host to a great number of diverse microorganisms, and researchers have only recently begun to appreciate the many influences of these microorganisms on human health. Rapidly advancing technologies now allow scientists to investigate the human microbiome—the microorganisms, their genes, and the environmental conditions that surround them—and to elucidate the important roles that it might play in a wide array of diseases, such as diabetes, asthma, and inflammatory bowel disease. Because the human microbiome has been shown to metabolize environmental chemicals and could itself be affected by chemical exposure, some have argued that it should be included as a component in human health risk assessment (Dietert and Silbergeld 2015). The US Environmental Protection Agency (EPA) and the National Institute of Environmental Health Sciences (NIEHS) recognize the possible importance of the human microbiome in human health and the complexity of incorporating interactions between the human microbiome and environmental chemicals into a risk-assessment framework. Given the complexity and resource constraints, EPA and NIEHS asked the National Academies of Sciences, Engineering, and Medicine to develop a research strategy to improve our understanding of the interactions between environmental chemicals and the human microbiome and the implications of those interactions on human health risk. As a result of that request, the National Academies convened the Committee on Advancing Understanding of the Implications of Environmental-Chemical Interactions with the Human Microbiome, which prepared the present report. This chapter briefly discusses the human microbiome and the risk-assessment framework and provides the committee's statement of task, its approach to the task, and the report organization.

THE HUMAN MICROBIOME

Human microbiome is an all-encompassing term that refers to all microorganisms on or in the human body, their genes, and surrounding environmental conditions (see Box 1-2). The microorganisms are found in large numbers on skin and mucosal surfaces and can exist as attached, mixed-species biofilms and as detached, free-swimming cells—two distinct states of microbial life that strongly influence gene expression and microbial activity (Singh et al. 2010). The human microbiome collectively encodes more genes, by several orders of magnitude than the human genome (HMP Consortium 2012a,b; Li et al. 2014). Because of the sheer amount of microbial life that colonizes the human body—the gut microbiota, for example, is composed of several trillion microbial cells—and its vast diversity, human beings are now regarded as ecosystems that are comprised of distinct ecologic niches or habitats, each housing a discrete collection of coevolved bacteria, archaea, viruses, and lower and higher eukaryotes (Oh et al. 2014) that interact extensively with each other and with the human host (Belkaid and Segre 2014).

Coevolution has led to interdependence: the human microbiome contributes a vast array of essential functions to the human host and influences a variety of physiologic, immunologic, and metabolic processes. For example, the gut microbiome ferments dietary complex carbohydrates, and this results in the production of anti-inflammatory short-chain fatty acids that modulate adipose, skeletal, and liver tissue and improve glucose homeostasis (see Figure 1-1; Canfora et al. 2015). In contrast, gut microbial metabolism of L-carnitine produces trimethylamine, which is oxidized in the liver to trimethylamine-*N*-oxide,

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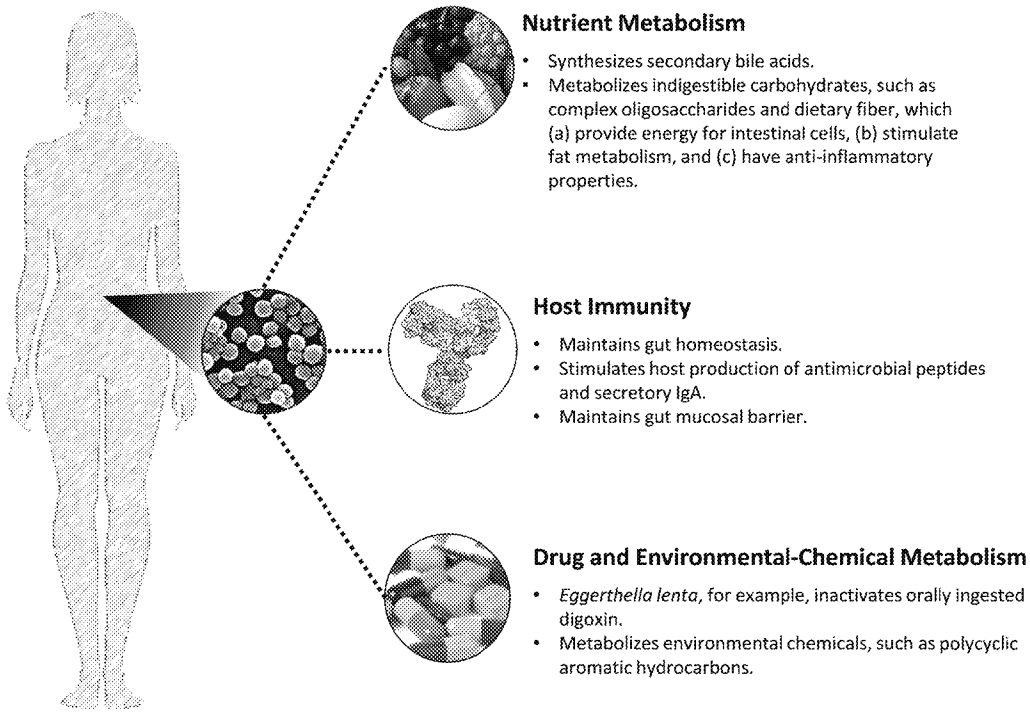

FIGURE 1-1 The gut microbiome plays important roles in human physiology and metabolism and functions as an ecologic niche that has an interface with the environment.

increased concentrations of which promote atherosclerosis (Koeth et al. 2013). The metabolic products of the microbiome, such as those described above, also shape the microenvironment, which exerts a strong selective pressure on microbial colonization. For example, *Lactobacillus* species in the vagina produce lactic acid, which promotes a low vaginal pH and inhibits several vaginal pathogens, including herpes simplex 2 virus (Conti et al. 2009), *Neisseria gonorrhoeae* (Graver and Wade 2011), and uropathogenic *Escherichia coli* (Juárez Tomás et al. 2003). Thus, research is showing that the human microbiome is fundamental in the maintenance of human health, and microbial perturbations are being linked to an ever-increasing array of neurologic, gastrointestinal, metabolic, oncologic, hepatic, cardiovascular, psychologic, respiratory, and autoimmune disorders (Lynch and Pedersen 2016).

Since completion of the first phase of the Human Microbiome Project sponsored by the National Institutes of Health, three basic truths that are generally accepted as important for human biology have emerged, as described below.

- First, the human microbiome has considerable body-site specificity. For example, the oral microbiome is distinct in composition and function from the microbiomes of the distal gut, various skin sites, and the vagina (HMP Consortium 2012a,b). Even within anatomic sites—for example, within the oral cavity or the vagina or along the length of the gastrointestinal tract—there are distinct patterns of microbiota composition. Although there is some consistency in bacterial phyla that inhabit the sites, species or strain variation related to age, geography, genetics, diet, and health status is also present (Lozupone et al. 2012; Greenhalgh et al. 2016).
- Second, perturbations of the composition and function of niche-specific microbial communities are associated with disease, both locally at the site of the perturbation and distally. For example, studies in mice have shown that perturbations of the composition and function of the gut microbiome can lead to neurologic dysfunction characteristic of autism-spectrum disorder (Hsiao et al. 2013), and a perturbed gut microbiome in early life in humans has been associated with asthma development in childhood (Arietta et

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al. 2015; Fujimura et al. 2016). Furthermore, rodent studies have indicated that metabolites derived from gut microorganisms influence precursor immune cells derived from bone marrow (Trompette et al. 2014); these findings support a mechanism by which the gut microbiome might exert a systemic and pervasive effect on host immunity through programming of hematopoietic populations. The research indicates that the composition and activities of at least the gut microbiome have the potential to elicit both local and systemic effects, and this underscores the critical role that it plays in defining host health.

- Third, increasing evidence indicates that the human microbiome expands and diversifies in a niche-specific manner from early life to the senior years, when it loses diversity. The precise timescale over which that occurs is still a matter of much debate; recent reports suggest appreciable functional diversification and microbial niche specialization as early as about 4–6 weeks of life (Chu et al. 2017). That finding implies that exposures before and around conception, during gestation, and throughout early development are likely to have a lasting effect and that those periods are fundamentally important. The senior years are also important when characteristic compositional instability and loss of community diversity correlate with declines in immunocompetence (Claesson et al. 2012).

The early research indicates the important role that the human microbiome might play in human health and raises the question of whether some consideration needs to be incorporated into risk assessment.

RISK ASSESSMENT

The 1970s saw a growing awareness and concern that some environmental chemicals could cause adverse health effects. Government programs were created to protect against harmful exposures, and agencies developed methods for estimating risks posed by chemical exposure. However, controversies arose over the various methods and their results, and Congress asked the National Research Council to evaluate risk-assessment practices. The request resulted in the report *Risk Assessment in the Federal Government: Managing the Process*, which established a framework for risk assessment (NRC 1983). Over the years, many articles and reports have been published on risk assessment, including some from the National Academies, the most recent being *Science and Decisions: Advancing Risk Assessment* (NRC 2009). However, the core elements of risk assessment—hazard identification, dose–response assessment, exposure assessment, and risk characterization—have remained the same.

Animal toxicology studies have traditionally provided the data for hazard identification and dose–response assessment for exposures to environmental chemicals, but epidemiology (human) studies have provided the primary evidence on some chemicals, such as arsenic and formaldehyde. In vitro assays and computational approaches are also being developed in light of scientific and technologic advances in biology and related fields and substantial increases in computational power. The hope is that the new approaches can predict toxicity on the basis of an understanding of the biologic processes that lead to adverse effects.

Regardless of the approaches used to provide data for various risk-assessment elements, none has explicitly considered or incorporated the human microbiome. As noted above, the gut microbiome can affect chemical metabolism, and there is growing evidence that perturbations of the human microbiome can affect health. Those findings lead to many important questions; the answers to which could have profound implications for risk assessment. Are potentially adverse health effects of chemicals that can be transformed by the human microbiome or can directly affect its composition and function being missed or mischaracterized because the human microbiome is not being explicitly considered? Because animals and humans have intact microbiomes, are any adverse effects that would involve the microbiomes already being captured in animal and human studies? If animal and human microbiomes differ substantially, do the differences themselves need to be considered? If a microbiome component needs to be incorporated into a risk-assessment framework, how should that be done? One question leads to another, and the complexity soon becomes clear. EPA and NIEHS recognized the challenges and asked the National Acade-

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mies to develop a research strategy to improve understanding of the interactions between environmental chemicals and the human microbiome and the implications of the interactions for human health risk.

THE COMMITTEE AND ITS TASK

The committee that was convened as a result of the request included experts in microbiology, metabolomics, clinical medicine, exposure science, toxicology, and risk assessment (see Appendix A for the committee's biographic information). As noted, the committee was asked primarily to develop a research strategy but was also asked to identify possible barriers to understanding and to describe opportunities for collaboration. The committee's verbatim statement of task is provided in Box 1-1.

THE COMMITTEE'S APPROACH TO ITS TASK

To accomplish its task, the committee held five meetings, which included two open sessions to hear primarily from sponsor representatives and a few invited speakers on various topics. The committee found, as it began to draft its report, that different people attach different meanings to various terms. To ensure clarity in this report, Box 1-2 contains the committee's definitions of several terms used throughout the report. Regarding the terms *variability* and *variation*, the committee acknowledges that there clearly is overlap of the terms as it defines them. However, the key distinction between the terms is that *variability* is used when one would not expect there to be substantial differences between states or conditions, such as the microbiome compositions of the same body sites of healthy people, and that *variation* is used when one would expect there to be differences between states or conditions, such as the microbiome compositions of different body sites, life stages, or species.

Although not included in Box 1-2, *exposure* and *dose* are used in this report. NRC (2012) noted that *exposure* can be considered as "stressors, receptors, and their contacts in the context of space and time." For the present report, the stressors of primary concern are environmental chemicals, and the receptors in the case of external exposures might be populations, individual humans, laboratory animals, or their microbiomes. In the case of internal exposures, the receptors might be host cells, tissues, organs, or individual microbes. As discussed in Chapter 5, some expansion of exposure-science concepts might be needed to incorporate the possible role of the human microbiome in modulating the health risks associated with exposure to environmental chemicals. Like NASEM (2017), this report uses the term *exposure* primarily but also uses *dose* in conventional phrases, such as dose–response relationship.

BOX 1-1 Statement of Task

An ad hoc committee will develop a research strategy to better understand the interactions between environmental chemicals and human microbiomes, including the intestinal, skin, and lung microbiomes, and the implications of those interactions on human health risk. The committee will assess the state of the science regarding the health implications of chemical metabolism by microbiota and chemical exposure on microbiota diversity and function. It will also assess what is known about how effects might differ depending on, for example, life stage or interindividual differences. The committee will then develop a research strategy that identifies the types of studies needed to improve understanding of how different microbiome communities can affect chemical absorption and metabolism, how population variation in microbiome activity might affect individual chemical exposure, and the effect of chemical exposure on microbiome functions and possible implications for human health risk. The committee will also identify methodological or technological barriers to advancing the field, discuss possible opportunities for coordination or collaboration, and indicate which research investments might provide the most information for improving understanding of microbiome implications for human health risk.

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BOX 1-2 Definitions of Selected Terms

Biomass refers to the quantity of microorganisms as a cell count or density in a given region or sample.

Environmental chemicals are chemicals that have entered the environment as a result of human activity and are subject to regulation (see Chapter 6 for further discussion).

Ex vivo refers to an experimental process that is carried out by removing biologic specimens or materials—such as primary cells, tissues, or organs—from an organism and using them directly in an artificial setting.

Gnotobiotic animal describes an animal maintained in the absence of any microorganisms (that is, germ-free conditions) or a germ-free animal that is colonized with a microbial strain or a defined multi-species community of microbes.

In vitro refers to an experimental process that is carried out in an artificial setting by using biologic specimens or materials that have not been directly isolated from an organism—such as immortalized cell lines, laboratory microbial strains, or purified proteins—or by using microbial communities outside their typical setting or by using any combination of those components.

In vivo refers to an experimental process that is conducted in a whole organism, such as a rodent or primate.

Metabolomics: The scientific study of small molecules (metabolites) that are created from chemicals that originate inside the body (endogenously) or outside the body (exogenously) (NASEM 2016).

Metagenome refers to all genomes or genes encoded by a microbiota.

Microbiome refers to "the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes and viruses), their genome (i.e., genes), and surrounding environmental conditions" (Marchesi and Ravel 2015). The term *microbiome* is often used in conjunction with a specific body site—such as the gut, skin, or respiratory microbiome—or as an all-encompassing term to refer to all microbiomes on or in the human body, that is, the human microbiome.

Microbiota or microbial community is a collection of microorganisms in a habitat.

Resilience is the ability of a microbial community to maintain or return to a steady state in the presence of or after some stress on or perturbation of its composition or function.

Variability refers to a measurable distribution of a state or condition that would typically be considered nominally homogeneous. For example, differences in the function or composition of the gut microbiome in a population of healthy adults would be described as variability.

Variation refers to differences or patterns of change between in two or more conditions or states. For example, differences between species, life stages, or body niches would be described as variation.

Several points should be noted regarding the focus of the present report. First, this report is not a comprehensive review of all microbiome research and is focused on answering the questions set forth in the committee's task. Accordingly, the research strategy that the committee proposes is directed at addressing questions about the interaction of the human microbiome with environmental chemicals and the implication of the interactions for human health risk. It is not a research strategy for directly investigating associations between the human microbiome and various diseases. Second, the statement of task asks for a research strategy to improve understanding of "how population variation in microbiome activity might affect individual chemical exposure." To address that point, the committee has focused on understanding how exposure is modulated by the microbiome and how variation in microbiome activity affects chemical–microbiome interactions or human health risk, which is referred to explicitly in the opening statement of the committee's task and is seen as the ultimate goal of the overall research strategy. Third, although the committee acknowledges that some interactions of environmental chemicals and the human microbiome might be beneficial, the primary focus of the present report is on the potential for adverse effects of such interactions because that is the traditional focus of risk assessment. Fourth, the committee acknowl-

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edges that the report appears to focus on the gut microbiome and the bacterial components of the human microbiome, but that focus reflects the current state of the science and the sparseness of the literature on other body-site microbiomes and on the viral and fungal components of the human microbiome.

ORGANIZATION OF THE REPORT

The committee's report is organized into six chapters and one appendix. Chapter 2 further describes the human microbiome and focuses on its variation and variability. Chapter 3 explores how the human microbiome can affect chemical exposure. Chapter 4 discusses methods for studying the human microbiome, and Chapter 5 continues the discussion of risk assessment and the impetus to include a human-microbiome component. Chapter 6 presents the committee's research strategy and discusses possible obstacles to the research and opportunities for collaboration. Appendix A provides biographic information on the committee members.

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2

Microbiome Variation

Traditional notions in which microorganisms were viewed solely as pathogens or etiologic agents of acute infectious diseases have been challenged. Today, scientists recognize that such a "foe" view neglects the growing evidence that many microorganisms reside in a symbiotic and likely mutually beneficial relationship with the host. The Human Microbiome Project, which characterized the microbial composition of multiple body sites in healthy people of different ethnicities in two cities (St. Louis, MO, and Houston, TX) in the United States (HMP Consortium 2012a,b; Aagaard et al. 2013), has established a body of information that has sparked numerous investigations to understand the link between human health and disease. Research now suggests associations between microbial perturbations and such diseases as obesity, type II diabetes mellitus, ulcerative colitis, Crohn's disease, and colorectal cancer (Mangin et al. 2004; Ley et al. 2005; Gophna et al. 2006; Manichanh et al. 2006; Turnbaugh et al. 2006, 2008, 2009; Bäckhed et al. 2007; Cani et al. 2007; Willing et al. 2009; Larsen et al. 2010; Schwiertz et al. 2010; Wu et al. 2010; Joossens et al. 2011; Lepage et al. 2011; Marchesi et al. 2011; Sobhani et al. 2011; Qin et al. 2012; Wang et al. 2012; Devaraj et al. 2013). Although associations have been reported, causal relationships have yet to be fully established in most cases, and environmental and host modifiers need to be defined. Furthermore, questions remain concerning when in the lifespan the host–microbial interactions that lead to various health or disease states are first established and to what extent they can be modified.

The Human Microbiome Project also demonstrated greater bacterial diversity and body-site specificity than expected on the basis of previous studies. Specifically, microbial community composition and function have been found to vary substantially over the human life span (Palmer et al. 2007; Aagaard et al. 2012; Yatsunenko et al. 2012; Bäckhed et al. 2015; Hollister et al. 2015) and to exhibit extensive body-site specificity with remarkable variation between sites but considerably less interindividual variation within given sites (Turnbaugh et al. 2007; Costello et al. 2009; Grice et al. 2009; HMP Consortium 2012a,b; Aagaard et al. 2013; Franzosa et al. 2015; Voigt et al. 2015). It is the variation in the human microbiome that is the primary focus of this chapter. General factors that contribute to variation in the human microbiome are discussed first and then variation specifically in the gut, skin, and respiratory microbiomes. The chapter concludes with a discussion of variation between human and animal microbiomes and implications for using animal models to study the human microbiome. It is important to note that the variation that is typically studied and is the focus of this report is not due to day-to-day fluctuations but actually measurable perturbations that are independent of circadian rhythm. Furthermore, some examples of variation or changes in the human microbiome that affect function or health are provided here, but a comprehensive review of the literature on the human microbiome and health and disease states is beyond the scope of this report.

FACTORS THAT CONTRIBUTE TO VARIATION IN THE HUMAN MICROBIOME

Population-based studies have identified multiple factors that relate to the observed variation in the composition, gene content, and function of the human microbiome. They include body site (Grice and Segre 2011; HMP Consortium 2012a,b; Costello et al. 2013), age (Yatsunenko et al. 2012), environmental exposures (chemical and microbiologic), disease state (Huang et al. 2015; Mar et al. 2016), genetics

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(Goodrich et al. 2014; Ma et al. 2014a; Imhann et al. in press), sex (Aagaard et al. 2013; Markle et al. 2013), socioeconomic status (Levin et al. 2016), geography (Yatsunenko et al. 2012), pregnancy status (Aagaard et al. 2012; Koren et al. 2012), and diet (Yatsunenko et al. 2012; David et al. 2014b).

Body site is a key factor in the variation observed in the human microbiome. Different body sites contain microbiomes that differ in microbial composition and function. As a result, each body site can be characterized by specific bacterial species and other microorganisms that have adapted to the site's environment, and the differences in microbial composition yield differences in metabolic capacity and in aggregate function of the human microbiome. Specifically, no bacterial taxon has been found to be present in all body sites, and a given taxon might be absent from a specific body site in one person but dominate corresponding microbial communities in another person (Zoetendal et al. 2012). At higher levels of microbial classification, however, individual body sites do exhibit characteristic phylum-level distributions. Such sites as the skin, respiratory tract, and reproductive system that have a low biomass exhibit fewer taxa but often have microbial communities with diverse functions (Grice et al. 2009; Yatsunenko et al. 2012; Aagaard et al. 2014; David et al. 2014a; Ordiz et al. 2015). In light of the substantial variation in microbial composition and function between body sites, the following discussion of the human microbiome is organized according to body site.

THE GUT MICROBIOME

The greatest microbial biomass in humans is in the gastrointestinal tract (the gut). Colonization of the gut by microorganisms probably begins in utero, although the exact timing of colonization during development is not known (Jiménez et al. 2005; Steel et al. 2005; Dominguez-Bello et al. 2010; Gerritsen et al. 2011; Rautava et al. 2012; Aagaard et al. 2014; Ma et al. 2014a; Collado et al. 2016; Fallani 2016; Gibson et al. 2016; Yassour et al. 2016; Chu et al. 2017). As a result, the neonatal gut is not sterile but rather harbors pioneer species in a somewhat simplified community that expands by the age of 4–6 weeks (Chu et al. 2017). Early influential factors in gut microbiome development include gestational age at delivery (Gibson et al. 2016), infant feeding patterns (Graham-Rowe 2011; Walker et al. 2011), maternal dietary fat intake throughout gestation and lactation (Ma et al. 2014a; Chu et al. 2016), antibiotic use (Dethlefsen et al. 2008), and environmental exposures (MacFarlane and Cummings 1999; Roager et al. 2014).

Data on the effect of mode of delivery on neonatal and infant microbiome composition and function are mixed. Although initial small studies suggest a link between vaginal (vs cesarean) birth and neonatal gut microbiota (Dominguez-Bello et al. 2010), longer-term longitudinal studies conflict as to whether the robustness of the association holds true throughout infancy (Bäckhed et al. 2015; Yassour et al. 2016; Chu et al. 2017; Levin et al. 2016). Several studies have suggested that the underlying medical indication for a cesarean might be more influential than the cesarean surgery itself (Azad et al. 2013; Chu et al, 2016, 2017). In recognition of that suggestion, the American College of Obstetricians and Gynecologists (ACOG 2017) recently released an opinion that stated that "much of the research that exists regarding the link between cesarean delivery and incidence of allergies and autoimmune diseases has found an association with mode of delivery. However, there is still not enough evidence to prove causation due to the environmental, dietary and genetic factors that also impact the development of conditions, such as asthma. Additionally, the microbiota of infants born by C-section will cause a variation in the research findings because some may have had contact with maternal vaginal bacteria if the procedure was performed after the onset of labor or rupture of membranes."

Soon after birth, the neonatal gastrointestinal tract is exposed first to colostrum and then to breast milk, formula, or both; these exposures result in the development of microbial communities (Harmsen et al. 2000; Morelli 2008; Biesbroek et al. 2014). Over the first year of life, bacterial taxonomic diversity in the gut expands in parallel with contraction of fungal diversity in healthy infants (Fujimura et al. 2016). Several studies have indicated that at the age of about 3 years the phylum-level distribution of bacteria in

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the gut resembles that of adults (Palmer et al. 2007; Yatsunenko et al. 2012), but interindividual differences are substantial at lower taxonomic levels (Armugam et al. 2011). In addition to microbial composition, the functional attributes of the gut microbiome in infants, children, and adolescents differ substantially from those of adults (Lynch and Pedersen 2016). For example, infants, children, and adolescent gut microbiomes are richer than adult gut microbiomes in microbial pathways involved in microbial folate biosynthesis (Hollister et al. 2015). The compositional and functional differences indicate that the microbiome adapts as the human host develops and ages (see Figure 2-1).

Compared with the period of dynamic gut microbiome development in early life, healthy adults exhibit relatively stable gut microbiota composition and metagenomic content (Yatsunenko et al. 2012). Factors known to be associated with variation in the community and structure of the gut microbiome include body site, age, environmental exposures, health status, genetics, socioeconomic status, geography, pregnancy, and diet. In addition, such other factors as exercise (O'Sullivan et al. 2015), antibiotic use (Dethlefsen and Relman 2011), and surgical interventions (Tremaroli et al. 2015) also play a role in shaping the gut microbiome. Of those factors, diet exerts a key and modifiable influence on the gut microbiome (Penders et al. 2006; Wu et al. 2011); both long-term and short-term eating habits have been shown to alter the microbiota of healthy adults (David et al. 2014b).

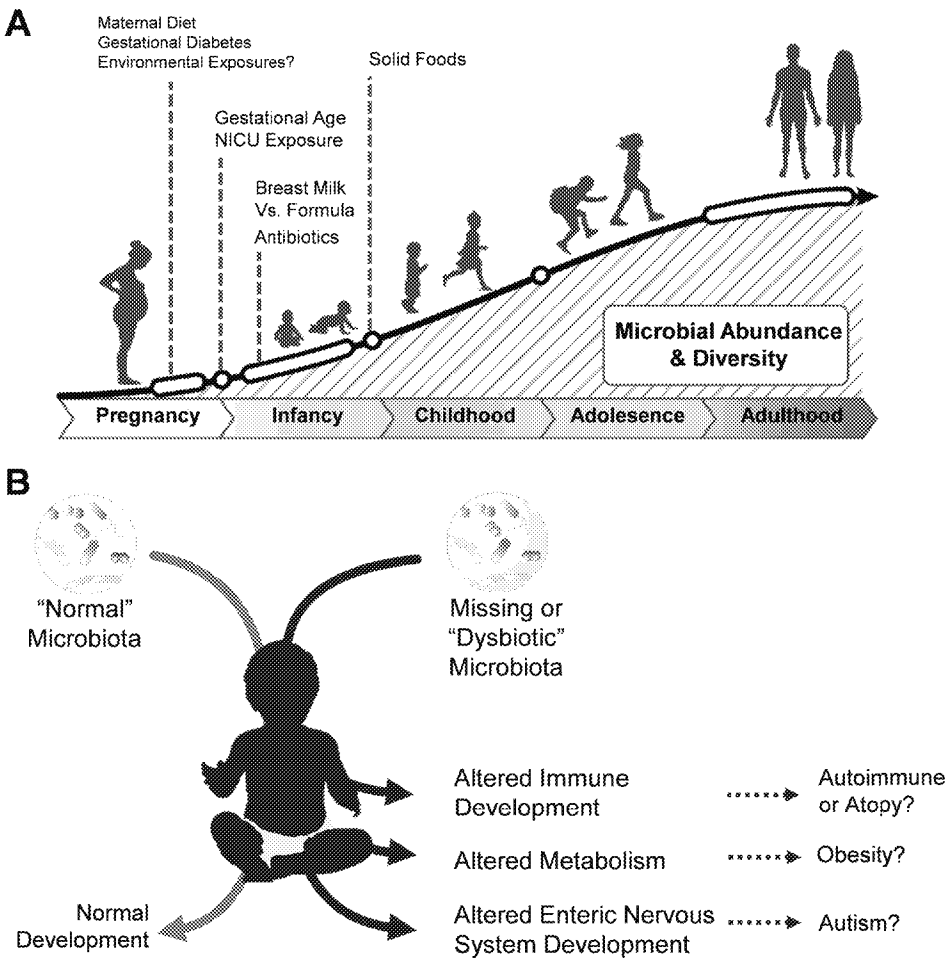

FIGURE 2-1 (A) Gut microbiome development in infancy is influenced by early-life events. (B) Acquisition of microbiota in early life is thought to shape infant development.

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Dietary inputs provide nutritional substrates for the gut microbiota (Krajmalnik-Brown et al. 2012) and can also be a source of live microorganisms (David et al. 2014a,b). The amounts and composition of carbohydrates, proteins, and fats affect gut microbiota composition. Although all are important, resistant carbohydrate polymers—dietary fiber that is resistant to degradation by human enzymes—have been more widely accepted as an important microbiota-determining factor (Walker et al. 2011; David et al. 2014a). For example, De Filippo et al. (2010) showed that gut microbiota composition varied with dietary fiber consumption in children in Italian and African populations. Specifically, the high-fiber plant polysaccharide-based diet consumed by children who lived in Burkina Faso correlated with a greater diversity and relative abundance of cellulose-degrading *Prevotella* and *Xylanibacter*. In contrast, lower-fiber diets of children raised in Florence, Italy, were associated with lower abundances of *Prevotella* and increases in opportunistic gastrointestinal *Enterobacteria* (*Shigella* and *Escherichia*), which resulted in an increased capacity for simple-sugar uptake and metabolism. In a separate study, increased relative abundance of *Prevotella* species was also observed after a 10-day low-fat–high-fiber dietary intervention (Wu et al. 2011); a high-fat–low-fiber diet was correlated with increased relative abundance of *Bacteroides*. In addition to differences in composition, functional attributes of the gut microbiome are strongly influenced by diet, as evidenced by the enrichment of gut microbial genes that encode α-amylase (responsible for degradation of plant polysaccharides) in Malawian populations, whose primary dietary starch is maize, and the comparative enrichment of bacterial α-L-fucosidase in age-matched populations in the United States, where dietary simple sugars are abundant (Yatsunenko et al. 2012).

