MICROBIOMES

Health and the Environment



Dylan Parks

Microbiomes: Health and the Environment

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DYLAN PARKS

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About Mavs Open Press

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About This Project

Overview

Microbiomes: Health and the Environment was created to provide accessible insight into the novel and complex world of polymicrobial community interactions. As we push forward into the future of medicine and environmental health, it is imperative that we learn from each other, from history, and keep up to date with the latest advances in research and technology. This book not only provides content from the latest microbiome studies, but contains interactive tools, videos, and thought-provoking questions to help the reader hone and truly understand the respective topic. Though there is much overlap between themes due to the ubiquitous nature of microbes, the book is broken down into sections pertaining to both human health (e.g., gut health and disease, as well as other organ-specific niches) and aspects of the environment (e.g., nutrient cycling and climate change, marine health, soil and plant health, etc.) influenced by microbes. However, the content is designed to bridge ideas and aspects between these themes to support the One Health concept: that the health of people, animals, and the environment are all interconnected. This project will continue to grow with new findings, and adapt with the ever-changing world of microorganisms.

Creation Process

This project was completed by thorough review of published literature from reputable resources and written in a style accessible

to both experienced scholars and newcomers to the subject. Specific excerpts and articles are provided throughout the book to give the reader first-hand insight into microbiome research. Supporting content such as images and videos are also implemented to give the audience a well-rounded and multifaceted view into each topic. Interactive tools, quizzes, and critical thinking questions make each section entertaining and informative. A section with case studies and directions for original synthesis make information in the book applicable to real world situations.

About the Author

Dr. Dylan Parks is an Assistant Professor of Instruction at the University of Texas at Arlington in the Biology Department. As a microbiologist, he has worked in both academia and industry, serving as laboratory technician at a local brewery. His research interests include microbiomics, biomedical sciences, fermentation science, and microbial symbiotic interactions.

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PART I

AN INTRODUCTION TO MICROBIOMES

1. An Introduction to Microbiomes

An Introduction to Microbiomes

Microorganisms represent the fundamentals of life and interact with almost every facet of it. Yet, for the majority of their existence they have been largely ignored, or rather unseen by humans. The unraveling of the complex interactions between all life forms is an endless duty and seems to generate more questions than answers. However, the cracks in knowledge produced by peering into the unknown offers insight into our life and the world around us. These minute creatures have shaped Earth's evolution since their dawn almost three-and-a-half billion years ago, and continuously affect our environment and health. The importance of the planet's collection of microbes is realized more and more each day, and our unceasing investigation of them will surely unlock secrets of life we could never imagine.

The study of microbiomes is fairly novel in the context of understanding and applying their communal existence to ourselves and surroundings. Though scientists have recognized symbiotic relationships and traditionally focused on individual microbes and their interactions with human health, environmental impact, industrial applications, etc., their respective communities and influence as a whole in these areas have only just begun to be elucidated. That is in no small part due to the daunting task of cataloging the immense and complex interplay between the multitude of different microorganisms in a given environment, though rapid advances in technology have begun to ease analysis.

What is a microbiome?

A microbiome can be best described as a collective polymicrobial community, or 'microbiota', and its associated activity with genetic and physio-chemical constituents in a defined spaciotemporal habitat (Figure 1). These members of the microbiota include bacteria, archaea, algae, protozoa, fungi, and viruses (though the latter is somewhat debated since viruses and their derivatives aren't technically living). Within this symbiotic context with a particular eukaryotic host, the entire entity is termed a 'holobiont' and the aggregate of genetic material termed the 'hologenome'. Interactions between these partners may have long occurred, shaping the evolution of each, whereas others may be novel or transient, sometimes resulting in prompt change and infectious diseases. The change in the normal microbiota, or dysbiosis, can result in a variety of different diseases. As so, their study has been especially important in the fields of life sciences, human health, and medicine.

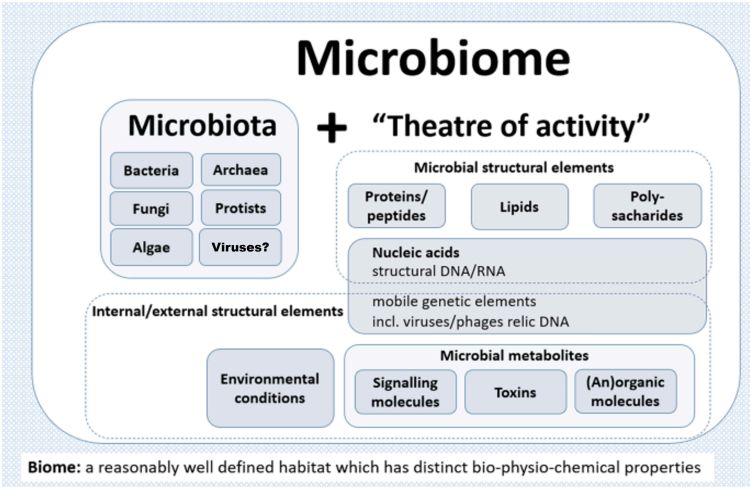


Figure 1. A schematic highlighting the composition of the term microbiome containing both the microbiota (community of microorganisms) and their "theatre of activity" (structural elements, metabolites/signal molecules, and the surrounding environmental conditions). (Berg et al., 2020 adapted by Dylan Parks).

Quick Quiz



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https://uta.pressbooks.pub/microbiomeshealthandtheenvironment/?p=1529#h5p-1

Our fascination with microorganisms began before we even fully understood them or were even able to see them. Their implications concerning human health were primarily explored during the 'golden age of microbiology' with the work of Louis Pasteur and Robert Koch. Their experiments and discoveries shed light on not only the ubiquitous nature of microbes, but their importance in our everyday lives. Other significant milestones and historical microbiological development can be viewed in Figure 2. Human health and infectious diseases were central to the field of microbiology, though food microbiology, industrial applications, and microbial ecology became increasingly explored. Over the last couple centuries, a microbial catalog of knowledge has slowly grown, but much of these findings were limited to those organisms that could be cultured and measured. The advent of sequencing

and 'multi-omics' technologies has since allowed researchers to document microorganisms that were previously missed or ignored with traditional techniques, and with further advances, larger microbial communities and symbioses can be better understood. The 'microbiome' was first defined in the late 1980s when a group of microbial ecologists were studying the rhizosphere, which provided context to better describe these polymicrobial communities (Whipps et al., 1988). Many other similar definitions have been published since then with varying specifics on genetic expression, symbioses, and ecological interactions (Lederberg & McCray, 2001, Marchesi & Ravel, 2015, Berg et al., 2020). The 'holobiont' concept stems from Adolf Meyer-Abich's 'theory of holobiosis' proposed in 1943 and was independently conceived and popularized in the early 1990s by Lynn Margulis, though it only described the host and a single symbiont (Margulis, 1991, Baedke et al., 2020). Since then has been expanded to include the entire microbiota in multiple symbiotic contexts (Simon et al., 2019). In recent decades there has been a steady increase in microbiome publications as the subject has grown in popularity. Along with that, there has been more analytical breakdown as certain microbiomes are being described with emphasis on specific members, such as the 'bacteriome', 'archaeome', 'mycobiome', 'protistome', and 'virome', and these terms are best used to refer to the distinct contribution of those particular microbes within the entire microbiome context. In general, though, most microbiomes are delineated by their specific host or type of environment, with the human microbiome being the most popular example.

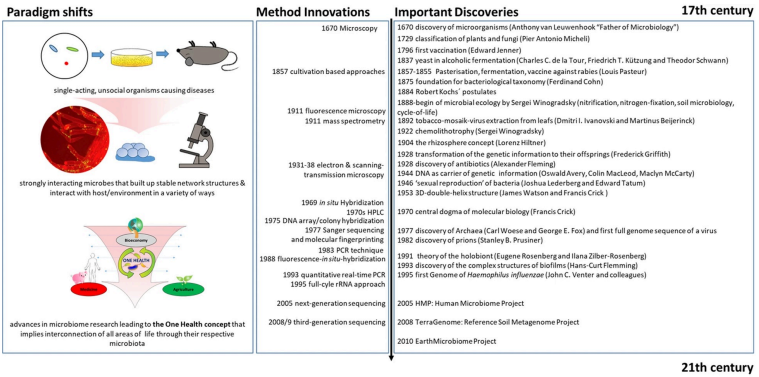


Figure 2. The history of microbiome research from seventieth century until our days, highlighting the shift of the paradigm from microbes as unsocial organisms causing diseases to the holistic view of microorganisms being the center of the One Health Concept: positively interconnecting all areas of our lives. (Berg et al., 2020).

The Human Microbiome

It was evident that the human microbiome and its involvement in a micro and macro scale needed to be characterized. The Human Microbiome Project (HMP) set out in 2007 with this as one of its primary goals (Turnbaugh et al., 2007). The program also set out with initiatives to develop a set of microbial genome sequences, explain the relationship between disease and microbiome changes and evaluate the data with multi-omics approaches, develop new tools and technology for computational analysis, establish a data analysis and coordinating center and research repositories, as well as address ethical, social, and legal implications of HMP research (Human Microbiome Project). The second phase of the HMP launched in 2014, called the Integrative Human Microbiome Project (iHMP), having the main mission to completely characterize the human microbiota with a key focus on human health and disease

using three projects: pregnancy and preterm birth, onset of inflammatory bowel disease (IBD), and onset of type 2 diabetes (NIH Human Microbiome Project, The Integrative HMP (iHMP) Research Network Consortium, 2019). Aside from these, the human microbiome and disruption of the microbiota has been linked to several other important conditions and diseases including multiple sclerosis, diabetes (types 1 and 2), allergies, asthma, autism, and cancer (Backhed et al., 2012, Hsiao et al., 2013, Petersen and Round, 2014, Trompette et al., 2014, Garrett, 2015, Lloyd-Price et al., 2016).

It makes sense that the human microbiome can have such an impact on human health and behavior if you consider that we are essentially a collection of organisms forming a living entity. In a way, our symbionts may even actually define more of who we are than just our own unique biological makeup. For instance, the ratio of microbial cells associated with a human body could equal, if not exceed (traditional estimates were tenfold), the number of human cells (Sender et al., 2016). Even more interesting is viewing our genetic makeup; the human genome contains about 20,000 genes, but its hologenome contains >33 million genes brought by its microbiota (Huttenhower et al., 2012, Lloyd-Price et al., 2016, Simon et al., 2019). Furthermore, the composition and rate of change of each person's microbiota is distinctive from one individual to another since it is influenced by variables like age, lifestyle, diet, antibiotics, occupation, environment, etc. (Gilbert et al., 2018). The genetic wealth and member diversity contributed from the microbiota has roles in adaptation, survival, development, growth, and reproduction of the holobiont and can affect fitness in the short term as well as have long lasting effects concerning the evolution of both partners (Rosenberg, and Zilber-Rosenberg, 2011).



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Co-evolution of the host-microbiota symbiosis can be considered even more unique when viewing the microbial consortium at different locations or organs in the host, as their makeup is governed by and reflects specific physiological processes in those areas. For example, the bacteria found in the human gut microbiota are primarily from the phyla Bacteroides and Firmicutes, whereas Actinobacteria and Proteobacteria command the skin microbiome, though there is some overlap and it is important to note that there are differences depending on exact location (e.g. dry vs. moist areas of the skin) (Grice and Segre, 2011, Jandhyala et al. 2015). Though there are differences between various microbiota within a holobiont, they can still influence each other to some degree. In the case of the gut and skin microbiotas in humans, deemed the 'gut-skin axis', there are indications that both the health of the gastrointestinal (GI) tract and skin, as well as their response to stressors, are correlated (Levcovich et al., 2013, O'Neill et al., 2016, Salem et al. 2018). Even more interesting is the effects certain microbiota can have on germ-free organs like the brain. Studies on the 'gut-brain axis' show that the microbiota in the GI tract, and in some cases disruption of it, are associated with many mental illnesses and neurodegenerative disorders including depression, anxiety, autism, schizophrenia, Parkinson's disease, and Alzheimer's disease (Clapp et al. 2017, Foster et al. 2017, Cryan et al. 2019). A variety of different 'axes' which demonstrate interplay between microbiota, organs, and locations have been identified in the human body and much of what is known about their connections is novel and early in its research.

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Environmental Microbiomes

Not only can our microbiome regulate who we are, but those communities in the surrounding environment can affect us, our microbiome, and others. Environmental microbiomes can directly or indirectly affect our health through ecological interactions. For example, soil microbiomes in the rhizosphere of plant roots and plant microbiomes of economically important crops have implications in agriculture, human health, and ecology (Saleem et al., 2019, Hirt, 2020). Plant growth, health, soil nutrient cycling and availability, and defense against potential pathogens are dictated by their own symbionts as well as their microbial neighbors in the ground, which include a variety of bacteria, protists, viruses, and network of fungi known as mycorrhizae (Busby et al., 2017, Hannula et al., 2017, Pratama and van Elsas, 2018, Zhong et al., 2019,). By better understanding the functional interactions of all the players in these environmental microbiomes, it may be possible to address problems like agricultural soil fertility, plant disease, and pollution. Thus, harnessing better microbiomes either by specifically engineering a microbial consortia for a targeted area/specimen or transplanting a natural community could improve sustainable agricultural practices which are desperately needed to feed the expanding population of humans while protecting the environment (Elhady et al., 2018, Arif et al., 2020, Hernandez-Alvarez et al., 2022).

Studying animal-microbiome systems can also give us

information about our environment. For instance, how anthropogenic-induced changes have impacted things like climate-change and approaches to maintain homeostasis with various environmental factors. A promising reservoir of research is the microbiota of marine animals since most of the planet is covered in water. Corals, sponges, various fish, and marine mammals have all been investigated to document the response of their microbial symbionts to changing environmental factors (Apprill, 2017). Other animal microbiome models are being used in an analogous means to understand how the microbiota could potentially influence human health and fitness. The microbiomes of classic biomedical models such as the fruit fly, zebrafish, and nematode worm are being investigated since these organisms are well known and readily available to work with in many labs (Douglas, 2019). Though, it is important to consider a variety of animal host-microbiome models as each could contribute unique insights to beneficial microbial interactions within the human holobiont. For example, the gut microbiome of honeybees, the skin microbiome of freshwater polyps, and the individual interaction of *Vibro fischeri* and the Hawaiian bob-tailed squid have provided valuable information to understanding host-microbiome relationships (Douglas, 2019). Further research of these and other systems are needed for the pursuit of technological and medical advances or cultural/societal changes necessary for overall human and planetary benefit.

Microbiome Analysis

Traditionally, it has been difficult to characterize complete microbial communities, as most projects require substantial time and resources. Though, advancements in NGS and 'omics' technologies have begun to allow researchers to tackle microbiome analytics using a variety of approaches including metagenomics, metatranscriptomics, proteomics, metabolomics, culturomics, etc.(Integrative HMP (iHMP) Research Network Consortium, 2014,

Bashiardes et al., 2016, Daliri et al., 2017, Janson and Hofmockel, 2018, Lin et al., 2019, Diakite et al., 2020,). Each technique is selected and applied depending on the experimental setup and what questions are being addressed. For instance, does the research care about the identification of members of the microbiome, how they are interacting with the host or each other, what macromolecules are present, what genes are being expressed, what is the functional potential, etc. These different types of investigations can then be integrated together in network analyses to establish linkages and correlations within the microbiome datasets, though statistical models and analytical tools must be carefully selected to avoid false outcomes and shortcomings (Jiang et al., 2019). Due to the particular limitations of these approaches and the unavoidably large datasets produced by microbiomic studies, it is paramount to continually develop novel technologies to unearth the knowledge buried in these microbiomes.

Considering the abundance of microbiomes that no doubt play role in human health, the extent to which microorganisms sway our lives is almost impossible to foresee, and the opportunities they present for improvement to industry, agriculture, environmental and human health is potentially unlimited.

Check Your Understanding

- What is the difference between a microbiome and microbiota?

- In what ways does a microbiome influence a holobiont?
- How can an environmental microbiome indirectly impact human health?

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PART II

ANALYZING MICROBIOMES

2. Analyzing Microbiomes

Analyzing Microbiomes

Choosing the correct approach when characterizing a microbiome is important when considering what questions are trying to be answered. Who are the players? What is the function? What genes are being expressed? How do they interact?

Even though when assessing a microbiome all members of the microbiota should be considered, some insight can be gained by viewing individual groups, such as the prokaryotic component, which can be further compartmentalized into the bacteriome and archaeome. In some cases, it is difficult to completely characterize all of the members due to inherent biological differences between these communities, and in other situations, certain groups may be absent or irrelevant to the study. In so, analyzing these individual pieces could help reveal their distinct importance and contribution to the overall big picture.

The type of analysis also depends on the research question. If the sole interest is to identify all the bacteria in a sample, then a metagenomic approach may work best. Whereas, if an interest lies in what the microorganisms are doing, then a metatranscriptomics or metaproteomics approach would be more appropriate. If there is an interest in one or a few particular members of the microbiome, culture-based techniques (culturomics) could be implemented, which can often catch rare microbes that are missed using sequencing techniques because of data filtering protocols (Lagier et al., 2012, Allaband et al., 2019). However, it is usually difficult to characterize an entire microbiome based on just a few microbes and achieving multiple isolations is extremely difficult as a 'one-size-fits-all' type media does not exist.

Sample Collection

Depending on the type of research questions and the particular microbiome of interest, there are different ways to collect microbial communities that will be subjected to analysis. That is, the approach to collecting microbes from the human gut is much different than getting a soil sample. Really, there is no perfect method for any one microbiome, though some may be more difficult to obtain than others, and will depend on a variety of factors such as feasibility, cost, patient acceptance, and downstream analytical methods (Allaband et al., 2019). It can also be very difficult to capture all microbes within a microbiome sample, as inevitably some will be missed or cannot be collected by a particular technique. For example, when analyzing the gut microbiome, stool collection is preferred due to its ease and frequent accessibility, but this method often misses microbes in the small intestine and others which are mucosally adherent as opposed to excreted (Eckburg et al., 2005, de Carcer et al., 2010, Allaband et al., 2019). Additionally, collection and storage materials, storage time, and transport options should be considered as these can compromise sample integrity and create problems for various analytical approaches (Costello et al., 2009, Caporaso et al., 2011, McDonald et al., 2018).

Approaches to Characterization

What is the Difference between Culture-Dependent and Independent Methods of Microbial Community Characterization?

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Up until the 1980s, microbiologists routinely relied on culture-dependent methods for microbial isolation, identification, and characterization. Colony morphology, stains (i.e., Gram stain), biochemical characteristics (i.e., coagulase test), motility tests, antibiotic resistance profiles, and other characteristics guided bacterial and/or fungal identification and taxonomy. However, this approach has several limitations, including an inability to mimic in vivo conditions and selection against slow-growing and/or fastidious organisms. With recent advances in sequencing technologies and development of bioinformatics tools and reference databases, researchers are now better equipped to capture microbial diversity without the biases of culture-based approaches.

Culture-independent methods of microbial identification rely on a targeted amplicon strategy, which employs highly conserved microbe-specific molecular markers and does not rely on growing isolates in pure culture. The 16S ribosomal RNA (rRNA) gene is used for bacterial identification, while fungi and other microeukaryotes are identified using either the 18S rRNA gene or the Internal Transcribed Spacer (ITS) region. A complementary approach to amplicon-based surveys is whole genome shotgun metagenomics. With this approach, one can identify the microbiota present and gain insight into the functional potential of the microbiota in an untargeted manner.

Within the culture-independent targeted approach, PCR

amplification techniques, like quantitative PCR (qPCR) and reverse transcriptase PCR (RT-PCR) can be implemented to detect and quantify specific organisms, genes, or expression values within the microbiome sample. Untargeted approaches typically give whole community characterization and include metagenomics, metatranscriptomics, metaproteomics, and metabolomics which have a variety of applications depending on the type of study.

Culturomics

Cultivating all the microorganisms within a certain microbiome can be quite challenging and time-consuming, but nonetheless can provide important information that would otherwise be missed with other approaches. Creating media that mimics the original environment can help to capture organisms that exist at lower concentrations than that of the detectable threshold of molecular tools (Lagier et al., 2012). The application of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has enhanced culture-dependent studies by allowing fast and reliable identification of bacterial taxa in a cost-effective manner (Seng et al., 2009, Lagier et al., 2012). Even though there have been advances in culturomics, the approach still has certain biases based on the types of microbes that can be easily grown (e.g. aerobic microbes are much easier to grow than anaerobic ones in most cases), as well as the selection for those that grow more rapidly and outcompete the others in a sample (Allaband et al., 2019). Thusly, it is imperative to close the gap between microbial richness observed in nature versus what is cultivable in the lab in order to better understand *in vivo* microbial functioning within a microbiome (Sarhan et al., 2019).

Metagenomics and DNA sequencing

Next-generation sequencing (NGS), also known as high-throughput sequencing (HTS), allows the generation of thousands of sequencing reads in a cost-effective manner (Nkrumah-Elie, et al., 2018). These reads can then be bioinformatically processed and annotated to identify microbial taxa, genes, potential functions, etc. depending on the sample type and experimental conditions (Rivera-Pinto et al., 2018). However, differences in wet lab work, sequencing platforms, processing pipelines, and statistical approaches can affect data output, which make reproducibility come into question (Gloor et al., 2016). Usually, information about microbiome compositions are provided as relative abundances instead of the true abundances due to sequencing platform imitations. This data is considered an arbitrary sum and is referred to as compositional data (Jiang et al., 2019). As sequence generation and pipelines improve and become standardized, the development and accessibility for analytical tools can help to streamline and produce consistent and more accurate results while aggregating data for microbiome characterization and meta-analysis without advanced expertise (Markowitz et al., 2007, Dhariwal et al., 2017, Gonzalez et al., 2018, Chong et al., 2020, Mitchell et al., 2020, Bharti and Grimm, 2021, Chen et al., 2021).

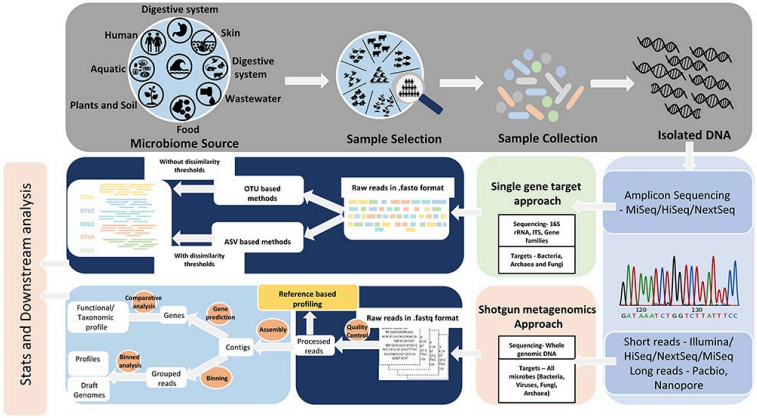


Figure 1. *An illustration of targeted amplicon and metagenomic sequencing approaches. A schematic overview demonstrating diverse sample types along with commonly utilized sequencing platforms, as well as systematic and stepwise data processing steps. (Bharti and Grimm, 2021).*

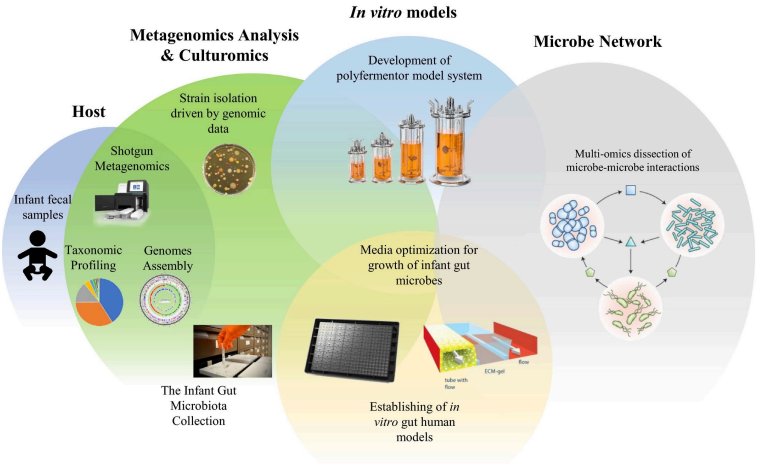


Figure 2. *Schematic representations of the in vitro models used for underpinning the microbe–microbe and host–microbe cross talks.*

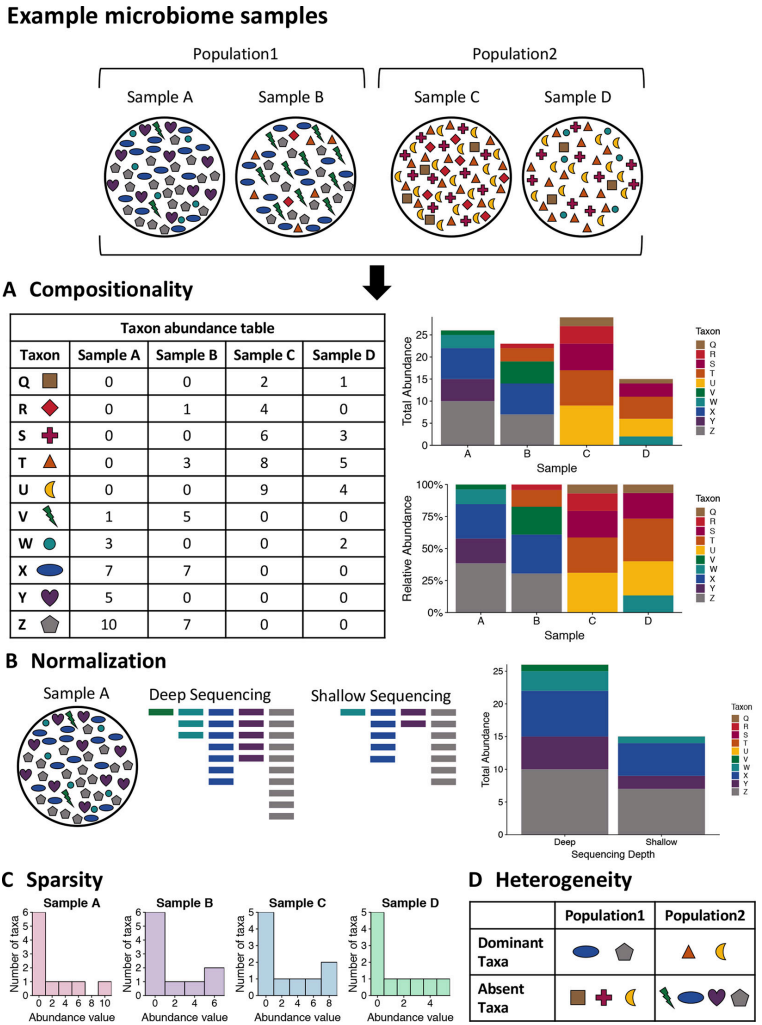


Figure 3. *Visualizing the unique challenges of microbiome data. A mock set of bacterial samples from two populations where each colored shape is a bacterial taxon. (A) Compositionality. The taxon abundance table depicts the count of each observed taxon in each sample. When sequencing microbiome samples, the resulting counts of taxa are not representative of the actual taxa counts in the sample due to constraints of sequencing. Due to this, relative abundances are generally used in analysis of microbiome data. The bar plots illustrate the difference in community representation between raw counts (top) and relative abundances (bottom). (B) Normalization. Due to the constraints of sequencing, the overall sequencing depth of a sample can impact the results. For example, shallow sequencing may miss rare taxa such*

as the green taxon V in the example sample A that is present in low abundance in the community. (C) Sparsity. Microbiome data are often very sparse, where most observations are zero. This is illustrated by the histogram of taxa counts for each sample where most counts are zero and there are few taxa with high counts. This can also be seen in the table for part A, where many entries are zero. (D) Heterogeneity. The table summarizes the taxonomic heterogeneity in the mock dataset between the two populations. Each sample has a unique taxonomic composition, but there are also population specific signatures. The samples in each population are dominated by a few taxa, and these dominant taxa are different for the two populations. Additionally, there are taxa that are highly abundant in one sample and absent from the rest, such as the purple taxon Y in sample A.



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Metatranscriptomics and RNA sequencing

While metagenomic studies have been instrumental in identifying various microbes and genes in a given sample, it does not differentiate between viable cells or when those genes are being expressed (Gosalbes, et al., 2011, Bashiardes et al., 2016). Metatranscriptomic strategies of microbiome analysis allow researchers to capture gene activity which can delineate the functional dynamics of a particular community and it's interactions with its host or environment. These RNA-based approaches can complement metagenomics, as the extent of expression of those annotated genes can be measured (Franzosa et al., 2014). This is considerably important concerning certain disease states because the presence of a pathogen does not always produce deleterious

conditions, but rather it is the functional activity of certain organisms.

RNA-seq experiments usually begin with isolation of total RNA in a sample, then selection is based upon whether it is prokaryotic or eukaryotic in origin, and mRNA must be isolated from other RNA species (i.e. rRNA and tRNA) (Bashiardes et al., 2016). cDNA is then synthesized from the mRNA, adapters are ligated to create a library, amplification and sequencing follows, and the generated reads are mapped to a reference genome where expression can be measured (Giannoukos et al., 2012, Bashiardes et al., 2016). These experiments can be difficult and sensitive, as care must be taken with sample collection, and avoiding contamination and degradation from ribonucleases is important to maintain integrity of the samples as well. Furthermore, metatranscriptomics may not always give the whole picture of community expression due to the high complexity of organisms, the delicateness of RNA, the wide range of transcript expression, and various limitations with current technology (Shakya et al., 2019).

Metaproteomics

Metaproteomics gives a snapshot of the entire protein content of a microbiome sample under various conditions, which can further provide insight into gene activity and metabolic functions. This strategy identifies peptides by matching tandem mass spectrometry (MS/MS) data to protein sequence databases, which can be further assigned to functional groups via annotation databases (Sajulga et al., 2020). This approach can be convoluted though, as many peptides match to similar proteins produced by different organisms, and proteins can be multi-functional making it difficult for assignment to any one group. Additionally, bioinformatic analysis of metaproteomes isn't exactly standardized which makes reproducibility and comparison of studies problematic (Schiebenhoefer et al., 2019, Sajulga et al., 2020).

Metabolomics

Metabolomics analyzes the non-protein metabolites that are produced and/or regulated by a given microbiome, which adds another angle of characterization to community-host-environmental interaction and functional dynamics. By understanding how various microbes process metabolites like natural products, nutrients, and medications, we can better determine how they influence their ecosystem through molecular interactions. Researchers typically obtain these molecules through gas or liquid chromatography and subsequently analyze them by mass spectrometry (Allaband et al., 2019, Bauermeister et al., 2021). Metabolomics strategies can either be targeted or untargeted, where the former is more sensitive and compares metabolites to a pre-determined bank with reference standards, however, it cannot detect novel molecules. Since most metabolites produced by microbiomes are unknown, an untargeted approach using tandem mass spectrometry is useful in detecting many small molecules, though annotation of modified metabolites is challenging (Watrous et al., 2012, Wang et al., 2016, Allaband et al., 2019). As with other microbiome analytical approaches, various data analysis tools and pipelines of the metabolome need to be standardized to a degree to improve overall research context. Moreover, major advancements in the known inventory of microorganism-derived metabolites and their functions are required to enhance metabolomics studies (Lee-Sarwar et al., 2020, Bauermeister et al., 2021).

Drag and Drop Quiz



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Conclusion and Future Directions

While various approaches to characterizing a microbiome each have their advantages and drawbacks, network analyses integrating all the -omics (i.e. metagenomics, metatranscriptomics, metaproteomics, and metabolomics) could be best to gain a holistic view of compositional and functional dynamics (Bashiardes et al., 2016, Jiang et al., 2019). Novel and improved approaches to collect and analyze various microbiomes will help to minimize loss of information and provide a better picture of how these communities interact with and influence their host or environment. Also, the establishment of more comprehensive publicly available databases, and protocols for streamlining computational procedures will allow more consistent and reproducible research for upon which knowledge can be accumulated.

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Check your Understanding

- What is the difference between culture-dependent and independent methods of microbiome analysis?
- What is the importance of culturomics in the scope of microbiome analysis?
- How can inaccurate or inconsistent results be produced from different metagenomic approaches to microbiome analysis?
- When would a metatranscriptomic analysis be more appropriate than a metagenomic approach? How do these approaches complement each other?
- What are the benefits and limitations of metabolomic analysis?

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3. Environmental Metagenomics

Environmental Metagenomics

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1. Introduction

Metagenomics can be defined as the techniques and procedures that are used for the culture-independent analysis of the total genomic content of microorganisms living in a certain environment [2]. It has many useful applications with very promising potential in both medical and environmental microbiology. The most common use of metagenomics in environmental microbiology is studying the diversity of microbial communities in particular environments through the analysis of rRNA genes and how these communities change in response to changes in physical and chemical properties of these environments [3].

Metagenomics also provides an opportunity to obtain and

identify novel enzymes with industrial applications from extreme environments where unculturable extremophiles live. In such circumstances, functional metagenomics enables the isolation of genes coding for extremozymes, enzymes that are capable of being catalytically active in extreme conditions, or genes that will allow for better understanding of the mechanisms that make such organisms resistant to extreme environmental conditions [4].

Metagenomics has special importance when it comes to studying soil microbiology. It is estimated that the number of distinct microorganisms in 1 gram of soil exceeds the number of microbial species cultured so far [5]. Therefore, metagenomics seems to be the ideal culture-independent technique for unraveling the biodiversity of soil microorganisms and to study how this biodiversity is affected with continuously changing conditions.

2. Sequencing technologies and metagenomics

Recently, taxonomic profiling, characterization, and analysis of microbial communities are being mostly performed using different next-generation sequencing (NGS) platforms. Metagenomic samples are high-throughput, short-read sequences, and the cost is relatively decreasing. In addition, these platforms are advantageous, avoiding the need for cloning of DNA fragments [6].

Recent advances in NGS technologies were developed to suit various numbers of applications, cost, and capabilities

[7]. The most commonly used platforms are the 454 Life Sciences (Roche) and Illumina systems (Solexa) [8]. The 454 sequencing technology, which was the first commercially available next-generation technology, is based on the pyrosequencing technique. It provides high throughput and relatively cheap analysis [9]. During the sequencing reaction in this technique, nucleotide incorporation into the growing chain is detected by the capture of the released pyrophosphate, which is converted into a light through an enzymatic reaction. Different nucleotides are sequentially added into each nucleotide incorporation event; therefore the light signal can be attributed to a specific nucleotide. Finally, the light signals are converted into sequence information. In the 454 pyrosequencer, the DNA fragments are amplified after being fixed on beads in a water-oil emulsion [10]. Pyrosequencing has been employed widely in the analysis of microbial diversity in many environments including marine environments [11] and different soil environments [12, 13].

Illumina sequencing technology relies on the use of fluorescently labeled reversible terminator nucleotides. Instead of being chemically modified to prevent further DNA synthesis (dideoxynucleotides) which is the case with Sanger sequencing, the terminator nucleotides are attached with blocking group that can be removed from the nitrogen base in a single step. DNA synthesis takes place on a chip where primers are attached. After each cycle, the dyes attached to each nucleotide are excited by laser followed by scanning of the incorporated bases. In order for the next synthesis cycle to proceed, the blocking group and the dye are first removed by a chemical reaction. Illumina

sequencing platform was successfully used to study microbial diversity in many environments [14, 15, 16].

In addition to the abovementioned technologies, recently developed sequencing technologies are available and being employed in metagenomic studies. These include SOLiD 5500 W Series developed by Applied Biosystems, single-molecule real-time (SMRT) DNA sequencing from Pacific Biosciences, and Ion Torrent semiconductor sequencing [8]. More innovative technologies are being developed that could be of great use for metagenomic studies in the near future. Strand sequencing technologies, currently being developed by Oxford Nanopore technologies, enable the sequencing of intact DNA strand that passes through a protein nanopore [17]. Irys Technology, developed by BioNano Genomics, represents one of the very promising new technologies in genomics era [8].

3. The metagenomic approaches

Metagenomics research strategy starts with selecting a proper ecological or biological environment of interest that hosts a wide variety of microbial communities which may have potential biotechnological applications. Environments that attract metagenomic researchers are mainly those characterized with extreme conditions or unique environmental conditions. These include environments with highly acidic or alkaline pH; high metal concentrations, pressures, or radiation; and high salinity or extreme temperatures [4].

Metagenomic analysis starts with isolating genomic DNA

that represents the whole community in the soil sample, constructing a DNA library from the isolated DNA, and screening the available library for a target gene. It is important here to select the DNA extraction method that will provide enough yield and DNA that represents the diversity of the whole microbial community in the target environment. This is still one of the most challenging steps of metagenomic analysis. The chemical and physical characteristics of soils are very wide and complex, depending on the type of the soil examined, that will make it difficult to develop a reference method for DNA extraction from soils. Besides, soils contain many substances that are co-extracted with genomic DNA and harbor inhibitory effects on the downstream processing of the extracted DNA. Examples include humic and fulvic acids [18]. Therefore, optimization and comparison between different extraction methods are usually required for each type of soil [19, 20, 21, 22].

A DNA library is then constructed from the genomic DNA isolated from the target environment. This is performed by fragmenting the isolated DNA into fragments with appropriate sizes that would allow for their cloning. This is performed by either using restriction enzyme digestion or mechanical shearing. DNA fragments obtained from such processes are cloned into the proper cloning vector. Plasmid vectors are used for small DNA fragments, and the libraries generated are called small-insert genomic libraries. Large inserts are cloning into cosmid or fosmid vectors which can hold inserts up to 40 kb in size or BAC vector which can carry inserts with sizes that exceed 40 Kb [23].

DNA libraries are usually constructed in a microorganism that is well-studied and is easy to manipulate inside the

laboratory such as *Escherichia coli*. In case there is a need for expressing the genes within the DNA inserts in other microorganisms, shuttle vectors are used to transfer the libraries into a proper host [24].

Finally, a screening assay is applied to search for a gene of a particular function, and the gene product is functionally analyzed. There are two different metagenomic strategies that are commonly used in research. The first one is focused on the use of marker genes such as the ribosomal genes 16S rRNA [25] and 18S rRNA [26] to study the composition of the microbial community in a certain environment or specific protein-coding gene with medical or industrial importance [27, 28, 29]. Such a strategy is called targeted metagenomics. The second approach is the shotgun metagenomics, in which a wide coverage of genomic DNA sequences is achieved using high-throughput next-generation sequencing to assess the entire taxonomic structure or functional potential of microbial communities [30].

The most challenging aspect of the screening process in metagenomics is the analysis of a huge amount of sequence data that are generated from the constructed library. A wide range of bioinformatic tools has been developed over the years to help analyze the metagenomic data and compare it to available online databases.

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PART III

HUMAN HEALTH AND DISEASE

4. Human Health and Disease

Human Health and Disease

The human microbiome plays a vital role in maintaining homeostasis of various organ systems and protecting against infectious agents. It can be subcategorized into local or regional microbiomes throughout the body, such as the gut, oral, skin, lung, and vaginal microbiomes. These manicured microecosystems are highly organized and complex, with each person having their own distinct makeup and distribution of various microorganisms. Though, individual microbiomes are unique, there is still capacity to understand key role players in the community and their potential for adaptation to improve human health in general.

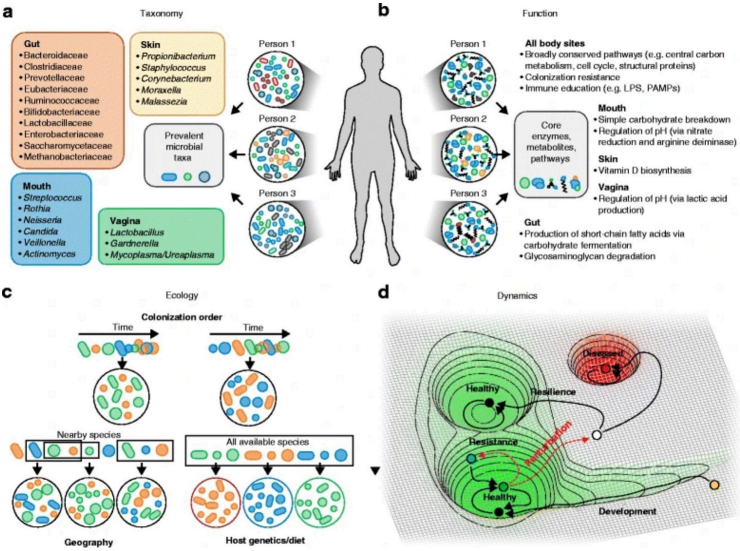


Figure 1. Possible definitions of a healthy microbiome: composition, function, dynamics, and ecology. a *Early definitions of a "healthy" microbiome generally focused on sets of taxa that might be expected to be found prevalently in healthy people. While purely taxonomic cores of any type have remained elusive, even in relatively narrowly defined populations, each body-site habitat possesses strong phylogenetic enrichments. Typical genera (or families in the gut) in healthy populations at different sites are shown here.* ***b*** *Metagenomic measurements have allowed the functional potential of the microbiome at different sites to be assessed. These studies have yielded more consistently shared functional cores of body-wide and niche-specific pathways that are maintained in health. LPS lipopolysaccharide, PAMP pathogen-associated molecular pattern.* ***c*** *Ecological assembly patterns provide another possible definition of a healthy microbiome, because each host may draw from a "typical" meta-population of potential microbes through a mix of partially stochastic processes. These processes may include the order in which microbes colonize their respective human habitat (affected by geography and early exposures, for example), the prolonged availability of each microbe in the host's local environment, and host selection.* ***d*** *The healthy microbiome can also be characterized in terms of its dynamics, depicted here in a simplified model as a conceptual energy landscape. The infant microbiome (yellow point) starts out in an unstable state and gradually descends towards one of potentially several healthy adult attractor states. Perturbations (dashed red arrows) can either be resisted (green point) or can move the microbiome out of the healthy state, after which a resilient microbiome will return to a healthy state (not necessarily the original healthy state) or fall into an unhealthy state (red). (Lloyd-Price et al., 2016).*

Disruption, or dysbiosis, of the microbiome can cause serious diseases and allow for opportunistic infections to occur. This disturbance can be caused by a variety of factors including changes in diet, exercise, geographical location, age, habits, as well as medical intervention like antimicrobial chemotherapy. This change in microbial composition subsequently leads to the development and exacerbation of a number of diseases impacting essentially every aspect of human physiology including the digestive, respiratory, integumentary, central nervous, and reproductive systems. Alternatively, dysbiosis may be the result of a particular disease, and in other cases disease-dysbiosis may be bidirectional (Silverman et al., 2019). The human microbiome can be influenced by many of the above mentioned factors, but also others like life partners, pet ownership, and occupation, which do not necessarily correlate with or contribute to dysbiosis (Kiecolt-Glaser et al., 2019, Kates et al., 2020).

A major driving feature of many of these elements is the host's genetics, which can also directly impact their microbiome (Tabrett and Horton, 2020). Part of the gut microbiome and even individual types of bacteria have been shown to be heritable (Kurilshikov et al., 2017), and it is likely that other areas of the human microbiome, like the skin microbiome, could be passed from parent to offspring as well (Si et al., 2015). The host genotype is intimately linked to its microbiome and has been shown to affect dysbiosis-induced diseases such as inflammatory bowel disease and atopic dermatitis (Knights et al., 2013, Dabrowska and Witkiewicz, 2016, Woo and Sibley, 2020). Host genetics and their microbiome can even indirectly affect one's susceptibility to other diseases. For example, the attractiveness of mosquitos to particular individuals is an heritable trait which is influenced by the many factors, including the skin microbiome; therefore altering the potential of contracting a mosquito-borne pathogen, such as a *Plasmodium* parasite which causes malaria (Martinez et al., 2020). It seems there is a combination between environmental factors, host genetics, behavior, and others that continually build and adapt the human

microbiome, however, is worthy to note that the former may play a larger role in shaping particular microbiomes, such as the gut microbiome (Rothschild et al., 2018).



One or more interactive elements has been excluded from this version of the text. You can view them online here: https://uta.pressbooks.pub/microbiomeshealthandtheenvironment/?p=1526#oembed-1

Media Attributions

- Figure 1 – Possible definitions of a healthy microbiome: composition, function, dynamics, and ecology by Lloyd-Price et al., 2016 adapted by Dylan Parks. Licensed under Creative Commons Attribution 4.0 International License http://creativecommons.org/licenses/by/4.0/
- Video 1 – You have the microbiome you deserve by Research Square. Licensed under Creative Commons: By Attribution 3.0 License https://creativecommons.org/licenses/by/3.0/

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5. The Gut Microbiome

The Gut Microbiome

The microbiome associated with the human gastrointestinal tract, termed the gut microbiome, may arguably be the most important component of the collective human microbiome. It has been shown to affect numerous other regions of the body and serves a role in many types of diseases throughout it. This is because many of the microbial products are absorbed in the alimentary canal and distributed throughout the body via the cardiovascular system.

Sample procurement to characterize the gut microbiome usually comes in the form of fecal matter, which readily available and less invasive. However, there are novel attempts to characterize gut microbiome constituents by sampling the mucosal-luminal interface (Yan et al., 2020). DNA and other features of interest can then be extracted from the microbes to provide information regarding gut microbiome composition and function.

Composition

The gut microbiome is quite versatile and its composition can vary widely among individuals with different ethnicities, across geographic locations, and with age (Yatsunenko et al., 2012, Gaulke and Sharpton, 2018). The host's genetics do play a role in the composition of the gut microbiome, as certain members are heritable, while other's abundances are the causal result of congenital diseases (Xu et al., 2020). Though diet, which is closely related to the aforementioned variables, may be the single strongest influencing factor when it comes to structuring this digestive community and alterations are reflected in both short-term and

long-term dietary interventions (David et al., 2014, Xu and Knight, 2015). Indeed, the type of diet, such as a high-fat diet, will have a direct impact on the gut microbiome. More specifically, the unique macromolecules within the gut can modify microbial abundance and predicted functions. For example, a certain oil in the diet could result in higher microbial richness, however, this diversity may not alone be a predictor of better health outcomes (Abulizi et al., 2019). Dietary vitamin content and even receptors required for processing them are in part modulated by the microbiome. For example, vitamin D deficiency and downregulation of its affiliated receptor is associated with pathogenesis of various diseases and their restoration promotes healthy host-microbe homeostasis (Jin et al., 2015). It is also interesting to note that diet usually changes throughout the year as certain food items become available or absent depending on the season. An increased gut prevalence of Bacteroidetes, which can digest complex plant carbohydrates, could be explained by a diet consisting heavily of produce during a harvest season (Davenport et al., 2014). The composition and diversity of the gut microbiome can even be linked to personality traits. Those individuals with larger social networks have a more diverse gut microbiome, and those affected by stress and anxiety show an altered composition with reduced diversity (Johnson, 2020). The connection between the gut microbiome and mental states and behaviors will be discussed more in the section “Mental Health”.

Video Quiz



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Dysbiosis and Disease

The gastrointestinal mucosal immune system modulates and responds to the gut microbiome, where the resident members aid in its development and transient pathogens cause dysfunction, leading to various diseases (Shi et al., 2017). Inflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis, IBD, and ankylosing spondylitis are implicated in the impaired interaction between the intestinal microbiota and mucosal immune system (Arbuckle et al., 2003, Mikuls et al., 2012, Costello et al., 2015). Microbial dysbiosis within these cases are marked by changes in composition and diversity of specific groups of organisms (Shi et al., 2017). So, the gut microbiome can not only serve as an indicator

for such diseases, but may also eventually become a target for treatment, with maintaining proper homeostasis a major goal.

Gut microbiome dysbiosis, whether it causes a particular disease or manifests as a result of it, is quickly becoming the focus of many gastrointestinal illnesses. Specific members of the gut microbiome (e.g. bacteriome, mycobiome, virome) can even vary and be affected differently depending on the disease and its severity. For example, while much of the focus is usually on bacteria in the gut, it is important to not discount the contributions of other microorganisms like bacteriophages and fungi. The gut phageome can vary in diversity, complexion, and in so has been shown to contribute to diseases like IBD, malnutrition, and AIDS (Norman et al., 2015, Reyes et al., 2015, Monaco et al., 2016, Shkoporov and Hill, 2019). Similarly, the gut mycobiome has shown to have roles in IBD, colorectal cancer, and even neurological diseases (Forbes et al., 2019, Gu et al., 2019, Qin et al., 2021). Though, it is likely the complex interactions between all members of the gut microbiome with the host undoubtedly play a role in various degrees for the progression of gastrointestinal and other diseases.

Those with Inflammatory Bowel Disease (IBD) experience substantial fluctuations in the gut microbiome, which is implicated due to signs and symptoms of the disease state, diet, as well as increased medication during flare ups (Walters et al., 2014, Halfvarson et al., 2017). IBD is a blanket term for two disorders, ulcerative colitis and Crohn's disease, which are characterized by chronic inflammation of the gastrointestinal tract and commonly results in frequent diarrhea, abdominal pain, bloody stool, weight loss, and fatigue. It is likely that the gut microbiome plays both a role in the development of these conditions and is affected by the induced changes. The gut virome component responds to disease-induced environmental change of IBD patients by shifting from virulent to temperate bacteriophage core, which subsequently affects the bacteriome and dysbiosis condition (Clooney et al., 2019). A familial study of patients with Crohn's disease showed an increase in the number of pathogenic bacteria, and a decrease in beneficial

bacteria. In particular, three potentially pathogenic biofilm-forming species from both the bacteriome (*Serratia marcescens* and *Escherichia coli*) and mycobiome (*Candida tropicalis*) interact with each other and impact the host immune system by increasing levels of proinflammatory cytokines and mucolytic enzymes which cause oxidative and tissue damage (Hoarau et al., 2016).



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Diabetes mellitus is another disease in which its progression is partially in response to gut microbiome dysbiosis. While there are other factors that play into diabetes such as culture, genetics, age, lifestyle, etc., this can be interlinked with an individual's microbiome. Studies over both type 1 and type 2 diabetes have shown that a change in the gastrointestinal microbial ecology is associated with diabetic subjects as compared with their healthy counterparts (Giongo et al., 2010, Larsen et al., 2010, Musso et al., 2011, Sohail et al., 2017). Type 1 diabetes stems from destruction of pancreatic beta cells, which results in decreased insulin production and elevated blood glucose levels. The gut microbiome may contribute to the disease by dysbiosis-associated immune regulation causing destruction of the beta cells and/or gut leakiness, endotoxemia, and chronic low-grade inflammation associated with certain enteric microbes (Cani et al., 2007, Lee et al., 2011, McDermott and Huffnagle, 2014, Sohail et al., 2017). Type 2 diabetes is characterized by the body's improper regulation and secretion of insulin and is associated by hyperglycemia.

Physiological changes in the GI tract could be induced by dysbiosis in the gut microbiome leading to gut permeability and insulin resistance (Everard and Cani, 2013). In general, the gut microbiota composition is less in terms of diversity and richness for those with type 2 diabetes, though an increase in abundance of certain groups like *Bifidobacterium* could improve conditions associated with pathogenesis (Cani et al., 2007, Sohail et al., 2017). It seems that an alteration of the microbial gut profile has considerable effects on host metabolism, gastrointestinal physiology, gut fermentation capabilities, and immunity which can have many other downstream implications (Boulange et al., 2016).

Obesity is commonly associated with type 2 diabetes as well as other comorbidities that are linked to gut microbiome dysbiosis. As mentioned earlier, diet strongly affects the composition and function of the gut microbial community and subsequently impacts the host's metabolic capabilities. In fact, a high-fat/calorie or improper diet that results in dysbiosis is evident earlier than the signs of the host's metabolic abnormalities, and so gut microbiome dysbiosis may be the principle ingredient responsible for the progression of obesity and type 2 diabetes (Nagpal et al., 2018). A high-sugar diet seems to promote an abundance of *Mollicutes*, a class within Firmicutes, which in turn suppresses Bacteroidetes (Turnbaugh et al., 2008), and this higher ratio of Firmicutes/Bacteroidetes has been proposed as a biomarker and hallmark indicator for obesity (de Bandt et al., 2011, Zou et al., 2020). However, it is important to consider other factors such as physical activity and medication that could cause a variation in diversity of the gut microbiome, as this ratio does not always definitively denote obesity (Magne et al., 2020). Though, there are similarities between many of the microbiome-linked contributing factors of pathogenesis progression for diet-induced diseases. For example, a high-fat diet induces increased gut permeability that allows exogenously produced bacterial compounds (e.g. lipopolysaccharides) to diffuse through the intestinal barrier, which then can interact with immune cells and lead to inflammation (Cani et al., 2007, Nagpal and Yadav,

2017). However, diet isn't the sole factor that can promote gut leakiness, and it seems that obesity in general causes an altered gastrointestinal state (Nagpal et al., 2018).



