Kingdom-Agnostic Metagenomics and the Importance of Complete Characterization of Enteric Microbial Communities
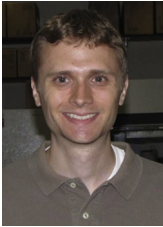
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Advanced sequencing techniques have shown that bacteria are not the only complex and important microbes in the human intestine. Nonbacterial organisms, particularly the virome and the mycobiome, are important regulators of intestinal immunity and inflammation. The virome is mucosal and systemic; it can alter the host response to bacteria and interact with host genes and bacteria to contribute to disease pathogenesis. The human mycobiome is also complex and can contribute to intestinal inflammation. We review what has recently been learned about the nonbacterial and nonarchaeal microbes in the gastrointestinal tract, discussing their potential effects on health and disease and analytical approaches for their study. Studies of associations between the microbiome and intestinal pathology should incorporate kingdom-agnostic approaches if we are to fully understand intestinal health and disease.

Keywords: Metagenomics; Mycobiome; Virome.

The microbes that inhabit the intestine can be broadly divided into prokaryotes (bacteria and archaea), bacteriophages, eukaryotic viruses, and the meiofauna. Of these, bacteria have been the most thoroughly studied. However, it has become increasingly evident that transkingdom interactions in the intestinal tract influence health and disease (Figure 1). Harboring 100 trillion prokaryotic cells at densities of 10^{11} to 10^{12} cells/mL, the human gastrointestinal (GI) tract is one of the most complex microbial ecosystems on Earth.[1] Bacteriophages, viruses that infect prokaryotes, are routinely observed to be ~10-fold more abundant than prokaryotes in the same environment and exhibit extensive diversity.[2,3] These complex communities interact in a predator-prey relationship, creating a dynamic community structure. Moreover, bacteriophages are a source of horizontal gene transfer among prokaryotic communities.[4] An increased understanding of prokaryotes and their bacteriophages is required to fully understand the intestinal metagenome.

There is increasing evidence for the presence of a eukaryotic virome in asymptomatic subjects. This virome includes a range of viruses (an average of nearly 10 infections per healthy person) that permanently infect the host.[5] These systemic viruses have profound effects on innate and adaptive immunity. This is particularly evident for herpesviruses, which have been shown to confer protection against influenza virus, adenovirus, and *Listeria* and *Yersinia* infections,[6–8] exacerbate symptoms of experimental autoimmune encephalomyelitis,[9,10] and reduce autoimmunity in models of lupus.[11] Therefore, systemic and local viral infections likely influence the nature of immunity and inflammation in tissues, including those of the GI tract.[6–12]

