

The Neglected Gut Microbiome: Fungi, Protozoa, and Bacteriophages in Inflammatory Bowel Disease

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The gut microbiome has been implicated in the pathogenesis of inflammatory bowel disease (IBD). Studies suggest that the IBD gut microbiome is less diverse than that of the unaffected population, a phenomenon often referred to as dysbiosis. However, these studies have heavily focused on bacteria, while other intestinal microorganisms—fungi, protozoa, and bacteriophages—have been neglected. Of the nonbacterial microbes that have been studied in relation to IBD, most are thought to be pathogens, although there is evidence that some of these species may instead be harmless commensals. In this review, we discuss the nonbacterial gut microbiome of IBD, highlighting the current biases, limitations, and outstanding questions that can be addressed with high-throughput DNA sequencing methods. Further, we highlight the importance of studying nonbacterial microorganisms alongside bacteria for a comprehensive view of the whole IBD biome and to provide a more precise definition of dysbiosis in patients. With the rise in popularity of microbiome-altering therapies for the treatment of IBD, such as fecal microbiota transplantation, it is important that we address these knowledge gaps to ensure safe and effective treatment of patients.

Lay Summary

Fungi, protozoa, and bacteriophages are often neglected in gut microbiome research of inflammatory bowel disease. Here, we review what is currently known of these microbes in inflammatory bowel disease and how they can be studied using high-throughput DNA sequencing methods.

Key Words: inflammatory bowel disease (IBD), microbiome, fungi, protozoa, bacteriophage

Introduction

Inflammatory bowel disease (IBD) is an umbrella diagnosis for a group of chronic inflammatory disorders of the gastrointestinal tract; the 2 most commonly diagnosed forms are ulcerative colitis (UC) and Crohn's disease (CD).¹ IBDs are multifactorial diseases that arise from complex interactions between genetic, environmental, and microbial factors.^{2–4} Among the microbial factors, the gut microbiome (Box 1) has been implicated in the disease.^{5,6} Patients with IBD generally have gut microbiomes that are less diverse in species and function compared with unaffected individuals,^{6,7} a microbial signature often referred to as dysbiosis.⁸ Research has not arrived at a consensus on the role of dysbiosis in IBD—whether it is a causal factor in IBD development, a perpetuating factor, or simply a result of the disease or lifestyle changes in response to the disease.^{9,10}

Bacteria are easily identified in gut microbiome studies due to their abundance and have become the focal point of most IBD research (Figure 1), to the exclusion of other intestinal microorganisms such as fungi, protozoa, and bacteriophages.^{11,12} This myopic investigation of the microbiome has caused the usage of terminology to shift to a point of imprecision and opaqueness; the terms *microbiota* and *microbiome* are often used to denote only the bacterial portion of a

microbial community.^{13–15} This usage becomes increasingly ambiguous when researchers describe IBD patients with dysbiosis, yet only the bacterial microbiome has been investigated.^{9,16,17} Currently, nonbacterial microorganisms are mainly associated with pathogenicity.¹² Despite this, many are found in the gastrointestinal tracts of healthy individuals, and there is still conflicting evidence on whether these species have direct and consistent proinflammatory effects.^{6,12,18–21} There is even evidence to suggest that the absence of some nonbacterial species is associated with disease.²² In this review, we seek to address this knowledge gap by discussing what is known of nonbacterial gut microorganisms, namely fungi, protozoa, and viruses, in IBD. We draw attention to new high-throughput sequencing methods used to study these microbes to develop a more comprehensive understanding of the gut microbiome in IBD.

Gut Fungi in IBD

Are gut fungi pathogenic or protective?

Fungi are found on every skin and mucosal surface of the human body,^{26,27} with the skin, vagina, oral cavity, small intestine, and large intestine harboring the highest abundance and diversity of fungal species. Most of these species are yeasts such as *Candida*, *Malassezia*, and *Saccharomyces*,^{27,28}

Box 1. Definitions of terms**Microbiota**

A collection of microbes, including prokaryotes (bacteria and archaea), eukaryotes (microbial parasites and fungi), and viruses, found in a specified environment.²³ The term is often used as a shortened replacement for *bacterial microbiota*, which may cause confusion if not explicitly stated.

Microbiome

The combined microbiota, their genes and gene products, and their surrounding microenvironment.^{23,24} Like *microbiota*, this term is often confusingly used to describe only bacterial populations. However, by default, it denotes the wider population of microbial types (prokaryotes, eukaryotes, and viruses), their genes, and environment.

Amplicon Sequencing

Sometimes referred to as metabarcoding, this high-throughput sequencing method can be used to survey the prokaryotic microbiome community by targeting the bacterial or archaeal 16S ribosomal RNA gene or to survey the eukaryotic community by targeting 18S and ITS (ITS1 and ITS2) ribosomal RNA genes.