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Chemotherapeutic Intervention and C. diff

Medication and antimicrobial drugs can also have drastic effects on the gut microbiome that invoke risk of secondary infections, allergies, and other diseases like obesity (Becattini et al., 2016). Though many of these prescribed treatments are necessary to combat infectious diseases, the aftermath may have more serious consequences. Not only does antimicrobial therapy disrupt the resident microbiome, but misuse, suboptimal dosing, and patient noncompliance can create conditions conducive to fostering antimicrobial resistance through selective pressure.

Clostridioides difficile (commonly called C. diff) infections are directly associated with antibiotic-induced dysbiosis in the gastrointestinal tract. *C. difficile* is part of the normal microbiota, however, as an opportunistic pathogen it can invade or colonize empty niches brought about by dysbiosis and cause potentially fatal episodes of pseudomembranous colitis, which is associated with abdominal cramping, pain, sepsis, and bouts of diarrhea (Kho and Lal, 2018). Infection and transmission of this organism has been well known for its prevalence in hospital settings, primarily affecting the elderly and immunocompromised, however, community-associated infections have recently increased in what was once considered

low-risk populations (Rouphael et al., 2008, Baker et al., 2010, Hensgens et al., 2012, Benson et al., 2015, Johanesen et al., 2015). It is also alarming that this organism has resistance mechanisms to many commonly prescribed antimicrobials, including β-lactams, aminoglycosides, lincomycin, tetracyclines, and erythromycin (George et al., 1978), and more recently 'hypervirulent' strains have developed resistance to fluoroquinolones (He et al., 2013, Johanesen et al., 2015). *C. difficile* infections have an enrichment of fungi that associate with the bacteriome and perhaps antifungal therapy could help improve treatment success if administered in conjunction with specific antibacterial drugs (Stewart et al., 2019). Though, these infections can have lingering impacts on the gut microbiome, as further antimicrobial therapy that is usually required can perpetuate the situation. The inflammation as a result of the disease induces the production of antimicrobial peptides by epithelial cells and neutrophils which inhibit the growth of the natural resident commensal microbes (Leber et al., 2015).

While many events of gut dysbiosis are directly linked to the chemotherapeutic effects on microbes since they are prescribed to target microbes responsible for the infection, some medications which are meant to address other diseases, like antidepressants for mental health, have undesired effects on the microbiome (Maier and Typas, 2017). In the cases of multi-drug combinations (e.g. non-steroidal anti-inflammatory drugs (NSAIDs), antidepressants, laxatives, proton-pump inhibitors (PPIs), etc.), it is not the number of drugs that affect gut microbiome diversity, but rather the types of drugs (Rogers and Aronoff, 2016). Though these scenarios become complicated as it is difficult to ascertain whether the alterations observed on the microbiome are from the drug's mechanism of action, a downstream side effect, or originate from the condition that is being treated, and it is likely a complex combination of all factors for each disease and medication (Rogers and Aronoff, 2016, Maier and Typas, 2017, Jackson et al., 2018).

Fecal Microbiota Transplant

Although pharmaceutical drugs are of dire importance to treat various diseases, whether they are infectious in nature or not, other avenues must be pursued for those that may benefit from restoration of the gut microbiome. Probiotics and fecal microbiota transplant (FMT) can serve as viable options for the prevention and treatment of gut microbiome dysbiosis. Probiotics are considered foodstuffs with microorganisms, usually bacteria (many being lactic acid bacteria) and yeast, and their byproducts that have a beneficial effect on human health when introduced into the body. Many probiotics are commercially available to consumers in the forms of products like yogurt, kefir, buttermilk, sauerkraut, pickles, premade vitamin supplements and many others. Specifically, probiotics can be used for the treatment and prevention of many of the aforementioned gut microbiome dysbiosis-associated diseases, especially those induced by antibiotics (Kim et al., 2019). The beneficial microbes outcompete pathogens for resources or prevent them from establishing a niche in which to grow (Ouwehand et al., 1999).

In more extreme cases of gut microbiome dysfunction and disease, like those from *C. difficile* infection (CDI) in which antibiotics are ineffective and can potentially exacerbate the problem, other measures must be taken. Fecal microbiota transplant therapy takes a stool sample containing the gut microbiome from a healthy donor and relocates it into the infected patient's colon. The introduced microbiota then helps move the gut microbiome towards homeostasis by restoring the structure of beneficial microbes and metabolites (Fujimoto et al., 2021). This procedure is usually reserved for those patients with recurrent CDI and has shown to be highly successful and is considered safer and more effective than prolonged antibiotic usage (Mattila et al., 2012, Cammarota et al., 2015), though is also being investigated for first-line treatment of CDI (Camacho-Ortiz et al., 2017). FMT has gained traction for its success and is being further considered as a

therapeutic option in other treatment protocols, such as those for cancer patients undergoing cancer immunotherapy to help improve response or manage toxicity (McQuade et al., 2020), and individuals undergoing allogenic hematopoietic stem cell transplant for hematological disorders that experience graft-versus-host disease complications from it (Zhang et al., 2021). However, precautions must be taken for FMT, as the donor's sample could potentially harbor other pathogenic microbes, like multi-drug resistant *Escherichia coli*, that can result in pathogenesis, further complications, and even death for the recipient (DeFilipp et al., 2019, Martinez-Gili et al., 2020). More comprehensive research and FMT trials must be performed in order to optimize this procedure to better match donors with recipients and to further understand the exact mechanisms of microbiome rehabilitation.



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Conclusion

Gut microbiome intervention may be the key to future treatments of diseases associated with dysbiosis like IBD, diabetes, obesity, colorectal cancer, etc., and offers a viable alternative to many traditional pharmaceutical interventions. Though, the gut microbiome is plastic and continually changes with its host's environment and lifestyle, so stabilization is constant work. Additionally, creating an ideal 'cocktail' of microbes that will maintain homeostasis when implemented can be challenge. While there are general members of the gut microbiome that exist at a constant level and show some correlation to normal health, there may not be a true 'standard' gut microbiome due to the vast differences between people across the world. So, this type of therapy may require a more unique and individualized approach that depends on the disease and characteristics of both the host and their microbiome.

Check Your Understanding

- What factors influence the composition of the gut microbiome?
- How is gut microbiome dysbiosis brought about?
- What diseases are associated with gut microbiome dysbiosis? Are features of pathogenesis always a cause or effect of these events? Explain.
- Explain the treatment options that are available for

gut microbiome dysbiosis.

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6. The Oral Microbiome

The Oral Microbiome

The oral microbiome as with other site-specific microbiomes on and inside the human body is very distinct for each individual and its makeup and function is reflective of a variety of factors. Even within the context of the oral cavity, there are several unique niches with their own microbial ecosystem, including saliva, tongue, teeth, gingiva, throat, tonsils, and others. Each of these habitats exhibit diverse and complex interactions between bacteria, archaea, fungi, viruses, and protozoa, where dysfunction can lead to a number diseases, both rare and common (Wade, 2013, Sampaio-Maia et al., 2016).

Factors Affecting Composition

The composition of the oral microbiome and its respective niches is inherently dependent on host genetics, but other factors and habits such diet and smoking have a substantial effect on diversity. Members of the oral cavity demonstrate more heritability than the gut microbiome, and higher abundances of certain heritable organisms, such as *Prevotella pallens*, are associated with a lack of dental caries, while on the other hand, *Streptococcus mutans* and *Lactobacillus* species are linked to cavities (Davenport, 2017, Xiao et al., 2018). Detection of the specific groups of microbes has traditionally been difficult since many are fastidious and cannot be cultivated. So other techniques like metagenomics, metatranscriptomics, and proteomics have been implemented to better characterize the microorganisms present and their respective roles (Parahitiyawa et al., 2010, Grassl et al., 2016). These

studies have found that the oral microbiota is quite different even between healthy individuals, and so it may be the microbial functionality that is more important in the progression of various diseases, such as the role of biofilm formation, plaque buildup, and sugar metabolism in the development of dental caries (Segata et al., 2012, Takahashi et al., 2010, Duran-Pinedo and Frias-Lopez, 2015, Sato et al., 2015, Davenport, 2017).

Dysbiosis and Disease

The oral cavity is such a dynamic location that constantly experiences a variety of different foods, drinks, oral hygiene products, and other environmental stimuli with each composed of a multitude of macromolecules, compounds, and potentially other microorganisms, and so it makes sense that its microbiome often fluctuates in diversity (Parahitiyawa et al., 2010). Though there are differences in composition between individuals, specific sites, and over periods of time, species such as *Streptococcus mitis* and *Granulicatella adiacens* are conserved and generally present throughout the oral microbiome, while the presence of other specific microbes are associated with a particular disease (Aas et al., 2005). However, diseases like Periodontitis, which is the chronic inflammation of the gums and tooth supporting structures and one of the most common oral diseases, has many organisms associated with the condition, so discerning which ones are primarily responsible is a complex task. There are a variety of viruses that cause oral-related conditions, such as the Human papilloma virus (HPV) which is known for causing lesions and warts in the mouth, as well as head and neck squamous cell carcinoma (Kumaraswamy and Vidhya, 2011). An increase of the protozoa *Entamoeba gingivalis* and *Trichomonas tenax* are observed in patients with gingival disease, but are not the causative agents, rather just taking advantage of the increased food sources (bacteria and food debris) from poor hygiene (Wantland et al., 1958). There is also a variety

of fungi present in the oral cavity with *Candida* species being the most common, and many of the members in the oral mycobiome are responsible for chronic diseases, however correlation isn't exactly clear (Ghannoum et al., 2010). Archaea, primarily methanogens, are also present in the oral microbiome, and while there aren't technically any known pathogens in this domain, there is an increase in abundance observed in patients with periodontitis (Lepp et al., 2004, Mattarazo et al., 2011, Wade, 2013, Willis and Gabaldon, 2020).

Generally, the oral microbiome is linked with aspects oral health and has primary implications in dental and periodontal diseases, however it can contribute to vitality and diseases in other parts of the body such as cardiovascular disease, stroke, Alzheimer's disease, cystic fibrosis, rheumatoid arthritis, diabetes, pneumonia, and preterm birth (Seymour et al., 2007, Duran-Pinedo and Frias-Lopez, 2015, Kori et al., 2020, Willis and Gabaldon, 2020). Additionally, the oral microbiome is implicated in various forms of cancer including esophageal, pancreatic, gastric, liver, colorectal, and oral (Willis and Gabaldon, 2020, Bakhti et al., 2021, Mohammed et al., 2021). In many of these types of cancers the Gram-negative anaerobe, *Fusobacterium nucleatum*, is a primary culprit as it can promote cancer by activation of cell proliferation, promotion of cellular invasion, induction of chronic inflammation and immune evasion (Al-hebshi et al., 2017, McIlvanna et al., 2021). Another contributing factor to cancer development is the use of tobacco products which cause oral microbiome dysbiosis (Al-habshi et al., 2017, Gopinath et al., 2021, Sajid et al., 2021). As with other microbiomes, deviation from the normal composition can result in altered function and progression of disease.

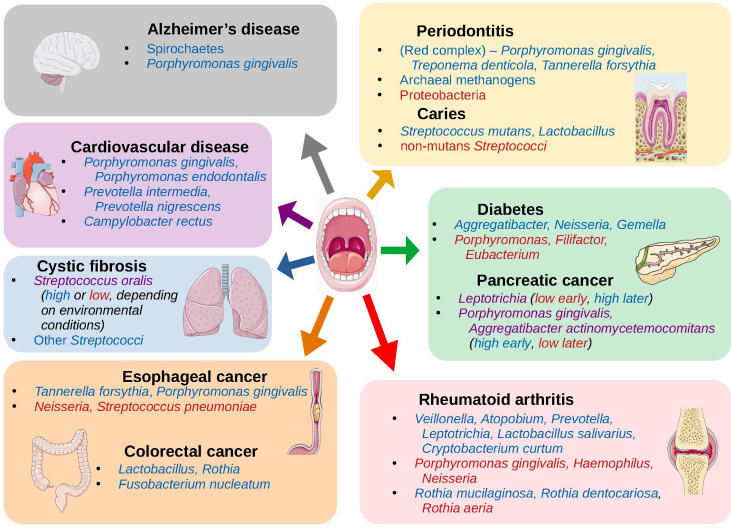


Figure 1. Oral and systemic diseases associated with the oral microbiome. A *representation of the associations found between diseases with increases or decreases of the abundances of organisms in the oral cavity. Organisms listed in blue have been shown to be increased in abundance in the oral cavity in individuals presenting with the noted disease, and organisms listed in red have been shown to be decreased. Those in purple may be either increased or decreased depending on the conditions or progression of the disease.* (Willis and Gabaldon, 2020).

Disease	Associated Organisms	Inhibited Organisms
Periodontitis	Phyla: Spirochaetes, Synergistetes and Bacteroidetes Classes: Clostridia, Negativicutes and Erysipelotrichia Genera: *Prevotella, Fusobacterium* Species: *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Filifactor alocis, Parvimonas micra, Aggregatibacter actinomycetemcomitans* Archaea: *Methanobrevibacter oralis, Methanobacterium curvum/congolense*, and *Methanosarcina mazeii*	Phyla: Proteobacteria Classes: Bacilli Genera: *Streptococcus, Actinomyces, Granulicatella*
Dental caries	Genera: *Neisseria, Selenomonas, Propionibacterium* Species: *Streptococcus mutans, Lactobacillus* spp. Fungi: *Candida albicans*	Species: non-mutans *Streptococci, Corynebacterium matruchotii, Capnocytophaga gingivalis, Eubacterium IR009, Campylobacter rectus, Lachnospiraceae sp. C1*
Oral cancer	Species: *Capnocytophaga gingivalis, Prevotella melaninogenica* and *Streptococcus mitis, Peptostreptococcus stomatis*, Streptococcus salivarius*, Streptococcus gordonii*, Gemella haemolysans*, Gemella morbillorum*, Johnsonella ignava* and Streptococcus parasanguinis I**	Species: *Granulicatella adiacens**
Esophageal cancer	Species: *Tannerella forsythia, Porphyromonas gingivalis*	Genera: *Neisseria* Species: *Streptococcus pneumoniae*

Figure 2. Examples of metagenomic studies of associations between the oral microbiome and oral diseases. *The first column indicates a disease, the second indicates organisms that have been found at higher abundances in individuals presenting with the disease, and the third indicates organisms at lower abundances. (*) indicates taxa associated with oral cancer from a study in which samples were from tumor and non-tumor sites in the same patients and disease treatment is not specified. References:* Matarazzo et al., 2011, Lepp et al., 2004, Vartoukian et al, 2009, Griffen et al., 2012, Costalonga and Herzberg, 2014, Liu et al., 2012, Jorth et al., 2014, Haubek, 2010, Koo and Bowen, 2014, Gross et al., 2010, Wang and Ganly, 2014, Pushalkar et al., 2012, Mager et al., 2005, Peters et al., 2017, Willis and Gabaldon, 2020.

Saliva

Saliva is an important component in maintaining homeostasis in the oral cavity, as it lubricates food, initiates the digestive process, and defends against bacteria. Disruption in secretion can lead to changes in the oral microbiome which promotes progression of oral and other diseases (Grassl et al., 2016). Since saliva contacts virtually all surfaces within the oral cavity it is involved with all other duties of the mouth, and adaptive and innate immune defense mechanisms can be considered the most important in terms of microbiological clinical relevance. The protein and gycoprotein content regulates the oral microbiome by promoting the colonization of commensal

microbiota while helping eliminate pathogenic microbes (Cross and Ruhl, 2018). These macromolecules aid in bacterial adhesion and biofilm formation so that they aren't dislodged by salivary flow and other oral physiological processes (Mandel, 1987). The establishment of beneficial microbes prevents pathogenic bacteria from gaining a foothold, and the agglutinins found in saliva aid in removal through binding and then swallowing (Scannapieco, 1994). The co-evolution of the microbiota and humans has cultivated the development of specific bacterial adhesins for colonization of the preferred microorganisms, thus establishing a mutualism, though pathogenic microbes are quick to adapt to changing binding motifs (Springer and Gagneux, 2013, Cross and Ruhl, 2018).

Saliva can harbor numerous microbes, with one milliliter containing approximately one hundred million microbial cells (Marsh et al., 2015) and over 600 different species (Dewhirst et al., 2020, Willis and Gabaldon, 2020). In one study, the prominent genera found across various types of saliva samples (i.e. spit, drool, and oral rinse) from healthy individuals were *Streptococcus* (17.5%), *Prevotella* (15.5%), *Veillonella* (15.3%), *Neisseria* (12.7%) and *Haemophilus* (10%) (Lim et al., 2017). Though it can be difficult to differentiate a core salivary microbiota from other specific oral niches since it coats the oral cavity.

For those suffering from various diseases, the relative abundance of certain microorganisms and general composition of the oral microbiota is altered as compared to healthy controls. For example, patients suffering from chronic obstructive pulmonary disease (COPD) and periodontitis have varying abundances of *Veillonella*, *Rothia*, *Actinomyces*, and *Fusobacterium* in saliva samples (Lin et al., 2020). Periodontitis and COPD are comorbid diseases that are commonly associated with other conditions like rheumatoid arthritis, diabetes mellitus, and cardiovascular diseases (Scher et al., 2014, Wang et al., 2014, Chrysanthakopoulos and Chrysanthakopoulos, 2014, Lin et al., 2020). Dysfunction of bacterial ecology in saliva is exacerbated by COPD and periodontitis, and

so restoration of the salivary microbiota may treat or reduce the severity of these diseases and their comorbidities (Jeffcoat et al., 2014, Zhou et al., 2014, Lin et al., 2020).

Alterations of the salivary microbiome are also associated with certain human viral infections like the herpes virus, influenza, and SARS-CoV (Blostein et al., 2021, Miller et al., 2021). Saliva generally is beneficial to oral health, though changes in it's makeup could be detrimental and further aggravate disease. For instance, the saliva microbiota and their byproducts could be responsible for increased susceptibility of infection of gum tissues with herpes simplex virus 1, especially in individuals with periodontitis lesions (Zuo et al., 2019). Eukaryotic viruses can also directly interact with oral bacteria and affect disease severity, as is the case with streptococci and influenza which results in an increased viral load (Kamio et al., 2015). Similarly, among patients with COVID-19 there are differences in the salivary bacterial community based on SARS-CoV-2 viral load, though the exact dynamics and repercussions of the interaction isn't yet well understood. It is possible that COVID-19-induced inflammation could directly impact the oral microbiome and contribute to other diseases connected with dysbiosis (Miller et al., 2021).

Dysbiosis of the fungal component in the salivary microbiome also contributes to overall oral microbial community changes and detrimental effects to the human host. The mycobiome is an important constituent of the oral microbiome though its member's abundance is much less than that of the bacteriome. There are two main genera in the salivary mycobiome: *Candida* and *Malassezia*, where the former is associated with dental plaque bacteria, carbohydrate-rich microbial communities, and acidic pH conditions which contribute to dental caries (Hong et al., 2020). Other interactions between the mycobiome and bacteriome in saliva has been observed in the chronic inflammatory disease oral lichen planus (OLP). OLP causes swelling, discoloration, and open sores of the mucosal membranes in the oral cavity, primarily affecting the buccal region (cheek), but the lips, gingiva, and tongue may also be affected. Similar to plaque buildup and cavity formation, this disease

is characterized by an increased abundance of genera *Candida*, but also *Aspergillus*, as well as a decrease in biodiversity (Li et al., 2019).

While diversity and abundance of specific groups within the salivary microbiome are associated with various diseases, it is important to consider other factors like diet, lifestyle habits, and genetics that can influence the progression of any particular disease.

Teeth, Plaque, and Cavities

Plaque formation occurs when a multispecies biofilm builds layers on the surface of teeth over time. This structure not only contains a variety of microbes, some of which are pathogenic, but proteins, carbohydrates, minerals, antimicrobial peptides and other compounds that dictate its structure and activity (Amerongen and Veerman, 2002, Flemmig and Beikler, 2011, Zarco et al., 2011). For normal healthy individuals, plaque biofilms are important in maintaining oral homeostasis and good tooth conditions as they can trap pathogens or prevent them from thriving due to competitive inhibition. However, regular detachment of these biofilms through oral hygiene and salivary flow are necessary to prevent pathogen establishment and their escape from immune responses and antimicrobial therapy (Avila et al., 2009, Filoche et al., 2009, Van Essche et al., 2010, Flemmig and Beikler, 2011, Zarco et al., 2011).

The persistence of oral biofilms contributes directly to cavity formation as carbohydrate-fermenting microbes within produce acidic byproducts which lower oral pH and damage tooth enamel (Selwitz et al., 2007, Ling et al., 2010). Cavities form once the surface layers of the tooth wear away and lesions form in the dentin, resulting in oral pain, tooth decay and loss (Selwitz et al., 2007, Zarco et al., 2011). Dental caries are the most prevalent disease for children worldwide, and dental care is the most common unmet need among children in the United States (Loesche and Grenier, 1976, Acs et al., 1999, Low et al., 1999, Peterson et al., 2013).

The fastidious nature of several members of plaque polymicrobial communities have made it traditionally difficult to characterize an exact consortium responsible for dental caries, however, recent studies using NGS technologies have detailed a few signature genera. *Streptococcus mutans* and *Lactobacilli* spp. are the primary culprits, but other genera such as *Fusobacterium*, *Bifidobacterium*, and *Actinomyces* are found in high abundance in people with cavities (Munson et al., 2004, Chhour et al., 2005, Corby et al., 2005, Peterson et al., 2013). The fungal yeast, *Candida albicans*, is also found regularly in children with severe early childhood caries (S-ECC) (Xiao et al., 2018). Interestingly, other members of *Streptococcus* including *S. parasanguinis*, *S. mitis*, *S. oralis*, and *S. sanguinis* are associated with individuals exhibiting good dental health (Corby et al., 2005, Peterson et al., 2013). Overall, during the progression of caries there is a reduction in species diversity in these communities (Peterson et al., 2013).

Gingivae and Periodontitis

Aside from dental caries, periodontitis, a.k.a. 'gum disease', is the other most common oral disease in humans, and it also results from alterations in oral microbial ecology. This inflammatory condition affects the supporting structures surrounding the teeth where the microbial communities that inhabit the subgingival area serve to trigger its onset (Hajishengallis and Lamont, 2012, Hong et al., 2015). Though progression of the disease comes in episodes, the continual breakdown of periodontium tissue (gingiva, periodontal ligament, cementum, and alveolar bone) leads to alveolar bone breakdown, formation of pocket lesions, and tooth loss (Listgarten, 1986). This condition is very difficult treat as specific antibiotics must be administered, though they are often unsuccessful as pathogens can hide in plaque, develop resistance, and quickly recolonize through reserves in the mucous membranes of the oral cavity. Once pockets form in the periodontium, periodontitis becomes irreversible, as the

tissues are unable to reattach to the bone and the pathogens within cannot be completely eradicated or removed (Pihlstrom et al., 2005, Horz and Conrads, 2007, Van Essche et al., 2011, Zarco et al., 2011).

Culture-based studies have shown that periodontitis is associated with varying levels of abundance of three bacterial species: *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, which are collective referred to as the 'red complex' (Socransky et al., 1998, Rocas et al., 2001, Socransky and Hafajee, 2005, Teles et al., 2010). More recent genetic analysis has revealed other bacteria associated with the disease, including those from the classes Clostridia, Negativicutes, and Erysipelotrichia; the genera *Synergistes*, *Prevotella*, and *Fusobacterium*; and the species *Filifactor alocis* (Vartoukian et al, 2009, Griffen et al., 2012, Costalonga and Herzberg, 2014, Willis and Gabaldon, 2020). Some methanogenic archaeal species have even been implicated in the disease, and perhaps serve a metabolic role as a 'hydrogen sink' for secondary fermenters (Lepp et al., 2004, Matarazzo et al., 2011). Viruses, both eukaryotic and bacteriophages, are also thought to play a part in the etiology of periodontitis. While bacteriophages manage biofilm formation through bacterial predation, the role of eukaryotic viruses found in the oral cavity such as those in the Herpesvirus family, is more enigmatic (Martinez et al., 2021). The protist *Trichomonas tenax* is frequently found in patients with severe periodontitis, though it is not known if its presence is a cause or a result of the disease (Benabdelkader et al., 2019. It is generally thought that this condition arises from events of dysbiosis causing an increase in community diversity and richness which then alters antagonistic/synergistic relationships, metabolic functions, and the oral environment (Kolenbrander et al., 2006, Shi et al., 2015, Ng et al., 2021). Those bacteria that may be absent or inhibited during dysbiosis conditions, and are associated with good periodontal health include those from the phylum Proteobacteria and the Firmicutes, class Bacilli, and the genera *Streptococcus*, *Actinomyces*, and *Granulicatella* (Griffen et al., 2012, Liu et al., 2012, Willis and Gabaldon, 2020).

Periodontitis resulting from oral microbiome dysbiosis has been linked to other diseases like chronic kidney disease, as well as several types of cancers including oral, esophageal, gastric, lung, pancreatic, prostate, hematologic, and breast (Fitzpatrick and Katz, 2010, Ioanndiou and Swede, 2011, Michaud et al., 2017). These associations stemming from the oral cavity could be due to dysbiosis-induced microbiome changes and recruitment of disease causing pathogens, production of harmful microbial-derived byproducts, and/or modulation of the immune system causing an increase in proinflammatory cytokines (Abnet et al., 2005, Kurkivuori et al., 2007, Chalabi et al., 2008, Meurman, 2010, Willis and Gabaldon, 2020). In reality, there are likely a multitude of factors and interactions between microbial communities and the host that lead to the progression of these diseases, however, it is still important to determine certain organismal signatures or functions that could indicate a condition and perhaps aid in diagnosis or treatment.



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Tongue

The tongue is another niche for a variety of microbial communities, and though it is a maneuverable oral centerpiece that comes into contact with the rest of the cavity, it has its own unique makeup of microbes. The tongue microbiome serves as an ideal model to analyze changing microbial consortia and understand microbial community dynamics. By employing a novel computational method

known as oligotyping, which relies on identifying certain nucleotide content of genetic sequences, a better resolution of microbial taxonomic distribution can be achieved (Eren et al., 2014, Welch et al., 2014, Wilbert et al., 2020). As expected, the tongue experiences large fluctuations in relative abundance across taxa, but this isn't completely explained by obvious human behavior such as oral hygiene and food/liquid intake. The complex microbial organization that is observed is likely a combination of many factors, like other microbiomes, such as host immunity, physiology influenced by circadian rhythm, epithelial cell renewal, other microbial (e.g. bacteriophage) interactions, as well as certain host behaviors and lifestyles (Welch et al., 2014, Wilbert et al., 2020).

The changing microbiome of the tongue can also be linked to various diseases and conditions in both the oral cavity and throughout the rest of the body. Viewing the tongue for diagnosis isn't a novel approach either, as traditional Chinese medical practices have viewed tongue phenotypes to discern various illnesses for thousands of years, and currently physical aspects of the tongue are being connected with its microbiome composition to better diagnosis certain diseases like oral, liver, gastric, colorectal, and pancreatic head cancers (Jiang et al., 2012, Han et al., 2014, Mukherjee et al., 2017, Cui et al., 2019 Lu et al., 2019, Mohammed et al., 2021). It is also interesting that members of the tongue microbiome can help regulate blood pressure and cardiovascular health through metabolism of dietary nitrate, and oral hygiene (e.g. tongue cleaning) can help promote the growth of these beneficial organisms (Tribble et al., 2019). While good habits like proper oral hygiene can cultivate a healthy oral microbiome and improve overall health, bad habits like smoking negatively affect microbial communities which contributes to various diseases. For example, metagenomic analysis investigating bacterial species and their gene content showed significant differences of certain species, strains, and metabolic pathways within the tongue microbiome between smokers and never smokers (Sato et al., 2020). This further demonstrates the usefulness of tongue microbiome analysis as a

reliable technique to explore and diagnosis certain diseases and conditions.

Conclusion

Though not as well characterized as the gut microbiome, the oral microbiome has a significant impact on human health. Within the oral cavity, specific niche microbiomes of saliva, teeth, gums, and tongue are each compositionally unique and implicated in various conditions and diseases. Moreover still, other oral niches like the buccal mucosa, palate, pharynx, and tonsils not detailed here are distinctive and have certain associated diseases initiated with respective microbiome dysbiosis (Gao et al., 2014, Fukui et al., 2018, van der Meulen et al., 2018). Areas within the oral microbiome may eventually become routine sites for medical observation since samples are easily acquired, especially saliva, and microbial analysis can serve as a fast and reliable option for diagnosis and potential treatment of oral disorders as well as other diseases.

As the oral cavity is the initial point of contact and entrance for foreign microbial loads into the body, oral disease and its associated microbial ecology appear to serve as a conduit for a multitude of other diseases. In a sense the oral microbiome can be considered a precursor to the gut and lung microbiomes, as microbes are undoubtedly mixed with food, drink, and air before being swallowed or inhaled and taken further into the respective system. It is therefore important to understand and better characterize the oral microbiome so that medical diagnosis and intervention can be improved. However, with vast differences in microbial community diversity and composition between humans across the word, this will be quite the challenge to determine normalized healthy consortia respective to people of various geographic regions, ages, lifestlyes, etc.

Hotspot Quiz



An interactive H5P element has been excluded from this version of the text. You can view it online here:
https://uta.pressbooks.pub/microbiomeshealthandtheenvironment/?p=679#h5p-4

Check Your Understanding

- What aspects of the oral microbiome allow for the formation of unique niches?
- How does dysbiosis within the oral microbiome contribute to certain diseases? (What aspects of changes in microbial community diversity and richness could cause oral and other diseases?)

- Why may saliva sampling be considered a viable option for diagnosis of oral and other diseases?
- How do oral biofilms contribute to cavity formation and tooth decay?
- Why are microbial infections associated with periodontitis hard to treat?
- How do certain lifestyles influence various niches of the oral microbiome and lead to disease?

Media Attributions

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- Figure 2 – Examples of metagenomic studies of associations between the oral microbiome and oral diseases by Willis and Gabaldon, 2020, caption adapted by Dylan Parks. Licensed under Creative Commons: By Attribution 4.0 License http://creativecommons.org/licenses/by/4.0/

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7. The Skin Microbiome

The Skin Microbiome

The microorganisms that inhabit the largest human organ are important in a variety of ways in regard to host health. The skin microbiome defends against pathogens, educates the immune system, helps wound healing, and moderates progression of diseases, and in return receives nutrients and real estate for colonization, creating a symbiotic establishment (Byrd et al., 2018). As the primary external interface to the environment, the skin serves as the initial physical barrier against invasion of potential pathogens. The territory of the skin itself is harsh for most microbes; its cool, acidic, dry, and considered nutrient poor, and so only those organisms that have adapted to these conditions can successfully colonize it. A majority of these microbes rely on obtaining nutrients from sweat, sebum, and dead skin cells by using proteases and lipases to break apart various compounds to liberate usable resources (Byrd et al., 2018). Though, there are adverse conditions and a general lack of food for microbes on the skin, there is still quite a diverse community which is unique to certain individuals and body locations.

Better characterization of the skin microbiome and its site-specific diversity can allow for greater understanding of skin diseases such as atopic dermatitis, acne, rosacea, and psoriasis that are associated with dysbiosis (Grice and Segre, 2011). The role of resident and transient microorganisms are important in the onset and progression of these types of diseases, and their study may help in diagnosis and treatment protocols.



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Composition and Stability

Within a single square centimeter on the skin there can be up to one billion microorganisms, and this mixed community of bacteria, viruses, protozoa, fungi, and mites can be both good and bad for host health (Grice and Segre, 2011, Weyrich et al., 2015). Overall, there are four main bacterial phyla that constitute the human skin microbiome: Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes (in descending order of abundance) (Grice et al., 2009). Microbial composition primarily depends on the specific location of skin site, and particularly whether it is dry, moist, or sebaceous (i.e. oily). Moist areas like the bend of the elbow or the feet harbor bacteria like *Staphylococcus* or *Corynebacterium* species and tend to have higher species diversity, while sebaceous locations promote the growth of *Propionibacterium* species and are lower in diversity (Grice et al., 2009, Oh et al., 2016, Byrd et al., 2018).

The composition of fungi on the skin also depends on location and physiological properties. Fungal members of the skin microbiome primarily consist of species from the genus *Malassezia*, with some from *Penicillium* and *Aspergillus*, and less of a few other genera. Specifically, the community of fungi on the feet have a high amount of diversity and it tends to change more over time as compared with other locations on the body. This may be due to environmental exposure, sock and shoe usage, or the fact that specific sites like plantar heels, toe webs and toenails are commonly infected by

fungal pathogens, which can be difficult to treat. (Findley et al., 2013).

Small arthropods less than half a millimeter also colonize the skin and makeup part of the microbiota. Two species of mites from the genus *Demodex* utilize lipids produced in the sebaceous regions of the skin; the larger species *D. folliculorum* tends to cluster around hair follicles while the smaller *D. brevis* situated near the eyelid rim is more antisocial (Schommer and Gallo, 2013). These lipid-eating mites normally have a symbiotic relationship with humans, however, they could potentially serve as mechanical vectors for transport of pathogenic bacteria, and their population buildup and/or associated events of dysbiosis could promote inflammatory reactions (Lacey et al., 2009, Lacey et al., 2011).

Eukaryotic viruses on the skin are primarily transient, exhibit lower site-specific affinity, and the most different from person to person. This is likely due to the fact that they are obligate intracellular pathogens with special constraints. Also, maybe as expected, various bacteriophage abundance in certain sites is dependent on the corresponding bacterial genera (Oh et al., 2016).

Microbial communities on the skin remain stable despite the constantly changing external environment that humans bring about. Over both short- and long-term time intervals, sebaceous sites are the most stable, and moist areas like the feet are the least stable. Interestingly, dry sites with high environmental contact and disruption, like the palms of hands, exhibit community stability over time (Oh et al., 2016). Though, variability and stability may be dependent on individual host habits and lifestyles. At an early age, however, there are drastic changes in the skin microbiome until it becomes established. At birth, the baby transitions from an essentially sterile environment in the womb, to open air and constant microbial exposure where initial colonization of the skin begins. The method of delivery also affects initial community composition on the skin. When a baby is born conventionally through the vaginal canal, the members of the skin microbiome reflect the vaginal microbiome, and if born via cesarean section, the

infant's skin community more closely resembles that of the mother's skin (Dominguez-Bello et al., 2010). In the initial few months, the skin community predominately consists of *Streptococci* and *Staphylococci* bacteria, but as aging ensues the abundance of these genera decrease and diversity and numbers of others begins to increase and level out (Capone et al., 2011). This has long-term effects on health, as the evolution of an infant's skin microbiome helps to regulate and mature both the skin and immune system.

Hot Spot Quiz

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Immune System Interaction

Exposure to potentially pathogenic microorganisms at a young age can help educate the immune system. Immediately after childbirth, initial skin colonization by microorganisms is allowed without the classical inflammatory response, though shortly after this period,

these microbes promote the development of distinct components of the immune system for future pathogen encounters (PrabhuDas et al., 2011, Naik et al., 2012, Naik et al., 2015, Belkaid and Harrison, 2017). This has been observed in infant's skin microbiome and an early colonization of *Staphylococcus aureus* being associated with a lower risk of developing atopic dermatitis, as the immune system is prepared for this organism that can exacerbate and perpetuate the disease (Kennedy et al., 2017, Blicharz et al., 2019). *S. aureus* can further modulate the immune system through toxin production and colonization, which initiates leukocyte responses and stimulates the adaptive and innate immune systems (Niebuhr, et al., 2011, Nakamura et al., 2013, Nakatsuji et al., 2016). Skin colonization of *Staphylococcus epidermidis* elicits similar immune responses, and a pre-association with this microbe could help defend against certain fungal and parasitic skin infections (Naik et al., 2012, Naik et al., 2015). Further investigation of microbe and immune system interactions will help uncover exact molecular functions and relationships, which could have future implications in therapy and treatment of skin diseases (Byrd et al., 2018).

Pathogens, Dysbiosis, and Skin Diseases

The skin microbiome also contributes to human health and combating infectious diseases in a preventative measure. In addition to educating the immune system, the commensal microorganisms that call the epidermis home help to prevent pathogen invasion by physically taking up niches and secreting certain antimicrobial compounds. For example, *S. epidermidis*, which is a part of the normal microbial flora of the skin, produces antimicrobial peptides and proteases that selectively targets and inhibits growth of pathogens such as *S. aureus* (Cogen et al., 2010, Iwase et al., 2010, Schommer and Gallo, 2013).

Though many commensal microorganisms on the skin can help to prevent pathogen colonization, these microbes themselves may be

opportunistic pathogens and can cause an infection when certain conditions arise. For example, S. *epidermidis* is a common cause of infections when transmitted from the skin into the body, usually through a medical procedure like catheterization (Otto, 2012). Staphylococci are known for their ability to form biofilms on medical devices, which make them more difficult to treat (Otto, 2009). The fungi, *Candida albicans*, is another normal skin resident that can cause opportunistic infections. It is thought that disruption of the normal skin flora, through means such as antibiotic therapy, could induce virulence factor production by the yeast, resulting in penetration of epidermal tissue and a subsequent case of candidiasis (Kuhbacher et al., 2017). Other skin diseases associated with dysbiosis, or various skin pathogens, include atopic dermatitis, acne, rosacea, psoriasis, and chronic wound healing.

Atopic dermatitis (AD), the most common form of eczema, is a chronic inflammatory condition of the skin that is caused by a mutation in several genes, including the fillagrin gene responsible for encoding a protein that helps maintain epidermal health (Bierber, 2008). AD is characterized by itchy, dry rashes that become vulnerable to infection when the skin barrier is breached by constant scratching, and occurs more frequently in areas that become lower in microbial diversity due to an altered physiology (Weyrich et al., 2015). S. *aureus* colonization is directly related to disease severity of AD, as it produces virulence factors that disrupt the integrity of the skin barrier. There is also an increase in abundance of S. *epidermidis*, and *Clostridium* and *Serratia* species, and this increase of select species suggests that a lower amount of microbial diversity plays a role in the progression of AD (Kong et al., 2012, Oh et al., 2013, Williams and Gallo, 2015). Selectively targeting pathogens such as S. *aureus* for treatment of AD proves to be difficult as the normal microbiota also suffers resulting in dysbiosis.

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Psoriasis is similar to AD in that the disease results in inflamed, scaly skin plaques that are itchy and painful. This condition is also associated with altered microbial diversity, and there is an association with the development of psoriasis and oral streptococcal infections, though the connection is not exactly known (Norlind, 1955, Owen et al., 2000). Within psoriatic lesions, there is an increase Proteobacteria and Firmicutes, a decrease in Actinobacteria, and specifically a decrease within the genera *Propionibacterium* (Gao et al., 2008, Fahlen et al., 2012, Statnikov et al., 2013). Though there is a decrease in general of microbial diversity in psoriatic lesions, there hasn't been any specific microbial causative agent identified for the disease.

Rosacea is a common chronic dermatosis which primarily manifests as persisting erythema (redness), telangiectasia (dilated or broken blood vessels), bulging, swelling, and/or raised patches in superficial facial skin (Picardo and Ottaviani, 2014). Like other cutaneous diseases, development of rosacea is linked with skin microbiome composition, and how those communities influence skin immune responses. In particular, an increase in *Demodex* mite abundance and density is observed in those with rosacea. They potentially contribute to the disease state through immune system activation, damaging epithelial tissue, and/or the exposure of antigenic proteins of bacteria released from their digestive tract (Forton and Seys, 1993, Georgala et al., 2001, Lacey et al., 2007, Koller et al., 2011, Casas et al., 2012, Forton, 2012,). The induced reactions from microbiome shifts, genetics, and environmental factors then likely invoke other inflammatory triggers, which

includes overgrowth of certain bacteria like *S. epidermidis* (Schommer and Gallo, 2013).

Acne (acne vulgaris) is a skin condition that results from hair follicles and sebaceous glands that are clogged with oil, bacteria, and dead skin cells, which creates whiteheads and blackheads. *Propionibacterium acnes* is a primary etiological agent in acne; it's secretion of lipases, proteases, and hyaluronidases damage skin pores and induce inflammatory responses (McKelvey et al., 2012). Although this species of microbe is part of the normal skin microbiota, there are differences in certain strains of *P. acnes* that may explain differential virulence. Some disease-associated strains also have genes for antibiotic resistance, making treatment options other than chemotherapy a necessity (Fitz-Gibbon et al., 2013). Other commensal microorganisms, like *S. epidermidis*, could interact with *P. acnes* and be implicated in acne formation also, further demonstrating that residents can become pathogenic when opportunistic conditions arise (Bek-Thomsen et al., 2008, Weyrich et al., 2015, Dreno et al., 2017).

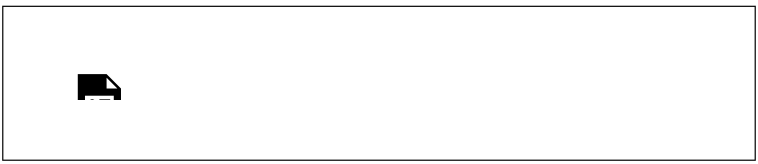
Chronic skin wounds (duration longer than three months) and their capacity to heal are also affected by the skin microbiome, especially in those individuals who are elderly, obese, immunocompromised, or diabetic (Weyrich et al., 2015, Byrd et al., 2018). Though the lesions or ulcers may not be initially caused by a microorganism, their presence, infection, and polymicrobial biofilm formation can be deleterious to the healing process and cause further complications (McKelvey et al., 2012, Wolcott et al., 2013). Analysis of skin wound microbiomes have shown a compilation of a diverse array of genera, but that microbial diversity is lower as compared with healthy skin (Gardiner et al., 2017, Kalan et al., 2019). Perhaps a higher microbial diversity allows for easier elimination of potential pathogens from the wound and promotes faster healing. An increase in facultative anaerobes, specifically the genus *Enterobacter*, are significant indicators in the persistence of chronic wounds and their lack of healing, possibly due to their versatile metabolism (Verbanic et al., 2020). Antibiotic therapy is an option to

eliminate certain bacterial pathogens for chronic wounds, however, the resulting changes in the microbiome and addressing fungal and viral constituents may necessitate multiple different treatment approaches (Price et al., 2009). Further studies are needed in order to see whether individual therapy and targeted therapeutic intervention would promote faster healing in cases with chronic wounds (Kong, 2011, Weyrich et al., 2015, Verbanic et al., 2020).

It is likely that changes in microbial community composition and the elicited immune response work in combination with host genetics and other environmental factors to cause various cutaneous disorders (Weyrich et al., 2015). This makes treatment of these complex conditions difficult, and targeting a few potential pathogens is rarely successful, especially if it is not known whether their presence is a cause or effect. So, future therapeutic efforts may focus on a similar approach to treating gut dysbiosis with FMT, and as so, use healthy skin microbiota transplants to repair and stabilize the skin microbiome for patients inflicted with skin diseases (Williams and Gallo, 2015).

Conclusion

The skin microbiome is incredibly complex, resilient, and has a strong influence on health and disease. Characterization and defining a normal skin microbiome could help in future diagnosis and treatment of disease, although factors like differences in host genetics, lifestyles, and particular skin locations must be accounted for. As research and medical technologies advance, it may be possible to utilize these microbial neighbors on the skin in efforts to promote better overall health.





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Check Your Understanding

- What features of human skin affect the composition and stability of its microbiome?
- How is the skin microbiome influenced in early life, and what implications does this have in human health?
- What diseases are associated with dysbiosis of the skin microbiome? How do these come about and what particular microorganisms are associated with each?
- How do certain members of the skin microbiome impede the healing process of chronic wounds?

Media Attributions:

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8. The Respiratory Microbiome

The Respiratory Microbiome

The respiratory microbiome (specifically the lower portions) was once thought to be nonexistent, as many considered healthy lungs to be a sterile environment. Like other microbiomes though, the upper and lower respiratory system are environments rich with bacteria, fungi, archaea, and viruses, and because of the novelty of study many of the members in these groups have not been identified. However, it is known that many of such organisms are not only responsible for or exacerbate a number of pulmonary diseases, but that the respiratory microbiome can help to mitigate infection and influence treatment of certain illnesses (Unger and Bogaert, 2017, Watson et al., 2019).

The human respiratory system begins with the upper respiratory system; starting with the nose and nostrils, then proceeds to the nasal cavity, pharynx, and larynx. The lower respiratory system then starts with the trachea, moves to the bronchi and bronchioles, and ends with the lungs. Each of these structures and areas have their own microbial niche which serve various roles in maintaining health, but also have the potential to be compromised. The respiratory system is similar to the oral and digestive system, in that they interact intimately with external stimuli and bring in foreign matter and microbes during regular biological processes, like inhalation. These systems also have overlapping parts (e.g. oral cavity and pharynx), where their respective microbiomes serve similar roles such as resistance to disruptive environmental factors and host defense (Dickson et al., 2014, Zaura et al., 2014, Hakansson et al., 2018). The mucosal surfaces of the respiratory tract also have

a natural healthy biofilm where the resident microbiota reside to maintain homeostatic functions, however, as an extension of dysbiosis, these biofilms can become altered and obtain pathogenic microbes which contribute to respiratory diseases (Hamilos, 2019).

Composition

Immediately after birth the respiratory microbiome becomes colonized, though the collection of microbes found here during infancy is not much different than other locations in the body, and generally reflect the mode of delivery (Dominguez-Bello et al., 2010). The respiratory microbiome, as well as others, become differentiated over time when the different species of microbes adapt and outcompete each other based on their niche, host genetics, and environmental factors (Bosch et al., 2016). Colonization additionally depends on microbial immigration, microbial elimination, and relative reproduction rates of its members (Figure 1) (Dickson and Huffnagle, 2015). With age, the respiratory microbiome of each particular individual reflects the aforementioned factors as well as other lifestyle habits, and so they can be vastly different between people. Although these respiratory microbiomes are unique, a core set of microbes can be defined, with most of them being aerobic or facultatively anaerobic (Stearns et al., 2015, Hakansson et al., 2018).

Microbial composition also depends on the specific structure or niche within the respiratory system. The nasal microbiome largely consists of *Staphylococci*, *Corynebacteria*, and *Streptococci*, which is most likely due to its similarity and proximity to the skin (Mika et al., 2015, Shilts et al., 2016). Communities within the paranasal sinuses are highly diverse and include lactic-acid producing bacteria such as *Lactobacilli*, *Enterococcus*, and *Pediococcus*, and once in the nasopharynx the collection of microbes becomes more complex and favors oxygen-utilizing groups (Abreu et al., 2012, Biesbroek et. al, 2014, Teo et al., 2015). In the lower respiratory system, the

microbiome resembles a bit of a mix from both the nasal and oral cavities, with a majority of bacteria from the genera *Streptococcus*, *Fusobacteria*, *Pseudomonas*, *Veillonella*, and *Prevotella* (Cui et al., 2014, Beck et al., 2015, Dickson and Huffnagle, 2015, Hakansson et al., 2018).

While much focus of the respiratory microbiome has been on bacteria (like many other microbiomes), study of the mycobiome and virome components have revealed their importance in pulmonary health and disease. Fungal spores are commonly inhaled, especially in high numbers during peak seasons, and depending on the particular organism and host immune health, they can cause infection and respiratory complications (Pashley et al., 2012, Denning et al., 2014, Nguyen et al., 2015). For healthy people, the respiratory mycobiome is predominated by environmental fungi including *Aspergillus*, *Cladosporium*, and *Penicillium* species (Charlson et al., 2012, van Woerden et al., 2012, Underhill and Iliev, 2014). However, members from these genera and other more well-known fungal pathogens, like *Candida albicans*, can be implicated in respiratory illnesses due to microbial community changes (Nguyen et al., 2015). The respiratory virome can also modulate various pulmonary diseases through respective bacterial interactions and host immune response. For bacteriophage composition, there are observed differences between healthy individuals and patients suffering from diseases associated with respective bacterial infections (Wilner et al., 2009). There is a diverse range of eukaryotic viruses that make up the normal human and respiratory virome, with the majority consisting of adenoviruses, herpesviruses and human papillomaviruses (HPVs), though these may only be transient and quickly cleared by the immune system or via the mucociliary escalator (Wilner et al., 2009, Popgeorgiev et al., 2013). More research needs to be conducted over the normal respiratory virome to determine its functionality and importance, though usually more focus is catered to those viruses directly involved in pathogenesis and dysbiosis.

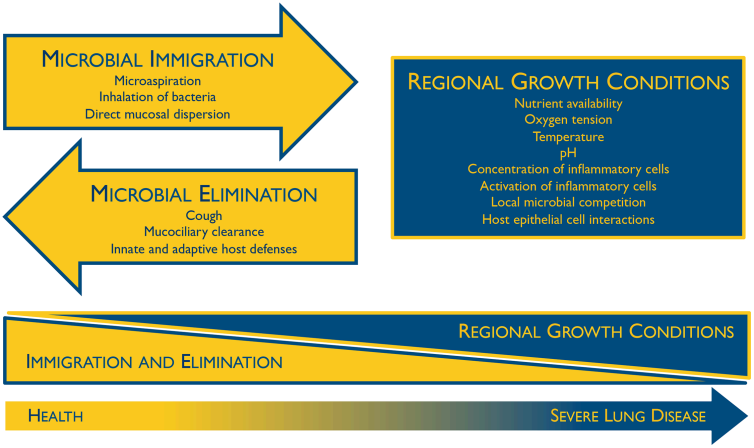


Figure 1. Ecological determinants of the respiratory microbiome. *The constitution of the respiratory microbiome is determined by three factors: microbial immigration, microbial elimination, and the relative reproduction rates of its members. In health, community membership is primarily determined by immigration and elimination; in advanced lung disease, membership is primarily determined by regional growth conditions. Adapted from Dickson et al., 2014.*

Dysbiosis and Disease

The normal matrix of organisms in the respiratory system maintain homeostasis through their complex interactive network with each other and their host. Pathogens like *Streptococcus pneumoniae* and *Haemophilus influenzae* are actually part of the resident respiratory microbiome and interact with commensal microbes, however, they can incite infection when they are not kept in check due to community perturbation (de Steenhuijsen et al., 2015). During events of dysbiosis, pathogens and opportunistic pathogens can contribute to and exacerbate several respiratory diseases including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, pneumonia, otitis media, and other acute infections like influenza.

Asthma is a chronic lung disease that usually develops at an early

age, and its complex etiology makes it difficult to treat. People with asthma experience inflammation and tightening of their airways and a production of extra mucus impairs breathing, talking, and being active. The prevalence of asthma has increased in recent decades, and a decrease in microbe exposure during youth could be partially responsible (Ober and Yao, 2011, Prescott, 2013). Proper development of the immune system is reliant on contact with microbes, and those individuals who are regularly subjected to a diverse variety of microorganisms at an early age have a reduced risk of developing asthma (Ownby et al., 2002, Fujimura et al., 2010, Fall et al., 2015). Aside from grooming the immune system, the intake of microbes influences the composition of the mucosal microbiota that lines the air passageways, which could directly affect asthma development (Durack et al., 2016). Indeed epidemiologic studies have shown that the disruption of the microbiome, such as in the case of antibiotic use during childhood, create a predisposition for this allergic disease (Khalkhali et al., 2014). Specifically, the increased presence of genus *Haemophilus* and other *Proteobacteria*, as well as the fungus *Aspergillus fumigatus* in the respiratory system are signature of asthma patients (Hilty et al., 2010, Teo et al., 2015, Urb et al., 2015). These pathogens would normally be cleared by the immune system or stifled by the resident microbiota, however, early childhood dysbiosis resulting in hampered immune functions and/or a discontent microbial consortium in the respiratory system contribute to the development of this condition.

Chronic obstructive pulmonary disease (COPD) is another chronic inflammatory lung disease that causes obstructed airflow to the lungs and is exacerbated by microbial dysbiosis. Emphysema, a condition where the alveoli are damaged due to cigarette smoke and other irritants, and chronic bronchitis, a condition characterized by inflammation of the lining of the airways, are the most common contributors to COPD. An alteration of the lower respiratory microbiome causing reduced diversity or the presence of specific organisms can further aggravate COPD causing a worsening of symptoms (Huang et al., 2014). Viral infections, like those caused by

the rhinovirus, are commonly detected (in about half the patients) during COPD exacerbations (Seemungal et al., 2001, Rohde et al., 2003, Mallia et al., 2011). Also during these bouts, there is an increase in pathogens from the bacterial genera *Haemophilus*, *Pseudomonas*, and *Moraxella* and a general shift towards the phylum Proteobacteria in the respiratory microbiome (Huang et al., 2014, Millares et al., 2014). Additionally, patients with COPD exhibit a decline or absence of certain normal lung organisms that could contribute to mucosal homeostasis and prevention of pathogen overgrowth, such as Firmicutes (*Streptococcus spp.*), as well as Bacteroidetes (*Prevotella spp.*) (Fukata and Arditi, 2014, Sze et al., 2015, Hakansson et al., 2018). These compositional changes are at least partially brought about by current COPD treatments like steroid and antibiotic usage, and so future therapy may need to take the respiratory microbiome into further consideration for various approaches (Wang et al., 2016).