Rapid diet-related changes in gut microbiota have been detected after long-term and short-term intervention studies of healthy adults (Jumpertz et al. 2011; David et al. 2014b; Zeevi et al. 2015). Although changes in fiber intake constitute one of the most important factors in daily microbiota fluctuations, fat consumption and protein consumption are also related to microbiota composition (David et al. 2014a). High-fat diets increase the concentration of bile acids delivered to the colon; a shift from a high-carbohydrate to a high-fat diet increased the abundance of bile-tolerant *Alistipes, Bilophila,* and *Bacteroides;* increased excretion of fecal short-chain fatty acids; and reduced the abundance of plant-polysaccharide degraders, such as *Roseburia, Eubacterium rectale,* and *Ruminococcus bromii* (Fava et al. 2013). Calorie content also influences the gut microbiome (Jumpertz et al. 2011); an increase by 1,000 kcal/day in caloric intake was associated with an increase in the relative abundance of *Firmicutes* and increased host-energy extraction (Jumpertz et al. 2011), a feature also associated with the gut microbiome of obese people (Ley et al. 2006; Turnbaugh et al. 2006).

Short-term and long-term dietary intervention studies have focused exclusively on healthy populations; however, in more heterogeneous and presumably more representative human populations, people exhibit distinct metabolic responses to identical meals (Zeevi et al. 2015). Using a machine-learning algorithm applied to blood glucose concentrations, dietary habits, and gut microbiome data, Zeevi et al. (2015) could predict a person's postprandial glycemic response to specific meals. Their results indicate that the metabolic fate of dietary components is tightly linked to the activities of the gut microbiome. It has been demonstrated that chemicals ingested via the diet exert an effect on the gut microbiota and that consumption of artificial sweeteners alters microbiota composition and can lead to insulin resistance (Suez et al. 2014).

Substantial differences in microbiome composition and function have been described among human populations distinguished by geography or ethnicity (De Filippo et al. 2010; Yatsunenko et al. 2012; Ma et al. 2014b). The differences have been attributed largely to distinct diets because diet clearly exerts a major selective pressure on the gut microbiome, as discussed above. However, confounding factors, such as host genetics and environmental microbial exposures, might also play a substantial role in the gut microbiome differences observed among populations of geographically segregated humans. Indeed, mounting evidence indicates that environmental microbiota exposures through residential house dust in early life (up to the age of 3 months) are related to development of or protection against allergy and asthma outcomes at the age of 3 or 7 years (Fujimura et al. 2010; Lynch et al. 2014; O'Connor et al. in press).

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Moreover, evidence from mouse models indicates that exposure to such microbiologically distinct residential house dusts differentially shapes gut microbiota composition and function in a manner that promotes or prevents development of protective airway and hematopoietic immune function after allergen or viral respiratory insult (Fujimura et al. 2014; Fonseca et al. 2017). For example, increased prevalence of allergic asthma in Hutterite children in the United States is associated with reduced exposure to environmental microorganisms in house dust, and nasal exposure of mice to house dust from Hutterite homes promoted proallergic responses after airway sensitization (Stein et al. 2016). The studies offer a plausible mechanism by which environmental microbial exposures in early life contribute to or protect against childhood disease development. Hence, the combination of environmental exposures and dietary selective pressure, particularly during the early-life period of microbiome development, appears to play a key role in determining how a person responds to later environmental exposures.

THE SKIN MICROBIOME

The skin makes up a vast and variable ecosystem that is comprised of about 1.8 m^2 of discrete habitats that are both physically and chemically distinct (Grice and Segre 2011) and form a physical interface with the external environment. Using 10 healthy adult subjects, Grice et al. (2009) demonstrated that although microbial biomass is typically low on the skin surface, relatively reproducible patterns of bacterial and fungal microbial colonization are apparent at specific body sites. The site-specific community states are driven primarily by local ecologic conditions, particularly water availability and nutrition, which are relatively consistent in healthy humans at specific body sites but vary widely between body sites. Grice et al. (2009) also examined temporal stability of the skin microbiome by performing a repeat sampling of five subjects 4–6 months after the initial sampling. Microbiota composition was relatively stable at some sites—such as those associated with the ear canal, groin, and nose—but varied at others, including the armpit, forearm, and buttock. Those findings suggest that skin-associated microbiota compositional stability is site-specific.

On the healthy human skin surface, sites with high water availability are typically enriched in members of the *Staphylococcus* and *Corynebacterium* genera (Costello et al. 2009; Grice and Segre 2011), and sebaceous sites are selectively enriched in *Propionibacterium* (Leeming et al. 1984). The site-specific selective enrichment in *Propionibacterium* is due largely to its multiple encoded lipases that catalyze degradation of sebaceous lipids and provide this genus with a competitive colonization advantage. Degradation of sebaceous lipids produces free fatty acids (Marples et al. 1971), which both lower the microenvironmental pH (Elias 2007) and inhibit the growth of potentially pathogenic species, including *Staphylococcus aureus* and *Streptococcus pyogenes*, while promoting the growth of coagulase-negative species, such as some members of the *Corynebacterium* and *Staphylococcus* genera (Korting et al. 1990). Studies of fungal species that colonize the skin surface are less common than bacterial investigations, but the studies that have been performed indicate that *Malassezia* forms a large portion (53–80%) of the skin-associated fungal biomass; variations in relative abundance depend on skin site (Gao et al. 2010).

A more recent metagenomic study of 18 skin sites in 15 healthy adults revealed that only about 30% of microbial functional gene content, primarily encoding processes essential to microbial growth and metabolism, was conserved (maintained) across body sites (Oh et al. 2014). The remaining functional gene content exhibited substantial variation between skin sites. Microbial metabolic diversity was lowest in sebaceous sites, which coincidentally exhibited lower taxonomic diversity than nonsebaceous skin sites (Oh et al. 2014). However, microbial communities in sebaceous sites exhibited a preponderance of fungal pathways, including those involved in the cell cycle, DNA replication, transcription, translation, protein degradation, and fungus-encoded vitamin D2 biosynthetic genes. Microbial communities in nonsebaceous sites exhibited increased capacity for sulfate, glutamate, aspartame, L- or branched amino acids, and sorbitol transport and putrescine or spermidine biosynthesis and transport. The results indicate that there is great chemical diversity, which the microorganisms in those anatomic niches use to their advantage.

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Although most studies have examined topographic variation in the skin microbiome in healthy populations, studies that have examined site-specific dermal microbiomes in healthy and diseased states have demonstrated that disease is associated with perturbations of the composition of the microbiota and its metagenome—findings consistent with those in other anatomic sites (Barnard et al. 2016). Thus, although the prevailing conditions at a given skin habitat influence the microbial colonization pattern and the functional genetic capacity of the communities in a relatively predictable manner, individual skin-associated microbial signatures have a remarkable range. That observation suggests that the microbial potential to transform or sequester dermal environmental chemical exposures depends on the body site, the individual, and the individual's health status.

Age, sex, and geography are also associated with skin microbiota heterogeneity. Microbial colonization of the skin is thought to begin during the perinatal and postnatal period. Costello et al. (2013) demonstrated in a small cohort of premature neonates that were sampled repeatedly over the first 3 weeks of life that of all the sites sampled (skin, saliva, and stool), the skin microbiota most resembled that of an adult. More recently, a study of mother–infant pairs demonstrated that the skin microbiota, although similar to the oral and fecal microbiota at birth, exhibits site-specific differentiation as early as the age of about 4–6 weeks (Chu et al. 2017). Culture-based studies have demonstrated that puberty-associated alterations in sebum production correspond with the quantity of skin-associated lipophilic bacteria (Somerville 1969). Moreover, sex-based differences in skin microbiota have been described and are associated with physiologic and anatomic differences in sweat, sebum, and hormone production that occur in males and females, particularly during puberty (Marples 1982; Fierer et al. 2008; Giacomoni et al. 2009). Exogenous factors that influence the skin microbiota include prevailing temperature and humidity, increases in which are associated with increased bacterial numbers on the underarms, back, and feet (McBride et al. 1977). Conversely, exposure to ultraviolet (UV) radiation is bactericidal; thus, gradients of UV exposure associated with longitude or latitude are thought to contribute to geographic variation in skin microbiota, although a large number of confounding factors co-vary with UV exposure in spatially separated geographic locales.

THE RESPIRATORY MICROBIOME

Research on the respiratory microbiome, particularly in the lungs, is still relatively sparse compared with investigation of the gut microbiome. In fact, knowledge regarding site-specific microbiota composition in both the upper respiratory tract and lower respiratory tract has increased rapidly in the last 5 years.[1] Invasive sampling of the lower airways for research studies is difficult to justify in otherwise healthy infants and children, so nasopharyngeal sampling that uses swabs, aspirates, or brushings has been pursued. Studies of healthy children reveal significant changes in nasopharyngeal bacterial composition related to age and delivery method (Biesbroek et al. 2014; Bosch et al. 2016); given age-related variation, age is an important factor to consider in the design of cross-sectional respiratory microbiome studies. The focus, however, has been largely on early life (such as up to 24 months), and the dynamics of the nasopharyngeal microbiota in healthy older children and adults are underexplored.

Recent studies that used bronchoscopy to sample the lower respiratory tract or lungs in healthy people and that used culture-independent analyses have consistently demonstrated the presence of a microbial community (Dickson et al. 2014, 2015, 2017; Bassis et al. 2015). However, the overall dynamics of community stability in the lower airways of healthy people without evident lung disease remains a matter of debate. Numerous physiologic and anatomic factors play a role in determining the composition of the respiratory microbiota and its regional variation in the respiratory tract (Dickson et al. 2014). Factors include differences in oxygen tension, airway luminal temperature, mucociliary clearance mechanisms, and other innate defenses. Moreover, microaspiration of upper airway and oropharyngeal secretions is

[1]As in clinical practice, the upper respiratory tract and lower respiratory tract are distinguished here by partitioning relative to the epiglottis.

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common and often asymptomatic even in healthy persons and likely leads to microbial colonization of the respiratory tract (Huxley et al. 1978).

Given the much lower microbial biomass found in the lungs than in the oropharynx or intestinal tract, it is important to emphasize that substantial attention must be paid to study design, sample collection, processing protocols, and collection of reagent controls in analyzing and interpreting findings. In light of those considerations, researchers have conducted detailed topographic study of the microbiota along the tracheobronchial tree and demonstrated microanatomic variability (Bassis et al. 2015; Dickson et al. 2017). Bacterial load and ecologic measures of mouth–lung similarity peak at or near the tracheal bifurcation, and this finding supports the hypothesis that in healthy persons microaspiration is the most likely route by which the lower respiratory tract receives and becomes colonized by bacteria (Dickson et al. 2017). However, studies on bacterial burden, community diversity, and mouth–lung similarities can yield different results that depend on the sampling methods used; the varied results reflect differences in the sampled surface areas, regardless of health or disease status and differences between the lower and upper respiratory tract (Denner et al. 2016; Perez-Losada et al. 2016; Dickson et al. 2017). The collective findings in healthy people highlight some of the intrinsic factors in the variability in data from studies of the respiratory microbiome that must be considered in developing study protocols and designs.

Emerging evidence suggests that bacteria commonly found as part of the "normal" lung microbiome might shape immune responses in the lung. For example, two recent studies suggest that a lung bacterial community that is enriched primarily in members of the *Veillonella* and *Prevotella* genera (supraglottic bacteria often found in the oropharynx) is associated with lung inflammation, as manifested by increased lymphocytes and neutrophils in bronchoalveolar-lavage fluid. Moreover, it is striking and somewhat counterintuitive that studies have not shown associations between cigarette-smoking history or smoking cessation and alterations in lower-airway bacterial microbiota composition; instead, changes are associated with the oral microbiota composition (Morris et al. 2013; Einarsson et al. 2016; Munck et al. 2016; Segal et al. 2016).

The composition of the microbiome in the lower respiratory tract of people who have chronic airway disease clearly differs from that of healthy people. Most studies have focused on patients who have cystic fibrosis, chronic obstructive pulmonary disease (COPD), or asthma. Those people all have impaired or dysregulated immune responses that might magnify the microbial perturbations observed in studies that examine the respiratory microbiome in healthy and diseased people. Intrinsic airway defenses—such as mucociliary clearance, epithelial barrier function, and innate immune functions, including the secretion of antimicrobial peptides—all work to mitigate potentially detrimental inhaled exposures. Those mechanisms become impaired in chronic airway disease to various extents. It is also well recognized that chronic airway diseases are clinically heterogeneous. Indeed, differences in underlying immune-response profiles and molecular phenotypes distinguish some presentations of asthma and COPD. Thus, it is likely that complex interactions among a variety of factors—including environmental exposures, genetic risk, and immune phenotype—shape airway-disease susceptibility and clinical manifestations and prognosis (Han et al. 2010; Huang and Boushey 2015; Huang et al. 2017).

Adding to that knowledge base, recent studies of the respiratory microbiome in cohorts of airway-disease patients have revealed important interindividual heterogeneity in microbiota composition in the upper respiratory tract and the lower respiratory tract (Cox et al 2010; Bogaert et al. 2011; Erb-Downward et al. 2011; Biesbroek et al. 2014; Zhao et al. 2014; Huang et al. 2015; Einarsson et al. 2016; Durack et al. 2017). Moreover, significant associations between clinical features of host disease and patterns of microbiota composition and predicted microbial functions have been shown and suggest potential mechanistic links. Lower-airway enrichment in members of the *Proteobacteria* phylum, in particular, has consistently been associated with chronic airway disease and with clinical outcomes in COPD or asthma. Those relationships between the microbiome and disease phenotype include microbiota enrichment patterns linked to worse lung function, airway reactivity, and symptom control, and to different airway immune-response profiles, including type 2 and nontype 2 inflammatory responses (Huang et al. 2011, 2015; Denner et al. 2016; Wang et al. 2016; Durack et al. 2017). Moreover, different medications can have profoundly different effects on the airway microbiome, as has been reported with antibiotics and corticosteroid administra-

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tion (Huang et al. 2014; Wang et al. 2016; Durack et al. 2017). In addition, the bronchial microbiome of asthmatic people who did not respond to a trial of inhaled corticosteroid therapy was enriched in predicted microbial pathways involved in chemical metabolism, and this finding suggests that the presence of some airway microorganisms could influence biotransformation of synthetic therapeutic drugs (Durack et al. 2017). The recent insights from studies of patients who have chronic respiratory diseases highlight the importance of understanding microorganism–host interactions in well-characterized clinical contexts because both the microbiome and the host phenotype can vary greatly.

VARIATION BETWEEN HUMAN AND ANIMAL MICROBIOMES

Animal models have long been a mainstay of experimental biology because of their intrinsic similarities to humans in anatomy, physiology, and genetics. They also provide genetically and microbiologically manipulable systems for studies that are untenable in humans. Most host–microbiome studies in animal models have been performed in mice. Their relatively short reproductive and life cycles make them an economical option for study of microbiome perturbations in a controlled experimental setup that allows the assessment of causality. A large number of mouse-based microbiome studies have contributed invaluable information on host–microbiome interactions. However, translation of results of microbiome studies from mouse to human systems can be difficult because of differences among mammalian species. For example, mouse and human skin surfaces clearly differ substantially. A recent genomewide transcriptomic study of skin-specific expression of human or mouse genes identified only a 30% overlap, which the authors offered as an explanation of why results generated with skin-associated mouse models fail to translate to humans (Gerber et al. 2014). Likewise, although the mouse gut and the human gut have similarities—for example, the ratio of mucosal to body-surface area is similar among species (Casteleyn et al. 2010)—distinct sections of the gut have substantial differences in this ratio. There are other differences in human and mouse gut anatomy: the mouse gut lacks an appendix and has a nonglandular foregut and glandular stomach, taller villi, fermentative metabolism in the cecum, a smooth colon with no divisions, paneth cells only in the small intestine, and abundant goblet cells in the proximal colon (Casteleyn et al. 2010). In addition to spatial differences between the mouse gut and the human gut in the distribution of antimicrobial-producing paneth and mucin-secreting goblet cells, mice encode additional toll-like receptors (proteins on cell surfaces that sense and respond to microorganisms). Mouse immune development is also distinct from that of humans; for example, the CD4+ population in mice develops in the postnatal period (Landreth 2002), whereas human CD4+ populations begin to mature in utero (Zlotoff et al. 2008). Other established immunologic distinctions between humans and mice include the relative ratio of leukocytes (humans have relatively more neutrophils and fewer lymphocytes than mice); the types of antimicrobial defensins (humans express only two intestinal defensins whereas mice express more than 20); the induction of nitric oxide synthase, which is inconsistently induced from human macrophages but reproducibly induced by IFN-γ and LPS in mouse macrophages; and differences in signaling molecules and B-cell and T-cell development and regulation (Mestas and Hughes 2004).

Not surprisingly, the anatomic and immunologic distinctions are associated with important differences in the composition of the mouse and human microbiomes. Using 16S rRNA profiling, Frorath et al. (1991) found that about 85% of bacterial genera that were detected in the mouse gastrointestinal tract were not detected in humans. However, using a higher-resolution approach and 32 gut samples from 16 human subjects and 88 samples from three mouse strains, Krych et al. (2013) found that 89% (80 of 89) of the prevalent bacterial genera were present in both humans and mice. A more recent meta-analysis of mouse gut and human gut microbiotas identified 79 genera that were detected in both, but the study revealed that the relative abundances of many of the dominant organisms were distinct (Nguyen et al. 2015). Alterations in the relative quantities of distinct species affect microbial interspecies interactions, which rely on quorum signaling—the process of sensing and responding to concentrations of microbial-derived chemical signals that allow species to determine the burden and activities of the species in their immediate environment and alter their gene expression accordingly (Papenfort and Bassler 2016). Such

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compositional alterations in both the species and their relative distribution in microbial communities could have important effects on the functional output of the microbiome. It should be noted that no study has examined overlap in fungal or viral population among humans and mammalian model systems, nor have the functional attributes of these communities been assessed. Furthermore, because previous studies have compared only the taxonomic composition of mouse and human microbiomes, functional attributes of the microbial communities might be more similar than their taxonomic composition would suggest. Nonetheless, given the breadth of microbial diversity known to exist in humans and the differences between human and mouse models, observations made in mice, although informative and foundational, might not capture the full breadth of microbial interactions that exist in situ in the human host.

To overcome the issues of microbial differences between mice and humans, several studies have used "humanized" mice—previously germ-free mice that have been inoculated with microbial species found in human stool (Chung et al. 2012; Smith et al. 2012; Ridaura et al. 2013). Personalized culture collections have been valuable in the experimental approach in validating the results of human-to-mouse fecal transfers and in providing a platform to determine which components are important (Ridaura et al. 2013). The studies also have been instrumental in, for example, understanding gut microbial responses to dietary changes. However, humanized mice, like bioreactor systems, do not fully recapitulate the microbial diversity of the human gut microbiome (Auchtung et al. 2015; Griffin et al. 2017). Therefore, although humanized model systems might be useful in promoting a fundamental understanding of causality or microbial dynamics in response to perturbation, the relevance of such mouse models to microbiome responses in humans must be interpreted cautiously.

FINDINGS

- Population-based studies have identified multiple factors related to the observed life span and body-site variation in the composition, gene content, and function of the human microbiome. The factors include age, environmental exposures, disease state, genetics, sex, socioeconomic status, geography, pregnancy status, and diet.
- Body site is a key factor in the variation observed in the human microbiome, and each body site can be characterized by specific bacterial species and other microorganisms that have adapted to the specific environment. The site-specific differences in microbial composition yield differences in metabolic capacity and in aggregate function of the human microbiome.
- Age and diet play primary roles in the variation observed in the gut microbiome. However, the combination of environmental exposures and dietary selective pressure, particularly during the early-life period of gut microbiome development, might play a key role in determining how a person responds to later environmental exposures.
- The site-specific community states in the skin microbiome are driven primarily by local ecologic conditions, particularly water and nutrient availability, which are relatively consistent in healthy humans at a specific body site but vary widely between body sites. However, skin-associated microbiota compositional stability appears to be site-specific.
- Numerous physiologic and anatomic factors play a role in determining the composition of the respiratory microbiota and its regional variation within the respiratory tract. This field of study is relatively new, but research indicates that important factors include differences in oxygen tension, airway luminal temperature, mucociliary clearance mechanisms, and other innate defenses.
- Mice and other animal models have been useful in studying host–microbiome interactions, including "humanized" mice that allow researchers to test the effects of a specific human microbiome on host biology. However, differences between humans and mice in anatomy, immunology, and microbiome composition can present challenges for translating results between these hosts. Observations made in mice and other animal models, although informative and foundational, might not capture the full breadth of microbial interactions that occur in human hosts.

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3

Characterizing Interactions Between the Human Microbiome and Environmental Chemicals

Scientific research is beginning to illuminate the various ways in which the human microbiome can interact with environmental chemicals. As discussed earlier, multiple studies suggest that exposure to environmental chemicals can alter microbial composition and potentially affect function. Research has also indicated that the human microbiome can modulate exposure to environmental chemicals. The idea that microbiota in and on the host can contribute to host metabolism is deeply rooted in the field of drug metabolism. Early observations regarding the fate of the antibacterial prodrug[1] Prontosil cemented the need to improve our understanding of how microorganisms metabolize chemicals and how these processes might affect the host, favorably or unfavorably (Spink et al. 1940). The concept of the microbiota and its contribution to host metabolism was further strengthened by the father of modern drug metabolism, R.T. Williams, and later expanded by his contemporaries who investigated the fates of simple aromatic chemicals, such as benzoic acid (Gingell et al. 1971; Williams 1972). However, technical limitations in identifying and cataloging the responsible microbiota have severely hindered progress in understanding underlying mechanisms. Only recently with the advent of high-throughput approaches, including sequence-based community profiling and metabolomics, has the contribution of microbiota to drug metabolism transitioned from basic observation to a more mechanistic understanding (Scheline 1968a,b; Patterson and Turnbaugh 2014; Spanogiannopoulos et al. 2016), although our understanding of its metabolic capacity remains limited (Idle and Gonzalez 2007).

Given that health risk is a function of both toxicity (dose-response) and exposure, a critical consideration for risk-assessment frameworks is how the activities encoded by the human microbiome influence the dose of toxicologically active chemicals at the ultimate target site (tissue, cell, or macromolecule). Knowledge of how the human microbiome modulates the pharmacokinetics and metabolism of environmental chemicals generally lags behind knowledge of how the microbiome modulates drugs. Still, there is compelling evidence on gut microbiome involvement in the metabolic transformation of environmental chemicals in broad chemical classes, including metals, polycyclic aromatic hydrocarbons (PAHs), pesticides and persistent organochlorines, nitroamines and aromatic amines, and other toxicant classes (Cerniglia et al. 1984; Van de Wiele et al. 2005; Van de Wiele et al. 2010; Claus et al. 2016).

Many molecular mechanisms probably underlie microbiome interactions, and incorporating such molecular-level detail into the risk-assessment framework for each environmental chemical is a daunting challenge. Nonetheless, research suggests that the human microbiome might modulate the exposure–response relationships of environmental chemicals through a few general mechanisms, which might directly or indirectly influence the pharmacokinetics of the chemicals. The mechanisms include direct metabolic transformation of environmental chemicals and secondary transformation, such as deconjugation of host-generated metabolites; regulation of epithelial-barrier permeability, with implications for transport or excretion of chemicals; and regulation of the expression or activity of endogenous host metabolic pathways (for example, in the host liver) via signaling processes that involve microbial products (Figure 3-1).

[1]A prodrug is a chemical whose metabolism forms a biologically active drug.

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As noted above, there is also a potential for direct effects of environmental chemicals on the composition of a microbiome itself. Although such changes might lead to adverse physiologic consequences through mechanisms that are independent of alterations of a chemical's pharmacokinetics, disruptions in the composition or abundance of a microbial species has the potential to affect all other mechanisms that are mediated by the microbiome.

Conceptually, each interaction can have favorable or unfavorable influences on chemical exposure, and the role of the interactions in modifying susceptibility to toxicity in connection with environmentally relevant exposures remains uncertain. Furthermore, extensive metabolic cooperation and exchange of metabolites that occur between microbial species in a community and with the host is not apparent when species are studied in isolation (Phelan et al. 2011; Traxler et al. 2013). Understanding of the toxicologic significance of the interactions requires strategies that include the microbiome as an integrated part of a multiorgan host response. It should also be emphasized that research on the roles of microbiomes in metabolism of environmental chemicals has focused on the gut microbiome. Examples of the general mechanisms are provided here, but there is a general need to expand knowledge of potential mechanisms of interaction for other body sites. This chapter explores the mechanisms highlighted in Figure 3-1 and concludes with a discussion of interindividual variability and microbiome metabolism of environmental chemicals.

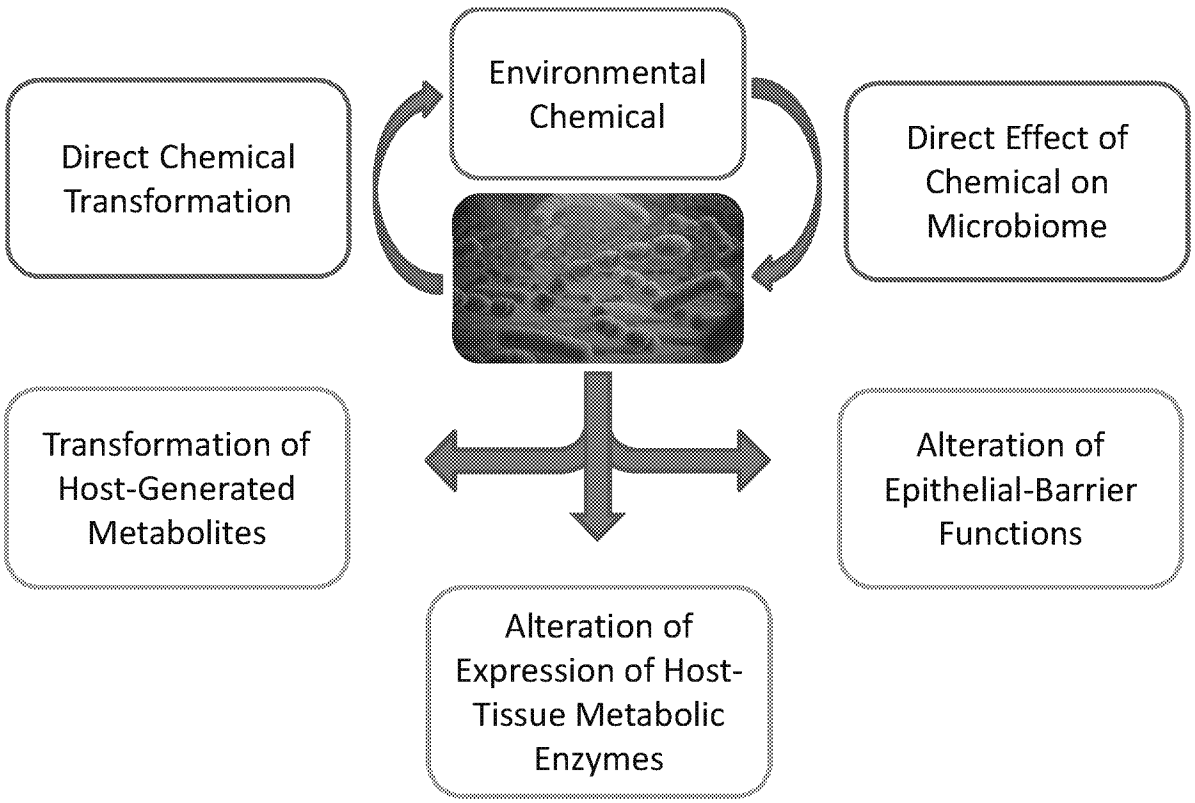

FIGURE 3-1 General mechanisms by which a microbiome might directly or indirectly modulate the exposure–response relationship of an environmental chemical. It should be noted that direct effects of chemicals on the microbiome have the potential to affect all other mechanisms that are mediated by the microbiome.

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DIRECT EFFECTS OF A CHEMICAL ON MICROBIOME COMPOSITION

One way that interactions between the microbiome and an environmental chemical can influence host health is through direct chemical-induced changes in the microbiome. Such changes can be detected by assessing changes in community membership, relative abundance of existing members, spatial organization of the community, microevolution within particular member species, gene expression, or activity of particular metabolic pathways. It is well established that microbiomes of specific composition can have distinct causal effects on host biology. If exposure to an environmental chemical or any other factor leads to alterations in microbiome composition, the altered microbiome itself might have distinct direct effects on the host, although not all changes will contribute causally to host phenotype. It is also conceivable that changes induced by environmental chemical exposure will change the capacity of the microbiome to metabolize chemicals directly or indirectly. As described below, various experimental strategies can be used to test potential effects of chemicals and other environmental factors on the microbiome.

The capacity of environmental chemicals to induce microbiome changes in animals has been demonstrated with a variety of pesticides, metals, artificial sweeteners, and drugs (Patterson and Turnbaugh 2014; Claus et al. 2016). Most studies have relied on analysis of microbial community composition, but additional insights can be gained through combination with other assays. For example, exposure of mice to arsenic in drinking water at 10 ppm for 4 weeks induced consistent alterations in the gut microbiome, including changes within the *Clostridiales* order (reductions in *Clostridiaceae* and *Catabacteriaceae* families and increases in Family XIII *Incertae Sedis*). Fecal-metabolite analysis identified a distinct signature of arsenic treatment, including 370 molecular features, many of which—such as bile acid, indole, and isoflavone derivatives—are predicted to be directly generated or modified by gut bacteria. Correlations between affected bacterial taxa and fecal metabolites were also detected; for example, Family XIII *Incertae Sedis* was correlated negatively with indolelactic acid and dihydrodaidzein (Lu et al. 2014). It is important to note that the drinking-water arsenic concentrations used in Lu et al. (2014) far exceed the drinking-water standard for arsenic (10 ppb). Others have examined effects of low concentrations of arsenic on microbiome composition (Dheer et al. 2015), but administration of arsenic as a sodium salt without appropriately paired controls might be a confounding factor in the experiments. Despite a growing number of experimental studies that report that environmental chemicals can alter microbiome composition, the use of high doses that are of questionable relevance to human environmental exposures is a common limitation of the literature (Claus et al. 2016). However, such results do suggest potential metabolic functions of specific chemical-sensitive microorganisms.