Shotgun Sequencing

A high-throughput sequencing method in which the DNA of a sample is fragmented and sequenced at random. When used to explore the microbiome, it is often referred to as metagenomic sequencing.²⁵ Instead of targeting a small proportion of the total genes in a sample like amplicon sequencing, shotgun sequencing captures random fragments of any DNA in the sample, including both host and microbial DNA.

Mycobiome

Like *microbiome*, this term includes the fungal community in a specified environment, and its genetic and environmental information. It is synonymous with *fungi microbiome*.

Protozoa

Unicellular eukaryotic microbes often referred to as parasites in the context of human health.

Virus

A nonliving biological entity that infects cells in order to persist and replicate. Viruses are divided into 2 coarse groupings: those that infect bacteria are termed *bacteriophages* (shortened to *phages*), and those that infect eukaryotic cells, including host cells and microbial eukaryotes, known simply as viruses.

Virome

A term for all viral DNA in a specified environment.

and these species have been associated with IBD.^{12,29} Elevated levels of anti-*S. cerevisiae* antibodies have been found in IBD patients,^{30,31} and these antibodies have been associated with an early risk for surgery in CD patients,³² and used to help differentiate³³ and predict the development of CD and UC.³⁴ Higher abundances of *Candida* species, particularly *C. albicans*, have been found in CD patients and their first-degree relatives, and in a mouse model of colitis.^{35,36} This has sparked the hypothesis that intestinal inflammation leads to a compromised mucosal barrier that allows *C. albicans* and potentially other opportunistic bacteria and fungi to proliferate and/or penetrate the mucosal barrier, thus driving interaction with the host immune system.³⁷ Additionally, this

fungus-induced inflammation may be exacerbated in IBD patients with inherited genetic mutations in antifungal immune genes (eg, *DECTIN-1*, *Card9*).³⁸

However, there is also evidence that *Candida* and *Saccharomyces* are not exclusively pathogenic. For example, the presence of *Candida* species had an alleviating effect in a mouse model of acute colitis³⁹ and *S. cerevisiae* has been shown to have an attenuating effect on *Escherichia coli*-induced mouse colitis,⁴⁰ suggesting that fungi may have a protective effect against IBD. Another species, *S. boulardii*, also had an attenuating effect in mice with carcinogenic colitis, a condition that can develop from IBD.^{41,42} The protective effect of *S. boulardii* has been trialed in CD patients with mixed success rates.^{43,44} Thus, there is evidence that some fungi may be beneficial in IBD, but inconsistent findings exemplify the complexity of fungi-host interactions. Fungal species may have a spectrum of effects that depend on multiple factors related to an individual's physiology and microbiome.¹¹ These complexities cannot be gleaned from single-species interrogations, and instead, methods examining the whole microbial community are now being favored.

Insights into gut fungi in IBD from high-throughput sequencing

Initial studies on fungi were limited to species that could be isolated and cultured, wherein researchers characterized a cultured species by sequencing its genome, or generating antibodies to its cellular components.²⁷ This limitation historically biased the reporting of microbes to only culturable species. This may be one of the reasons why *Candida* and *Saccharomyces* are most often reported in IBD research, as many species from these genera are readily isolated, cultured, and identified.^{45,46} Bacterial research previously suffered from similar limitations, and high-throughput DNA sequencing technologies, such as amplicon sequencing and shotgun sequencing, were developed to help overcome these challenges.²⁵ A major advantage of these methods is that they can indiscriminately capture DNA from several fungal taxonomic groups directly from a sample, without the tedious requirement of culturing each fungal species.⁴⁷ They can also capture DNA from unculturable fungal species in samples dominated by bacterial and human DNA, and thus may give a more representative depiction of the fungal community of a sample.¹¹

With the rise of high-throughput sequencing, the past 10 years have seen a steady increase in studies of the human fungal microbiome, known as the mycobiome.¹¹ The 2 most common sampling types for surveying the mycobiome are fecal samples, either in the form of whole stool or swab, and mucosal biopsies. Fecal samples are used as a proxy for the intestinal microbiome due to the invasiveness of acquiring biopsies,⁴⁷ although it is expected that the microbiome composition of these 2 sample types will somewhat differ.⁴⁸ It is now apparent that the gut mycobiome can include species from several dozen genera of fungi (eg, *Alternaria*, *Aspergillus*, *Candida*, *Cladosporium*, *Cryptococcus*, *Debaryomyces*, *Fusarium*, *Galactomyces*, *Malassezia*, *Penicillium*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Trichosporon*), dominated by yeast species from the family *Saccharomycetaceae*.⁴⁹⁻⁵² Gut mycobiomes differ between individuals and seem to be more temporally variable than gut bacterial microbiomes.^{49,51}