Cystic fibrosis (CF) is a genetic disease that causes normal cellular secretion of mucus, sweat, and digestive juices to become thicker and viscous. In the respiratory system, the buildup of sticky mucus can clog airways, impair lung function, and make breathing difficult. Bacterial pathogens, like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Haemophilus parainfluenzae*, tend to grow in the sputum and can cause infections which worsening CF conditions (Keogh and Stanojevic, 2018). Their biofilm-forming capabilities also contribute to the excess mucus production, further causing complications as infectious agents which can be resilient and persistent (Høiby et al, 2010, Orazi and O'Toole, 2017). While changes in the microbiome are not typically responsible for exacerbations of CF, antibiotic administration is the primary treatment option to eliminate these pathogens residing in the mucus, however, prolonged use can result in decreased microbiome diversity and dysbiosis in the respiratory system as well as other areas (Zhao et al., 2012, Carmody et al., 2013 Price et al., 2013, Dickson et al., 2014).

Pneumonia is a microbial infection of the lungs that can cause the alveoli to fill with pus or other liquids, and severe cases can

be life-threatening, especially to children, the elderly, and those who are immunocompromised (Wardlaw et al., 2006, Chalmers et al., 2011, Valley et al., 2015). The intensity of the infection is directly correlated with the microbial load and diversity, so etiologic determination is important for proper treatment, as the onset of pneumonia can have bacterial, fungal, or viral origins (Dickson et al., 2014, Iwai et al., 2014). Viral pneumonia is commonly caused by influenza or rhinovirus, however, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has now also become a major cause post 2019 (Jain et al., 2015, Zhou et al., 2020, Pettigrew et al., 2021). Respiratory viral infections are the most common cause acute respiratory illnesses, and often times, these viral infections then lead to secondary bacterial infections, like pneumonia, due to host immune reactions and induced dysbiosis in the respiratory tract and gastrointestinal system (Hanada et al., 2018). *Streptococcus pneumoniae* is the most common culprit for bacterial pneumonia, and while it regularly resides in the upper respiratory tract, under dysbiotic conditions this organism can proliferate and spread to the lower respiratory tract and cause illness (File, 2003, de Steenhuijsen Piters et al., 2014). In patients with pneumonia, there is a decrease in many Gram-negative anaerobic bacteria that are apart of the resident microbiota, some of which are associated with a reduced risk of hospital-acquired pneumonia and clearance of *S. pneumoniae* (Bousbia et al., 2012, de Steenhuijsen Piters et al., 2014, Krone et al., 2014). Although, changes in the respiratory microbiome are more apparent in bacterial pneumonia rather than viral, it is not known whether this alteration in microbial consortia is a cause or effect of the disease, though it is likely conditional of both circumstances (Ramos-Sevillano et al., 2019, Pettigrew et al., 2021).



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Conclusion

The respiratory microbiome is an up-and-coming avenue for research, diagnosis, and treatment of several pulmonary illnesses. While much of its characterization is still in it's infancy, proper identification of microorganisms is important for management of respiratory diseases (Nguyen et al., 2016). As antibiotic resistance continues to surge, novel approaches are necessary to mitigate not only respiratory illnesses, but to maintain general health. And similarly to other microbiome-related treatments, like fecal microbiota transplant for the gut, eventually oral or aerosol administered microbiota may be implemented to treat pulmonary conditions (Huang and Boushey, 2015). Continual research detailing both core and individual respiratory microbiomes will push the advancement of these 'futuristic' medical motions.

Drag and Drop Quiz

Drag each contributing factor of the respiratory microbiome composition into the appropriate category.

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Check your Understanding

- How is the colonization of the respiratory microbiome influenced, and what factors affect its composition over time?
- What factors of the respiratory microbiome affect the development of asthma?
- Which microorganisms are implicated in COPD and periods of exacerbation? How does dysbiosis contribute to this chronic condition?
- Why do you think a greater change is observed in the respiratory microbiota in patients with bacterial pneumonia versus viral?

Media Attributions

- Figure 1 – Ecological Determinants of the Respiratory Microbiome by Dickson and Huffnagle, 2015. Licensed under Creative Commons: Attribution 4.0 International (CC BY 4.0) License https://creativecommons.org/licenses/by/4.0/
- Video 1 – The Lung Microbiome: Challenging Old Paradigms about Microbes by National Human Genome Research Institute. Licensed under Creative Commons: By Attribution 3.0 License https://creativecommons.org/licenses/by/3.0/

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9. The Vaginal Microbiome

The Vaginal Microbiome

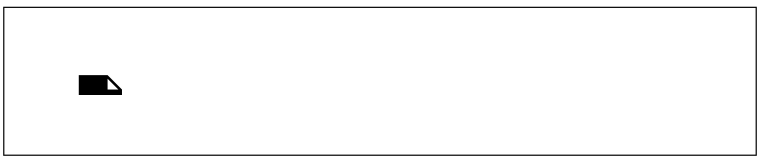
The vaginal microbiome is a reproductive organ-specific niche that harbors a unique collection of microorganisms that are important in a variety of aspects to human health. This microbial community has significance in maintaining vaginal homeostasis, protecting against urogenital infections, host immunity, and reproductive capacities. In recent years, next generation sequencing (NGS) techniques have been employed to classify the vaginal microbiome into community state types (CSTs), or vaginotypes, based upon composition, which can enhance epidemiological studies to make better associations between the microbiome and host-vaginal health (France et al., 2020, Mancabelli et al., 2021).

Composition and Role

The vaginal microbiome is composed of over 200 different bacterial species, though it is primarily dominated by members from *Lactobacillus* genus (Ma et al., 2012, Auriemma et al., 2021). The *Lactobacilli* protect the vagina from potential pathogen invasion through their fermentation of vaginal epithelial cell-produced glycogen into lactic acid, which lowers vaginal pH, as well as their production of various antimicrobial compounds, and resource/space competitive inhibition (Boskey et al., 2001, Amabebe and Anumba, 2018, Chee et al., 2020, Jang et al., 2019). Though, the bacteriome is usually the emphasized feature of the vaginal microbiome, it also harbors protists, fungi, archaea and viruses, with each occupying their own niche in the normal healthy network (Belay et al., 1990, Bradford and Ravel, 2017, Happel et al., 2020,

Chacra and Fenollar, 2021). The composition isn't always fixed however, as factors like the menstrual cycle, hormone fluctuation, sexual partners, hygiene, genetics, age, the environment, drug use, and other aspects of lifestyle can affect the microbial makeup (Aagaard et al., 2012, Fettweis et al., 2014, Hyman et al., 2014, Zapata and Quagliarello, 2015, Martin and Marrazzo, 2016, Diop et al., 2019, Chacra and Fenollar, 2021).

As mentioned earlier, using 16S rRNA sequencing, the vaginal microbiome has been categorized into CSTs and allowed for deeper analysis, where comparisons of the consortia within these classifications can be used to make associations with host and vaginal health. There are 5 main CSTs each predominated by a particular species of *Lactobacilli*, except for group IV, which has been further dissected into additional subgroups: CST I—*L. crispatus* dominated, CST II—*L. gasseri* dominated, CST III—*L. iners* dominated, and CST V—*L. jensenii* dominated (Ravel et al., 2011, France et al., 2020, Chacra and Fenollar, 2021, Mancabelli et al., 2021). CST IV is not dominated by any particular species, and contains a mixture of both strict and facultative anaerobes including *Gardnerella*, *Atopobium*, *Lactobacillus*, *Bifidobacterium*, etc., where certain controversial subgroups (e.g. CST IV-A, CST IV-B, CST IV-C, CST IV-D, CST IV-G, etc.) have various combinations depending on the study (Gajer et al., 2012, Albert et al., 2015, France et al., 2020, Mancabelli et al., 2021). Each of the five groups has correlations with vaginal pH, microbial colonization and biodiversity, as well as host characteristics such as pregnancy, ethnicity, and age, though it is not always absolute (France et al., 2020, Mancabelli et al., 2021). With reproducible organization of the vaginal microbiome, connections can be made with medical conditions, which could benefit treatment and diagnosis of vaginal diseases and associated affairs.



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Dysbiosis and Disease

As with other microbiomes associated with the human body, disturbance of the resident vaginal microbiome can result in complications of health. Many diseases of the urogenital tract such as bacterial vaginosis (BV), urinary tract infections (UTIs), yeast infections, and several sexually transmitted infections (STIs) are caused by pathogenic microbes associated with dysbiosis (Taha et al., 1998, Donders et al., 2000, Wiesenfeld et al., 2003; Lai et al., 2009, De Seta et al., 2019, Mancabelli et al., 2021). Normally, the local flora maintains homeostasis, however, changes in composition provide opportunistic pathogens a window to proliferate and invade.

Bacterial vaginosis, associated with CST IV, stems from a perturbance of the vaginal flora, specifically a decrease in *Lactobacilli* and an increase in other microbes like *Gardnerlla vaginalis*, *Atopboium vaginae*, *Ureaplasma urealyticum*, and others that are only usually found in low numbers (Gajer et al., 2012, Margolis and Fredricks, 2015, Onderdonk et al., 2016, Zozaya et al., 2016). This condition can be asymptomatic in up to half the women with BV, and in the others can be diagnosed by observed changes in vaginal discharge or by the Nugent scoring system which utilizes a Gram stain (Figure 1) (Nugent et al., 1991, Amsel et al., 1983, Schwebke, 2000). While not exactly considered a sexually

transmitted disease, bacterial vaginosis is commonly associated with certain sexual practices, though other factors such as hygiene, nutrition, intrauterine devices, hormonal changes, and certain comorbidities can contribute to susceptibility (Avonts et al, 1990, Calzolari et al., 2000, Neggers et al., 2007, Verstraelen et al., 2010, Zabor et al, 2010, Margolis and Fredricks, 2015). Women with this disease also have a higher risk to contract sexually transmitted infections, and it can be linked to reproductive complications and poor infant health (Wiesenfeld et al., 2003, Prince et al., 2015, Chacra and Fenollar, 2021). Typically, antibiotics targeting anaerobic bacteria are administered to treat BV, however recurrence is common, likely due to the antimicrobial-resistant nature of biofilms formed by the pathogens and/or regular exposure to external reservoirs (Swidinski et al., 2008, Oduyebo et al., 2009, Marrazzo et al., 2012, Bradshaw and Sobel, 2016). Probiotics, specifically containing *L. crispatus*, may be the better option, and vaginal microbiota transplantation from a healthy donors is a promising treatment course of action (Hemmerling et al., 2010, Ma et al., 2019).

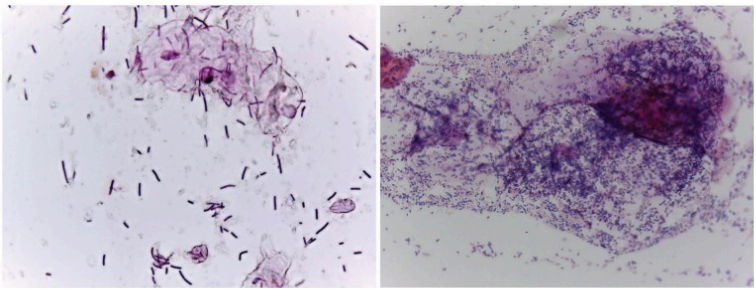


Figure 1. Examples of Gram-stained slides from women with (right, Nugent score = 9) and without (left, Nugent score = 1) BV. *In women with a lactobacilli-dominated microbiome (left), long purple rods are the main morphotype. In contrast, women with BV tend to have a lot more bacteria overall, with a significant fraction that do not stain as Gram-positive (i.e. pink). Morphotypes in BV tend not to be long Gram-positive rods. Although not part of the Nugent scoring system, women with BV tend to have large conglomerations of bacteria associated with epithelial cells as seen in the upper right quadrant (Lewis and Gilbert, 2020).*

Urinary tract infections are a common problem, especially for females, which are disproportionately affected. This infection is most likely to occur in the urethra or bladder (though in severe cases the kidneys can be affected), which causes pain in the pelvic area and during urination, the frequent urge to urinate, and the presence of blood in urine (Lee and Neild, 2007, Sheerin, 2011, Hooton, 2012). The vaginal microbiome, or more specifically some normal residents, like *Escherichia coli*, can move to the urogenital tract and cause issues resulting in a UTI, which often occurs via sexual activity (Nicolle et al., 1982, Foxman, 2014, Stapleton, 2016, Lewis and Gilbert, 2020). Other fastidious microorganisms, like those abundant in women experiencing BV, contribute to a higher risk of contracting an infection in the urinary tract as compared with those not suffering from dysbiosis in the vagina (Hillebrand et al., 2002, Sumati and Saritha, 2009). In these cases, the vaginal opening may harbor potential uropathogens (Figure 2) and transient exposure to the urinary tract could prompt colonization or other reactions (e.g. immunomodulation) that results in a UTI (Lewis and Gilbert, 2020). Antibiotics are traditionally used to treat UTIs, however probiotics and estrogen administration could help to restore the *Lactobacillus* colonization and protect against complicated and recurrent infections (Raz and Stamm, 1993, Eriksen, 1999, Prais et al., 2003, Stapleton et al., 2011, Tan and Chlebicki, 2016, Lewis and Gilbert, 2020).

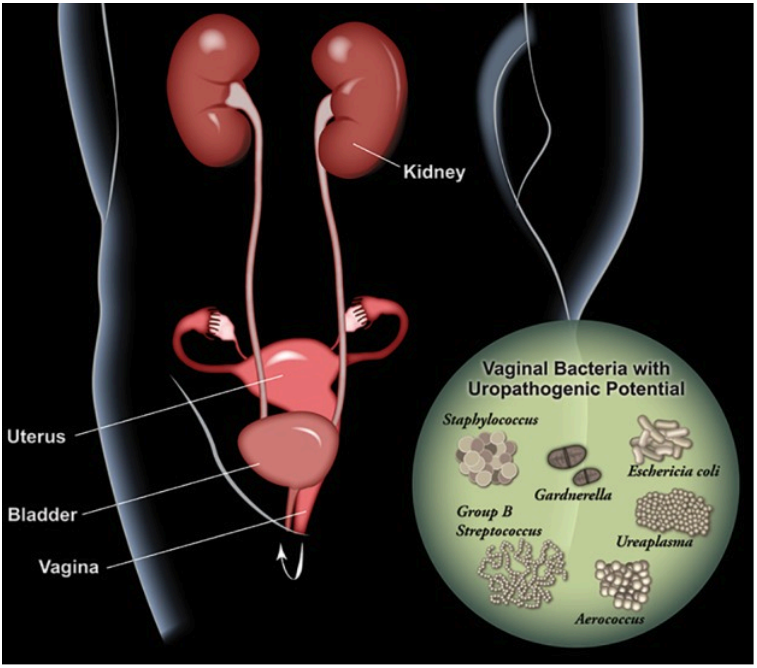


Figure 2. Schematic illustrating vaginal bacteria with potential to impact the urinary tract. *The vagina can serve as a reservoir for several bacterial species known to be causes of UTI (E. coli, GBS, Staphylococcus) as well as underappreciated potential uropathogens (G. vaginalis, Aerococcs Ureaplasma) that can cause UTI and have been associated with urological conditions such as urgency incontinence and "sterile" pyuria (Lewis and Gilbert, 2020).*

Yeast infection of the vaginal region caused by *Candida* species, also known as vulvovaginal candidiasis (VVC), is a common condition with severe symptoms and a high recurrence rate (Oerlemans et al., 2020). Those affected experience vaginal itchiness or soreness, dyspareunia (painful intercourse), abnormal vaginal discharge, redness, swelling, and thinning of the vaginal wall (Chew and Than, 2016, Oerlemans et al., 2020). While it is the second-most common infection of the vagina behind BV, this disease primarily

affects premenopausal women with a low vaginal pH value under 4.5 (Kim and Park, 2017, Gupta et al., 2019). The exact cause of VVC isn't exactly clear, though it is thought it can come as a result of microbiome dysbiosis induced by prolonged antibiotic usage, which allows various *Candida* species to overgrow and establish an infection (Goldacre et al., 1979, Mitchell, 2004, Peters et al., 2014, van de Wijgert and Verwijs, 2020). Traditionally, antifungal medication has been used to treat VVC, however administration of probiotic vaginal microbes may be more prudent as their mechanisms of pathogen colonization and biofilm formation inhibition are more effective to prevent disease recurrence (Petrova et al., 2016, Tachedjian et al., 2017, Allonsius et al., 2019, Oerlemans et al., 2020, van de Wijgert and Verwijs, 2020).

Individuals with vaginal microbiome dysbiosis characterized by a decrease in abundance of *Lactobacilli* species (i.e. BV) are at a higher risk of contracting sexually transmitted infections, such as those caused by *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Chlamydia trachomatis*, and *Mycoplasma genitalium* (Martin et al., 1999, Cherpes et al., 2003, Peipert et al., 2008, Brotman et al., 2010, Molenaar et al., 2018, De Seta et al., 2022). Infections caused by these organisms usually result in symptoms similar to other genital infections such as vaginal itching, pain, unusual discharge, rash, etc. which like other conditions (e.g. vaginitis), result in decreased epithelial integrity and can exacerbate pathogen invasion (Miller and Shattock, 2003, Greenbaum et al., 2019). In those individuals with BV and non-inflamed tissue, the increased risk for STIs could be due to the negative effects dysbiosis-related bacteria have on the innate immune system (Murphy and Mitchell, 2016, Liebenberg et al., 2017). Similarly, contraction of sexually transmitted viral infections like human immunodeficiency virus (HIV), herpesviruses, and human papillomavirus (HPV) are frequently associated with vaginal dysbiosis, immunomodulation, and disruption of the epithelial barrier (Sewankambo et al., 1997, Borgdorff et al., 2016, Siqueira et al., 2019, Torcia, 2019, De Seta et al., 2022). While there is correlation between vaginal dysbiosis and STIs, the protective

mechanisms of the resident vaginal microbiota are unknown and still need to be uncovered (De Seta et al., 2022).



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Reproduction, Pregnancy, and Infant health

The vaginal microbiome has further implications in host immunity, fertility, pregnancy, spontaneous preterm birth, and infant health (Anahtar et al., 2015, Fettweis et al., 2019, Gupta et al., 2020, Xu et al., 2020). Indeed, there are a multitude of factors that affect reproduction like age, genetics, hormone levels, fallopian tube blockage, menstrual cycle, and vaginal pH, however only recently has the vaginal microbiome been studied for its association with various fertility factors (Xu et al., 2020, Fan et al., 2022).

Changes in the resident vaginal flora and infections by certain pathogens can cause complications for reproductive health and pregnancy. For example, infection by Group B *Streptococcus* (GBS) has been associated with a decline in ovarian function, pregnancy loss, preterm delivery, and is the leading cause of bacteremia and meningitis in newborns (Zaleznik et al., 2000, Phares et al., 2008, Kolter and Henneke, 2017, Tazi et al., 2019, Xu et al., 2020). The vaginal microbiome of pregnant women is less rich and diverse than non-pregnant individuals, likely caused by changes in sex hormone levels (Farage et al., 2010, Aagaard et al., 2012). These alterations can cause shifts in the vaginal microbiome that could then result in

infection and a risk of preterm or spontaneous labor (Wylie et al., 2018, Fettweis et al., 2019, Feehily et al., 2020, Gupta et al., 2020).

Infants become introduced to the microbial world via their mother, and predominately right after birth where vaginal versus cesarean delivery has a great impact on composition (Chu et al., 2017). However, exposure may happen earlier *in utero*, as this environment may not be as sterile as once thought as some studies have shown the presence of microbes in the placenta and amniotic fluid (Aagaard et al., 2014, Collado et al., 2016, Kolter and Henneke, 2017). The establishment of a newborn's microbiome has profound effects on the development of immunity and metabolism as well as the onset of diseases like atopic dermatitis and obesity in later life (Rautava et al., 2012, Collado et al., 2016, Ta et al., 2020).

Conclusion

The vaginal microbiome is a complex community which affects many facets of human health and disease including urogenital, reproductive, immune, and infant. Characterization of the vaginal flora has allowed categorization into community state types which can help to predict and diagnose disease states. Within so, these dynamic changes that occur under various conditions produce a unique fingerprint for the vaginal microbiome which can be analyzed and potentially treated in a specific manner (Ceccarani et al., 2019, Lagenaur et al., 2020, Abou Chacra and Fenollar, 2021). While antibiotic administration has its benefits, novel approaches using targeted application of probiotic microbiomes (e.g. a gel containing specific *Lactobacilli* species) could help in treating diseases associated with vaginal dysbiosis by restoring those disrupted communities, as well as alleviating certain negative consequences of chemotherapy (Pino et al., 2019, Lagenaur et al., 2020 Oerlemans et al., 2020). More in-depth and continued research of the vaginal microbiome is necessary to illuminate the interactions and connections of it members to human health.

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Drag and Drop Quiz

Drag the species of bacteria that dominates each vaginal community state type.

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Check your Understanding

- Explain vaginal microbiome CSTs. Why are they important?
- Which CST is associated with BV, and which genus is typically absent?
- How does BV contribute to the development of other conditions like UTIs, VVC, and STIs?
- How does the vaginal microbiome influence reproduction?
- Where does a newborn acquire its microbiome, and what factors affect the composition?
- What alternatives to antimicrobials are promising for the treatment of vaginal microbiome associated diseases?

Media Attributions

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10. Mental Health and Multi-Microbiome Interactions

Mental Health and Multi-Microbiome Interactions

It may seem obvious that localized microbiomes are responsible for diseases related to their respective areas (e.g., IBD and the gut microbiome or AD and the skin microbiome), however, it is a fascinating phenomenon that various microbiomes can affect each other and influence health in different parts of the body. Even more captivating is the link between certain microbiomes and mental health conditions presuming the brain is devoid of microbes.

These links between microbiomes are referred to as axes, and initial connections began with the most well-studied microbiome, the gut. Since many gut-derived microbial-produced molecules and compounds are spread through the bloodstream, associations with various organs are formed, such as the gut-brain axis, gut-skin axis, gut-lung axis, or a combination of multiple; gut-brain-skin axis. There are likely overlapping connections between all microbial components that form the human holobiome, thus teasing apart the exact members and their functions is a dutiful task.

Gut Interaction with Other Microbiomes

As mentioned earlier, the dissemination of molecules and compounds from the gut to the rest of the body creates an interconnected highway affecting most, if not all, parts of the body.

Certainly, juxtaposed regions such as the oral cavity and the proximal portions of the GI tract have a relationship between their microbiomes, which it termed the gut-oral axis (GOA). These linked microbiomes have been shown to have immunomodulatory roles in the development of rheumatoid arthritis (RA) and osteoarthritis (OA) under dysbiosis, where the abundance of oral *Porphyromonas gingivalis* and intestinal *Prevotella copri* could be responsible (Drago et al., 2019, du Teil Espina et al., 2019). It has also been proposed that dysbiosis of the gut-oral microbiome axis is implicated in cirrhosis of the liver through pathogen invasion, resultant systemic inflammation, and impaired immunity and liver function (Acharya et al., 2017). Even gastrointestinal conditions like IBD and cancer of the colon, liver, and pancreas are linked with both the gut and oral microbiome dysbiosis, further demonstrating this strong interorgan connection to human health (Park et al., 2021).

The gut and the skin microbiomes are also linked via the gut-skin axis (GSA), and as the primary interface to the environment, they have major roles in physiological health (Figure 1). Dysbiosis of the gut-skin microbiome axis has influence on both GI and cutaneous disorders such as IBD, celiac disease, atopic dermatitis, psoriasis, acne, and other dermatologic issues, where each of these diseases can be associated with the 'partner disease' (i.e. a disease in one system linked to a disease in another system) (O'Neill et al., 2016, Salem et al., 2018, De Pessemier et al., 2021). Though crosstalk is bidirectional, more research seems to have focused on skin disorders and homeostasis as a result of gut microbiome health, where diet, immunomodulation, intestinal permeability, and metabolite secretion are major contributing factors (De Pessemier et al., 2021). However, there is evidence that *Mallassezia restricta*, a fungal member of the skin microbiota, is associated with Crohn's disease and can exacerbate colitis (Limon et al., 2019, Sinha et al., 2021).

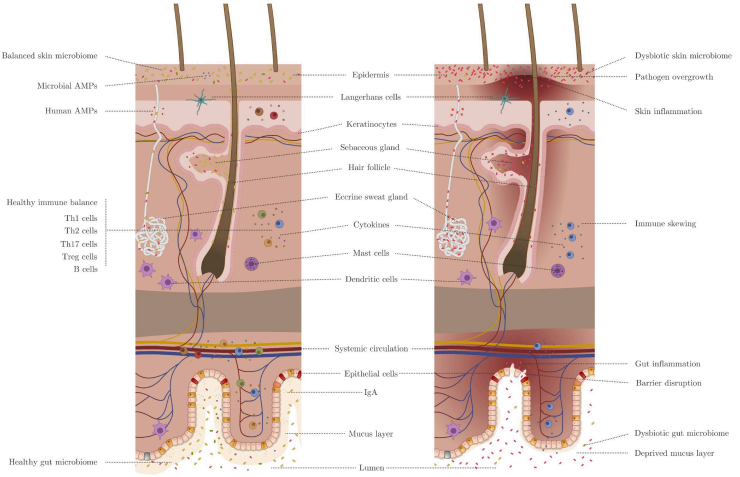


Figure 1. Inflammatory and microbial influences between the gut and skin for a healthy state (left) and a dysbiotic state (right): The intestinal and epidermal barriers are connected through the systemic circulation (blood and lymph) and are visualized here together in a simplistic manner. The dysbiotic state is characterized by an impaired gut barrier (imbalance in gut microbiome, reduced mucus layer, reduced IgA secretion, barrier disruption, intestinal permeation into the bloodstream, and gut inflammation) and an impaired skin barrier (imbalance in skin microbiome, reduced human and microbial antimicrobial peptides (AMP) production, skin rashes/thickening/lesions, and skin inflammation). Gut and skin dysbiosis are connected through an immune imbalance (Th2 skewing in this example), whereas crosstalk can be bidirectional (De Pessemier et al., 2021).

The gut-lung axis (GLA) has roles in the regulation of immunity and the development of various respiratory diseases. (Frati et al., 2019). The respiratory system and GI tract are connected by the mesenteric lymphatic system, where intact or fragmented microbes and their metabolites enter systemic circulation after passing through the intestinal barrier and can migrate to the pulmonary system to modulate immune responses (Enaud et al., 2020). The pathophysiology of diseases such as atopy and asthma are complicated and can be contributed to a variety of factors, however, there is evidence that dysbiosis of the gut microbiome contributes

to the development of asthma, particularly in youths who exhibit a decrease in *Lachnospira* and increase in *Clostridium* spp. (Penders et al., 2007, Watson et al.,2019). Gut dysbiosis is also involved in chronic obstructive pulmonary disease (COPD) exacerbation, where fiber deficiency in an individual's diet can contribute to chronic inflammation. Metabolism of fiber by gut microbes produces anti-inflammatory short-chain fatty acids (SCFAs), which could reduce inflammation both systemically and in the lungs, and so targeted dietary intervention for these patients may be a viable treatment addition for COPD and other respiratory diseases associated with inflammation like COVID-19 (Li et al., 2018, Vaughan et al., 2019, Allali et al., 2021). Moreover, SCFAs also have an important role in the defense against secondary infections in those afflicted by viral respiratory infections, further demonstrating the importance of gut microbiome health in connection with the GLA (Sensio et al., 2020).

Like other axes, immunomodulation by the gut microbiome also has influence on the gut-vagina axis (GVA), though it is less extensively studied. One promising avenue of treating vaginal diseases, specifically cervical cancer associated with human papilloma virus (HPV), is the use of mucosal lactic-acid bacteria (LAB)-based vaccines to modulate the gut microbiome. The approach could work as a prophylactic or for direct therapy, and be more easily administered via an oral route instead of parenteral (Taghinezhad-S, et al., 2021). Endometriosis and infertility are also disorders associated with sex hormone levels and inflammation, which once again are influenced by the gut microbiome composition and state. Specifically, there are some members in the gut who can affect the levels of circulating estrogen through metabolic processes and an altered state could lead to increased risk or symptom severity of these disorders (Salliss et al., 2022). Other female reproductive-associated diseases such as polycystic ovarian syndrome (PCOS), also associated with gut dysbiosis, could be ameliorated by diet modification and restoration of gut homeostasis. Here, the use of flaxseed oil could increase diminished

levels of SCFAs observed in those with PCOS and protect against inflammation characteristic of the disease (Wang et al., 2020).

The maternal microbiome, including gut, vagina, and breast milk, can greatly influence the colonization and health of the infant after birth, but also affect the fetus prior to delivery. Fetal immune development is most likely influenced in the womb from translocation of maternal gut microbes and/or their metabolites across the placental barrier or ingestion of amniotic fluids (Walker et al., 2017, Nyangahu and Jaspan, 2019). Post-reproduction, the infant gut microbiome is continually developed through initial diet, and more specifically vertical transmission of the contents in the mother's breast milk which includes its own unique microbiome (Ojo-Okunola et al., 2018, Ojo-Okunola et al., 2019, Quin et al., 2020). The human milk microbiome promotes infant gut colonization of probiotic strains which have roles in programming the immune and metabolic systems, as well as anti-infective, anti-allergic, and anti-tumor properties (Heikkila and Saris, 2003, Olivares et al., 2006, Lara-Villoslada et al., 2007, Civardi et al., 2015, Hassan et al., 2015, Walker and Iyengar, 2015, Boix-Amoros et al., 2016, Ojo-Okunola et al., 2018).



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Mental Health Axes

The thought of microorganisms controlling aspects of cognitive function in their host, especially humans, is fascinating to say the least. While connections between other microbiomes, organ

systems, and diseases may be less surprising, the microbial link to psychiatric, neurodevelopmental, age-related, and neurodegenerative disorders is very much intriguing. The human microbiome can communicate with the brain in a variety of ways; through the immune system, metabolism, endocrine system, circulatory system, and the nervous system, where microbes, their induced immune response, and their metabolites such as short-chain fatty acids, branched chain amino acids, and peptidoglycans are involved (Figure 2) (Liang et al., 2018, Cryan et al., 2019, Olsen and Hicks, 2019, Hadian et al., 2020, Bear et al., 2021). Several local microbiomes each contribute affects to the mental state, and whether it is gut-, skin-, oral-, lung-, etc. derived can dictate roles in various disorders. This is not just a one-way street though, as microbes around the body can alter mental states but also be affected by governance of the brain in a bi-directional manner (Ma et al., 2019).

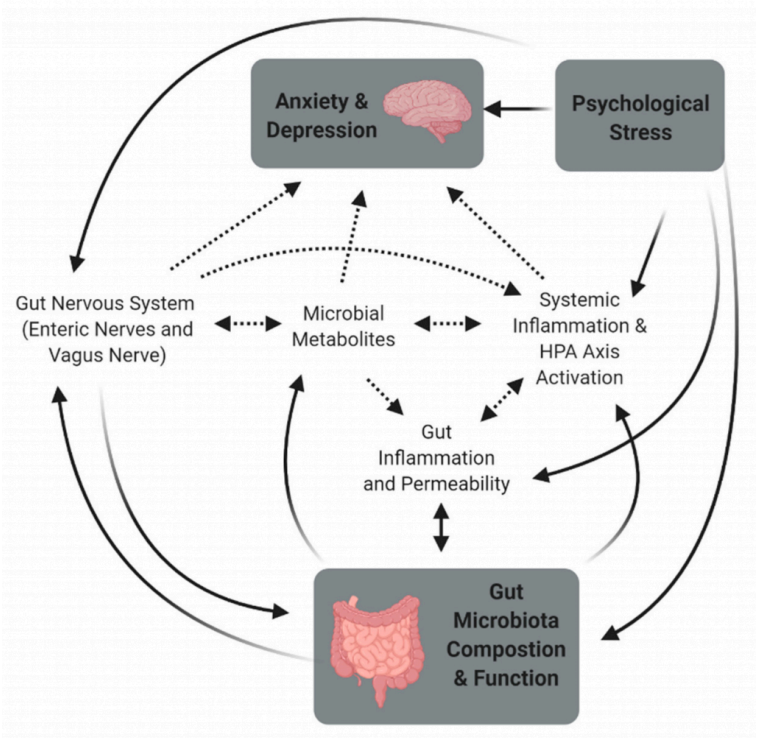


Figure 2. The proposed mechanisms of the microbiome-gut-brain axis (MGBA) are complex and intertwined. Emerging research shows that psychological stress interacts not only directly with the brain and mood, but also with many of the MGBA mechanisms thought to contribute to changes in mood with alteration of the gut microbiota. Solid lines indicate strong evidence of an effect, and dotted lines show proposed mechanisms with limited but emerging evidence. Abbreviations: HPA; Hypothalamic-Pituitary-Adrenal (Bear et al., 2021).

Gut-Brain

Similar to other types of microbiome and organ interactions, the gut's role in mental health is central and most well studied, which makes sense as it is the largest repository of microorganisms associated with the human body. The gut-brain axis (GBA; and gut

plus essentially every other microbiome and brain axis, e.g., gut-skin-brain) is implicated in a number of cognitive functions and disorders including autism, anxiety, depression, stress, pain sensitivity, learning capacities, memory loss, moods and emotions, behavior (dietary, social, and reproductive), schizophrenia, Parkinson's disease, and Alzheimer's disease (Desbonnet et al., 2013, Stumpf et al., 2013, Dash et al., 2015, Gareau, 2016, Luczynski et al., 2016, Hoban et al., 2017, Liang et al., 2018, Manderino et al., 2017, Nishida and Ochman, 2017, Vuong et al., 2017, Cowan et al., 2018, Cryan et al., 2019, Bear et al., 2021, Narengaowa et al., 2021). The gut microbiome actually develops in sync with the brain and psychology, and disturbances during different stages of growth can result in the onset of different diseases (Figure 3) (Borre et al., 2014, Gur et al., 2015, Sampson and Mazmanian, 2015, Dinan and Cryan, 2016, Luczynski et al., 2016, Sharon et al., 2016, Kundu et al., 2017, Vuong et al., 2017, Carlson et al., 2018, Liang et al., 2018).

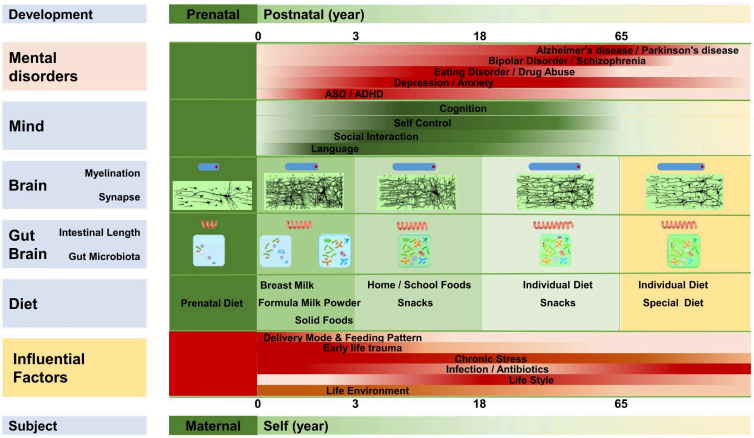


Figure 3. The gut-brain, brain, and mentality develop almost synchronously throughout the lifespan. The gut-brain, brain, and mentality undergo similar developmental patterns; all three are susceptible to several factors that influence the gut microbiota. Myelination, intestinal length, and the gut microbiota develop almost synchronously. Diet plays an important role in the maturation of the gut-brain and brain, and mentality is regulated by the development of the brain and gut-brain. Microbiota disruption at different stages is likely to increase the incidence of different mental disorders (Liang et al., 2018).

Early perturbation of the gut microbiome during the post-natal period, and even within the womb, can especially increase susceptibility to developing mental disorders since these are critical stages for development of the gut-brain axis and mind (Borre et al., 2014, Gur et al., 2015, Diaz Heijtz, 2016, Mika et al., 2016, Slykerman et al., 2016, O'Mahony et al., 2017, Liang et al., 2018). Even up through senescence, an abnormal gut microbiota is linked with several mental disorders, though the good news is that they can be remedied or improved by returning the gut to a homeostatic state. As the prevalence of mental disorders and neurological diseases have been steadily increasing, healing mental health by exploring and implementing options such as fecal microbiota transplant, diet intervention, probiotics, prebiotics, and psychobiotics are a must (Cryan and Dinan, 2012, Dinan et al., 2013, Liang et al., 2015, Pirbaglou et al., 2016, He et al., 2017, Kang et al., 2017, Mika et al., 2017, Bruce-Keller et al., 2018, Liang et al., 2018, Yang et al., 2018, Kesika et al., 2021, Margolis et al., 2021).

There are several ideas as to why there has been an increase in mental and neurological disorders in relation to the human microbiome. The "Gut Microbiota" hypothesis suggests this escalation is a direct result from gut microbiome dysbiosis due to factors of modern society such as diet, antibiotics, and stress (Liang et al., 2018b). The "Old Friends" hypothesis proposes that the co-evolution of early humanity with its microbiota in a less civilized and more natural ecosystem promoted the development of a stronger immune system, but now changes in lifestyle and environment leads to weaker immunity and subsequent disorders (Strachan, 1989, Rook and Lowry, 2008, Kramer et al., 2013, Rook, 2013). Lastly, the "Leaky Gut" theory implies that damage to the mucosal barrier of the gut increases intestinal permeability, which allows biomolecules and microorganisms to gain access various parts of the body that they normally couldn't, and thereafter cause disease (Leclercq et al., 2012, Smythies and Smythies, 2014, Kelly et al., 2015, Potgieter et al., 2015, Slyepchenko et al., 2017, Liang et al., 2018). All of theses propositions are clear in their indication that the gut microbiome and its part

in immune development has a definite role in the functioning of the mind, though the exact extent and mechanisms remain to be discovered.

> *One or more interactive elements has been excluded from this version of the text. You can view them online here: https://uta.pressbooks.pub/microbiomeshealthandtheenvironment/?p=695#oembed-2*

Other Microbiomes and Mental Health

The majority of human microbiome interaction with regards to human health usually includes the gut to some degree, though there are some questions as to whether certain microbiomes create their own axes with the brain.

The oral microbiome can potentially get direct or indirect access to the brain through the olfactory tract, or via the circulatory system where blood can transport microbes to the blood-brain barrier (BBB), perivascular spaces, and circumventricular organs (Figure 4) (Olsen and Singhrao, 2015, Ranjan et al., 2018, Olsen and Hicks, 2019). Microbial access to the bloodstream can commonly occur during dental procedures, and pathogen invasion of the brain can impact neuro-immune activity and inflammation (Olsen, 2008). These oral-brain axis-derived infections could contribute to mental disorders such as Alzheimer's disease (AD), dementia, Down's syndrome, bipolar disorder, and autism spectrum disorder (ASD; which can lead to oral dysbiosis) (Ilievski et al., 2018, Olsen and Hicks, 2019, Maitre et al., 2020). Oral dysbiosis can also promote the development of ASD by affecting the metabolome and thus can create a troublesome positive feedback loop (Mussap et al., 2016, Wang et al., 2016).

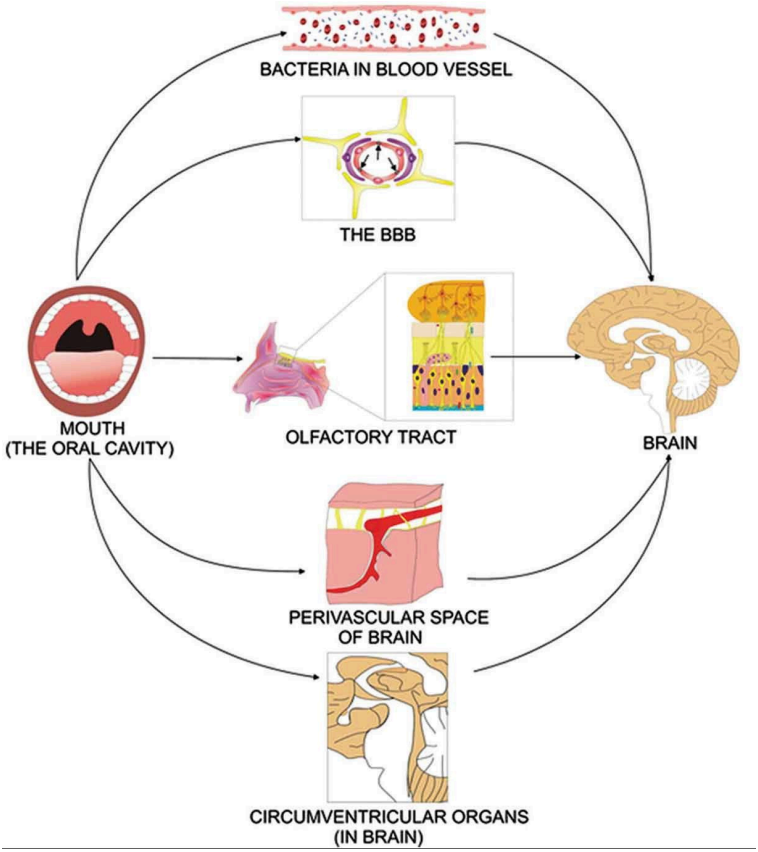


Figure 4. Direct and indirect mechanisms of infecting the brain. In the direct mechanism, the oral cavity infects the olfactory tract, and the olfactory nerve transfer the bacteria to the brain. In other mechanisms, bacteria inside the mouth infect the blood and find their way via blood, blood–brain barrier (BBB), *perivascular spaces and circumventricular organs to the brain* (Olsen and Hicks, 2019).

Concerning the skin-brain axis, dysbiosis of the skin microbiome and chronic wounds can elicit systemic effects and induce neural responses that eventually affect the central nervous system (Figure 5) (Hadian et al., 2020). Chronic wounds exhibit persistent inflammation and can have various etiologies including diabetic foot

ulcers (DFU), venous ulcers, arterial ulcers, pressure ulcers, surgical wounds, and other traumatic wounds (Green et al., 2014, Renner and Erfurt-Berge, 2017, Bui et al., 2018, Pedras et al., 2018, Hadian et al., 2020). In these cases, a compromised skin barrier can allow pathogens and their derivatives to enter the bloodstream, and if the skin is in a dysbiotic state, then abundant pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* can amplify this effect by inducing epithelial permeability (Roy et al., 2014, Basler et al., 2017). These microbes, metabolites, pro-inflammatory mediators, and other constituents circulate within the blood, potentially induce permeability in the blood-brain barrier (BBB), and eventually reach the brain to cause disorders such as wound-associated depression, anxiety, and other cognitive disorders (Wang et al., 2011, Zhang et al., 2015, Hadian et al., 2020). The skin-brain axis acts in a bi-direction manner too, with studies showing connections between certain mental disorders like post-traumatic stress disorder, and skin diseases associated with dysbiosis like psoriasis, chronic urticaria (hives), and atopic dermatitis (Gupta et al., 2017, Beri, 2018).

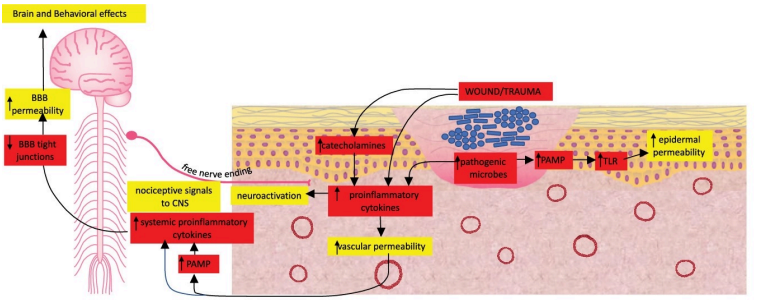


Figure 5. The chronic wound with its microbiota induces localized inflammatory alterations that can result in systemic effects by increasing both epidermal and vascular permeability and activating neurons to generate nocioceptive signals—all ultimately culminating in central nervous system effects. Red: Proposed mediators. Yellow: Proposed downstream events. BBB: blood brain barrier, CNS: central nervous system, PAMP: pathogen associated molecular pattern, TLR: toll like receptor (Hadian et al., 2020).

The lung-brain axis connects pulmonary microbes to neurodegenerative disorders and behavior characteristics, and though not much research has focused on this specific link, there are indications that air pollution can be a trigger. Pollutants in the air come from a variety of sources including engine emissions, coal combustion, biomass burning, and secondary photochemical products, such as ground level ozone (O_3) (Mumaw et al., 2016). These compounds can be a source of chronic neuroinflammation and persistently affect microglial cells in the central nervous system, which in turn can increase risk of diseases such as Alzheimer's disease, Parkinson's disease, and autism, as well as elicit a decline in cognitive function in the elderly (Power et al., 2011, Wellenius et al., 2012, Roberts et al., 2013, Volk et al., 2013, Heneka et al., 2014, Jung et al., 2015, Kirrane et al., 2015, Mumaw et al., 2016). It is likely that air pollution also negatively affects the lung microbiome, and dysbiosis could further aggravate immune responses and cognitive disruption (Mousavi et al., 2021, Whiteside et al., 2021).

Is there a brain microbiome?

That is, are there resident microbes residing with a living brain? With many neurodegenerative and neuropsychiatric diseases lacking a clear etiology, determining any and all potential associations could help in therapeutic efforts (Link, 2021). The healthy brain is an assumedly sterile environment, though the same was once thought about other organs like the lungs. Interestingly, one study found evidence for the existence of viable bacteria in the human brain. Here, researchers were interested to see whether microbial invasion accompanied damaged brains observed in HIV/AIDS. After sequencing total RNA from cerebral white matter, they found bacteria and phage sequences in both experimental and control brain samples, which was further validated by 16S rRNA gene

target amplification and *in situ* staining (Branton et al., 2013, Link, 2021).

If there were resident microbes in the brain, they would certainly be at much lower abundances as compared with other regions such as the gut or the oral cavity. Though there has been much interest in characterizing the presence of pathogens in unhealthy brains for disorders like Alzheimer's disease, which is an arduous feat in itself, finding residents in a healthy state could prove even more difficult (Zhan et al., 2016, Alonzo et al., 2018, Dominy et al., 2019, Link, 2021). That is, identification approaches could easily miss novel or fastidious microorganisms, and contamination is hard to avoid. Furthermore, a wide-range of controlled and unbiased studies are necessary to catalogue a possible brain microbiome, however, completing this task in humans brings about ethical implications and sampling limitations (Link, 2021). It is fascinating to consider the prospect of symbiotic brain microorganisms, and how they could influence our health or who we are.

Conclusion

Mental faculties contribute greatly to human health, and therefore characterizing the involvement of the human microbiome is of substantial importance. Many of these select microbiomes overlap, and the disorders associated with each are likely entangled in a complex array. Brain functions are extremely complex as is, and piecing together the microbial puzzle within their reach further complicates matters. However, there is great optimism in considering the human microbiome as a source or target for advanced therapy to resolve mental disorders and other diseases, which could drastically change the way we think about medicine.

Discussion Concept Mapping

With a partner or as a class, connect two or more microbiomes using a concept map. Explain how and/or why the various microbiomes are linked (e.g., disease cause vs. effect, direct vs. indirect, pathways, consortia translocation, factors affecting composition, etc.).

Check your Understanding

- In what ways does the gut microbiome connect with other microbiomes and regions of the body? How does it influence the development of diseases in these areas?
- How could early use of antibiotics influence the development of asthma in children?
- How could gut microbiome metabolites (SCFAs) contribute to healthy human states?
- In what ways does the human microbiome communicate with the brain?

- What options are available to treat mental disorders through the gut microbiome?
- Why have mental illnesses been increasing in prevalence and how could the gut microbiome be involved?
- How are microbiomes, other than the gut, implicated in mental health?
- What role does skin microbiome dysbiosis and chronic wounds have in the development of cognitive disorders?

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PART IV
ENVIRONMENTAL MICROBIOMES

11. Environmental Nutrient Cycling and Human Health

Environmental Nutrient Cycling and Human Health

The importance of microorganisms is unquestionable in regard to how nutrients circulate throughout each ecosystem. There are direct and indirect links between Earth's ecosystems and human health, though like other microbial networks, they are sometimes unfathomably complex.

Climate change may be the most obvious association between environmental and human health, however, the solution to balance is not one likely easily achieved. Each type of ecosystem is unique, and reflects different changes to anthropogenic activity. Global soil microbiomes and organic foliar litter have great impact on worldwide biogeochemical cycling, plant health, and bioremediation, where environmental changes can disrupt microbial taxonomic distribution and functional profiles (Albright et al., 2020, Naylor et al., 2020). The ocean microbiome is vast considering it is the largest ecosystem on the planet, and plays a tremendous part in biogeochemical cycling, ecosystem dynamics, and response to climate change(Moran, 2015, Acinas et al., 2019, Marz et al., 2021). Also, since much of the ocean microbiome is uncharted, it could also serve as a major untapped reservoir for novel and progressive biosynthetic products (Paoli et al., 2021). Other aquatic environments such rivers, lakes, wetlands, and freshwater systems and their interaction with sediments and plants greatly influence carbon and other nutrient cycles in their respective ecosystems (Amado and Roland, 2017, Avila et al., 2019, Trevathan-Tackett et al., 2021).

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In a cyclic manner, climate change can also greatly affect microbiome dynamics of large ecosystems like glaciers, tundra, permafrost, and even dry deserts, which can further exacerbate disturbances in that region and beyond. (Hamilton et al., 2013, Tripathi et al., 2019, Vigneron et al., 2019, Hough et al., 2020, Ray et al., 2020). These environments harbor several dormant microorganisms that can produce greenhouse gases like carbon dioxide and methane, and if they become metabolically active en masse, this could drastically increase contribution to climate change (Feng et al., 2020). Not only can ecosystem biodiversity be affected, but these changes can impact human society and health. Thus, it is important to consider analytical strategies to better understand global change so future actions can be coordinated to mitigate any negative consequences, and evaluating microbiomes may be part of the solution.

Global Change and the Soil Microbiome: A Human-Health Perspective

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The importance of the gut and the soil microbiomes as determinants of human and ecosystem health, respectively, is gaining rapid acceptation in the medical and ecological literatures. This suggests that there is a wealth of highly transferable knowledge about the microbial ecology of human and non-human ecosystems that is currently being generated in parallel, but mostly in isolation from one another. I suggest that effectively sharing this knowledge could greatly help at more efficiently understanding and restoring human health and the functioning of ecosystems, which are currently under wide-spread pressure. I illustrate this by comparing the effects of nitrogen deposition on ecosystem carbon sequestration with unhealthy dietary habits and human disease. The deposition of N, a key nutrient for plant growth, may increase carbon sequestration (equivalent to obesity) through several mechanisms, including a reduction in the ability of soil microbes to process organic matter, which some argue could help mitigate climate change. However, this usually results in a degradation of ecosystem health and, thus, cannot represent a real solution. Similarly, human obesity is linked to an alteration of the composition and functioning

of microbial communities inhabiting the gut, which is often attributed to unhealthy dietary habits, including ingesting high amounts of simple sugars and processed foods. Finally, I advocate for the explicit recognition of the many commonalities between the functioning of the gut and ecosystems and a broader multidisciplinary collaboration among experts in ecology and human health, including the engineering of soil microbial communities designed *ad-hoc* to restore ecosystem health.

Nitrogen Deposition and Carbon Sequestration in a Changing Climate

It has been widely proposed that atmospheric nitrogen (N) deposition could help mitigate climate change by increasing the rates of carbon (C) sequestration in terrestrial ecosystems (Knorr et al., 2005; Reich et al., 2006; Yue et al., 2016). Two commonly observed responses are typically proposed as mechanisms: first, a greater amount of N usually implies a higher capacity for plant growth, which would result in a greater amount of C retained within the system (Magnani et al., 2007; de Vries et al., 2009; Laubhann et al., 2009). Of course, for this to be true, it is necessary that the increase in the rates of C uptake and accumulation exceed the C emission rates, whatever the main route by which the latter happens, including plant and/or microbial respiration and changes in fire dynamics due to an excess of biomass accumulation (Dezi et al., 2010; Fenn et al., 2010). The second main mechanism is linked to a reduction in decomposition rates, particularly of recalcitrant organic matter, which would, therefore, accumulate within the system (Knorr et al., 2005; Waldrop and Zak, 2006).

Otherwise, this accumulated C may be lost to the atmosphere in the form of CO_2 after being respired by soil microorganisms (Janssens et al., 2010). Of course, the relative importance of these mechanisms depend on how plant communities and soil microorganisms respond, directly and indirectly, to the additional inputs of N which, in any case, usually ends up resulting in a disruption of the interaction between these two key components of the ecosystem (Liu et al., 2014).