The effects of environmental chemicals on the composition of host-associated microbiomes can be modulated by the host. For example, exposing mice to polychlorinated biphenyls (150 μmol/kg for 2 days) led to alterations in gut microbiota in sedentary animals but not in exercised animals (Choi et al. 2013). Effects of environmental chemicals on microbial composition and metabolite profiles can also be affected by sex, as demonstrated recently in mice exposed to diazinon (Gao et al. 2017). And host genotype contributes to microbial composition (Benson 2016). For example, dietary exposure to 2,3,7,8-tetrachlorodibenzofuran (24 μg/kg for 5 days) led to gut microbiome perturbations, inflammation, and alterations in bile-acid metabolism and signaling in wild-type mice but not in those lacking the arylhydrocarbon receptor (Zhang et al. 2015); this finding suggests that a host receptor-dependent mechanism is involved. However, our understanding of the role of host genotype in determining the effects of environmental chemicals on microbial composition is limited. To define the effects of chemicals on a microbiome independently of host effects, complex microbial communities (Joly et al. 2013; Maurice et al. 2013; Suez et al. 2014) or individual microbial strains (Shehata et al. 2013) have been cultured and exposed to chemicals in vitro to reveal effects on microbial growth, gene expression, and community composition.

Although those and other studies have shown that environmental chemicals can induce microbiome changes, the ability of the altered microbial communities to contribute causally to host phenotypes remains largely unknown. Studies that have analyzed other environmental factors provide instructive experimental strategies for addressing that question. For example, feeding mice a high-fat diet (Turnbaugh et

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al. 2008) or treating mice with low doses of penicillin from early in life (Cox et al. 2014) leads to changes in the gut microbiome and other host phenotypes. In each study, transplantation of the altered microbiome into germ-free recipient mice induced phenotypes that were observed in the donor animals. Such microbiome-transplantation experiments are important because they can help to determine whether the microbiome changes have causal effects on host phenotypes. They can also help to answer the question of whether the host phenotypes are induced directly by the environmental factor or indirectly through the altered microbiome. Another experimental strategy for determining direct and indirect effects on the host is to compare the host phenotypic response to the environmental factor in the presence and absence of a microbiome. For example, administration of a high-dose broad-spectrum antibiotic cocktail in mouse models caused host responses (such as immune downregulation and mitochondrial-dependent epithelial-cell death) that could be explained by loss of antibiotic-sensitive microorganisms, by the remaining antibiotic-resistant microorganisms, or directly by the antibiotics in the absence of microorganisms (Morgun et al. 2015).

Several reports have shown that a chemical challenge can be sufficient to alter host physiology and microbiome composition and that the alteration of the microbiota is sufficient to change the physiology of germ-free recipient hosts after microbiome transplant (Cox et al. 2014; Suez et al. 2014; Chassaing et al. 2015). However, the reported experiments alone do not clearly distinguish between direct causal effects of the chemical on the microbiome and indirect effects of the chemical acting first on the host and altering selective pressures on the microbiome that change microbiome composition. Direct causal relationships between a chemical-induced change in the microbiome and host phenotype has been demonstrated only for noncaloric artificial sweeteners (Suez et al. 2014). That study demonstrated that drinking-water administration of saccharin at doses equivalent to the acceptable daily intake for humans (5 mg/kg-day) altered microbiome composition and induced glucose intolerance in mice. Fecal microbiomes from unexposed mice were also exposed to artificial sweeteners in vitro and then used to colonize germ-free mice; higher glucose intolerance was observed in the colonized mice.

ALTERATIONS IN THE FUNCTIONS OF EPITHELIAL BARRIERS

Epithelial barriers form the interface between many host tissues and the external environment. In addition to their roles as protective barriers, epithelia regulate sensory perception, absorption, surface transport, immune function, and excretion of molecules, ions, and water. Increasing evidence suggests that there are intimate bidirectional interactions between the microbiota and epithelial cells wherein the composition and activity of the gut microbiota, for example, modulate the structure and function of the intestinal epithelium and vice versa (Ulluwishewa et al. 2011; Peterson and Artis 2014; Kelly et al. 2015). Direct manipulations of the gut microbiota via gnotobiotic rearing, antibiotic treatment, or probiotic treatment have been causally linked to changes in intestinal permeability in animal models (van Ampting et al. 2010; Everard et al. 2013; Grover and Kashyap 2014; Leclercq et al. 2014; Tulstrup et al. 2015; Thevaranjan et al. 2017). Perturbations of the microbiota after exposure to such factors as infection, stress, and dietary changes have also been linked to changes in gut-barrier integrity. Clinical associations between microbial changes and "leaky gut syndrome" (increased intestinal permeability) in various gastrointestinal, immune, metabolic, and neurologic disorders raise the question of whether microbiota–epithelium interactions contribute to the cause and development of disease symptoms (Hartmann et al. 2012; Marchesi et al. 2016; Richards et al. 2016). Overall, the gut microbiome is emerging as a key regulator of epithelial permeability and integrity with important implications for the absorption, transport, and excretion of environmental chemicals.

Exactly how the microbiota modifies epithelial-barrier integrity is poorly understood, but some evidence suggests that microbial regulation of tight-junction proteins, mucus-layer structures, and transport systems could contribute. Epithelia are comprised of a continuous layer of squamous, cuboidal, and columnar cells that are interconnected by tight-junction complexes that join adjacent cell membranes and regulate paracellular and transepithelial passage of solutes. Various probiotic treatments and microbiome

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manipulations have altered expression of tight-junction proteins concurrently with changes in intestinal permeability (Turner 2009). For example, in a mouse model of metabolic syndrome, probiotic administration of *Akkermansia muciniphila* increased small-intestine expression of the tight-junction proteins claudin 3 and occludins that correlated with decreases in concentrations of serum lipopolysaccharide, a surrogate measure of permeability (Everard et al. 2013; Plovier et al. 2017). Likewise, in a mouse model of autism spectrum disorder,[2] treatment with the commensal *Bacteroides fragilis* altered colonic expression of claudins 8 and 15 that correlated with decreases in translocation of the fluorescent tag FITC-dextran, an indicator of enhanced barrier integrity (Hsiao et al. 2013).

Epithelia of many internal organs contain specialized mucus-secreting cells that cover the epithelia with protective layers of viscous colloidal fluid. Some studies suggest that the microbiota can influence mucus secretion, thickness, or density. For example, *Akkermansia muciniphila*-mediated improvements in intestinal barrier integrity, described above, also correlated with increases in intestinal mucus-layer thickness. In addition, biophysical forces resulting from microbial fermentation of complex polysaccharides can regulate physical compression of the mucus hydrogel (Datta et al. 2016). Such changes in mucus-layer structure would probably alter solute transport dynamics. Taken together, microbial influences on epithelial-barrier integrity could be mediated by various biologic pathways.

DIRECT CHEMICAL TRANSFORMATIONS

Databases arising from the bioremediation literature have cataloged over 1,500 chemical reactions that involve the biotransformation of chemicals by environmental microorganisms (Gao et al. 2010). Research relevant to environmental-chemical exposures of humans and animals, however, is largely limited to the gut microbiome, which probably has less complex pathways than environmental microbiomes because the gut is primarily an anaerobic environment and has less microbial diversity than environmental microbiomes (Thompson et al. 2017). In contrast with the mammalian liver, in which metabolism of environmental chemicals commonly involves oxidations by cytochrome P450 enzymes, chemical transformations mediated by the gut microbiome favor reactions that do not rely on oxygen or reactions whose products provide a substrate for microbial metabolism and growth. Accordingly, Spanogiannopoulos et al. (2016) broadly categorized the direct microbial metabolic transformations commonly observed for chemicals as reduction and hydrolysis reactions. Other investigators have classified the reactions further into at least five major core enzymatic families—azoreductases, nitroreductases, β-glucuronidases, sulfatases, and β-lyases—that are expressed widely by different phyla in the microbiome (Claus et al. 2016). Examples of major classes of metabolic transformation pathways of environmental chemicals in mammalian host-associated microbiomes are provided here to illustrate the current state of knowledge. Detailed descriptions and examples can be found in several comprehensive reviews (Sousa et al. 2008; Tralau et al. 2015; Claus et al. 2016; Spanogiannopoulos et al. 2016).

Much of the evidence on the direct actions of microbial enzymes on environmental chemicals is derived from studies of drugs at high therapeutic concentrations. However, inasmuch as microbial enzymes often have broad substrate specificities, parallel examples can be drawn to illustrate the potential importance of the enzymes for classes of environmental chemicals of concern in relation to human exposure. For example, azoreductases that are found in several bacterial phyla in the human gut are associated with reduction and inactivation of azo-bonded prodrugs used in treatment of ulcerative colitis, such as 5-aminosalicylic acid (Sousa et al. 2014). Bacterial azoreductases are also implicated in production of mutagenic and carcinogenic aromatic amines via reduction of azo dyes that are common in foods, textiles, and other consumer products (Rafii et al. 1990; Xu et al. 2007). Considerable variability in azoreductase

[2]Autism is associated with increased gut permeability and a higher incidence of gastrointestinal disorders, including irritable-bowel syndrome and disease (Coury et al. 2012).

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activity on different bacterial isolates has been reported (Rafii et al. 1990). However, the specific bacterial genera in the gut that are responsible for those activities are not clearly known.

Gut-microbiome involvement in the metabolism of mutagenic and carcinogenic chemicals that are commonly formed as byproducts of combustion, such as urban air-pollution emissions and emissions associated with flame-based food processing, has also been demonstrated in vitro and in vivo (Möller 1994; Möller et al. 1994). In the presence of human fecal bacteria in vitro, the direct mutagenic activity of 2-nitrofluorene (2-NF)[3] is diminished, presumably because of reduction to a less mutagenic aminofluorene product (Hirayama et al. 2000). Such findings imply that gut microbiota might have a protective role against the toxicity of those chemicals. In contrast, studies that compared germ-free and conventional mice illustrated that the presence of gut microbiota enhances the potential of 2-NF to form DNA adduct and mutagens. The discrepancies might be explained by the more complex metabolism of aminofluorene that occurs in vivo, which involves additional systemic metabolism to mutagenic products that are not replicated in vitro. Studies that used simulated in vitro human gut microbiomes reported that gut microbiota can also convert PAHs, such as naphthalene and benzo[a]pyrene, into hydroxylated metabolites that have new estrogenic activity (Van de Wiele et al. 2005). The relative extent of the formation of those metabolites in vivo in the anaerobic environment of the gut compared with metabolic pathways that occur in other organ systems is not clear. Other early work suggests that the microbially mediated hydroxylation of naphthalene observed in vitro might occur through mechanisms different from those observed in vivo (Bakke et al. 1985).

Studies of human and rodent gut bacteria in vitro also show that gut microbiomes have the capacity to modify bioavailability and toxicity of metals in multiple complex ways (Diaz-Bone and Van de Wiele 2009). For example, methyl mercury can be demethylated to elemental form by fecal bacteria, and fecal excretion of mercury after administration of methylmercuric chloride is lower in germ-free mice and mice treated with antibiotics than in control mice (Nakamura et al. 1977; Rowland et al. 1980). In humans, the complete methylation of inorganic arsenic to dimethyl arsenic is thought to be a key urinary elimination and detoxification pathway that is catalyzed by methyltransferase activity encoded by the host AS3MT gene, which is polymorphic in human populations (for review, see Hughes et al. 2011; Hall and Gamble 2012). However, in vitro studies that used human gut bacteria show that inorganic arsenic can be reduced and undergo methylation to intermediate forms that are more toxic, including monomethylarsonic and monomethylarsonous acids and other multi-methylated forms (Rowland and Davies 1981; Van de Wiele et al. 2010). Despite the transformations observed in vitro, the contribution of the methylated forms to arsenic toxicity in vivo is not clearly established. As noted by Hughes et al. (2011), the significance of gut microbiome-mediated metabolism of arsenic in human health risk depends on whether the bioavailability of the metabolites is different from that of the parent compounds, and this has yet to be resolved. It is noteworthy that physiologically based pharmacokinetic (PBPK) models for estimating tissue-level arsenic metabolism and dosimetry have been developed for multiple species (El-Masri and Kenyon 2008; Evans et al. 2008). However, the PBPK models do not explicitly distinguish between microorganism-specific metabolism and its influence on biodistribution and host-dependent processes, such as those mediated by gut enterocytes. Including microorganism-specific parameters in PBPK models could provide a framework for quantifying the specific role of the microbiome in modulating the pharmacokinetics of arsenic and would facilitate comparison of effects among species.

TRANSFORMATION OF HOST-GENERATED METABOLITES

Microbially mediated hydrolytic reactions can play important roles in modulating the pharmacokinetics and bioavailability of environmental chemicals. In particular, phase II conjugation reactions medi-

[3]2-NF is a common mutagen found in diesel-exhaust emissions and is formed during incomplete combustion processes (Moller 1994; Moller et al. 1994).

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ated by host liver enzymes, which often promote the detoxification and biliary elimination of environmental chemicals and drugs, can in some cases be reversed by microbial hydrolases in the gut. For example, the herbicide propachlor is conjugated with glutathione in the liver, which protects against hepatic toxicity of propachlor. Early studies have reported that the gut microbiota of rats can further metabolize the glutathione conjugates and thus potentially interfere with a key detoxification step (Bakke et al. 1980).

Deconjugation reactions by gut β-glucuronidases promote reabsorption of some drug metabolites, potentially altering pharmacokinetic profiles, toxicity, or efficacy of the parent drugs. A notable example is the colorectal cancer drug irinotecan (CPT-11), which is metabolized to an active ester that is later glucuronidated in the liver and eliminated by biliary excretion to the intestines. Microbial β-glucuronidases in the gut can cleave the glucuronide conjugate and promote enterohepatic recirculation of a parent drug molecule. The increased systemic drug concentrations and extended exposure in the gastrointestinal tract resulting from enterohepatic recirculation are thought to be responsible for gastrointestinal toxicity of CPT-11 observed in some cancer patients (Roberts et al. 2013; Wallace et al. 2015). Similar mechanisms have been associated with common nonsteroidal anti-inflammatory agents, such as indomethacin (Higuchi et al. 2009; Saitta et al. 2014). Intestinal β-glucuronidase activity has also been linked to metabolism of nitrated PAHs, which are common byproducts of incomplete combustion processes (Möller 1994). For example, 2-NF is metabolized after inhalation exposure to hydroxylated nitrofluorenes (OH-NFs) that have increased mutagenic potency. OH-NFs circulate systemically and can be further detoxified and excreted as glucuronide conjugates, but intestinal β-glucuronidase can regenerate OH-NFs and expose the intestine to increased mutagenic risk. In contrast, after oral exposure, 2-NF is reduced to the corresponding amine by intestinal microbiota and acetylated to form acetylaminofluorene, which can undergo further ring hydroxylation to products that have less mutagenic potency and are ultimately excreted. The broader influence of microbial β-glucuronidase activity on the toxicity of environmental chemicals is only beginning to be understood. However, because a wide variety of environmental chemicals might be subject to biliary elimination via β-glucuronidation, interactions with the gut microbiome through this mechanism might be more common than now appreciated.

There is a paucity of information on the potential for gut microbiota to catalyze conjugation reactions similar to that of phase II metabolism in the liver directly, such as glutathionylation, acetylation, and sulfation. However, the gut microbiome favors cleavage reactions that provide substrates for microbial growth (Spanogiannopoulos et al. 2016). A caveat to that observation is that metagenomic sequencing indicates the presence of homologues of phase II genes, such as glutathione *S*-transferases and *N*-acetyltransferases in human gut microbiomes, and this finding suggests a potential for such enzymatic activities (Das et al. 2016). Those metabolic pathways play important roles in detoxification and can vary substantially among individuals and human populations, so future research on their potential role in modifying chemical metabolism is warranted.

ALTERATIONS IN EXPRESSION OF HOST-TISSUE METABOLIC ENZYMES

In rodents and humans, metabolism (such as cytochrome P450 activity) is not fully developed at birth but continues to change throughout adolescence and after puberty (Hines 2013). Specifically, biotransformation reactions, including those associated with phase I and phase II metabolism, vary substantially throughout development. For example, substantial differences in protein concentrations and activity of cytochrome P450s (CYP), flavin monooxygenases, sulfotransferases (SULT), glutathione *S*-transferases, and uridine 5'-diphosphoglucuronic acid gluconysyltransferases have been reported in studies of fetal, postnatal, and adult liver tissue (reviewed in Hines 2008), and some members in each enzyme family are influenced by development more strongly than others or differently from others (for example, SULT1A1 vs SULT1E1 or CYP3A4 vs CYP3A7). Thus, a detailed understanding of the developmental events is critical for safe drug development, delivery, and dosing to neonates, infants, and young children: given the critical developmental windows, pharmacovigilance of these groups is essential (Fabiano et al. 2012). Similarly, early-life developmental changes in metabolism might constitute a critical window

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when risk of adverse responses to environmental chemicals is greatest; that observation is supported by gray baby syndrome, which results from the toxic effects of a lack of liver enzymes in newborns to metabolize the antibiotic chloramphenicol (Knight 1994). Important species and sex differences in the timing and expression of numerous chemical metabolizing enzymes should also be noted (Moscovitz and Aleksunes 2013).

Layered on top of developmental events are genetic influences that are the focus of pharmacogenomicists and their study of people who are poor, intermediate, extensive, and ultrarapid metabolizers identified through genetic screens (Ma and Lu 2011). Despite the extensive body of literature on the developmental and genetic influences on metabolism, gaps in understanding of how metabolism is developmentally regulated remain, and some have suggested that the gut microbiome is an important factor in this development (Selwyn et al. 2015). The discussion below deals largely with the relationship between chemical-metabolism development and the gut microbiome, but interactions between the skin microbiome and the lung microbiome might similarly influence the expression of host genes involved in chemical metabolism. Unlike the gut microbiome and chemical metabolism, however, how the skin or lung microbiome influences metabolism of chemicals has received little attention.

Observations of germ-free rats dating back to the 1960s provided some of the first evidence that the gut microbiota is an important contributor to host liver metabolism (Danielsson and Gustafsson 1959; Björkhem et al. 1970; Eriksson and Gustafsson 1970). Conventionally raised rats excrete much higher concentrations of free or unconjugated steroids (those lacking sulfate) than germ-free rats because their gut microbiota has deconjugation enzymes (bile salt hydrolases) that are important for reducing bile salt toxicity (Ridlon et al. 2016). The early reports also provided some of the first evidence of the important role of the gut microbiota in the process of enterohepatic circulation (Dawson and Karpen 2015), a process of signaling and exchange of nutrients, chemicals, and other substances between the small intestine and the liver. Others have demonstrated that colonization of germ-free mice with microbiota derived from conventionally raised mice is associated with important changes in liver gene expression (CYP8b1), particularly through modification of bile acid synthesis (Claus et al. 2011). It is intriguing that the modification of bile acid pools by the gut microbiota regulates the community composition of the gut microbiome and host physiology.

Recent analyses based on comprehensive studies that used RNAseq profiling of the intestinal epithelium and liver show that the gut microbiota indeed contributes to the development and regulation of genes involved in chemical metabolism (Li et al. 2016; Selwyn et al. 2016). Comparison of gene expression from livers of conventionally raised mice and germ-free mice revealed significant differences in the expression of chemical metabolism genes in the liver (expression of 21 genes increased, and expression of 34 genes decreased under germ-free conditions) (Selwyn et al. 2015). Most notably, CYP3a expression was significantly decreased under germ-free conditions; on colonization of germ-free mice with a probiotic cocktail, CYP3a expression could be restored to levels measured in conventionally raised mice (Selwyn et al. 2016). Those observations are important for two reasons: (1) CYP3a (and CYP2d6) enzymes are important for metabolizing over 50% of known drugs, and (2) regulation of CYP3a expression occurs via the pregnane X receptor, a nuclear receptor that is thought to serve as an important signaling conduit between the gut microbiota and the host (Björkholm et al. 2009). Additional research is needed to understand how the microbiome and its products interact with host nuclear receptors—including peroxisome proliferator-activated receptors α, β, and γ (Nicholson et al. 2005), constitutive androstane receptor (Björkholm et al. 2009), farnesoid X receptor (FXR) (Wahlström et al. 2017a), and the aryl hydrocarbon receptor (Zhang et al. 2015).

Recent developments in understanding FXR function have shed light on how host-gut microbiome interactions in the small intestine regulate gene expression in the liver. FXR is a ligand-activated nuclear receptor that is important for bile acid metabolism and for maintenance of glucose and lipid homeostasis (Gonzalez et al. 2016). Studies comparing germ-free mice and conventionally raised mice have identified FXR as a central mediator of the interactions between the liver, the small intestine, and the gut microbiota (Li et al. 2013; Sayin et al. 2013; Jiang et al. 2015a,b; Parséus et al. 2017; Wahlström et al. 2017b). Specifically, the gut microbiota can modulate liver metabolism by altering the composition of the intestinal

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bile acid pools (for example, FXR agonists include chenodeoxycholic acid and taurcholic acid, and FXR antagonists include tauro-β-muricholic acid) and thus influence intestinal FXR signaling back to the liver (Wahlström et al. 2017b). That process is critical for regulating bile acid secretion in the liver and uptake in the ileum that the microbiota tightly controls in such a way as to favor optimal growth conditions; strong evidence from rodent and human studies has implicated the gut microbiota–FXR signaling axis as a key contributor to metabolic disease (Gonzalez et al. 2016; Zhang et al. 2016; Xie et al. 2017). Observations from bariatric-surgery patients has provided additional support linking the gut microbiota, the small intestine, and changes in liver metabolism (Kuipers and Groen 2014).

Studies of other models of metabolism—including zebrafish (*Danio rerio*, Rawls et al. 2004), nematodes (*Caenorhabditis elegans*, Scott et al. 2017), and fruit flies (*Drosophila melanogaster*, Combe et al. 2014)—have similarly identified how microbiota colonization activates or contributes to development of chemical metabolism pathways. Important chemical metabolizing enzymes in the cytochrome P450 family were upregulated after microbiota colonization; however, these transcriptional changes were not well conserved and appear to be species-specific. Regardless, the microbiota-dependent upregulation of chemical metabolism genes in the model organisms further supports the evolutionary importance of the host–microbiota interaction in modulating environmental chemical exposures.

INTERINDIVIDUAL VARIABILITY AND MICROBIOME METABOLISM OF ENVIRONMENTAL CHEMICALS

As discussed in Chapter 2, many factors affect the human microbiome and lead to substantial differences in composition. How those compositional differences translate to functional variability in processes that influence the metabolism and disposition of environmental chemicals has received little attention. There are few experimental strategies to evaluate pharmacokinetic variability, and they have relied heavily on culture-based methods, which have limitations in their application to large human cohort studies. More recently, studies that leverage metagenomics sequence databases arising from the Human Microbiome Project have begun to identify microbial gene homologues for major families of chemical metabolism enzymes (Saad et al. 2012; Das et al. 2016). For example, a computational analysis of 397 individual gut metagenomes identified over 800 bacterial genera that potentially can metabolize environmental chemicals, and it predicted individual variability in the abundance of metabolic enzymes on the basis of geography, age, and average drug use (Das et al. 2016). The authors suggested that differences in abundance patterns imply distinct roles of the microbiome in pharmacokinetic variations observed among individuals and predicted that gut microorganisms could be stratified into three groups on the basis of their capacity to metabolize drugs and environmental chemicals. Although the biologic implications of such genome-enabled strategies await future experimental validations, there is a need to develop similar analyses and databases for predicting environmental-chemical metabolic pathways in microbiomes at other body sites, such as oral, lung, and skin.

FINDINGS

- Although knowledge of how microbiomes modulate the pharmacokinetics and metabolism of environmental chemicals generally lags behind that of drugs, there is compelling evidence of gut microbiome involvement in the metabolic transformation of environmental contaminants in broad chemical classes.
- Research suggests that microbiomes might modulate the exposure–response relationships of environmental chemicals through a few general mechanisms, including regulation of epithelial-barrier permeability, with implications for transport or excretion of chemicals; direct metabolic transformation of environmental chemicals and secondary transformation, such as deconjugation, of host-generated metabolites; and regulation of the expression or activity of endogenous host metabolic pathways (such as in the host liver) via signaling processes involving microbial products. There is also a potential for direct effects of environmental chemicals on the composition of a microbiome itself.

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- It is important to note that each interaction conceptually can increase or decrease chemical exposure and that the role of the interactions in modifying human susceptibility to toxicity of environmentally relevant exposures remains largely uncertain.
- Although research has provided important clues regarding microbial transformation of environmental chemicals and vice versa, there are substantial gaps in the understanding of how chemical exposure changes activity or function of a microbiome and of the breadth of potential metabolic pathways of environmental chemicals in a given microbiome.
- The community composition of the microbiome varies widely among species, individuals, and life stages, and how phylogenetic variability translates to functional variability in processes that influence the metabolism and disposition of environmental chemicals has received little attention.
- In vitro experiments have provided important information on microbial metabolism, but caution is needed in interpreting results solely from in vitro studies; the toxicologic significance of microbiome-mediated metabolism of chemicals needs to be evaluated as part of an integrated, multiorgan host response.

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4

Current Methods for Studying the Human Microbiome

The human microbiome has evolved to be a remarkably diverse, finely balanced, and highly environment-specific ecosystem (Lloyd-Price et al. 2016). Each body site constitutes a specific habitat that can include trillions of microbial cells and hundreds of strains that differ nearly completely from one site to another throughout the body (HMP Consortium 2012a,b). Using techniques from molecular epidemiology, microbial ecology, and microbiology, researchers have demonstrated that changes in typical immune interactions, biomolecular activities, or pathogen exclusion are associated with such diseases as inflammatory bowel disease, autism, and cancer (Bäckhed et al. 2012; Hsiao et al. 2013; Petersen and Round 2014; Trompette et al. 2014; Garrett 2015). Culture-independent population studies of the human microbiome follow an approach similar to that of Franzosa et al. (2015), which includes collecting stabilized microbial biomass specimens at various times from people who have various exposures or phenotypes of interest; assaying the collected samples with one or more molecular profiling technologies (Segata et al. 2013); bioinformatically profiling the resulting raw data to quantify microbial features of interest, such as species abundances, strains, and biomolecular functional elements; and statistically associating changes in those features with population phenotypes or exposures. That approach is similar to other types of molecular epidemiology studies, such as gene-expression biomarker discovery or genome-wide association studies, and can be combined with experimental approaches that change or challenge the microbiome.

Because of the nature of human microbiome studies, the resulting associations are most often correlative rather than clearly causal; however, additional targeted assays can be used to establish causality and mechanism. The most common targeted assays might involve gnotobiotic transfer of human microbiome samples into controlled model organisms (such as mice) or change-inducing treatments, such as administration of antibiotics, to knock down or alter the composition of the microbiome (Morgun et al. 2015). In addition to transferring whole communities, individual microbial strains that are identified from whole-community profiling can be targeted for isolation (Faith et al. 2010) by using classical microbiology techniques or engineered systems, such as microfluidics. That approach allows the microbial physiology or biochemistry of individual strains of interest (such as secretion products or biomolecular repertoires) to be finely measured and manipulated. Such in vitro systems can be scaled up to include laboratory profiling of entirely synthetic communities, particularly in continuous-culture systems. Detailed properties of host immune sensing and control of a microbiome can be profiled from human tissues (Honda and Littman 2016) by measuring T-cell and B-cell populations, immunoglobulins, cytokine pools, small molecules, and gene expression. The profiling is most often conducted on microbial communities in the gut but can be done for any site-specific community, such as the oral cavity or the skin (Belkaid and Segre 2014). Computational analyses can complement any of the approaches discussed.

Each method for human microbiome profiling—epidemiology, animal, or in vitro studies—has benefits and drawbacks, generally similar to those of other methods in translational molecular research. Human population studies are expensive and difficult to control experimentally at each stage (sample collection, data generation, and data analysis), and they are not generally amenable to interventional studies to establish causality. However, direct measurements of exposures and health risks are possible. Animal models can rarely precisely recapitulate human-associated microbial community structure (Chung et al. 2012), and gnotobiotic facilities can be expensive and difficult to maintain. However, various gnotobiotic systems—including ones that use mice, fish, pigs, and even fruit flies—are now available for modeling

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different aspects of the human microbiome (Fritz et al. 2013); each can be colonized and perturbed in a targeted experimental manner. In vitro microbial systems, including ones that contain host cells in the microbial culture, have the longest history, are widely available, and present one of the most controllable environments for mechanistic and molecular profiling. However, continuous culture of many anaerobic organisms presents challenges, and in vitro systems are physiologically the least relevant.

This chapter continues the discussion and provides greater detail on the approaches and methods used today to study the human microbiome. The discussion is divided into three parts. First, systems for studying the human microbiome are described; aspects of sampling the human microbiome are considered, and then animal models, engineered in vitro and ex vivo systems, and culture systems are described. Second, technologies for assaying the microbiome—nucleotide sequencing, other molecular profiling techniques, and direct observation methods—are addressed. Third, methods and approaches for analyzing the data are discussed. The chapter concludes with a discussion of strengths, weaknesses, and gaps in the technologies.