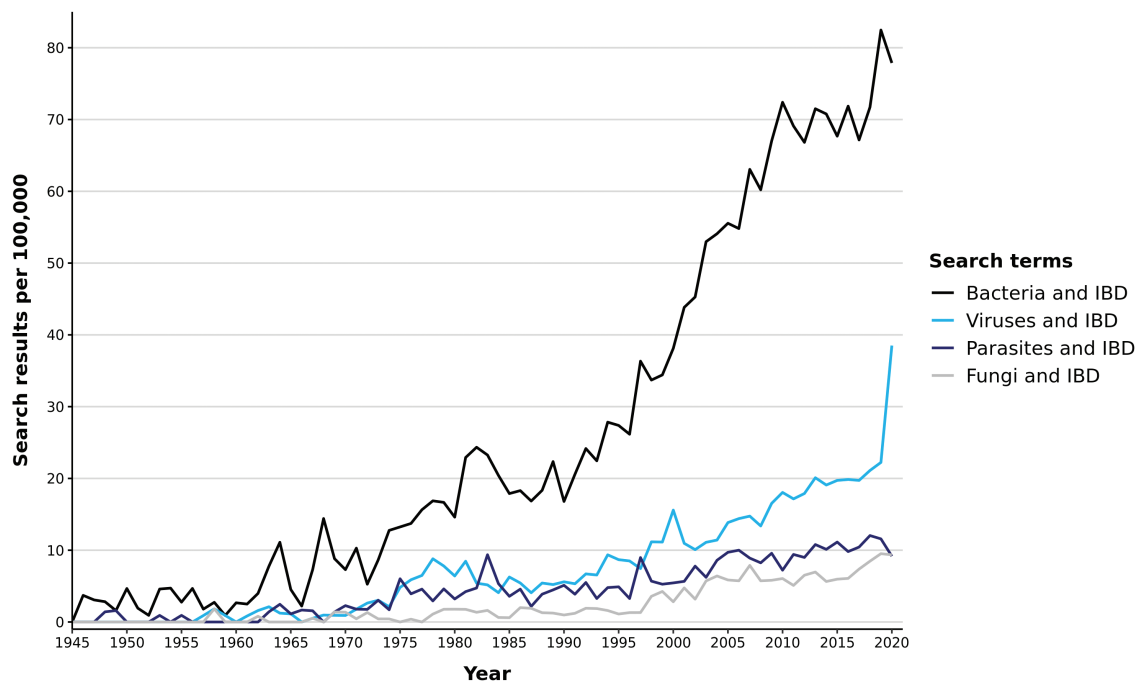


FIGURE 1. Search results of citations featuring microbial types and inflammatory bowel disease (IBD) on PubMed from 1945 to 2020. The total number of search results for bacteria and IBD (15 354) outnumbers results on IBD and viruses (4570), parasites (2314), and fungi (1516). Search conducted on November 1, 2021. Figure generated with PubMed by year (<https://esperr.github.io/pubmed-by-year/>).

Several mycobiome studies indicate that our intestinal mycobiome, like the bacterial microbiome, differs due to environmental factors such as mode of delivery during birth, age, diet, and geographical location.⁵³⁻⁵⁶

High-throughput sequencing approaches have revealed that the gut mycobiome differs in IBD patients, a microbial signature sometimes referred to as *fungus dysbiosis* (Table 1). Amplicon sequencing studies of colonic biopsies have shown that adult CD patients have a higher number of fungal species compared with UC patients and unaffected control subjects,⁵⁷ whereas adults with active UC have fewer species and less abundant mycobiomes in both colonic biopsies⁵⁸ and stool.⁵² CD patients in flare also have a higher fungal load in both inflamed and uninfamed mucosa than CD patients in remission and healthy individuals.⁵⁹ Both CD and UC patients also have an altered abundance of different yeast species, namely *C. tropicalis* in stool of CD patients,⁶⁰ *D. hansenii* in inflamed mucosa of CD patients,⁶¹ *Aspergillus* in colonic biopsies from UC patients,⁵⁸ and an increase in *C. albicans* and decrease in both *M. sympodialis* and *S. cerevisiae* in stool from a cohort of CD and UC patients in flare.⁵² The fact that some yeasts are more abundant in IBD patients has been incorporated into the hypothesis that IBD may be caused or perpetuated by an overgrowth of opportunistic intestinal fungi.^{12,29,37} These fungi may achieve this through interkingdom interactions, such as the biofilm formed between *C. tropicalis* and the bacterial species *E. coli* and *S. marcescens*, which induces the expression of pathogenic fungal hyphae.⁶⁰ Other fungal species may be able to exert their pathogenicity without the help of bacteria. For example, *D. hansenii* was shown to preferentially localize to inflamed mucosa in colonic tissue isolated from biopsy-injured mice and ileal tissue isolated from CD patients.⁶¹ *D. hansenii* prevented repair of colonic mucosa in the absence of bacteria, which was established using gnotobiotic mice.