The Need for a New Perspective

In this article, I will adopt a human health perspective, hardly used in the discipline of global change ecology, to substantiate why atmospheric N deposition cannot represent a positive (i.e., healthy) alternative to mitigate climate change. In the medical literature, it is now widely recognized that human beings are like ecosystems (in fact, some consider us as living ecosystems) in which the eukaryotic cells that form part of our bodies and the prokaryotic cells that live in and on us are deeply interconnected, whereas the enormous importance of our microbiome to human health is also increasingly gaining acceptation (Bengmark, 1998; Berendsen et al., 2012; Ha et al., 2014; Alivisatos et al., 2015; Tilg and Adolph, 2015; Blaser, 2016; Blaser et al., 2016). The fact that many modern diseases, including conditions of the nervous and circulatory systems, skin and heart and allergies (including atopic dermatitis and food allergies), are directly caused by alterations in the microbial communities that live in our interior and exterior is also gaining rapid acceptation (Ha et al., 2014; Tilg and Adolph, 2015; Chang et al., 2016; Tang and

Lodge, 2016). In this sense, the word ecosystem is widely used in the current literature of integrative medicine and gastroenterology. However, the opposite does not frequently happen in ecology [i.e., (cautiously) comparing ecosystems with the human body], despite the wealth of knowledge in the medical and human health literature that we, as ecologists, could apply in, for example, issues related to understanding the functioning (i.e., metabolism) of ecosystems and plant-soil-microbe interactions subjected to human pressure (Berendsen et al., 2012; Blaser et al., 2016; Table 1; Figure 1). Therefore, I will finally defend the need to approach problems in ecology from a more multidisciplinary, fresher perspective.

Characteristics associated with a healthy gut	Impacts associated with an unhealthy diet	References	Characteristics of healthy soils and ecosystems	Impacts associated with nitrogen deposition	References
Higher bacterial diversity and abundance that are able to metabolize more food sources and provide essential molecules such as vitamins, hormones, etc.	Functionally and compositionally impoverished gut communities	Thomas et al., 2014	Higher microbial diversity and abundance	Functionally and compositionally impoverished soil communities	Leff et al., 2015; Berg et al., 2017
Protection against disease through the stimulation of the immune system. More longevous individuals	Disease (obesity, diabetes, depression, inflammation etc.). People die younger	Berendsen et al., 2012; Ha et al., 2014; Thomas et al., 2014; Alviatos et al., 2015; Tilg and Adolph, 2015; Xu et al., 2015; Blaser, 2016	Higher stability and resistance to disturbance	Die-back due to long-term N toxicity, altered fire regimes, pest outbreaks, less tolerance to frost, heat waves, etc.	Bobbink et al., 2010
Lean phenotype	Obese phenotype	Joyce and Gahan, 2014	Rapid processing, transformation and stabilization of litter inputs in the long-term soil pool	Accumulation of intact and partially decomposed leaf litter (i.e., carbon accumulation)	Knorr et al., 2005
High-energy feeling	Low-energy feeling	Umu et al., 2013	Higher ecosystem functionality and supply of key services such as air and water purification, food resources for pollinators, protections against extreme events such as floods, heat waves, etc.	Lower functionality and reduced supply of key services	Costanza et al., 1997; Jones et al., 2014
Well-trained immune system. No medication required. This is also associated with a smaller social cost	Weak immune system. Medication is very often required to treat diverse conditions. Development of drug-resistant strains. Higher social cost	Round and Mazmanian, 2009; Blaser, 2016	High ability to self-regenerate. No or very little management required. No extra cost involved	Management required, including applying herbicides, weeding, etc. High monetary cost	Costanza et al., 1997; Chiquoine et al., 2016
Higher Bactericidetes to Firmicutes ratio	Lower Bactericidetes to Firmicutes ratio	Mathur and Barlow, 2015	Higher fungal to bacterial biomass and activity ratio. Higher abundance and diversity of mutualistic mycorrhizal strains	Lower fungal to bacterial ratio. Less mutualistic mycorrhizal strains	Frey et al., 2004; Treseder, 2004; Waldrop et al., 2004; de Vries et al., 2006; Wallenstein et al., 2006

Table 1. The importance of the gut and the soil microbiomes as determinants of human and ecosystem health, respectively, is gaining rapid acceptation in the medical and ecological literatures.

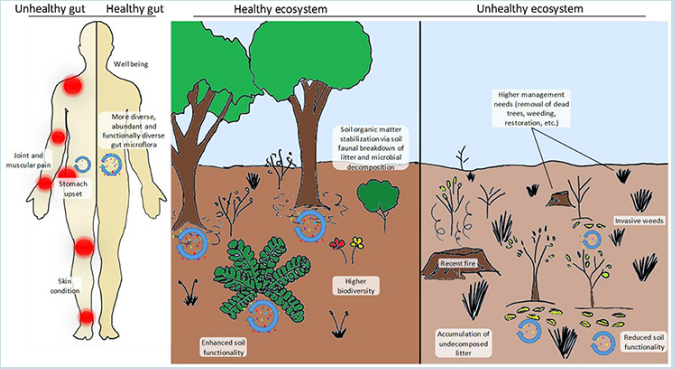


Figure 1. Comparison between a healthy and an unhealthy gut (left-hand side panel) and a healthy and an unhealthy ecosystem (right-hand side panel). In the case of the human individual, his/her gut microbiota is less diverse ("red" microbes are completely absent), less abundant and contains more pathogenic taxa ("yellow" microbes). The individual with the healthy gut has a more functional and more abundant microbiota that provides him/her with essential nutrients, hormones, amino acids, etc. and stimulates his/her immune system. A healthy gut is also less prone to become infected by pathogens and can process toxic compounds (i.e., detoxify) more easily. The disturbed (i.e., unhealthy) ecosystem shown here has recently been affected by a devastating fire fuelled by the accumulation of N-loving exotic grasses that have altered the natural fire dynamics of the system. Facilitated by the altered fire dynamics, the system has also become chronically dominated by weedy grasses, therefore requiring intensive (and costly) management practices (dead tree removal, weeding and restoration), also posing a threat to nearby human populations and their properties. Biodiversity (in terms of microbial, faunal and plant communities) is remarkably higher in the healthy ecosystem, which also has a higher potential to process organic matter inputs and stabilize them in the long-term soil pool. In contrast, undecomposed or partially decomposed litter accumulates on the functionally impoverished soil of the unhealthy ecosystem. This pattern is in agreement with reported observations of higher soil carbon sequestration under increased nitrogen deposition scenarios due to the inhibition of soil enzymes and the reduction of microbial biomass but poses relevant questions such as: Is this type of N deposition-induced carbon sequestration desirable?

And, does it really represent a long-term (or even short-term) solution?

Why Nitrogen Deposition Cannot be the Solution to Climate Change

The reason why I think that a temporary, N deposition-induced increase in the rate of C sequestration will not contribute to mitigating climate change in the long term is equivalent to the reason of those that argue that an increase in obesity rates in human populations derived from a diet rich in simple sugars and processed food and the consequent alteration of their microbiome will not successfully and permanently solve any public health problem of today's societies. Ingesting large amounts of simple sugars, processed foods, sugary drinks and saturated fats is definitely better than starving, but that does not mean that it is a healthy practice. And the same happens with N deposition and C sequestration. In ecosystems where N is still a limiting nutrient, which is quite common worldwide (LeBauer and Treseder, 2008), an increase in the availability of N can increase ecosystem productivity to levels comparable to human obesity (Tian et al., 2016), but that does not mean that the ecosystem is healthier and, therefore, that this will result in a long-term benefit (Bobbink et al., 2010; Jones et al., 2014). In this sense, a healthy ecosystem may be defined here as a highly multifunctional ecosystem that can maintain an adequate supply of services, at least as compared to a previously defined reference state.

In medicine, the term dysbiosis refers to changes in the composition of the microbiome that are not beneficial to the individuals, including a loss of abundance and diversity of beneficial microorganisms and increased number of pathogens, and that result in the development of a condition (Ha et al., 2014; Tilg and Adolph, 2015). This term could also be used to describe ecosystems that are dysfunctional due to alterations of their microbial communities. In this sense, it has been repeatedly shown through experimental studies and meta-analyses that increased N deposition is typically associated with changes in soil microbial communities (usually related to a decrease in abundance and biodiversity; Treseder, 2004, 2008; Ramirez et al., 2010; Zeng et al., 2015), reduced ecosystem functionality (alterations of energy metabolism; Waldrop and Zak, 2006; Treseder, 2008; Liu et al., 2014) and short- to mid-term increases in C sequestration, especially in aboveground biomass, but also in the soil and roots (comparable to obesity, as previously mentioned; Xia and Wan, 2008; Yue et al., 2016). Given that metabolic disorders and obesity in humans are clearly associated with a deterioration in the health status of individuals that may even result in cases of fatality due to chronic diseases, sudden death or, quite commonly in the natural world, to increased sensitivity to other environmental stresses (Mathur and Barlow, 2015; Monteiro et al., 2015), I think that we would do well to be cautious when we consider, perhaps naively, the potential benefits of a N that, after all, is the result of the atmospheric pollution derived from our activities (Gruber and Galloway, 2008).

The "Deceptively Simple" Solution

The connections between human health, disease, and the microbiome, especially in the case of the gut, are becoming increasingly apparent and are attracting the public attention, especially because of the high social cost of unhealthy dietary habits and lifestyles and the "deceptively simple" solution of the problem (Mathur and Barlow, 2015; Tilg and Adolph, 2015; Blaser, 2016). In the case of both people and ecosystems, (i) ensuring a healthy supply of nutrients derived from the breakdown and cycling of unprocessed food/organic matter, (ii) minimizing the use of antibiotics (particularly those associated with the livestock industry in the case of ecosystems; Park and Choi, 2008) and chemicals (including herbicides and pesticides in the case of ecosystems) that destroy the microbiome, unless this is strictly necessary, and (iii) promoting practices that favor the system's ability to self-regenerate, something that living systems do wonderfully well, and that increase its resilience against pathogens and extreme events could be part of the solution, if not all, of the problem.

Of course, there are opportunities to aid in the recovery of our damaged and degraded ecosystems as well as there are possibilities to recover the lost or damaged intestinal flora (Brudnak, 2002; Sheth et al., 2016). This can be achieved by the use of properly designed probiotics or fecal transplants or, in the case of ecosystems, inocula assembled in the lab from pure cultures or soil samples obtained in the field from healthy ecosystems (Bowker, 2007; Chiquoine et al., 2016; Wubs et al., 2016) in conjunction with a balanced

nutrient supply (i.e., organic matter inputs, the equivalent to prebiotics; Mathur and Barlow, 2015; Sheth et al., 2016). In this sense, the concept of synbiotics (i.e., synchronous administration of probiotics and prebiotics) could represent a particularly promising benchmark borrowed from the human health literature to successfully restore degraded ecosystems (Tang and Lodge, 2016) and, thus, the human probiotics industry has an opportunity to play a key role in this development.

Concluding Remarks

Recognizing and understanding the similarities and deep connections between the gut and the belowground world, where roots are the equivalent to our gut and the rhizosphere is the gut microflora (Berendsen et al., 2012) can help us advance the understanding of ecosystems by leaps and bounds through the search of similar microbial indicators of disease (e.g., Bacteroidetes to Firmicutes ratio in humans; Mathur and Barlow, 2015) and, therefore, to implement quick and successful measures in ecosystem management rather than relying, perhaps naively, on that the very same thing that caused climate change (i.e., pollutant emissions to the atmosphere) will also be part of the solution. From here, I advocate for the development of a new field of research that specifically aims at recognizing and make practical use of the profound links between the functioning of the gut and the ecosystems that extend beyond our bodies and that benefits from a truly multidisciplinary collaboration among experts in the areas of global change ecology and human health.

Author Contributions

The author confirms being the sole contributor of this work and approved it for publication.

Conflict of Interest Statement

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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- Which microbiome is an under-explored source for the discovery of biosynthetic products?
- How could global warming's effect on permafrost and tundra further increase greenhouse gases?
- What are the pros and cons of nitrogen deposition to counteract climate change?
- What are some suggested solutions to improve environmental and human health concerning microbiomes?

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12. The Ocean Microbiome and Marine Life

The Ocean Microbiome and Marine Life

The ocean is teeming with life, both macro and micro, where their adaptation and success are dependent on both a local and global scale. Oceanic microorganisms have major roles in nutrient cycling, ecological interactions, and influence and respond to environmental changes (Doney et al., 2012). Temperature is the strongest factor that determines microbial community composition, which parallels depth stratification, and these communities can serve as indicators for anthropogenically-induced climate changes (Sunagawa et al., 2015). It is predicted that carbon fixation by microbial primary producers will decrease which will have downstream effects on ocean microbiome structure and overall trophic level interactions (Moran, 2015).

While free-living marine microorganisms can provide relevant information and predictive models about the ocean, those host-associated microbes can also give environmental insight into the largest ecosystem on the planet.

Marine Animal Microbiomes: Toward Understanding Host–Microbiome Interactions in a Changing Ocean

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All animals on Earth form associations with microorganisms, including protists, bacteria, archaea, fungi, and viruses. In the ocean, animal–microbial relationships were historically explored in single host–symbiont systems. However, new explorations into the diversity of microorganisms associating with diverse marine animal hosts is moving the field into studies that address interactions between the animal host and a more multi-member microbiome. The potential for microbiomes to influence the health, physiology, behavior, and ecology of marine animals could alter current understandings of how marine animals adapt to change, and especially the growing climate-related and anthropogenic-induced changes already impacting the ocean environment. This review explores the nature of marine animal–microbiome relationships and interactions, and possible factors that may shift associations from symbiotic to dissociated states. I present a brief review of current microbiome research and opportunities, using examples of select marine animals that span diverse phyla within the Animalia, including systems that are more and less developed for symbiosis research,

including two represented in my own research program. Lastly, I consider challenges and emerging solutions for moving these and other study systems into a more detailed understanding of host–microbiome interactions within a changing ocean.

Introduction

Marine animals are the icons of life in the oceans. They represent about two million species (Mora et al., 2011) and include a wide range of body designs, from the highly simplistic sponges lacking true tissues and organs to the complex vertebrates containing specialized tissues and organs, such as fish and marine mammals, with some iconic representatives presented in Figure 1. The bodies of marine animals span several orders of magnitude in size, from the abundant planktonic copepod (1–2 mm) to the highly mobile blue whale (30 m), the largest animal on Earth. Marine animals are key members of ocean ecosystems and serve as both prey of and predators for other animals within the complex ocean food web. In contrast to terrestrial animals, marine animals have developed strategies for osmoregulation as well as highly specialized approaches for maintaining homeostasis within diverse temperature, oxygen and pressure gradients of the ocean (Graham, 1990; Knoll and Carroll, 1999). Marine animals also possess sophisticated specializations and functions that promote their success on or within their benthic or pelagic habitats, including specializations for living or enduring depths (outlined in Figure 1) that vary widely in factors such as light availability, access to food and predator exposure.

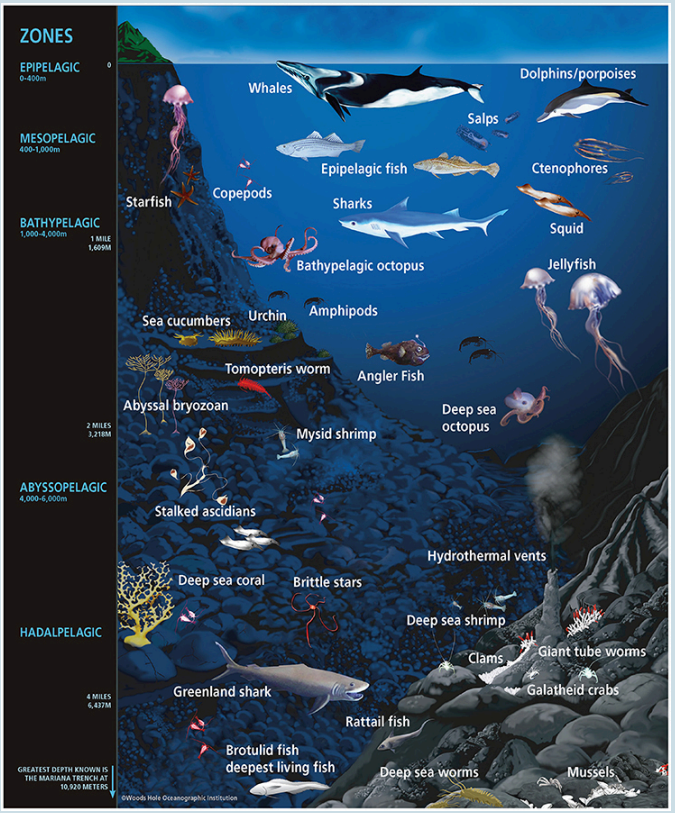


Figure 1. Illustration and common names of representative ocean animal life within their approximate depth-defined ecological habitats. Microorganisms exist on the surfaces and within the tissues and organs of the diverse life inhabiting the ocean, across all ocean habitats. Animals are not drawn to scale.

Marine animals share the sea with a vast diversity of microorganisms, including protists, bacteria, archaea, fungi, and viruses which comprise millions of cells in each milliliter of the 1.3 billion km^3 of water comprising the oceans (Eakins

and Sharman, 2010). These microorganisms are several micrometers or smaller in size, but collectively their roles in oxygen production, nutrient cycling, and organic matter degradation provide critical functions to the oceans and Earth (Arrigo, 2005; Falkowski et al., 2008). Microorganisms that associate with marine animals are part of the animal's microbiome, or collection of microorganisms that reside on or within the animal. Some of the microorganisms comprising the microbiomes of marine animals are thought to originate from this surrounding supply of seawater-associated cells (e.g., Nussbaumer et al., 2006), while other cells appear to have strict inheritance patterns, passed on through generations from the host (Sharp et al., 2007).

Over the past two decades, the widespread application of genomic and more integrative microbiological approaches have advanced our understanding of animal microbiomes (reviewed within McFall-Ngai et al., 2013). Symbiotic relationships between microorganisms and marine animals have been studied for decades, but technological advancements are providing new insights into the sheer diversity of microbial life in association with animals in the sea (Smith, 2001; Douglas, 2010). For example, reef-building corals are acknowledged as the icons of animal–microbial symbiosis in the sea, with corals hosting photosynthetic symbionts that make critical contributions to host nutrition (Muscatine et al., 1981). New reports of diverse protists, bacteria, archaea, and viruses in association with corals provide insights into the role of these cells for fulfilling diverse functional processes within the different niches of the coral host (reviewed within Thompson et al., 2015; Bourne et al., 2016). In fact, for many terrestrial animals, new reports of microbial symbioses provide

insights into the variety of genetic and biochemical interactions and the ways that microorganisms contribute to animal health, behavior, and ecology (e.g., Ley, 2010; Cho and Blaser, 2012).

Understanding the microbiomes of marine animals is a growing research area within the field of marine science. Currently, the science is heavily focused on identifying consistent or "core" microbial members of the microbiome (Shade and Handelsman, 2012). After first gaining an understanding of "who's there" generally using diversity-based surveys targeting the small subunit (SSU) ribosomal RNA (rRNA) gene, these microbiomes are then often examined as a whole or in smaller units to understand the function of the cells, the nature of the associations and ultimately gain insight into the role of the microbiome in animal health, physiology, ecology, and behavior (Ezenwa et al., 2012; McFall-Ngai et al., 2013). Additionally, the ocean environment is changing at unprecedented rates due to climate-related and anthropogenic-induced impacts (Halpern et al., 2008; Doney et al., 2012), and the microbiome is also being investigated for its possible role as a sentinel of a changing host (Ainsworth and Gates, 2016).

How environmental changes and animal life history events affect the microbiomes of marine animals is growing area of research, and there is an emerging focus on better understanding interactions between the animal, microbiome, and ocean environment, including the elements that may define their exchanges (e.g., Meron et al., 2011; Lesser et al., 2016; Webster et al., 2016). Therefore, this review considers the symbiosis and dissociated stages of animal–microbiome associations, and discusses factors and causes that may alter interactions between animals and

their microbiome. Next, this review discusses current research examining animal–microbiome relationships and interactions, by focusing on select systems that represent diverse marine animal phyla and which span the range of being more to less developed for microbiome research. Two of these systems, corals and marine mammals, are represented in my own research program. Lastly, this review concludes with a discussion of challenges in marine animal–microbiome research and opportunities available to further advance knowledge of animal–microbiome interactions in the ocean.

Conceptual Model of Factors Contributing to Host-Microbiome Interactions

Host–microbiome dynamics are generally described as falling into two main categories: symbiosis, in which the organisms are involved in a normal metabolic and immune signaling interactions, and secondly dysbiosis, in which the relationship or interactions are heavily altered, possibly related to a major stress or infection event. While host–microbiome symbiosis and dysbiosis has been mostly considered in humans and humanized models (Hamdi et al., 2011; Nicholson et al., 2012; Scharschmidt and Fischbach, 2013), many of the same concepts are applicable to organisms in the sea (Egan and Gardiner, 2016), and are being explored in various systems (discussed below). The exact factors and mechanisms tipping the scale between symbiosis and dysbiosis will probably vary with complexity of the host anatomy and immune functioning (e.g., simplistic sponges and corals compared to more complex fish and

sharks) as well as with the complexity of interactions that may occur between the members of the microbiome.

A normal animal–microbiome relationship in the ocean could be referred to as a "symbiotic" state, although the exact nature of the relationship may vary for each cell in the association. For example, cells residing on the surface or within the gut cavity of an animal are physically associated, yet do not share as intimate of an association as those microbes residing intracellularly with the host's cells. This normal symbiotic state is subject to a variety of environmental fluctuations, which are generally defined by the characteristics of the habitat (Figure 1). For example, in the ocean's upper photic zone, animals are exposed to variations in temperature and light, and host–symbiont interactions, especially in ectothermal animals, could alter on cycles such as seasons that generally control the temperature and light environment. Normal fluctuations in animal-specific patterns could also alter host–microbiome relationships. For example, changes in diet, possibly due to short-term prey availability, can alter gut microbiota and host–microbiome metabolic exchanges in other systems (e.g., David et al., 2014), and similar diet trends may also affect marine animals. Stress is another factor more complex animals encounter on a daily basis (e.g., squid, crabs, fish), which could be related to social/territorial encounters or chasing or fleeing from prey, and the short-term production of stress hormones such as cortisol can influence host–microbiome relationships (e.g., Moloney et al., 2014).

There are also normal animal life events that occur on longer time frames or that are more drastic in scope, such as animal development, aging, and reproduction. In non-

marine animals, these factors have been shown to cause alterations in animal-microbial relationships (e.g., Heintz and Mair, 2014). These changes can be drastic enough to cause a state of "altered symbiosis" that could extend for short or longer term. For example, the gut microbiome of women generally becomes altered during pregnancy (Koren et al., 2012). Events resulting in normal animal stress may also lead to a more altered symbiotic state, for example if social conflict was more chronic, perhaps due to the pressures of a particular habitat. Data from humans and humanized models suggests that the microbial community and associated genes do fluctuate with the normal variations and animal life events, and both may be considered "healthy" fluctuations (Nicholson et al., 2012). However, how these fluctuations affect exchanges between the host and microbiome is much less understood.

If symbiosis and altered symbiosis are considered as normal host–microbiome variations throughout an organism's life, dysbiosis is the breakdown in the relationship, generally related to one or more major stressors, and can greatly alter host health and lead to a disease state (Holmes et al., 2011). The stressor may come from an external source, such as a pollutant, infective agent, or a longer-term natural environmental change—and there are probably countless other factors that could fit this category (Figure 2). For example, one of the most visible signs of host–microbiome dysbiosis is with scleractinian corals, whose relationship with unicellular algae breaks down after long-term yet small increases in seawater temperature, causing the coral to become "bleached" (Brown, 1997). In humanized models, major stressors such as malnutrition are related to less physically visible changes

in innate immunity, which are linked to microbial ecology (Hashimoto et al., 2012). Understanding the relationship between symbiosis, dysbiosis and host health and functioning are general topics of research in most host–microbiome studies, but the environmental changes occurring in the ocean environment have made this area of research more pressing for marine animals. Overall, the concepts behind the model presented in Figure 2, as well as variations of this model, are generally driving much of the current research examining animal-microbial relationships in the ocean.

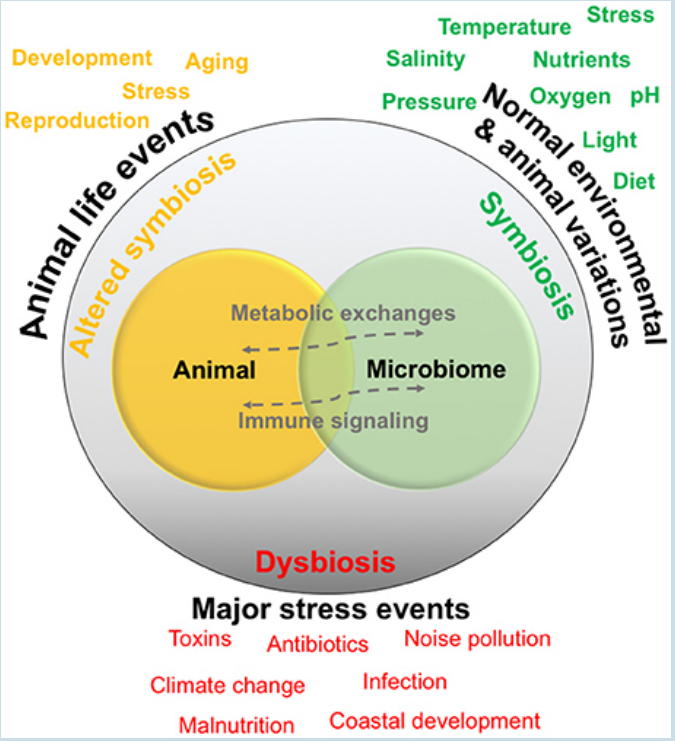


Figure 2. Conceptual diagram of a host-microbiome relationship. Relationships are generally thought to exist in a symbiotic state, and are normally exposed to environmental and animal-specific factors that may cause natural variations. Some events may change the relationship into a functioning but altered symbiotic state, whereas extreme stress events may cause dysbiosis or a breakdown of the relationship and interactions.

Overview of Diverse and Emerging Animal-Microbiome Study Systems

The microbiomes of diverse marine animals are currently

under study, from simplistic organisms including sponges (e.g., Webster et al., 2010) and ctenophores (Daniels and Breitbart, 2012) to more complex organisms such as sea squirts (Blasiak et al., 2014) and sharks (Givens et al., 2015). Below I present some of the current study systems that represent a diverse cross-section of marine animal phyla, and trends of research in these systems including focus on symbiosis and dysbiosis. The organisms are generally presented in order from increasing to decreasing knowledge about the host-microbiome relationship.

The relationship between the Hawaiian bobtail squid *Euprymna scolopes* (phylum Mollusca) and the bioluminescent bacterium *Vibrio fisheri* (also recognized as *Aliivibrio fisheri*) is one of the best studied symbiotic relationships in the sea and is a choice system for general symbiosis research (Figures 3A,B). The *E. scolopes*-*V. fisheri* relationship has provided insight into fundamental processes in animal-microbial symbioses, and especially biochemical interactions and signaling between the host and bacterium (McFall-Ngai, 2000, 2014). Much of this research focuses on establishment of the symbiosis, with less focus on dysbiosis. Additionally, because *V. fisheri* exists in the light organ, these studies have been primarily limited to this one isolated relationship, with the remainder of the squid's microbiome virtually unstudied (but see Barbieri et al., 2001; Collins et al., 2012). The *E. scolopes*–*V. fisheri* system offers simplicity for the study of host–microbial interactions and numerous helpful developments in animal husbandry, genomic tools, and experimental design that could be applied to ask more comprehensive questions about squid–microbiome

interactions, including the conditions leading to dysbiosis of relationships.

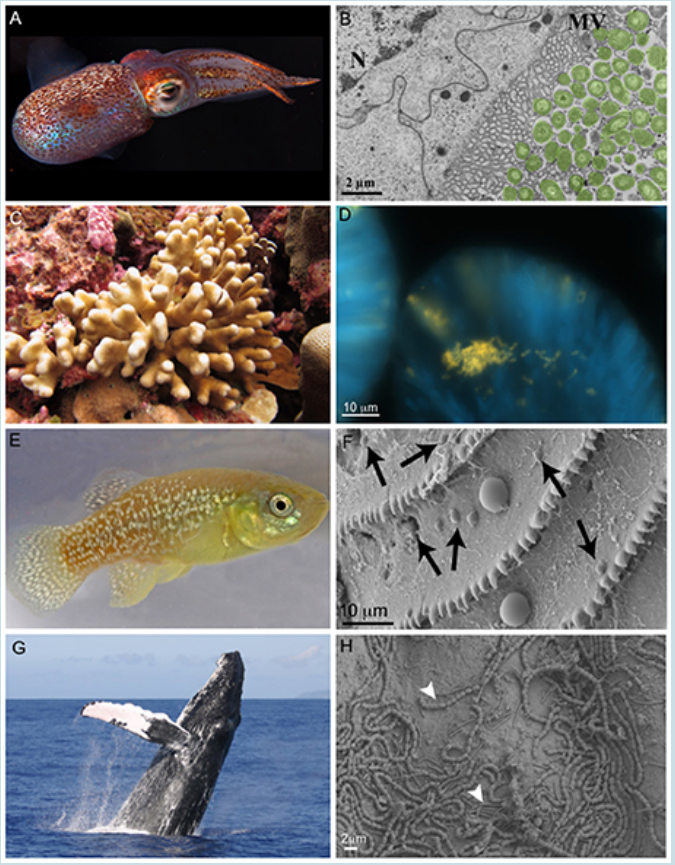


Figure 3. Photographs of marine animals and their associated microbiomes from select study systems. Photographs include: the Hawaiian bobtail squid Euprymna scolopes (A) and a transmission electron micrograph of Vibrio fisheri cells associating with dense microvilli (MV) and in proximity to the epithelial nucleus (N) within the light organ (B); the reef-building coral Stylophora pistillata (C) and a microscopy image of Endozoicomonas cells (probed yellow using in situ hybridization) within the tentacles of a S. pistillata host (D); the Atlantic killifish (Fundulus heteroclitus) (E) and a scanning

electron microscopy (SEM) image of the surface and scales of the fish, with arrows pointing to bacterial-sized cells and larger cells (which are not noted) are presumably phytoplankton (F); a humpback whale (Megaptera novaeangliae) breaching (G) and a scanning electron microscopy image of a humpback's skin surface associated bacteria, with arrows indicating two different cell morphologies (H). Photographs (A,B) were produced by M. McFall-Ngai and were previously published photographs (McFall-Ngai, 2014), (C,H) were previously published by the author Neave et al. (2016) and Apprill et al. (2014), photograph (D) was taken by Liping Xun and photograph (E) by Evan D'Alessandro.

Similar to *E. scolope*, the gutless marine oligochaete worm *Olavius algarvensis* (phylum Annelida) is another relatively well-studied marine host to microbes. One major difference is that it has been studied within the context of a larger consortium of microorganisms compared to *E. scolope*. These 3 cm long worms reside within shallow marine sediments of the Mediterranean Sea. The worms do not contain a mouth or a digestive or excretory system, but are instead nourished with the help of a suite of extracellular bacterial endosymbionts that reside upon coordinated use of sulfur present in the environment (Dubilier et al., 2001). This system has benefited from some of the most sophisticated 'omics and visualization tools (Woyke et al., 2006). For example, multi-labeled probing has improved visualization of the microbiome (Schimak et al., 2016) and transcriptomics and proteomics have been applied to examine host–microbiome interactions, including energy transfer between the host and microbes (Kleiner et al., 2012) and recognition of the consortia by the worm's innate immune system (Wippler et al., 2016). The major strength of this system is that it does offer the ability to

study host–microbiome interactions with a low diversity microbial consortium, and it also offers a number of host and microbial genomic resources (e.g., Woyke et al., 2006; Ruehland et al., 2008). Dysbiosis has not been heavily investigated in this system, and given the growing knowledge of host–microbial interactions, *O. algarvensis* could be an imperative animal for dysbiosis research.

As mentioned above, corals (phylum Cnidaria) (Figure 3C) are one of the most common examples of an animal host whose symbiosis with microalgae can turn to dysbiosis, and is visibly detected as bleaching. Coral microbiomes have been examined in a variety of studies, which demonstrate how variations in the ocean environment, most notably temperature, light, and inorganic nutrients, affect the abundance and performance of the microalgal symbionts, as well as calcification and physiology of the host (Dubinsky and Jokiel, 1994; Anthony et al., 2008). Studies have also suggested that resident bacteria, archaea, and fungi additionally contribute to nutrient and organic matter cycling within the coral, with viruses also possibly playing a role in structuring the composition of these members, thus providing one of the first glimpses at a multi-domain marine animal symbiosis (reviewed in Bourne et al., 2016). The gammaproteobacterium *Endozoicomonas* is emerging as a central member of the coral's microbiome, with flexibility in its lifestyle (Figure 3D) (Neave et al., 2016, 2017). Ocean disturbances including elevated temperature and ocean acidification have been shown to disrupt the coral's associated bacteria (Thurber et al., 2009; Meron et al., 2011), including relationships with *Endozoicomonas* (Morrow et al., 2015). However, some members of this microbiome appear

to be stable across large environmental gradients (Hernandez-Agreda et al., 2016). In addition to nutrition, the microbiome plays a role in coral health and stress. Temperature and light stress to corals can result in overproduction of reactive oxygen species (ROS), which can be detrimental to *Symbiodinium* and result in bleaching, but the associated bacteria have also recently been shown to contribute extracellular ROS (Diaz et al., 2016; Zhang et al., 2016), which could play a signaling role with the host or within the microbiome. Given the recent mass bleaching occurring on reefs (Hughes et al., 2017), corals will likely continue to be a useful and popular system for symbiosis and dysbiosis research. There are number of resources available to further promote study of the coral microbiome, including integrated databases (Franklin et al., 2012; Madin et al., 2016), a growing number of host and microbial genomes (Shinzato et al., 2011; Bayer et al., 2012; Neave et al., 2017), and laboratory amendable "model" systems (Weis et al., 2008; Baumgarten et al., 2015).

Sponges (phylum Porifera) are common members of the ocean's diverse benthic habitats and their abundance and ability to filter large volumes of seawater have led to the awareness that these organisms play critical roles in influencing benthic and pelagic processes in the ocean (Bell, 2008). They are one of the oldest lineages of animals, and have a relatively simple body plan that commonly associates with bacteria, archaea, algal protists, fungi, and viruses (reviewed within Webster and Thomas, 2016). Sponge microbiomes are composed of specialists and generalists, and complexity of their microbiome appears to be shaped by host phylogeny (Thomas et al., 2016). Studies have shown that the sponge microbiome contributes to nitrogen cycling

in the oceans, especially through the oxidation of ammonia by archaea and bacteria (Bayer et al., 2008; Radax et al., 2012). Most recently, microbial symbionts of tropical sponges were shown to produce and store polyphosphate granules (Zhang et al., 2015), perhaps enabling the host to survive periods of phosphate depletion in oligotrophic marine environments (Colman, 2015). The microbiomes of some sponge species do appear to change in community structure in response to changing environmental conditions, including temperature (Simister et al., 2012a) and ocean acidification (Morrow et al., 2015; Ribes et al., 2016), as well as synergistic impacts (Lesser et al., 2016). Understanding the effect of these altered host–microbiome interactions on sponge growth and ecology are topics for further research. As such, there are a number of resources to support research on sponges including a curated database of sponge–microbial sequences (Simister et al., 2012b), cultivated microbial isolates and sponge cell cultures from some species (Taylor et al., 2007) to facilitate investigations.

Atlantic killifish, (*Fundulus* spp., Phylum Chordata) (Figure 3E) are one of the most abundant estuarine fishes in North America, and are related to other families with more global distributions in coastal areas (Fritz et al., 1975; Lotrich, 1975). The killifish have a broad North American geographic distribution yet limited subpopulation movement, and thus the Atlantic killifish have become a useful field-residing model species for examining biological and ecological responses to natural environment conditions (salinity, oxygen, pH, and temperature) as well as chemical pollutants (Burnett et al., 2007). While the killifish microbiome (Figure 3F) has not been extensively studied,

there is work examining the influence of pollutants on the skin and mucus of the fish, which suggests that this skin microbial community is relatively resistant to change (Larsen et al., 2015). Populations of the fish offer a unique host genetic resistance to toxicity (Hahn et al., 2004), and it is possible that this resistance is also facilitated by features of the microbiome. The Atlantic killifish appear to be an ideal study species for microbiome investigations and especially the response of the host–microbiome symbiosis to changing ocean conditions. Specifically, the killifish can be maintained in laboratory aquaria, they are hardy and amendable to experimental manipulation, and spawning material can be acquired for developmental (Burnett et al., 2007).

The microbiomes of marine mammals (phylum Chordata) (Figure 3G) have recently been investigated and offer a comparative study system to terrestrial mammals (reviewed within Nelson et al., 2015). Marine mammals are often viewed as sentinel species of the ocean, because they appear to rapidly respond to ocean conditions, disturbances, and pathogens similarly to humans (Bossart, 2011). Several studies have examined the skin (Figure 3H), gut and respiratory microbiomes of diverse marine mammal species, and describe species-specific relationships (Johnson et al., 2009; Apprill et al., 2014; Bik et al., 2016). Connections between the community composition of the microbiome and animal health (Apprill et al., 2014) and diet (Nelson et al., 2013; Sanders et al., 2015) have been made, and more detailed studies are needed to understand these specific connections. While there are very limited resources available for studying host–microbiome interactions in marine mammals, there are some animals in captivity as well as well-studied populations that will heighten investigations

of host–microbiome symbiosis and dysbiosis in these sentinel species.

Challenges and Emerging Solutions to Studying Animal–Microbiome Interactions

A number of the systems highlighted above are currently examining animal–microbiome interactions, but these are generally most developed in systems such as *O. algarvensis* that offer lower complexity microbiomes, or within the single host–symbiont relationship between *E. scolopes* and *V. fisheri*. As such, a major challenge to the field is exploring host–microbiome interactions within the context of a diverse microbiome, and especially if the microbiome includes members such a uncharacterized protists, fungi, and viruses, which have generally not been described in most marine animal systems. Therefore, a through description of the microbiome is a first necessity, but this still presents many challenges on a variety of levels. For example, amplifying or shotgun sequencing microbial DNA with the presence of abundant host cells often requires optimization or high sequencing output (e.g., Rocha et al., 2014; Weber et al., 2017). Taxonomic databases generally contain few microbial sequences from many of these animals, and therefore simple tasks such as assigning taxonomy can be challenging. Developing animal-specific databases (Simister et al., 2012b), which include the next-generation supplied sequences generally not available in curated taxonomic databases, could help alleviate this problem. There are also a number of new tools for metagenomics-based analysis, including advancements in binning genomes from complex samples (Kang et al.,

2015; Graham et al., 2017) as well as new visualization methods for comparing genomes (Eren et al., 2015; Wagner et al., 2017). A challenging issue that has received less attention is how to gain information from unknown genes and gene families, which can make up over half of the environmental microbial genomes. Algorithms utilizing gene function predictions do provide some assistance with this problem (Mi et al., 2015), and these tools may improve as more environmental microbial genomes are available. Lastly, computational tools are emerging to facilitate identifying associations between host genetic variation and microbiome composition (Lynch et al., 2016).

Once some of these hurdles are overcome and a comprehensive view of the microbiome is available, researchers can then explore the nature of the host–microbiome relationship. Visualization using a variety of different microscopy-based techniques is a powerful tool to recognize the physical relationship between a host and the microbiome, as well as the organization of cells within the microbiome. Electron microscopy provides the most detailed information about this organization, but this is less useful for complex microbiomes because taxonomically distinct microbial cells with similar appearances cannot be distinguished. Fluorescent *in situ* hybridization (FISH), and especially using a multi-taxonomic, simultaneous probing technique such as Combinatorial Labeling and Spectral Imaging FISH (CLASI-FISH) (Valm et al., 2011) can provide significant insight into host–microbe and microbe–microbe interactions. FISH techniques do require optimization for some animal systems, such as corals that possess autofluorescent host tissues (Wada et al., 2016). Visualization techniques can also be paired with isotope probing, to

provide opportunities to trace the transfer of specific molecules between the host and microbiome, as well as within the microbiome using Nano-SIMS and Nano-SIP approaches (Musat et al., 2016). There have also been many recent instrumental and database advances in the field of metabolomics (Beisken et al., 2015), and this approach is beginning to be applied to examine host–microbiome interactions (Gomez et al., 2015; Sogin et al., 2016). An understanding of specific microbial metabolites will help facilitate targeted investigations of how these products affect the host nutritional and immune systems.

Lastly, experimental manipulation is a challenge to the study of host–microbial interactions in the ocean. Studying the animals in their natural environment is the most ideal approach because it ensures that the surrounding seawater microbial community is maintained. However, natural experiments are only as helpful as the natural variability in the host-microbe system, and generally only afford the opportunity to study events such as seasonality, animal growth or other life history events. Artificial systems such as aquaria or mesocosms offer opportunities to manipulate environmental conditions or expose the animal to antibiotics or other molecules that are difficult to dose in the wild. However, not all animals are ideal for these systems (e.g., large whales, hydrothermal vent worms), and it can be challenging to reproduce some environmental conditions. Advances in aquaria design that offer consistency in environmental conditions and the ability to manipulate complex environmental interactions, such as the Australian Institute of Marine Science's National Sea Simulator, provide opportunities to conduct more realistic experiments. As the need to understand how host–microbiome interactions will

alter with the forecasted changes in ocean temperature and pH, facilities such as this will become critical to animal–microbiome research in the ocean.

While studies of marine animal–microbiome interactions are certainly plagued by a number of challenges, the future is also very bright for this emerging field. Many of the new bioinformatics and methodological advancements now available to marine biologists stem from the biomedical field, and thus marine animal microbiome research, as well as other environmental-based fields, are profiting from the elevation in microbiome research funding and attention. There could also be growing interest in using marine animals as models for examining resilience, promoted by the fact that alterations in the ocean conditions are often outpacing those in terrestrial environments. Given the phylogenetic breath of animals in the ocean, coupled with the many diverse ocean environments, there is certainly a wealth of research opportunities available to study host–microbiome interactions in the ocean.

Author Contributions

The author confirms being the sole contributor of this work and approved it for publication.

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Conflict of Interest Statement

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Studying Ocean Microbiomes Data

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https://uta.pressbooks.pub/microbiomeshealthandtheenvironment/?p=713#h5p-10

Prevalence of certain marine topics appearing in combination with the term holobiont in the title, abstract, and/or keywords of scientific publications from 1990 to February 2021 (N = 1269). Bars indicate the percentage of publications that contain terms related to a certain marine topic (cnidarians, corals, sponges, etc.). It is important to note that the different topics may overlap in several

publications. For example, many publications treat of the topics of coral and sponge holobionts together. Data retrieved from the curated citation and abstract database Scopus on the date 10/03/2021. (Stevenne et al., 2021)

Check your Understanding

- Which environmental factor is the biggest driver of change for the community composition of free-living oceanic microbes?
- What factors affect the normal symbiotic state between marine host and their microbiome?
- Describe one well-studied marine animal-microbial symbiosis and its importance.
- What are some challenges to studying marine host-associated microbiomes?

Media Attributions

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- Figure 3 – Photographs of marine animals and their associated microbiomes from select study systems by Apprill, 2017. Licensed under the terms of the Creative Commons Attribution License (CC BY).

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13. Soil Microbiomes

Soil Microbiomes

The terrestrial landscape on Earth harbors countless microbes that have major responsibilities in shaping their surrounding environment. One gram of soil can contain up to 10 billion microorganisms and consist of thousands of different species. Each ecosystem has unique soil properties that cultivate a diverse array of microbial communities, which are primarily composed of bacteria, however archaea, protists, fungi, viruses, and other microscopic organisms can be found in varying abundances too. The composition and health of the surrounding environment and its macro inhabitants are dependent on their soil borne microbial partners, and a disturbance in the balance of the soil microbiome can have far-reaching effects (Omotayo and Babalola, 2021).

Environmental and agricultural sustainability is important in a number of regards, especially maintaining biodiversity, plant and animal health, ecosystem homeostasis, and even human health. Anthropogenic effects on the climate and environment have become abundantly apparent, and it is imperative to adjust mindsets to become more environmentally conscious, not only to preserve aspects of nature, but to accommodate an increasing human population. The soil is foundational to agricultural practices, and therefore food production, and so a better understanding of various soil microbiomes could serve to benefit future applications and practices (Tosi et al., 2020). One possibility, is to become less reliant on synthetic nitrogen fertilizers for soil, and utilize symbiotic nitrogen-fixing bacteria as sources for plant nitrogen.

One or more interactive elements has been excluded from this version of the text. You can view them online here: https://uta.pressbooks.pub/microbiomeshealthandtheenvironment/?p=717#oembed-1

Microbe to Microbiome: A Paradigm Shift in the Application of Microorganisms for Sustainable Agriculture

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Light, water and healthy soil are three essential natural resources required for agricultural productivity. Industrialization of agriculture has resulted in intensification of cropping practices using enormous amounts of chemical pesticides and fertilizers that damage these natural resources. Therefore, there is a need to embrace agriculture practices that do not depend on greater use of fertilizers and water to meet the growing demand of global food requirements. Plants and soil harbor millions of microorganisms, which collectively form a microbial community known as the microbiome. An effective microbiome can offer benefits to its host, including plant growth promotion, nutrient use efficiency, and control

of pests and phytopathogens. Therefore, there is an immediate need to bring functional potential of plant-associated microbiome and its innovation into crop production. In addition to that, new scientific methodologies that can track the nutrient flux through the plant, its resident microbiome and surrounding soil, will offer new opportunities for the design of more efficient microbial consortia design. It is now increasingly acknowledged that the diversity of a microbial inoculum is as important as its plant growth promoting ability. Not surprisingly, outcomes from such plant and soil microbiome studies have resulted in a paradigm shift away from single, specific soil microbes to a more holistic microbiome approach for enhancing crop productivity and the restoration of soil health. Herein, we have reviewed this paradigm shift and discussed various aspects of benign microbiome-based approaches for sustainable agriculture.

Introduction

The health of soil plays an essential role in the ability of plants to produce food, fuel, and fiber for a growing world population. To keep pace, the total area of cultivated land worldwide has increased over 500% in the last five decades with a 700% increase in fertilizer use and a several-fold increase in pesticide use (Banerjee et al., 2019). In addition to being the world's largest agricultural producers and exporters, the EU, Brazil, United States, and China also are some of the world's largest pesticide users – each using 827 million, 831 million, 1.2 billion, and 3.9 billion pounds of pesticides, respectively, in 2016 (Donley, 2019). However, these numbers are not sustainable from either a supply-

chain or environmental perspective. Thus, because natural resources are limited and their overuse pollutes the environment, the continued use of fertilizers and water to meet the demand of future global food requirements is not sustainable. Of relevance here is that agricultural intensification with high resource use and low crop diversity can negatively affect soil- and plant-associated microbiota (the so-called "phytobiome") with subsequent impacts on critical ecosystem services (Matson et al., 1997).

There is growing evidence that aboveground plant diversity supports belowground microbial biodiversity, primarily through root exudation and rhizo-deposition (Bais et al., 2006; Eisenhauer et al., 2017; Morella et al., 2020). These more simple carbohydrates released into the soil primarily feed bacteria (Gunina and Kuzyakov, 2015) and are the most abundant near the root surface and diffuse along a gradient as distance from the root increases (Gao et al., 2011). The microbial composition is more abundant and complex in the rhizosphere, the narrow zone surrounding plant roots, with up to 10^9 cells per gram in typical rhizospheric soil, comprising up to 10^6 taxa (Lakshmanan et al., 2017). The more complex carbohydrates (e.g., lignin, cellulose) are largely degraded by decomposer fungi that break down these recalcitrant compounds into forms that can be used by other microbes. This conversion is largely decoupled from conventional agricultural practices, wherein the organic matter content is often lost to the system (Craven and Ray, 2019), and the carbon flux is at least partially unregulated in this regard. Again, defining nutrient fluxes with techniques like Stable Isotope Labeling (SIP) holds great potential to define and construct resilient, functioning and beneficial microbiomes that can contribute

to future holistic agriculture. Thus, applying an efficient and diverse soil microbiome backed by these new technologies can facilitate and promote sustainable agriculture and can effectively contribute to meet the triple requirements of economic, social and environmental sustainability (Ray and Craven, 2016).

Historically, microorganisms that promote plant growth and nutrient acquisition have been used largely as single strains in agriculture to offset such fertilizer inputs as nitrogen and phosphorous. However, studies of natural populations suggest that groups of microbes with distinct function niches play pivotal roles in adhering and desorbing inorganic nutrients to physical surfaces, as well as breaking down organic residues and incorporating them into the soil (Lakshmanan et al., 2014; Finkel et al., 2017; Kumar and Dubey, 2020). Conceptually, such observations support the idea of the microbiome as a second genome or an extended genome of the plant (Vandenkoornhuyse et al., 2015). It is now evident that improving plant performance in a sustainable manner is beyond the binary interaction between a specific microbe or a consortium of beneficial microbes and a targeted host plant. This is a much more complex set of interactions than previously thought that requires modeling for improving predictable outcomes. In this review, we will highlight the current state of the art for the incorporation of specific plant growth-promoting microorganisms and discuss the principles and management practices for next-generation, microbiome-based approaches for sustainable agriculture.

Application of Beneficial Microbes in Sustainable

Agriculture: Past, Present and Future

Since the early 1800s, the United States Department of Agriculture has recommended the use of certain rhizobacteria to improve nitrogen fertility in leguminous crops (Schneider, 1892). Since that time, a great deal of research has been conducted on this relationship between legumes and these bacteria, now termed rhizobia, that inhabit unique structures, the nodules, that form on the roots. Rhizobia infecting these nodules are now capable of "biological nitrogen fixation," whereby di-nitrogen is fixed into forms that can be used by the plant. Symbiotically, the bacteria trade these nitrogenous compounds to the host plant in exchange for photosynthetically derived carbon. Despite these limited applications, much remains to be learned regarding both the functional and taxonomic diversity of these symbiotic bacteria and their host plants, the role they play in the global nitrogen cycle, and ultimately, how they can best be harnessed for improving plant productivity. This is particularly true for marginal lands that are not suited for row crop production but will need to be incorporated into global food and forage production approaches moving forward. Further, such degraded lands must but regenerated with the goal of restoring soil health and productivity. Any successful endeavor in this regard must include a characterization of the soil microbiome, both taxonomically and functionally. Attempts currently are underway to fix nitrogen in such non-legumes as wheat, corn and other staple crops that produce the bulk of human food by engineering symbiotic relationships using synthetic biology approaches (Rogers

and Oldroyd, 2014; Ryu et al., 2020). Such approaches would significantly impact global food supplies, and may function adequately to reduce the arable land required to meet productivity goals.

Plant growth-promoting microbes not only play critical and diverse roles in growth promotion *per se*, but also in improving various aspects of plant resilience against a wide array of biotic and abiotic stresses (Arnold et al., 2003; Sun et al., 2010; Agler et al., 2016; Azad and Kaminskyj, 2016; Singh, 2016; Oleńska et al., 2020; Rai et al., 2020). In this context, researchers globally have worked over the last several decades on plant growth-promoting microorganisms, such as root-associated mycorrhizal fungi, across a broad range of crops and encompassing a wide range of agro-climatic conditions. For perspective, Brundrett and Tedersoo (2018) recently reviewed 135 years of mycorrhizal research and reported that merely 8% of the vascular plants are non-mycorrhizal, suggesting that plant families associating with mycorrhizae have been very successful over the evolution of the plant kingdom.

Traditionally, agricultural application of beneficial microorganisms involves a few types of well-characterized microbes, such as mycorrhizal fungi or rhizobia bacteria, for which the mechanisms underlying the plant growth promotion effects are well understood. Further, most of these studies focused solely on the ability of the applied microorganisms to facilitate such specific plant growth-promoting traits as phosphate solubilization, nitrogen fixation, ACC deaminase production (Sarkar et al., 2018), siderophore production, biofilm formation, plant hormone production, biotic, and abiotic stress tolerance or

resistance, among others (Weyens et al., 2009; Bhattacharyya and Jha, 2012; Singh et al., 2019). While these beneficial microorganisms can impart considerable benefits to plant growth and fitness, they are typically documented in simple, one-on-one studies, often conducted in sterile soils in greenhouse conditions. As a consequence, the effects found in such simplified conditions often fail to translate to more complex field situations (Chutia et al., 2007; Nicot et al., 2011; Parnell et al., 2016). Soil in field plots have more complex microbial environments that are presumably adapted to the local eco-environment.