SYSTEMS FOR STUDYING THE HUMAN MICROBIOME

Considerations in Sampling the Human Microbiome

The first step in a microbiome study typically involves the collection of stabilized microbial biomass specimens that will be used and analyzed in various assays. Each sampling method for human-associated microbial community types has strengths and weaknesses that are driven by the dramatically different microbial ecologies in or on the body. The methods that have been established for gathering a sample of sufficient biomass (referring to the quantity of microorganisms needed for an assay) for each major body site are described here, and limitations of each approach are noted.

The gut microbiome is most commonly sampled from stool, which represents well the microbial community of the colonic lumen and to a smaller degree that of the small intestine (Yasuda et al. 2015). Stool is easily obtained for sampling, has extreme microbial density and minimal human genetic contamination (HMP Consortium 2012a,b), and contains material that can be assayed with a variety of molecular techniques. Because microbial characteristics can change rapidly with environmental conditions (such as a sudden decrease in temperature and exposure to air), it is important to take steps to preserve samples by, for example, immediately freezing them or using various laboratory protocols and commercial kits to fix them (Franzosa et al. 2014; Song et al. 2016). It is possible to culture many microorganisms from frozen stool samples, whereas fixatives typically kill microorganisms (preventing culture) and might not be compatible with conducting some molecular assays at a later time. Fixatives do, however, allow convenient collection and shipping of samples. In a clinical setting, mucosal biopsies are common and provide a more precise and biogeographically resolved snapshot of the mucosally associated microbial community (Morgan et al. 2012), but they are more challenging to obtain and can be assayed only with technologies that are not affected by the presence of human cells in the sample. Other sample types, such as mucosal brushing or rectal swabs, are also possible but are less well studied with respect to protocol consistency and community representation (Tong et al. 2014).

Skin sampling is limited primarily by the low microbial biomass that is found on typical surfaces. The moist, dry, and sebaceous sites across the body can have substantially different ecologies that are difficult to differentiate without detailed profiling (Grice and Segre 2011). Swab sampling is easiest but retrieves the smallest biomass, and microbial adhesion can be surprisingly affected by the type and material of swabs used (Aagaard et al. 2013). A combination of razor scraping and swabbing is the most practical for retrieving samples with greater biomass but requires training and care to perform safely (Oh et al. 2014). Biopsies obtain the greatest microbial and human biomass and, as in the gut, are typically amenable only to assays that are not affected by the inclusion of human genetic material. However, skin microbiome samples in general are often characterized as having high human nucleotide fractions—as much as about 90% of the sample (HMP Consortium 2012a,b)—and require more extensive sequencing and care

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during analysis. Because of the low biomass of skin microbiome samples and the challenges associated with collecting them, assays that evaluate skin microbiome samples must include special consideration of negative controls to ensure appropriate interpretation of sampling results (Oh et al. 2014).

Similar issues are encountered in connection with sampling methods for the respiratory microbiome. Clinically, the respiratory tract is divided into the upper and lower regions relative to the epiglottis; each region experiences different exposures to the external environment and has different mucosal-epithelial barrier properties (Wolff 1986). Given the variation, it is not surprising that different sampling approaches can provide different readouts and information. Varied clinical approaches and sampling tools have been used to obtain material from the nasal passages, sinus cavities, oral cavity and pharyngeal region, and the tracheobronchial tree. Although surgical specimens, such as those collected during sinus surgeries or from explanted lungs, offer the greatest opportunity for detailed sampling, less invasive approaches are necessary for larger studies (Perez-Losada et al. 2016; Dickson et al. 2017). Swabs, aspirates, sputum, lavage, and brushings have all been used in respiratory microbiome studies. Swabs—most often used to sample the upper respiratory tract—recover different amounts of material compared with aspirates, sputum, lavage, and brushings. Sputum can be spontaneous or collected via induction protocols, such as inhalation of hypertonic saline. Aspirates tend to collect secretions already present, whereas lavage involves instillation of saline into an airway passage and withdrawal of the fluid with suction. In the lungs, the volume returned from bronchoalveolar lavage can be highly variable and depend on disease state; for example, less volume is returned in cases of severe obstruction or emphysema. Thus, measurements based on lavage fluid need to consider dilution as a factor. Small brushes can also be inserted to obtain cells and secretions from the mucosal surface, but care is required to perform this method. Finally, as above with the skin, the respiratory tract is less microbially dense, and it is essential to use protocols that have carefully controlled elements to minimize sample contamination by nontarget tissue (Charlson et al. 2012; Salter et al. 2014; Lauder et al. 2016). Such elements include proper staff training; preparation of work materials, surfaces, and instruments; and collection of controls, including within-subject biologic controls (such as paired upper-airway and lower-airway samples) for accurate interpretation of microbial sequence data.

All human microbiome sampling protocols are sensitive to batch effects—technical, not biologic, differences that arise from many stages of the sampling and data-generation process (Salter et al. 2014). Such effects can make data from multiple studies difficult to compare and, in the worst case, can introduce subtle differences that result in misleading conclusions. Gross differences in population structure, geography, or environmental conditions can change measured microbial communities. Differences in how samples are collected and processed can strongly influence microbiome assays. Differences in the protocols used to assay the samples can obfuscate biologic effects. And differences in data handling, quality control, and taxonomic, functional, or molecular profiling techniques can contribute to unwanted technical artifacts (Sinha et al. 2015). All those factors are important considerations during study design and data analysis when one dataset is compared with others (Sinha et al. 2017). It is difficult today to compare multiple microbiome datasets reliably because not all datasets can be combined. To enhance comparability, research programs need to make every effort to standardize protocols in advance, run cross-protocol controls throughout, and statistically meta-analyze any remaining systematic differences between datasets.

Understanding the Human Microbiome by Using Model Organisms

Insights into the microbiome and its interactions with human hosts and their chemical environments can be obtained or refined by using diverse nonhuman model systems. Although no nonhuman model system will fully recapitulate all aspects of the human microbiome, each has distinctive strengths that can be leveraged selectively to address scientific questions that would be difficult or impossible to answer with human studies alone. Overall, nonhuman models provide valuable opportunities to gain insights into molecular pathways, physiologic processes, host microbial genotypes, and microbial–chemical stimuli that might be relevant and translatable to the human microbiome and human health.

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Animal models are widely used to investigate the human microbiome for several reasons. First, it is much easier to manipulate animal models than human subjects experimentally. Animal studies allow the careful control of experimental variables, scalability, and reproducibility that is often impossible in human studies. Second, ecologic and physiologic attributes of the animal body are highly complex and dynamic and cannot be comprehensively recapitulated in in vitro or in silico models. Finally, the common ancestry of humans and other animals has resulted in the conservation of many genomic, molecular, cellular, and physiologic traits across animal lineages and allows many (not all) findings derived from animal studies to be extrapolated to humans. The advantages of using animal models are counterbalanced by important caveats, including salient differences among animal lineages in anatomy, physiology, and microbiomes (Ley et al. 2008). Although the caveats might limit the relevance of animal models for understanding some aspects of the human microbiome, animal models are important in the larger field of microbiome science.

Several fundamental experimental strategies can be used to study microbiomes in animal models. First, animals can be used to test whether the microbiome composition and function correlate with such variables as host age, host genotype, host body site, diet, and chemical or other experimental exposures. The experiments are typically performed on laboratory or wild animals that are colonized by complex microbial communities. Second, animals can be used to study the effects of the presence or composition of a microbiota on host phenotypes. To test whether microbiome composition contributes to host phenotypes, animals with an intact microbiome can be treated with broad-spectrum antibiotics to reduce microbial abundance and alter community composition. That is a relatively inexpensive and rapid way to disrupt the microbiome, but its disadvantage is that it does not distinguish between the effects induced by loss of antibiotic-sensitive microorganisms, by the remaining antibiotic-resistant microorganisms, or directly by the antibiotics (Morgun et al. 2015). Third, another inexpensive and rapid approach for testing the effect of a particular microbial community or strain is to introduce it directly into conventionally reared animals that are already colonized with a microbiota (a probiotic or super-colonization approach). Introduction can also be achieved by co-housing animals that initially contain distinct microbial communities or strains. However, introduction of microorganisms to compete with the pre-existing microbiota and establish stable colonization has had a low success rate and has resulted in considerable variation in experimental outcomes.

The effect of the presence or composition of a microbiota on host phenotypes can be addressed with substantial experimental control by using gnotobiotic animal models. As noted in Chapter 1, the term *gnotobiotic* refers to an animal that has no microorganisms (a germ-free animal) or an animal whose composition of associated microorganisms is fully defined by experimental methods. Germ-free animals can be colonized with microbial communities or strains of interest and then evaluated to assess effects on the host. The donor microbial communities can be derived from various sources; "humanized" animal models that are more relevant to the human condition are produced when a human source is used (Ridaura et al. 2013). Although gnotobiotic animal models provide strong experimental control, they are accompanied by distinct challenges and caveats, such as the relatively high cost and labor needs of gnotobiotic-animal facilities; developmental, immunologic, and physiologic anomalies of gnotobiotic animals; and augmented nutritional requirements of gnotobiotic animals (Falk et al. 1998).

The different experimental approaches described above have been used in a broad array of animal species, including mice, zebrafish, fruit flies, and *Caenorhabditis elegans*. Each species has a unique set of characteristics related to its relative size; transparency; microbiome complexity, composition, and function; genetic variance; and evolutionary distance from humans (Leulier et al. 2017). For example, using mice offers some advantages, such as powerful genetic resources that include inbred lines to reduce the effect of genetic variability, an extensive array of knockout strains, and their relatively close evolutionary distance and physiologic similarity to humans. But the disadvantages of using mice include the difficulty of in vivo imaging and the relatively high cost and low scalability of gnotobiotic and conventional husbandry. In contrast, zebrafish have such advantages as facile in vivo imaging owing to their optical transparency, small size that permits genetic and chemical screens, and scalable and inexpensive husbandry requirements that are easily adjusted for gnotobiotic methods. But the disadvantages of using zebrafish

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instead of mice include greater evolutionary distance from humans and smaller overlap in bacterial taxa[1] in their microbiomes (Rawls et al. 2006; Hacquard et al. 2015). For all those animal models, best practices are emerging to promote interpretability and reproducibility of experimental results, partly by accounting and controlling for interfacility and interindividual variation in microbiome composition (Macpherson and McCoy 2015; Stappenbeck and Virgin 2016).

Engineered Systems for Studying Host–Microbiome Interactions In Vitro and Ex Vivo

Using in vitro and ex vivo experimental systems for studying host–microbiome interactions allows greater manipulation of experimental conditions and increased ability to examine interactions that are too complex to study in vivo. As defined in Chapter 1, the terms *in vitro* and *ex vivo* differ mainly in the source of the samples being used in the assay. Both require the use of an artificial setting for conducting an experiment: in vitro systems typically rely on such samples as cell lines or laboratory microbial strains whereas ex vivo systems typically rely on samples that are directly isolated from a host organism. The main systems currently in use for in vitro and ex vivo cultures that examine host–microbiota interactions include co-culture of microorganisms with or without host primary epithelial cells, tissues, or cell lines; microfluidic co-culture of microorganisms with or without engineered tissue; and organoid[2] culture. Those systems are used primarily to examine bidirectional signaling between microorganisms or between target host tissue or cell types and a body-site microbiome. Perhaps central among the challenges of using the systems in an artificial setting is the propensity of microbial cultures to become ecologically imbalanced, with components either dying or overgrowing and preventing the cell-culture system from reflecting the in vivo community accurately. Although the following discussion focuses on gut-centric applications, analogous systems exist for the lung and, to a lesser extent, for the skin.

In the context of studying the gut microbiome, polarized epithelial monolayers are grown from primary or immortalized small intestinal or colonic cells on transwell membranes[3] (Kauffman et al. 2013; Moon et al. 2014) or three dimensional scaffolds (Chen et al. 2015), and microorganisms are seeded on the apical face. Changes in the quality of the epithelial layer can be measured by assessing permeability, transmembrane resistance (used to measure how tightly connected neighboring cells are), active transport, absorption, and excretion. Miniaturization of culture systems to microliter or nanoliter scales renders them amenable to microfluidic manipulations, such as isolation of single bacterial cells from complex microbial communities and their study with imaging, gene-expression profiling, or mass spectrometric readouts (Ma et al. 2014a,b). Limitations of those techniques include their lack of secondary epithelial structures, such as villi and crypts; the absence of additional epithelial-cell subtypes, such as goblet, endocrine, and immune cells; the lack of mucus layers between host and microbial cells; and the difficulty of incorporating realistic multiorganism microbial-community components.

Some limitations are overcome by building structured epithelial layers with microfluidic and tissue-engineering approaches. Gut-on-a-chip technology uses microfluidic platforms to grow intestinal epithelial cells and mimic the movement of fluids through the gut (Kim and Ingber 2013); this promotes the formation of intestinal-tissue structures with specialized cell types, such as absorptive, goblet, enteroendocrine, and Paneth cells. The structures exhibit barrier properties, including mucosal linings and peristaltic motion. Continuous movement of fluids can enable persistent microbial microcolonization as a continu-

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[1]A *taxon* (plural, *taxa*) is a taxonomic group of organisms, such as a family, genus, or species.

[2]An organoid is "an in vitro 3D cellular cluster derived exclusively from primary tissue, embryonic stem cells, or induced pluripotent stem cells, capable of self-renewal or self-organization, and exhibiting similar organ functionality as the tissue of origin" (Fatehullah et al. 2016).

[3]Transwell membranes are inserts that can be placed inside a standard tissue-culture dish that has a permeable membrane on which the cells sit; this arrangement allows separation of the area above the cells (the apical face) and the area below the cells (the basolateral face). When cells are growing under ideal conditions, the cells control the passage of solutes between the two areas.

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ous-culture system (Kim et al. 2012, 2016). Limitations include the need for customized chip fabrication, specialized equipment and technical expertise, and difficulties in introducing diverse microbial components. Furthermore, the technology has thus far been tested and used only with immortalized cell lines and does not account for varied host genetics.

Growth of intestinal organoids, spheroids, or "mini-guts" is relatively accessible compared with that of microfluidic approaches and allows personalized organoid lines from different clinical donors or animal models to be generated. Several protocols have been developed and generally introduce specialized factors into cell-culture media to differentiate embryonic or induced pluripotent stem cells into clusters of villous epithelia or equivalent differentiated cell clusters of other body sites, such as the lung (Wilson et al. 2015; Nigro et al. in press). However, studying microorganisms in organoids requires careful microinjection into each cluster. Furthermore, the enclosed structures and lack of physiologic flow can result in rapid disruption of injected microorganisms, and this limits experimentation to relatively short timescales.

Microfluidic and organoid culture systems reproduce epithelial structures and various differentiated cell subtypes but typically lack integrated immune, muscle, and neuronal cells that are important for many host-microorganism interactions. No in vitro system faithfully captures all those elements in a unified technology. However, ex vivo culture systems can enable careful control of microbial colonization, luminal perfusion, and chemical exposures (Roeselers et al. 2013). Intestinal tissues can be isolated from model organisms and maintained in ex vivo culture for short durations. Chemicals, microorganisms, or both can be introduced into the systems particularly in combination with perfusion methods; this approach yields physiologic or molecular readouts that in the best cases closely mimic their in vivo counterparts. However, they have not yet been extensively explored to support multimicrobial model communities. As a technical intermediate between animal and culture-based models, ex vivo systems trade controllability for model accuracy. Advances in the development of parallel ex vivo multiculture systems that have increased experimental control and prolonged culture times are being explored.

Analogous tools are available to study host–microbiome interactions in the respiratory tract. Primary airway epithelial cells and cell lines are well-established tools in respiratory-disease research, but their application to study microbiota interactions has been limited. Recently, microfluidic platforms and organoid culture models for studying respiratory biology have been developed (Dye et al. 2015; Benam et al. 2016a,b). The former include lung-on-a-chip and small-airway-on-a-chip technologies that parallel the gut-on-a-chip platform. Substantial advances have also been made in ex vivo lung-perfusion models (animal and human), which are being used to conduct translational research on lung diseases. The ex vivo perfusion techniques now available have been so successful that clinical studies are investigating their use as a preservation method for donor lungs in human lung transplantation (Nelson et al. 2014; Tane et al. 2017).

Synthetic models of the skin microbiome are likewise in early development. One recent medium-throughput model system of the human stratum corneum (outermost skin layer) that uses collected sloughed human cells was used to evaluate survival of skin pathogens and commensals (van der Krieken et al. 2016). A commercial three-dimensional in vitro skin model is also available and can be populated with human skin microbiota and used to evaluate the effects of chemical exposure on skin colonization (Bojar 2015). These systems do not yet cover the diversity of microbial biochemical environments on skin, nor has their microbial suitability or modeling accuracy been ascertained.

Overall, the in vitro and ex vivo systems for examining host–microbiota interactions vary in experimental throughput, physiologic relevance, and experimental control. Conventional co-culture with primary epithelial cells or cell lines enables moderate experimental throughput that can be precisely controlled and manipulated. Microfluidic and engineered tissue systems are relatively high-throughput with potentially moderate physiologic relevance but require more technical infrastructure and are harder to manipulate. Organoid cultures offer moderate experimental throughput, moderate to high physiologic relevance, and moderate experimental control, whereas ex vivo perfusion systems are low-throughput and highly physiologically relevant and therefore offer more moderate control.

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Culture Systems for Characterizing the Human Microbiome

The longest-standing in vitro technique for studying host-associated microorganisms is microbial culture. In tandem with the rise of culture-independent profiling, culture-based techniques have been refined to capture a wider array of organisms from the human microbiome than previously possible, including anaerobes and nonbacterial members, under ever more accurately controlled conditions. Bioreactors that contain microbial cultures, for example, can be used to test specific hypotheses about microorganism–microorganism interactions, microbial production of metabolites, microorganism–chemical transformations and kinetics, and effects of chemicals on microbiome structure and function. Studying microorganisms without the host component has several advantages: the system has increased reproducibility, microorganism–microorganism interactions can be studied in a more defined way, environmental conditions that affect microbiome composition and interactions can be easily controlled, and microbial biotransformations and metabolites can be precisely identified.

Studies have used bioreactors to simulate gut microbial communities to learn more about fermentation processes (Miller and Wolin 1981), biofilm formation (McDonald et al. 2015), and microbial-community responses to perturbations resulting from exposure to antibiotics (McDonald et al. 2015), nanoparticles (Taylor et al. 2015), metabolites from polyphenol transformations (Gross et al. 2010), and polycyclic aromatic hydrocarbons and polybrominated diphenyl ethers (Cui et al. 2016). For any culturing technique to be successful, knowledge of optimal environmental conditions for the desired microorganism is required. Important conditions include pH, oxidation–reduction potential, temperature, and nutrients (Browne et al. 2016; Lagier et al. 2016; Lau et al. 2016). Microorganisms cultured from the human gut have been used to test biotransformations of specific pollutants, such as *Eubacterium limosum* metabolism of the insecticides methoxychlor and DDT (Yim et al. 2008).

Although development of in vitro host-microbiome simulator devices or bioreactors is in its infancy, several devices have found their way into basic and translational research. First, the simulator of the human intestinal microbial ecosystem (SHIME) (Van den Abbeele et al. 2012) is a model of the small and large intestines that contains stable and functional microbial communities similar to those found in the human (Joly et al. 2013). It is one of the earliest types of linked continuous culture systems that mimic the human digestive tract microbiome by controlling compartmentalization, nutrient availability, pH, and other environmental conditions. Another version of the SHIME model is the mucosal SHIME (M-SHIME); it permits the study of mucosa-associated microorganisms (Van den Abbeele et al. 2012). A simpler model is the minibioreactor array, which, unlike the SHIME model, is amenable to high-throughput screening, although it does not model multiple regions of the gastrointestinal tract (Auchtung et al. 2015).

Recent advances in culturing techniques that have been enhanced by sequencing and metabolomics techniques have increased the percentage of host-associated cultivable microorganisms (Browne et al. 2016; Lagier et al. 2016; Lau et al. 2016). As noted, however, culture conditions are critical. And as expected, culture outcomes are affected by collection and storage procedures and such factors as oxygen exposure, potential microbial growth, and changes resulting from freezing and thawing (Lau et al. 2016). Using selective culture media and choosing appropriate environmental conditions are critical for success. For example, a combination of anaerobic and microaerobic[4] conditions at the correct pH is needed to isolate gut microorganisms. Isolation of anaerobes requires oxygen depletion in the media and airspace of the culture chamber and defined growth requirements, such as specialized media and targeted nutrient supplementation. Other challenges are the existence of syntrophic (mutually dependent) relationships, and the presence of many microorganisms in the host as a biofilm that is difficult to replicate externally. Special culture methods—such as the roll tube method in which the culture medium is rolled inside a test tube until it forms a thin film around the internal wall of the tube and methods that use soft agar plates in

[4]A microaerobic environment is one in which the oxygen concentration is lower than that found under standard atmospheric conditions.

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which the culture medium has a lower concentration of gelatin, which allows the detection of mobile microorganisms—can be used to encourage the growth of difficult microorganisms further (Dickson et al. 2017). Microfluidic devices that allow droplet separation and sequencing in tandem have been developed and used to isolate gut microorganisms that were previously considered uncultivable (Leung et al. 2012; Brouzes et al. 2015), and a microfluidic streak plate platform has been developed to facilitate cultivation of dominant and rare species in a microbial community (Dickson et al. 2017). Such novel platforms will allow physiologic microbial characterization and help to decipher the important roles of individual microorganisms, including their possible biotransformation pathways.

As noted above, there are clear advantages of studying microbial cultures and isolates that use the systems described. However, there are also some disadvantages: the host is not considered, syntrophic interactions are difficult to replicate, cultures or isolates rarely capture the physical structure of biofilms or other structured communities, enrichment and isolation techniques are often lower-throughput than molecular techniques, ideal culture conditions are not always known for many microorganisms of interest, and they can require more diverse expertise or facilities than do molecular techniques.

TECHNOLOGIES FOR ASSAYING THE MICROBIOME

Assaying the microbiome as described above can use various technologies as highlighted in Figure 4-1. The following sections describe nucleotide sequencing of DNA and RNA, other molecular profiling techniques, and methods for direct observation of the human microbiome.

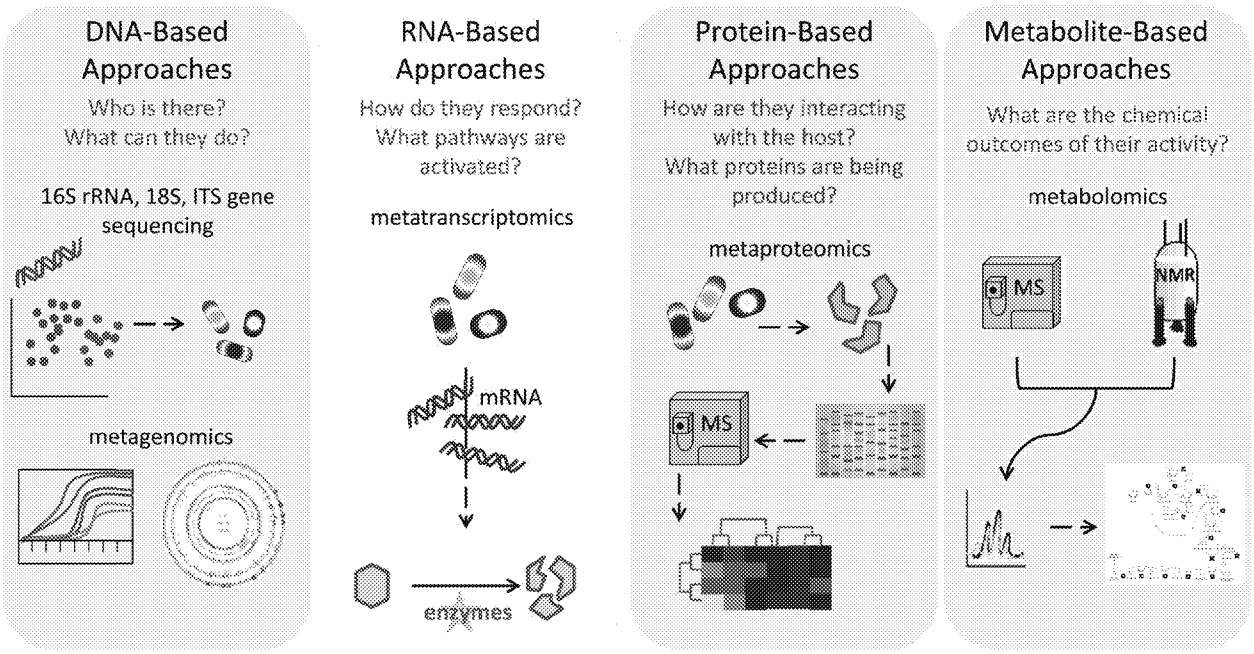

FIGURE 4-1 Culture-independent molecular approaches to study host–microbiome interactions. Several aspects of the central dogma—the flow of genetic information from DNA to RNA to protein—can be assessed to study host–microorganism and microorganism–microorganism interactions at the molecular level in human populations, animal models, and in vitro models. Current technologies readily support small molecular proteomic and metabolite surveys (targeted or untargeted) and nucleotide sequencing of RNA and DNA to assess host and microbial gene expression, taxonomic profiles, and genomes. Source: Adapted from Ilhan (2016). Reprinted with permission; copyright 2016, *Nature*.

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Nucleotide Sequencing

The decreasing cost and increasing accessibility of nucleotide sequencing unquestionably boosted human-microbiome studies in population health, and it is still the primary tool used to study the microbiome (Franzosa et al. 2015). One of the earliest and most widespread techniques is amplicon[5] sequencing, in which a single genomic locus is targeted for polymerase chain reaction (PCR) amplification; the chosen locus must be largely conserved throughout microorganisms of interest but contain sufficient variation to allow distinction of individual strains or species. Resulting PCR products are sequenced and compared with known reference sequences in a database. Amplicon sequencing most commonly targets the 16S rRNA gene (Hamady and Knight 2009), which is almost universal among bacteria, whereas the 18S rRNA gene and internal transcribed spacer (ITS) sequence variants are increasingly common for eukaryotic profiling[6] (Findley et al. 2013). The methods rely on conserved targets of the PCR primers that are adjacent to sequences that are sufficiently variable to differentiate organisms of interest. As the price of sequencing technologies have decreased, whole-community metagenome sequencing of arbitrary short reads has become more common and today can provide billions of sequence reads (many gigabases) per community. Practical methods have also recently been developed to apply long-read metagenomic sequencing to RNA metatranscriptomes[7] in the human microbiome (Franzosa et al. 2014), and protocols that use long-read high-throughput sequencing (Tsai et al. 2016) and single-cell sequencing (Gawad et al. 2016) are also emerging.

Amplicon sequencing, metagenome sequencing, and metatranscriptome sequencing have different strengths and weaknesses. All are sensitive to the specific protocols used for nucleotide extraction from samples, which requires care to avoid biasing experimental results. Microorganisms vary in their sensitivity to the reagents used for the extraction of genomic material, so researchers must be cautious to avoid destroying sensitive subsets of microorganisms while still extracting genomic material from more hardy or resistant organisms. If RNA is the desired genetic material, extra caution will be needed to avoid destroying the RNA during sample processing. Amplicon sequencing can be inexpensively carried out by using samples that have extremely low microorganism biomass or mixed samples that have, for example, substantial human or other nonmicrobial nucleotides (Hamady and Knight 2009). However, it provides information on only a relatively small region of a single gene. In most cases, that information is sufficient to generate taxonomic or phylogenetic profiles at about genus-level resolution. In some cases, more careful analysis makes it possible to get species-level or strain-level information. Amplicon sequencing can be highly sensitive to the details of amplification, primer composition, polymerase enzyme, and the PCR program (Gohl et al. 2016).

Shotgun metagenomics (a nontargeted sequencing process) can readily resolve species-level and strain-level classification and provide genome content, functional potential, and some genome assembly for organisms of even modest abundance. However, it remains more expensive than amplicon sequencing, it is less tolerant of low biomass or contaminated samples, and it requires substantially more complex and computationally expensive analytic pipelines.

Metatranscriptomics is in its infancy. In addition to being more expensive because of challenging protocols and the scarcity of computational tools, it is not yet established in which environments or for which health-relevant phenotypes microbial community transcription will prove to be most informative (Franzosa et al. 2014).

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[5]An amplicon is a segment of DNA or RNA that is amplified during a replication event in the cell or during a polymerase chain reaction.

[6]The 18S rRNA gene sequence variants are particularly well-suited for broad-spectrum assays, and the ITS sequence variants are particularly well suited for fungi.

[7]A metatranscriptome is the entirety of the RNA sequences expressed by the microbiome as identified by sequencing.

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Finally, most molecular techniques do not differentiate between current molecular activity (living microorganisms) and previously generated biomolecular pools (dead microorganisms), but those distinctions can be resolved better with culture-based or direct observation methods.

Other Culture-Independent Molecular Profiling Techniques

Metabolomic and metaproteomic techniques that use mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry are among the most prevalent non-sequencing-based, culture-independent approaches to molecular profiling of the human microbiome. To date, MS-based and NMR-based profiling has been used to identify secreted and intracellular microbial products and metabolites, including fatty acids, vitamins, bile salts, and polyphenols. As a subset of the metabolome, lipids from microbiome samples have been profiled with MS-based detection methods after lipid extraction and separation. MS-based detection methods can be used after protein extraction and fractionation by two-dimensional electrophoresis or isotope tagging to profile metaproteomes from microbiome samples. Those approaches enable the quantification of cellular proteins from microbial cells and their post-translational modifications as the direct functional products of microbial metatranscriptomes and metagenomes (Kolmeder and de Vos 2014; Soufi and Soufi 2016). Emerging technologies for localized or in situ metabolomics profiling with such approaches as MS imaging, topographic mapping, and rapid evaporative ionization MS coupled with surgical diathermy devices enable spatial resolution of metabolic profiles within the microbial-community structures (Rath et al. 2012; Bouslimani et al. 2015; Golf et al. 2015).