The GI tract is also colonized with less abundant microbial eukaryotes. These include a fungal "mycobiome" and other microbial eukaryotes, such as the protozoa *Blastocystis*.[13,14] Combined, these microbial eukaryotes constitute the meiofauna.[15] Meiofauna are metazoans with body sizes between 45 μm and 1 mm. This definition also includes eggs and juvenile stages of larger species, including those of parasitic helminths such as *Ascaris*. The effects of meiofaunal disease have been studied extensively in humans and mice.[16–18] Meiofauna have been reported to protect against several immune-mediated diseases, including inflammatory bowel disease (IBD), multiple sclerosis, rheumatoid arthritis, type 1 diabetes mellitus, and asthma (reviewed by Elliott and Weinstock[19]). A full catalogue of meiofauna present in healthy and unhealthy subjects has yet to be completed. This catalogue would assist in identifying
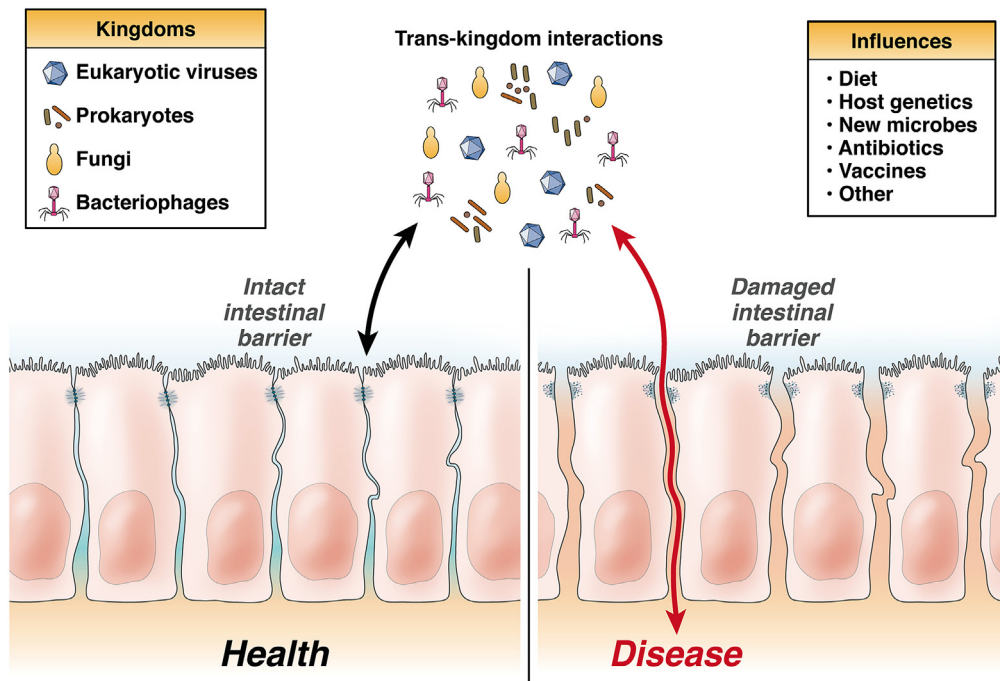
Figure 1. Model of trans-kingdom interactions between microbial taxa and the intestine. Trans-kingdom microbial interactions and environmental factors influence the composition of the intestinal flora. These microbes and microbial products interact with healthy hosts and those with disease and can gain access to new tissues and organs when the integrity of the epithelial barrier is compromised. Shifts in flora would lead to changes in host-microbe interactions.

appropriate models in which to perform mechanistic studies designed to elucidate their effects on human health.

The GI microbiome is therefore a complex community comprising prokaryotic and nonprokaryotic microbes that includes bacteriophages, the eukaryotic virome, and the meiofauna. Studying such a complex community offers new opportunities and challenges. Many of these challenges can be met by taking advantage of modern nucleic acid sequencing techniques. These techniques will expand our understanding of the total metagenome in GI health and disease and could lead to new therapeutic and diagnostic strategies.

The Prokaryotic Virome

Recent studies have shown that a complex array of viruses inhabit the intestine and could be important for gut homeostasis.[20,21] The most common of the enteric viruses in the human gut, the bacteriophages, infect prokaryotes. Viruses that infect archaeal organisms have been less well studied. Bacteriophages are rapidly evolving and have been shown to outnumber their hosts 10 to 1, thereby applying constant evolutionary pressure.[22] Bacteriophages are classified by the nature of their nucleic acid and virion morphology. There are 2 broad categories: the tailed, double-stranded DNA viruses of the order *Caudovirales* (families *Podoviridae*, *Siphoviridae*, and *Myoviridae*) and the nontailed, cubic or filamentous viruses, which are largely composed of single-stranded DNA viruses (family *Microviridae*).[23] The *Caudovirales* and *Microviridae* are the most abundant bacteriophages in the human gut, although several other bacteriophages are readily detected.[24] GI bacteriophages have not been completely cataloged, but studies have shown the extreme diversity of these populations.[3,25]

Bacteriophages infect their hosts via virulent (lytic) or temperate (lysogenic) strategies. Temperate bacteriophages (prophages) latently integrate genetic material into prokaryotic genomes or reside as extrachromosomal plasmids. Prophage induction often results from environmental stress such as nitric oxide, antibiotics, and nutrient availability, which leads to rapid viral replication and bacterial cell lysis.[26] Many of these environmental stresses and additional metabolic stresses or signals are likely present during development of GI disease.[27,28]

In addition to inducing cell lysis, bacteriophages transfer genetic material from one prokaryote to another. This may result in transmission of virulence factors and antibiotic resistance genes and alter the pathogenic and resistance landscape of a prokaryotic community.[4] Bacteriophage-induced changes in prokaryotic community structure and function may have broad implications for health and GI disease. For example, prophage lysogeny might provide *Enterococcus faecalis* with a competitive advantage in vivo.[29] Acute changes in the microbiome have also been observed after administration of bacteriophages to mice,[30] suggesting that bacteriophages may be developed to manipulate the microbiome as therapeutic agents.