In recent years, next-generation sequencing has revolutionized our understanding of microbial community composition and function, and together with improved culturing methodologies has greatly facilitated the use of biologicals in the field (Schweitzer et al., 2008; Panke-Buisse et al., 2014; Mueller and Sachs, 2015). Specifically, metagenomics-based approaches have uncovered vast, previously unrecognized populations of microbes that may have new or enhanced properties that could be used for agriculture, bioremediation, and human health. For example, comparative analyses of rhizosphere metagenomes from resistant and susceptible tomato plants enabled the identification and assembly of a flavobacterial genome that was far more abundant in the resistant plant rhizosphere microbiome than in that of the susceptible plants. Such findings certainly reveal a role for native microbiota in protecting plants from phytopathogens, and pave a way forward for the development of probiotics to ameliorate plant diseases akin to human health (Kwak et al., 2018). In another study, a 16S rRNA gene amplicon sequencing analysis of maize root microbiome led to the identification

of bacteria that promote growth under low temperature conditions (Beirinckx et al., 2020). Additionally, principles of consortium design that rely on cross-talk, cross-feeding and/or substrate channeling between different microorganisms offer new opportunities for "intelligent" consortia design (Calvo et al., 2014; Vorholt et al., 2017; Paredes et al., 2018). We propose that the manipulation of the plant microbiome holds tremendous potential for agricultural improvement (Table 1). Through recent years of research, it is elucidated how microbes worked in nature before, and how decades of chemical fertilizer use have silenced their ability to improve plant fitness and soil health. Therefore, designing a microbial consortium that carefully weighs and evaluates the relationship between inoculants and the resident microbiome would substantially improve the plant growth-promoting potential and resilience of agricultural biologicals to boost plant growth. In this review, we will discuss the key considerations that would improve the likelihood of microbial products to improve crop yield, decrease disease severity and/or ameliorate abiotic stress response. Further, it is likely that such considerations would reduce the inconsistency between the performances of beneficial microbes from controlled greenhouse conditions and more natural environments.

Category	Salient findings	Reference
Plant growth promotion	Chilling temperatures critically affects growth of Maize in N. hemisphere. This study reported enrichment of *Comamonadaceae* and the *Pseudomonadaceae* in the root endosphere of maize grown under chilling conditions. Additionally two bacterial strains were identified from the root endosphere that could boost maize growth under chilling conditions.	Beirinckx et al., 2020
	A root endophytic bacteria Sphingomonas sp. Cra20 improved growth of Arabidopsis thaliana under drought stress by stimulating the growth of lateral roots and root hairs. Additionally, the relative abundance of Sphingomonas increased in the rhizosphere bacterial community in the water-deficit treatment, suggesting the role of Sphingomonas sp. Cra20 in alleviating drought induced stress.	Luo et al., 2019
	A community-based culture collection (CBC) approach was undertaken to isolate bacteria from the stalks and rhizosphere of Sugarcane. Subsequently, a synthetic community was designed by cross-referencing the CBC with the sugarcane microbiome profile that comprised of highly abundant bacterial groups from roots and stalks. The synthetic community could successfully improve the biomass of sugarcane, and was found to displace the native rhizosphere bacteria community.	Armanhi et al., 2018
	Willows (Salix spp.) were grown in gamma-irradiated petroleum-contaminated soils. Plants were inoculated with contaminated rhizosphere soil from a willow that grew well, or with contaminated bulk soil in which the plants had died. Willows inoculated with bulk soil performed better than those inoculated with rhizosphere soil. Microbiomes of different treatments were divergent at the beginning, but had converged at the end of the study, suggesting lasting effect of inoculated microbiome on plant growth, but not on the rhizosphere microbiome.	Yergeau et al., 2015
Plant defense response	Microbiome structure of Banana endosphere in the roots and shoot tips were investigated during plant growth and wilting processes. The keystone bacterial species belonging to the family *Enterobacteriaceae* family were isolated and further engineered to express ACC deaminase. Plants inoculated with engineered *Enterobacteriaceae* strains increased resistance to the Fusarium wilt disease. The findings illustrate that the keystone species in the banana microbiome plays functional role in the wilt resistance.	Liu et al., 2019
	Composts represent a sustainable way to suppress diseases and improve plant growth. Compost derived microbial communities enriched in the rhizosphere of Tomato were analyzed for antifungal activity against soil-borne fungal pathogens. Subsequently, microbial synthetic communities (SynComs) were designed with an overarching aim to improve plant fitness. SynComs were found to promote tomato growth as well as suppressed Fusarium wilt symptoms in controlled conditions.	Tsolakidou et al., 2019
	Tomato variety Hawaii 7996 is resistant to the soil-borne pathogen *Ralstonia solanacearum*, whereas the Moneymaker variety is susceptible to the pathogen. Rhizosphere microbiome analysis revealed clear differences in community profile of these two varieties. Transplantation of rhizosphere microbiota from resistant plants suppressed disease symptoms in susceptible plants. Additionally, a flavobacterium strain isolated from resistant plant rhizosphere microbiome was found to suppress R. solanacearum in susceptible plants.	Kwak et al., 2018
	A simplified synthetic bacterial community based on maize rhizosphere microbiome was designed representing most dominant phyla to study their functional attributes in maize seedlings. This synthetic community inhibited the phytopathogenic fungus *Fusarium verticilloides*, both *in planta* and *in vitro*. This study indicates how community profile information can be utilized to design beneficial microbial consortia for improving plant fitness and productivity	Niu et al., 2017
Abiotic stress response and nutrient use efficiency	Recognition of microbes by plant immune system mediated by phosphate stress was investigated. A representative synthetic bacterial community (SynCom) was designed that comprised of 35 bacteria isolated from the roots of Brassicaceae. SynCom enhanced the activity of PHR1, the master transcriptional regulator of the PSR, in Arabidopsis thaliana grown under limited phosphate. Additionally PHR1, repressed plant's immune system in phosphorous starved regime, validating plant's prioritization of nutritional stress over defense.	Castrillo et al., 2017
	Nitrogen-use efficiency of indica varieties of rice is superior to that of japonica varieties. Root microbiome analysis of these two varieties revealed that microbiota of indica and japonica highly distinct. Further, it was found that this distinctness was associated with a rice nitrate transporter and sensor NRT1.1B. Based on microbiome analysis, microbial synthetic communities (SynComs) were designed. It was found that indica-enriched SynCom improved rice growth in organic nitrogen conditions compared with a japonica-enriched SynCom.	Zhang et al., 2019
	Root associated microbiome of drought-sensitive pepper plant (*Capsicum annuum* L.) were analyzed focusing on role of microbes conferring plant growth under water limitation. Subsequently pepper root associated culturable bacteria were isolated and evaluated for plant growth promotion and drought tolerance abilities. The composition of the cultivable community associated to rhizosphere and root surrounding soil fractions shared a high similarity. Most of these isolates were able to promote plant growth and alleviate drought-induced stress with enhanced abilities observed in *Bacillus* and the *Rhizobacteria* strains.	Marasco et al., 2012
	Community profiling microbiome associated with superior halo-tolerant seepweed Suaeda salsa revealed that rhizospheric and endophytic bacterial community were enriched in genes responsible for salt stress acclimatization. This suggest that S. salsa preferentially recruit halotolerant taxa to confront soil salinity. Based on root endosphere core microbiota, halotolerant bacterial and fungal strains belonging to Pseudomonadales and Montagnulaceae were isolated. It was demonstrated that these core microbiome members were successfully able to improve growth and salt tolerance in the non-host rice plant.	Yuan et al., 2016

Table 1. List of recent publications in plant and soil microbiome focusing on plant fitness and productivity.

Microbes for Plant Growth Promotion: A Reductionist Approach

Sustainable agriculture primarily focuses on reducing the dependency of plants on chemical fertilizers and improving

their ability to grow on marginal soil types. For such purposes, individual microorganisms for plant growth-promotion have largely focused on those that facilitate growth and development by enhancing acquisition of nutrient resources from the environment, including fixed nitrogen, iron and phosphate, or modulating growth by altering plant hormone levels (Figure 1) (Hayat et al., 2010). Another approach aimed at reducing yield losses to disease relies on microbes that decrease or prevent the deleterious effects of plant pathogens by several different mechanisms (Glick, 2012), i.e., by acting as a biocontrol agent. Microbe-based plant growth-promoting products, more popularly marketed as biofertilizer, has been commercially available in many countries since the 1950s (Timmusk et al., 2017). Application of such plant growth-promoting microbes in agricultural context and more specifically as inoculants has been nicely reviewed by Souza et al. (2015). However, under certain cases, the results obtained in the laboratory could not be reproduced in the field primarily due to the presence of many crop species and crop varieties, variable environmental conditions between fields, (Timmusk et al., 2017; Saad et al., 2020), occasionally due to the low quality of the inocula, and their inability to compete with the indigenous population. In that context, it is important to consider the fact that there is always greater likelihood of success by introducing mixed cultures of compatible microorganisms, rather than single, pure cultures. This is simply because each strain in the multi-strain consortium can compete effectively with the indigenous rhizosphere population and enhance plant growth with its partners. For example, sequential inoculation of nitrogen fixing bacterium *Azotobacter vinelandii*, followed by plant growth-

promoting root-endophytic fungus *Serendipita indica* demonstrated better growth in rice (Dabral et al., 2020). Dual inoculation of *S. indica* and *Mycolicibacterium* strains boosted the beneficial effects in tomato (del Barrio-Duque et al., 2019) and that of arbuscular mycorrhizal fungus with plant growth-promoting bacteria *Bacillus subtilis* demonstrated better growth in wheat (Yadav et al., 2020) as compared to the singly inoculated plants. There also are numerous other reports that showed two strains used in a consortium promoted plant growth in a more effective manner (Nadeem et al., 2013; Fatnassi et al., 2015; Priyadharsini and Muthukumar, 2016). Nevertheless, to unlock the full potential of soil microbes for such nutrient cycling as nitrogen or phosphorus and providing plant protection against biotic and abiotic stress microbiomes, it is necessary to develop strategies to comprehend the functional capabilities of soil microbial communities. Irrespective of the approach, persistence is the first and foremost principle underlying the design of a successful microbial consortium for conferring plant growth promotion. This is not surprising, as the survival and activity of microbes in any soil system face a monumental task of competing with the myriad of microbes naturally adapted to that same soil. Thus, in addition to establishment of a compatible interaction with the host, a successful microbial inoculant has to subsequently compete and persist in the context of indigenous microbes as well as local abiotic conditions (Finkel et al., 2017). It has been reported that bacterial inoculations can persist in soil up to 7 weeks, but whether this inoculum also can provide plant growth benefits is not clear (Schreiter et al., 2014). While persistence or resilience

of any microbial inoculum is more dependent on biotic components of a specific soil type, their persistence can be improved by inoculating crops with consortia rather than single strains (Verbruggen et al., 2012; Nemergut et al., 2013). Thus, it can arguably be stated that the diversity of a microbial inoculum, in addition to its plant growth-promoting traits, is critical for enhancing productivity and longevity (Cordero and Polz, 2014).

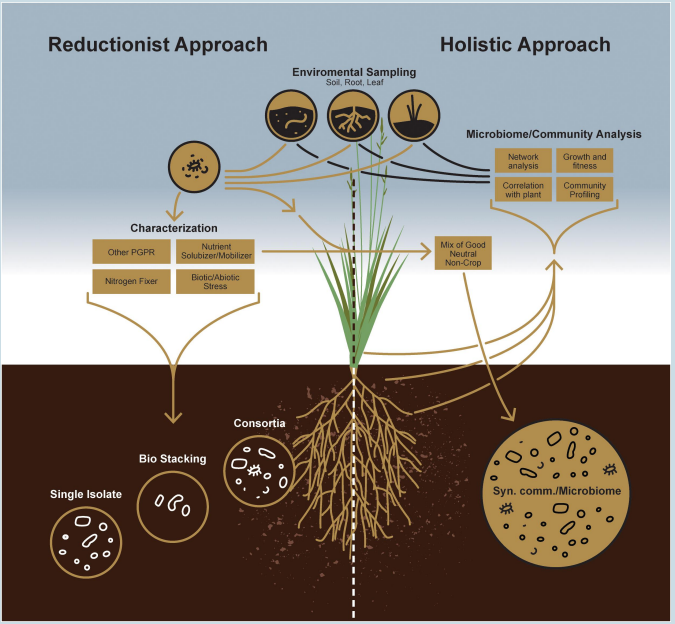


Figure 1. A schematic comparison between individual microorganism-based reductionist approach and microbial community-based holistic approach.

To improve the likelihood of success for such a management strategy, *a priori* knowledge of indigenous

microbial populations competing with the introduced plant growth-promoting agent(s) is critical. While a reductionist approach can define the currency of individual plant-microbe interactions, the concepts of microbial community survival and functioning require, a more holistic, microbiome-based approach empowered by next-generation sequencing technology to study plant-microbe interactions at the community level (Figure 2). Indeed, this will enable researchers to design more robust, synthetic microbial consortia capable of reliably enhancing agricultural productivity.

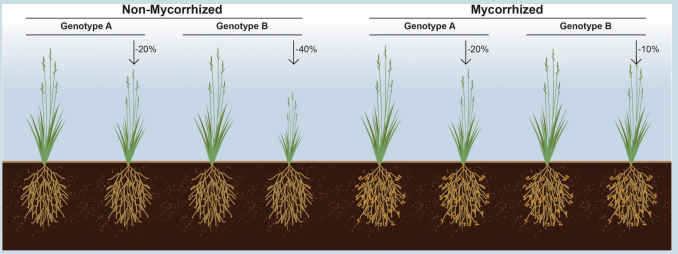


Figure 2. Responsiveness as the% gain in plant fitness attribute in response to symbiosis over un-colonized cohorts. This figure illustrates a hypothetical situation wherein genotype A loses less biomass (−20%) in response to soil nutrient limitation than does genotype B (−40%). However, if genotype B for its inherently associated rhizosphere microbiome responds optimally to a mycorrhizal symbiont, then it may be that it loses the least biomass (−10%) due to soil nutrient limitation, if the symbiont is present.% denotes loss in biomass due to soil nutrient limitation.

Microbiomes for Plant Growth Promotion: The Holistic Approach

Soil is a vastly heterogeneous growth medium, providing a wide spectrum of ecological niches for microorganisms

that enable diverse strains to coexist and form complex microbial communities. When the earliest plants extended their roots into primordial soils, they encountered a habitat already teeming with bacterial and fungal life (Bulgarelli et al., 2013; Kemen, 2014). Since that early time, plants have interacted with rhizosphere microbes, evolving strategies to forge beneficial alliances with some while keeping others at bay. Such early associations certainly had consequences on plant growth and development. Therefore, a more holistic approach is needed to understand better these microbes and the roles they play in the overall health of plant and soil (Figure 1). Again, recent advances in next-generation sequencing technology and the decreasing costs associated with that technology now allow us to evaluate how microbial populations fluctuate in both space and time or to identify core microbiomes that appear conserved among host genotypes or species (Sergaki et al., 2018). Thus, although culture-independent methods have contributed tremendously to our understanding of plant-associated fungal and bacterial community structures, the study of microbiome functions remains challenging because of the inherent noise of plant-associated microbial communities. It is now well known that there are core sets of microbes that, depending on the host, are recognized as keystone taxa that consistently associate with healthy plants (Banerjee et al., 2018). Consequently, researchers working with specific plant-microbe interactions have increasingly acknowledged the mitigating impact these larger microbial communities have on individual plant-microbe outcomes for plant growth promotion or fitness. Now, plant-associated fungal and bacterial stains from various plant species are being isolated, which will provide in the near future an inestimable resource for assembling taxonomically defined microbial

communities with increasing complexity. Therefore, it is now imperative to take advantage of this knowledge to design consortia of microbes to maintain a sustainable rhizosphere community, with key functional properties that include plant protection, nutrient acquisition, and alleviating biotic and abiotic stress responses. From that perspective, synthetic community (SynCom) approaches can provide functional and mechanistic insights into how plants regulate their microbiomes (Figure 1). Not surprisingly, recent culture-independent analyses thus have paved the way for developing SynComs more often (Bodenhausen et al., 2014; Armanhi et al., 2018; Carlström et al., 2019).

Mycorrhizal fungi, at least the arbuscular type, were early symbiotic partners of most land plant species, improving nutritional conditions through soil exploration and pathogen resistance of host plants (Klironomos et al., 2000). In reward for the essential physiological services, they receive ca. 20% of net photosynthetic products from plants (HoÈgberg et al., 2001). Other mycorrhizal systems may have different nutritional benefits and costs, as has been proposed for the serendipitous system (Craven and Ray, 2019). Additionally, third-party partners can modulate the outcome of the tripartite interaction, such as the case of mycorrhizal helper bacteria (Frey-Klett et al., 2007), fungal endobacteria (Bonfante and Desirò, 2017; Bonfante et al., 2019) like *Candidatus Moeniiplasma glomeromycotorum* within the spores and hyphae of Glomeromycotina (Naito et al., 2017), *Rhizobium radiobacter* within *Serendipita indica* (Guo et al., 2017), and N_2-fixing endobacteria *Pseudomonas stutzeri* inside basidiomycetes yeast endophyte *Rhodotorula mucilaginosa* (Paul et al., 2020). Hence, it is imperative to

consider the composition and functioning of these microbe–microbe interactions to understand plant–microbiome associations in a holistic manner.

Principles and Management of Rhizosphere Microbiomes for Sustainable Agriculture

Competence and Resilience of the Rhizosphere Microbiome: Impact of Introduced Microbes on Native Microbiomes

In 1904, the German agronomist and plant physiologist Lorenz Hiltner coined the term *rhizosphere* (Hartmann et al., 2008) to describe the area around a plant root inhabited by a unique population of microorganisms. Since then, numerous studies have been undertaken to decipher the interplay between plants and rhizosphere microorganisms, encompassing a wide variety of plant growth-promoting bacteria, fungi, insects, protozoans, viruses, etc. (Marschner, 2012; McNear, 2013). The majority of these studies have traditionally followed a simple principle for maximizing successful host infection by pre-inoculation onto the targeted crop of choice to provide a competitive advantage for a desired microbe. Conceptually, this increases the relative abundance of a given beneficial microbe in the rhizosphere, at least temporarily, to achieve the desired benefit. Such studies typically take place in a controlled, artificial condition, such as a defined growth medium in a greenhouse, where competition from a native rhizosphere community is relatively low or non-existent. As mentioned above, this approach occasionally has failed once field

application is attempted or the benefits are dramatically reduced in amplitude and/or endurance.

As an example, Lekberg and Helgason (2018) conducted a literature survey of research papers published on mycorrhizal functioning spanning a 30-year period (1987–2017). The most striking finding of this survey was that less than 5% of the work scientifically manipulated mycorrhizal abundance in the field. While we are not arguing the merit of greenhouse-based studies where the number of variables can be controlled and accounted for, yield gains in field conditions will continue to be modest with such an approach. Rhizosphere competence must be evaluated in a field situation if the true power of this approach is to be realized.

Over the last few decades, mycorrhiza-based biofertilizers containing one or several species of fungi were developed in forestry and agriculture (Jeffries and Rhodes, 1987; Baraza et al., 2016; Igiehon and Babalola, 2017). These inoculants are generally effective in plant growth promotion under controlled lab and greenhouse conditions. However, few targeted efforts have been made to measure interactions between the introduced microbe(s) and the native mycorrhizal community, let alone the more complex rhizosphere microbiome (Svenningsen et al., 2018; Turrini et al., 2018). To optimize outcomes from these interactions, targeted research must be undertaken to understand how such mycorrhiza-based biofertilizer integrate themselves within the context of the native microbiome.

Integration of Rhizosphere Microbiomes in

Plant-Microbe-Nutrient Relationships

The soil microbial community often assists plants by weathering minerals from rock surfaces and degrading recalcitrant soil organic matter whereby soil microbes break down soluble and insoluble organic matter and convert it into inorganic, plant-available forms. Soil organic matter turnover is thus considered a net positive, as it liberates the nutrients locked up in organic matter. For this reason, conventional farming has always relied heavily on soil tillage, along with such other intensive agricultural practices as usage of inorganic fertilizers, herbicides and pesticides. However, it is already clear that such practices have negative consequences on the functional diversity of soil microbiomes. Long-term chemical fertilization has been shown to dramatically decrease the soil pH, which leads to a decrease in bacterial diversity and other changes in microbial community structure (Sun et al., 2015). This was well documented in the work of Kumar et al. (2017), who showed that long-term application of high doses of inorganic nitrogenous fertilizers severely reduces relative abundance, diversity and structure of diazotrophs, which play a key role in converting atmospheric N_2 to plant-available ammonium.

As mentioned above, soil bacterial communities play a pivotal role in soil organic matter decomposition. In particular, soil carbon and nitrogen are critical factors for bacteria that rely on soil organic C and N decomposition to obtain energy (Chen et al., 2014; Wild et al., 2014; Tian et al., 2018). Further, different types of soil C selectively

manipulate soil microbial community composition, resulting in changes in such belowground ecosystem functions as decomposition and nutrient transfer and creating feedbacks that may affect overall plant growth and productivity (Orwin et al., 2006). For example, bacteria belonging to the genera *Chloroflexi*, *Nitrospirae*,
and *Planctomycetes* preferentially feed on recalcitrant organic C, whereas *Proteobacteria* and *Bacteroidetes* prefer labile organic C present in the soil (Nie et al., 2018). For this reason, amending the soil with such organic fertilizers as compost or manure contributes to higher microbial diversity and biomass compared to mineral-fertilized soils, which in turn positively impacts soil health (Schmid et al., 2018; Banerjee et al., 2019). Unfortunately, only a few agroecosystem experiments exist that compare organic and conventional management strategies over an extended period for evaluation of impact on soil health and restoration (Raupp et al., 2006; Khatoon et al., 2020). Hartmann et al. (2015) took a metagenomics approach to assess microbial diversity of soil in response to more than 20 years of continuous organic and conventional farming. Not surprisingly, they found that organic farming increased richness, decreased evenness, and shifted the structure of the soil microbiota when compared with conventionally managed soils under mineral fertilization (Hartman et al., 2018; Li et al., 2020b). There also are reports of significant alterations in the microbial community composition of both summer maize and winter wheat in response to increased nitrogen fertilization dose (Wang et al., 2018; Li et al., 2020a). Clearly, a better understanding of the interactions between the soil microbiome and conventional agricultural practices

is crucial for the development of sustainable management of soil fertility and crop production.

Managing the Rhizosphere Microbiome to Induce Disease Suppression in Soil

Disease suppressive soils were originally defined by Baker and Cook (1974) as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil." Disease suppressive soils are the best examples of microbiome-mediated protection of plants against root infections by soil-borne pathogens. Such disease-suppressive soils have been described for various soil-borne pathogens, including fungi, bacteria, oomycetes, and nematodes (Mazzola, 2007; Kwak et al., 2018). To date, several microbial genera have been proposed as key players in disease suppressiveness of soils, but the complexity of the microbiome, as well as the underlying mechanisms and microbial traits, remain elusive for most disease suppressive soils (Toyota and Shirai, 2018).

Recently, Carrión et al. (2019) showed that upon pathogen invasion, members of the *Chitinophagaceae* and *Flavobacteriaceae* became enriched within the plant endosphere. They proposed that this bacterial population shift led to the induction of enzymatic activities associated with fungal cell-wall degradation, as well as secondary metabolite biosynthesis, all aimed at accelerating and augmenting the plant defense response(s). Although the disease suppressive abilities of

certain soils can be at least partially attributed to their physico-chemical properties, the capacity of a soil to suppress disease progression is more often attributed to agri-management practices and crop rotation (Weller et al., 2002). In classic studies by Gerlagh (1968) and Shipton et al. (1973), the authors have shown soil to become disease suppressive after mono-culturing wheat over time. More recently, a comparative metatranscriptome analysis of wheat rhizosphere microbiome grown in fields suppressive and non-suppressive to the plant pathogen *R. solani* AG8 clearly revealed distinct dominant taxa in these two soil types. Additionally, suppressive samples showed greater expression of polyketide cyclase, terpenoid biosynthesis, and cold shock proteins (Hayden et al., 2018). While development of probiotics for the human gut microbiome has already been an established field of research, the use of probiotics that comprises naturally occurring bacterial antagonists and competitors that suppress pathogens has recently emerged as a promising strategy for disease suppression in soil. A study on application of probiotic consortia that comprised predefined *Pseudomonas* species reported suppression of the bacterial plant pathogen *Ralstonia solanacearum* in the tomato rhizosphere microbiome (Hu et al., 2016). In another study, amendment of *Metarhizium*, an insect-pathogenic fungus that is commonly employed as biological control agents against crop pests, in the rhizosphere of common bean (*Phaseolus vulgaris*) significantly increased the relative abundance of plant growth promoting such taxa as *Bradyrhizobium*, *Flavobacterium*, *Chaetomium*, and *Trichoderma* while suppressing the root rot disease symptoms *Fusarium solani* (Barelli et al., 2020). Soil

suppressive properties are mostly derived from the biological functions of soils. Therefore, elucidation of microbial functions in suppressive soils by a next-generation sequencing approach will facilitate the development of effective, consistent and durable disease management tools.

Impact of Agriculture Management Practices on the Soil Microbiome

One important context for plant-microbe interactions is soil structure, as it can vary greatly depending on land-use history, plant species composition and successional stage (Erktan et al., 2016). Besides playing pivotal roles in soil organic matter decomposition, carbon cycling, nutrient mobilization, etc., saprotrophic fungi also are involved in creating soil structure through the secretion of extracellular compounds and physical binding of soil via hyphal networks (Bergmann et al., 2016). Interestingly, studies on the impact of tillage on the soil fungal communities have shown mixed results. Reports in no-till systems have varied from increased ratios of fungal to bacterial biomass (Acosta-Martínez et al., 2010) to decreased ratios (Mbuthia et al., 2015), as well as no change at all (Mathew et al., 2012). More recent studies have shown that soil fungal communities are negatively impacted by tillage, as they typically would be responsible for degrading crop residue left on the surface with no-till (Yin et al., 2017). More specifically, soil bacterial communities were primarily found to be structured by tillage, whereas soil fungal communities responded mainly to management type with additional effects by tillage (Hartman et al., 2018). Additionally, it is acknowledged that

organically managed systems increased taxonomic and phylogenetic richness, diversity and heterogeneity of the soil microbiota when compared with conventional farming systems (Lupatini et al., 2017). In a simple definition, organic farming system consists of low-input agro-ecosystem farms in which plant productivity and ecosystem functionality are based on the natural availability of plant nutrients (Lammerts van Bueren et al., 2002). A study aimed at comparing the soil microbiome in conventional and organic farming systems in central Europe revealed no major differences among the main phyla of bacteria between the two farming styles (Armalytë et al., 2019), whereas another study that investigated the effects of 12 years of organic farming on soil microbiomes in northern China reported shifting of the community composition of dominant phyla and significant alterations of functional groups associated with ammonia oxidation, denitrification and phosphorus recycling when compared to conventional farming systems (Ding et al., 2019).

In addition to tillage, crop rotation also plays a pivotal role in increasing belowground microbial diversity compared to intensive mono-cropping practices. Although the United States Department of Agriculture has advocated [via the Conservation Reserve Program (CRP)] crop rotation to improve eroded land as early as 1985 (Allen and Vandever, 2005), its benefit on soil health has only been recognized recently. Several studies reported increases in such soil quality parameters as organic matter content, microbial biomass and respiration under crop rotation management when compared with a mono-cropping system (Campbell et al., 1991; Luce et al., 2013). A meta-analysis of 122 studies that examined crop rotation revealed similar findings, namely

that adding one or more crops in rotation to a monoculture substantially increased the soil microbial biomass along with increases in total soil C and N, respectively (McDaniel et al., 2014). In another study, soil microbial communities of corn and switchgrass in mono-cropping systems when compared with mixed prairie grasses demonstrated that bacterial and fungal biomass, especially arbuscular mycorrhizal fungi, were higher in plots with mixed prairie grasses (Jesus et al., 2016). A 16S amplicon-based metagenomic analysis of an almost 20-year-old field trial in Bernburg, Germany revealed a significant effect of tillage practice and the preceding crop on prokaryotic community structures (Babin et al., 2019)

Cover crops are typically unharvested crops planted between cash crops that augment C provisioning to the soil system not only via unharvested residues, but also as root exudates that can support many rhizosphere microbes during the active growing season of the cover crop. Other benefits attributed to cover cropping include improved N fertility by incorporating legumes as a cover crop, reduced soil compaction via deep-rooted plants, and reduced erosion by keeping a plant and its root system in the field year round (Fernandez et al., 2016). Of various crop rotation management practices, those that include cover crops sustain soil quality and productivity by enhancing soil C, N and microbial biomass (Kim et al., 2020), making them a cornerstone for sustainable agroecosystems. Nonetheless, very few studies have assessed the relationship between cover crop stands and their associated belowground microbial communities. Early research in unfertilized grasslands demonstrated that fungal communities respond positively to plant-derived C inputs, suggesting that

inclusion of cover crops in a rotation may promote fungal community development (Denef et al., 2009). More recently, a field study tested this hypothesis by specifically examining the impact on soil microbial communities of eight fall-sown cover crop species grown singly and in multispecies mixtures following a spring oats (*Avena sativa* L.) cropping season and found that certain cover crops selectively favored particular microbial functional groups. Arbuscular mycorrhizal fungi were more abundant beneath oat and cereal rye (*Secale cereale* L.) cover crops, while non-AM fungi were positively associated with hairy vetch (*Vicia villosa* L.) (Finney et al., 2017). Beyond positively affecting soil C and increasing the diversity of such beneficial fungi as arbuscular mycorrhiza, clover as a cover crop is often reported to suppress the relative abundance of pathogenic fungi (Benitez et al., 2016). Contrarily, in a 2-year field study, cover crops reportedly increased overall phylogenetic diversity of fungi but did not change the relative abundance of saprophytes, symbionts or pathogens, implying that cover cropping does not always appear to contribute to functional changes in the fungal community (Schmidt et al., 2019).

Reassessment of Plant Responsiveness to Symbiosis

It is now increasingly evident that plants employ fine-tuned mechanisms to shape the structure and function of their microbiome, with different genotypes of the same plant species growing in the same soil yet associating with distinct microbial communities (Berendsen et al., 2012). This is demonstrated in the findings of Bazghaleh et al. (2015), who clearly demonstrated the importance of intraspecific host variation in the association of chickpea cultivars with

AM and non-AM fungi. Therefore, specific traits of a plant that modulate its microbiome should be considered as a trait for plant breeding (Wallenstein, 2017).

Despite the obvious importance of beneficial microorganisms for plant growth and fitness, and the impact of plant genotype on shaping their microbiome composition, plant germplasm is typically screened in the absence of microbes, and the selection of best breeding lines made solely based on the interaction between plant genotype and performance under various abiotic factors. We propose that an *a priori* examination of the interaction between a plant genotype(s) and the symbiotic microbes upon which it likely depends is an important factor in the selection of plant breeding lines. It seems very likely that a subset of rejected germplasm could outperform others, but only when coupled with a beneficial microbe or microbiome (Figure 2). Arguably, current breeding and selection efforts most likely result in decoupling of the soil microbiome from plant fitness. As a result, modern varieties may have lost their ability to support diverse microbiomes and thus, fail to gain the most from these interactions (Wallenstein, 2017).

It is now acknowledged that transitioning from a highly intensive mono-cropping system to a more diversified cropping system consisting of multiple host genotypes leads to increased bacterial and fungal diversity (Calderon et al., 2016). Hence, future plant breeding efforts should incorporate plant characteristics that are related to microbiome diversity. For example, efforts focusing on manipulating plant root exudates likely play a critical role in selective recruitment of the rhizosphere microbiome (Bakker et al., 2012). In support of this notion, it has been shown that plants can select which microbial populations

receive the lion's share of root exudates, demonstrating a capacity by the host to refine its microbial composition. Hence, an unbiased screening of plant genotypes for responsiveness in the presence of a beneficial microbe or microbiome can set forth a new and potentially transformative paradigm in selecting microbes for plant growth promotion (Figure 2).

Significance of Mycorrhizas: A Critical Component of Healthy Soil Rhizospheres

Mycorrhizae are mutualistic associations between soil fungi and plant roots that gradually evolved to be reciprocally beneficial to both partners (Brundrett, 2002). The benefits are generally assumed to involve an exchange of photosynthetically derived carbon from the host plant in exchange for soil nutrients provided by the foraging mycorrhiza. While likely true of arum-type arbuscular types of mycorrhizae, there are other types that can derive carbon from organic matter in the soil, or even "steal" it from one host plant to supply to another (Allen and Allen, 1991). A recent study has reported that in contrast to *Arum maculatum*, in which carbon is entirely derived from photo-assimilation, the green leaves of *Paris quadrifolia* contain a striking 50% carbon of fungal origin. Such partial mycoheterotrophy could thus potentially be widespread among the roughly 100,000 plant species that are known to develop a Paris-type AM, with far-reaching implications for our understanding of C trading in plant-microbe communities (Giesemann et al., 2019). Exactly what the mycorrhiza gains from this interaction is still under debate, but benefits may involve a safe haven from the open, more

competitive soil space and a second, more reliable carbon source (Sapp, 2004).

Mycorrhizae not only shape plant communities, but they also affect the functional diversity of their cohabitants in the rhizospheric microbiome. The mycelium of mycorrhizal fungi transports plant-derived carbon into the soil in the form of sugars, amino acids and polyols to help sustain the microbiome (Tarkka et al., 2018). More recent studies focusing on soil microbial ecology revealed that mycorrhizal fungi mediate many diverse interactions within the soil "mycorrhizosphere," including pathogens and mutualists that fix atmospheric nitrogen, take up phosphorus, produce vitamins, and/or protect against antagonists (Buée et al., 2009; Tedersoo et al., 2020). The "ectomycorrhizosphere," which forms a very specific interface between soil and many trees, hosts a large and diverse community of microorganisms that likely play roles in mineral weathering and solubilization processes (Uroz et al., 2007). This carbon-rich mycorrhizosphere also supports large communities of root-associated microorganisms that further accelerate weathering of minerals by excreting organic acids, phenolic compounds, protons, and siderophores (Drever and Vance, 1994; Illmer et al., 1995).

Similarly, the extraradical hyphae of arbuscular mycorrhiza provide a direct pathway for the translocation of photosynthetically derived carbon to the soil, leading to the development of nutrient-rich niches for other soil microorganisms, particularly bacteria. A quantitative real-time PCR method detected significantly higher 16S rDNA abundance in both the bulk and the rhizosphere soils of zucchini (*Cucurbita pepo* L.) inoculated with *Acaulospora laevis* and *Glomus mosseae* (Qin et al., 2014). Additionally,

arbuscular mycorrhizae have been reported to increase the relative abundance of *Firmicutes*, *Streptomycetes*, *Comamonadaceae*, and *Oxalobacteraceae* inhabiting the mycorrhizosphere (Offre et al., 2007; Nuccio et al., 2013). While there is clear evidence that microbial communities in the rhizosphere function cohesively with their mycorrhizal partner in nutrient mobilization from soil minerals, nitrogen cycling and protection of plants against root pathogens, such bidirectional synergy is not always universal. There are reports that indicate suppressive effects of bacterial communities on mycorrhizal functioning and vice versa. While one study reported (Svenningsen et al., 2018) that soil with a higher abundance of *Acidobacteria* suppresses the normal functioning of extra-radical mycelium in arbuscular mycorrhizae, another study found that *Glomus intraradices* and *Glomus mosseae* suppressed most of the associated soil microbial community (Welc et al., 2010).

A Novel Type of Endophytic Symbiont: The *Serendipitaceae*

A diverse group of fungi in the Basidiomycota, the *Serendipitaceae* (formerly Sebacinales Group B) (Oberwinkler et al., 2014) encompasses endophytes and lineages that repeatedly evolved ericoid, orchid and ectomycorrhizal types. Accordingly, in many natural ecosystems these fungi form mycorrhizal symbioses with an astounding variety of host plants – every mycorrhizal type, in fact, except for arbuscular. Previous research performed in our lab with a strain of this group, *Serendipita vermifera*, demonstrated plant growth-promoting properties in a

variety of plants (Ghimire and Craven, 2011; Ray et al., 2015; Ray and Craven, 2016; Ray et al., 2020). Unfortunately, the agronomic utility of these fungi is hampered by the paucity of strains available, the large majority isolated from Australian orchids. We have begun to address this constraint by isolating the first North American strain of *Serendipita*, named *Serendipita vermifera* subsp. *bescii* NFPB0129, from the roots of a switchgrass plant in Ardmore, Oklahoma (Craven and Ray, 2017; Ray et al., 2018).

As mentioned above, soil organic matter has a tremendous influence on the biological, chemical, and physical properties of soils, making it a vital component of healthy agricultural systems. Whether a natural soil or an agricultural one, the release of the nutrients locked within SOM requires decomposers, primarily insects, fungi, and bacteria, to secrete organic acids and enzymes that can loosen and break down the cellulose and the recalcitrant lignin into nutritive forms that can be used by other microbes and plants. Unlike arbuscular mycorrhizae, which exchange inorganic, mineralized nutrients mined from the soil for carbon derived from host photosynthesis, members of the *Serendipitaceae* studied thus far have a complete arsenal of carbohydrate-active enzymes (CAZymes), representing approximately 4% of the entire gene set and rivaling the more well-studied saprophytic white and brown wood rotters, and much more than other symbiotic fungi. Additionally, genome analysis of *S. bescii* and *S. vermifera* suggests that *Serendipitaceae* fungi have the metabolic capacity to assimilate N from organic forms of N-containing compounds (Ray et al., 2019). We hypothesize that this carbohydrate-degrading enzyme complement endows these *Serendipitaceae* fungi with saprotrophic

abilities (Craven and Ray, 2019). Unlike free-living decomposers that maintain a solitary lifestyle, seeking only dead or dying plant tissues as their source of subsistence, *Serendipitaceae* fungi seem to maintain a largely symbiotic lifestyle with the roots of living host plants. It currently is unclear whether there is expression of CAZymes while strains of *Serendipita* are in symbiosis with host plants, and if so, whether there is spatial or temporal separation from more mutualistic traits. Still, the capacity of some strains to form mycorrhizal relationships with orchids, where the seeds require carbon from the fungus for germination and often well into the plant's lifespan, suggests that these *Serendipitaceae* symbionts may be less of a carbon cost to their host plant. Presumably, this saved carbon could potentially be used for other symbiotic relationships or developmental processes. In any case, these intriguing fungi and their seemingly unlimited host range provide a novel symbiosis that could be used in a broad variety of cropping systems.

Conclusion

Soil-dwelling microorganisms are critical components of soil health, itself a determinant of plant productivity and stress tolerance. Deploying microbes to improve agriculture productivity is an extremely attractive approach that is non-transgenic and can be viewed collectively as the extended plant genome. Because these same microbes can contribute to restoring soil health and productivity, they have a bright future in low-input, sustainable agriculture that extends beyond more classically defined plant-microbe symbioses.

Author Contributions

PR and KC conceived and planned the overall idea of the review manuscript. PR, VL, JL, and KC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Hotspot Quiz



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Check your Understanding

- What roles does the soil microbiome have in the movement towards sustainable agriculture?
- What is a rhizosphere?
- What type of soil fungi are associated with promoting plant health as well as the soil microbiome? How do they do so?
- What are the challenges with studying plant growth promoting microorganisms?
- Explain the difference between a reductionist vs. holistic approach for improving plant growth with microorganisms.
- What types of agricultural practices can alter the soil microbiome?
- How could the use of 'probiotics' suppress disease-causing soil microorganisms?

Media Attributions

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14. Plant Microbiomes

Plant Microbiomes

Plant health is influenced by a variety of environmental factors, and though the soil content (including the microbial community present) is a major element, the distinct plant microbiome serves an important role. Similar to organ systems in the human body (e.g. skin, gut, etc.), the components of various plants (e.g. internal tissues, leaves, roots, etc.) can have unique microbial communities that contribute to its respective and overall vitality. A better understanding of the corresponding roles of plant microbiomes can be combined in a synergistic effort with other environmental microbiomes to maintain and promote sustainable agricultural practices, ecosystem biodiversity, and research model studies.



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Plant microbiome–an account of the factors that shape community composition and diversity

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Abstract

Plants live in association with diverse microorganisms, collectively called the microbiome. These microbes live either inside (endosphere) or outside (episphere) of plant tissues. Microbes play important roles in the ecology and physiology of plants. Significant progress has been made in revealing structure and dynamics of plant microbiome in the last few years. Various factors related to host, microbes as well as environment influence the community composition and diversity of plant microbiome. This review aimed to provide a general account of the factors (host, microbe and environment) that drive the microbial community composition in plant. First, we gave an overview of the aboveground and belowground plant microbiomes. Next, we discussed which host factors are involved in variation in plants followed by importance of microbe-microbe interactions and the elements of environment that influence

composition and community structuring of plant microbiomes.

1. Introduction

A diverse kind of microorganisms associated with a higher organism (human, animal, plants etc.) is together defined as its microbiome. All higher organisms examined to date, including plants, insects, fish, rats, apes, and humans, harbor microbiomes [1,2]. Research on the human microbiome has progressed very quickly. Recently, researchers have also paid much attention to elucidating the composition and functions of plant and soil microbiomes. It is now believed that plants are not separate entities, but rather they live in association with a large variety of microbes. These microbes live either inside (endosphere) or outside (episphere) of plant tissues. Among these microorganisms, bacteria and fungi are predominant. About a few thousand bacterial and fungal taxa have been reported from plant tissues [3,4]. They play important roles such as increased nutrient availability, uptake by plants and increased plant stress tolerance. Thus, plant fitness (growth and survival) is the result of physical and physiological functions of the plant *per se* as well as the associated microbiome, which together are known as a plant holobiont [5].

The study of the association of plants with microorganisms precedes that of the animal and human microbiomes, notably the roles of microbes in nitrogen and phosphorus uptake. The most notable examples are plant root-arbuscular mycorrhizal (AM) and legume-rhizobial symbioses, both of which greatly influence the ability of

roots to uptake various nutrients from the soil. Some of these microbes cannot survive in the absence of the plant host (the 'obligate symbionts' including viruses, some bacteria and fungi), which provides space, oxygen, proteins, and carbohydrates to the microorganisms. The association of AM fungi with plants has been known since 1842, and over 80 % of land plants are found associated with them [6]. It is thought that AM fungi helped in the domestication of plants [7]. Traditionally, culturable microbes have been used for plant-microbe interaction studies with the enormous unculturable microbes remain uninvestigated and consequently, our knowledge of the roles of these unculturable microbes remains largely unknown.

Unraveling the types and outcomes of plant-microbe interactions has received considerable interest among ecologists, evolutionary biologists, plant biologists, and agronomists [4,8,9]. Recent developments in meta-omics and the establishment of large collections of microorganisms have dramatically increased our knowledge of the plant microbiome composition and diversity. The sequencing of marker genes of entire microbial communities, referred to as metagenomics, sheds light on the phylogenetic diversity of the microbiomes of plants. It also adds to the knowledge of the major biotic and abiotic factors responsible for shaping plant microbiome community assemblages [8].

However, our understanding on the roles of microbiomes, with respect to their impact on plant ecology and physiology, is still far from complete, and we are at the beginning of knowing their functions [10]. The outcome of this improved knowledge will have significant bearings on a variety of experiments and applications, such as

development of biofertilizer and biopesticides for sustainable agricultural production with less reliance on agrochemicals, while augmenting yield and nutritional value [11].

In this review, we will present an account of recent studies and prospects of studying the plant microbiome. Firstly, we will present an overview of aboveground and belowground plant microbiomes. Next, we will discuss the factors driving the composition and community structuring of plant microbiomes. We will not address plant pathogenic microbes, although inclusion of this fraction of microbiome would make sense from a broader and more holistic viewpoint.

2. Rhizosphere microbiome

The rhizosphere comprises the 1–10 mm zone of soil immediately surrounding the roots that is under the influence of the plant through its deposition of root exudates, mucilage and dead plant cells [12]. A diverse array of organisms specialize in living in the rhizosphere, including bacteria, fungi, oomycetes, nematodes, algae, protozoa, viruses, and archaea [13]. The most frequently studied beneficial rhizosphere organisms are mycorrhizae, rhizobium bacteria, plant growth promoting rhizobacteria (PGPR), and biocontrol microbes. Gans, Wolinsky [14] projected that one gram of soil could harbor more than a million distinct bacterial genomes. İnceoğlu, Al-Soud [15] reported 55,121 OTUs (operational taxonomic unites) from the potato rhizosphere. Among the prokaryotes in the rhizosphere, the most frequent bacteria are within the

Acidobacteria, Proteobacteria, Planctomycetes, Actinobacteria, Bacteroidetes, and Firmicutes [3,16]. In some studies, no significant differences were reported in the microbial community composition between the bulk soil (soil not attached to the plant root) and rhizosphere soil [17,18]. Certain bacterial groups (e. g. Actinobacteria, Xanthomonadaceae) are less abundant in the rhizosphere than in nearby bulk soil [3].

Mycorrhizal fungi are abundant members of the rhizosphere community, and have been found in over 200,000 plant species, and are estimated to associate with over 80 % of all plants [19]. These mycorrhizae–root associations play profound roles in land ecosystems by regulating nutrient and carbon cycles. Mycorrhizae are integral to plant health because they provide up to 80 % of N and P requirements. In return, the fungi obtain carbohydrates and lipids from host plants [20]. Recent studies of arbuscular mycorrhizal fungi using sequencing technologies show greater between-species and within-species diversity than previously known [21].

3. Phyllosphere microbiome

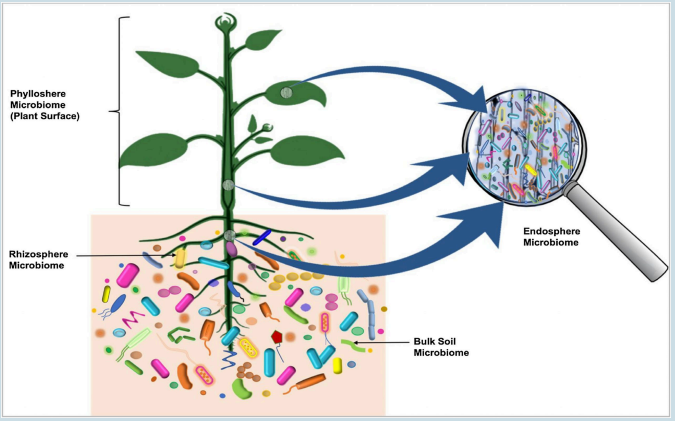
The aerial surface of a plant (stem, leaf, flower, fruit) is called the phyllosphere and is considered comparatively nutrient poor when compared to the rhizosphere and endosphere. The environment in the phyllosphere is more dynamic than the rhizosphere and endosphere environments. Microbial colonizers are subjected to diurnal and seasonal fluctuations of heat, moisture, and radiation. In addition, these environmental elements affect plant

physiology (such as photosynthesis, respiration, water uptake etc.) and indirectly influence microbiome composition. Rain and wind also cause temporal variation to the phyllosphere microbiome [22]. Overall, there remains high species richness in phyllosphere communities. Fungal communities are highly variable in the phyllosphere of temperate regions and are more diverse than in tropical regions [23]. There can be up to 10^7 microbes per cm^2 present on leaf surfaces of plants, and thus the bacterial population of the phyllosphere on a global scale is estimated to be 10^{26} cells [24]. The population size of the fungal phyllosphere is likely to be smaller [25]. Phyllosphere microbes from different plants appear to be somewhat similar at high levels of taxa, but at the lower levels taxa there remain significant differences. This indicates that microorganisms may need finely tuned metabolic adjustment to survive in phyllosphere environment [24]. Proteobacteria seems to be the dominant colonizers, with Bacteroidetes and Actinobacteria also predominant in phyllospheres [26]. Although there are similarities between the rhizosphere and soil microbial communities, very low similarity has been reported between phyllosphere communities and those in open air [27].

4. Endosphere microbiome

Some microorganisms, such as endophytes, penetrate and occupy the plant internal tissues, forming the endospheric microbiome (Fig. 1). The AM and other endophytic fungi are the dominant colonizers of the endosphere [28]. Bacteria, and to some degree Archaea, are important members of endosphere communities. Some of these endophytic

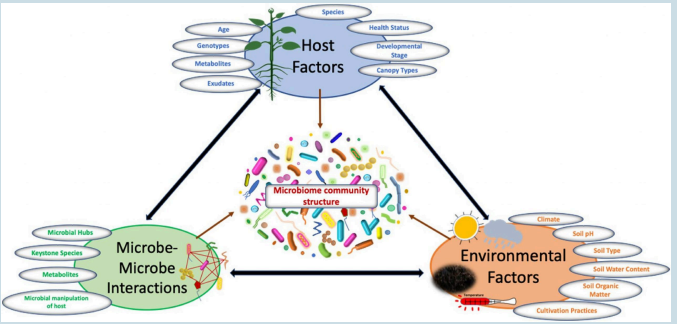
microbes interact with their host and provide obvious benefits to plants [[29], [30], [31]]. Unlike the rhizosphere and the rhizoplane, the endospheres harbor highly specific microbial communities. The root endophytic community can be very distinct from that of the adjacent soil community. In general, diversity of the endophytic community is lower than the diversity of the microbial community outside the plant [18]. The identity and diversity of the endophytic microbiome of above-and below-ground tissues may also differ within the plant [28].



The 'Plant Microbiome' consists of diverse microbial communities on the outside surface and in internal tissues of the host plant. The rhizosphere, endosphere, and phyllosphere constitute the major compartments of plant microbiome. The soil microbiome is an important source of the plant microbiome.

5. Drivers of plant microbiome composition

Plant microbiome structure is influenced by complex interactions between hosts, microbes, and associated environmental factors such as climate, soil, cultivation practices etc. (Fig. 2). Below, we provide an assessment of current knowledge of these factors, providing insight to plant-microbe interactions in a broader-sense.



A simple schematic representation of various factors that shape microbiome community structures. The host, microbe, and environment all factors are interconnected and together build the microbiome community in plants.

6. Host factors that influence plant microbiome community composition

6.1. Plant species

The identity of the host plant has a significant influence on the identity of its microbiome. Different plant species growing adjacent to one another can harbor distinct microbiomes. A comparative survey of root microbiomes in maize, sorghum, and wheat showed different community composition among these plants [32]. Samad et al. [33] investigated the microbiome compositions of roots and rhizospheres using 16S rRNA gene from grapevines and some weed species growing in the same field, and their findings suggested that these species hosted significantly different microbiomes in the roots and rhizosphere, with the more pronounced difference in the root communities. Plants that are distantly-related phylogenetically show greater variation in associated microbiome compositions, suggesting a role of plant phylogeny in structuring root microbiomes [32]. Plant species also influences the identity and diversity of endophytic communities. Manter et al. [34] reported differences in endophytic community composition in potato and *Eucalyptus* plants. The most abundant bacterial root endophytes in potato were rare or absent in *Eucalyptus* and vice-versa [34] suggesting that the host plant selects its endophytic microbes. Analysis of endophytic fungi of three native Australian *Nicotiana* species revealed that they are host-specific but not plant organ- or host location-specific [28].

In addition to the rhizospheric and endophytic microbiomes, phyllosphere community composition also depends on plant identity [24]. Kembel et al. [35] showed that the leaf microbiome community is highly correlated with plant evolutionary relatedness similar to the endospheric microbiome.

The effects of host plant species in recruiting microbes from the surrounding environment indicate that plants have evolved traits that govern root microbiome assemblages [36]. For example, endosphere, rhizosphere community composition are correlated with host taxonomy [36]. Xiao et al. [37] found that the rhizosphere and root microbiomes are mostly influenced by soil type, and the nodule and root endophytes are influenced by plant species. Differential microbiome assembly in different plant species is attributed to variation in plant resource consumption [36]. Plant traits such as leaf permeability, wettability and topography and physicochemical properties, cuticle chemistry, root exudates, antibiotic production, and inherent plant immunity to invasion by microbes may also to play a role.

6.2. Plant genotypes

Evidence suggests a difference in microbe community composition between genotypes of a particular species [17,26,38]. Genetics of the host is one of the factors that shape the plant-microbiome structure. For example, OTUs in three different potato varieties were cultivar-specific [36]. Similarly, cultivar-dependent effects have been reported for the bacterial communities in young potato rhizospheres [15]. Peiffer et al. [39] demonstrated that OTU

richness and β-diversity are influenced by plant genotypes in maize. Bulgarelli et al. [38] reported that genotype contributed to about 6% of the variation of the microbiome composition in the rhizosphere region. A larger influence of host genotype on community composition has been reported [40]. Genotype-dependent microbiome community structuring has been reported for sweet potato, wheat, pea, and oat [9,41]. Bacteria such as *Acinetobacter*, *Chryseobacterium*, *Pseudomonas*, *Sphingobium*, and *Stenotrophomonas* were more abundant in low-starch cultivars than those having high-starch contents [41]. The rhizosphere communities of different genetic clones of wild-type and transgenic lines have been reported to be distinct in *Populus* [42]. Within-species genetic variability can influence microbiome composition in leaf tissues [43]. Wagner et al. [44] conducted an extensive field experiment to unravel drivers of community composition of bacteria associated with leaves and roots of *Boechera stricta*. Their findings suggested that the host genotype influences leaf community, but the root microbiome was variable at different collection sites.