Because the mycobiome shows a marked alteration in IBD, it has the potential to be used as a diagnostic tool. For example, the fecal mycobiome was used to discriminate between CD and UC by combining fungal load with bacterial load, clinical biomarkers (fecal calprotectin and C-reactive protein), and demographic data (age, gender, BMI, and smoking habit) in a random forest predictive model.⁶⁵ The fecal mycobiome also differs in patients experiencing a flare^{52,65} compared with patients in remission who may have gut mycobiomes that more closely resemble a healthy mycobiome,^{64,65} and was better able to predict relapse in CD and UC patients when fungal load was incorporated into the predictive model described previously. Therefore, the fecal mycobiome may be a minimally invasive diagnostic tool for predicting IBD subtype and relapse.

Mycobiome shifts also exist in pediatric IBD patients (Table 1). Pediatric patients showed a reduction in overall gut fungal diversity and an increase in *Cyberlindnera jadinii* and *C. parapsilosis* in stool samples compared with healthy adult and pediatric control subjects.⁶² In one of the few shotgun sequencing studies of the IBD mycobiome, pediatric patients with active CD undergoing a formula diet (exclusive enteral nutrition) or anti-tumor necrosis factor therapy also had elevated *C. jadinii*, as well as elevated *S. cerevisiae*, *Clavispora lusitanae*, *C. albicans*, and *Kluyveromyces marxianus* at baseline compared with healthy pediatric control subjects.⁶³ This elevation in yeast species subsequently decreased following 8 weeks of nutrition therapy, suggesting that diet is an effective modifier of the mycobiome in patients.

It is clear that differences exist between the IBD mycobiome and unaffected individuals. However, a lack of research and studies incorporating different populations, sample types, and methods existing studies means that it is still too early to ascertain clear trends (Table 1). Research thus far suggests that the IBD mycobiome varies between CD and UC,^{52,57,65}

TABLE 1. Current insights from high-throughput sequencing of the nonbacterial microbiome in IBD

Study	Study Population(s)	Microbiome Sample(s) and Methods	Key Findings
Fungi			
⁵⁷	Active CD (n = 31), active UC (n = 26), non-IBD intestinal inflammation (n = 15), healthy individuals (n = 32)	18S rRNA-based amplification, denaturing gradient gel electrophoresis, and clone library analysis of stool and biopsies from inflamed colon	↑ fungal species in active CD biopsies compared with active UC and control subjects
⁶²	Pediatric IBD (26 CD, 4 UC, and 2 indeterminate colitis), healthy adult and pediatric control subjects (n = 90)	ITS1 sequencing of stool	↓ fungal diversity (Shannon index) in IBD ↑ abundance of <i>Cyberlindnera jadinii</i> and <i>C. parapsilosis</i> and ↓ abundance of <i>Cladosporium cladosporioides</i> in IBD
⁶³	Pediatric active CD (n = 90), healthy pediatric control subjects (n = 26)	Shotgun whole metagenome sequencing of stool	↑ abundance of <i>C. jadinii</i> , <i>S. cerevisiae</i> , <i>Clavispora lusitaniae</i> , <i>C. albicans</i> , and <i>Kluyveromyces marxianus</i> in active CD, which decreased following 8 weeks of exclusive enteral nutrition
⁶⁰	Active and inactive CD (n = 20), CD relatives (n = 28), unrelated healthy individuals (n = 21)	ITS1, ITS2, and 16S rRNA sequencing of stool	↑ abundance of <i>C. tropicalis</i> in CD patients, positively correlated with <i>Serratia marcescens</i> and <i>Escherichia coli</i>
⁵⁹	Active CD (n = 16), inactive CD (n = 7), healthy individuals (n = 10)	ITS2 rRNA sequencing of ileocolonic biopsies with quantitative PCR	↑ fungal load of both inflamed and inflamed mucosa in active CD compared with inactive CD and healthy control subjects
⁵⁸	Active UC (n = 14), healthy individuals (n = 15)	18S and ITS2 rRNA sequencing of biopsies from inflamed colon; quantitative PCR of 18S rRNA for fungal load	↓ fungal species count and abundance in active UC ↑ abundance of <i>Aspergillus</i> in active UC
⁵²	Active CD and UC patients (n = 106), inactive CD and UC patients (n = 129), healthy individuals (n = 38)	ITS2 rRNA sequencing of stool	↑ abundance of <i>C. albicans</i> and ↓ abundance of <i>S. cerevisiae</i> and <i>Malassezia sympodialis</i> in active IBD compared with remission ↓ fungal species count in UC
⁶⁴	PSC patients with IBD in remission (n = 27), PSC patients without IBD (n = 22), IBD patients in remission without PSC (n = 33), and healthy individuals (n = 30)	ITS2 and 16S sequencing of stool	No difference in fungal diversity (Shannon and Chao1 indices) between IBD remission and healthy individuals ↑ fungal diversity (Shannon) index in PSC compared with IBD remission
⁶¹	Mice injured by colonic biopsies and treated with antibiotics to impair healing, control mice injured but not treated with antibiotics Patients with active CD (n = 7) and healthy individuals (n = 10)	Quantitative PCR of ITS of murine mucosal wounds and patient ileal biopsies	↑ <i>Debaryomyces hansenii</i> abundance in mucosal wounds of antibiotic-treated mice compared with control subjects ↑ <i>D. hansenii</i> abundance in inflamed mucosa of CD patients compared with uninfamed mucosa in same patients
⁶⁵	Patients with 3-month remission of UC (n = 31), and ileal or ileocolonic CD (n = 34), patients with active CD (n = 55), UC relatives (n = 29), CD relatives (n = 29), healthy unrelated individuals (n = 28)	Quantitative PCR of ITS2 and 16S rRNA of stool, random forest predictive modelling	↑ fungal load in relapsed CD compared with patients who remained in remission ↑ fungal load in relapsed UC compared with UC remission, CD remission, and CD relapsed Fungal and bacterial load combined with clinical markers (C-reactive protein and fecal calprotectin) and demographic data distinguished UC from CD and could predict relapse
Protozoa			
⁶⁶	Active and inactive CD and UC patients (n = 100), healthy individuals (n = 96)	Culture and PCR of stool	↓ <i>Blastocystis</i> and <i>Dientamoeba fragilis</i> prevalence in active CD and UC than inactive ↓ <i>Blastocystis</i> prevalence in both active and inactive IBD than control subjects