Advances in Analysis of Intestinal Bacteriophages

A glimpse into the diversity of bacteriophages in human stool was made possible by deep sequencing virus-like particles (VLPs).[31] Nearly 60% of VLP sequences were found to differ from anything within sequence databases (a common theme in virome studies, discussed further in the following text). Of the sequences similar to reference

bacteriophages, more than 80% were from temperate prophages and members of the *Siphoviridae* family, and the remainder largely consisted of other *Caudovirales* and *Microviridae*. To date, the fecal virome studies in healthy subjects have led to several important observations: (1) there is little intrapersonal variation in bacteriophages, (2) there is a high degree of interpersonal variation in bacteriophages, even among genetically related subjects, (3) temperate bacteriophages are prevalent, (4) the diversity of bacteriophages increases in adulthood, and (5) diet affects the bacteriophage community structure.[3,24,25,32] Furthermore, antibiotic resistance gene annotations were observed in VLP preparations from mice given antibiotics.[33] Enrichment of VLPs and modern sequencing technologies allow for investigation of virome diversity at an unprecedented level and will be valuable for studies of host-associated viromes.

Despite advances in sequencing technology, we have been unable to classify most sequences obtained from VLP preparations. The studies discussed in the preceding text were unable to classify 60% to 87% of VLP sequences or contigs.[3,25,33] Development of more extensive bacteriophage reference databases or new classification schemes is required to better describe bacteriophage gene content and compare bacteriophage communities between individual subjects or groups. Given the importance of bacteriophage regulation of the bacterial microbiome, a major effort is warranted.

How Do Bacteriophages Affect Human GI Function?

Intestinal health is associated with a diverse prokaryotic community; reduced bacterial diversity is associated with diseases such as IBD.[34] The exchange of fitness genes, including antibiotic and nitric oxide resistance genes, is increased by bacteriophage-mediated transmission of genetic material.[29,33] Intestinal inflammation, a feature of IBD, might stimulate prophage induction, based on reports of abnormal enteric bacteriophage communities in patients with IBD.[35,36] Microscopy and nucleic acid sequencing studies have shown increases in *Caudovirales* and bacteriophages in ileal tissues from patients with IBD compared with controls. Bacteriophages might therefore disrupt bacterial communities or even act directly on GI cells to promote the development of IBD.

Little is known about interactions between bacteriophages and mammalian cells. Bacterial translocation is associated with intestinal disease and results from breakdown in the intestinal barrier,[37] yet bacteriophage translocation and its association with disease is less clear. A study of 19 patients with Crohn's disease (CD) and 18 healthy controls provided evidence that bacteriophages can spread systemically; *Mycobacterium* bacteriophages were isolated from blood samples from patients with CD and controls.[38] Studies have reported bacteriophage translocation in animals (reviewed by Górski et al[39]). In the circulation, bacteriophages would be readily available for immune detection; they are known to induce humoral immune responses.[40,41]

Additionally, bacteriophages have been reported to have adjuvant-like effects during virus infection of eukaryotic cells[42] and to stimulate cytokine production by macrophages in vitro.[43] Furthermore, bacteriophage capsid proteins adhere to mucin glycoproteins,[44] putting them in proximity with the intestinal epithelium, where they might interact with the immune system. Given their abundance in the intestine, their ability to stimulate immune responses, and their effects on the prokaryotic community, bacteriophages should continue to be evaluated for their roles in maintenance of GI health and development of disease.