Various platforms for targeted or untargeted metabolomic surveys and quantification of small molecules from biofluids include gas chromatography, liquid chromatography, capillary electrophoresis coupled with MS, Fourier transform infrared spectroscopy, and NMR spectroscopy (reviewed in Smirnov et al. 2016; Vernocchi et al. 2016). The methods differ in how specifically they can identify analytes, how well analytes can be distinguished, how sensitive the methods are to low molecular concentration, and their dynamic range of detectable molecules, data acquisition speed, and technical complexity of protocols.

MS-based profiling and NMR-based profiling are powerful tools for evaluating metaproteomic and metabolomic functional outputs of microbial activity and host–microorganism interactions. A primary advantage of those techniques over nucleic-acid–based microbiome profiling is the potential to identify microbial molecules that mediate microorganism–microorganism and host–microorganism signaling. However, methodologic limitations include the need to tailor sample preparation to target molecules and the inability to identify a wide array of molecule types simultaneously with a single sample-collection, handling, and preparation protocol. Furthermore, resources for determining accurate molecular identities and for differentiating between host-derived and microbially derived molecules are lacking. Further methodologic, technologic, and resource development is needed to create standardized protocols for metaproteomic and metabolomic profiling of microbiomes.

Direct Observation of the Human Microbiome

Most microbiome analyses have focused on DNA or RNA sequencing or metabolomic analyses, but useful insights into microbiome composition, function, and spatial organization can be gained by using a variety of imaging technologies. Transmission and scanning electron microscopy can be used to visualize microbial community organization in fixed samples but is not well suited to resolving individual taxa or traits in a complex community. Fluorescence in situ hybridization (FISH) can be used to evaluate the taxonomy, location, and organization of microbial community members in fixed microbiome samples. In the FISH method, fluorescently labeled DNA probes that recognize a gene sequence within targeted microbial taxa are hybridized to a fixed intact microbiome sample and imaged to visualize the location of the

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microbial cells that contain the corresponding DNA sequence with micrometer resolution. It can be performed with probes that recognize single taxa or multiplexed to target diverse taxa in a single sample (Earle et al. 2015; Mark Welch et al. 2016). Fluorescence-activated cell sorting can be used similarly to quantify and sort microbial cells that are dissociated from a microbiome sample and that display a phenotype that is detectable with a fluorescent marker, such as an exogenous fluorescent probe or genetically encoded fluorescent protein (Maurice et al. 2013; Ambriz-Aviña et al. 2014).

The above methods require fixation or dissociation of a microbial community, but other methods can be used to visualize microbial location and behavior in live animals. In mice, microbial taxa engineered to encode fluorescent reporter proteins can be visualized, although spatial resolution is low because of the opacity of host tissues (Wiles et al. 2006). In contrast, the optical transparency of the zebrafish permits high-resolution and longitudinal in vivo imaging of microbial cell location and behavior (Rawls et al. 2007; Jemielita et al. 2014) and location of nutrients (Semova et al. 2012).

When viable microbial community samples are available,[8] their physiology can also be directly evaluated with enzymatic assays, which can measure growth (such as changes in optical density), colony (or microcolony) structure, or metabolic activity (such as pH or oxygen use). Direct enzymatic activity screens are more challenging to apply to microbiome samples but are practical in assessing the physiology of individual isolates from the microbiome that can be cultured (Tasse et al. 2010; Cohen et al. 2015; Koppel and Balscus 2016). There are high-throughput platforms for enzymatic assays (Jiang et al. 2015; Kaiko et al. 2016; Biggs et al. 2017), but they are not as well developed as high-throughput molecular profiling assays.

Finally, genetic screens and modifications can be used to observe microbial communities. Functional metagenomics (Lam et al. 2015) uses phenotypic screens that generally involve isolating large DNA fragments from a microbiome and generating a library of clones in a species, such as *Escherichia coli*, that lacks the function of interest. The library of clones can then be cultured under selective conditions, for example, with antibiotics. Assaying for a desired trait, such as antibiotic resistance or enzymatic activity, can identify the DNA sequence fragments that confer the trait and can potentially identify the microbiome member that encodes the given trait. Other single-organism genetic tools that can be extended to communities include transposon mutagenesis, forward and reverse genetics, and the introduction (or removal) of entire organisms (wild isolates or engineered organisms) to assess the resulting genetic or organismal effects on community phenotype. Recent advances in genetic manipulation, such as CRISPR-based editing and chemical mutagenesis, have begun to be applied to microbial communities (Mimee et al. 2015; Bae et al. 2016) and are expected to increase the ability to manipulate host-associated microbial interactions experimentally.

Direct observation of microbial communities can provide extremely precise, spatially detailed information regarding host-microbial interactions (Mark Welch et al. 2016). Likewise, microbial genetic manipulation has an extremely long and powerful history and allows precise molecular hypotheses to be tested in situ. Both techniques can be technically challenging in the human microbiome or associated models. Direct microscopy does not typically resolve more than tens of different organisms, for example, and taxa typically not higher than the genus. Likewise, genetic manipulation in whole microbial communities requires careful recolonization of a model by modified organisms, completely gnotobiotic manipulation in animal systems, or comprehensive transformation of community members in situ, all of which are technically challenging to conduct and verify. When they are appropriate, however, these systems offer among the most targeted mechanistic molecular tests in reductionist models of human microbial biotransformations.

[8]Viability is surprisingly difficult to assess in a culture-independent manner, but sequencing has now been successfully coupled with a variety of DNA-intercalating dyes, such as propidium monoazide, for determining whole-community viability (Emerson et al. 2017).

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ANALYZING MICROBIOME POPULATION AND EXPOSURE DATA

The Human Microbiome and Molecular-Epidemiology Analytic Approaches

As noted earlier, most current analytic methods for studying the human microbiome use techniques related to molecular epidemiology, which generally follow a strategy in which features of interest are bioinformatically quantified from culture-independent data and then statistically associated with environmental or health-related covariates and outcomes (Franzosa et al. 2015). Features used to describe the microbiome can include operational taxonomic unit[9] counts or abundances derived from amplicon sequencing (Hamady and Knight 2009); species or strains detected with metagenome sequencing (Truong et al. 2015; Donati et al. 2016); functional profiles (gene or pathway quantifications) in metagenomes or metatranscriptomes (Abubucker et al. 2012); ecologic summary statistics, such as species distributions or diversity (Hamady and Knight 2009); or partial to near-complete genome assemblies and annotations (Sangwan et al. 2016). Ultimately, any feature can be quantitatively modeled as a matrix of abundances or presence-or-absences, and samples can be additionally annotated with metadata, including outcome measures (health status or clinical phenotypes); host demographics or biometrics; population structure, such as ethnicity or genetic background; covariates, such as medications and diet; other molecular measures, such as microbial metabolites or gene expression; or environmental exposures.

Multivariate statistical modeling techniques—such as generalized linear modeling, factor analysis, variations on ordination, correspondence analysis, partial least-squares analysis, or nonparametric analysis of variance—are then applied. Such statistical or machine-learning methods are not unique to microbial-community epidemiology but are shared with other high-dimensional population analyses. For example, linear modeling is typically adapted to associate multiple population variables—such as health outcomes, demographics, biometrics, and chemical exposures—with microbial variables (Morgan et al. 2012, 2015), taking into account the mathematical properties of typical microbial measurements (sparse, zero-inflated, count-based, or proportional data). Nonparametric tests originally developed for quantitative ecology (Excoffier et al. 1992; Zapala and Schork 2006) are appropriate for determining whether overall variance in microbial community structure, as opposed to individual microbial features, is explained by covariates. Predictive models, such as random forests or support vector machines (Pasolli et al. 2016), can also be used to link microbial features to health outcomes or covariates. All the tests essentially detect microbial feature associations with covariates, including chemical exposures or exposure-related health outcomes, that occur more strongly than would be expected by chance (Paulson et al. 2013; Foxman and Martin 2015); these associations are similar to ones that can be observed and studied for gene expression or human genetic variation in other statistical -omics settings. The methods are typically well suited to large population studies that can indicate associations and can contribute to the generation of hypotheses that need to be probed in more detail with other methods to gain insight about causality and mechanisms.

Ecologic and Systems-Biology Analyses of the Human Microbiome

Other common analyses of the human microbiome use a systems-biology approach with the goal of identifying functional relationships among microorganisms, cells, or molecules. They might target molecular-interaction networks or ecologic structures in microbial communities directly (Faust et al. 2012; Friedman and Alm 2012; Kurtz et al. 2015) or in association with human immune-cell subsets (Amit et al. 2011). Molecular-network reconstruction techniques include identifying functionally related gene products by using co-expression data; this has been particularly successful in recovering human molecular regulatory programs during microbial exposure in immune-cell subsets (Haberman et al. 2014; Morgan et

[9]Operational taxonomic units are used to cluster sequences on the basis of similarity (Nguyen et al. 2016).

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al. 2015; O'Connell et al. 2016). Similar data and techniques can be used to reconstruct regulatory and metabolic networks within microbial communities themselves, typically relying more on genomic potential (metagenome annotations) than on transcriptional profiling (Carr et al. 2013; Nielsen et al. 2014). The co-variation approach or other types of guilt-by-association approaches to identifying related molecules within a network can be extended to include phylogenetic information or profiling (Eisen 1998; Carr et al. 2013; Lan et al. 2014) or inferred metabolic capabilities by flux balance analysis (Zengler and Palsson 2012; Khandelwal et al. 2013; Hanemaaijer et al. 2015; Zelezniak et al. 2015). However, all the methods can be challenging to carry out in the microbiome, where, in contrast to the human genome, most microbial gene products are not annotated with well-characterized molecular or biochemical roles.

Analyses intended to characterize ecologic structure include models of microbial dispersion (such as entry of microorganisms into a community) (Costello et al. 2012), transmission (movement of microorganisms between communities) (Blaser and Falkow 2009; Funkhouser and Bordenstein 2013; Milani et al. 2015), and co-occurrence (ecologic relationships, such as symbiosis or competition between microorganisms) (Faust et al. 2012; Friedman and Alm 2012; Kurtz et al. 2015). Because nearly all molecular assays measure relative abundance (compositions) rather than absolute cell counts, spurious correlations make it difficult to infer truly functional co-occurrence patterns (Tsilimigras and Fodor 2016). Dynamic systems models capture relationships in abundance patterns among organisms over time and have also been used to describe microbial interaction patterns. Examples of dynamic systems models include differential equations—for example, modified Lotka-Volterra systems (Stein et al. 2013; Marino et al. 2014; Bucci et al. 2016)—and probabilistic graphical models, for example, Gaussian processes (Tonner et al. 2017). Again, the level of detail can be difficult to reach with current data and modeling techniques because of the lack of taxonomically precise (strain-level) profiles sampled sufficiently densely over time to construct models outside simplified, in vitro systems.

STRENGTHS, WEAKNESSES, AND GAPS IN TECHNOLOGIES FOR STUDYING RELATIONSHIPS BETWEEN THE MICROBIOME AND CHEMICAL EXPOSURE

Systems

The microbiome field has available a diverse spectrum of experimental-animal systems that offer rigorous experimental control and provide distinct opportunities to define causality within host-microbiome–chemical interactions. However, as in all fields, researchers need to understand the strengths and weaknesses of each system and choose from among them appropriately. A persistent challenge in the use of nonhuman experimental systems to study the microbiome is to define which aspects of human-microbiome–chemical interactions can be effectively modeled and examined in each setting. To address that challenge, researchers need to improve their understanding of which aspects of each model system are reflective of humans, which ones are not, and which ones are likely to be relevant to host-microbiome–chemical interactions. Because the field relies heavily on microbiome transplant studies in animal models, experiments that include chemical treatment and microbiome transplantation will need to determine how to account and control for potential carryover of a chemical from the chemically exposed donor to the unexposed recipient via the transplanted microbiome. Finally, inasmuch as understanding of the field is based largely on cross-sectional sequence-based data, increased efforts need to complement the data with information on additional molecular activities and the spatial or temporal dynamics of microbial communities.

In vitro microbial-community model systems share many of the strengths and weaknesses of animal models but to a greater degree. For example, they are easier to manipulate and control, but they are less physiologically similar to a human, particularly because they lack host cellular and immune responses. Attention must be paid to how a chemical is introduced into the experimental systems and how the resulting exposure is measured and characterized. Specifically, in vitro systems that use static or flow-through

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technology present challenges in delivering specific, known amounts of chemical to the target organelles, cells, or tissues. In culture-based systems, genomic methods are well established for bacteria and their communities but less established for fungi, archaea, and viruses. Gaps for the other microorganisms include a lack of reference genomes, culture conditions for isolates, and adaptability for genetic manipulation. However, in vitro systems are often extremely cost-effective and scalable, and they are particularly well suited to screening assays, such as microorganism–microorganism or microorganism–chemical interaction testing. In vitro systems allow, for example, the introduction of potentially bioactive (positively or negatively) chemical exposures into a controlled microbial (typically not host-associated) setting with accompanying readout of microbial metabolism.

It is important in all such model systems to consider and integrate information from systems at various levels of reductionist scale, that is, from single microbial isolate cultures through human population measurements. A striking challenge in integrating results from systems across all scales is the small extent to which microbial gene products have been characterized. The lack of knowledge limits interpretation in vivo and manipulation in vitro.

Analyses

Analysis of human microbiome data, regardless of their source or assay method, can benefit from the approaches that have been developed over the last 2 decades in other fields that use molecular -omics approaches. Specifically, many associative studies share designs and methods with those in molecular epidemiology, such as genome-wide association studies or cancer-biomarker discovery that analyzes gene expression. With small statistical changes, computational methods and lessons learned from those other fields can be directly applied in microbiome research. The availability of individual microbial isolate reference data (primarily genome sequences) to contextualize microbial-community data is both a strength and a weakness: tens of thousands of reference genomes are available and constitute a powerful resource with which to interpret the microbiome, but these reference genomes are primarily bacterial, and there is a major gap if one wants to study viruses, fungi, and other microorganisms. Another major gap in the field is that most sequenced microbial genes and microorganism-associated chemicals that have been detected are not functionally or biochemically characterized; it is not even clear what fraction of them has been detected. That situation leads to a pool of biochemically functional "dark matter" with as-yet-unknown effects on microbial ecology or human health. Finally, as in most fields of molecular -omics, new computational methods will continue to be needed for integrating many types of microbial-community data; new methods will lead to increasingly accurate methods for identifying associations between molecular activities in the assays and human health outcomes.

FINDINGS

- Various animal models that have extensive conserved molecular and immunologic mechanisms provide appropriate experimental environments for controlled manipulation of host-associated microbial communities, although none mimics humans perfectly.
- Gnotobiotic animal models are particularly amenable to studies of the effect of microbial-community composition on host phenotype. Their use would benefit from more study of which aspects are shared (or not) with humans under different manipulations at each body site.
- Animal experiments that include chemical treatment and microbiome transplantation will need to determine how to differentiate carryover of a chemical from an exposed donor to an unexposed recipient via the transplanted microbiome.
- In vitro and ex vivo techniques can be usefully adapted to characterize diverse human-microbiome members and representative communities, but identifying appropriate culture conditions and models poses technical challenges.

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- Human-microbiome experimental systems remain less developed outside the gut.
- As microbiome research is a young field, diversity in experimental protocols can make comparability of results among human-microbiome studies difficult.
- Computational methods and quantitative best practices of other -omics technologies can generally be applied to microbiome data with appropriate adaptations of statistical techniques.
- Most microbial genes and microbially associated chemicals in the microbiome are not functionally or biochemically characterized, and it is not even clear what fraction of them has been detected.

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5

Risk Assessment: Incorporating Chemical–Microbiome Interactions

Enormous advances have been made in the last several decades in the sciences devoted to understanding the health effects of environmental chemicals, but substantial knowledge gaps still leave large uncertainties in health risk assessments. Studies of chemical–microbiome interactions and their consequences indicate that further research could advance understanding of human health risk posed by exposure to environmental chemicals. Specifically, understanding chemical–microbiome interactions is likely to improve the use of results of studies in epidemiology, toxicology, and exposure science in carrying out risk assessments. Knowledge of chemical–microbiome interactions might also help to explain differences between animal toxicity studies and human responses, to extrapolate research findings from animal studies to humans, and to identify unrecognized health consequences of environmental exposures. The large variation in the microbiome compositions in populations of different life stages, sexes, and ethnicities might inform the extrapolation of findings of studies of laboratory animals to human populations. Epidemiology studies in different populations might sometimes reveal different responses to chemical exposure, and it is possible that those differences might be explained by population variation in microbiome composition. It is reasonable to hypothesize that adequate consideration of the roles of human microbiomes will improve understanding of the health risks posed by exposures to environmental chemicals.

This chapter discusses aspects of the integration of microbiome considerations into risk assessment. First, the risk-assessment process and data sources are briefly reviewed. Next, major risk-assessment issues in chemical–microbiome interactions are identified. Because exposure assessment is a key element of the risk-assessment process, exposure-assessment challenges are discussed in the context of the human microbiome, and several examples are provided to illustrate the challenges. A discussion of research needed to address risk-assessment needs concludes the chapter and sets up the committee's research strategy described in Chapter 6.

THE RISK-ASSESSMENT PROCESS

Risk assessment used in regulatory programs in the United States and globally has been undergoing considerable reform and advancement in recent years. Much of the reform is aimed at moving from intensive chemical-by-chemical assessment to large-scale assessments that might more easily determine which of the thousands of chemicals used in industry pose health risks that should be assessed in depth. Changes also have been proposed that will improve the usefulness of risk-assessment results for making risk-management decisions (Schaafsma et al. 2009; Krewski et al. 2014).

Science and Decisions: Advancing Risk Assessment (NRC 2009) provides widely accepted guidance on ensuring the scientific adequacy of risk assessments and their utility for decision-making. Effective decisions begin with development of a clear and complete understanding of the problem for which a decision is needed. That initial problem formulation is then used to guide the development of a risk assessment that is certain to be useful for decision-making. The risk assessment is then conducted by using a general framework first proposed in a 1983 National Research Council study (NRC 1983). That framework, illustrated in Figure 5-1, is still considered valid and is used by the US Environmental Protection

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Agency (EPA) and related agencies. In addition, EPA has developed numerous guidelines for the conduct of risk assessment (EPA 2014a), including guidelines for addressing the recommendations in *Science and Decisions*. EPA guidelines (EPA 2016) describe the optimal evaluation and use of data that often contain inconsistencies and that require proper treatment of uncertainty in extrapolation of results from animal or human studies of limited scope to policies designed to protect the general public. Other federal agencies have developed guidelines to meet their risk-assessment needs.

It is important to note that the *Science and Decisions* model can be used to guide the development of a research program of the type outlined in Chapter 6 of the present report. The problem to be addressed—understanding the role of chemical–microbiome interactions in human health risk—leads to the formulation of research questions whose answers make risk assessments that include consideration of the microbiome and its influences feasible.

DATA SOURCES AND REQUIREMENTS FOR RISK ASSESSMENT

Most of the environmental toxicology data used for hazard identification and dose–response assessment (Figure 5-1) are derived from studies of experimental animals. Internationally standardized protocols for animal studies are available to investigate general toxicities and a wide array of effects, including effects on reproduction and development and effects on the immune, nervous, and endocrine systems. Epidemiology studies have contributed valuable information on some important environmental pollutants; often, they are based on exposures in occupational settings. Toxicity data from fundamental research are also used when available. Much of the basic toxicology research has focused on specialized end points and underlying mechanisms of toxic action. Various technologies have become available to study chemical interactions and responses at the molecular and cellular levels, and this knowledge provides information on toxicity mechanisms (NRC 2007; NASEM 2017). Such approaches are being scaled to high-throughput formats for rapid evaluations of large numbers of chemicals, including chemicals that have not been studied previously to any substantial degree (Kavlock and Dix 2010). Environmental toxicology is also changing with the use of gene-editing technologies that allow rapid probing of the genetic aspects of toxicity mechanisms (Shen et al. 2015).

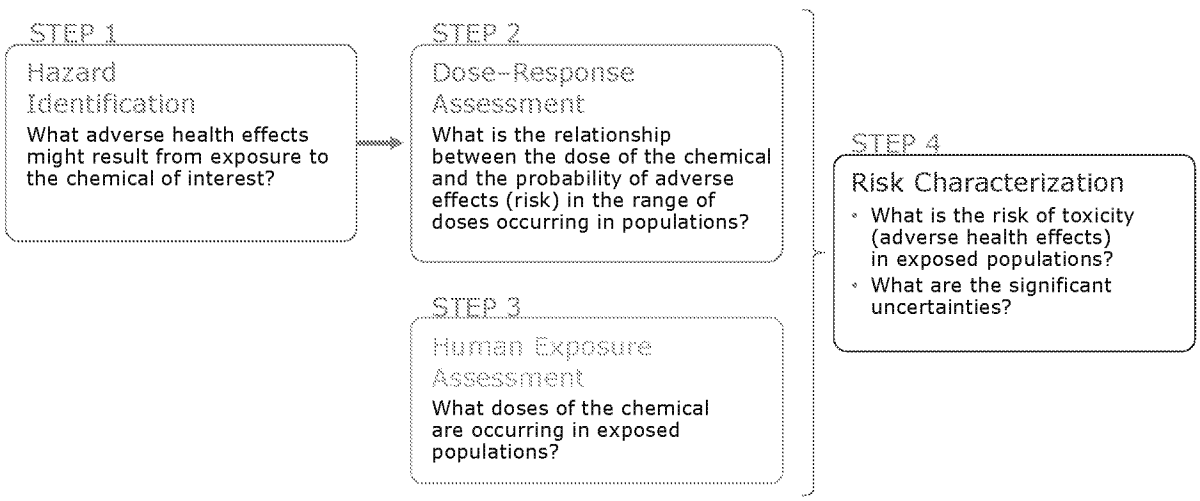

FIGURE 5-1 The standard four-step framework for risk assessment.

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Exposure science, essential for understanding human health risk, has undergone remarkable advances in the last few decades (NRC 2012; NASEM 2017). The science has moved understanding of human exposures to chemicals from simple descriptions of the presence of a chemical in air, water, food, or a consumer product to far more complete depictions of multiple chemical exposures on and in the body and of the variations in these exposures over life stages and in different population groups. The technologies for developing more rapid and complete exposure profiles, from the use of remote and personal sensors to the identification and sampling of key biomarkers, are contributing copious new data for environmental risk assessment. Characterization of animal and human exposure (and effects) has advanced through the use of biomonitoring, biomarkers, and physiologically based pharmacokinetic models (Vandenberg et al. 2010), which facilitate elucidation of the absorption, distribution, metabolism, and excretion of chemicals and have become especially important in informing interspecies extrapolations and characterizing interindividual variability.

EPA usually initiates a risk assessment only when there is sufficient and convincing evidence from whole-animal or epidemiology studies that exposure to a substance is causally related to one or more adverse health effects and when those studies also provide information on dose–response relationships. Research will be needed to develop and test protocols for microbiome health-effects studies that yield dose–response information; current protocols for developing toxicity data do not explicitly take into account a role of the microbiome in affecting outcomes.

MAJOR RISK-ASSESSMENT ISSUES RELATED TO CHEMICAL–MICROBIOME INTERACTIONS

As discussed in Chapter 3, research with pharmaceuticals has shown that some chemicals (for example, antimicrobial pharmaceuticals) can harm or alter human and animal microbiomes. And research with environmental chemicals and pharmaceuticals has shown that the microbiome can alter internal exposures to some chemicals by, for example, transforming a chemical to a more or less toxic form or altering uptake of a chemical. Those types of effects might not be fully evaluated in current risk-assessment practice (Dietert and Silbergeld 2015). As a result, a risk assessment might fail to provide adequate protection of the general population if chemical–microbiome interactions are not incorporated into studies implicitly or are not explicitly addressed, particularly when results from studies in animals or in a specific population are used to characterize risk to another species or population that has a different microbiome composition and function from that of the studied population. The outcome might be a mischaracterization of the nature of a hazard associated with exposure to an environmental chemical—for example, chemical–microbiome interactions might produce a different health effect from the chemical itself—or an overestimation or underestimation of the risk associated with exposure. The following sections consider the implications for each study type used in risk assessment.

Epidemiology Studies

The chemical–microbiome interaction of whatever form and magnitude is presumably integrated into epidemiology studies that are conducted in large populations and include health and exposure assessments throughout the subjects' lifetimes (or key life stages) of exposure and in a variety of potentially confounding disease states. However, current understanding of the microbiome suggests that the results of such epidemiology studies might be useful only in describing risks to similar populations. An understanding of chemical–microbiome interactions in a population might be critical when using epidemiologic results from studies conducted in populations of different cultures, locations, life stages, and other factors that affect the microbiome. When chemical–microbiome interactions are substantive in modifying exposure or harming the microbiome, researchers might find that an incomplete understanding of the composition and role of the microbiome has complicated and limited the use of epidemiology studies in risk assessment. If more knowledge of the role of differences in the microbiome among populations in

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influencing chemical sensitivity were available, the information could indicate whether a study population is more or less sensitive than the general US population and thereby inform decisions regarding the appropriate magnitude of uncertainty factors.

Animal Toxicity Studies

Chemical–microbiome interactions are integrated into whole-animal toxicity studies. However, such studies typically are conducted with a homogeneous, in-bred group of animals that are maintained in standardized laboratory conditions that will affect their microbiomes. Animal studies are rarely carried out from preconception through natural death, so the temporal changes in microbiomes that contribute to risks and benefits over a lifetime might not be seen. They also rarely use experimental designs that control for common variation in microbiome composition between and within animal facilities. Furthermore, housing and test conditions are intended to minimize nonchemical stress, including exposure to pathogens.

If there are chemical–microbiome interactions that affect toxicity, a thorough understanding of the limitations in extrapolating the laboratory-animal results to humans might be necessary. For example, the mode of exposure of research animals could affect the microbiome in ways that influence risk in humans and animals differently; a gavage dose administered as a bolus, even when equivalent in milligrams per kilogram per day, might affect the microbiome of the digestive tract in ways that dietary or environmental exposure would not. Furthermore, the vehicle of administration could influence the microbiome in laboratory animals, and the temporal pattern of exposure could influence the microbiome in a manner that differs between laboratory animals and humans. A wide range of doses from low to high will need to be investigated, and risk assessors will need to know whether current default uncertainty and variability factors that have been used to extrapolate from animals to humans are sufficiently protective of public health. As with epidemiology studies, an understanding of the microbiome in the population targeted for public-health protection will be important throughout all susceptible life stages and disease conditions.

In Vitro Studies

Data derived from in vitro studies and from high-throughput testing alone are not considered a sufficient basis for risk assessment of new chemicals. However, risk assessors are interested in how to use those results in risk assessment, and the data have been recognized as valuable for providing important insights on toxicity mechanisms and setting priorities for in-depth toxicity testing (NRC 2007; NASEM 2017). In the context of the microbiome, however, the challenge of using newer techniques to screen chemicals for toxicity and exposure is exacerbated by the likelihood that the data do not incorporate chemical–microbiome interactions. New methods will be needed to expand in vitro and high-throughput testing to include the effects of the microbiome in mediating toxicity. Some types of in vitro studies might be well suited to testing the direct effect of chemicals on the microbiome and its functions (see Chapter 4).

ADDRESSING EXPOSURE CHALLENGES

In considering how the interactions between environmental chemicals and the human microbiome might influence human health risk, proper characterization of exposures plays a central role. As defined by Zartarian et al. (2005), exposure constitutes the "contact between an agent and a target. Contact takes place at an exposure surface over an exposure period." A 2012 National Research Council report, *Exposure Science in the 21st Century: A Vision and a Strategy*, stated that "exposure science addresses the intensity and duration of contact of humans or other organisms with … chemical, physical, or biologic stressors … and their fate in living systems" (NRC 2012). A key idea is that to capture its influence on risk, exposure must be characterized both conceptually and quantitatively.

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How the human microbiome might mediate health risk associated with exposure to environmental chemicals, however, is barely addressed in the exposure-science literature. For example, NRC (2012) aimed to define the scope of exposure science and stated that "a central theme of this report is the interplay between the external and internal environments and the opportunity for exposure science to exploit novel technologies for assessing biologically active internal exposures from external sources"; the report does not mention the human microbiome. Similarly, exposure science has emphasized the use of information on exposures to environmental chemicals to support quantitative assessments of the associated human health risks (Fenske 2010). An important tool in risk assessment is the EPA *Exposure Factors Handbook* (EPA 2011); the latest edition, which spans more than 1,500 pages in 19 chapters, contains only brief mentions of microorganisms. It is evident that the exposure-science research community has devoted relatively little attention to microbial exposures of humans; although there is some work defining risk associated with pathogens, the community has been nearly silent on the human microbiome.

The current state presents a challenge and an opportunity. There is a need to expand the scope of exposure science to incorporate the emerging understanding of the roles of the human microbiome as an agent that influences exposures to and risks posed by environmental chemicals. Because knowledge is developing rapidly in this field, there will be a need to refresh the effort on a regular basis.