Table 1. Continued

Study	Study Population(s)	Microbiome Sample(s) and Methods	Key Findings
67	Patients with active CD (n = 76) and UC (n = 31), healthy individuals (n = 616)	18S rRNA sequencing of stool	↓ <i>Blastocystis</i> prevalence in IBD
Viruses			
68	Patients with active (n = 10) and inactive (n = 1) ileocolonic CD, healthy individuals (n = 8)	454 pyrosequencing of stool	↓ virome diversity (Shannon index) in CD
69	Pediatric patients with CD (n = 6), and healthy individuals (n = 6)	454 pyrosequencing of ileal and colonic biopsies, and gut washes	↑ abundances of viral species in CD, <i>Caudovirales</i> most abundant
63	Pediatric active CD (n = 90), healthy pediatric control subjects (n = 26)	Shotgun whole metagenome sequencing of stool	No difference in bacteriophage species between groups
70	Patients with CD (n = 18), UC (n = 42), healthy individuals (n = 12)	454 pyrosequencing of stool	↑ abundances of <i>Caudovirales</i> bacteriophage species in CD and UC
71	Patients with new-onset active CD (n = 12), healthy individuals (n = 12)	454 pyrosequencing of colonic biopsies	↑ viral species in active CD
72	C57BL6/J <i>Rag1</i> ^{-/-} mice with colitis induced by injection of CD4+ CD45RB ^{High} T cells (n = 3) and control mice injected with saline (n = 3)	Shotgun whole metagenome sequencing of stool	↑ abundances of <i>Caudovirales</i> bacteriophages species in murine colitis

Abbreviations: CD, Crohn's disease; IBD, inflammatory bowel disease; PCR, polymerase chain reaction; PSC, primary sclerosing cholangitis; rRNA, ribosomal RNA; UC, ulcerative colitis.

between patients in remission and in flare,^{52,64,65} and between sites of inflamed mucosa and adjacent uninflamed tissue.⁶¹ Therefore, it is important that we continue to have different disease subtypes and disease states represented in future IBD mycobiome datasets. Longitudinal sampling will also help uncover whether compositional changes are a cause or effect of flare.⁷³

Limitations and future directions of gut mycobiome research

Preliminary work shows that the IBD mycobiome differs from unaffected individuals, though inconsistencies in findings and, more importantly, an overall lack of research means that much work is still needed in this area. Fungi remain underexplored in sequence-based approaches, likely due to the low abundance of fungal DNA relative to bacterial DNA in gut microbiome samples.^{51,65} It has been hypothesized that the ratio of fungal to bacterial cells changes throughout the gastrointestinal tract and that parts of the upper gastrointestinal tract (stomach and duodenum) have a higher ratio of fungi to bacteria than lower parts (jejunum, ileum, and colon).^{11,74} The proportional influence of fungi may thus differ considerably throughout the gastrointestinal tract, and further research is needed to understand such differences in interactions. The anatomical variation in fungal interactions might indeed be one reason that we see an anatomical restriction in where CD and UC occur in individuals, and in the diseases themselves.