Agler et al. [45] proposed that host genotype influences on keystone microbes, which then pass these effects onto the total microbiome by changing microbe–microbe interactions and altering plant fitness [3]. The host specificity of plant microbiome could also be attributed to the nutrient preferences of plants [46]. However, whether the observed host influences are heritable, how strong the effect is, and whether these associations will be actionable for plant breeding is yet to be established.

6.3. Plant organ

Different plant tissues host distinct microbiome communities. Edwards et al. [47] demonstrated that each surface and internal tissue of plants may harbor distinct microbial communities and that the role of tissue-type was greater than host type and the microbiome of the soil. This may be because the adaptation strategies of various tissues may affect the microbes in colonizing them for community composition. For instance, surface tissues are exposed to constant fluctuations of weather and have relatively poor nutritional status compared to the root or internal tissues. Therefore, microbes colonizing the leaf surface need to be adapted in these conditions [25]. Other studies found very little or no effect of plant organ in community composition of fungi and bacteria [28].

Study with agaves determined that prokaryotes are largely influenced by the plant compartment, whereas the rhizosphere, the phyllosphere, and endosphere communities are clearly different from each other and from adjacent soils [48]. Many studies have reported that fungal communities show a different pattern, where the biogeography of host were the major influencing factor [48]. This may be explained by the dispersal limitation in fungi [49], because fungi are eukaryotes like plants and animals, but bacteria are different in this respect.

6.4. Plant age and developmental stage

In the cases of interactions between plants and pathogens, ontogenic resistance (age-related resistance) is widely reported and correlated with plant developmental stage [50]. Symbiosis research has also indicated that plant age and developmental stage are important factors affecting microbial communities [51]. Analysis of the bacterial rhizosphere community of *Arabidopsis* revealed that the seedling stage selects distinct microbiomes at developmental time points. Plants produce mixtures of compounds and specific phytochemicals in the root exudates. Some of these chemicals are indeed distinct at plant developmental stages and appear to shape microbiome community assemblages [52]. The effect of the plant age on microbiome using DGGE fingerprint analysis revealed that it significantly influences bacterial community composition of all groups investigated for all three sweet potato cultivars [41]. The effect of plant age on the composition of bacterial microbiome in the rhizosphere has been demonstrated in potato, maize and soybean [15,53,54]. Age-related microbiome differentiation may be associated with root growth, physiology, root architecture, root morphology, root exudate, and its composition [51,52]. However, further research is needed on the identity and effect of root exudates at different plant developmental stages to determine how plants communicate in the rhizosphere. This knowledge might offer a basis for augmenting agricultural crops by the application of rhizosphere microbes.

6.5. Plant canopy type

Plant canopy type also influences microbiome community composition. For example, bacterial communities in sugar maple leaf samples are correlated with canopy composition [55]. The microbial migration through rain runoff may be an important factor for variation in microbial colonization in different canopy types. Canopy structure influences the composition of endophytic community but not the rhizospheric community, indicating less effect of rain runoff, and there may be other mechanisms such as soil factors or some unknown factors involved in this variation [55].

6.6. Plant immunity and signaling

Plant health status may influence microbiome composition. Plants employ two layers of defense against pathogens: pattern-triggered immunity (PTI), which is triggered by conserved molecular structures such as microbe/pathogen-associated molecular patterns (MAMPs/PAMPs), and damage-associated molecular patterns, which are recognized by plasma membrane-localized pattern recognition receptors [56]. It is unknown whether plants recognize non-pathogenic microbes in the similar way as they recognize pathogens and modulate their response. When plants are challenged with herbivory or pathogens they release hormones and exogenous volatiles that alter the composition of root exudates (for review, see [57]), and these in turn modify the microbiome community.

Aphid infestation and pathogenic microbial infection increaseed populations of the non-pathogenic rhizobacterium *Bacillus subtilis* in the sweet pepper rhizosphere [58]. In *Arabidopsis thaliana* plants infected by *Pseudomonas syringae*, the expression of root malate transporter is altered, indicating a change in secretion of malic acid that increased the number of the beneficial rhizobacterium *Bacillus subtilis* [59,60]. Cucumber roots infected by *Fusarium oxysporum* f. sp. *cucumerinum* exhibited augmented secretion of fumaric and citric acid, which led to the formation of biofilms (aggregates of living bacteria in a slimy extracellular polymeric substances) of *Bacillus amyloliquefaciens*. Most investigations, however, have focused on one-to-one interactions (plant-microbe), although in reality, plants are subjected to attack by numerous microbial pathogens and insect pests. Therefore, it would be interesting to see how multiple herbivory and/or pathogens modify the community composition of plant microbiomes. Recent studies have reported a change in rhizosphere microbiome community composition as influenced by specific compounds such as sugars, sugar alcohols, or mixtures of various chemical compounds in root exudates [52]. Plants use various strategies in response to pathogenic infection and insect attack. One of them is activation of defense responses in roots, which may influence microbiome composition in the rhizosphere and roots [61]. When aphids feed on foliage both SAR (Systemic Acquired Resistance) and ISR (Induced Systemic Resistance) signaling are activated throughout the plants, which elicits sweet pepper plants to attract *B. subtilis* in the rhizosphere [58]. Again, increased JA signaling in plant either by injury or exogenously

in *Medicago truncatula* caused higher colonization of beneficial mycorrhizae [62]. Different root and phyllosphere endophyte microbial communities have been reported when altered SA signaling was induced [63]. Plants lacking in jasmonate-mediated defense have shown more diverse epiphytic colonization [64]. It is evident from these studies that the role of plant defense systems on the microbial composition are inconstant, and that SAR is an important factor in regulating some bacterial community composition. Chemical signals released by plants for example, flavonoids, activate varied responses in plant rhizosphere microbiomes [65]. Branching in mycorrhizal hyphae is affected by strigolactones, which enhance and promote seed germination by parasitic plants [66].

6.7. Plant derived compounds

A diverse array of antimicrobial compounds are produced in plants [67]. Some of them, such as different alkaloids, phenolics, and terpenoids, are common in plants. Some are specific to particular groups [68], for instance, Brassicales produce glucosinolates. It was found that transgenic *Arabidopsis* producing an exogenous glucosinolate had different microbiomes in the rhizosphere and root tissues [69]. Voges et al. 2018 reported a significant role of plant derived coumarins in structuring the rhizosphere community. They suggested that iron-mobilizing coumarins are involved in redox reactions that can mobilize ferric iron and generate reactive oxygen species (ROS) with detrimental effects on microbial proliferation and thus selectively inhibit certain microbial growth while allowing proliferation other more beneficial

partners. Triterpenoid saponins, which are known as avenacins, are found in oat (*Avena strigosa*), and have antifungal properties [70]. Oat plants lacking avenacin production attracted different culturable fungi in roots [71] and were more vulnerable to pathogenic infections than wild-type oat [72]. Interestingly, however, a recent comprehensive analysis of the rhizosphere community of these two genotypes reported little difference between the fungal communities. The effect of avenacins on the Amoebozoa and Alveolata was profound but has not been reported for bacterial communities [73]. This revealed that a small difference in plant genotype might exert multifaceted and unpredicted effects on the plant microbiome composition and diversity. These plants derived compound may affect microbiome assembly in different ways. For example, root exudates may be specific for host plant and can modulate rhizosphere community as well as selects specific root microbiome and thus contributing host specific plant microbiome. Also, antimicrobial compound may selectively enhance microbial growth by restricting certain microbes which is a kind of 'balance' in mutualism.

7. Microbial factors in shaping plant microbiome structure

Microorganisms play important roles in shaping microbial community structures in plants. However, our knowledge on how microorganisms influence microbiome structuring is limited.

7.1. Microbial manipulation of hosts

Microorganisms can affect host plants, for example host root exudations, which in turn affect the permeability of roots and root metabolism. Some microbes in soil where the plant if growing can also absorb certain compounds in root exudates and excrete other compounds. Soil microbes can produce compounds that affect plant signaling and metabolism, which lead to production of microbe-derived compounds in plants. Some microbes produce antibiotics (e.g., penicillin and polymyxin) which increase the exudation of organic materials, altered cell permeability, and increased leakage [74] and results in a variable microbiome assembly.

7.2. Microbe-microbe interaction

The extent to which microbe–microbe interactions can play roles in the microbiome composition is not well understood. The outcome of microbe-microbe interactions could be explained as cooperation, parasitism, and competition. In cooperation, at least one species benefits, while others are not harmed. When both species benefit, the term mutualism is used, whereas, when one partner benefits while the other is not affected, the term commensalism is used. In contrast, parasitism and competition are harmful for at least one species [75].

We know from recent studies that microbial communities harbor highly connected taxa called keystone taxa [76]. These taxa independently or in a group show a substantial

effect on microbiome composition and functions regardless of their spatial and temporal dynamics. They play a unique and vital role on microbiomes, and their absence could cause a significant alterations in microbiome composition and functioning [76]. They use various strategies to impact on host microbiome. For instance, they might cause changes in intermediate or effector groups which in turn regulate microbiome community composition and functioning [77]. Production of a secondary metabolite (2,4-diacetyl phloroglucinol) was reported for some strains of *Pseudomonas fluorescens* that suppress *Gaeumannomyces graminis* var. *tritici*, responsible for wheat take-all [78]. Similarly, many fungi (e.g. *Trichoderma* and bacteria (e.g. *Bacillus*) produce various secondary metabolites that suppress microbial growth [79,80]. The keystone taxa may produce bacteriocins to shift microbiota structure selectively. Again, by synergism, keystone taxa may alter the abundance of their partners, and influence community structure and performance. For example, certain species of *Burkholderia* are symbiotic with arbuscular mycorrhizae and may change abundance and community composition of AM fungi, thereby influencing plant community richness, diversity, and production [19]. Agler et al. [45] studied the roles of microorganisms (bacteria, fungi and oomycetes) in the community composition of phyllosphere microbiomes of Arabidopsis thaliana using a systems biology approach. They described an interkingdom interactions network with a profound influence on community structure. They identified a few taxa, termed “microbial hubs’, which are highly interlinked and have a significant impact on communities. They used two “hub” microbes (Albugo, an oomycete pathogen and Dioszegia, a basidiomycete yeast)

in detail. Albugo strongly affected epiphytic and endophytic bacterial colonization. Many symbiotic microbes (including pathogens) produce effector proteins to suppress, activate, or alter host defense mechanisms [81], and some can entirely reprogram the host metabolism [82]. These host adjustments can lead to alterations of microbiome composition because some microorganisms and not others can benefit from altered conditions. Actually, the niches of some microorganisms is dependent on others. For instance, primary colonizers can aid subsequent colonizers against hazardous abiotic factors [83] or can enhance the competitive ability of following colonizers by producing secondary metabolic compounds [84]. There can be direct microbe-microbe interactions, such as the hyper-parasitism (parasite of parasite) of primary colonizers [85] and opportunists that utilize host's compromised plant defenses to colonize them [86]. Such phenomena point out why some colonizers can affect the development of microbes on the host even if they may be distantly related [84] and highlight a crucial functions of such interactions in shaping microbiome composition and structure.

One of the major strategies by which PGPR augments plant growth is by its influence on rhizosphere microbes. For example, *Pseudomonas* sp. DSMZ 13134 alters the composition of dominant bacteria in barley roots [87]. In some cases, however, the effects of PGPRs on resident microbiome may not be prominent. For example, no substantial changes in rhizosphere community were noticed after the application of *Bacillus amyloliquefaciens* FZB42 [88]. Supporting results have also been reported in soybean with the application of *B. amylolique faciens* BNM122 [89]. The recent investigation on the effect of *B. amyloli*

quefaciens on the lettuce microbiome using 454-amplicon sequencing revealed no or only transient and minor effects in the rhizosphere zone [90]. Interestingly, a decrease in the bacterial number was reported. In the field only 55 % of the inoculated bacterial DNA could be traced after a month [91]. The effects of *Bacillus subtilis* strain PTS-394 on the rhizosphere microbiome has been examined by metagenomic profiling. Similar to the results above for *B. amyloliquefaciens* FZB42 [90,91], only a minor effect on the composition was reported. However, up until now, the impact of *Bacillus* PGPR on other plant microbiota, such as fungi, has not been investigated, and investigation on this could reveal a more general effect of inoculated bacteria on resident microbiota and thus on host physiology and ecology.

8. Environmental factors as drivers of plant microbiome assembly

Environmental elements, such as soils, climatic conditions, geography, farming activities, and plant domestication, could result in the differences of plant associated microbial community composition [92]. A change in an environmental component results in plant phenotypic changes (e.g., [93], which consequently also change the assemblage of distinct microbiomes harboring plant compartments [94]. Plants grown under controlled conditions provide specific environments for microorganisms. For instance, when lettuce was grown under a glasshouse, a distinct bacterial signature was found from those grown in open fields [95]. Whitaker, Reynolds et al. [96] reported the community composition of endophytic

fungi local environment (i.e., site), but not by host ecotype, pointing that environmental factors are major drivers of the endophytic mycobiome of switchgrass.

8.1. Soil

Plants recruit root microbes mainly from the soils where they grow. Various soil factors viz. soil types, soil pH, and the C/N ratio, as well as available P and K are frequently reported to be the determinants of root microbial community composition by affecting plant growth and immunity [8,9]. Innumerable studies using high throughput sequencing have proved that soil type is a major factor for root microbiome structure, which is evident from the differential initial microbial inocula present in different soil types [18,47]. Dombrowski et al. [97] collected the arctic-alpine *Arabis alpine* samples from the native location as well as from those grown under controlled conditions and investigated the root microbiome by 16S rRNA amplicon sequencing analysis. They reported that soil type and length of time the plants remained in the soil are the most important drivers, causing variation of up to 15 % of root microbiota. In addition, in the same soil, the root microbiome of perennial A. *alpina* was similar to A. *thaliana* and *Cardamine hirsuta*, the annual relatives of A. *alpina*. The root microbiome communities are strongly influenced by the composition of the soil microbiome close to roots. A strong correlation between the soil and root bacterial communities in A. *thaliana* has been reported by various authors [3,18]. Similarly, the structure of fungal communities is influenced more predominately by soil type than by host plant [98]. The type of soil influenced the

rhizosphere bacterial microbiota composition in lettuce [99], oak [16], *Arabidopsis* [3], and maize [100].

Several studies have shown that environmental variability, such as soil pH, C: N ratio, soil carbon, water content and biogeography may influence the microbiota composition [39]. Lauber et al. [101] described that the impact of soil pH on total community composition was obvious even at a very high taxonomic level. Analyses revealed that pH has substantial correlation with the structure of these microbiome phyla in all soil types studied. The effects of soil pH on soil bacterial community composition has also been reported in other studies using various methods [102]. Zarraonaindia et al. [103] reported that the composition of soil and root microbiome of grapevine significantly influenced by soil pH and C:N ratio but leaf- and grape-associated microbiota were mostly influenced by soil carbon. Hartman, Richardson et al. [104] reported from their study that pH was the most important factor in predicting the alteration of soil bacterial communities, and they detected changes in phylum-level abundances across the pH levels. However, contrasting results have been reported by Fierer et al. [105], where they noted soil carbon was more important than soil pH for Bacteroidetes, Betaproteobacteria, and Acidobacteria. This difference of the findings was possibly linked to sample sizes and number of soil types investigated as well as to differences in methodologies. The exact mechanism(s) of the role of soil pH on microbial community composition and diversity is unknown. Two general explanations have been given [101]. Firstly, soil pH may indirectly alter bacterial community structure with its influences on soil characteristics. Secondly, soil pH may directly impose a physiological limit

on soil bacteria, changing outcomes of competition or reducing survival of taxa intolerable to condition. In addition, we hypothesize that soil pH may alter plant microbiome by its influence on plant growth and physiology, which also may influence soil microbiome composition. Other soil properties, for example soil temperature and contaminants in the soil, also influence microbiome composition. Heat disturbance of soil results in a shift in rhizobacterial microbiome composition [106]. Heat disturbances due to natural wildfires can cause a decrease in microbial activity and significant alterations in microbial communities [107]. Increasing petroleum hydrocarbon contamination levels results in the alteration of willow microbiome structure. These alterations were less extreme in the rhizosphere and plant tissues, but they were prominent in the bulk soil. These could be because plants provide more controlled conditions and shield microbes against an enhanced contamination gradient [108].

8.2. Cultivation practices

Land use and cultivation practices are the most important causes of declines in biodiversity, leading to undesirable consequences for the environment [109]. Changes in the vegetation influence the diversity and structure of soil microbiomes. Agricultural activities do not necessarily have negative consequences in soil bacterial community diversity and structure, but they may have positive or neutral feedback (effect not perceived) [110]. For example, the intensity of land use (LUI) has been reported to influence the pattern of bacterial communities. Estendorfer et al. [111] found that under low LUI, there remains a strong interaction

between plants and adjacent soil. In contrast, no influence of LUI on microbial diversity has been found in the rhizosphere, which indicates that plant species have much more influence on the rhizosphere community than soil properties do [112]. However, Suleiman et al. [113] reported that plants may have robust core microbiome compositions that are less prone to alteration due to variation of land use, soil type or edaphic factors. It was found that microbiome of in relatively untouched deciduous forest and long-term mowed grassland soils were comparable, although there were significant differences in soil properties and vegetation [114], Continuous cultivation causes changes in soil properties, which in turn might affect the soil microbiome communities. As per the reports of Allison and Martiny [115], there are three potential impacts caused by land disturbance such as the microbial structure might be affected, it might be changed but quickly return to the original composition (resilient), or might remain unchanged.

8.3. Climatic variables

Climate is an important driver of plant and soil microbiome composition. The role of climate in influencing plant microbiome community composition has to date been largely unheeded or found to be of little importance [116]. A recent study, however, across various ecosystems in Britain showed that rainfall and temperature gradients are two major climatic factors in shaping the bacterial community composition in plants [117]. Researchers de Vries et al. [118] reported that precipitation is an important driver of soil microbial community composition. The biomass of fungi and bacteria has been reported to increase with increasing

mean annual precipitation (MAP), and the effect was more prominent for fungi, resulting in comparatively higher fungal abundance (increased F/B ratio) under higher precipitation levels. The above trends could be linked to higher soil organic matter contents in higher rainfall areas. In particular, in uplands where harsh climatic conditions prevail, a higher organic matter build-up is noticeable, which leads to fungi-dominated microbiome communities [119].

9. Conclusions and future perspective

The factors that influence microbiome assemblages and dynamics in plant and soil are now better understood, and research in this aspect is increasing. However, our knowledge on the underlying mechanism(s) of microbiome assemblages and how they influence the host plants is still lacking. Connecting the microbiome composition and diversity to their function is a great challenge for future research. For instance, to what extent could we use and manipulate the plant microbiome to boost sustainable agricultural production and environmental protection? We now know that that host genetic factor has a significant influence on microbiome diversity and structure, indicating that breeding and trait selection provide opportunities to select for desired microbiomes [45]. To have a more profound and broader knowledge on plant microbiome, there is a need to integrate novel molecular approaches (e.g., meta-omics), ecological models (e.g., food web theory, assembly of communities, or coexistence theory), and recent bioinformatics and statistical advances with a view

to correlating community assemblages with ecological functions.

In the future, more emphasis should be placed on identifying the underlying mechanisms that drive microbiome community composition and assembly. We need to know the contributions of (1) the microbe-microbe interactions, (2) soil and other environmental variable and (3) various host traits in shaping community structure. Future research will direct toward solving some pertinent questions. For example, how stable are the drivers of microbiome community? To what extent do agricultural practices affect the microbiome of plant species? How predictable are these divers? With high throughput technologies, such as next-generation sequencing and metagenomics, we can begin to study endophyte microbiomes across hosts, environmental conditions, and at different time points and focus on the mechanisms of the plant-endophyte association.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Check your Understanding

- What are the various types of plant-associated microbiomes and how does each affect plant health?
- How are the compositions of plant microbiomes influenced by the environment?
- What aspects of the host plant can shape its

microbiome?

- In what ways can the plant microbiome be governed by microbe-microbe interactions?

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15. Pollution and Bioremediation

Pollution and Bioremediation

Achieving sustainable life, for humans, animals, and the environment, requires a plan of action to mitigate anthropogenically-induced damage and develop future practices to maintain planetary homeostasis. One of the biggest threats to ourselves and the environment is the buildup of human waste and pollution (e.g. plastics, oil, synthetic products, etc.) that take a tremendously long time to naturally breakdown. With the human population predicted to continually climb, continuing the same destructive practices will only result in more waste generation in a fraction of the time that it takes for decomposition. One alternative means to solve the pollution problem is to use specialized microbiomes for bioremediation. That is, a specially designed microbial consortia could clean up (i.e. breakdown and assimilate) various toxic molecules much more quickly. However, this will take time to understand how and which type of microbes could perform these tasks and if there are any associated (negative) effects.

Alternative Strategies for Microbial Remediation of Pollutants via Synthetic Biology

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Continuous contamination of the environment with xenobiotics and related recalcitrant compounds has emerged as a serious pollution threat. Bioremediation is the key to eliminating persistent contaminants from the environment. Traditional bioremediation processes show limitations, therefore it is necessary to discover new bioremediation technologies for better results. In this review we provide an outlook of alternative strategies for bioremediation via synthetic biology, including exploring the prerequisites for analysis of research data for developing synthetic biological models of microbial bioremediation. Moreover, cell coordination in synthetic microbial community, cell signaling, and quorum sensing as engineered for enhanced bioremediation strategies are described, along with promising gene editing tools for obtaining the host with target gene sequences responsible for the degradation of recalcitrant compounds. The synthetic genetic circuit and two-component regulatory system (TCRS)-based microbial biosensors for detection and bioremediation are also briefly explained. These

developments are expected to increase the efficiency of bioremediation strategies for best results.

Introduction

The remediation processes aided by microorganisms present at the various contaminated scenarios constitute bioremediation (Basu et al., 2018; Kumar et al., 2019). Microbial remediation uses multiple metabolic pathways responsible for enzyme production (Sharma B. et al., 2018; Dangi et al., 2019). These enzymes mainly take part in the degradation pathways of xenobiotics (Junghare et al., 2019). There are different customary methods for bioremediation, primarily based on the site of bioremediation, *in* and *ex situ* (Tomei and Daugulis, 2013). *In situ* is applied to the site to minimize soil disturbance. This method is mostly adopted due to less expenditure from avoiding excavation and transport of contaminated soil (Khan et al., 2004). According to Khan et al., 2004 less disruption in *in situ* bioremediation causes less dust dispersion and hence better degradation (Joshi et al., 2016) of contaminant. Bioaugmentation, bioventing, biosparging, and engineered *in situ* bioremediation are main *in situ* bioremediation methods (Azubuike et al., 2016). *Ex situ* bioremediation methods are solid phase system (composting, landfarming, and biopiling) and slurry phase system (bioreactors) (Kumar et al., 2011). Transportation of soil to accelerate microbial degradation are done by solid and slurry phase systems, whereby treatments of domestic, industrial, and organic waste are done by *ex situ* bioremediation (Juwarkar et al., 2010). These traditional bioremediation methods take time and consume much cost

expenditure, giving less result output. Traditional bioremediation (Duarte et al., 2017) processes showed the above limitations of extra time taking, less removal or dissimilation of pollutants, (Bharagava et al., 2019) disturbance to nature delicacy such as more land coverage for a long time, and a foul smell in the environment (Dangi et al., 2019; Kumar, 2019). Therefore, researchers are eager to discover new bioremediation technologies for best results. Dvoøák et al. (2017) described bioremediation via synthetic biology for boosting bioremediation strategies. This approach can catch the catabolic (Jacquiod et al., 2014) and metabolic complexities for reviewing the potential of the microbial population synthetically. The preliminary information for developing synthetic microbial models for bioremediation can be obtained by mining genes from the databases (Fajardo et al., 2019). The computer logics involvement can determine the microbial cell interactions with recalcitrant compounds (Kim et al., 2015). These strategies can together grasp the natural metabolic potential of microorganisms to transform into novel biological entities of interest (Dangi et al., 2019). Furthermore, the regulation of metabolic pathways (Alves et al., 2018) in a controlled manner can also be achieved for bioremediation processes (Rochfort, 2005). This transition via synthetic biology application (Figure 1) for remediation purposes would improve the bioremediation processes via the involvement of potent (Zhu et al., 2017) dissimilating particular contaminants (Trigo et al., 2008). Synthetic biological systems mediate cellular modulations for efficient functioning and working of existing processes. They permit the modification of cellular processes *viz.* metabolic pathway acting for a particular chemical compound. The

advancement of synthetic biology for bioremediation of various contaminants is attaining the focus of scientists and researchers. For instance, a sustainable synthetic microbial community's establishment for bioremediation is being investigated. Microbial interactions and quorum sensing within communities are vastly studied for application in the area of bioremediation with synthetic biology applications. Achievement of the synthetic genetic circuit of *Pseudomonas putida* proved to be the golden gadget for degradation studies. Besides this, genome editing by CRISPR-Cas, TALEN, and ZFNs adds knowledge for reviewing the progression in bioremediation studies. Synthetic microbial biosensors and metabolic engineering of cellular processes for utilization and detection of contaminant residues will remediate the environment from persistent recalcitrant pollutants. This review is focused on the above mentioned strategies and their elements (Figure 2) applicable for bioremediation purposes and research.

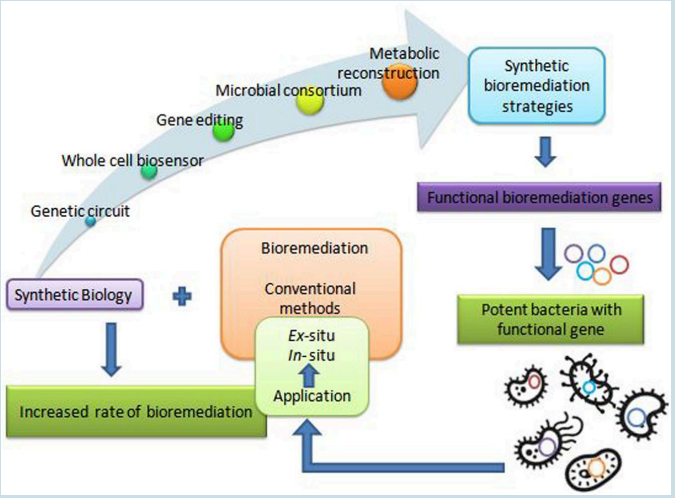


Figure 1. The strategies of synthetic biology applicable for bioremediation.

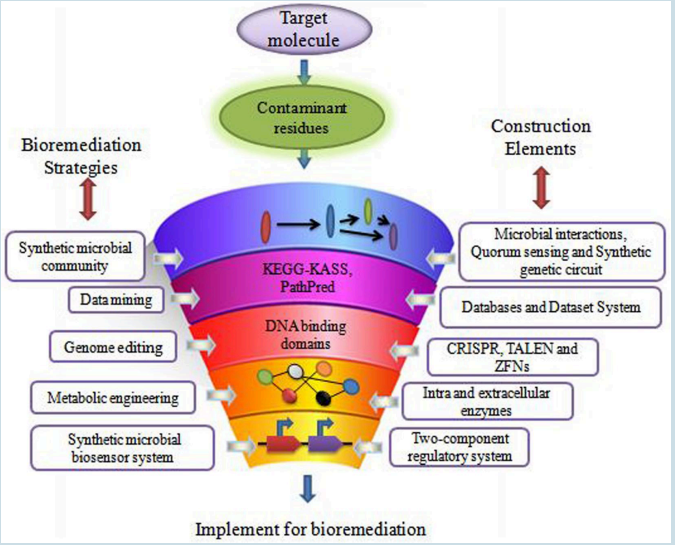


Figure 2. The components and their construction elements of synthetic biology for bioremediation studies.

Metabolic Reconstruction for Designing Synthetic Models

A computational platform is utilized for the reconstruction of cellular metabolism (Agapakis et al., 2012) via metabolic pathway analysis (MPA) (Banerjee et al., 2016). MPA mathematically represents the reactions of metabolism. This method is based on stoichiometric balance reactions so as to propose steady-state metabolic flux during cellular growth. The stoichiometry matrix imposes constraints of flux, making the consumption and production of the compound at a steady state (Bordbar et al., 2014). The maximum and minimum flux of a reaction can also be

determined by providing the topper and least bound. This helps to define the extent of permissible flux supply (Richelle et al., 2016; Rawls et al., 2019). The next step is defining the objective according to the biological problem to be studied. This objective mathematically represents the reactions responsible for the phenotype appearance. The mathematical reactions and phenotype are combined with linear equations and solved by computational algorithms such as COBRA Toolbox11 and Matlab toolbox (Orth et al., 2010; Bordel, 2014). FBA is fundamentally simple, having immense applications in studying gaps, physiology, and genomes via systems biology approach (Kim et al., 2015; Hellweger et al., 2016). These gaps are missing metabolic reactions, making the genome partially known. FBA uses computational algorithms that can predict missing reactions *viz*. OptKnock and OptCom, which can knock out the genes responsible for producing the desired compound (Biggs et al., 2015). These approaches are beneficial for constructing microbial communities for bioremediation of particular contaminants (Khandelwal et al., 2013). However, MPA is the most challenging method when metabolic information is incomplete, making it difficult to obtain a real model (Covert et al., 2001). But this method shows cellular functions in the dynamic community, and thus is very useful for the prediction and exchange of metabolic flux in communities of microorganisms (Khandelwal et al., 2013). Recently, a metabolic model has been constructed by using two *Geobacter* species with parameterized electron transfer and metabolic exchange to characterize syntrophic growth dynamics. Such a system may have useful applications in the field of bioremediation and degradation of particular contaminants (Butler et al., 2010). A computational platform

is also needed for better prediction of engineered genetic pathways for community dynamics. A graph-based tool Metabolic Tinker was developed by McClymont and Soyer to identify thermodynamically feasible biochemical routes for compounds deterioration (Johns et al., 2016). This may be applied to identify the routes for degradation of recalcitrant compounds by microbial consortia. These computational tools are utilized along with omics (Kim et al., 2014; El Amrani et al., 2015) and biological data for desired output (Berger et al., 2013) and toxicity prediction (i.e., META-CASETOX System) (Peijnenburg and Damborský, 2012). These are also applied for functional gene identification and their profile analysis, PCR analysis and drug discovery, etc (Dangi et al., 2019). Computer-aided drug discovery and development (CADDD) is used effectively with chemical and biological aspects, i.e., chemical structures accounting the biological role and its activity via ligand-based drug design, structure-based drug design, quantitative structure-property relationships, and quantitative structure-activity (Kapetanovic, 2008). Furthermore, Table 1 depicts similar methodologies applicable to bioremediation studies. De Jong (2002) analyzed the multicellular feedback control strategy in a bacterial consortium (Bruneel et al., 2011) to define the robustness conceivable under desired conditions.

S.no.	Purpose	Approach	Methodology	References
1.	Degradation study	Functional gene identification for bioremediation	PCR (Polymerase Chain Reaction) product sequence analysis	Jagwani et al., 2018
2.	Cell behavior study	Whole-cell simulation	A computer model for bacterial cell in response to the contaminated environment	O'Brien et al., 2015; Panigrahi et al., 2019
3.	Toxicity of chemicals	Analysis of chemical and biological properties	*In silico* toxicology (IST) protocols for toxicity assessment; QSAR (Quantitative Structure Activity Relationship) model	Pavan and Worth, 2006; Myatt et al., 2018
4.	Identification of functional bioremediating microbe	Target identification	Protein structure prediction, Protein – protein interaction (PPIs)	Shukla, 2017
5.	Remediation of textile dyes	Interaction of protein-ligand	Molecular docking	Sridhar and Chandra, 2014; Kumar et al., 2016
6.	Bioremediation of toxic pollutants	Structure prediction	Biodegradability evaluation and simulation system	Das et al., 2016

Table 1. The methodologies applied for bioremediation research.

They utilized an ordinary differential equations (ODE)-based model and agent-based simulation on a consortium (Hawley et al., 2014; Atashgahi et al., 2018) of interacting species population for increasing the efficacy of the proposed feedback control strategy. The application of bioinformatics (Arora and Bae, 2014) resources is a prerequisite dimension for obtaining the data to begin the microbial bioremediation studies of recalcitrant compounds (Gong et al., 2012; Ofaim et al., 2019). This involves the information related to the degradation of xenobiotics by microbes and their pathways for dissimilation (Dao et al., 2019; Salam and Ishaq, 2019; Thelusmond et al., 2019; Wei et al., 2019). The data related to end products and intermediate metabolites released throughout degradation pathways can also be retrieved (Dvoøák et al., 2017). An extended information source linked to degradation is MetaRouter, allowing data (Singh and Gothalwal, 2018) for life sciences laboratories to explore degradation possibilities of recalcitrant compounds (Mohanta et al., 2015). The information on oxygenic degradation of xenobiotics can be retrieved from OxDBase, a biodegradative oxygenase database (Chakraborty et al., 2014; Shah et al., 2018). Oxygenase is a class of enzyme which transfers the oxygen molecule for oxidizing the chemical compound. They play a role in the degradation of organic compounds by aromatic ring cleavage (Jadeja et al., 2014). OxDBase is very particular in providing knowledge of oxygenases-catalyzed reactions, and is a powerful tool applicable to bioremediation studies (Singh, 2018). Bioconversion and biodegradation of persistent and toxic xenobiotics (Desai et al., 2010; Bao et al., 2017) compounds catalyzed by oxygenases decrease the

compound sustainability and toxicity in the environment (Kües, 2015; Kondo, 2017). Therefore, OxDBase is very helpful in acknowledging the degradation processes involved in bioremediation (Shah et al., 2012). The transcriptional characterization of genes responsible for the biodegradation and biodissimilation of a particular compound has great significance in proposing molecular methodologies. This can be done by the Bionemo (Biodegradation Network Molecular Biology) database (Libis et al., 2016a). Bionemo contains the entries for sequences of genes coding for biodegradation (Carbajosa and Cases, 2010). It also links the gene transcription and its regulation (Libis et al., 2016b). The data retrieved from Bionemo can be used for designing cloning experiments and primers (Arora and Shi, 2010). Garg et al. (2014) used eMolecules and the EAWAG-BBD PPS database for the prediction of pathways involved in the biodegradation of 1-naphthyl-N-methyl carbamate. These above findings empower the researchers to analyze and establish what prerequisites must be fulfilled for developing synthetic bioremediation models.

Designing the Synthetic Microbial Communities

Recent advancements in the field of synthetic biology for environmental issues have shown a great impact. The use of GMOs in environmental biotechnology for remediation (Malla et al., 2018) of toxic compounds, xenobiotics, and pesticidal compounds are being done. To design a synthetic community, it is important to understand natural microbial communities (Schloss and Handelsman, 2008). In a natural community, it is difficult to find out which species are actually taking part in bioremediation (Großkopf and Soyer,

2014). Thus, a synthetic microbial community is a promising method for constructing an artificial microbial community with function-specific species for bioremediation purposes. These communities may act as a model system for the study of functional, ecological, and structural characteristics in a controlled manner. Großkopf and Soyer (2014), defined synthetic community by the culturing of two microbial species under well-defined conditions on the basis of interaction and function (Bruggeman and Westerhoff, 2007). These factors determine the dynamics and structure of the community. It is based upon the identification of processes and patterns engaged in by bacterial species. These microbial interaction patterns are metabolism-driven and responsible for community interaction (Wintermute and Silver, 2010). Social-based microbial interactions (i.e., mutualism, cooperation, and competition, etc.) and the total outcome of these interactions between two microbial populations can be +/+ and −/+ or +/−, respectively (Foster and Bell, 2012). It is said that community structure and function majorly depends on cooperation. The effect of cooperation on community dynamics is determined by engineered cooperation resulting in the synthetic community. Engineered cooperation between two microbial strains (Singh et al., 2016) can be done by manipulation of environmental conditions, i.e., knocking the genes out and in Zuroff et al. (2013). Beyond this, other interaction patterns have been analyzed with engineered microbial species in the synthetic community. Such an application of engineered interaction is highly recognizable in bioremediation strategies (Sharma, 2012). Synthetic biology provides greater potential for the sustainable existence of microorganisms (Dellagnezze et al., 2014) acting together in a large population. Thus, synthetic microbial communities are

proved as a key strategy for the bioremediation of contaminants, i.e., pesticides, petroleum (Kachienga et al., 2018), oil spill, acid drainage (Serrano and Leiva, 2017), etc. For building the synthetic microbial communities, the engineered interspecies and intraspecies interactions can make cellular functions robust and enhance the capabilities of microbial consortia in various contaminated scenarios. Quorum sensing is a bacterial signaling mechanism, which is a density-dependent phenomenon via cell-cell communication and population level behavior. The signaling is done by the release and reception of chemical compounds by microbial candidates in a population. This leads to multicellular behavior (Obst, 2007), offering engineerable tasks to design function specific synthetic communities. These synthetic models can also be exploited to obtain a rational design that can lose the function when subjected to competition with other species in the natural environment. With the evolution of genomic constituents and gene transfer, the possibility of the gradual extinction of genetic circuits is present. Thus, strategies are required to maintain the robustness of the synthetic community, achieved via the synthetic models by the development of synergistic and cooperative properties that reduce instability and loss of function (Johns et al., 2016). A recent study by Coyte et al. (2015) suggests that competition among species is significant in determining the stability of communities, acting as a limiting factor in the stability of the synthetic community. Thus, these dynamics must be accelerated in order to design particular function specific synthetic communities for bioremediation purposes (Coyte et al., 2015).

Genetic and Metabolic Engineering

Enríquez (2016) said that genome editing is an umbrella term that refers to "scientific technological advances that enable rational genetic engineering at a local (gene) or global (genome) level to facilitate precise insertion, removal, or substitution of fragments of Deoxyribonucleic acid ("DNA") molecules, comprising one or more nucleotides into the cell(s) of an organism's genome." Transcription-activators like effector nucleases (TALEN), clustered regularly interspaced short palindromic repeats (CRISPR-Cas), and zinc finger nucleases (ZFNs) are major gene editing tools used (Table 2). The most efficient and simple technique of gene editing has been described as CRISPR-Cas (Kanchiswamy et al., 2016). These tools can boost the process of bioremediation. TALEN has a DNA-binding modular which is sequence-specific for the host genome (Utturkar et al., 2013). TALEN binding to DNA creates a double stranded break (DSB) in the target sequence and leaves sticky ends for stability. Similarly, ZFNs is also a DNA-binding domain composed of 30 amino acids. It introduces DSB at the target site of the host genome by the Fok1 cleavage domain. A new sense of using hybrid nucleases containing TALENs and ZFNs nucleases came to act for solving the molecular complications. The CRISPR-Cas system, on the other hand, has unique action properties of high sequence specificity and multiplex gene editing. This unique property is derived from bacteria *Streptococcus pyogenes* as immunity against the virus. The CRISPR-Cas system consists of crisper derived RNA (crRNA) and trans acting antisense RNA (trcRNA) joined by guide RNA (gRNA).

gRNA directs the Cas9 enzyme to introduce DSB in the target DNA sequence by recognizing it. These gene editing tools create the knock-in and knock-out and are under processing for implementation in bioremediation studies (Kumar V. et al., 2018). Recent reports indicate though that the CRISPR-Cas system is mostly adopted and performed by researchers in model organisms i.e., *Pseudomonas* (Karimi et al., 2015; Nogales et al., 2020) or *Escherichia coli* (Chen et al., 2018; Marshall et al., 2018; Pontrelli et al., 2018). Nowadays, the new insights toward CRISPR tool kits and designing gRNA for expression of function-specific genes related to remediation in non-model organisms (i.e., *Rhodococcus ruber* TH, *Comamonas testosteroni* and *Achromobacter* sp. HZ01) are also suggested in the field of bioremediation (Mougiakos et al., 2016; Jusiak et al., 2016; Hong et al., 2017; Stein et al., 2018; Tang et al., 2018; Liang et al., 2020). For gene editing and metabolic engineering, the contaminant-inhabited bacteria are the most suitable candidates because they are used to survive and harbor in stress conditions of various toxic, recalcitrant and non-degradable xenobiotics, as discussed above. Moreover, understanding metabolic pathways seems to be important in studying the microbial bioremediation (Plewniak et al., 2018), i.e., bioremediation of toxic pollutants by the haloalkane dehalogenases production pathway and decontamination of pyrethroid from the soil via the biodegradation pathway of fenpropathrin studied in *Bacillus* sp. DG-02 (Chen et al., 2014). Metabolic engineering leads to modification of the existing pathway for the betterment of the bioremediation process (Michel et al., 2007). This approach majorly covers the study of microbial enzymes, i.e., oxidases, esterases,

monooxygenases, oxidoreductases, phenoloxidases involved at various degradation steps (Figure 3A; Mónica and Jaime, 2019; Mujawar et al., 2019). Enzyme-based bioremediation has many advantages because it is an eco-friendly process. The genetic approach increases the perspective of getting recombinant enzymes. There are research reports of extracellular enzymes having a role in enzymatic bioremediation. For instance, arsenic bioremediation (Andres and Bertin, 2016; Choe and Sheppard, 2016; Akhter et al., 2017; Biswas et al., 2019) (bioaccumulation and biotransformation) is achieved *via* arsenite oxidase coded by *aio*A gene of *Klebsiella pneumonia* (Mujawar et al., 2019); enzymes released by white rot fungi degrade PAHs (polycyclic aromatic hydrocarbon) (Zhao and Poh, 2008; Košnár et al., 2019), dyes, TNT (2,4,6- Trinitrotoluene) and PCBs (polychlorinated biphenyls) (Gupte et al., 2016; Kutateladze et al., 2018; Sadańoski et al., 2018). Esterase D enzyme acts on β-endosulfan (organochlorine pesticide), producing simpler compounds (Mehr et al., 2017; Chandra et al., 2019). *LiPs* encoding hemoproteins from *Phanerochaete chrysosporium* degrade PAHs. However, incomplete or partial degradation of contaminants lead to simpler non-toxic degradable compounds which can be consumed by microbes (Kumavath and Satyanarayana, 2014) as intermediates in biological pathways or substrate, i.e., *LiP* (lignin peroxidase) dissimilate benzopyrene to three compounds of quinine, namely 1,6- quinone, 6,12- quinine and 3,6- quinine (Gupta and Pathak, 2020). Furthermore, *MnP* (Manganese peroxidase) oxidizes organic compounds in the presence of Mn(II) (Xu et al., 2018; Singh et al., 2019). Laccase, MFO (mixed function oxidases), glutathione S transferase, cytochrome P_{450} also acts in

biodegradation of recalcitrant compounds (Singh, 2019; Boudh et al., 2019). Catechol 1,2-dioxygenase (intracellular enzyme) from *Pseudomonas* NP-6 dissimilate catechol to muconate compounds (Guzik et al., 2011). Also, enzyme immobilization (Cavalca et al., 2013; Sharma B. et al., 2018) increases the half-life, stability, and enzyme activity at a notable level. The enzymatic bioremediation is an elementary, expeditious, and environmental friendly method for microbial removal and degradation of persistent xenobiotics compounds (Sharma B. et al., 2018). Isolation and characterization of microorganisms with enzymatic capabilities have been done with the limitation of less productivity (Rayu et al., 2012). Organophosphates (OP) and organochlorines (OC), major constituents of insecticides, accumulate in the agricultural soil (Panelli et al., 2017) and reach the water bodies via agricultural run-off. Effective bioremediation of γ-hexachlorocyclohexane (OC) and methyl parathion (OP) has been reported by genetically engineered microorganisms (Gong et al., 2016). Moreover, bioremediation of organophosphates and pyrethroids has been experimented with using genetically modified *P. putida* KT2440 (Zuo et al., 2015). With the advent of metabolic engineering, the catabolism and degradation of various persistent compounds has been reported. The degradation pathways of methyl parathion and γ-hexachlorocyclohexane in *Sphingobium japonicum* and *Pseudomonas* sp. WBC-3 witnessed the bioremediation strategy (Liu et al., 2005; Miyazaki et al., 2006). Furthermore, 1, 2, 3-trichloropropane, a persistent constituent of fumigant, is dissimilated into the environment (Techtmann and Hazen, 2016) via heterologous catabolism by the assembly of three enzymes from two

different microorganisms in *E. coli* (Dvorak et al., 2014). A metabolic pathway (Bertin et al., 2011) degrading organophosphorus and paraoxon is engineered by inserting the organophosphorus hydrolase gene (opd) and pnp operon encoding enzymes that convert p-nitrophenol into β-ketoadipate in *P. putida* (de la Pena Mattozzi et al., 2006). A study showed *pob*A and *chcpca* gene clusters of *Rhodococcus opacus* R7 take part in the bioremediation of naphthenic acid; more specifically, expression *ali*A1 gene codes for fatty acid CoA ligase for degrading long chains of linear as well as alicyclic naphthenic acid (Zampolli et al., 2020). To minimize the accumulation, the above-mentioned strategy is attained using microbes for partial or complete mineralization of persistent compounds (Miyazaki et al., 2006).

Features	Gene editing tools			References
	CRISPR	**TALEN**	**ZFNs**	
System	Adaptive immune system	Pathogenic *Xanthomonas*	Gene expression system	Kumar N. M. et al., 2018
Specificity	crRNA	TALE Domain	Zn finger Domain	Jaiswal et al., 2019b
Cleavage	Cas9	*FokI* nuclease	Nuclease	Kumar V. et al., 2018; Jaiswal et al., 2019b
Nucleases per target per experiment	Single or more sgRNA; singleCas9	Single TALEN pair	Single ZFN pair	Hamilton et al., 2019
Activity	High	High	Moderate	Shanmugam et al., 2019
Designing and screening	Easy	Difficult	Difficult	Dangi et al., 2019
Multiple gene editing	Suitable	Not suitable	Not suitable	Sinha et al., 2019

Table 2. Comparative features of CRISPR, TALE, and ZFNs.

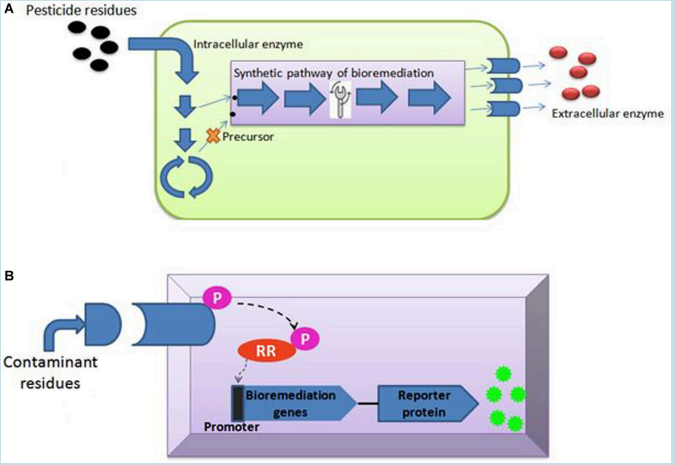


Figure 3. Schematic presentation (A) intracellular and extracellular enzymes production; (B) TCRS based biosensor.

Synthetic Genetic Circuit and Microbial Biosensor

The synthetic genetic circuit requires chassis for implantation. The *P. putida* is a HVB (Host Vector Biosafety) strain recognized as safe by the Recombinant DNA Advisory Committee. It is also referred to as GRAS (Generally Recognized as Safe) to release in the environment. It is ideal for the next generation of synthetic biology chassis panel because it can withstand high intolerant changing conditions including temperature, pH, solvents, toxins, osmotic, and oxidative stress. Also, *P. putida* has versatile metabolism and low nutrient requirements (Pabo and Nekludova, 2000). These qualities make this organism the best bacterial model for environmental bioremediation applications (Tanveer et al., 2018). Recently, the *P.*

putida synthetic genetic circuit has been established for the designing of promoter genes and expression of the gene responsible for the degradation of persistent compounds (Adams, 2016). An extension of synthetic biology is the integration of genome with reporter system, and synthetic promoters of *P. putida* may be developed for synthetic bioremediation pathways. Elmore et al. use serine integrases for synthetic genetic circuit development. Microbial cells have the advantage of a cellular system, which controls cell growth and response to external factors like temperature, light, pH, and oxygen (Tropel and Van Der Meer, 2004). The external environment of microbes inhabiting the contaminated site will respond to concentrations of various persistent compounds present (Ray et al., 2018; Antonacci and Scognamiglio, 2019). Whole cell biosensors for checking the presence, detection and biodegradation potential of xenobiotics compounds (pharmaceutical residues, pesticides, paraffin, PAHs and PCBs, etc.) present (Adhikari, 2019) in environmental samples are attaining attention (Wynn et al., 2017; Heng et al., 2018; Patel et al., 2019). The reporter proteins acting microbe makes a color signal at the detection of particular contaminants *via* transducer (Zhang and Liu, 2016). A biosensor aiming for detection and bioremediation purposes must have enhanced contact between microbe and contaminant (Dhar et al., 2019). This helps the bacterium to adjust their cellular pathways in response to external environmental conditions and encodes the genes for utilizing the recalcitrant compounds as substrate (Bilal and Iqbal, 2019; Skinder et al., 2020). Synthetic biology strategies are feasible for removing a particular toxic compound because the genetic circuits can be developed against the exogenous environmental toxin

(Checa et al., 2012; Tay et al., 2017). The synthetic genetic circuits are assembled via a two-component regulatory system (TCRS) in bacteria (Futagami et al., 2014; Uluşeker et al., 2017). This system acts upon environmental change, and thereby, cells respond to these changes. A prokaryotic TCRS has histidine kinase (HK) and response regulator (RR). The HK is a sensor domain with an extracellular loop present as an integral membrane protein. HK also has a transmitter domain in the last cytoplasmic transmembrane, which is a highly conserved domain. Histidine phosphotransfer (DHp) and catalytic ATP-binding domain (CA) acts for molecular recognition of RR and ATP hydrolysis. The transmitter domain transmits the signal from periplasm to cytoplasm via PAS (Periodic circadian proteins, Aryl hydrocarbon nuclear translocator proteins, and single minded proteins), HAMP (HKs, Adenyltatecyclases, Methyltransferases, and Phosphodiesterases) and GAF (cGMP-specific phosphodiesterases, adenylyl cyclases, and formate hydrogenases) (Casino et al., 2010). Thus, HK senses the external environmental change and adds phosphate to conserved histidine. The HK also regulates RR by phosphorylating the aspartate residues. This promotes the promoter (Figure 3B) binding to activate the gene expression or vice-versa (Ravikumar et al., 2017). Therefore, TCRS-based synthetic biology application for biosensor development for cell-mediated detection and bioremediation can prove to be a new advancement.

Ecological Safety and Risk Assessment

The scientists and researchers are performing the experimental setup to study the bioremediation (Yergeau et

al., 2012) potential against various pollutants like oil spill, plastics, synthetic dyes, organic hydrocarbons (Yadav et al., 2015), pesticides (Jaiswal et al., 2019a), heavy metals (Hemmat-Jou et al., 2018; Lebrazi and Fikri-Benbrahim, 2018), and other xenobiotics, etc (Rucká et al., 2017; Paniagua-Michel and Fathepure, 2018; Wu et al., 2020). Considering that bioremediation is performed in an open environment rather than in a closed fermentation tank, the ecological safety of bioremediation performing bacteria must be considered. Economic safety is justified by the metabolic aptness (Gillan et al., 2015) of microorganisms as compared to other traditional physical and chemical bioremediation methods. Besides, regulation for using genetically and metabolically modified bacteria is released to evaluate the possible risks (Khudur et al., 2019). The risk assessment is mainly done by regulatory agencies, i.e., Organization for Economic Cooperation and Development (OECD) at the application level for environmental safety (Russo et al., 2019; Alam and Murad, 2020; Pastor-Jáuregui et al., 2020). The possible risks are gene contamination in the native member of microbial consortium, leading to mislaying of the natural trait (Mills et al., 2019; Pineda et al., 2019; Rycroft et al., 2019). The competitiveness between natural and genetically modified species can give rise to selection pressure on non-target microflora (Kumar N. M. et al., 2018; Mohapatra et al., 2019). Moreover, environment and ecosystem risk assessments infer unpredictable and adverse effects, as discussed above (Cervelli et al., 2016; van Dorst et al., 2020). Particularly, the ecological risk assessment behind addition of GEMs (Genetically Engineered Microorganisms) (Benjamin et al., 2019; Ahankoub et al., 2020) to the native environment rather than a laboratory (Fernandez et al., 2019) is done mainly because of unnecessary delivery of

antibiotic resistance marker along with recombinant genome of interest (Davison, 2002; Nora et al., 2019), and unintentional uptake or transfer of induced genes to other microorganisms (French et al., 2019; Janssen and Stucki, 2020). This phenomenon is definitely disturbing the microbial native genome entity (Gangar et al., 2019; Petsas and Vagi, 2019). Another aspect to consider is the change in microbial metabolism (Okino-Delgado et al., 2019), which may release uncertain toxic compounds into the environment, indirectly affecting (negatively) microbial candidates in this context (Myhr and Traavik, 2002). Under the TSCA (Toxic Substances Control Act) (Gardner and Gunsch, 2020), the Office of Pollution Prevention and Toxics (OPPT) programs (Pietro-Souza et al., 2020) of the United States Environmental Protection Agency (Leong and Chang, 2020; Saxena et al., 2020) monitors the environmental and health risks and releases premanufacture legal notice for the accreditation of field research outlines and prototypes (McPartland and McKernan, 2017; Khan et al., 2020). A magnificent example is given by University of Tennessee. In 1995, they gave application and suggested the risk evaluation of microbial bioremediation agents (mainly *Pseudomonas fluorescens* HK44) on the environment and health (Sayler et al., 1999; Khan et al., 2016; Sharma J. K. et al., 2018; El Zanfaly, 2019). Remarkably, the literature survey points toward a biowar weapon for humanity (Gómez-Tatay and Hernández-Andreu, 2019; Wang and Zhang, 2019), stating that gene editing tools left in bad hands could mislead ethical and moral duties (Khan, 2019; Thakur et al., 2019; Sharma et al., 2020).