In the near term, risk assessments will likely continue to incorporate risk factors derived from laboratory studies of animal models. A critical feature for generating accurate risk factors is the proper characterization of exposures in the test animals. Exposure science has made and can continue to make important contributions to such efforts. Doing that well for circumstances in which health risks are influenced by microbiomes will require amendment of some of the core ideas in exposure science. For example, it will be necessary to rethink the concepts of "external" and "internal" in relation to exposure. Traditionally, an external exposure is related to interactions that occur at contact surfaces. Hence, exposure science would seek to quantify the nature and extent of interaction between a chemical and a human receptor at the boundaries that separate the environment from the human body, such as in lung tissues, on skin surfaces, and at the gastrointestinal epithelium. Those ideas could be readily extended to address cases in which a surface-resident microbiota mediates exposure by transforming the chemical or changing the permeability of the epithelium. The alteration of the chemical might influence the associated risk for many reasons, for example, by changing the rate of uptake across the body's tissues, influencing chemical fate within the body, and changing the toxicity of the agent. In such cases, it could be appropriate to consider the composition and function of the human microbiome as an exposure factor. However, a greater challenge is to incorporate within the framework of exposure science the potential for chemical exposures to alter the human microbiome itself and thereby influence risks. For the specific case of the gastrointestinal tract, it is not clear how to define where the contact surface occurs. How to apply the conceptual differentiation between external and internal exposure is not apparent when the target is a human-associated community of microorganisms that might be influenced by the chemical and interacts with its human host.

The following sections provide examples that illustrate some of the challenges and opportunities in integrating exposure-science principles into studies of how the human microbiome influences risks posed by exposure to environmental chemicals. The examples highlight chemicals that are recognized as environmental health risks and about which there is at least suggestive evidence that microbiome–chemical interactions could modulate their exposure or health risk. One or two examples are provided that pertain to each of the major microbiome sites listed in the committee's statement of task (gut, skin, and respiratory tract).

Formaldehyde and the Upper Respiratory Tract

Formaldehyde is a widely used industrial chemical. Indoor formaldehyde concentrations can be increased by emissions from indoor sources, most notably urea-formaldehyde resins that are used in the manufacturing of wood-based construction materials, such as plywood (Salthammer et al. 2010). Historically important concerns about formaldehyde exposure have been associated with the use of a spray-foam

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insulation material in the 1970s (L'Abbé and Hoey 1984) and with trailers used for emergency housing in the aftermath of flooding associated with Hurricane Katrina (Murphy et al. 2013). EPA recently issued regulations limiting emissions of formaldehyde from wood products (81Fed. Reg. 89674 [2016]).

Formaldehyde has a low molecular mass, high vapor pressure, and high water solubility (Salthammer et al. 2010). Because of its high mobility and strong tendency to partition into aqueous solutions, the primary sites of exposure to formaldehyde are the upper respiratory tract and the eyes. In California, the chronic-exposure reference concentration is 9 $\mu g/m^3$ (OEHHA 2016), a concentration that is routinely exceeded in indoor environments (Salthammer et al. 2010). Furthermore, the International Agency for Research on Cancer (IARC 2012) concluded that "formaldehyde is *carcinogenic to humans,*" and the National Toxicology Program (NTP 2016) concluded that formaldehyde is "known to be a human carcinogen."

Formaldehyde is used as a disinfectant and sterilant. In liquid form, it has a wide range of effectiveness by "alkylating the amino and sulfhydral groups of proteins and ring nitrogen atoms of purine bases" (Rutala et al. 2008). The question is whether inhalation exposure to formaldehyde at high concentrations indoors could disrupt the human microbiome associated with upper airways? If so, would such disruption alter health risks posed by the exposure? The literature contains no clear evidence on that point. However, given the importance of known adverse health effects of formaldehyde, a relatively straightforward exposure pathway, and the antimicrobial properties of formaldehyde, it seems to be a strong candidate for studies to investigate whether and how exposure to an environmental chemical might interact with the microbiota of the upper airways in a manner that influences health risks. What is particularly germane is whether exposures to formaldehyde at concentrations encountered (or potentially encountered) in the environment interact with the microbiota in the upper airways in a manner that materially influences associated health risks, considering both irritancy responses associated with acute exposures and cancer risk associated with cumulative exposures.

Phthalates and the Transdermal Pathway

Phthalates are a class of semivolatile organic compounds widely used in commercial products, including vinyl flooring and many consumer products. One important application of phthalates is as plasticizers: they are added to polymeric materials to provide flexibility. In that function, the phthalates are not bound to the host polymeric material but instead can migrate into other media. Indoor concentrations of several phthalates are commonly much higher than outdoor concentrations (Rudel et al. 2010), including butyl benzyl phthalate, bis(2-ethylhexyl) phthalate (DEHP), diethyl phthalate (DEP), dibutyl phthalate (DBP), and diisobutyl phthalate. Among the health concerns associated with phthalate exposures are reproductive toxicity and developmental toxicity (Kavlock et al. 2006; Lyche et al. 2009; Kay et al. 2014).

Human exposure to phthalates can occur through multiple pathways, including ingestion (dietary and nondietary), inhalation, and transdermal routes (Colacino et al. 2010; Bekö et al. 2013). Recent research has shown that transdermal permeation can make a contribution to human intake of the relatively volatile species DEP and DBP that is quantitatively similar to that of inhalation (Weschler et al. 2015). And clothing has been found to be an important moderator of dermal exposure. Initially clean clothing can inhibit dermal exposure, whereas previously worn clothing exposed to airborne phthalates at high concentrations can be a vector for increased uptake (Morrison et al. 2016).

In the case of DEHP, the effectiveness of uptake has been linked to its chemical conversion to the monoester, mono(2-ethyhexyl) phthalate (MEHP). Lipases are known to play an important role in that process, and the presence of lipases in microorganisms has been noted (Nakamiya et al. 2005; Kavlock et al. 2006). Although, the extent to which such conversion occurs because of gastrointestinal or skin microbiota is unknown, Hopf et al. (2014) have shown that when DEHP is applied to viable skin in aqueous emulsion, the DEHP is converted to MEHP, which can permeate the skin more effectively. Several microbial species have been shown to convert DEHP to MEHP. It appears worthwhile to investigate further whether skin-associated and other microbiomes mediate phthalate uptake and thereby influence risk through chemical conversion of the diesters to monoesters.

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Triclosan and the Microbiome

Triclosan presents a potentially important case to consider. It was created as an antimicrobial agent for use in health-care settings. Because of concerns about outbreaks of new diseases, such as severe acute respiratory syndrome, triclosan began to be widely incorporated into a broad array of items, including many cleaning agents and personal-care products. The combination of deliberate, nonspecific antimicrobial action and widespread distribution into the uncontrolled environment raises general and specific concerns. Halden (2014) effectively summarized the issue as follows: "The polychlorinated aromatic antimicrobials triclosan and triclocarban are in widespread use for killing microorganisms indiscriminately, rapidly, and by nonspecific action. While their utility in healthcare settings is undisputed, benefits to users of antimicrobial personal care products are few to none. Yet, these latter, high-volume uses have caused widespread contamination of the environment, wildlife, and human populations."

Because triclosan is widely used in liquid products applied on the body surface, the general population experiences a high degree of exposure intimacy. On the basis of US production and import data and biomonitoring evidence, Nazaroff et al. (2012) estimated that about 1–2% of all the triclosan used in US commerce enters human bodies and is excreted in urine. Research of Csiszar et al. (2016) substantiates that finding: considering 518 chemicals used in personal-care products, they found that the median product intake fraction was 2% for chemicals in wash-off products and 50% for chemicals in leave-on products. Exposures of the human microbiome as a consequence of inadvertent ingestion (for example, of toothpaste and mouthwash), dermal product use (for example, soaps), and inhalation (Mandin et al. 2016) are certain to occur.

Research is beginning to probe whether exposure to triclosan can disrupt the microbiome. Recent animal studies indicate that triclosan exposure can affect the gut microbiome. For example, exposure of mice to triclosan via drinking water caused an alteration in gut microbial composition that favored the selection of bacteria that had genes related to "triclosan resistance, stress response, antibiotic resistance and heavy metal resistance" (Gao et al. 2017). And exposure of zebrafish to triclosan via the diet altered composition and ecologic dynamics of the gut microbiota (Gaulke et al. 2016).

Human studies have also explored the possible effects of triclosan on the microbiome. Poole et al. (2016) conducted a double-blind crossover study in which 13–16 healthy subjects used household and personal-care products that did or did not contain triclosan and triclocarban for 4-month periods. They concluded that "although there was a significant difference in the amount of triclosan in the urine between the [trial] phases, no differences were found in microbiome composition, metabolic or endocrine markers, or weight." However, Yee and Gilbert (2016) summarized the evidence about the possible role of triclosan in shaping the human microbiome. They highlight the importance of considering hospitals that provide maternity services and note that more than 98% of infants "are particularly naïve to microbes [and] their microbiota is vulnerable at this developmental stage." Given widespread human exposure, research to investigate the effects of triclosan on the human microbiome and to answer such questions as whether early-life exposure to triclosan is predisposing infants to adverse health outcomes appears to be warranted.

Nitrate, Arsenic, and the Gut Microbiome: A Case for Re-evaluation?

As discussed in Chapter 2, the gut is the site with the greatest mass of microbiota, and it is the best studied. A rapidly developing literature describes the many ways in which the gut microbiome influences human health. A smaller literature is emerging on how the gut microbiota mediates health risks posed by exposures to environmental chemicals. Two examples are discussed briefly here to illustrate the nature and significance of how transformations of environmental chemicals that are influenced by the gut microbiome might alter health risk.

Nitrate exposure is linked to the blood disorder methemoglobinemia (blue-baby syndrome), a potentially fatal condition in neonates. EPA's current health risk assessment of nitrate is based on infant susceptibility (EPA 1991). Although fetal hemoglobin, intestinal pH, and other factors increase susceptibility (Nelson and Hostetler 2003), EPA's supporting data include a concern that nitrate toxicity appears to be

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exacerbated by gastrointestinal illness in infants. Research has shown that multiple factors influence bacteria of the infant intestinal tract and nitrate bioactivation (Jones et al. 2015). However, research has not been conducted to characterize and quantify the relationship between the gut microbiome, nitrate exposure, and the risk of methemoglobinemia. Research could be conducted to test the current concern that infant intestinal health status is a key component of nitrate risk assessment. Tools are available to characterize the microbiome in healthy infants and to quantify ex vivo bioactivation of nitrate and nitrite under various conditions. Such research would establish a baseline with which microbiome composition and metabolic capacity of infants who have intestinal illness could be compared. New information on the role of the microbiome in altering susceptibility to nitrate toxicity in infants could be important in refining the outdated assessment that focused on infants or in altering future health risk assessments for nitrate exposure at other life stages.

Arsenic is a ubiquitous contaminant of natural systems with important potential for harming human health. Attaining public drinking-water standards and soil guidance concentrations has been problematic (ATSDR 2007; EPA 2010; NRC 2013; Carlin et al. 2016). Accurate risk assessments of arsenic exposure are important both to protect public health and to ensure that expenditures for water treatment and soil remediation are warranted. Arsenic risk assessments have been based partly on epidemiology studies conducted in multiple countries, including Taiwan, Chile, Argentina, and Bangladesh (EPA 2010; FDA 2016). Those studies are based on populations whose dietary intake can be increased by arsenic in water, rice, and foods cooked in arsenic-contaminated water. As EPA conducts a new arsenic risk assessment, the role of arsenic ingestion will continue to be a key scientific issue (EPA 2014b). Current risk assessments do not incorporate the emerging evidence, described in Chapter 3, that the gut microbiome affects the bioavailability and metabolism of arsenic in animal models and in human microbiome cultures (Diaz-Bone and Van de Wiele 2010; Van de Wiele et al. 2010). New research in mice shows that exposure to arsenic alters the microbiome, perhaps in ways that harm health (Lu et al. 2014). Furthermore, a recently published study found that mice exposed to arsenic at environmentally relevant dietary concentrations had alterations in the gut-microbiome composition and in a variety of important bacterial functional pathways (Chi et al. in press). Characterizing microbiomes in populations from different geographic locations and with different cultural practices (for example, food sources and preparation methods) might reveal different exposure profiles. Differences in the microbiomes of study populations might become as important in interpreting epidemiology study results as measuring arsenic intake in exposed populations.

RESEARCH TO ADDRESS RISK-ASSESSMENT NEEDS AND IMPLICATIONS

Whether interactions between some environmental chemicals and the microbiome have adverse health consequences cannot be known without substantial new research. Accordingly, research is needed to address when the microbiome is a direct target of chemical toxicity and is perturbed by chemical exposures in ways that have measurable adverse health effects on the host, when the microbiome is not itself perturbed in harmful ways but modulates exposure to environmental chemicals, and how variability and variation of the human microbiome influence the consequences of microbiome–environmental-chemical interactions. Research to inform risk assessment could focus on the following elements:

- The extent to which harm to the microbiome is incorporated into or detectable in conventional animal testing.
- The extent to which microbiomes differ substantially among animal strains and species and between humans and animals.
- Characterization of the degree to which microbiomes can recover from insult or adapt to continuing insult.
- How different microbiomes of the body of most relevance to environmental exposures—gut, lungs, and skin—are affected and evaluated.

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- Understanding exposure pathways and how physicochemical properties of environmental chemicals influence exposure and mediate uptake.
- How differences among humans in their microbiomes affect their susceptibility or resistance to environmental chemicals.

In addition to the elements listed above, new approaches might be needed to evaluate dose–response relationships that might be affected simultaneously from chemically induced changes in the microbiome, chemically induced toxicity to the host, and microbiome-induced effects on host health. Understanding and integrating the relative effects on the dose–response relationships will likely pose a key risk-assessment challenge. Chapter 6 describes the committee's research strategy to begin to address the important topics noted.

It is not likely that definitive answers to the important risk-assessment issues will emerge unless a substantial research program is under way; even then, one can expect answers to emerge slowly. Thus, as in all current risk assessment efforts, default assumptions will continue to be used to address data gaps and other uncertainties. Moreover, it might not be possible to develop clear criteria for adverse effects on the microbiome itself, so other targets of toxicity (ranging from intracellular components to organ systems) will remain the subjects of risk assessment even if a chemical has an adverse effect on the human microbiome. Nonetheless, understanding that the microbiome might be adversely affected is important because such knowledge might provide new insights into health effects and human population sensitivities. Uncertainty factors that have traditionally been used in risk assessment should be able to accommodate new knowledge regarding interactions of environmental chemicals and the human microbiome.

IDENTIFYING HEALTH RISK ASSESSMENTS THAT MIGHT NEED RE-EVALUATION

As data on chemical–microbiome interactions emerge and are used in risk assessments, it is likely that some previous risk assessments will be considered outdated and will need to be re-evaluated. The merits of health-risk re-evaluation are well established and based on optimizing public-health benefits, either to provide greater protection from potential health effects or to reduce the expenditure of resources on unnecessary exposure-management actions. Identification of new findings and evaluation of the likelihood that they will alter assessments are activities that are already being pursued in federal and state risk-assessment programs. Indicators of the magnitude of changes in risk that would present opportunities to improve public-health protection have not been uniformly established.

Findings that might result in a re-evaluation of chemical risk assessments include such outcomes as magnitude of change (lower or higher) in toxicity or exposure and the discovery of a previously unrecognized but highly susceptible population or life stage (that is, one that is highly sensitive to the chemical or is highly exposed). Increased susceptibility might be a consequence of such factors as sex, age, behavior, or health status. As new study protocols that account more completely for chemical–microbiome interactions are developed, the resulting data might reveal previously unknown exposures or health outcomes that are important to consider in applying research results to protect public health. Results from the types of studies described in Chapter 6 could inform current risk-management practices and help to guide priorities for future research on chemical–microbiome interactions relevant to health risk assessment. The work could result in changes in variability and uncertainty factors that could be applied to past risk assessments to adjust for a new understanding of chemical–microbiome interactions. It could also provide important information that would help in setting priorities for retesting or re-evaluation. Classes of chemicals, disease states, life stages, health end points, or other generalizable groupings of data could be identified for re-evaluation because the chemical–microbiome interaction has not been fully included in past studies or the interaction indicates greater exposure and health consequences than previously recognized.

A chemical-specific assessment of risk could be undertaken whenever emerging evidence of toxicity or exposure uniquely related to perturbation of the microbiome becomes available. Such work might already be possible in emerging research on arsenic and on nitrate. Results can be compared with past as-

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sessments to begin to gauge the impact that future re-evaluations might have on risk management. On a larger scale, existing risk assessments can be evaluated to determine the extent to which known or possible chemical–microbiome interactions are likely to alter the assessments. A screening evaluation can be used to set priorities for chemicals that warrant reassessment, and new assessments can follow.

Answering the question of whether past assessments of health risk were sufficiently "robust" requires a science-policy finding of the change in health or exposure measures that provides opportunities for public-health protection. A small increment or decrement that is identified in a risk assessment is unlikely to drive wide-scale research in chemical–microbiome interactions or methodologic changes in risk assessment protocols. However, risk managers might support research if the increased risk were found for a highly specific population or site or for an easily regulated chemical.

FINDINGS

- Adequate consideration of the roles of the human microbiome will improve understanding of the health risks posed by exposures to environmental chemicals.
- Data used for hazard identification and dose–response assessment are derived from studies of experimental animals; however, it is not clear that current methods for generating animal data or extrapolating from animals to humans can incorporate the influence of the microbiome on adverse health outcomes properly.
- Characterization of animal and human exposure and health risk has advanced through the use of biomonitoring, biomarkers, and physiologically based pharmacokinetic models. Those methods have not been consistently applied to or do not encompass aspects known to be important for the microbiome, such as life stage, sex, and disease state.
- A risk assessment might fail to provide adequate protection of the general population if chemical–microbiome interactions are not incorporated implicitly into studies or explicitly addressed, particularly when results from studies in animals or in one population are used to characterize risk to another species or population that has a different microbiome composition and function. The outcome might be a mischaracterization of the nature of a hazard associated with exposure to an environmental chemical or an overestimation or underestimation of the risk associated with exposure.
- There is a need to expand the scope of exposure science to incorporate the emerging understanding of the roles of the human microbiome and its components as agents that influence exposures to and risks posed by environmental chemicals.
- Studying how the human microbiome is affected by chemicals requires a clear understanding of the nature and magnitude of change in the microbiome that might result in adverse health effects.

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6

Research Strategy

Developing a research strategy to understand the interactions between environmental chemicals and the human microbiome and the implications of the interactions for human health risk is a complex task. Understanding of how perturbations of the human microbiome might cause or contribute to the development of various diseases is in its infancy, so the task of understanding how environmental chemicals fit into the picture is even more difficult. Initially, the committee envisioned a research strategy that was similar to a flowchart or decision tree in which one or more experiments would lead naturally to a next set of experiments. However, such a straightforward approach is not feasible today given the state of the science. Thus, the committee determined that the research strategy should address broadly the three general topics highlighted in its statement of task: the effects of environmental chemicals on the human microbiome, the role of the human microbiome in modulating environmental-chemical exposure, and the importance of population variability or variation in modulating chemical–microbiome interactions. The committee addresses each of those in this chapter by describing the scientific value of the research, recommending experimental approaches for conducting the research, and identifying possible barriers specific to the research. It then describes the need for specific tool development to conduct microbiome research and finally identifies opportunities for collaboration. Because selection of chemicals for the experimental approaches is germane to all research topics, the committee first provides recommendations for selecting candidate chemicals for research. The committee emphasizes that the research strategy described in this chapter is not meant to be undertaken all at once, and the committee's strategy will be influenced by research on the relationships between microbiome perturbations and disease. Furthermore, as discussed in this chapter, the research will be a collaboration of many agencies and organizations, each with its own priorities and interests in conducting specific research.

SELECTION OF CHEMICALS FOR EXPERIMENTAL APPROACHES

Development of research programs to study whether and how the human microbiome might modulate health risks posed by exposure to environmental chemicals requires decisions regarding the specific chemicals to be investigated and the appropriate exposure routes. The universe of chemicals that could be labeled environmental is large; it includes naturally occurring and synthetic chemicals, chemicals produced as byproducts of industrial activity and energy production, and those resulting from transformation of parent chemicals in the environment. A subset of that universe of chemicals consists of those subject to the requirements of major laws and regulations that are intended to protect human health from harmful exposures to chemicals that occur in environmental media (air, water, food, and soils), in consumer products of all types (including foods and pharmaceuticals), and in the workplace. For purposes of the present report, the committee has defined environmental chemicals as comprising the chemical subset noted above with emphasis on those regulated by the US Environmental Protection Agency (EPA).

It is clearly impossible to investigate all environmental chemicals that fit the committee's definition. Moreover, in the absence of much more knowledge than is available now, it is impossible to specify the numbers and types of chemicals that would have to be investigated to provide an unequivocal answer to the broad question regarding the interaction between environmental chemicals and the microbiome and associated human health risks. If, for example, a clear and uniquely microbiome-mediated form of toxicity were identified for a few important chemicals, that might be sufficient to demonstrate the importance

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of this new branch of toxicology and the need for further study. But it is not at all clear how many failures to demonstrate such a role of the microbiome would be needed to conclude that the subject should not be further pursued.

It is important to consider criteria for selecting chemicals to be investigated carefully. In the bulleted statements below, the committee presents recommendations for appropriate criteria. Not all criteria need to be satisfied for any particular chemical to be considered suitable for study. And the need for additional criteria might become apparent as data are generated. For example, if emerging research indicates that children differ substantially from adults in their vulnerability to chemical–microbiome interactions, selecting chemicals to which children are heavily exposed or highly sensitive could be given top priority.

- Chemicals should be selected to represent the important categories of environmental chemicals regulated by EPA, such as pesticides, heavy metals, organic solvents, air and water criteria pollutants, persistent organic pollutants, consumer-product chemicals, and pharmaceuticals and veterinary drugs that have entered the environment.
- Chemicals in groups that have the highest priority for regulation because they have been shown to pose substantial health risks (substantial toxicity and widespread exposure) should be strong candidates for initial investigation.
- Chemicals that have been assessed in studies of short duration (14 and 28 days) and medium duration (90 days) would be strong candidates for initial investigation because replication of those studies to investigate chemical–microbiome interactions would be less expensive and less time-intensive than studies of longer duration. Longer-term studies will likely follow as microbiome research develops a body of knowledge and inquiry.
- Some chemicals that are known to have toxicity end points similar to health effects that have been associated with perturbed microbiomes (for example, immune-system effects, nervous-system effects, metabolic effects, and perhaps reproductive effects) should be selected.
- The candidate chemicals should include ones that have known capacity to perturb microbiomes or that can be readily studied for that property before full-scale toxicity investigations begin. This information will be important in defining doses to be used in the studies. Antibiotics that have been found in the environment could be candidates for such studies.
- The candidate chemicals should include those known to undergo transformation by the human microbiome.
- Chemicals that have produced large interindividual variability in dose–response studies are also strong candidates for investigation.

Chemicals that satisfy most of those criteria can be selected before experimental studies (animal and in vitro experiments) are conducted. In the case of observational epidemiology studies, it will not be possible to select chemicals according to the same criteria. Rather, it will be necessary to identify opportunities for fruitful studies and to make decisions about whether they involve environmental chemicals as defined in the present report. The committee recommends that, when possible, the same chemicals and methods be used for studies in whole animals, in vitro systems, and human populations to allow comparisons and integration of findings. Such an approach would maximize the possibility of reaching generalizable conclusions from the total body of research.

EFFECTS OF ENVIRONMENTAL CHEMICALS ON THE HUMAN MICROBIOME

A research priority is investigation of the effects of environmental chemicals on the human microbiome and consequent changes to human health. The question is whether environmental-chemical exposures or doses that are in the range of known or anticipated human exposures can induce microbiome perturbations that modulate adverse health effects. This section explores the scientific value of the research, recommended experimental approaches, and research barriers.

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Scientific Value of the Research

As discussed in Chapter 2, the human microbiome has important effects on host biochemistry and physiology, and research over the last decade has associated disruptions in the microbiome with various disease outcomes. For example, regulation of immune-system, nervous-system, and metabolic functions occurs under the influence of gut microbiome metabolites, and alterations in gut metabolite profiles have been associated with aberrations in the functioning of these systems. Such aberrations can lead to both short- and long-term adverse health consequences. There is recent evidence that exposures to some environmental chemicals can alter microbiome composition but little evidence that those alterations have adverse effects on health status. There is, however, evidence that long-term, low-level exposures to some antibiotics alter animal microbiomes so as to increase capacity to extract energy from food and lead to obesity (Cox et al. 2014). That finding is consistent with the use of low-level antibiotic treatment to promote more rapid growth of farm animals. Thus, it is reasonable to hypothesize that some environmental chemicals might alter microbiome composition and result in aberrations in health status. Most important, assessment of whether environmental chemicals can cause microbiome disruptions has the potential to identify and prevent or ameliorate adverse health outcomes caused by such disruptions.

Full exploration of the association between environmental-chemical exposure, microbiome disruptions, and adverse health outcomes is contingent on a deeper understanding of exactly what a disrupted or "unhealthy" microbiome is—a topic that extends well beyond the scope of this research program. As understanding grows, however, determining whether environmental-chemical exposures can cause such structural or functional disruptions will become a high priority because the exposures constitute a cause that potentially can be regulated and mitigated. Ultimately, greater understanding should stimulate new toxicology concepts and testing protocols that include the effects of chemicals on the microbiome.

Experimental Approach

A research program that addresses the question of how environmental chemicals affect the microbiome and the possible consequences could consist of defining toxicity end points for the microbiome, identifying environmental chemicals that can perturb (structurally and functionally) the microbiome, and using animal and epidemiology (human) studies to demonstrate that microbiome perturbations by environmental chemicals cause or modulate a change in health. The research program will require using short-term, high-level experiments—for example, using established study protocols to screen chemicals for effects on the microbiome—and conducting more detailed follow-up studies that require new population cohorts or that aim to elucidate toxicity mechanisms.

Defining Toxicity End Points for Microbiomes

The dose–response relationship is central to toxicology in that it quantitatively reflects the effect that a given exposure has on a given biologic system. The dose–response relationship relies heavily on quantitative measures of health outcomes or end points, such as gene expression, enzyme activity, or alterations in cellular physiology. If a microbiome is considered the "biologic system" for which a dose–response relationship needs to be defined, the question of which end points best reflect microbiome toxicity arises. Accordingly, end points that exhibit dose-dependent properties and act through known mechanisms will need to be established. Because no known end points of microbiome toxicity have been established, comprehensive approaches—including 16S rRNA or internal transcribed spacer (ITS) gene community profiling, metatranscriptomics, metaproteomics, metabolomics, and other measures of physiologic activity—will be needed to capture all aspects of the microbiome response to a given toxicant. For example, although 16S rRNA and ITS sequencing approaches will capture changes in community structure, measures of the microbiome stress response—both general and specific to a particular environmental chemical—will be captured best through metatransciptomic approaches. However, the committee empha-

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sizes that an integrated approach that includes the collection of data from multiple -omics assays will be important for establishing the most comprehensive view of the microbiome response to an environmental chemical.

To establish quantifiable end points, the committee recommends studying the effects of chemicals with different mechanisms on mouse and human microbiomes by using bioreactors, such as the simulator of the human intestinal microbial ecosystem (SHIME), described in Chapter 4.[1] Many antimicrobial agents are good candidates for this investigation because they have known mechanisms—for example, inhibition of DNA replication and transcription, protein synthesis, or cell wall biosynthesis—and exhibit predictable dose–response relationships. Furthermore, some antimicrobials are bacteriostatic (they restrict growth and reproduction) whereas others are bactericidal (they cause cell death). Therefore, antimicrobials should help to establish quantitative end points that ultimately could be used to understand or predict the toxic effects of environmental chemicals on a microbiome. The feasibility of using bioreactors has been demonstrated, but several important factors must be carefully considered before these studies are undertaken. First, the source of the microbiome needs to be considered. Human stool samples and rodent fecal or cecal contents are popular microbiome sources; however, how accurately they reflect the microbiome of a particular gastrointestinal niche remains a topic of intense debate (Dantas et al. 2013), and how accurately a rodent-specific microbiome reflects what might be observed in the human microbiome is unclear. Regardless, for the purpose of establishing testable end points, those microbiome sources are ideal because they are easily collected and stored and can be collected longitudinally. Second, although variability that results from diet, age, or sex can be strictly controlled in rodents, it cannot be in human studies, so experiments will need to be designed with consideration of the variation and variability associated with the human microbiome. Third, antimicrobials could influence community structure through selection via antibiotic resistance that could be especially important during long-term incubations. Therefore, acute, short-term dosing schemes will be essential for developing signatures of microbiome toxicity, and long-term chronic dosing studies should be interpreted with caution.

Once a stable bioreactor system is established, increasing doses of antimicrobials that have different mechanisms can be added, and samples can be collected longitudinally. Use of a longitudinal study design allows comparisons of acute and chronic dosing schemes. Samples can be subjected to comprehensive analysis by a suite of -omics tools. Changes in microbial membrane potential, membrane permeability, and DNA replication can also be assessed (Maurice and Turnbaugh 2013). Next, extensive statistical and bioinformatic analyses can be applied to determine patterns in gene expression, metabolite concentrations, or other physiologic measures that are consistently altered in comparison with unexposed microbiomes and hence can serve as end points for studies of effects of environmental chemicals. Data are likely to identify specific members of the microbiome that contribute to specific end points; thus, defined culture systems (such as monocultures or cultures that are representative of the major taxa in structure and function) could provide an avenue to clarification of the mechanistic role of specific taxa of bacteria or fungi. Having established the identities of microorganisms that are most sensitive, one can conduct more detailed studies to increase understanding of a chemical's mechanism of action.