Owing to the low abundance of fungi in intestinal microbiome samples, deep sequencing is required to capture the fungal genomic component of these samples, which can be costly and time-consuming.¹¹ There are, however, methods to mitigate this difficulty. Samples can be enriched for fungal DNA prior to sequencing via several options of protocols and kits, to reduce the sequencing effort required to capture fungal

DNA.^{47,75} Additionally, computational tools have been developed that specifically recognize fungal DNA sequences. There are now several bioinformatic pipelines available to recover and taxonomically assign fungal DNA from amplicon data (eg, RiboTagger)⁷⁶ and shotgun data (eg, FindFungi, EukRep, HumanMycobiomeScan, EukDetect).⁷⁷⁻⁸⁰

High-throughput sequencing methods for studying the mycobiome can circumvent some of the limitations of previous technologies, but they also suffer from their own limitations. Though amplicon sequencing is a useful method for determining fungal abundances and coarse phylogenetic groupings, the approach does not always yield good resolution to the species level, or even to the genus level, and is generally less sensitive than 16S sequencing for bacteria.^{27,81} Shotgun sequencing is more sensitive than amplicon sequencing; however, it is more expensive and computationally intensive. Because shotgun sequencing indiscriminately captures all the DNA in a sample, human DNA contamination is common and must be dealt with in the laboratory and computationally.⁸² Another disadvantage of amplicon sequencing is that it does not allow for direct functional inference, as only ribosomal genes are sequenced with this method, and function is inferred with predictive tools.⁸³ Shotgun sequencing can recover partial or whole microbial genomes, so it enables direct functional inference. This method is additionally advantageous because one can profile both the bacterial and fungal portions of the microbiome in a single effort.

There are also limitations that affect both amplicon and shotgun sequencing. Gene and genome references available for fungi in databases are still biased toward already cultured organisms.²⁷ This is important to consider for both amplicon and shotgun studies wherein yeasts are still frequently reported over other fungi. Because fewer fungal species from the gastrointestinal tract have been cultured than bacteria, even less is known of their metabolic functions, and so predictive tools can be unreliable. Reference databases used to assign

taxonomy to fungi also contain thousands of unannotated and incorrectly annotated sequences.⁸¹ Fortunately, tools that facilitate de novo assembly of fungal genomes are available to characterize the fraction of the mycobiome that is both unculturable and lacking reference genomes, such as a recent pipeline used to identify novel fungal genomes from premature infants,⁸⁴ and EukCC, a tool to estimate the quality of eukaryotic genome assembly.⁸⁵ With these tools, reference databases for fungi are expanding faster than ever before. Researchers can now retrospectively mine metagenomic data for fungal DNA, and this can similarly be done for IBD cohorts. New metagenomic approaches such as these are critically important to answer remaining questions regarding the role of the mycobiome in IBD: Is there a characteristic mycobiome signature of IBD or its subtypes? Is there a tipping point at which yeasts such as *Candida* become more abundant and, potentially, proinflammatory? Which fungal and bacterial species or strains are involved in this process? Is an increase in these species a cause or a result of IBD?

Gut Protozoa in IBD

Gut protozoa: Falsely villainized?

Intestinal parasites are typically known for causing dysenteric infections.⁸⁶ These parasites have gradually been depleted with industrial-associated lifestyle factors such as improved sanitation, hygiene, and health care.^{87,88} However, an industrialized lifestyle has also been associated with the rise in incidences of IBD,^{89,90} and some hypothesize that exposure to certain intestinal parasites may be beneficial for maintaining a healthy and diverse microbiome.⁹⁰⁻⁹² Macroparasites, namely helminths, and their purified antigens have been used to treat IBD in mice⁹³⁻⁹⁵ and in controversial human trials with some success.⁹⁶⁻⁹⁹ Protozoa have received far less attention in relation to IBD, although there are several protozoan species that are able to commensally colonize and reside in the human intestine.¹⁸

Blastocystis species and *Dientamoeba fragilis* are the most common protozoa found in human stool and are primarily transmitted through the fecal-oral route.^{100,101} The prevalence of *Blastocystis* species in human stool ranges from 1% to 50% in developed nations and is generally >30% in developing nations.^{67,101,102} Similarly, the prevalence of *D. fragilis* varies greatly between regions of the world, with a higher prevalence in developing regions.^{103,104} *Blastocystis* and *D. fragilis* are often blamed for causing gastroenteritis-like symptoms, although they have been found in both symptomatic and asymptomatic individuals and their pathogenicity is thus still debated.¹⁸ More recently, largescale controlled cohorts have not found an association between *Blastocystis*, *D. fragilis*, and gastroenteritis.¹⁰⁵⁻¹⁰⁷ Rather, these protozoa were found to be more abundant in healthy individuals¹⁰⁸ and were also associated with increased gut bacterial diversity.⁵¹ These findings suggest that *Blastocystis* and *D. fragilis* may not be parasitic, but rather enteric commensals. In fact, the name “parasite” may be a misnomer for these species. This hypothesis is also supported in IBD patients, wherein both *Blastocystis* and *D. fragilis* have been found more frequently in unaffected individuals and UC patients with inactive disease than in UC and CD patients with active disease.^{22,66,67} Whether the lower prevalence of *Blastocystis* in patients was a cause or effect of the disease was not addressed in these studies, but we should consider if these protozoa are a hallmark of a healthy gut, and

whether administering antibiotics when they are found may be causing harm.