Conclusion and Future Perspectives

The microbial bioremediation process for removal and detoxification of contaminants from the environment has now emerged as the best option. Synthetic biology is addressing the decontamination and remediation strategies for xenobiotics and related compounds from the environment. It has been observed that the requisite for understanding existing metabolic pathways is a must for developing synthetic models of bioremediation. Moreover, genomics reconstruction methods (Luo et al., 2014; Marco and Abram, 2019) and technologies made a solid platform for bioremediation studies. Satisfactory progress has been witnessed in the field of bioremediation among various contaminants with the role of specific genes and enzymes applicable via synthetic biology methodologies. Therefore, it is concluded that microbial synthetic biology remediation strategies not only increase the efficiency of microbial bioremediation processes for a particular contaminant, but also provide the best methodologies for researchers.

Author Contributions

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Check your Understanding

- Explain bioremediation and the roles that microorganisms can have in this process.
- What is synthetic biology and how can it be applied for bioremediation?
- What are some important considerations and challenges for designing a synthetic microbiome?

- Compare the major techniques for genetic and metabolic engineering.
- How could microbial biosensors contribute to bioremediation efforts?
- What aspects of ecological safety and risk assessment must be addressed for application of synthetic microbiomes for bioremediation in open environments? Why?

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PART V

OTHER MICROBIOME APPLICATIONS

16. Forensic Microbiomes

Forensic Microbiomes

Characterizing human and environmental microbiomes is not only important to maintain host and ecological health, but can have other far-reaching applications such as forensic science. Determination of unexplained situations and phenomenon can be quite challenging, and with advances in microbiomics, there is a possibility to generate sufficient data and evidence to provide viable answers and solutions.

Forensic Applications of Microbiomics: A Review

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The rise of microbiomics and metagenomics has been driven by advances in genomic sequencing technology, improved microbial sampling methods, and fast-evolving approaches in bioinformatics. Humans are a host to diverse microbial communities in and on their bodies, which

continuously interact with and alter the surrounding environments. Since information relating to these interactions can be extracted by analyzing human and environmental microbial profiles, they have the potential to be relevant to forensics. In this review, we analyzed over 100 papers describing forensic microbiome applications with emphasis on geolocation, personal identification, trace evidence, manner and cause of death, and inference of the postmortem interval (PMI). We found that although the field is in its infancy, utilizing microbiome and metagenome signatures has the potential to enhance the forensic toolkit. However, many of the studies suffer from limited sample sizes and model accuracies, and unrealistic environmental settings, leaving the full potential of microbiomics to forensics unexplored. It is unlikely that the information that can currently be elucidated from microbiomics can be used by law enforcement. Nonetheless, the research to overcome these challenges is ongoing, and it is foreseeable that microbiome-based evidence could contribute to forensic investigations in the future.

Introduction

For over 100 years, microbiology has played a relatively diminutive role in forensic science (MacCallum and Hastings, 1899). In the early 1990s, the sequencing of amplified viral DNA was used to support a case alleging the transmission of Human Immunodeficiency Virus from a dentist to several patients in Florida, United States (Smith and Waterman, 1992). The emergence of PCR-mediated genotyping of bacteria was considered to be a valuable forthcoming tool in forensics—e.g., van Belkum

(1994) suggested that forensic science would soon be a major area for the application of PCR-mediated genotyping due to the rapidity of technological advances at the time (van Belkum, 1994). In the mid-1990s, fungal and pollen spore analyses were also developed, allowing investigators to differentiate between soil types, which in turn allowed linking substrate items to particular sites (Bruce and Dettmann, 1996; Bryant and Mildenhall, 1998). However, it was not until the early 2000s and the rise of bioterrorism that *microbial forensics*—the "scientific discipline dedicated to analyzing evidence from a bioterrorism act, biocrime, or inadvertent microorganism/toxin release for attribution purposes"—emerged in response to the new threat (Budowle et al., 2003; Carter et al., 2017).

Many forensic applications have been limited to individual taxa analyses, and *microbial forensics* has, historically, been constrained by a lack of available and cost-effective sequencing technologies (Berglund et al., 2011; Kuiper, 2016). This approach has changed dramatically in the last decade as advances in genomic sequencing technology, and new methods for processing complex community datasets (and often low biomass samples) have led to the advent of a new field of microbiomics. The science and study of the microbiome (Statnikov et al., 2013; Capasso et al., 2019) combined with metagenomics (all genomes from a sample) have enhanced the development of the microbial forensic toolkit (Clarke et al., 2017; Hampton-Marcell et al., 2017).

As of March 2019, the conviction rate for homicides in England and Wales (United Kingdom) was only 79% (Office for National Statistics, 2019), slightly higher than in the US (~70%) (Bureau for Justice Statistics., 2019). Across the

globe, there is also a high prevalence of wrongful convictions and often insufficient evidence to convict a perpetrator of a crime (Sangero and Halpert, 2007; Ingemann-Hansen et al., 2008; LaPorte, 2017; Walsh et al., 2017). According to the Innocence Project, a national litigation and public policy organization dedicated to exonerating wrongfully convicted individuals, to date, 375 people in the United States have been exonerated by DNA testing, including 21 who served time on death row (Innocent Project, 2020). There is thereby a strong interest from the public, lawmakers, and the law enforcement system to augment and expand the forensic toolkit, including molecular methods. Microorganisms are abundant in and on the human body (microbial cells can outnumber or equal the total number of human somatic cells) (Noel et al., 2014; Sender et al., 2016; Vázquez-Baeza et al., 2018), in surrounding environments, and on objects associated with a crime (Desmond et al., 2018; Oliveira and Amorim, 2018). A growing body of evidence suggests that forensically relevant microbial profiles could be used as evidence or, at the very least, complement traditional investigative methods (Metcalf et al., 2017; Schmedes et al., 2017; Richardson et al., 2019; Phan et al., 2020). This use of microbial profiles as evidence is done using computational tools that are being developed alongside new approaches in bioinformatics, processing tools, and refined protocols. However, since the field is still in its infancy (Goudarzi et al., 2016; Komaroff, 2018) and historically underfunded (Morgan and Levin, 2019), there is much uncertainty as to the true potential of microbiomic tools in forensics.

In this review, we provide an overview of past, current, and future potential applications of microbiomics in

forensics. Specifically, we will discuss the six most comprehensively researched themes (Figure 1): including geolocation (e.g., substrate analysis and different spatial dimensions and the power of machine learning), personal identification, biological sex determination, trace evidence, manner and cause of death (e.g., death by drowning), PMI, and other applications (e.g., localization through animal microbiomes).

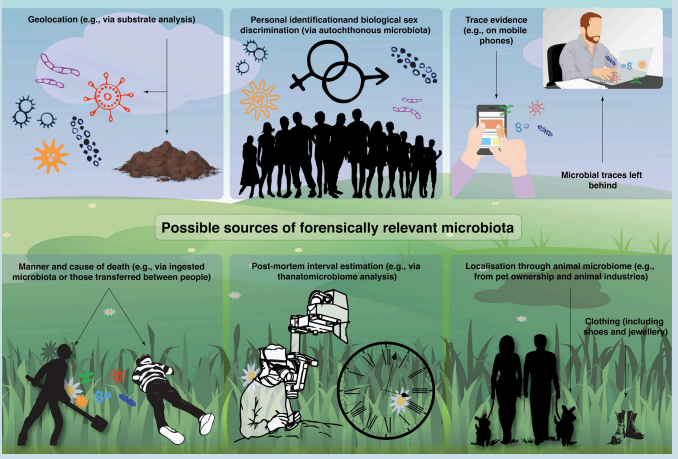


Figure 1. A summary of possible sources of forensically relevant microbiota identified by the literature review.

Major Themes in the Forensic Microbiology Literature

Geolocation

In the past few years, intensive work has been carried out to characterize environmental microbiome, particularly in urban environments and transit systems. These studies have demonstrated that unique community profiles may exist in certain areas of a city (Afshinnekoo et al., 2015; Rosenfeld et al., 2016), as well as "molecular echoes" of environmental events, and even a forensic capacity for geospatial microbiomic data (MetaSUB International Consortium, 2016; Danko et al., 2019a). In the following, we focus on two leading aspects of geolocalization.

Substrate Analysis

The potential of analyzing microbial profiles from the soil is increasingly being recognized in forensic microbiological research. Both the rhizosphere and bulk soil microbiomes exhibit a high level of heterogeneity between different sites. As such, with methodological refinement, soil microbiome samples could provide valuable biogeographic data to localize the origin of the soil sample. Another potential application is the acquisition of information to help determine the provenance of an item(s) associated with a crime.

Habtom et al. (2019) demonstrated distance-decay

relationships between microbial samples from the soil at a local scale (25–1,000 m) (n = 5 sites, n = 2–4 soil types, and five replications). The results showed that the greater the distance between the samples, the more they differed, suggesting that both soil type and geographic location are important factors in determining microbial community composition. Indeed, patch discrimination using the soil microbiome has previously been demonstrated (Macdonald et al., 2011), and Jesmok et al. (2016) correctly classified 95.4% of soil bacterial profiles to their location of origin using various methods including abundance charts, non-metric multidimensional scaling, analysis of similarity, and k-nearest neighbor. However, this was a feasibility study with a modest sample size (n = 19). Further studies with larger sample sizes and replications are needed to explore the full potential of this approach.

Evaluating the microbial communities in an assemblage of soil samples (e.g., soils from a crime scene or alibi site and other intermediary sites) could be useful in forensics. Samples originating from a mixture of different soil substrates have been correctly differentiated by using a combination of Ribosomal Intergenic Spacer Analysis and 16S rRNA gene sequencing (Demanèche et al., 2017). Recent evidence suggests that 18S rRNA gene sequencing can also provide greater discriminatory power over traditional Mid-Infrared spectroscopy at fine scales for eukaryotic species (Young et al., 2015). Furthermore, Sanachai et al. (2016) demonstrated that the site origin of soil, obtained from the sole of a shoe, could be elucidated by comparing the similarity of soil bacterial 16S rDNA profiles acquired by the denaturing gradient gel electrophoresis technique.

Despite the potential in this field, further limitations

need to be identified and addressed. For example, Pasternak et al. (2019) identified several potentially limiting factors to consider when interpreting the results of microbiomic analyses. For instance, soil samples are incredibly complex and highly heterogeneous even at short spatial scales, which presents a major issue to using these in a forensic context, and microbiomes exhibit a high level of physical, chemical, and biological diversity in both space and time. Pasternak et al. (2013) showed that actinobacterial fingerprints significantly differed between two seasons (summer and winter) at the same sites—implying that temporally-associated issues could arise. Keet et al. (2019) have also pointed out that soil microbiome composition can change as a result of abiotic soil conditions and plant community patterns—which poses a considerable challenge to the accuracy of results.

Metagenomic analysis of gravesoil and the soil around human and non-human animal cadavers has been undertaken for forensic purposes. Carter et al. (2015) investigated microbial community succession in soils associated with swine cadavers across two seasons (summer and winter). They demonstrated that postmortem microbial communities changed in specific and reproducible ways, but decomposition effects on soil microbial communities differed significantly between seasons. The authors suggest that the ecological succession of microbial communities will be useful for forensic investigations, but future research should aim to gain a greater understanding of seasonality on decomposition. The sample size for this study was not explicitly stated, but according to the ordination plots, it appears to be modest

($n \leq 10$ per treatment). Therefore, results should be interpreted with caution.

Adserias-Garriga et al. (2017) investigated daily thanatomicrobiome changes in the soil as an approach to estimate PMI. They collected soil samples from around human cadavers ($n = 1$ male and $n = 2$ females) and demonstrated successional changes on a daily basis. Rapid growth of *Firmicutes* was observed from the bloat stage to advance decay (<5% relative abundance at day 1 to 75% relative abundance day 12), and the authors proposed a *Firmicutes* growth curve to estimate PMI. However, the authors state that the growth curve results may only apply under Tennessee summer conditions and that confirmatory research is needed using a larger number of cadavers and under different environmental conditions. The results do, however, corroborate those of Finley et al. (2016), who evaluated microbial communities associated with gravesoil human cadavers ($n = 18$). The researchers allowed the cadavers to decompose over a range of decomposition time periods (3–303 days) and showed increases in the relative abundance of *Firmicutes* in surface bodies over the decomposition period (from ~10% at 0–3 months to ~40% at 7–9 months).

Singh et al. (2018) investigated the spatial (0, 1, and 5 m) dynamics of human cadaver decomposition on soil bacterial community structure. They collected soil samples from each spatial buffer ($n = 14$ for the 0 m, $n = 17$ for both 1 and 5 m) and observed evidence of a predictable response to cadaver decomposition that varied over space. Bacterial community composition (beta diversity) at 0 m was significantly different from the 1 and 5 m communities, whereas there were no significant differences between the

1 and 5 m communities. The researchers also found that bacterial alpha diversity was significantly lower in the 0 m samples, suggesting that the additional nutrient input from the cadavers may reduce bacterial alpha diversity. This study provides additional spatial-compositional insights to complement the growing body of knowledge in this area of forensic microbiome applications.

Soil microbiome analysis has the potential to be used in forensics; however, additional research is required to validate the sensitivity and reproducibility of results (Young et al., 2017). Overall, to gain a greater understanding of both spatial and temporal dynamics associated with the microbiome and to develop techniques to mitigate similar pitfalls, further microbiome surveys are essential.

Different Spatial Dimensions and the Power of Machine Learning

The growing interest in sampling and predicting environmental microbiome profiles at different spatial scales and orientations (e.g., between households, cities, states, and altitudes) to provide information on the location and provenance of people and objects resulted in the development of a multitude of approaches. Chase et al. (2016) identified the three cities where nine offices were located with 85% accuracy based on analyzing office microbiome samples using sampling plates, and although this study suffers from a small sample size (n = 3 per office), it demonstrates potential with further refinement. Lax et al. (2014) analyzed samples from household occupants (n = 1625 from 18 participants in 10 houses) and their built

environments. The authors matched feet microbiome samples to the house with 82.9% accuracy—a relatively low degree of accuracy from an evidentiary perspective but demonstrating the potential of such methods for fine-scale biolocalization. Walker and Datta (2019) analyzed whole-genome sequenced microbiota sampled from 12 cities in seven different countries as part of the 2018 CAMDA MetaSUB Forensic Challenge. The CAMDA dataset (n = 30) included three mystery samples. The authors applied machine learning techniques to identify the geographical provenance of the microbiome samples. Up to 90% of the samples were correctly classified, demonstrating the potential of machine learning applications to biogeography, although further evidence is necessary to employ these applications in an evidentiary context. In a related study, Ryan (2019) applied a random forest classifier built on a dataset of 311 city microbiome samples. Their method correctly classified 83.3% of the mystery samples. Grantham et al. (2019) presented a different algorithm for predicting the geolocation of fungal samples from dust (n = 1300) in the United States using deep neural network classifiers. Applied to a global dataset of samples from 28 countries, the authors state that their algorithms make "good point predictions" with >50% of the geolocation errors under 100 km for US-wide analysis and nearly 90% classification accuracy of a sample's country of origin for the global analysis. This particular field, combining microbiomics and machine learning, is in its infancy, and future studies would benefit from larger sample sizes and improved classification accuracy before such approaches can be used with confidence in a forensic context.

Another important spatial factor to consider is that microbiome compositions do not only differ in horizontal space. Skin microbiomes have also been shown to differ between humans living in high and low altitudes. For example, Zeng et al. (2017) collected skin microbiome samples from humans (n = 99) and pigs (n = 82) in Tibet. They found enrichments of several bacterial taxa (e.g., *Arthrobacter* sp., *Paenibacillus* sp., and *Carnobacterium* sp.) in samples collected from higher altitudes. Alpha diversity was also significantly lower in skin samples collected from individuals living at higher altitudes. This suggests a potential future route to determine geolocation based on altitudinal parameters via the analysis of skin microbiome samples in the future—although here too, methodological refinement will be essential. Furthermore, understanding how skin microbiomes may fluctuate throughout the life course will also be an essential factor to consider.

Overall, all the models prioritize classification over prediction abilities. To enable real-time prediction of geographical coordinates from sampling data, increasing the sample sizes geographically and temporally, and developing more rigorous methods is essential.

Personal Identification

A growing body of evidence suggests that human individuals may be uniquely identified based on stable autochthonous (i.e., native to a given environment) microbial profiles. This could have a substantial impact on forensic science—for example, in situations where the

investigator cannot retrieve sufficient amounts of human DNA (i.e., from human somatic and germ cells). Yet it is unknown whether the variation in microbial communities between people is sufficient to identify individuals within large populations uniquely or stable enough to place them over time.

To answer some of these questions, Franzosa et al. (2015) tested different body site-specific microbial profiles and attempted to match them with 25–105 microbiome profiles during the person's first and second visits to the sampling site. The authors reported that these profiles were useful in distinguishing individuals at the initial sampling time point and that 30% of the individuals were still uniquely identified several months later. In this study, gut microbiome samples were used to pinpoint 80% of individuals (n = 120) up to a year later. These results are encouraging—particularly in shorter timescales—however, they still suffer from relatively high variability. As such, greater improvements, e.g., in methods and sampling effort, will be needed before such approaches can be useful in a forensic setting.

High resolution melting analysis that targeted the 16S rRNA gene from oral swab samples have also been used to demonstrate its potential in distinguishing between individuals (Wang et al., 2019), albeit with a very small sample size in this study (n = 5). Schmedes et al. (2018) demonstrated accurate identification of individuals (n = 12) based on skin swab samples from different body sites (n = 14). They achieved 97% accuracy by sampling shirts and 96% accuracy using palm samples based on 1-nearest neighbor classification on nucleotide diversity of the bacterial genome. In another recent study, the

researchers utilized a similar approach to identify individuals (n = 51). They analyzed microbiome samples collected from three different body sites—the manubrium (i.e., the upper-most segment of the sternum), the palmar surface of the hand, and the ball of the foot (Woerner et al., 2019). The researchers achieved 100% classification accuracy when conditioned on a maximum nearest neighbor distance for diversity, suggesting strong potential should these results be replicable in studies with much larger sample sizes.

Watanabe et al. (2018) suggested that minor taxa are one of the key factors for distinguishing between individuals. Their study analyzed microbiome samples (n = 66) from individuals (n = 11) over 2 years and achieved 85% accuracy in distinguishing individuals. They also used the same analytical methods to classify publicly available skin microbiome samples from individuals (n = 89) with a 78% identification accuracy. However, this level of accuracy is unlikely to be sufficient for forensic applications. The authors suggested that although personal identification is possible, the estimation of the accuracy decreases for larger cohorts due to increments of similar microbiome patterns. Overall, the use of microbiomics as a forensic tool to determine personal identification shows potential and technological viability and might be useful in situations where the investigator is unable to retrieve sufficient amounts of human DNA. Nonetheless, the findings fall short of the burden of proof. Improvements in the model's sensitivity and specificity are required, and a methodology to address potential contamination issues. Furthermore, a better understanding of the microbial dynamics across time

and space is essential for the findings to have a forensics value.

Biological Sex Determination

Recent evidence supports another contribution of microbiomics toward personal identification –the discrimination of biological sex, which could be useful where sufficient quantities of human DNA are unable to be retrieved. For example, airborne bacteria communities have previously been characterized in indoor environments (Chan et al., 2009). Luongo et al. (2017) investigated airborne bacterial and fungal diversity (i.e., constituents of the "aerobiome") from different University dormitory rooms (n = 91). They used machine learning techniques and were able to predict the biological sex of room occupants with 79% accuracy based on relative abundances of the microbiota. Curiously, rooms occupied by males exhibited higher relative abundances of the microbiota. The authors suggested that it could be because males may shed more biological particles or use fewer cosmetic barriers such as skin lotions.

Biological sex-related differences in the human thanatomicrobiome—the microbial communities colonizing organs following death (*thanatos*, Greek for death) (Zhou and Bian, 2018)—have also been demonstrated by Bell et al. (2018). The authors compared amplicon signatures (using the 16S rRNA gene V1-2 and V4 regions) in the corpse heart tissue of 10 individuals and discovered key differences between males (n = 6) and females (n = 4). For example, *Streptococcus* sp. was found exclusively in male

heart tissues, whereas females had a significantly higher prevalence of *Pseudomonas* sp. With refinement, such an approach could help to determine the biological sex of a corpse and the provenance of body parts.

In a study by Tridico et al. (2014), the authors "readily distinguished" male (n = 3) and female (n = 4) subjects based on the analysis of their pubic hair microbiomes. They identified *Lactobacillus* spp. that were unique to female participants. They also suggested that pubic hair is relatively insulated from the environment and colonized with niche-specific microbiota, which could be useful in forensic investigations. Unfortunately, the modest sample size of this study limits the conclusions that can be drawn from it. Nonetheless, the findings were supported by another small study by Williams and Gibson (2017), who identified individuals (n = 9) and their biological sex from pubic hair microbiota with an error ratio of 0.027 ± 0.058 and 0.017 ± 0.052, respectively. However, the sample sizes for all these studies are modest, and as such, further validation studies with larger sample sizes are needed before reliable conclusions can be drawn.

Interestingly, Phan et al. (2020) analyzed skin microbiome samples from both genders (n = 45) and found that the absence of the bacterial genus *Alloiococcus* could be useful in predicting female biological sex. The study showed a correlation between certain bacterial species and personal characteristics (e.g., biological sex). They specifically explored the presence/absence of microbiota from fingermarks left behind on surfaces and achieved a relatively modest 67% sex prediction accuracy using leave-one-out cross-validation analysis. Improvements in sample sizes and machine learning accuracy are necessary to

explore the potential of this approach further. Additional research into whether certain bacteria (and other microorganisms) are distinct to females or simply related to external factors (such as cosmetic products on hands) would also be necessary.

Trace Evidence

There is an increasing interest in studying forensically relevant microbial profiles left behind on objects and surfaces. For instance, several studies showed that there is often a high level of bacterial presence on personal objects such as mobile phones (Koroglu et al., 2015; Kõljalg et al., 2017; Koscova et al., 2018; Kurli et al., 2018). Furthermore, human-associated items such as shoes and mobile phones have been shown to support distinct microbiomes (Lax et al., 2015; Coil et al., 2019).

Meadow et al. (2014) investigated the potential utility of mobile phones as "personal microbiome sensors." They selected 17 individuals and collected three samples (the cell phone's touch surface, their index finger, and their thumb). They demonstrated that bacterial communities sampled from mobile phones were more similar to their owners than other people. They found that about 22% of the taxa on participants' fingers were also found on their phones, whereas only 17% were shared with other people's phones. An individual's index finger shared approximately 5% more OTUs with their mobile phone than with everyone else's mobile phone in the study. Furthermore, 82% of the OTUs were shared between a person's index finger and their

phone. Although promising, here again, the sample size and accuracy of results need to be increased in future studies.

Kodama et al. (2019) found that postmortem skin microbiomes could be associated with personal objects with a high degree of accuracy. Several of the objects in the study were associated with 100% accuracy (i.e., medical devices, eyeglasses, bottles, and steering wheels), whereas objects like computer devices, remote controls, and cell phones were associated with over 67% accuracy, suggesting that with refinement, skin microbiome samples could be reliably linked to objects at the scene. Furthermore, studies have found that the postmortem skin microbiomes were stable and similar to antemortem skin microbiomes for up to 60 h postmortem (Kodama et al., 2019).

Salzmann et al. (2019) investigated the microbial profiles of different bodily fluids (n = 22). They identified source-specific microbial signatures from various bodily fluids. For example, the phyla *Proteobacteria* was associated with skin and semen sources, whereas *Firmicutes* showed a higher prevalence in saliva and vaginal secretions. Dobay et al. (2019) suggest that even when body fluid is exposed to indoor conditions for 30 days, samples continue to harbor body-site-specific microbial signatures. Hanssen et al. (2017) also demonstrated promising results, albeit with a small sample size (n = 6), for the microbially-mediated classification of body fluids. They performed pattern recognition by fitting a linear discriminant analysis model using Principal Component scores and were able to classify saliva samples in 94% of the cases.

Neckovic et al. (2020) recently investigated the potential transfer of skin microbiomes between individuals and substrates (i.e., allochthonous microbiota). They found that

skin microbiota has been reliably transferred through direct contact, that is, between individuals shaking hands. Microbiota also transmits through indirect contact, as demonstrated by individuals rubbing a substrate and then swapping substrates with another person. The authors suggested that such analysis could be useful to corroborate sexual assault cases or other contact-related crimes. They also suggested that further research should consider the relative surface area of contact, pressure, friction, and the duration of the contact.

Manner and Cause of Death

The 'manner of death' is a determination made by an expert following an investigation (e.g., a coroner, the police, or a medical examiner). Five manners of death are generally considered: natural, accidental, suicide, homicide, and undetermined (Advenier et al., 2016). Lutz et al. (2019) recently collected microbiome samples from 265 corpses from Finland, Italy, and the United States. The inspected cadavers differed in the manners of death: accidental death (n = 88), natural death (n = 106), homicide (n = 23), and suicide (n = 45). Their results suggested that *Lactobacillus*, *Enterobacteriaceae*, *Sediminibacterium*, *and Rhizobiales* were associated with different manners of death. With further research, these associations could be developed into predictive markers that help to determine the manner of death. However, as noted by the authors, *Sediminibacterium* and *Rhizobiales* bacteria may also represent environmental contamination, which needs to be controlled, and further validation through controlled

experiments is needed to improve the reliability of their approach to determine the manner of death.

The potential of this microbiomics approach to determining the manner of death was corroborated in a recent study by Zhang et al. (2019) who, by obtaining samples during routine death investigations at the Wayne County Medical Examiner's Office (Detroit, Michigan, United States), found different biomarkers associated with the manner of death. In this study, *Xanthomonadaceae* was more prevalent in cases related to hospital deaths, whereas *Actinomyces* sp. tended to be more prevalent in suicide cases. Increasing the numbers of samples generally increased the accuracy of the models. The authors cautioned that the prediction accuracy depends on the machine learning methods used and the number of anatomical sites analyzed. The authors suggest this study provides baseline information, and it could be possible to use machine learning to develop reference databases that allow microbially-mediated manner of death predictions in the future.

Kaszubinski et al. (2020) modeled beta-dispersion to test for manner and cause of death association using a microbiome data set of $n = 188$ postmortem cases (five body sites per case). The researchers demonstrated that beta-dispersion and demographic data could distinguish among manner and cause of death. In particular, they found that cardiovascular disease and drug-related deaths were correctly classified in 79% of cases. They found that binary logistic regression models were most effective at improving model success. This was an improvement over multinomial logistic regression models, which confirmed the manner and cause of death assessment only 62% of the time. The

results of this study show promise for using postmortem microbiomes to indicate the manner of death. However, as the researchers' highlight, sample sizes need to be greater. Moreover, the development of large databases will likely be required to train models with high success rates prior to being used in practical forensic contexts.

In terms of cause of death (i.e., the disease or injury that produces physiological disruption in the body leading to death), researchers such as Christoffersen (2015) have investigated the importance of microbiological testing. Studying autopsy results (n = 42), the author reported that the cause of death could be determined in 42% of the cases via microbiological analysis. The study highlighted factors indicative of a microbiologically related cause of death, such as a raised CRP measurements. Raised CRPs have also been implicated in SIDS as a cause of death (Rambaud et al., 1999; Szydlowski et al., 2013) and even for astronauts returning from space (Garrett-Bakelman et al., 2019). Deadly bacterial infections, such as infection or sepsis, may also occur following neonatal circumcision (Elhaik, 2016, 2019).

A specific forensic microbiome application for determining the cause of death is the diagnosis of 'death by drowning,' which is one of the leading causes of unnatural deaths worldwide (Domínguez et al., 2018; Cenderadewi et al., 2019). Analyzing the presence of diatoms (single-celled algae) has been the 'gold standard' for well over a decade; however, its reliability has been questioned (Kakizaki et al., 2009; Huys et al., 2012). Several studies have provided support for death by drowning diagnoses by designing real-time PCR assays with primers to detect bacterial species associated with aquatic environments, such

as *Aeromonas* spp. (Aoyagi et al., 2009; Uchiyama et al., 2012; Rutty et al., 2015; Voloshynovych et al., 2019). These studies provided support for this cause of death diagnosis based on relatively high detection rates of microbiota, for example, 84% (n = 32), 75% (n = 20), and 84% (n = 43)—although to strengthen the cause of death diagnoses, the accuracy levels, and sample sizes could again be much improved. It has also been suggested that bioluminescent bacteria may be biomarkers for death by drowning in seawater. For example, Kakizaki et al. (2009) developed a simple assay targeting the 16S rRNA gene to identify bioluminescent colonies such as *Vibrio fischeri* and *Vibrio harveyi*. More recently, Lee et al. (2017) analyzed microbiome composition and pulmonary surfactant protein (SP-A) expression to develop a marker for diagnosis of death by drowning. They analyzed microbiota and histological appearance of both drowned and postmortem groups of experimental rats, comparing freshwater vs. marine water treatments. The authors found that 5513 and 5480 OTUs were unique to marine and freshwater, respectively. They also found that expression levels of SP-A were higher in the lungs of drowning victims compared to postmortem submersion. These findings could have important forensic value (e.g., determining both the type of environment and the timing of death) and demonstrate good potential for future applications. Marella et al. (2019) point out that other studies have focused on the presence of fecal bacteria, coliforms, and streptococcal bacteria to help determine the cause of death by drowning. These bacteria are sampled from the femoral artery and vein and the right and left ventricles. Fecal bacteria are considered to be always present in subjects who drowned

compared with those with other cause of death diagnoses (Lucci et al., 2008; Marella et al., 2019). For example, Lucci and Cirnelli (2007) found fecal streptococcal presence in 100% of the freshwater drowning cases they studied (n = 22) and coliforms present in 90.91%. In this study, the control subjects (n = 30) uniformly showed an absence of fecal bacteria. In a later study, Lucci et al. (2008) assessed if the presence of these bacteria in the drowning medium could be detected in victims submerged after death. The researchers collected samples from drowned victims (n = 5 freshwater and n = 5 in seawater) and victims who were submerged after death (n = 3). Coliforms and streptococci were detected in all drowned victims but not in those submerged after death. These findings suggest that fecal coliforms and streptococci could be used as markers of drowning. However, the minuscule sample sizes must be interpreted with caution and increased considerably in future studies.

Postmortem Interval

The Thanatomicrobiome

Determining the PMI (the time elapsed since a person has died) is often an essential part of a criminal investigation. To improve PMI prediction accuracy, researchers have begun examining the thanatomicrobiome (Javan et al., 2016; Burcham et al., 2019). Postmortem, these communities overwhelm the immune system allowing for subsequent colonization (Javan et al., 2019). Preliminary studies suggest that these microbial communities may

undergo important successional changes in organs that could aid in determining the PMI (Adserias-Garriga et al., 2017).

Early studies on model animals suggest that this is feasible. For over a 48-day period of decomposition, Metcalf et al. (2013) aimed to uncover a "microbial clock" to provide an estimate of PMI by sequencing the 16S rRNA gene for bacterial and archaeal communities and the 18S rRNA gene for microeukaryotes. Their model provided reliable PMI estimates (±3 days) (n = 223). However, the study was conducted in controlled conditions using experimental mouse models—thereby necessitating a degree of caution when extrapolating the data to 'real-life' situations. Another study investigated the decomposition of pig cadavers. Their model predicted the PMI within 2–3 h of the time of death with 94.4% accuracy (Pechal et al., 2014), demonstrating promise with further methodological refinement. Pechal et al. (2018) carried out a large-scale study of body microbiome samples (n = 188) that found postmortem microbiomes were stable, reflecting antemortem microbiomes 24–48 h after death. The researchers also found that specific bacterial taxa were important in predicting health status. For example, *Haemophilus* and *Fusobacterium* were twice as abundant in healthy individuals, whereas *Rothia* was 0.09 times more abundant in heart disease cases. With further development, this could be used to indicate the state of human health during clinical investigations into a range of deaths, from chronic and natural to sudden and violent (Pechal et al., 2018). It is important to note that, although appropriate at the time, the bioinformatics approach used to process OTUs and to make functional predictions (e.g.,

QIIME 1.8 and PICRUSt 1) is now considered to be outdated. Furthermore, Amplicon Sequence Variants (ASV) may provide a richer taxonomic picture (Callahan et al., 2017).

Studying human subjects, Johnson et al. (2016) sampled the skin microbiome of decomposing human cadavers and developed an algorithm to estimate PMI. The authors achieved low error rates for skins samples and a PMI estimation accuracy of ±2 days (n = 144 from 21 cadavers), a substantial improvement compared to prior efforts (e.g., via entomological analysis). Belk et al. (2018) used 16S rRNA amplicon sequencing and found that creating models with the class or phylum taxonomic levels provided the most accurate predictions of PMI. This finding corroborated the study by Johnson et al. (2016) and illustrated its potential usefulness for forensics.

Another study using 454 pyrosequencing to determine abundances and diversity of the postmortem microbiome in several key organs such as the brain, heart, liver, and spleen found varying PMIs ranging from 29.5 to 240 h (Can et al., 2014). This study revealed that the most abundant taxa in postmortem microbial communities were the anaerobic, spore-forming Firmicute bacteria, *Clostridium* sp. Javan et al. (2017) confirmed that *Clostridium* sp. dominated at long PMIs, adding evidence to support the use of microbiomics in PMI determination in the future.

Localization Through Animal Microbiomes

Several studies have shown that animals from different taxonomic groups and environments possess unique

microbial profiles. For example, Tibetan chickens *Gallus gallus*, Chinese Rhesus macaques *Macaca mulatta*, and plateau sheep *Ovis* spp. have unique gut microbiomes (Zhou et al., 2016; Huang et al., 2017; Zhao et al., 2018) shaped by genetic, geographical, and altitudinal factors. It has been demonstrated that the skin microbiomes of Estrildid finches, amphibians, bats, cetaceans, and dogs *Canis lupus familiaris* are unique (McKenzie et al., 2012; Avena et al., 2016; Erwin et al., 2017; Torres et al., 2017; Engel et al., 2018; Russo et al., 2018). Interestingly, Song et al. (2013) found that humans share microbial communities with their dogs.

With further investigations and methodological refinement, such capabilities point to the potential feasibility of linking a person with a site based on shared microorganisms with animals. Although further studies are needed, there is potential for forensic pathways to associate trace microbial profiles obtained from other species (unique to the given species) to a given environment and/or occupation (e.g., animal industries) or to pet ownership. For example, non-human animal-specific microbiota could potentially be detected on the body or clothing of a suspect or victim, which may be useful in the absence of sufficient animal DNA (i.e., from somatic and germ cells) evidence. This profile could then conceivably be traced to the point of contact with an animal or animal-based environments such as equine stables, pet shops, or zoos, thus complementing other traditional forensic evidence. However, this approach is mostly theoretical at the moment, and future research will be needed to test its feasibility.

Discussion

As of today, microbiome-based forensics are almost absent from criminal investigations and courts. To explain why this is so, we may divide the different possible applications of microbial forensics into two groups: first, reconstruction issues such as the cause of death and PMI, which ask "what happened?" and help elucidate the circumstances of the crime; and second, comparison issues such as geolocation and personal identification, which ask "how similar are these two DNA profiles?" and may (dis)connect a suspect from an object or a place (e.g., murder weapon or crime scene). Reconstruction applications for forensic use are easier to develop since competing propositions are usually well-defined and limited in number; if sufficient research is invested in ascertaining the microbial characteristics associated with each combination of possible environmental, spatial and temporal conditions, then reconstruction becomes straight-forward. For example, if a cadaver is found buried at a depth of 1 m in a desert in summertime, and the temporal succession of the gut microbial community for these environmental conditions has previously been established, then PMI can be inferred with a high degree of accuracy and certainty. Thus, reconstruction microbiomics can readily pass the *Daubert* standard set by the US supreme court (Daubert v. Merrell Dow Pharmaceuticals Inc, 1993) to be recognized as admissible evidence bearing sufficient scientific foundation, including general acceptance in the scientific community, known and acceptable rate of error, and so on.

Comparison microbiomic tools, on the other hand, may provide greater benefit to the criminal investigation but are harder to develop to a level that would satisfy the *Daubert* standard. The most beneficial way to employ such tools would be in a "one-to-many" configuration, similar to forensic human DNA analysis: a DNA profile from trace evidence is compared to all the profiles (from known persons and locations) in a database, and if it exists in the database, its frequency in the relevant population (e.g., of soils) is calculated to enumerate the probability of encountering this profile by chance (i.e., originating from a location or person unrelated to the crime). At this time, however, there are hardly any relevant forensic databases of microbiomes that can be compared to trace evidence. In their absence, the only way to proceed in a forensic context is in a "one-to-one" configuration. For each criminal case, questioned samples (e.g., from a suspect's shoe) are compared to context samples from the crime scene, alibi area, and other relevant sites. This approach provides less benefit to the investigation because it can only give conclusions of exclusion, that two samples do not share the same origin, or relative conclusions, such as "sample A is more similar to sample B than it is to sample C." Inclusionary conclusions such as "sample A is very similar to sample B; the probability of a different sample, from another place, person or time, being this similar to sample B at random is 1 in X Million" is impossible without either an extensive database (which is costly to build and maintain) or thorough theoretical knowledge (which we do not have yet) regarding the factors that shape microbiomes. However, even for "one-to-one" analyses, we can provide a statistical evaluation of the evidence (Habtom et al., 2019)

based on the Likelihood Ratio framework as recommended by the European Network of Forensic Science Institutes (Willis et al., 2015).

There are three more major hurdles to forensic tools of comparative microbiomics. The first one involves samples of mixed origins, containing substrates or DNA from different locations or persons. DNA analysis of mixtures is difficult even with simple human DNA profiles, and certainly more so with microbiomic DNA profiles which are far more complex. It is often impossible to tell with certainty which, or even how many, disparate microbiomes are present in the mixture, let alone accurately infer the DNA profile of each one. The famed 2016 report of the US President's Council of Advisors on Science and Technology [President's Council of Advisors on Science and Technology (US), 2016] found that the prevalent subjective analysis of complex human DNA mixtures by forensic experts is not a reliable methodology. Consequently, several computer programs were developed to interpret complex human DNA mixtures in an objective manner, and these are slowly being validated and accepted for routine forensic use. Software for objective analysis of microbiomic DNA mixtures may be built on this basis, but these are still years in the future. The second major hurdle is temporal variation. Contrary to human DNA, which can remain unchanged for years, microbial communities (both on the body and deposited as trace evidence) can fluctuate over time, often in correlation with changes in environmental parameters like moisture and pH (e.g., Pasternak et al., 2013). In cases where two samples for comparison are obtained at different times when markedly different environmental conditions prevail, mitigating the temporal changes in community structure is

needed before analysis can ensue. So far, only a few studies have addressed this topic experimentally, mainly by applying various carbon sources to force the different microbial communities to "converge" (Pasternak et al., 2019), but so far with limited success. The third, and perhaps most challenging hurdle, is the problem of DNA transfer. In the past decade, human DNA evidence gained widespread credibility and acceptance in the courts so that the identification of a DNA profile from trace evidence as originating from a specific person is rarely disputed nowadays. Instead, it is becoming more and more common for the defense to challenge the method of deposition of the DNA, suggesting that it reached the crime scene by a legitimate activity (before or after the crime occurred) or by DNA transfer (e.g., when the innocent suspect shook the hand of the real perpetrator). This hurdle can sometimes be overcome by using the likelihood-ratio approach with activity-level propositions (Mayuoni-Kirshenbaum et al., 2020); however, similar to the former hurdles, this one is also very much still an open question, and it will take more time, effort, and research before microbiomics is ready to be employed and accepted within the legal system.

Conclusion

Over the last decade, advances in genomic sequencing and bioinformatics have given rise to microbiomics, which fructified in a growing compendium of tools seeking to explore the panoply of microorganisms present in our bodies and environment. The evidence examined in this review indicates that microbiomics could be a forensically relevant and promising discipline with a multitude of

applications—from determining substrate provenance and acquiring trace evidence to identifying individuals and estimating PMI. These advances may allow various microbiomic data, like those obtained from thanatomicrobiome analysis, to be used by forensic scientists to address questions related to criminal investigations, or at least be used alongside other forensic methods.

Throughout their life-course, humans and their microbiomes undergo complex interactions and co-adaptation processes involving nutrient intake and resulting in the production of decomposition products such as metabolites. Following a person's death, these interactions change dramatically, and the microbiome composition and dynamics fluctuate accordingly. Understanding these colonizations and fluctuations represent major conceptual, methodological, and computational challenges—as do antemortem microbial dynamics. Related microbiome-based research in a forensics context and greater exploration of fungal and viral communities may also lead to an important enhancement in the forensic toolkit in the future.

Many challenges remain to overcome, such as contamination issues, modest study sample sizes, model over-specification and misspecification, prediction accuracies of machine learning techniques, understanding complex spatial and temporal variations in environmental microbiome dynamics, as well as risks and ethical concerns (Shamarina et al., 2017). Notably, even human DNA-based evidence, which is far better understood, is not error-proof, as indicated by the Phantom of Heilbronn case (Daniel and van Oorschot, 2011). Moreover, the vast majority

of published work used 16S or targeted sequencing approaches, which have known limitation for taxonomic resolution and could likely benefit from metagenomics methods (Ogilvie and Jones, 2015; McIntyre et al., 2017) and/or methods that utilize longer reads (Danko et al., 2019b; Foox et al., 2020). Also, more field-based testing and deployment of these sequencing methods could benefit from rigorous, titrated standards for ensuring accuracy (McIntyre et al., 2019). Given this, many of the applications reported in the literature should be considered proof of concepts rather than full-fledged forensic applications. Nonetheless, as Ogilvie and Jones (2015, p. 1) have summarized: "*it is clear that we remain in a period of discovery and revelation, as new methods and technologies begin to provide [a] deeper understanding of the inherent ecological characteristics of this [microbial] ecosystem.*"

Author Contributions

EE initiated the study. JR carried out the review. EE, JR, ZP, and CM wrote the manuscript. All authors approved the manuscript.

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Conflict of Interest

EE consults the DNA Diagnostics Center. CM is a co-founder of Biotia, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abbreviations

PCR, polymerase chain reaction; MetaSUB, metagenomics and metadesign of subways and urban biomes; PRISMA, preferred reporting items for systematic reviews and meta-analyses; CAMDA, critical assessment of massive data analysis; PMI, postmortem interval; OUT, operational taxonomic unit; CRP, C-reactive protein; SIDS, sudden infant death syndrome.

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Quick Quiz



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Check your Understanding

- What types of microbiomes can be analyzed in regards to geolocation for forensic science?
- How could microbiome analytical techniques be improved for personal identification?
- How are various microbiomes different between biological sexes?
- What are some common objects that can have microbiomes applicable for trace evidence?
- What is the thanatomicrobiome and how can it be used to determine postmortem interval?
- What are the major difficulties with comparative microbiomes concerning forensic applications?

Media Attributions

- Figure 1 – A summary of possible sources of forensically relevant microbiota identified by the literature review by Robinson et al., 2021 licensed under the terms of the Creative Commons Attribution License (CC BY).

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PART VI
CASE STUDIES

17. Case Study #1 - Human Health

Case study #1 – The Farmer's Flu

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Part I – Background and Problem

During one week at the end of January of 2019, a high number of flu-like cases have appeared in the small city of Bogart, Iowa which has a population of approximately 50,000 people. 157 individuals were hospitalized over a two-week period, and large proportion included children under the age of 5 years old. Many common symptoms were initially observed in the majority of the patients, however, other less common symptoms manifested in some ill patients after about a week and earlier in those who were immunocompromised and with co-morbidities.

-Common symptoms included:

- fever
- headache
- muscle pain or body aches
- shortness of breath

- vomiting
- diarrhea
- cough
- congestion or runny nose
- fatigue

-Less common symptoms included:

- stiff neck
- lethargy
- chest pain
- swelling of the throat
- severe joint pain
- green, yellow, or bloody mucus production
- facial redness and swelling



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Article 1: Respiratory Viral Infection-Induced Microbiome Alterations and Secondary Bacterial Pneumonia

Article 2: Allergic inflammation alters the lung microbiome and hinders synergistic co-infection with H1N1 influenza virus and Streptococcus pneumoniae in C57BL/6 mice

Initial epidemiological data showed that diseased individuals all were at or were in contact with someone who visited the farmer's

market the previous weekend. The market was established more than 20 years ago, and each weekend merchants open their stalls selling everything from pottery, jewelry, and linens to produce, homemade jams, and even livestock. The market is considered to be the staple of town commerce and entertainment, giving Bogart its cozy home-town feel, and even many consumers and merchants come in from the smaller surrounding towns to benefit from the commerce.

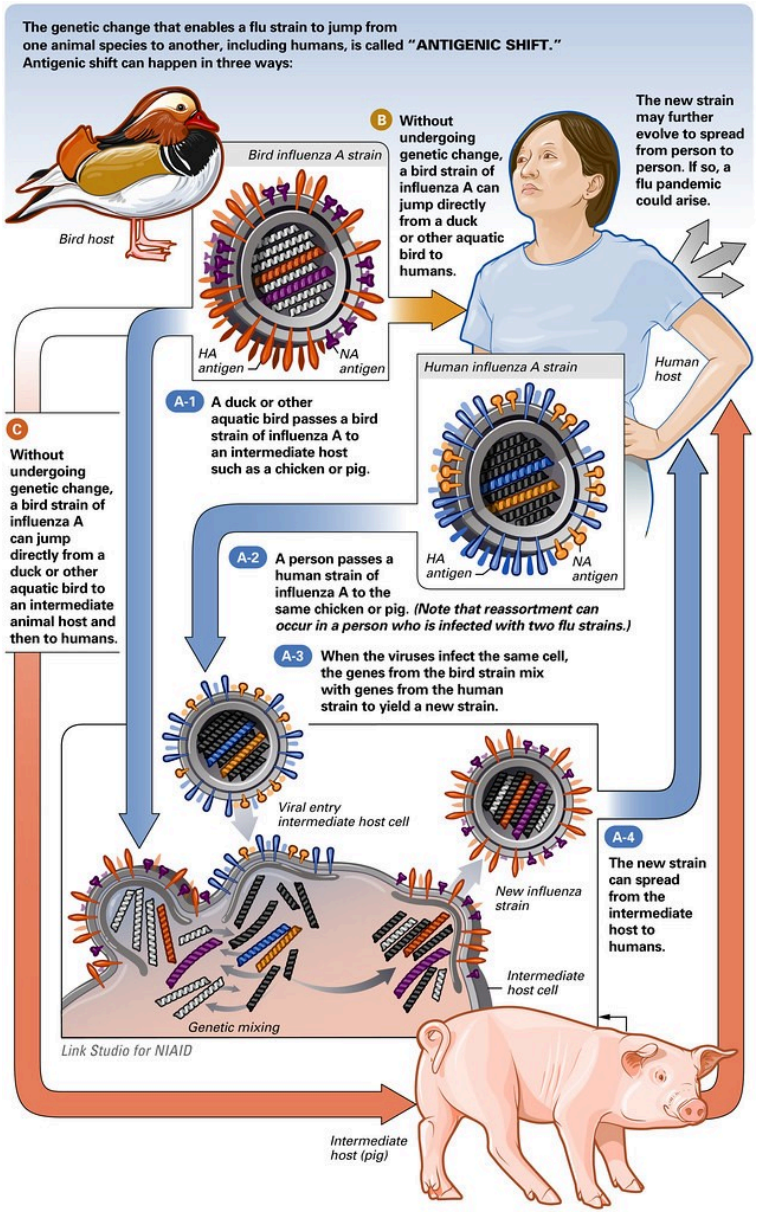


Figure 1. Antigenic shift of the Flu Virus. *The genetic change that enables a flu strain to jump from one animal species to another, including humans, known as "antigenic shift." This process can happen in 3 ways: (A) strain is*

passed to an intermediate host from both human and animal where genetic recombination occurs, creating new strain that can spread to other animals. (B) without undergoing genetic change, the flu jumps directly from animal to human. (C) Without undergoing genetic change, the flu jumps to an intermediate host, then to humans.

Questions:

1. What specific pathogens could be responsible for the observed symptoms and why would there be differences in the observed effects in different individuals?
2. Which microbiomes may be implicated in this disease and why?
3. Do you think this situation is a major public concern? Why or why not?

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Case study #1 – The Farmer's Flu

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Part II – Approach, Implementation, Reasoning

Medical personnel took nasopharyngeal (NP) swabs of sick patients for testing. For children and older adults and those who were averse to NP swabs, nasal and throat swabs or aspirate specimens were collected (Flu specimen collection – CDC). Physicians prescribed various antiviral medication; either two doses per day of oral oseltamivir, four oral doses per day of umifenovir, or inhaled zanamivir for 5 days, and for patients in the hospital, one dose of intravenous peramivir or oral baloxavir for one day. They also prescribed prophylactic antimicrobials, including quinolones (moxifloxacin), cephalosporins (ceftriaxone and cefepime), or macrolides (azithromycin), or a glycopeptide (vancomycin). Other over the counter drugs were suggested to treat symptoms like fever and headache. Public health officials advised the community to get the flu vaccine as well.

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Over the next few weeks, the number of cases increased and symptoms began to worsen for many. The initial assumption is a seasonal flu outbreak. Interestingly, more cases began to pop up in surrounding rural towns, and many patients were transported to the larger hospitals in the city to be put on ventilators. As mortality rates also began to rise, the CDC declared this situation a flu epidemic.

Article 1 – The respiratory microbiome and susceptibility to influenza virus infection

Article 2 – Secondary Bacterial Infections in Patients With Viral Pneumonia

Article 3 – Patterns in the longitudinal oropharyngeal microbiome evolution related to ventilator-associated pneumonia

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Questions:

1. What types of laboratory tests and analytical techniques do you think were being conducted? What problems could have arisen with the analysis of the patient's samples?
2. Why do you think both antibacterial and antiviral medication was prescribed? How would you explain the difference between treatments of a viral and a bacterial infection to a

patient?
3. What reasons or factors could cause a high mortality rate in those infected with the influenza virus?

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Part III – Discussion

After several weeks of patient study and a rising number of mortality and cases, infections were confirmed to be from a mutated variant of influenza A (H1N1; i.e. swine flu). Medical officials believe the high mortality rate and other severe cases may be due to a secondary bacterial infection caused by a pathogen that is resistant to antimicrobials.

Further epidemiological analysis shows that the initial original cases were specifically in individuals who visited the livestock section of the farmer's market for an extended period of time.

Article 1 – The distribution of microbiomes and resistomes across farm environments in conventional and organic dairy herds in Pennsylvania

Article 2 – Antimicrobial use and production system shape the fecal, environmental, and slurry resistomes of pig farms

Article 3 – Influence of Pig Farming on the Human Nasal Microbiota: Key Role of Airborne Microbial Communities



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Apologies, the video and audio are a bit glitchy.

Last line of defense antibiotics, polymixin (colistin) and carbapenems, were prescribed for patients not responding to the initial antibiotics. These patients were also put under quarantine in ICU wards as their symptoms began to worsen, which additionally included abdominal pain, bloody stool, and severe diarrhea that persisted in patients even after being discharged from the hospital.

Article 4 – Incidence, outcome, and risk factors for recurrence of nosocomial Clostridioides difficile infection in adults: A prospective cohort study



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Questions:

1. How do you think antibiotic resistance of the secondary bacterial pathogen came about?
2. How could chemotherapy affect various microbiomes and potentially contribute to pathogenesis and other disease symptoms?
3. How would you inform the public about this situation, and what measures would you suggest to prevent transmission or recurrence?

Attributions:

- Article 1 – The distribution of microbiomes and resistomes across farm environments in conventional and organic dairy herds in Pennsylvania by Pitta et al., 2020 under CC BY 4.0
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Part IV – Resolution

Research scientists, medical professionals, and epidemiologists finally pieced the 'Farmer's Flu' epidemic puzzle together using next generation sequencing technology and keeping detailed records of observational data.