Once a repertoire of end points—such as changes in physiology, gene expression, protein concentrations, or metabolite concentrations—is established for antimicrobial exposure, the experimental approach can be applied to environmental chemicals of concern. However, there are several caveats to the experimental approach outlined. First, it does not take into account metabolism by the host and so might miss compounds that undergo biotransformation or bioactivation through host-dependent mechanisms before having their effects on the microbiome. Second, it assumes that environmental chemicals of concern work through mechanisms analogous to antimicrobial chemicals (that is, by affecting DNA, protein, or cell-wall biosynthesis). Third, it assumes that bioreactor systems accurately model what is present in the gut or other body niches, faithfully represent the community structure and its metabolic activity, and are capable of growing even the most fastidious organisms. Fourth, the approaches do not fully capture differ-

[1]Although the focus here is on the gut microbiome, the experimental approaches could be adapted for skin and lung microbiomes and other body niches.

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ences that can occur through different routes of exposure, such as inhalation and dermal. Therefore, the committee recommends that model systems that faithfully recapitulate the host–microbiome interaction of the skin and lung be considered so that all exposure routes are captured fully.

Identifying Environmental Chemicals That Perturb Microbiomes

Other high-priority research would be aimed at developing a high-throughput bioreactor system that operates under physiologically relevant conditions to screen environmental chemicals in a uniform manner for their ability to perturb microbiomes. The goal is to provide a reproducible platform for assessing dose-dependent effects of environmental chemicals on defined microbial communities and on individual microbial species within a community through measures of physiologic activity (such as metabolic activity and membrane permeability) and biologic activity (such as DNA replication and transcriptional response). Once established, the measures of the microbiome response to environmental-chemical exposure could be used to populate a database and later to inform screening programs in mice and perhaps could be cross-referenced with signature responses in human populations.

Although development of bioreactors to investigate microbiome interactions is still in the early stages, several devices described in Chapter 4 have found their way into basic and translational research. Bioreactor systems permit flexibility in study design by using single strains or defined or complex communities, can be cultivated for various periods to assess acute and chronic exposures, and can be modified to include different host components, including mucin barriers or dietary constituents that more closely resemble in situ conditions. Such bioreactor systems can be designed to incorporate surfaces for microbial attachment so that the response of mixed-species biofilms and free-swimming microorganisms can be assessed, thereby recapitulating the primary modes of microbial lifestyle in and on the human host. In the bioreactor studies, it will be essential to use doses of chemicals relevant to human exposures, including concentrations typically associated with environmental or industrial accidents. However, dose estimates might need to be re-examined to take into account interactions with the microbiome at both internal and external body sites (Silbergeld 2017). For example, although estimates of arsenic exposure via drinking water typically reflect the absorbed dose (the dose passed from the gastrointestinal environment into circulation), the dose to the microbiome could be substantially higher. Furthermore, members of the microbial community are not likely to exhibit the same dose–response relationship with an environmental chemical. Therefore, experimental systems that range from individual strains of bacteria to complex microbiomes must be considered to investigate the potential of an environmental chemical to alter the microbiome. A final consideration is whether a mechanism of action is mediated by the host or is independent of the host. If it is host-independent, simpler bioreactors that require less investment can be developed because they do not require a host component for incorporation into the system.

A long-term goal would be to evaluate distinct microbiome configurations that are representative of different life stages or disease states, which might represent periods of increased susceptibility to environmental chemicals. Such platforms would provide important information regarding susceptible human populations and would be important in trying to capture human variation and variability.

Linking Microbiome Perturbations by Environmental Chemicals to Adverse Health Outcomes

Animal Studies

Evidence of adverse health outcomes caused by perturbations of microbiomes induced by environmental chemicals could be provided by animal experiments, especially for chemicals that require metabolism by the host. Although the bioreactor experiments can screen environmental chemicals rapidly, they cannot fully capture host-mediated processes that in many cases have been identified as key mechanistic components of environmental-chemical toxicity. The committee recommends starting with gnotobiotic animals that have a defined microbiome or standardized community (as described in Chapter 4) to reduce

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measurement and experimental variability. When diet and other environmental factors can be carefully controlled, it should be possible to assess the interactions of environmental chemicals with the microbiome and the host and their contribution to adverse outcomes. For example, if one observes a correlation between the environmental-chemical exposure, microbiome perturbation, and adverse outcomes, one could transfer the perturbed microbiome into germ-free mice and observe whether the adverse outcome is recapitulated in them. If so, that would be strong evidence that the microbiome perturbation induced by the environmental chemical is involved in manifestation of the observed adverse outcome. An important caveat to that approach is that only a portion of the microorganisms present in the donor community will be efficiently transferred to the germ-free host. The approach has been used in only a few experiments that use gut communities; therefore, it is unclear whether it will be an effective approach for all gut communities and for those from skin or lung.

A long-term goal is to screen environmental chemicals by using animal models to assess microbiome perturbations in inbred, transgenic, and outbred lines and established disease models. The outbred lines particularly allow assessment of the consistency of effects of chemical exposures in genetic and microbial gradients in such animals. The studies are not limited to rodents; for example, zebrafish, fruit flies, or nematodes might be best suited to studies that require high-throughput analysis. Unlike the defined gnotobiotic experiments discussed above, these studies will allow better understanding of realistic microbiome variation and consistency of effects. Ideally, the studies would also include multiple animal models, multiple animal facilities, and gnotobiotic transfers from defined communities or multiple human donors.

Epidemiology Studies

Epidemiology and population exposure studies that are already under way could be used to identify microbiome co-variation with an environmental chemical of interest. The approach could involve, for example, identifying a human population in which a chemical exposure of interest has been tracked and collecting new samples appropriate for microbiome analyses, generating new microbiome-relevant data from biobanked samples from such a cohort, or adding measurements of environmental-chemical exposures of a human population that is being followed for other purposes, including microbiome measurements. For short-term, proof-of-concept purposes, simple measures of microbiome structure might be sufficient to identify cases in which a perturbation occurs either in tandem with or after chemical exposure and manifestation of adverse health outcomes; the microbiome changes would then need to be investigated in more detail to characterize their functional or clinical consequences (if any). In such cases, it will also be crucial to separate health effects mediated by microbial activity from those induced by direct chemical exposures of the host. That research could use existing prospective cohort infrastructure, including banked specimens and could benefit particularly from collaboration among institutions, such as environmental- and population-health scientists and funding agencies.

Barriers

Defined, validated, and quantitative measures of host–environmental-chemical interactions exist but not for chemical interactions with microbial communities, although individual microbial physiology can be robustly detailed. Thus, defining measurable and quantifiable end points that reflect toxicity to the microbiome are of paramount importance. Many of the antimicrobial experiments that the committee describes are likely to require substantial investments of time and resources, are exploratory and thus unlikely to be supported through traditional funding mechanisms, and require unique expertise not found in a single laboratory. Successful studies will require a consortium of microbiologists, toxicologists, microbiome-analysis experts (those who have expertise ranging from sequencing to metabolomics), bioinformatics experts, and persons who have other relevant expertise, as appropriate. Only after clear, quantitative measures of microbiome toxicity have been established can the approaches be applied to representative

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environmental chemicals of concern. Identification of exposures or doses that are in the range of known or anticipated exposures will also be important, although a range of doses should be studied. Finally, a major challenge will be capturing human microbiome variation and variability that might not be apparent on the basis of sequencing but probably would be observed with metabolic output. Thus, more functional analyses of the human microbiome that use metatranscriptomics, metaproteomics, and metabolomics will be required.

There are several barriers to development of the bioreactor platforms. First, as discussed in Chapter 4, there is the difficulty of establishing and maintaining physiologic communities in vitro. Second, perturbations of the microbiome could require host-mediated chemical metabolism from such organs as the liver or some other host-mediated process that has not been incorporated into the bioreactor platform. Third, as discussed in Chapter 4, bioreactors might not be able to capture functional diversity, including interindividual, developmental and body-site variation. Fourth, although the research discussed above should help to identify end points to use, end points for assessing microbiome toxicity have not yet been established. Fifth, there is little understanding of how microbial-community composition and interaction depend on life stage and on the developing or aged host tissues.

Overall, an additional barrier to research to understand how environmental chemicals might affect the human microbiome is the unknown level of functional redundancy that could exist within the human microbiome. For example, many chemicals are capable of altering microbiome composition, but is the altered composition itself a response, and would one expect it to be monotonically dose-dependent? If alteration of the microbiome composition can be shown to be causally related to an adverse host response (for example, a change in the abundance of microorganisms that metabolize chemical X or in the abundance of microorganisms that produce a lipid mediator of inflammation), is it possible that the response would behave in a threshold-like manner because of the large potential for functional redundancy in the microbiome? As a hypothetical example, a detoxification product of a metabolized chemical could be generated by a broad class of enzymes represented by different genes throughout various taxa in a microbiome. In that case, a shift in the composition of the microbiome—even a robust shift—might have little consequence if sufficient redundancy in function remains in the microbiome. At high doses, where the microbiome is reduced in biomass and abundance, there could be threshold effects related to metabolism or elimination, but such high doses might not be relevant to environmental exposures. Answering questions about associations of adverse outcomes with changes in a microbiome induced by environmental-chemical exposures will require experiments with bioreactors (investigating the microbiome only), germ-free models (investigating the host only), and conventional animals (investigating the host and the microbiome, including their interactions).

THE ROLE OF THE HUMAN MICROBIOME IN MODULATING EXPOSURES TO ENVIRONMENTAL CHEMICALS

Another high-priority research topic is the effects of the human microbiome on exposure to environmental chemicals. Specifically, what is the role of a microbiome in modulating absorption, distribution, metabolism (activation or inactivation), and elimination (ADME) of environmental chemicals? This section explores the scientific value of the research, recommended experimental approaches, and barriers.

Scientific Value of the Research

As discussed in Chapter 3, there is increasing evidence that microbiomes can modulate the relationship between external exposure and internal dose of some environmental chemicals or their active metabolites. Conceptually, interactions between a microbiome and environmental chemicals might influence all aspects of the ADME profile of a given chemical. For example, some microorganisms present in the gut microbiome can metabolize foreign chemicals in ways similar to metabolism by the liver and other

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organs. Because the toxic properties of many environmental chemicals are influenced or directly caused by some of their metabolic products, the creation of metabolites by a microbiome could influence toxicity outcomes. Accordingly, understanding of the specific interactions between a chemical and a microbiome is particularly important in the context of assessing risk because it provides a means of quantifying the relationship between external chemical exposure and the target-tissue dose of the parent chemical or the active metabolite associated with an adverse effect.

Scientists have little understanding today of the total capacity of microbiomes to biotransform environmental chemicals; for most cases, the specific microbial enzymes and microbial species involved have not yet been elucidated. Thus, fundamental research should be aimed at broader identification of specific microbial enzymes and microbial species that mediate chemical transformation processes. Ultimately, linking the specific microorganisms, genes, and enzymes to a particular chemical transformation process is essential if substantive progress is to be made in addressing individual susceptibility and interspecies extrapolation questions at a mechanistic level and in understanding the degree of functional redundancy within a microbiome. Furthermore, if the effect of the microbiome on chemical exposure can be quantified, models can be developed by using a compartmentalized approach that could improve exposure assessment for specific chemicals in a hypothesis-driven manner without necessarily understanding the contributions of individual microbial species.

Experimental Approach

Determination of health risks associated with exposure to environmental chemicals and the potential roles of the microbiome in modulating such risks depends on an understanding of the biologic effects of the chemical, its distribution, its metabolism, and its clearance in model systems that permit analyses of the role of the microbiome in such processes. The committee has organized the experimental approach so that the data generated could feed directly into development of a microbiome component for physiologically based pharmacokinetic or pharmacodynamic (PBPK-PD) models (see Box 6-1). The goal of developing quantitative models provides a framework for guiding basic research toward outcomes that can be valuable for risk assessment in the near term. The emphasis on PBPK-PD modeling is not to imply that basic research should be a secondary part of the research strategy. Rather, the committee recognizes that the knowledge of microbiome roles in metabolism of environmental chemicals has progressed substantially. Through thoughtful selection of chemicals for study on which there is existing knowledge, there might be an opportunity to accelerate progress in understanding how much the microbiome might influence ADME processes.

The traditional PBPK-PD modeling approach follows a data-based parallelogram strategy that incorporates in vitro cell type-specific data on both animals and humans and in vivo data generated from model animals (see blue boxes in Figure 6-1). Those three data sources feed into development of the PBPK-PD model to permit prediction of human responses to chemical exposure (Goldsmith et al. 2012). The widely used framework can be adapted to incorporate data on microorganism-specific contributions to ADME. A successful strategy for PBPK-PD modeling of human-microbiome effects on chemical exposure would be enhanced by including information from existing human-microbiome databases on microbiome gene content (metagenomes), transcription, and metabolism and by efforts to improve existing reference databases. That information could be used to infer potential chemical-metabolism pathways in a microbiome and to formulate initial models. Opportunities to validate model predictions in existing human population-based studies or those initiated specifically for the purpose of such studies should also be pursued (green boxes in Figure 6-1). The committee notes that the initial focus of this research is on the gut microbiome because a large body of literature implicates it in chemical transformation processes. However, the overall strategy could be generalizable to other tissue sites, including the oral, respiratory, and skin microbiomes.

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Animal Studies to Generate Pharmacokinetic–Pharmacodynamic Data

Absorption and metabolism are two primary determinants of chemical kinetics in mammalian systems (Yoon et al. 2012). The human microbiome encodes a vast ancillary metabolic potential and plausibly plays a role in such processes. But few experimental animal studies have been designed explicitly to assess the specific role of the microbiome in ADME, and such microorganism-specific data have not been incorporated into PBPK-PD models. Integration of such data into current models could help to explain response variability within human populations and reduce uncertainties in current model predictions.

BOX 6-1 Physiologically Based Pharmacokinetic or Pharmacodynamic Models

Over the last several years, the use of PBPK-PD models has proved increasingly promising for predicting ADME and consequent biologic effects of chemical exposures for integration into risk-assessment frameworks. That approach combines in vitro and in vivo data on multiple biologic scales (from specific primary cell types to whole-animal models) and permits modeling of distinct exposure routes (oral, dermal, inhalation, and intravenous) and doses in multiple model species. Although PBPK-PD models are increasingly used for risk assessment, they typically do not explicitly include the microbiome as a distinct compartment. Some PBPK-PD calculations based on animal studies do implicitly incorporate the effect of microbiomes on ADME processes for some chemicals, such as PBPK-PD models that include enterohepatic recycling of a parent chemical due to intestinal β-glucuronidase-mediated cleavage of its metabolites. For the most part, however, current models lack the flexibility to simulate the effect that changes in microbiome structure or function have on a chemical's ADME profile.

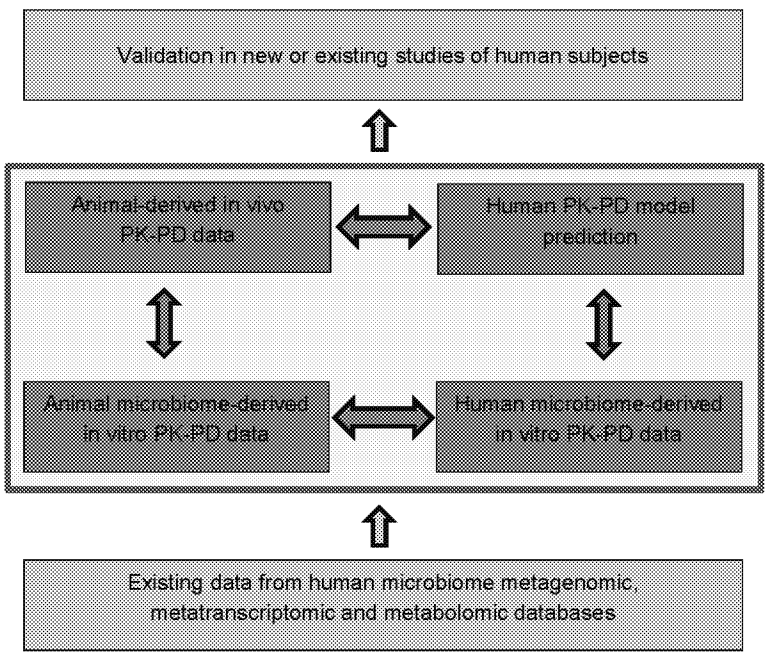

FIGURE 6-1 Parallelogram strategy (blue boxes) for predicting human response to chemical exposure that incorporates in vitro and in vivo data into PBPK-PD models. A strategy for examining the role of the human microbiome in modulating chemical exposures would generate microbiome-based data to inform model prediction. The modeling results would be enhanced by integrating human microbiome databases to predict microbial metabolic capacity and by using human cohorts to validate model predictions.

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To determine initially whether the microbiome plays a role in a chemical's kinetic behavior, comparative studies of conventional animals (ones that have an intact microbiome) and germ-free animals would allow assessment of the effects (and their magnitude) of the microbiome on ADME processes in vivo. In the simplest form, experimental animals are exposed (via oral, dermal, inhalation, or intravenous routes), the concentrations of the parent chemical and its metabolites are assessed in several target organs and in the circulation and urine, and binding of the parent chemical or its derivatives to receptors in target organs (if known) is investigated (Yoon et al. 2012). Although germ-free animal models have some caveats, as noted in Chapter 4 and in the section on barriers below, they offer a unique opportunity to consider host vs microorganism-derived chemical interactions in vivo and to some extent extricate host from microbial contributions to these processes.

To develop data that might be more directly relevant to human microbiome-derived chemical transformation, one could also consider experiments that compare germ-free animals with ones that have been colonized with a microbial inoculum derived from human feces (humanized) or colonized with specific human-derived microorganisms to study the functions of interest. Humanized animals offer an opportunity to evaluate effects by using complex microbiomes derived from heterogeneous sources that differ in their constitution, such as those from infants, adults, or people who have chronic diseases known to influence microbiome composition. Animals that have been colonized with a defined microbial community allow assessment of microorganisms that are suspected of playing a key role in chemical transformation. Both approaches offer an opportunity to generate information on the capacity of such organisms to modulate chemical exposures and influence ADME. Similarly, comparing untreated conventional animals with antimicrobial-treated animals would allow assessment of the effects of acute microbiome perturbation on ADME and toxicokinetics.

In Vitro Systems for Generating Pharmacokinetic–Pharmacodynamic Data

Once a microbiome has been implicated in modulating ADME processes in an animal model, in vitro systems, such as bioreactors and gut-on-a-chip, can be used to isolate the microbial component and compare mechanisms among species. In vitro experiments should be used to define functional traits of the microbial community that transform the environmental chemical, to identify microorganisms and microbial interactions implicated in chemical transformations, to identify microorganism-modified metabolites, and to obtain microorganism-specific chemical transformation rates, which should be compared with those obtained by using human microbiomes for incorporation into PBPK-PD models. Environmental-chemical metabolites formed in vitro should be reintroduced into animal models to test or verify their mechanism. As shown in Figure 6-1 (bottom blue bars), microbiomes obtained from mice exposed to an environmental chemical could be used in parallel with human microbiomes from exposed populations, if available, to determine whether the same metabolites are produced after chemical exposure and through similar types of microbial interactions.

A major advantage of in vitro systems is the potential to implement high-throughput studies. Development and standardization of high-throughput in vitro systems will require careful consideration of model microbial reference communities and reference strains that broadly represent the diverse metabolic functions of the unperturbed human microbiome, which are as yet poorly defined. As discussed below, further development of microbial reference strains will require continued effort to improve functional annotation of metagenomes with emphasis on identifying the specific enzymatic pathways that act on environmental chemicals.

Identifying Specific Microbial Enzyme Functions

New chemical probes and chemical screening technologies are emerging that could reduce the experimental effort and time needed to isolate and identify specific proteins and microorganisms that interact with environmental chemicals in the microbiome. For example, chemical probes designed to target

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enzyme active-site chemistries have been used to profile cytochrome P450 enzyme activities and drug–protein interactions in vivo (Wright and Cravatt 2007; Wright et al. 2009; Sadler and Wright 2015) and to identify microbial glycoside hydrolases and other enzymatic activities in bacteria (Chauvigné-Hines et al. 2012). Chemical probes that mimic structures of specific classes of environmental chemicals and have reactive tags (such as biotin) could also be useful for initial screening efforts to identify direct interactions between a chemical and a microbial species within a complex microbial community. In conjunction with chemical probes, genetically engineered bacterial reporter strains could be used as sensitive indicators of microbiome perturbations. The approach has been used extensively to sense environmental chemicals in the field (Roggo and van der Meer 2017), but reporter strains have been used less commonly to sense and record specific signals in the mammalian gut microbiome (Kotula et al. 2014). However, the potential for reporter strains to affect the microbiome-community structure and function should be carefully considered.

Emerging technologies that hold promise for characterizing how environmental chemicals are metabolized in a microbial community include stable-isotope labeling, which permits tracking of labeled chemicals, and advanced mass-spectrometry methods (Berry et al. 2013). Coupling those approaches with single-cell genomics strategies should prove useful for identifying the specific microorganisms responsible for chemical interactions (Lasken 2012; Berry et al. 2013; Koppel et al. 2017). Although such discovery-based studies might have a longer time horizon, their early inclusion as part of an integrated research strategy is critical for achieving the goal of assessing personalized microbiome status as a potential risk factor for environmental-chemical interactions.

Barriers

Although the components of this research strategy for assessing the role of a microbiome in modulating ADME of environmental chemicals are based on an established framework for PBPK-PD model development, several barriers to its implementation remain to be resolved, as outlined below.

- In situ conditions that might influence ADME, such as dietary interactions and pH and oxygen gradients, are largely unknown in human populations of potentially heightened susceptibility, such as infants and patient populations that exhibit changes in microbiome diversity in association with their underlying disease. Thus, it might be difficult to recapitulate such conditions in model systems accurately.
- Germ-free animal models are known to have altered host tissue physiology compared with conventionally raised animals, including adaptive changes in expressing enzymes that are critical for metabolic transformation of drugs and environmental chemicals. The extent to which adaptive changes in normal host metabolism occur in germ-free or other gnotobiotic systems is not broadly understood and requires rigorous evaluation. However, for some chemicals, the use of germ-free models could be problematic for measuring PBPK-PD parameters.
- To address variations in microbiome structure and function that are naturally present in human populations, large experimental design matrices might be required, whether animal models or in vitro systems are used, and might require large resource investments. In designing cost-efficient studies to identify sources of variability effectively, such statistical techniques as design of experiments could be used.
- Development of in vitro model systems, such as gut-on-a-chip, that include the microbiome is still in its infancy. There is still no consensus on microbial reference communities or strains that reflect the metabolic potentials of an unperturbed microbiome accurately. That knowledge gap might present challenges in obtaining comparable results from in vitro systems that can be directly extrapolated to the whole animal or to human systems.
- Because the human gut microbiome cannot be fully recapitulated—for example, in a germ-free rodent or in vitro system—some microorganisms will be missing from such studies. That limitation reiterates the need for fundamental studies to understand what gene products (enzymes and proteins) are encoded by the microbiome and are involved in metabolism of chemicals.

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THE IMPORTANCE OF MICROBIOME VARIATION AND VARIABILITY

As discussed in Chapter 2, the human microbiome structure and function vary with, for example, body site, life stage, genetics, geography, and health status. The human microbiome also differs from microbiomes of animal species. Variation and variability have important implications in assessing risk posed by environmental-chemical exposure.[2] This section explores experimental approaches to examine the importance of variation and variability among humans and then between humans and laboratory animals.

Assessing the Importance of Human Microbiome Variability and Variation

As noted, microbiome variability and variation within the human population are substantial, and a question is whether knowledge of population and life-stage variation and variability in the human microbiome will improve understanding of the susceptibility to environmental chemicals and of individual health risk. The subsections that follow discuss the scientific value of the research and recommend research designed to investigate this important topic.

Scientific Value of the Research

As discussed in Chapters 1 and 2, humans and their microbiomes have co-evolved to form an ecosystem that is comprised of distinct habitats whose microbial community structure and function vary. Many factors—such as age, race, genetics, health status, physical condition, diet (including early-life nutrition), and geography—affect microbiome structure and function. Susceptibility to environmental-chemical exposure and associated health risk might be modified not only by those factors but by the variation and variability of the human microbiome structure and function (see Figure 6-2). Understanding how the variation and variability of the human microbiome might affect chemical-microbiome interactions will be critical in assessing microorganism-mediated risk posed by environmental-chemical exposures. For example, the microbial community and its functions are sparser and less varied in the infant than in the adult; if one considers only the adult microbiome, one could miss identifying critical windows or periods of susceptibility. Explicitly considering human microbiome variation and variability might also substantially improve our capacity for identifying at-risk populations and for developing strategies to mitigate exposures and reduce associated disease incidence in these populations.

Experimental Approach

The goals of the research described are to understand the importance of human microbiome variability and variation at any given life stage or among specific populations and ultimately to ensure that studies consider such variation and variability adequately and appropriately in assessing the health risks to human populations posed by exposure to environmental chemicals. In conducting this (and other) human microbiome research, two points need to be emphasized. First, the respiratory, gut, and skin microbiomes vary in their taxonomic composition and function, so one needs to consider the environmental exposure route when selecting the specific organ and tissue system to study. For example, although studies of one community could inform those of another, studying the response of the gut microbiome to an environmental chemical that is absorbed mainly through the skin might not be directly informative for human risk assessment. Second, community composition and its function are not the same, so examining microbial function rather than only taxonomy should be encouraged. For example, subtle variations in low-biomass communities might impart important functional differences in metabolites or small-molecule intermediates; conversely, because functional redundancy is probable in many microbial communities, variations in community composition might not necessarily impart key functional differences.

[2]See Chapter 1 for a discussion of the definitions of variation and variability.

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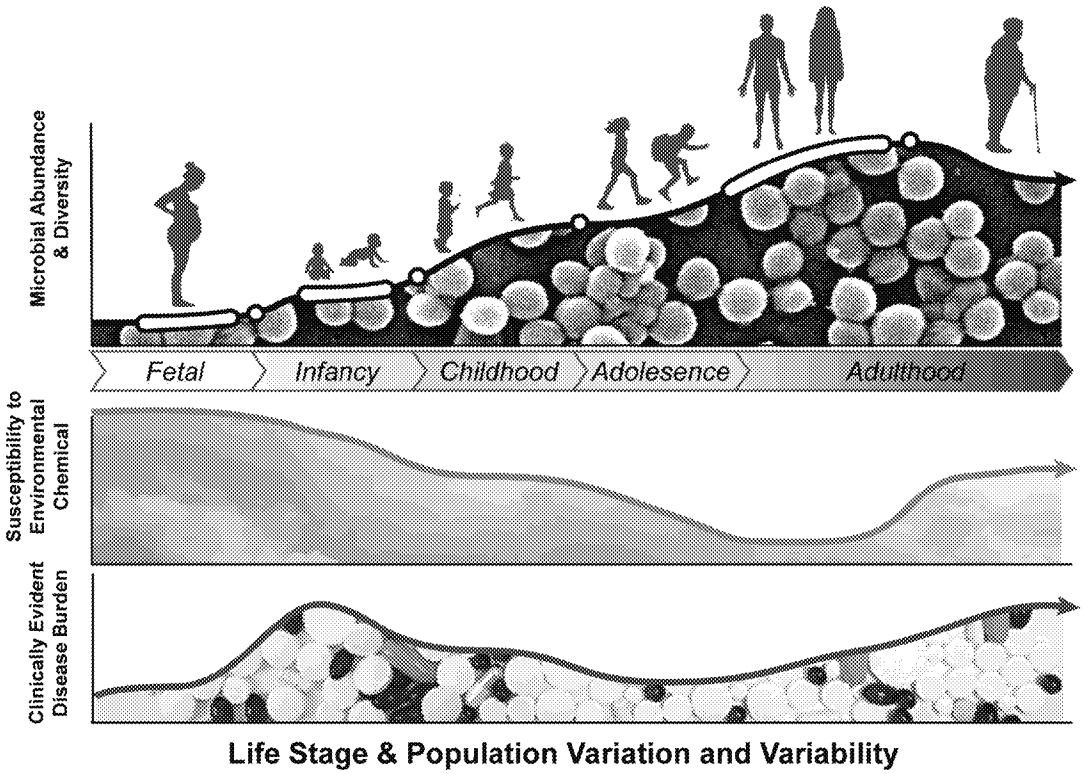

FIGURE 6-2 Susceptibility to environmental-chemical exposure and associated health risks might be affected not only by developmental stage and baseline health status but by the variation and variability in the human microbiome.

Variation and variability can be understood best by conducting comparative studies that assess functional similarities and differences of environmental exposure in the factors known or hypothesized to affect microbiome diversity. Specifically, the studies should characterize microbiome communities and their functional differences by such factors as race, sex, life stage, and health status and emphasize populations that represent key windows of potential vulnerability, such as infants, pregnant women, adolescents, and geriatric populations, and resiliency, such as healthy adults. Functions that would be strong candidates for evaluation include microbial activities and pathways for chemical metabolism, regulation of transport and barrier integrity, and modulation of factors relevant to host developmental, metabolic, immunologic and neurologic outcomes.

In the near term, large and well-characterized human population studies that are already under way could be used for conducting comparative studies. Sample collection for microbiome analysis could be added to current studies of large, longitudinally followed, and well-characterized cohorts in which toxicant exposures have been or could be readily assessed. The longitudinal component of such human studies offers an opportunity to examine short-term and long-term effects of exposure and could be particularly enlightening with respect to populations at heightened risk, for example, early-life acute or chronic toxicant exposures that have the potential to affect microbiome development in a manner that manifests as disease later in childhood. Such an effort is likely to yield valuable data at moderate cost. This approach could be enabled by developing rapid and agile funding opportunities for supplemental grants to awarded projects that are investigating chemical–microbiome interactions.