The Eukaryotic Virome

Viruses that infect eukaryotic cells in the GI tract are less abundant than bacteriophages but are the main etiologic agents of acute gastroenteritis,[45] persist in healthy and immunocompromised subjects,[46] and can cause intestinal disease.[47] There are several well-characterized enteric viral pathogens that cause acute enteritis or colitis (eg, rotavirus, norovirus, adenovirus, astrovirus, and coronavirus; reviewed elsewhere[45,48,49]). The issue of persistence of these viruses has been understudied, but a significant proportion of apparently healthy subjects may shed certain "pathogenic" enteric eukaryotic viruses. For example, noroviruses may be shed for extended periods and can be the source of outbreaks of disease among those exposed to the carrier.[50–54] Sequencing of fecal samples from healthy children has revealed a complex community of eukaryotic viruses that includes picobirnaviruses, adenoviruses, anelloviruses, astroviruses, bocaviruses, enteroviruses, rotaviruses, and sapoviruses.[46] A study of RNA viruses in the fecal material of 2 healthy donors revealed 42 viral species, including 35 plant RNA viruses (probably originating from digested food) and human picobirnavirus.[55] Picobirnaviruses have been widely detected in stool samples from subjects with diarrhea of unknown etiology,[56–58] in healthy subjects,[46] and in livestock,[59] although the pathogenic potential of picobirnaviruses is unclear. The detection of these viruses in humans indicates that many viruses capable of infecting eukaryotic cells reside within the human GI tract.

Anelloviridae and *Circoviridae* families have also been widely detected in the human gut[60–62] and in some cases systemically.[63,64] Anelloviruses and circoviruses are incredibly diverse and are present in many organisms, likely related to their status as ancient components of the tree of life and to the high rate of recombination among single-stranded DNA viruses.[65–67] Although it is unclear how these viruses are transmitted to humans, anellovirus genomes are readily detected within the first few months of life; nearly 100% of children seroconvert by the time they are 5 years of age.[64,68] Given the near ubiquitous presence of anelloviruses and circoviruses in plants and livestock,[69] transmission by the ingestion of contaminated food seems likely. This was recently reported for gyrovirus 4, which is a circovirus detected in poultry products and human stool.[70]

Although there is no strong evidence that anelloviruses cause diseases in animals, a relationship has been described between anellovirus DNA and fever in pediatric patients.[71]

In contrast, circoviruses cause a wide array of diseases in birds, pigs, and, more recently, dogs.[72–74] The diseases caused by dog and porcine circovirus increase in severity during coinfection with one or more other enteric pathogens,[75] so the pathogenesis of circovirus might depend on the pathogenic effects of other organisms.

Eukaryotic Virome Dynamics in Disease

The human immune system develops over the first several years of life.[76] Because of this and the increased susceptibility to viral gastroenteritis in newborns,[45] many researchers have used virus-specific approaches to associate eukaryotic virome expansion with immunosuppression and disease.[46,62] For example, stool samples from 2 healthy infant siblings were collected at 1-week intervals over a period of 1 year; virus shedding was analyzed using polymerase chain reaction.[46] Interestingly, the healthy infants shed a number of different eukaryotic viruses (eg, picobirnaviruses, adenoviruses, anelloviruses, astroviruses, bocaviruses, enteroviruses, rotaviruses, and sapoviruses) for extended periods without major clinical symptoms.[46] Another study of children with nonpolio acute flaccid paralysis and healthy controls identified several known and novel viruses in both groups of subjects.[62] Sequencing of stool samples from pediatric patients with acute diarrhea identified known enteric pathogens and several highly divergent viruses, with as little as 35% amino acid identity to the nearest relative in GenBank. The viruses identified included new astroviruses, picobirnaviruses, caliciviruses, nodaviruses, and anelloviruses.[57] These findings show that the human GI tract is colonized by a diverse set of viruses during early stages of development in healthy infants and those with diseases. The role of these viruses on the development and function of the immune system, as well as the overall generation of a healthy or diseased GI tract, requires further study.