Two farmers from a nearby rural town contracted the novel swine flu variant. They then brought the pigs to market in Bogart, where the flu spread. However, they not only spread the flu variant, but also a highly contagious antibiotic-resistant strain of *Haemophilus influenza* type B (Hib). One of the farmers was sick the week prior and therefore immunocompromised which allowed the development of this secondary infection. It is likely that this strain of *H. influenza* acquired antibiotic resistance via horizontal gene transfer from *Haemophilus parasuis*, which is commonly found in pigs.

As this respiratory disease was treated with multiple ineffective prophylactic antimicrobial drugs, patients' resident microbiomes became depleted and allowed for another infection by opportunistic pathogens. In the case of those who were hospitalized, many developed another secondary infection by *Clostridioides difficile*, resulting in gastrointestinal distress.

After a few months, with the use of both old and new treatment options, outreach to the public with information about the diseases, and proper community compliance, the epidemic came to an end. Bogart resumed as a quiet cozy town, and still holds its locally famed farmer's market.

Reading 1: H. *influenza* – CDC – Epidemiology and Vaccine-Preventable Diseases



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Article 1 – Basic Characterization of Natural Transformation in a Highly Transformable Haemophilus parasuis Strain SC1401

Article 2 – The Gut-Lung Axis in Health and Respiratory Diseases: A Place for Inter-Organ and Inter-Kingdom Crosstalks

Article 3 – Translating Lung Microbiome Profiles into the Next-Generation Diagnostic Gold Standard for Pneumonia: a Clinical Investigator's Perspective

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Questions:

1. How is the gut microbiome linked with the oral and lung microbiomes? Explain how both of the secondary infections by *H. influenza* and *C. difficile* in this case could be related by their respective microbiomes.
2. What other diseases, conditions, or treatments have similar multi-microbiome effects?
3. What are some examples of novel microbiome diagnostic and treatments that could work to restore the affected microbiomes?

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18. Case Study #2 - Environment

Case study #2 – The Land is Sick

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Part I – Background and Problem

Coffee production in South America has drastically decreased in the 2021-23 growing seasons. Specifically, the major farms in Columbia and Brazil which collectively contribute over half of each countries coffee (both specialty and commercial) production, have had severe crop failures. As a result, the price of a cup of coffee has noticeably increased, putting financial strain on suppliers and businesses.

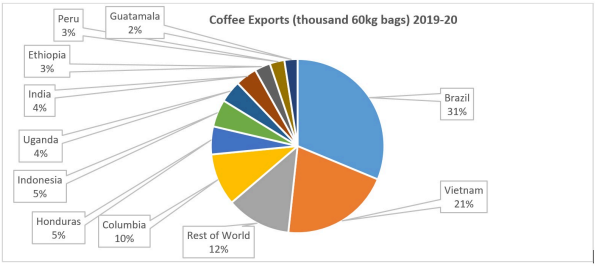


Figure 1. Coffee exports by country 2019-20. Source: International Coffee Organization

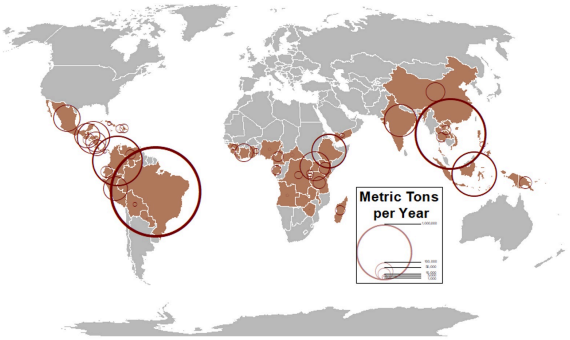


Figure 2. A world map of countries by coffee production, 2019.

Interestingly, the few years prior to this decline, coffee production was at an all-time high, with more coffee in the market than demanded. This dropped the price of coffee, and in many cases original farmers were not fairly compensated.

The International Coffee Organization (ICO) has tasked a team of researchers to identify potential problems to South American coffee plant failure and provide quick and efficient resolution.

Article 1 – The Bacterial Microbiome of *Meloidogyne*-Based Disease Complex in Coffee and Tomato

Article 2 – A review of three major fungal diseases of *Coffea Arabica* L. in the rainforests of Ethiopia and progress in breeding for resistance in Kenya

Article 3 – Structure and Dynamics of the Gut Bacterial Community Across the Developmental Stages of the Coffee Berry Borer, *Hypothenemus hampei*

Video 1 – The biggest threats to the coffee industry

Video 2 – #1 Specialty coffee and the price crisis | Producer Crossover 2019

Questions:

1. As a scientific researcher, what are some factors you would take into consideration when addressing this problem?
2. What type of environmental microbiomes could be implicated in coffee plant crop failure and why?
3. Should other coffee farms in countries besides those in South America be worried about similar failure in their crops? Why or why not?

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Case study #2 – The Land is Sick

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Part II – Approach, Implementation, Reasoning

Upon initial inspection, the unhealthy coffee plants exhibited yellowing of leaves, appearance of red-brown lesions, abnormal shape, damaged coffee berries, and little new growth or production. Researchers decided to take samples of healthy and unhealthy leaves and berries, as well as soil and root samples.

Article 1 – Prokaryotic diversity in the rhizosphere of organic, intensive, and transitional coffee farms in Brazil

Article 2 – A metagenomics approach in the evaluation of the soil microbiome in coffee plantations under organic and conventional production in tropical agroecosystems

Video 1 – #2 Producer Crossover 2019

Video 2 – How countries farm and make coffee differently

Over the next year, other plantations in Columbia and Central American begin to experience similar declines in coffee plant health. Farmers were questioned about any changes in practices, and while there have been some adjustments do to the fluctuating coffee prices, traditional farming techniques have remained the same for the most part in smaller plantations. Larger plantations with intensive farming made more changes due to trader suggestions on improving yields including application of different fertilizers and hiring less experienced workers.

Questions:

1. What type of analytical techniques do you think were or should be performed on the samples taken?
2. How could the observed symptoms of the unhealthy plants be connected to an associated microbiome?
3. How could human intervention exacerbate or ameliorate this situation?

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Part III – Discussion

Analysis of the negatively affected plantations' soil showed a decline in plant growth promoting bacteria and mycorrhizae and an increase in bacterial and fungal pathogens like *Pseudomonas syringae* and *Cercospora coffeicola*.

Over the next few years (2022-24) coffee plants still struggle to grow in Central and South America. Other parts of the world, such as Vietnam and other Asian countries, are seeing a decline in crop health also, further contributing to global coffee market troubles. Many of these farms switched from shade-grown to intense sun-

grown coffee to try to meet needs for demand. Larger corporations and roasters have purchased many of the smaller family farms to try to recoup losses and implement 'new and improved' growing strategies for increased production. This included switching to primarily sun-grown coffee, and application of large amounts of synthetic nitrogen fertilizer.



Figure 1. Shade grown coffee in Guatemala



Figure 2. Sun-grown coffee plantation in Brazil

Video 1- #3 The future of the price crisis

Video 2 – How coffee destroys the environment

Article 1 – Root endophytes of coffee (*Coffea Arabica*): Variation across climatic gradients and relationships with functional traits

Article 2 – Brazilian Coffee Production and the Future Microbiome and Mycotoxin Profile Considering the Climate Change Scenario

Article 3 – Effects of environmental factors on microbiota of fruits and soil of *Coffea arabica* in Brazil

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Questions:

1. What factors could cause these changes in the soil microbiomes?
2. Why do you think coffee plantations across the globe are beginning to fail as well?
3. How does natural biodiversity benefit ecosystem health on both a micro and macro scale?

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Part IV – Resolution

In 2030, most varieties of coffee are extinct, and only small amounts of coffee are produced on shade grown farms further from the equator. It appears that coffee plantations were severely impacted by increasing global temperatures, which altered ecosystem dynamics and promoted pathogen and pest invasion. Fungi, bacteria, and arthropods devastated already struggling coffee farms and with a major switch to sun-grown coffee, soil microbiomes were depleted and disease spread rapidly through monoculture crops. This switchover was prompted by an energy drink corporation, KAPOW!, which wanted to corner the caffeine market and boost production, though their expertise in growing coffee was lacking and the excessive use of synthetic fertilizers on already sun baked land which requires much more watering further doomed plantations. At least they now produce a cheap "coffee" flavored energy drink. It tastes terrible.

Article 1 – Coffee Microbiota and Its Potential Use in Sustainable Crop Management. A Review

Article 2 – Soil fungal communities differ between shaded and sun-intensive coffee plantations in El Salvador

Article 3 – One health relationships between human, animal, and environmental microbiomes: A mini-review

Video 1 – Teaching coffee farmers about the birds and the bees

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Questions:

1. In what ways are environmental microbiomes impacted by changing environmental factors and how can this promote diseases within an ecosystem?
2. How could various environmental microbiomes be utilized to improve sustainable practices and technology?
3. How are environmental and human microbiomes

interconnected?

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19. Case Study #3 - Synthesis (Create Your Own)

Develop your own case study related to microbiomes!

Group members (4-5) will develop and write an original case study involving a microbiome and health or environmental condition. Topics must be approved by the instructor beforehand. The case study should consist of 3 parts: (i) background and problem/issue, (ii) approach to solve, implementation, and reasoning, (iii) discussion of progression of the case study. The final part, (iv) resolution and conclusion will be completed by another group and presented over. The case study should have an interesting title, include references, images, figures, etc. from relevant resources (be sure to use in-text citations and include references at the end), and 3 critical thinking questions concluding each part (9 total).

PART VII

JOURNAL CLUB

20. Journal Club Articles

The following 'Journal Club' articles can be used as supplementary information for various microbiome topics and included in group discussions:

1. Microbiome definition re-visited: old concepts and new challenges by Berg et al., 2020 under a Creative Commons Attribution 4.0 International License
2. Role of the gastrointestinal tract microbiome in the pathophysiology of diabetes mellitus by Sohail et al., 2017 under a Creative Commons Attribution 4.0 International License
3. The Firmicutes/Bacteroidetes ratio: a relevant marker of gut dysbiosis in obese patients? by Magne et al., 2020 under a Creative Commons Attribution 4.0 International License
4. The trans-kingdom battle between donor and recipient gut microbiome influences fecal microbiota transplantation outcome by Kazemian et al., 2020 under a Creative Commons Attribution 4.0 International License
5. Saliva microbiome changes in patients with periodontitis with and without chronic obstructive pulmonary disease by Lin et al., 2020 under a Creative Commons Attribution 4.0 International License
6. The human oral microbiome in health and disease: from sequences to ecosystems by Willis and Gabaldon, 2020 under a Creative Commons Attribution 4.0 International License
7. The lung microbiome: new principles for respiratory bacteriology in health and disease by Dickson and Huffnagle, 2015 under a Creative Commons Attribution 4.0 International License
8. Viral and bacteriome characterization of children with pneumonia and asthma in Mexico City during winter seasons 2014 and 2015 by Romero-Espinoza et al., 2018 under Creative

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9. New insights into the intrinsic and extrinsic factors that shape the human skin microbiome by Dimitriu et al., 2019 under Creative Commons Attribution 4.0 International License
10. Probiotics and vaginal microecology: fact or fancy? by Buggio et al., 2019 under Creative Commons Attribution 4.0 International License
11. Human breast milk bacteriome in health and disease by Ojo-Okunola et al., 2018 under Creative Commons Attribution 4.0 International License
12. Synergies of systems biology and and synthetic biology in human microbiome studies by Ezzamouri et al., 2021 under Creative Commons Attribution 4.0 International License
13. Microbiomes associated with foods from plant and animal sources by Jarvis et al., 2018 under Creative Commons Attribution 4.0 International License

PART VIII

ADDITIONAL RESOURCES

21. The Integrative Human Microbiome Project

The Integrative Human Microbiome Project is an open access article published in *Nature* under the Creative Commons: By Attribution 4.0 License (http://creativecommons.org/licenses/by/4.0/).

The Integrative Human Microbiome Project

The Integrative HMP (iHMP) Research Network Consortium

Abstract

The NIH Human Microbiome Project (HMP) has been carried out over ten years and two phases to provide resources, methods, and discoveries that link interactions between humans and their microbiomes to health-related outcomes. The recently completed second phase, the Integrative Human Microbiome Project, comprised studies of dynamic changes in the microbiome and host under three conditions: pregnancy and preterm birth; inflammatory bowel diseases; and stressors that affect individuals with prediabetes. The associated research begins to elucidate mechanisms of host–microbiome interactions under these conditions, provides unique data resources (at the HMP Data Coordination Center), and represents a paradigm for future multi-omic studies of the human microbiome.

Main

Although the 'omics era has accelerated all aspects of biological research, its effects have been particularly apparent in studies of microbial communities and the human microbiome. In the 18 years since the publication of the first human genome, studies of the microbiome have grown from culture-based surveys of the oral cavity and gut to molecular profiles of microbial biochemistry in all ecological niches of the human body[1,2,3]. Epidemiology and model systems have been used to identify associations between changes in the microbiome and conditions ranging from autism[4] to cancer[5,6,7], and microbial and immunological mechanisms have been identified that affect, for example, the efficacy of drugs used to treat cardiac conditions[8] or survival during graft-versus-host disease[9].

Contemporary studies of the human microbiome have also been a source of basic biological and translational surprises, exposing a compelling range of novel findings and open questions. Every human being appears to carry their own, largely individual, suite of microbial strains[10,11], which are acquired early in life[12,13,14], differ between environments and populations[15,16], and can persist for years[17] or undergo relatively rapid transitions[18]. Microbial diversity manifests differently in different ecological niches of the body; for example, greater diversity is generally expected in the gut, but can be associated with dysbiotic states and risk of adverse events in the female reproductive tract. The microbiome can be perturbed by conditions such as inflammatory bowel disease and diabetes, but a variety of microbiome-linked health states, and the underpinnings of these links, remain unexplored. How dynamic is the microbiome during processes such as pregnancy or viral infection? Which changes in the microbiome represent causes rather than effects of changes in health? Which molecular elements of a personalized microbiome might be responsible for health outcomes, and how do they integrate with and maintain physiological processes such as the immune system and metabolism? And what ecological elements dictate the success of a microbiota transplant, and why are they

successful in treating some individuals and conditions, but not others?

The National Institutes of Health Human Microbiome Project was one of the first large-scale initiatives to address a subset of these questions[19] (Fig. 1). Launched in 2007[20], the first phase of the program sought to determine whether there were common elements to 'healthy' microbiomes, in the absence of overt disease. Studies of both a baseline adult population[21,22,23] and 'demonstration' populations with specific disease states established typical ranges (for some populations) of microbial membership and enzymatic repertoires across the body, combinations of metabolic functions that were either prevalent or strain-specific, and some of the host factors (such as race or ethnicity) that determine this variation. Studies of targeted populations identified ecological states of niches such as the vagina[24,25], skin[26,27,28], and gut[29,30,31,32,33], among many others (https://www.hmpdacc.org/health/projectdemos.php). This first phase of the HMP (HMP1) thus yielded a wealth of community resources: nucleotide sequences of microorganisms and communities from a large number of isolates, individuals, and populations (http://hmpdacc.org)[34,35,36,37]; protocols to support reproducible body-wide microbiome sampling and data generation[38,39,40]; and computational methods for microbiome analysis and epidemiology[41,42,43,44,45,46,47].

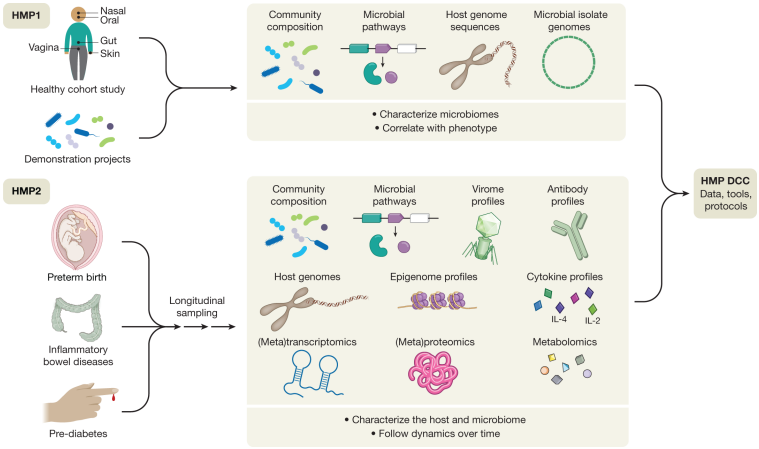


Fig. 1 The first and second phases of the Human Microbiome Project. The ten-year NIH Human Microbiome Project (HMP) program, organized into two phases (HMP1 and HMP2), developed reference sequences, multi-omic data sets, computational and statistical tools, and analytical and clinical protocols as resources for the broader research community. The HMP1 focused on the characterization of microbial communities from numerous body sites (oral, nasal, vaginal, gut, and skin) in a baseline study of healthy adult subjects, and included a set of demonstration projects that focused on specific diseases or disorders. The HMP2 expanded the repertoire of biological properties analysed for both host and microbiome in three longitudinal cohort studies of representative microbiome-associated conditions: pregnancy and preterm birth (vaginal microbiomes of pregnant women), inflammatory bowel diseases (gut microbiome) and prediabetes (gut and nasal microbiomes). These studies followed the dynamics of these conditions through multi-omic analyses of multiple measurement types over time, including changes in microbial community composition, viromics, metabolomic profiles, gene expression and protein profiles from both host and microbiome, and host-specific properties such as genetic, epigenomic, antibody, and cytokine profiles, along with other study-specific features. All sequences and multi-omic data, clinical information, and tools from both HMP1 and HMP2 are housed in the HMP Data Coordination Center (DCC) or referenced public or controlled-access repositories to serve as a central resource for the research community.

One of the main findings of the HMP1 was that the taxonomic composition of the microbiome alone was often not a good correlate with host phenotype—this tended to be better predicted by prevalent microbial molecular function or personalized strain-specific makeup[21]. This finding served as the foundation for the

development of the second phase of the HMP, the Integrative HMP (iHMP or HMP2)[48], which was designed to explore host–microbiome interplay, including immunity, metabolism, and dynamic molecular activity, to gain a more holistic view of host–microbe interactions over time. This multi-omic program sought to expand the resource base available to the microbiome research community, to begin to address the relationship between host and microbiome mechanistically, and to address the questions introduced above. Disease-targeted projects within the HMP2 were therefore encouraged to use multiple complementary approaches in order to assess the mechanisms of human and microbial activity longitudinally and to provide protocols, data, and biospecimens for future work. These projects included three studies that followed the dynamics of human health and disease during conditions with known microbiome interactions, thus addressing important health outcomes directly while also serving as models of 'typical' microbiome-associated conditions of broad interest to the research community. These comprised pregnancy and preterm birth (PTB); inflammatory bowel diseases (IBD); and stressors that affect individuals with prediabetes. These studies, which have now reached the first stage of completion[49,50,51], together provide a wealth of information and insights about not only microbial dynamics, but also associated human host responses and microbial inter-relationships. A collection of more than 20 manuscripts to date describe some of these results at https://www.nature.com/collections/fiabfcjbfj, and together they provide a rich multi-omic data resource to be mined by future work (http://www.ihmpdcc.org).

The vaginal microbiome, pregnancy and preterm birth

Preterm birth can have devastating consequences for newborn babies, including death and long-term disability. In the United States, approximately 10% of births are premature[52], and the

incidence is even greater in lower-resource countries. Environmental factors, including the microbiome of the female reproductive tract, are important contributors to prematurity. Notably, these factors have a greater effect in women of African ancestry, who also bear the highest burden of PTB[53]. Infant mortality has been reduced in recent decades, but the incidence of PTB has not decreased[54], and progress in predicting individual risk of PTB has stalled. During pregnancy, the maternal immune system maintains a delicate balance of pro- and anti-inflammatory effectors[55], and contributors to PTB include breakdown in maternal–fetal tolerance, vascular disorders, stress, cervical insufficiency, premature rupture of the fetal membranes, and intra-amniotic infection[56]. Microbial ascension into the uterus is thought to precipitate PTB by disrupting the maternal immune balance, leading to spontaneous preterm labour, and/or by the release of microbial products (for example, collagenases, proteases or toxins) that compromise the integrity of fetal membranes and lead to premature rupture of the membranes[57].

The Multi-Omic Microbiome Study: Pregnancy Initiative (MOMS-PI) research group, as part of HMP2, characterized the microbiomes of pregnant women to gauge their effects on risk of PTB (Fig. 2). The project followed 1,527 women longitudinally through pregnancy and involved the collection of 206,437 specimens, including maternal vaginal, buccal, rectal, skin and nares swabs, blood, urine, and birth products, as well as infant cord and cord blood, meconium and first stool, buccal, skin and rectal swabs. Subsets of these specimens underwent 16S rRNA gene taxonomic analysis, metagenomic and metatranscriptomic sequencing, cytokine profiling, lipidomics analysis, and bacterial genome analysis. The MOMS-PI team analysed 12,039 samples from 597 pregnancies to investigate the dynamics of the microbiome and its interactions with the host during pregnancy leading to PTB[50].

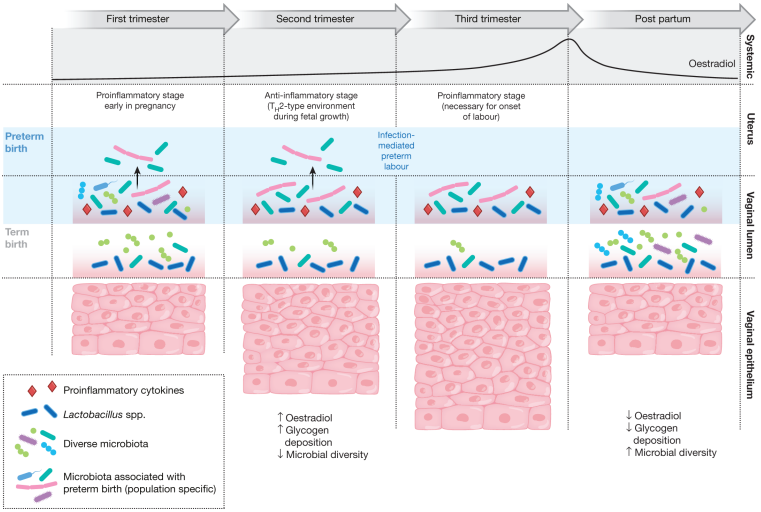


Fig. 2. The vaginal microbiome and its relationships with host factors in pregnancy and preterm birth. The MOMS-PI project followed 1,527 pregnancies longitudinally and involved the collection of 206,437 biospecimens for analysis of host and microbial factors (16S amplicon, metagenomic, and metatranscriptomic sequencing; cytokine profiling; metabolomics; proteomics; genomics; and microbial isolate culture). Around 600 pregnancies were analysed in depth to assess features that lead to preterm birth; this analysis identified both host (for example, cytokine) and microbial (for example, ecological and specific strain) factors. As pregnancy progresses, with predictable changes in systemic oestradiol levels, the uterine and vaginal environments undergo various changes. The uterus switches from an early pro-inflammatory condition to an anti-inflammatory condition in the second trimester, and then back to a pro-inflammatory condition before the onset of labour. Meanwhile, specific changes in the microbiome of the vaginal lumen can be associated with preterm birth, possibly through mechanisms involving microorganisms travelling from the vagina to the uterus. The figure depicts an overview of longitudinal changes in the vaginal mucosal ecosystem and uterus during pregnancy.

These multi-omic investigations identified temporal changes in the vaginal microbiome associated with full-term pregnancies. Women who often began pregnancy with a vaginal microbiome of greater ecological complexity generally converged towards a more homogeneous *Lactobacillus*-dominated microbiome by the second trimester[58]. Interestingly, this trend was most pronounced in

women of African ancestry with lower socioeconomic profiles. Although the overall MOMS-PI cohort was demographically diverse, most women who experienced spontaneous PTB at less than 37 weeks of gestation were of African ancestry. The MOMS-PI team (http://vmc.vcu.edu/momspi) also identified signatures of higher risk for PTB in women who experienced spontaneous preterm birth at less than 37 weeks of gestation[50]. Women who went on to experience spontaneous PTB were less likely to exhibit a vaginal microbiota dominated by *Lactobacillus crispatus*, as previously reported in other populations[59,60,61,62], and were more likely to exhibit an increased proportional abundance of several taxa including *Sneathia amnii*, *Prevotella*-related clades, a Lachnospiraceae taxon known as BVAB1, and a *Saccharibacteria* bacterium known as TM7-H1. Notably, these taxa were also associated with low levels of vitamin D[63], suggesting that the vaginal microbiome might mediate a link between PTB risk and vitamin D deficiency[64]. The signatures of PTB were also reflected in metagenomic and metatranscriptomic measurements, and vaginal pro-inflammatory cytokines (including IL-1β, IL-6, MIP-1β and eotaxin-1) were positively correlated with PTB-associated taxa. Conveniently for future possible interventions, the vaginal microbiomes of mothers who experienced PTB were most distinct from those of control mothers early in pregnancy, and a preliminary model to predict risk of PTB was most sensitive and specific using vaginal microbiome profiles from samples collected before 24 weeks of gestation.

The MOMS-PI research group identified intriguing associations between the vaginal microbiota, host response, and pregnancy outcomes that are consistent with the involvement of microorganisms ascending from the vagina in at least some cases of spontaneous PTB. As an essential next step, the contribution of racial and demographic background to the vaginal microbiome in pregnancy with relation to pregnancy outcomes must be fully explored through harmonized, large-scale studies[50]. It is clear that PTB has a complex aetiology[56]. The relative contributions of fetal

and maternal genetics and epigenetics, particularly as related to genetic variation of the innate immune system, should be explored. Harmonized large-scale studies would permit the development of population-specific risk assessment algorithms using vaginal microbiome profiles, features from genetic and prenatal (fetal) genetic screens, biomarkers such as cytokines and metabolites, and key clinical features from classic markers of risk including maternal age, body mass index, pregnancy history (including history of PTB), cervical length, and measures of stress and other environmental exposures. With the addition of new data from the microbiome, other environmental factors, and multi-omic inputs, new algorithms promise to improve our ability to predict risk of PTB early in pregnancy, to facilitate clinical trials by identifying high-risk patients, and ultimately to stratify patient populations into treatment groups.

The gut microbiome and inflammatory bowel disease

Studies of the gut microbiome in gastrointestinal disease have a particularly long and detailed history, especially in complex chronic conditions such as the inflammatory bowel diseases (IBD). IBD, including Crohn's disease and ulcerative colitis, affects millions of individuals worldwide, with increasing incidence over the past 50 years or more coinciding with multiple factors such as westernization, urbanization, shifts in dietary patterns, antimicrobial exposure, and many more that could influence host–microbiome homeostasis[65]. The microbiome has long been implicated in IBD, potentially as a causative or risk factor[66,67], as an explanation for heterogeneity in treatment response (that is, some individuals respond well to relatively benign aminosalicylates or corticosteroids whereas others still experience severe inflammation even after surgical intervention)[68], or as a novel point of therapeutic intervention (for example, by transplantation of faecal microbiota[69,70]). Although meta-omic techniques have been used

to identify functionally consistent microbial responses that help to explain the gut microbiome's role as part of a pro-inflammatory feedback loop in the gut during disease[71], and a few strains of microorganisms have been shown to be IBD-specific[72], no comprehensive model of specific microbial, molecular, and immune interactions yet exists to explain the disease's onset and dynamic progression.

Therefore, to better characterize mechanisms of host–microbiome dysregulation during disease, the Inflammatory Bowel Disease Multi'omics Database (IBDMDB) project followed 132 individuals from five clinical centres over the course of one year each as part of HMP2 (Fig. 3). Integrated longitudinal molecular profiles of microbial and host activity were generated by analysing 1,785 stool samples (self-collected and sent by mail every two weeks), 651 intestinal biopsies (collected colonoscopically at baseline), and 529 quarterly blood samples. To the extent possible, multiple molecular profiles were generated from the same sets of samples, including stool metagenomes, metatranscriptomes[73], metaproteomes, viromes, metabolomes[74,75], host exomes, epigenomes, transcriptomes, and serological profiles, among others, allowing concurrent changes to be observed in multiple types of host and microbial molecular and clinical activity over time. Protocols and results from the study, further information about its infrastructure, and both raw and processed[76,77] data products are available through the IBDMDB data portal (http://ibdmdb.org), from the HMP2 Data Coordination Center (DCC; http://ihmpdcc.org), and in the accompanying manuscript[49].

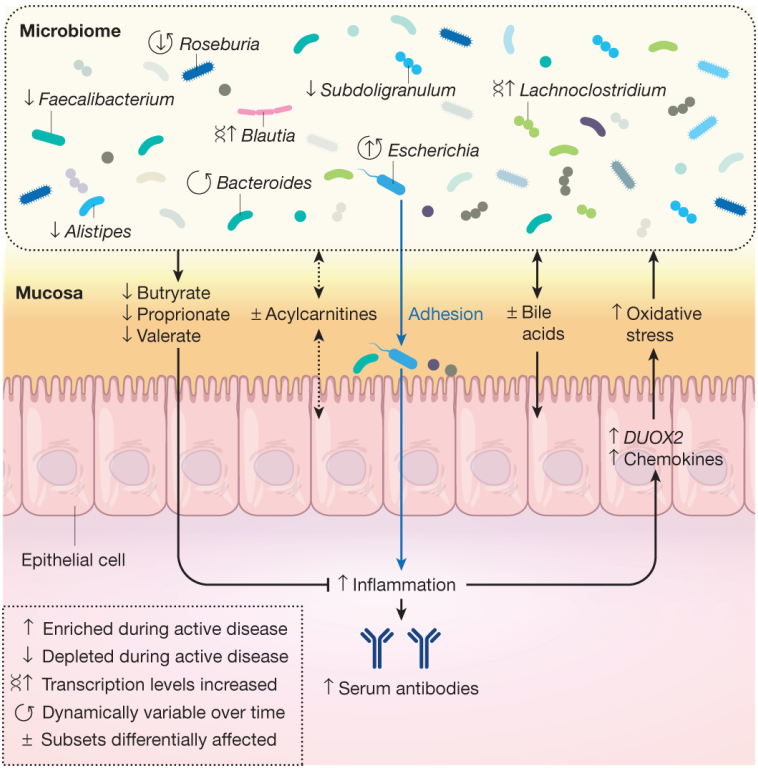


Fig. 3. Host–microbiome dynamics in IBD. The IBDMDB followed more than 100 participants with IBD (Crohn's disease or ulcerative colitis), as well control individuals without IBD, for one year each to assess host and microbial molecular activity during changes in disease activity and gastrointestinal inflammation. Nearly all measured host–microbiome properties showed changes in either activity or stability during disease, including those shown here—not only microbial taxa and microbial transcription, but also host- and microbiota-derived small molecules in the gut, epithelial transcriptional responses at multiple points along the colon, and circulating antibody levels in peripheral blood serology.

This unique study design allowed the IBDMDB to identify a variety of differences in the microbiome and host immune response over time during the course of the disease. Indeed, these dynamic changes were of much greater magnitude than were cross-sectional

differences among clinical phenotypes, which have been emphasized by previous studies[67,71,78,79]. This was due in part to the prospective nature of the cohort, which recruited patients with Crohn's disease or ulcerative colitis during both active and quiescent periods of disease, showing that microbial compositions in patients with IBD often revert to more control-like, 'baseline' configurations when the disease is not active. By identifying the gut microbial configurations that were most different from baseline—regardless of specific disease state—the study defined a dysbiosis score that called out highly divergent microbial compositions, which share many features common to an overall inflammatory response (for example, tolerance to oxidative stress). This dysbiosis was not unique to the microbial response to inflammation, however, and was associated with other host and biochemical alterations, pointing to new potential directions for management of systemic dysregulation in IBD. These included large shifts in acylcarnitine pools and bile acids, increased serum antibody levels, and alterations in transcription for several microbial species. Concurrent transcriptomics and 16S amplicon mucosal community profiling from biopsies also identified potential host factors that might be able to shape the microbial community, in particular several chemokines, highlighting these as being involved in a potentially dysregulated interaction during periods of disease activity[49].

The study's longitudinal multi-omic profiles further allowed researchers to characterize the stability and dynamics of host–microbiome interactions during disease, in particular highlighting ways in which community state and immune responses are distinctly less stable in participants with IBD than in control, healthy individuals. In numerous cases, the microbiome of a participant with IBD changed completely over the course of only weeks (measured as maximal Bray–Curtis dissimilarity to earlier samples from the same subject), whereas such shifts were rare in individuals without IBD. The main microbial contributors to these large-scale shifts from one time point to the next largely mirrored

the differences observed in dysbiosis, and the shifts frequently marked the entrance into or exit from periods of dysbiosis. Finally, the study's long-term, complementary molecular measurements enabled the construction of a network of more than 2,900 significant host and microbial cellular and molecular interactors during IBD, ranging from specific microbial taxa to human transcripts and small molecule metabolites. This network of mechanistic associations identified several key components that are central to the alterations seen in IBD, highlighting octanoyl carnitine, several lipids and short-chain fatty acids, the taxa *Faecalibacterium*, *Subdoligranulum*, *Roseburia*, *Alistipes*, and *Escherichia*, some at both the metagenomic and metatranscriptomic levels, and host regulators of interleukins[49]. Networks of mechanistic associations such as this may provide the key to disentangling the complex system of interactions that results in chronic inflammation in IBD and in other systemic microbiome-linked immune diseases.

Multi-omics profiling in prediabetes

Type 2 diabetes mellitus (T2D) affects more than 10% of the adult US population, and another 30% show early signs of the disease (referred to as prediabetes)[80]; 70% of the latter will develop diabetes in their lifetime. T2D is characterized by complex host–microbiome interactions[81,82], but little is known about systemic alterations during prediabetes, their effects on biological processes, or the critical transition to full-blown T2D. Prediabetes and T2D are often associated with insulin resistance, and thus studies of individuals with prediabetes or insulin resistance offer unique opportunities to investigate the earliest stages of diabetes. It is essential to create a global and simultaneous profile of both host and microbial molecules in individuals with prediabetes over time, in order to fully understand the molecular pathways that are affected in people with prediabetes and/or insulin resistance and how these conditions

affect both biological responses to environmental challenges (for example, viral infections[83,84]) and the onset of T2D.

To better understand T2D at its earliest stages, as part of iHMP, the Integrated Personal 'Omics Project (IPOP)[85] followed 106 healthy and prediabetic individuals during quarterly periods of health, respiratory viral infection (RVI) and other perturbations over about four years[51] (Fig. 4). In one such perturbation, a subset of 23 individuals underwent a directed weight gain followed by weight loss[86]. In total, 1,092 collections across all participants were profiled. For each visit, blood was assayed for host molecular 'omics profiling and two types of samples, nasal swabs and faeces, were collected for microbial profiling. Each participant's exome was sequenced once; otherwise, for each visit, 13,379 transcripts were profiled from peripheral blood mononuclear cells, 722 metabolites and 302 proteins from plasma, and 62 cytokines and growth factors from serum. In addition, thousands of gut and nasal microbial taxa and computationally predicted genes were profiled using 16S rRNA amplicons. All visits were also intensively characterized by 51 clinical laboratory tests. In addition, because of the focus on T2D, a number of glucose dysregulation tests were performed, including measurements of fasting glucose and haemoglobin A1C levels, oral glucose tolerance tests, and tests of insulin resistance.

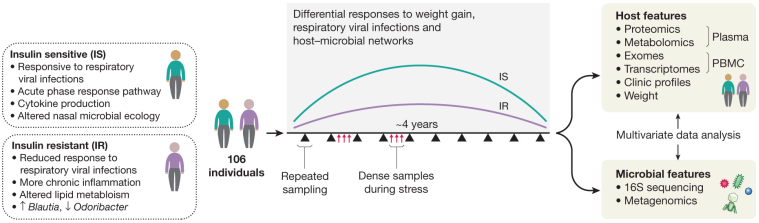


Fig. 4. Differential host and microbial responses to dietary perturbations and infectious disease in individuals with prediabetes. For integrated personal 'omics profiling of the microbiome during prediabetes, 106 participants were followed for up to four years, with samples (primarily blood and stool) collected quarterly and additional samples collected during periods of RVI and other stresses. The genomes of the participants were sequenced and measurements of transcriptomes, proteomes, metabolomes, and microbiomes taken at each visit, in addition to clinical details. Insulin-resistant individuals showed differences from insulin-sensitive participants in various measurements, both at baseline and in response to the stresses such as weight loss and RVI.

Baseline measurements were generally stable within individuals, even for long periods of time, with only some analytes changing significantly over time[51]. However, many analytes, such as clinical laboratory measurements, cytokine profiles, and gut microbial taxa (mostly those of low abundance) were highly variable between individuals. Participants who were ultimately insulin-resistant had distinguishable molecular and microbial patterns at baseline from those who were ultimately insulin-sensitive, and an analyte test was devised as part of the study in order to differentiate them. Notably, individuals undergoing RVI or changes in weight showed thousands of specific molecular and microbial changes during these perturbations, and insulin-resistant and insulin-sensitive individuals responded very differently to perturbations. For example, during RVI, insulin-resistant participants showed substantially decreased and delayed inflammatory responses (for example, the acute phase response and IL-1 signalling) and altered gut microbial changes when compared with insulin-sensitive participants (for example, in Lachnospiraceae and Rikenellaceae but

not bacilli). Accordingly, there were fewer changes in nasal microbiota in insulin-resistant participants, and both the richness and the diversity of nasal microorganisms decreased during RVI in insulin-sensitive but not insulin-resistant participants. Furthermore, global co-association analyses among the thousands of profiled molecules revealed specific associations in insulin-resistant individuals that differ from those seen in insulin-sensitive participants and vice versa, indicating different patterns of host–microbiome interactions in the two groups[51].

Another important goal of the study was to assess how host–microbiome multi-omics and related emerging technologies can be used to better manage patients' health. We found that taking millions of measurements per individual over time enabled the early detection of potential disease states[51,87]. These included early detection of T2D, which developed differently among participants and was better detectable with varied assays; for example, some individuals first exhibited measurements in the diabetic range on tests of fasting glucose, whereas others did so on tests of haemoglobin A1c, oral glucose tolerance tests, or even continuous glucose monitoring. These results, together with detailed characterization of glucose dysregulation over time, illustrate the heterogeneity of T2D development. Overall, the data led to microbially linked, clinically actionable health discoveries in a number of diseases in addition to T2D, including metabolic disease, cardiovascular disease, haematological or oncological conditions, and other areas; these signs were often present before symptom onset, demonstrating the power of using big data, including the microbiome, to better manage human health.

Resources from the HMP2

Together, the HMP1 and HMP2 phases have produced a total of 42 terabytes of multi-omic data, which are archived and curated by the DCC at at http://ihmpdcc.org and in public and/or controlled-

access repositories such as the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra), the Database of Genotypes and Phenotypes (dbGaP; https://www.ncbi.nlm.nih.gov/gap/), Metabolomics Workbench (https://www.metabolomicsworkbench.org/), and others (Fig. 5). All data on the DCC is available for unrestricted use, with a subset of project metadata also being shared when permitted by institutional review boards (IRBs), and other restricted data (for example, human genome sequences and protected metadata) available through controlled access at dbGaP (projects PRJNA398089, PRJNA430481, PRJNA430482, PRJNA326441, phs001719, phs000256, phs001626, phs001523, and others). The formal data models and associated entity relationship schemas produced by all phases of the HMP are freely available at https://github.com/ihmpdcc/osdf-schemas. The DCC website allows users to find, query, search, visualize, and download data from thousands of samples with associated metadata. Once a user has identified a set of files, conditions, subjects, or phenotypes of interest, he or she can add this set to a shopping cart for further operations. Files can then be directly downloaded for use at the user's local site or in the cloud. The HMP DCC efforts are thus by design consistent with the NIH's stated goals to make all data generated from NIH funding findable, accessible, interoperable, and reusable[88]. The success of these efforts is evidenced by a consistently high rate of user access to the web resources, with 9,000–12,000 user sessions each month, and a greater throughput anticipated after the publication of these resources here.

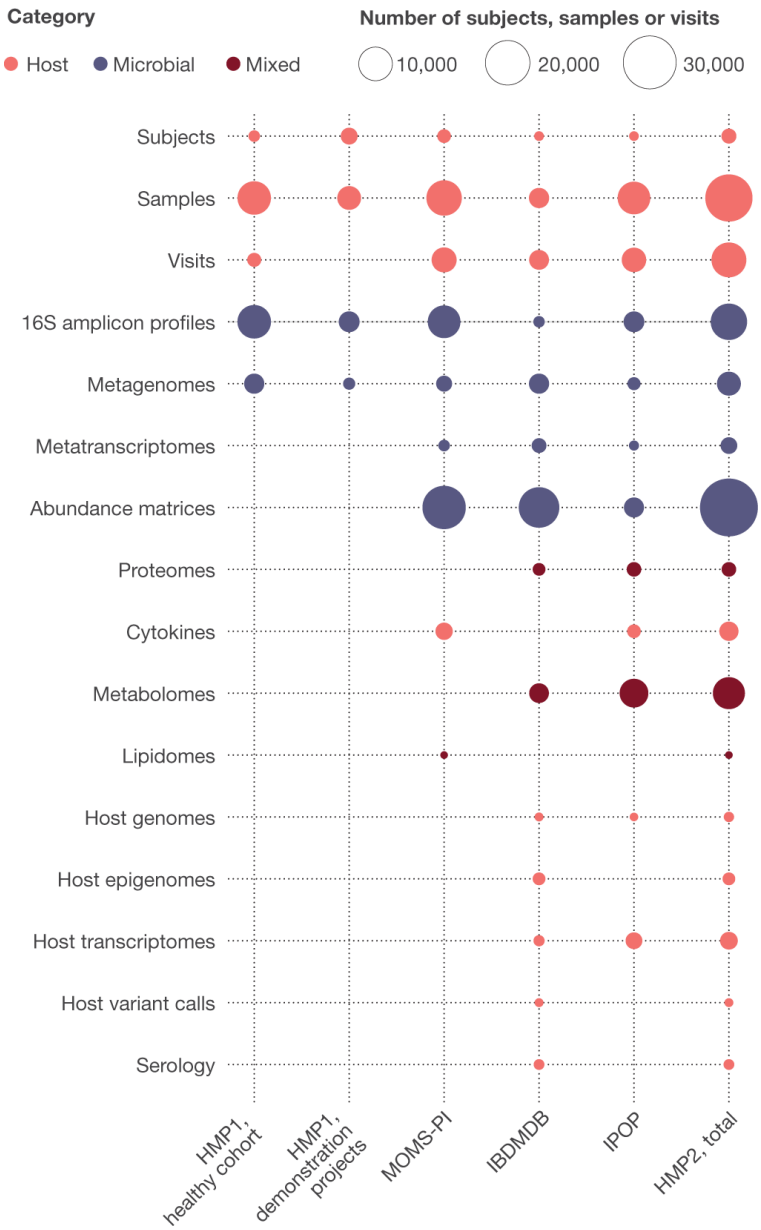


Fig. 5. Resources from HMP1 and HMP2 available at the DCC. The HMP DCC (http://ihmpdcc.org) hosts raw and processed data from both phases of the Human Microbiome Project, comprising in total more than 42 Tb of

multi-omic data. From the HMP1, these include 16S rRNA gene amplicons and metagenomes from the healthy human subjects (HHS) baseline cohort, as well as resources from demonstration projects (https://www.hmpdacc.org/health/projectdemos.php) and genomes of associated microbial isolates. From the HMP2, these include data that IRBs gave permission to be made publicly available from the pregnancy and preterm birth (MOMS-PI), inflammatory bowel disease (IBDMDB), and prediabetes (IPOP) projects, with links to raw data in other repositories. Additional data deposited elsewhere including microbial reference genomes, HMP1 human genomes, and controlled access data for all HMP2 projects is also linked from the DCC. Categories of data are colour coded, and the number of items in each dataset is indicated by the size of the circles.

Complementary host–microbiome interactions

Although each of the three HMP2 studies revealed new biology within their respective areas of health and disease, a surprising range of host–microbiome immune and ecological features were common among them. The combination of shotgun metagenomics, untargeted metabolomics, and immunoprofiling was particularly effective, as in all projects this subset of molecular measurements tended to efficiently capture interpretable host and microbial properties that are linked to disease. Conversely, genetic variants were generally difficult to link to the microbiome in such small populations, which were necessary in order to deeply profile multi-omics over time, and we anticipate host sequencing to be more useful when integrated into larger cross-sectional surveys. Another notable property was that, as in most microbiome studies, changes that occurred within individuals, populations, or phenotypes were often much smaller than baseline variation between individuals. This is particularly true at microbiome-relevant time scales, for which repeated measures as rapid as days to weeks were necessary to capture the most specific host–microbiome interactions. Health-associated microbiome interactions can thus manifest in extremely diverse ways among individuals, making a combination of large-scale population surveys with within-subject longitudinal profiles

essential for understanding the mechanisms of microbiome-linked disease.

As a result, other aspects of host–microbiome interactions were highly localized and subject-specific within each of the three studies. In all three conditions, microbial changes and associated host responses were strongest when captured at the time the changes occurred, and often within the tissue of origin. It is thus clear from these and other studies that host–microbiome interactions have both localized and systemic effects. Strong local perturbations initiated from either the host or microbial side can induce subsequent spatiotemporal responses that can continue over time and/or in other tissues, presumably with signals carried spatially by circulating small molecules and/or temporally by gene regulation or microbial growth, and involving regulatory circuits with both host and inter-microbial components. Continued coordinated efforts to measure the diverse host and microbial properties involved in each condition will thus be important for developing targeted and, when necessary, personalized therapies for microbiome-associated conditions, as well as for uncovering general principles that govern host–microbiome interactions. Other dynamic interactions that were not measured in all studies, such as an individual's first microbial exposures near birth and subsequent immune development, may also represent key contributors to baseline microbiome personalization and help to explain disease-linked dynamics based on events that took place years or even decades earlier.

Next steps in microbiome multi-omics

The collective results of the NIH HMP projects, alongside many other studies, show that the microbiome is an integral component of human biology, with a major role in health and well-being. Inter-individual variability and highly diverse host–microbiome responses over time have driven the development of new methods for

population microbiome studies using multiple, complementary longitudinal measurements, as well as highlighting the need to follow such studies up with mechanistic models in order to validate causative associations. The successful close of the HMP program itself has left an enduring legacy of multiple scientific generations of trained human microbiome investigators; provided the resulting community with a wealth of data, analytical, and biospecimen resources; and positioned the NIH and other funding agencies to continue work in a broad range of microbiome-linked conditions[89]. Funding for microbiome science, human and otherwise, is now being coordinated among NIH Centers and Institutes (https://www.niaid.nih.gov/research/trans-nih-microbiome-working-group); other US government agencies including the National Science Foundation, Environmental Protection Agency, Department of Energy, National Institute of Standards and Technology, Department of Agriculture, National Oceanographic and Atmospheric Administration, National Aeronautics and Space Administration, and Department of Defense (https://commonfund.nih.gov/hmp/programhighlights); philanthropic organizations including the Bill and Melinda Gates Foundation, the March of Dimes, the Burroughs Wellcome Fund, the Sloan Foundation, the Keck Foundation, the Juvenile Diabetes Research Foundation, the Crohn's and Colitis Foundation, and others; and industry and public–private partnerships. Moreover, as complex global projects are launched to tackle aspects of personalized medicine, it is now obvious that it is informative to include components focused on the effect of the human microbiome.

As with any large study, the HMP2 has raised more new questions than it has answered. The aetiologies of baseline inter-individual differences in the microbiome, and of its dynamic changes over time, were not apparent even from the wide range of measurement types incorporated into these three studies and populations. Many immune and biochemical responses appear to be associated with specific strains that are unique to one or a few individual hosts,

but it is not clear whether such strains are sufficient or necessary for their associated disease phenotypes. A few mechanisms were identified by which signals in the gut can be transmitted to systemic conditions such as diabetes, but not the specific small molecules or immune cell subsets by which they are likely to be transmitted—particularly in other health conditions that have not yet been studied in such detail. Finally, each HMP2 study was necessarily carried out within a geographically and genetically constrained population, and global differences in early life events, infectious disease exposure, or diet may change how microbiome dynamics contribute to human disease. Human-associated microbiology now clearly extends beyond infectious and gastrointestinal diseases to areas barely imaginable a few decades ago, including metabolism, neoplasia, maternal and child health, and central nervous system function. As the NIH HMP comes to an end, it is clear that its results have revealed a multitude of new avenues of research and technologies for future investigation, and we look forward to new discoveries based on resources from the program and exciting findings yet to come.

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Contributions

All authors contributed manuscript text and created or edited figures. Individual HMP2 projects discussed were implemented and managed by J.M.F., G.A.B. and J.F.S. (PTB); J.L.-P. and C.H. (IBD); W.Z., M.P.S. and G.M.W. (T2D); H.H.C., A.M. and O.W. (DCC).

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Ethics declarations

Competing interests

M.S. is a cofounder of Personalis, Qbio, Sensomics, January, Filtricine and Akna and advisor for Genapsys. The other authors declare no competing interests.

Additional information

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22. BMC Microbiome Open Collections

The following is a link to BMC Microbiome Open Collections where literature pertaining to various microbiomes can be accessed: https://microbiomejournal.biomedcentral.com/articles/collections

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Environmental Nutrient Cycling and Human Health

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The Ocean Microbiome and Marine Life

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- Hot Spot Quiz Figure – Plant and Soil Microbiomes by Gopal & Gupta, 2016 licensed under Creative Commons Attribution-Share Alike 4.0

Plant Microbiomes

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Pollution and Bioremediation

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Forensic Microbiomes

- Figure 1 – A summary of possible sources of forensically relevant microbiota identified by the literature review by Robinson et al., 2021 licensed under the terms of the Creative Commons Attribution License (CC BY).

Case study #1 – The Farmer's Flu

Part I – Background and Problem

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- Article 3 – Translating Lung Microbiome Profiles into the Next-Generation Diagnostic Gold Standard for Pneumonia: a Clinical Investigator's Perspective by Kitsios, 2018 under CC BY 4.0

Case study #2 – The Land is Sick

Part I – Background and Problem

- Figure 1 – Coffee exports by country 2019-20 by Dylan Parks. Data source: International Coffee Organization
- Figure 2 – A world map of countries by coffee production, 2019 by Cbahrs licensed under CC BY-SA 4.0
- Article 1 – The Bacterial Microbiome of *Meloidogyne*-Based Disease Complex in Coffee and Tomato by Lamelas et al., 2020 licensed under the terms of the Creative Commons Attribution License (CC BY).
- Article 2 – A review of three major fungal diseases of *Coffea Arabica* L. in the rainforests of Ethiopia and progress in

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- Article 3 – Structure and Dynamics of the Gut Bacterial Community Across the Developmental Stages of the Coffee Berry Borer, *Hypothenemus hampei* by Mejía-Alvarado et al., 2021 licensed under the terms of the Creative Commons Attribution License (CC BY).
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- Video 2 – #1 Specialty coffee and the price crisis | Producer Crossover 2019 by Coffee Circle licensed under a Creative Commons Attribution License (reuse allowed)

Part II – Approach, Implementation, Reasoning

- Article 1 – Prokaryotic diversity in the rhizosphere of organic, intensive, and transitional coffee farms in Brazil by Caldwell et al., 2015 licensed under a Creative Commons Attribution-ShareAlike 4.0 International (CC BY-SA 4.0) license.
- Article 2 – A metagenomics approach in the evaluation of the soil microbiome in coffee plantations under organic and conventional production in tropical agroecosystems by Rodriguez et al., 2020 licensed under a Creative Commons Attribution-NonCommercial 4.0 International License.
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- Article 2 – Brazilian Coffee Production and the Future Microbiome and Mycotoxin Profile Considering the Climate Change Scenario by dos Santos et al., 2021 licensed under the terms and conditions of the Creative Commons Attribution (CC BY) license.
- Article 3 – Effects of environmental factors on microbiota of fruits and soil of *Coffea arabica* in Brazil by Veloso et al., 2020 licensed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

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- Article 2 – Soil fungal communities differ between shaded and

sun-intensive coffee plantations in El Salvador by Rao et al., 2020 licensed under the Creative Commons CC0 public domain dedication.

- Article 3 – https://www.frontiersin.org/articles/10.3389/fpubh.2018.00235/full by Trinh et al., 2018 licensed under the terms of the Creative Commons Attribution License (CC BY).
- Video 1 – Teaching coffee farmers about the birds and the bees by VOA Learning English licensed under a Creative Commons Attribution License (reuse allowed)

Derivative Notes

Minor revisions not noted below include editing language for clarity, length, and flow as well as corrections to and additions of hyperlinks and citations. Other revisions not listed below include removing first person language and references to sections of the text that were not included in the book/article.

Analyzing Microbiomes

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    - Grammar and syntax edits made under the section 'Ecological Safety & Risk Assessment'.

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The Integrative Human Microbiome Project

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