In the longer term, improved computational approaches, advances in data science, and innovative human-study design will advance understanding of the implications of variation and variability of the human microbiome. Specifically, the advent of high-throughput DNA and RNA sequencing and high-throughput untargeted protein and metabolite profiling technologies with rapidly developing computa-

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tional methods will provide measures that, when integrated, will allow modeling of cause–effect relationships. When combined with detailed characteristics of the human host, a computational approach might identify modifiers (factors that affect variability and variation) that are important in manifestation of the health effect. However, tackling the integrated analysis of heterogeneous data types at the scale and complexity necessary will demand data-science innovation and computational advances that are outlined further in the section "Tool Development."

In conjunction with the human studies, it will be important to replicate or validate the findings or observations from those studies in bioreactors or gnotobiotic-animal models described earlier in this chapter. For example, a bioreactor or gnotobiotic-animal model could be used to investigate the responses of microbiomes that were isolated from groups (defined by some host factor) that did and did not manifest a given effect that resulted from exposure to an environmental chemical.

Assessing the Importance of Microbiome Variation Between Animals and Humans

Understanding the importance of the variation between animal and human microbiomes is critical. The central question is whether the differences are so great that effects are being missed or mischaracterized by using the animal models to predict health risks associated with environmental-chemical exposure. Furthermore, do the intraspecies uncertainty factors that are used to extrapolate effects in animals to humans account for the microbiome variation? The following text explores the scientific value of the research and recommends research that could be conducted to address such questions.

Scientific Value of the Research

For most health risk assessment, animal models have been the basis for determining the toxic effects of chemicals and estimating the potential for adverse health effects in humans. The extent to which toxicity studies have already accounted for mediating effects of microbiota on health effects is currently unclear. For environmental chemicals for which there is reasonable evidence or suspicion of microbiome-mediated health effects, it is important to determine whether chemical–microbiome interactions of consequence observed in model systems are similar in human microbiomes. If not, understanding why findings in model-system microbiomes differ from those in human microbiomes is imperative. For example, are the differences the result of an inability to recapitulate a microbiome in an in vitro system (that is, key microbial-community members are not present in the in vitro system), or do they result from true variation between an animal microbiome and the human microbiome? The research described below should produce new knowledge of microbial-community function that should allow assessment of the capacity of animal models to recapitulate the activities of human microbiomes.

Experimental Approach

Like research to investigate variation and variability within the human population, this research involves conducting comparative studies that focus on functional differences rather than only taxonomy. Ultimately, the goal would be to focus on functional capacity encoded by the human microbiome to identify the animal species and study designs most appropriate for extrapolating to humans. The comparative studies should focus on evaluating functional similarities and differences between native microbiomes from humans and test animals, such as mice, zebrafish, fruit flies, pigs, and nonhuman primates; native microbiomes from laboratory-reared and wild model organisms; and native human and animal microbiomes and microbiomes resulting from transplantation of human microbiota into test animals. Functions that would be strong candidates for evaluation would be microbial activities and pathways for chemical metabolism, regulation of transport and barrier integrity, and modulation of factors relevant to host developmental, metabolic, immunologic, and neurologic outcomes. Important considerations for the experiments include use of appropriate controls that take into account effects of the vehicle or chemical form administered and use of a

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range of doses that include environmentally relevant exposures to evaluate effects on the shape of the dose–response relationship. Near-term goals of the research would be the following:

- Identification of functional pathways, including chemical metabolism pathways, that are uniquely encoded by microbiomes from select model organisms and performance of multi-omics functional characterization of microbiomes from humans and animal models, including comparisons of animals from different colonies and genetic backgrounds to assess various potential microbiome compositions.
- Functional characterization of human microbiome samples in response to environmental-chemical exposure and conduct of comparative analyses of functional profiles after transplantation of microbiota into model organisms.
- Understanding of differences and similarities between model-organism and human-host responses (such as metabolism, absorption, elimination, immunity, and behavior) to environmental-chemical exposures by using defined microbial communities in in vitro or gnotobiotic models.
- Assessment of the redundancy and uniqueness of microbiomes of various model organisms and humans through comparative microbial functional genomic and metabolomic studies.

Model organisms will be essential for testing causal relationships between environmental exposures, microbiome perturbations, and health outcomes. They will enable the identification of the molecular and cellular underpinnings of observed interactions. Thus, model systems that can be used to represent the human condition faithfully are critical. Over the long term, model organisms or microbiomes that faithfully and stably encode functions relevant to human microbiomes might be engineered by using synthetic-biology or genetic-engineering strategies.

Barriers

Any model system for assessing microbiomes and even human-based studies have inherent and specific limitations. Knowledge of such limitations should inform decisions on experimental approaches to be used and what research cannot be adequately addressed with a single approach. Potential barriers include the following:

- There could be difficulties in obtaining detailed functional characterization of some microbiomes—for example, with multi-omics approaches—because of limitations related to sample collection, sample type, sample quantity, and the preponderance of host components, such as human DNA, RNA, or protein that is usually associated with tissue samples. Such microbiomes would include those from difficult-to-access host sites or from sites that have small amounts of retrievable material, which potentially limit their study with multiple analytic approaches.
- A related barrier is the understanding of how sample collection, processing, and storage could affect multilevel functional characterization of a given microbiome or data interpretation. Research study protocols should strive to harmonize tools and methods among systems whose microbiomes are to be compared, for example, gut microbiomes from an animal model and human subject.
- Detailed functional characterization of microbiomes could be difficult because of technologic limitations in generating reliable reference databases of microbial genomic and metabolomic annotations and the poor scalability and relatively high cost of some animal models.
- Inability to reproduce findings related to chemical–microbiome interactions derived from a given experimental approach because of lack of standardization is a barrier. Investigators will need to control and disclose variables relevant to microbiome assessments, including initial characterization of microbiomes, animal-care procedures and conditions, choices of laboratory reagents, and methods for sample processing and outcome measurements. If a lack of reproducibility is observed, the extent to which such an observation is due to microbiome differences rather than other variables unique to the models or human cohorts would be important to clarify.

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- Many experimental systems present important technical challenges in creating exposure conditions that appropriately mimic the human condition to be studied. Such challenges, which already exist in toxicology studies of health risks associated with environmental-chemical exposure, are amplified when microbiome-modulated influence is of central concern, and they can become even larger when variability and variation are of key interest.

TOOL DEVELOPMENT

The research strategy developed by the committee emphasizes the three main elements highlighted in the statement of task: the effect of chemical exposure on the human microbiome, the role that the human microbiome plays in environmental-chemical exposure, and the importance of population variation in modulating microbiome-mediated effects of environmental-chemical exposure. While deliberating on the three elements, the committee identified several important tool-development needs that are pertinent for addressing the research described in this chapter. Those needs are relevant to a much broader set of concerns throughout the field of microbiome research and therefore are beyond what is encompassed in the charge to this committee. Consequently, progress in those matters will not be the province solely of the research strategy set forth in this chapter. Progress in the areas discussed below should be monitored and applied where appropriate to improve knowledge about the influence of the microbiome on health risks associated with exposure to environmental chemicals.

In Vitro Models

As discussed in Chapter 4, in vitro models will be used mainly for three goals: to understand biochemical transformations of environmental chemicals by different body-site microbiomes (gut, lung, and skin) by using state-of-the-art analytic tools, to identify important interactions between environmental chemicals and the microbiome and their effects on microbial-community structures (diversity) and functions that could affect host health, and to understand host transformation of environmental chemicals that might affect microbiome composition or function. In vitro model systems that faithfully model the host gut environment—including protective mucus barriers, immune cells, and cellular architecture—have not yet been developed, despite such advances as the SHIME and mucosal-SHIME (M-SHIME) systems. As discussed, consideration of nutrient flow, oxygen tension, mechanical stress, and microbial biofilm formation are not yet captured in a single platform, so current in vitro model systems are unable to incorporate microbial communities that are fully representative of naturally occurring microbiomes—that is, ones that contain population, structural, and physiologic diversity, such as a mix of biofilm (or adherent) and free-swimming microbial populations. It is important to understand how various factors—such as nutrient and oxygen gradients, protective mucus barriers, epithelial cell types and architecture, mechanical stress, and fluid shear stress—change microbiome gene expression and metabolism, and which factors need to be recapitulated in an in vitro system. Once in vitro systems are able to incorporate complex characteristics, the effects of an environmental-chemical exposure on the microbiome can be tested with improved robustness and understanding of the chemical–microbiome interaction and its effects on the host. Beyond in vitro systems that can faithfully model the gut, there is a great need to develop systems for studying the skin, lung, and other body sites.

Microbial Reference Communities

Past initiatives of the Human Microbiome Project (HMP) have provided some initial healthy-adult reference community data. The HMP collected samples from at least 17 body sites from among 300 people who are representative of the variation in race, ethnicity, and sex of a healthy-adult cohort of the

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US population. Those data are being used to inform the generation of microbial reference communities and to standardize microbial populations that faithfully recapitulate the variation present in the human microbiome. However, additional work is needed to advance the microbiome field. The development and use of reference communities for in vitro and animal studies will allow comparison of results among institutions and increase reproducibility of results. It is likely that several representative reference communities beyond those informed by the HMP will be needed to account for the developmental, disease, and geographic, racial, and ethnic differences that determine the interindividual variation discussed earlier in the present report. Capturing key demographic, medical, social, and lifestyle factors that might also shape a person's microbiome will be important for the use of reference communities because these factors might affect interpretation of results and decision-making. It will be important to consider and incorporate not only the taxonomic variation observed in the human microbiome but the functional capacity and characteristics that continue to be discovered.

Reference Information and Annotation

The functional -omics data generated from, for example, metatranscriptomic and metabolomics approaches could help to elucidate time-resolved microbiome activity in response to environmental stresses that potentially lead to changes in host health. By understanding the time-course changes with high-complexity multi-omics longitudinal datasets, one could construct better predictive models that lead to the identification of higher-confidence biomarkers and targets. For those approaches to be used for understanding microbiome dynamics, the genomic, transcriptomic, and metabolic databases and libraries need to expand their coverage of relevant strains, genes, enzymes, and metabolite identities and functions. The vastness and complexity of the microbiome have resulted in genomic databases that contain scores of unannotated genes about which we know almost nothing. Similarly, there remains much to be annotated and identified in metabolomic databases, including chemical structure, metabolite source (human vs microbe), and metabolism pathway. Enriching the databases will facilitate clear identification of the potential for interactions among host and microbial states and for biotransformation of environmental chemicals.

As human metagenomics-sequence databases continue to expand, computational modeling strategies for reconstructing metabolic pathways and identifying enzyme homologues among metagenomes can provide an initial framework for inferring functions and chemical interactions of specific genes in the microbiome (Saad et al. 2012; Das et al. 2016). That approach was recently used to identify over 800 bacterial genera (from 397 human metagenomes) that might express enzymes that metabolize environmental chemicals (Das et al. 2016). Such estimates could be experimentally constrained by using metatranscriptomic and metaproteomic analyses to define which of the predicted gene products are expressed and under what circumstances.

Computational Models

An overarching goal is the development of computational models that can predict chemical–microbiome interactions and their consequences. Development of such models is in its infancy and will require large-scale data generation. At the molecular level, as noted above, most biochemically relevant microbial gene products are not yet characterized and need to be cataloged. Similarly, associations between specific microorganisms at the strain level and relevant phenotypes, such as biochemical activities or health outcomes, need to be bioinformatically identified and cataloged. Having that information will allow development of single-protein and single-microorganism models that can be extended to model biochemical activities arising specifically from microorganism–microorganism and host–microorganism interactions. The ultimate aim is to develop multiscale metabolic models that incorporate many different

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microbial members, human cell types, and even organ systems. The large number of interacting components and the likely stochasticity of the interactions make predictive computational models of host–microorganism interactions challenging.

It is not yet possible to predict the community-wide effects of most chemical exposures on the human microbiome; that is, how will the whole community structure be affected by a particular chemical exposure and with what temporal dynamics? Conversely, it is not yet possible to predict how the microbiome affects the ADME characteristics of a particular environmental chemical. Predictive models of microbial and chemical effects on human health outcomes will need to be developed to complement the predictive host–microorganism models. For example, computational associations have not yet been made between microbial products and most health-relevant human immune pathways or systemic metabolism. Long-term effects of microbiome activity on health, such as the induction of chronic disease, will be particularly difficult to model.

OPPORTUNITIES FOR COLLABORATION AND COORDINATION

In the United States, several agencies play roles in assessing health risks associated with exposures to environmental pollutants. Similarly, microbiome-related research is being conducted by several agencies and sectors. Progress in fields related to risk assessment and in microbiome research has occurred largely independently. The segregation of research programs in those fields, historically and currently, poses a major barrier to the advancement of knowledge on interactions between environmental chemicals and human microbiomes and the potential effects of such interactions to influence human health. Funding mechanisms that promote multidisciplinary research that specifically encourages collaboration between experts in such fields as exposure science, epidemiology, toxicology, risk assessment, human health, and microbiome research are crucial for the implementation of the research strategy described in the present report. To support such efforts effectively, agencies and research entities that conduct microbiome and human-health research are encouraged to develop collaborations with their counterparts in fields related to risk assessment and vice versa. For example, collaborations between the National Institutes of Health (NIH) and EPA or state environmental and public-health agencies that have a long history of assessing the health risks posed by environmental-chemical exposures are encouraged. That type of interdisciplinary collaboration should be sought out, encouraged, and supported to make the best use of existing knowledge and resources at each agency or organization. Likewise, initiatives similar to the Center for Children's Health, the Environment, the Microbiome and Metabolomics at Emory University, jointly funded by EPA and the National Institute for Environmental Health Sciences (NIEHS) could be considered as vehicles for stimulating and fostering the types of interdisciplinary research needed. Because pharmaceuticals and other products regulated by the Food and Drug Administration (FDA) enter the environment, collaboration between EPA and FDA might be valuable. The participation of experts in diverse research disciplines during the entire research cycle—planning and designing studies, conducting experiments, and analyzing data—is likely to result in studies that are better suited to addressing the research recommended by the committee. Moreover, such multidisciplinary initiatives could serve as an ideal training environment for the next generation of researchers whose expertise spans several fields.

To assist members of the various research communities, Box 6-2 lists some important resources that could serve as a starting point for identifying potential collaborators and notes where the resources could be leveraged to address the research described by the committee. The resources and related programmatic efforts present potential high-yield opportunities to advance the current understanding of the health consequences of environmental chemical–human microbiome interactions.

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BOX 6-2 Resources for Research Collaborations

Resources with Microbiome-Related Samples or Data

- NIH-sponsored *RoadMap* and *Common Fund* initiatives, such as HMP 1.0 and 2.0 and the NIH Common Fund Metabolomics Program. The HMP banked its extensive data in dbGaP (NCBI 2017), where further data projects are similarly stored and available. The initiatives include computational resources, and multi-omics studies are a part of HMP 2.0, the NIH Common Fund Big Data to Knowledge (BD2K) program, and the NIH Precision Medicine Initiatives.
- Microbiome research that is planned or under way in NIH institutes or other federal institutes or agencies that share interests in the developmental origins of health and disease, including the National Institute of Child Health and Human Development, the National Institute of Allergy and Infectious Diseases, the National Institute of Diabetes and Digestive and Kidney Diseases, NIEHS, the National Science Foundation, the Department of Defense, the Centers for Disease Control and Prevention, and the US Department of Agriculture (USDA). Large human studies funded by one institute could provide opportunities to add sample collections that would otherwise be outside the scope of the parent project. Because enrollment and sample collection usually occur in the first years of a grant, rapid-response supplemental funding could help to leverage and combine investments of multiple institutes or agencies.
- National Aeronautics and Space Administration initiatives on multi-omics analysis and microbial cross-talk (NASA 2017).

Resources with Samples or Data Related to Environmental-Chemical Exposure

- NIEHS Exposure Biology and Exposome research programs offer an opportunity for sample-sharing and coordination of data analytics for microbiome analyses. Research programs that seek to improve integrated exposure assessments, such as the Children's Health Exposure Analysis Resource (CHEAR), and programs that aim to develop biomonitoring sensor platforms and exposome databases offer important opportunities to integrate microbiome sampling and analysis as part of the overall research strategy.
- International programs—including projects focused on cohorts for early-life exposome assessment, such as HELIX[3] and EXPOsOMICs[4]—provide important opportunities to collaborate with the exposure-science community.
- Existing resources of stored and available data and specimens of vulnerable populations, such as pregnant women and children, can be found in the Data and Specimen Hub (NICHD 2017a), the Maternal-Fetal Medicine Units Network (NICHD 2017b), and the Human Placental Project (NICHD 2017c).
- Foundation resources of specific populations of likely interest include the March of Dimes, the Burroughs Welcome Fund Preterm Birth Initiatives, the Gates Foundation, and Global Alliance to Prevent Prematurity and Stillbirth programs.
- Partnerships or coordination with the environmental-bioremediation research and microbial-ecology communities stewarded by EPA, the Department of Energy, USDA, and other agencies could provide additional opportunities to catalog and cross-reference potential chemical–microorganism transformation pathways found in environmental microbial communities, such as soil (Gao et al. 2010), with those in human microbiomes and might create an important new knowledge base.

(Continued)

[3]See http://www.projecthelix.eu/index.php/en.
[4]See http://www.exposomicsproject.eu/.

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BOX 6-2 Continued

Examples of Risk or Exposure Data That Could Help to Inform Chemical Selection

- The EPA Integrated Risk Information System (IRIS) database (EPA 2017a) provides oral reference doses and inhalation reference concentrations for many environmental chemicals of concern. Analogous potency indicators for carcinogens are the oral slope factor and inhalation unit risk factor. The IRIS database includes descriptions of how the quantitative measures were derived, which is typically through an assessment of relevant published studies, most commonly involving human and animal exposures.
- Through various activities implemented at federal and state levels, the EPA Office of Ground Water and Drinking Water and the Agency for Toxic Substances and Disease Registry collect and evaluate data on population exposures, exposure pathways, and toxicology and produce results used by programs for making decisions on chemical selection or for updating risk assessments.
- State (for example, CALEPA 2016, 2017; MNDH 2017) and regional (for example, EPA 2017b,c) databases provide health values for environmental chemicals that are derived by using quantitative risk assessment. Agencies document the underlying scientific studies on which the values are based, and many programs describe the risk-assessment protocols that are used to develop health protective guidance.

CONCLUDING REMARKS

The committee believes that implementation of its proposed research strategy will substantially advance understanding of whether and to what extent the human microbiome affects the nature and magnitude of adverse health effects caused by exposures to environmental chemicals. In the relatively near term (2–4 years), results from the proposed research should allow judgments to be made about whether explicit consideration of microbiome interactions in the study of environmental-chemical toxicity yields information that is not available from traditional studies, that is, ones that do not explicitly consider microbiomes. Within a similar time frame, it should also be possible to gain some understanding of whether any such new information arises from the study of the effects of chemicals on the microbiome, from the study of the effects of the microbiome on chemical exposure, or both. Near-term results from the proposed research should thus allow judgments to be made about the need for and priorities to be assigned to continued pursuit of this new field of environmental research. Those results should also provide substantial guidance on preferred study methods.

If results from the near-term research provide relatively convincing evidence that explicit consideration of the microbiome in the development of chemical toxicity yields information that has previously been absent, the committee recommends that EPA begin to incorporate that information into human health risk assessments at least on an experimental basis. The longer-term research results should provide an understanding of the nature and magnitude of the sources of variation and variability that affect chemical–microbiome interactions and their health consequences. Those results will likely have the most important effects on the conduct of risk assessments. Ultimately, both the near-term and longer-term research should lead to the type of information that is needed to assess the importance of the microbiome as a contributor to the human health risks associated with exposures to environmental chemicals and thus allow informed decisions to be made about the need for and nature of continuing research.

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Appendix

Biographic Information on the Committee on Advancing Understanding of the Implications of Environmental-Chemical Interactions with the Human Microbiome

Ronald M. Atlas (*Chair*) is professor of biology at the University of Louisville. His early research focused on oil spills and bioremediation. He later focused on the molecular detection of pathogens in the environment, which informs the development of biosensors to detect biothreat agents. Dr. Atlas is the author of nearly 300 manuscripts and 20 books. He is a fellow of the American Academy of Microbiology and has received the American Society for Microbiology (ASM) Award for Applied and Environmental Microbiology, the ASM Founders Award, the Edmund Youde Lectureship Award in Hong Kong, and an honorary doctorate from the University of Guelph. He has served as a member of the Department of Homeland Security's Science and Technology Advisory Committee, the National Aeronautics and Space Administration's Planetary Protection Board, the Federal Bureau of Investigation's Scientific Working Group on Bioforensics, and the National Institutes of Health's Recombinant DNA Advisory Committee. He has also served as the ASM president and is co-chair of the ASM Biodefense Committee. Dr. Atlas has been the chair or a member of numerous National Academies committees. He received his PhD in microbiology from Rutgers University.

Kjersti M. Aagaard is a professor in the Department of Obstetrics and Gynecology, the Department of Molecular and Human Genetics, the Department of Molecular and Cellular Biology, and the Department of Molecular Physiology and Biophysics of Baylor College of Medicine. She specializes in maternal–fetal medicine, in which her research interests include basic-science investigations and translation into clinical research. She is specifically interested in microbiome interactions with preterm birth and in the role of the in utero environment and epigenetics in fetal programming and development. Dr. Aagaard earned her MD from the University of Minnesota Medical School in Minneapolis and her PhD from the Mayo Graduate School of Medicine.

Elaine Hsiao is an assistant professor in the Department of Integrative Biology and Physiology and the Department of Medicine, Digestive Diseases at the University of California, Los Angeles (UCLA). Her research interests include the microbiome, neurobiology of disease, neuroimmunology, and host–microorganism interactions. Specifically, her research explores the effects of the microbiota on the nervous system and communication between microorganisms and the nervous system. Dr. Hsiao also studies the particular functions of microbiome species and the effects of modification of the microbiome on neurologic disease. She was the De Logi Chair in Biological Sciences at UCLA, received a National Geographic Emerging Explorer Award, and was selected for the Forbes "30 under 30 in Science & Healthcare." Dr. Hsiao was the 2013 Caltech Everhart Lecturer and served on the White House Office of Science and Technology Microbiome Forum. She received her PhD in neurobiology from the California Institute of Technology.

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Yvonne Huang is an assistant professor of internal medicine at the University of Michigan in the Division of Pulmonary and Critical Care Medicine. Dr. Huang's research interests include the microbiome, asthma, chronic obstructive pulmonary disease (COPD), and interactions between therapeutics and the microbiome. Her work on the respiratory microbiome in asthma and COPD includes trials sponsored by the National Institutes of Health. She was a Yale/Johnson & Johnson Physician Scholar in International Health and served as associate director of the adult cystic fibrosis program at the University of California, San Francisco (UCSF). Dr. Huang earned her MD from the University of Alabama at Birmingham, trained in internal medicine at Yale, and completed a fellowship in pulmonary/critical care medicine at UCSF.

Curtis Huttenhower is an associate professor of computational biology and bioinformatics at the Harvard T.H. Chan School of Public Health and an associate member at the Broad Institute. Dr. Huttenhower's laboratory worked extensively with the National Institutes of Health Human Microbiome Project (HMP) to identify and characterize the microorganisms found in association with both healthy and diseased humans. In 2015, he co-led one of the follow-up HMP2 Centers for Characterizing the Gut Microbial Ecosystem in Inflammatory Bowel Disease. He received a National Science Foundation CAREER award in 2010 for his research on microbial communities and was awarded a Presidential Early Career Award for Scientists and Engineers in 2012. Dr. Huttenhower was also awarded the Overton Prize from the International Society for Computational Biology in 2015. He is a member of the editorial boards of the academic journals *Genome Biology*, *Microbiome*, and *BMC Bioinformatics*. Dr. Huttenhower received his PhD in genomics from Princeton University.

Rosa Krajmalnik-Brown is an associate professor in civil and environmental engineering and is part of the Swette Center for Environmental Biotechnology in the Biodesign Institute at Arizona State University. She specializes in molecular microbial ecology for bioremediation, the use of microbial systems for bioenergy production, and human intestinal microbial ecology and its relationship to obesity, bariatric surgery, and autism. She is an author of five patents and more than 70 peer-reviewed publications and has presented numerous talks and posters at national and international conferences. She was awarded a Fulbright scholarship and completed her PhD in environmental engineering at the Georgia Institute of Technology.

Susan Lynch is a professor of medicine at the University of California, San Francisco, where she is the director of the Colitis and Crohn's Disease Microbiome Research Core and the associate director of the Microbiome in Inflammatory Bowel Disease Program. Dr. Lynch's research program focuses primarily on the gastrointestinal microbiome and its role in the origins and maintenance of chronic inflammatory diseases, including airway diseases. She was awarded the Rebecca Buckley Lectureship by the American Academy of Allergy, Asthma and Immunology, was named one of *Foreign Policy* magazine's Global Thinkers in 2016, and serves as an American Society of Microbiology Distinguished Lecturer and a senior editor for *Microbiome*. Dr. Lynch earned her PhD in molecular microbiology at the University College Dublin.

William Nazaroff is the Daniel Tellep Distinguished Professor of Engineering in the Department of Civil and Environmental Engineering at the University of California, Berkeley. His research group studies the physics and chemistry of air pollutants in proximity to people, especially in indoor environments. The group also works in the domain of exposure science and stresses the development and application of methods for understanding mechanistically the relationship between emission sources and human exposure to pollutants. Dr. Nazaroff has served as president of the Academy of Fellows in the International Society of Indoor Air Quality and Climate and president of the American Association for Aerosol

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Research. For the National Academies, he chaired the Planning Committee for the Workshop on Health Risks of Indoor Exposure to Particulate Matter and served on the Committee on the Effect of Climate Change on Indoor Air Quality and Public Health and the Committee on Air Quality in Passenger Cabins of Commercial Aircraft. Dr. Nazaroff earned his PhD in environmental engineering science from the California Institute of Technology.

Andrew Patterson is an associate professor of molecular toxicology at Pennsylvania State University. He studies the metabolism of drugs and foreign chemicals by the human body and how chemicals in diets or nutrients derived from diets influence health and disease. Much of his research involves the use of metabolomics tools. Dr. Patterson was a research fellow of the National Cancer Institute, and he served on the National Academies committee responsible for planning the workshop Getting the Most from Microbiome Research in the Next Decade. He earned his PhD in genetics from the George Washington University.

John F. Rawls is an associate professor in the Department of Molecular Genetics and Microbiology at the Duke University School of Medicine and director of the Duke Microbiome Center. He has secondary appointments in Duke University's Department of Medicine, Center for Host–Microbial Interactions, and Cancer Institute. Dr. Rawls's laboratory uses zebrafish and mouse models to study how intestinal microbiota affect vertebrate health. He was recognized as a Pew Scholar in the Biomedical Sciences. Dr. Rawls received his PhD in developmental biology from Washington University.

Joseph V. Rodricks is a founding principal of Ramboll Environ. An expert in toxicology and risk analysis, he has consulted for hundreds of manufacturers and government agencies and for the World Health Organization in the evaluation of human health risks associated with exposure to chemical substances. Before Environ, Dr. Rodricks served for 15 years as a scientist at the US Food and Drug Administration; in his last 4 years, he served as associate commissioner for health affairs. His experience extends from pharmaceuticals, medical devices, consumer products, and foods, to occupational chemicals and environmental contaminants. He has served on the National Academies Board on Environmental Studies and Toxicology and on 30 other boards and committees of the National Academies, including the committees that produced the seminal works *Risk Assessment in the Federal Government: Managing the Process* (1983) and *Science and Decisions: Advancing Risk Assessment* (2009). Dr. Rodricks has nearly 150 scientific publications and has received honorary awards from three professional societies for his contributions to toxicology and risk analysis. He earned his PhD in biochemistry from the University of Maryland, College Park and was a postdoctoral scholar at the University of California, Berkeley.

Pamela Shubat is retired from the Minnesota Department of Health Environmental Health Division, where she supervised the work of the Health Risk Assessment Unit. She has worked on many aspects of risk assessment, toxicology, and exposure assessment. For example, she has been involved in work on sensitive subpopulations and life stages and drinking-water contaminants. Dr. Shubat's major responsibilities have included research on fish contaminants, childhood lead-poisoning prevention, population-based exposure assessment, and rules for groundwater contaminants. In addition to state work, she served as an appointed member and chair of the US Environmental Protection Agency (EPA) Children's Health Protection Advisory Committee and as a peer reviewer for EPA projects that involved methylmercury, polychlorinated biphenyls, and risk-assessment practice. Dr. Shubat is a member of the EPA Federal–State Toxicology Risk Analysis Committee. She earned a PhD in pharmacology and toxicology from the University of Arizona.

Brian Thrall is an associate director in the Biological Sciences Division and chief scientist for the Health Impacts and Exposure Science Group at Pacific Northwest National Laboratory (PNNL). He has over 20 years of experience in leading research programs focused on developing and applying systems-toxicology and exposure-science strategies to elucidate and ultimately predict biologic response pathways modulated by exposure to environmental agents of concern to human health. As director of PNNL's Center for

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Nanotoxicology, Dr. Thrall leads a multidisciplinary team that uses state-of-the-art genomics, proteomics, bioinformatics, and cell imaging to understand receptor-mediated interactions between biologic systems and engineered nanomaterials. In addition, he serves as the thrust leader for PNNL's Microbiomes-in-Transition research program, which includes a focus on developing tools and strategies to elucidate microbiome–exposome interactions. Dr. Thrall earned his PhD in pharmacology and toxicology from Washington State University.

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