Limitations and future directions of gut protozoa research

The study of intestinal protozoa has experienced similar biases to fungal research—some species have been heavily studied whereas others are scarcely discussed (Table 1). There is an evident ascertainment bias toward the reporting of culturable parasites, and very little is known about unculturable protozoan members of the human gut microbiome.¹⁰⁹ For example, *Blastocystis* species, though anerobic, can be readily cultured and they are commonly detected with microscopy following in vitro culture from stool.^{101,110} However, microscopic detection of *Blastocystis* subtypes in stool is less sensitive than sequencing methods, particularly when they are present in low abundances.¹¹¹ Capturing protozoan DNA can be achieved with 18S amplicon sequencing, and there are parasite-specific 18S primers that can capture DNA from several taxonomic groups.¹¹² Though as stated previously, this method is rarely sensitive enough for robust species-level resolution.¹⁰⁹ Shotgun sequencing can bypass some of the limitations of amplicon sequencing, but it is similarly limited by the low proportion of protozoa in the intestinal microbiome.⁵¹ Thus, deep sequencing, even deeper than required to detect fungi, is necessary to capture enough protozoan DNA for species identifications. It is therefore critical that samples are enriched for eukaryotic cells prior to sequencing. Fortunately, decreases in the cost of sequencing, enrichment for eukaryotic DNA, and improvements to computational methods and reference databases may soon help to provide insights into protozoa in IBD.

Gut Bacteriophages in IBD

Bacteriophages: contributors to the IBD gut microbiome

An assortment of viral particles exists in the gastrointestinal environment of many animals, including humans.^{21,113} Viruses of the gut microbiome include 2 major types: those that infect eukaryotic cells (eg, human cells) and phages that infect bacteria. While both types have been detected in the human gut,¹¹⁴ phages comprise most of the viral species present in the gut. Phages can transfer genetic content, such as antibiotic resistance genes, between bacterial cells,^{114,115} and cause rapid destruction of bacterial cells upon infection during the lytic cycle. Therefore, these viruses can regulate population levels of resident bacteria¹¹⁴ and should be recognized as able contributors to microbiome composition shifts, such as those seen in IBD.¹¹⁶

The gut virome in IBD studies

The gut virome is an emerging area of study in IBD research, and to date, the field contains only a handful of studies (Table 1). One small study of stool samples from CD patients (n = 11) and unaffected control subjects (n = 8) found that virome and bacterial diversity in stool samples was lower in the patients.⁶⁸ Conversely, in another study, colonic biopsies of 12 CD patients had more viral species compared with the 12 control subjects.⁷¹ This same study also found that the sample type and patient from which the sample originated had a greater impact on virome composition than the disease state, suggesting high inter-individual variation in virome composition. Bacterial composition was contrastingly less

variable within groups and was instead more affected by the disease state. Other studies suggest that *Caudovirales* phages, a grouping of over 350 double-stranded DNA viral species,¹¹⁷ may be more abundant in murine colitis, in pediatric CD, and in adults patients with CD and UC.^{69,70,72} However, not all studies have recapitulated this finding.⁶³ Given that virome research is newly emerging, discrepant findings between these studies may be largely influenced by methodological biases (discussed subsequently), in addition to confounding influences between cohorts.

Current limitations and future directions of virome research

Identifying and classifying viral DNA in microbial samples remains challenging.¹¹⁸ As they have incredibly high diversity, tiny gene content, and acquire new mutations rapidly, viral species are not easily assigned to closely related species. There is also no gene common to all viruses that can be used as a viral identity marker, and thus, sequencing viral DNA cannot be achieved with a targeted amplicon-like sequencing method.¹¹³ Additionally, viral DNA makes up a small proportion of the total DNA in a microbiome sample.¹¹⁹ Culturing viruses is equally challenging. Viruses cannot make their own energy because they are parasitic and rely on host cells for resources, so these hosts must be identified and cultured as well. As many microbes of the gastrointestinal tract cannot be cultured, it is difficult to culture their associated viruses.⁶⁸