It is intriguing to speculate that an immature immune system allows for prolonged eukaryotic virome expansion in children and the establishment of an enteric eukaryotic virome. However, it is not clear whether this would be an event that conditions a healthy immune system or a pathogenic process. A recent study of the enteric virome in monkeys infected with simian immunodeficiency virus[77] could shed light on the interactions between the GI virome and the immune system. In the study, infection with simian immunodeficiency virus led to expansion of enteric eukaryotic viruses in Rhesus macaques, including picornaviruses, adenoviruses, parvoviruses, circoviruses, and caliciviruses. This virome expansion corresponded with increased gut permeability and ileal epithelial pathology. Interestingly, virome expansion and disease was not observed in simian immunodeficiency virus–infected African green monkeys, which do not develop acquired immunodeficiency syndrome, suggesting that increased virus shedding, and potentially damage to the intestinal wall, is secondary to immunodeficiency.

Eukaryotic Viruses and IBD

The etiology of IBD is unclear, but environmental stimuli, microbiome composition, and genetics all appear to contribute.[78,79] Genome-wide association and metadata analyses have associated 163 loci with an increased risk of CD and ulcerative colitis.[80] These studies revealed a number of common pathways involved in the pathogenesis of IBD, including autophagy, barrier function, and adaptive and innate immunity. However, a study of monozygotic and dizygotic twin pairs revealed that the concordance rates for CD and ulcerative colitis were only 35% and 16%, respectively, indicating that environmental factors are major determinants of disease development.[81] Intestinal microbiome imbalance, or dysbiosis, is believed to be one important environmental determinant,[82] and enteric viruses could be involved.[83,84] A role for enteric virus infection in the pathogenesis of IBD was reported in mice deficient for the IBD susceptibility gene *Atg16L1*.[47] In this study, IBD-like pathology developed in mice with disruptions in *Atg16L1* after infection with murine norovirus but not in wild-type mice. Pathology was also dependent on the bacterial composition of the gut, shown by the reversal of virus-induced disease with antibiotic treatment. This finding indicates that a balance of enteric viruses, bacteria, and host genetic factors determine the health of the intestine and that it is important to consider the enteric virome in studies of intestinal diseases.

Introduction to Meiofauna

The diversity of meiofauna living on or in our bodies is vastly underappreciated. This is unfortunate because humans are constantly exposed to these organisms, particularly in developing countries.[85,86] These meiofauna have extensive biochemical capacities and can affect the activities of human cells and those of other commensal microbes. A detailed cataloging and functional analysis of our associated eukaryotic brethren is required for a full understanding of our personal microbial ecology.

Many members of the meiofauna significantly affect morbidity and mortality, including fungi (eg, *Candida*, *Aspergillus*[87]), unicellular Protozoa (eg, *Giardia*, *Entamoeba*[14]), and helminthic worms (eg, *Ascaris*[88]). Similar to studies on prokaryotic community structure, modern sequencing technologies have expanded our view of meiofauna abundance and diversity. Few of these studies have focused on identifying meiofauna in the human GI tract and have instead focused on other tissues such as skin.[89] The analysis of skin for fungi is particularly informative, because the skin contains a diverse mycobiome that varies by location on the body. This is parallel to the earlier discovery of the bacterial microbiome, which relied on the use of advanced molecular techniques. No study has shown sampling to saturation,[90,91] so our knowledge of meiofauna diversity in various environments will likely expand with continued analysis.

Meiofauna in the GI Microbiome

Similar to the study of eukaryotic viruses, extensive research has been dedicated to the examination of

meiofauna in the context of infectious disease, particularly when morbidity and mortality are high (eg, amoebiasis). However, these studies have focused primarily on epidemiology, host-pathogen interactions, and treatment.[92–94] Several studies have used low-throughput sequencing or culture-based techniques to show that commensal GI meiofauna exist and may be important in promoting health or disease.[95–98] A summarized overview of meiofauna regularly associated with the human GI tract is shown in Figure 2. *Blastocystis*, a single-celled protozoan, is a common GI inhabitant, as are fungi from the genus *Candida*.[97,98] Moreover, restriction fragment length polymorphism analysis of 18S ribosomal DNA clones identified 37 different fungal types, including several species of *Aspergillus* and *Saccharomyces*, among other diverse fungal genera in the human GI tract.[95] These findings were later supported and expanded to include members of *Penicillium* and *Pneumocystis*.[96] All of these studies were relatively limited in the number of samples processed across different demographic groups, but the trend is that high-throughput molecular techniques identified higher levels of diversity than low-throughput techniques.

Two important studies used modern metagenomic sequencing techniques to analyze the human intestinal meiofauna. The first study was an element of the reports from the Human Microbiome Consortium in 2012. In parallel to the efforts of the Consortium to characterize prokaryotic communities using 16s ribosomal RNA amplicon sequencing, there was an effort to use random, shotgun sequencing, which captured meiofaunal (and other) sequences. Using this approach, <1% of the reads mapped to nonbacterial taxa,[99] most of which were fungal, represented by the taxa *Ascomycota* (which includes *Saccharomyces* and *Candida*) and *Microsporidia*. The remaining eukaryotic assigned reads were represented by *Hexamitidae* (which includes the genus *Giardia*), Trichomonadidae, and *Entamoeba*.[100–102]

More recently, the fungal meiofauna of 96 stool samples from healthy subjects were analyzed using 454 pyrosequencing of the *ITS1* gene fragment.[103] In total, 66 fungal genera were identified and fungal sequences were detected in 100% of the samples. Per individual fungal sequence diversity was relatively low compared with prokaryotic diversity; most subjects had fewer than 10 detectable genera. Similar to early studies, *Saccharomyces* and *Candida* were the dominant fungal taxa. The investigators also analyzed populations of Archaea in the same stool samples and correlated abundance of Archaea with fungal taxa. In doing so, they correlated abundance of the Archaea *Methanobrevibacter* with *Candida* in samples from subjects with carbohydrate-rich diets. These findings indicate that diet can affect proportions of meiofauna and that there is potential for trans-kingdom interactions in the GI tract.

Meiofauna and GI Disease

The incidence of IBD is higher in developed countries than in the developing world.[104] This is likely due to several host and environmental factors. One widely considered environmental factor is decreased meiofauna exposure in industrialized communities. This is particularly evident for helminths and *Blastocystis*, which have been inversely correlated with the incidence of IBD[105] and protect animal models of IBD by suppressing inflammation.[106–108] This

Figure 2. Simplified taxonomic overview of meiofauna in the human GI tract. An overview of the common meiofauna present in the human intestine. Opisthokonta include animals, fungi, and many microbial eukaryotic organisms; Amoebozoa are unicellular amoeboid protozoa; Fornicata are unicellular flagellated protozoa; Alveolata contain eukaryotes such as the apicomplexa, ciliates, and dinoflagellates; and Stramenopiles are a major line of eukaryotes containing a wide range of species from algae to diatoms.
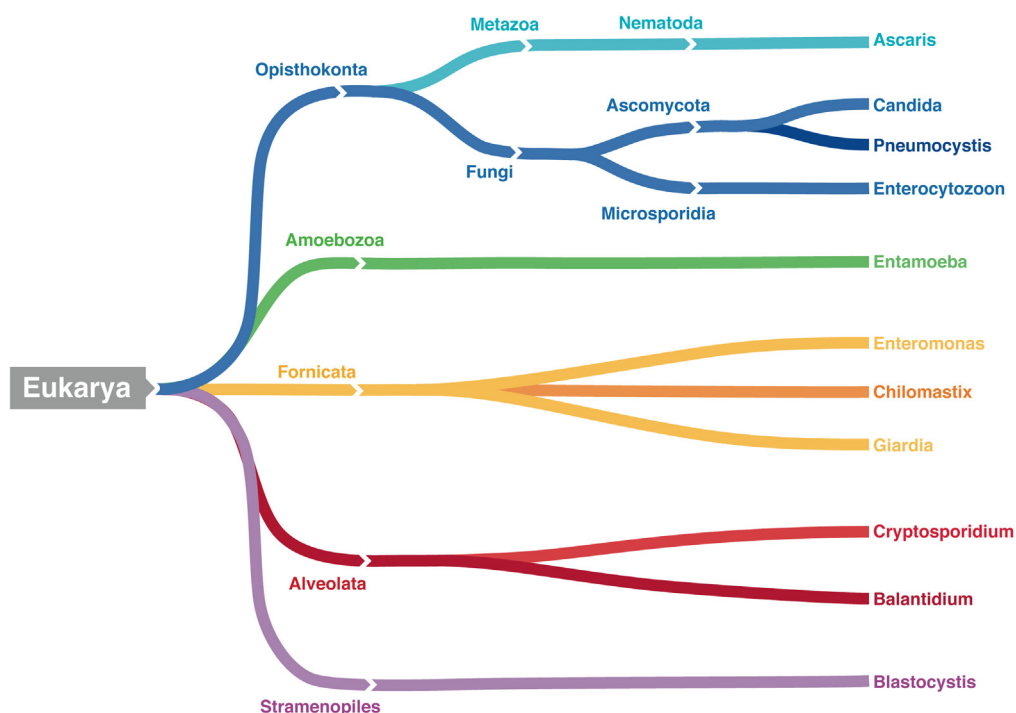
protection could result from meiofauna-mediated alterations in the intestinal bacterial community[109] or direct interactions between meiofauna and host cells.[106,107] A study of 78 patients with IBD and 75 healthy subjects (controls) revealed that T-cell responses to helminth antigen were greater in controls than in patients with IBD, suggesting that exposure or perhaps robust responses to antigens protect against intestinal inflammation.[110] In fact, helminth colonization might have therapeutic potential for patients with IBD.[111]