Embarking on a metagenomic study of the virome may seem like a daunting task, but there are some methodological strategies that can assist in managing the challenge. Prior to sequencing, viral particles can be isolated and purified from a microbiome sample by size selection via centrifugation, filtering (0.2- to 0.45- μm filters), and particle precipitation with polyethylene glycol.¹²⁰ Newer computational tools can also reduce the difficulty of studying the human virome, such as METAVIR, an online resource for annotating virus genes from metagenomic data,¹²¹ and VIP and VirFinder, which provide pipelines to map, filter, and identify viruses from metagenomic sequences.^{122,123} There are also several databases to identify viral genes (eg, National Center for Biotechnology Information viral genomes resource, IMG/VR, and ACLAME).¹²⁴⁻¹²⁶ Future gut virome studies can incorporate tools like these, following viral protein enrichment¹²⁷ or host DNA depletion, and high-throughput sequencing of patient microbiome samples.¹¹⁸

Fecal Microbiota Transplantation and the Neglected Microbiome

Given the observed link between the gut microbiome and IBD, researchers and clinicians have turned to microbiome-based therapies such as fecal microbiota transplantation (FMT) to treat the disease.¹²⁸⁻¹³⁰ FMT initially received attention for its high efficacy in treating *Clostridioides difficile* infections,¹³¹ and is a procedure that involves the transfer of stool or its microbial derivatives from a healthy donor to a patient, by means of enema, oral capsule, or nasogastric tube.¹³² This therapy is presumed to work by restoring a patient's microbiome to a healthy state.¹³³ FMT is an attractive alternative to other standard therapies, such as immunosuppressants, biologics, or surgery, as successful engraftment of

FMT offers the prospect of long-term symptom amelioration without the side effects of other treatment options. So far, FMT for IBD has seen moderate successes and low adverse events in UC patients.¹³⁴⁻¹³⁶ The average rate of clinical remission achieved sits below 50% which is on par with many other IBD therapies, though this number varies depending on factors such as FMT type, mode of administration, donor type (related vs unrelated), IBD subtype, and geographic location.^{134,135}

FMT success rates may also differ because the microbial composition of donor stools used in FMT is still poorly described.⁹ Further, we have only successfully characterized a fraction of the total gut microbiome, leaving many species yet to be described.¹³⁷ It is therefore critical to include all microbial types in microbiome analyses of FMT studies to provide a more precise depiction of the biological material each patient receives. This might allow for better discrimination between effective and ineffective donor and recipient microbiome samples. Currently, prospective donor samples are screened for agents previously regarded as putative pathogens, such as *Blastocystis* and *D. fragilis*,¹³⁸ and donors who are positive for these protozoa may be ruled out.¹³⁹ As we have previously discussed, the growing body of literature would suggest that these protozoa are innocuous colonizers of the human gut and may in fact indicate a healthy microbiome. For example, one study did not find different outcomes between *C. difficile* patients receiving FMT that was positive and negative for *Blastocystis*.¹⁴⁰ Donor-derived viruses may also be an important factor for FMT efficacy. One study found that *C. difficile* patients who received donor stool with a higher content of *Caudovirales* phages were more likely to respond positively to their transplants.¹⁴¹ Thus, further work on gut fungi, protozoa, and bacteriophages is required to reduce the likelihood of discounting commensal species in microbiome-based therapies.

Conclusions and Future Directions

Exploring the ill-defined, nonbacterial microbiome through high-throughput methods is the next logical step toward understanding the link between the gut microbiome and IBD. To this end, it is important that sequencing and computational methods for analyzing eukaryotes and viruses are accessible to clinicians, and that we continue to foster multidisciplinary collaborations to translate bioinformatic results to clinical diagnostics.⁴⁷ Future research should incorporate data from nonbacterial organisms with extensive patient information, such as disease state and lifestyle factors, to disentangle the interplay between microbial and host factors.^{11,116,142} Last, IBD microbiome research, as in many other fields, will benefit from statistical modelling to disentangle relationships between eukaryotes, prokaryotes, viruses, and host genomic data.¹⁴³ As interest in the gut microbiome and microbiome-based therapies continues to rise, studying these relationships will ensure greater precision of diagnostics and treatments for IBD patients.

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The references for this Review were identified using PubMed and Web of Science with Boolean search terms for bacteria (“bacter*”), viruses (“virus* OR viral OR virome* OR bacteriophage* OR phage*”), parasites (“parasit* OR protoz* OR helminth* OR protist*”), and fungi (“fung* OR mycobio* OR yeast*”) combined with the search terms for IBD (“IBD OR Crohn’s OR ulcerative colitis OR inflammatory bowel”). The results were narrowed to articles and reviews published in English. The final reference list was chosen for its novelty and relevance to the scope of this Review topic.

Author Contributions

G.L.G. wrote and conducted the research for the manuscript. L.S.W. and J.M.A. provided substantial discussion and editing of the manuscript.

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Conflicts of Interest

The authors declare no competing interests.

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