Several lines of evidence indicate that increased fungal diversity contributes to GI diseases, including IBD.[17] Serum samples from patients with IBD have increased levels of antibodies against *Saccharomyces* compared with controls,[112] which has proven useful for the diagnosis of IBD[113] but may also suggest fungal involvement in the disease process.[114] Decreases in fungal diversity correlate with an increase in healthy bacterial colonization after probiotic therapy,[115] suggesting a role for fungi in treatment strategies, and trans-kingdom interactions between fungi and bacteria. Studies in mice have identified an interaction between GI fungi and the immune system.[116] The C-type lectin receptor Dectin-1 recognizes B-1,3-glucans found on fungal cell walls. Their interaction leads to production of inflammatory cytokines and T-helper 17 cell-mediated immune responses.[16] Mice lacking Dectin-1 develop more severe chemical-induced colitis than wild-type mice and have altered responses to endogenous fungi.

One of the best examples of trans-kingdom interactions that affect human health is mucosal *Candida* infection after antibiotic treatment.[117] Yeast are not directly affected by antibiotics, but antibiotic treatment alters bacterial flora, which enables yeast to proliferate. This may be due to competitive exclusion of the yeast by bacterial production of short-chain fatty acids, which may be less prominent during antibiotic treatment.[118,119] These antibiotic effects can last for months and have been implicated in increases in rates of *Candida albicans* infection.[120,121] These studies highlight the importance of trans-kingdom interactions in intestinal health and disease.

Techniques for Trans-kingdom Metagenomics

Taxonomic studies of meiofauna are similar to those of prokaryotic communities in that amplicon-based sequencing has proven useful. Genes of the small subunit (18S), large subunit (28S), and internal transcribed spacer (*ITS*) regions have been regularly used in meiofaunal metagenomic studies.[16,103,122] The ITS region is of particular interest because of its high variability, which enables species-level differentiation.[123] However, variation in genome copy number makes the interpretation of eukaryotic amplicon sequencing difficult. Although 16S ribosomal RNA copy numbers also vary in prokaryotic genomes, the variation is more extreme in eukaryotes, which have tens or thousands of copies of ribosomal genes.[15] Thus, alternative techniques are required to confirm any quantitative interpretations.

The amplicon approach is particularly challenging for viral metagenomics. Viruses are not monophyletic and do not share a conserved gene at a high level of conservation across all or even most taxa. Therefore, a single amplicon-based approach does not identify all viral types in a microbiome. In addition, most viral genes are poorly conserved.[124] In fact, variation at the nucleotide level of up to 5% or even more occurs in quasispecies of a single virus indicating that nucleic acid conservation is not accurate as a differentiator between viruses when multiple related viruses are present. This contrasts strongly with analysis of the bacterial and meiofauna metagenome (at least to the extent that we understand them today), where sequence diversity is low in comparison.

In the absence of a single amplicon-based strategy, laboratory enrichment of VLPs, followed by random sequencing, has proven useful. This process focuses sequencing depth on viral genetic material and has enabled numerous discoveries in GI bacteriophage and eukaryotic virus populations.[25,31,125] Unfortunately, current VLP-based protocols likely remove or destroy certain types of viruses, depending on the laboratory methods. For example, during purification of VLPs, filtrates are frequently treated with chloroform before VLP nucleic acid extraction to remove contaminating materials.[20,21] This process is likely to remove or destroy enveloped viruses, producing less than comprehensive results. Although protocol modifications are required for comprehensive analyses of the total viral metagenome, the utility of these procedures is unquestionable.

Challenges for Kingdom-Agnostic Metagenomics

We must make several considerations in planning kingdom-agnostic metagenomic studies. Metagenomic sequencing randomly samples a sequence from a population of molecules, comprising full or partial microbial genomes and potentially host genetic contaminants in various relative abundances. In a GI microbiome, prokaryotes and bacteriophages are most abundant compared with relatively rare meiofauna and eukaryotic viral taxa.

There are large differences in cellular integrity among these groups (eg, the chitinous walls of yeast cells vs proteinaceous viral capsids) and nucleic acid encapsidation (eg, nuclear in many meiofauna vs cytoplasmic in bacteria). These differences require special consideration when the study is planned because variations in specimen storage and nucleic acid extraction may be required. Additionally, complementary DNA (cDNA) synthesis is required for detection of RNA-based organisms by modern sequencing technologies. Although cDNA synthesis itself is not challenging, the interpretation of results from metagenomic sequencing of a mixed pool of cDNA and DNA can be. For example, a single-copy gene in a cell may be represented by tens or thousands of transcripts. Our inability to distinguish genes from cDNAs made from transcripts makes it difficult to assign accurate taxonomic abundances.

In addition to differences in proportions of organisms, genome sizes vary greatly among organisms; viral genomes are as small as a few kilobases in size, whereas fungal genomes can be 100 megabases.[126] The probability of randomly sampling from a microorganism depends on genome size and its proportion. These properties have recently been explored using a generalization of the Steven's theorem to identify rare genomes in a mixed sample. Wendl et al[127] showed that the expected number of reads covering the target genome increases as the total number of supplied reads increases. For example, for a 50-kilobase target in 0.05% abundance and a 100–base pair read length, we would expect to identify 5 reads aligning to the target with just 18,000 input reads. This number increases as more reads are added, achieving 20 assigned reads with 55,000 input reads and so on. The investigators stated that most of these reads will exist as singletons and contig coverage will only occur as more reads are supplied. Because many reads and contigs in viral metagenomes are unique (they do not match anything in the reference database), the assembly of contigs is a highly desirable goal. Analysis of contig structure and shared contigs between samples has been informative, and until more comprehensive reference databases are generated, contig analysis will likely be the primary method for analysis of viral metagenomes.[3,25,128]

As with all studies that use modern sequencing technologies, computational time and personnel expenses are major limiting components of metagenomic studies. Fortunately, many modern bioinformatic tools are available for analyzing either amplicon or shotgun sequence data.[129,130] The time to complete any metagenomic analysis (be it amplicon or shotgun) depends on the questions at hand. Each type of project may require additional analyses, which are frequently unexpected, as well as expertise in the specific subject matter. This is particularly true for the non-prokaryotic members of microbial communities because of the scarcity of available reference resources, which makes annotation and classification particularly challenging. Therefore, trained computational biologists and microbiologists are required for the efficient and effective completion of a study.

Future Directions

Analyses of the bacterial component of the enteric microbiome have led to a greater understanding of intestinal health and disease. However, we coexist with microbes from all kingdoms of life and the importance of trans-kingdom interactions is becoming increasingly evident. Studies of intestinal health and disease must therefore consider the contributions of microbes from all kingdoms, including the virome and mycobiome. We hope this review brings to light ways to overcome some of the technical hurdles inherent to kingdom-agnostic metagenomics and the importance of a concerted effort to do so. The lessons from the analysis of the bacterial microbiome are profound for all of biology. It is extremely likely that the metagenome as a whole will contain both surprises and unexpected value.

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CONCEPTS IN MAMMALIAN GUT MICROBIOME