

REVIEW



Neglected gut microbiome: interactions of the non-bacterial gut microbiota with enteric pathogens

Kanchan Jaswal*, Olivia A Todd*, and Judith Behnsen 

Department of Microbiology and Immunology, University of Illinois Chicago, Chicago, IL, USA

ABSTRACT

A diverse array of commensal microorganisms inhabits the human intestinal tract. The most abundant and most studied members of this microbial community are undoubtedly bacteria. Their important role in gut physiology, defense against pathogens, and immune system education has been well documented over the last decades. However, the gut microbiome is not restricted to bacteria. It encompasses the entire breadth of microbial life: viruses, archaea, fungi, protists, and parasitic worms can also be found in the gut. While less studied than bacteria, their divergent but important roles during health and disease have become increasingly more appreciated. This review focuses on these understudied members of the gut microbiome. We will detail the composition and development of these microbial communities and will specifically highlight their functional interactions with enteric pathogens, such as species of the family *Enterobacteriaceae*. The interactions can be direct through physical interactions, or indirect through secreted metabolites or modulation of the immune response. We will present general concepts and specific examples of how non-bacterial gut communities modulate bacterial pathogenesis and present an outlook for future gut microbiome research that includes these communities.

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Introduction

The gut microbiota and its importance for health and disease is now widely accepted by scientists and has permeated into common knowledge within the general lay population. Over the past decades, a considerable amount of research has been performed to characterize the composition and functions of gut commensal communities in humans, but also in various other multicellular organisms. Gut microbes are critical to human health and their functions are highly diverse. They range from the production of nutrients for colonocytes to the modulation of critical brain functions and have been extensively reviewed elsewhere.^{1–5} This review focuses on neglected microbiome members and their interactions with enteric bacterial pathogens.

Enteric bacterial pathogens

In this review, we will focus on one particular function of the gut microbiota: colonization resistance

and modulation of the pathogenesis of enteric bacterial pathogens. These pathogens are often food- or water-borne and infect the gastrointestinal tract, frequently causing diarrhea and gut inflammation.⁶ Enteric bacterial pathogens are a highly diverse group that includes Gram positive and Gram negative bacteria. Common Gram positive enteric pathogens are *Clostridium difficile*, an anaerobic gut microbe, and *Enterococcus faecalis*, a facultative anaerobic bacterium. Both can expand after antibiotic treatment^{7,8} and cause difficult to treat gastrointestinal infections. The Gram negative bacteria *Campylobacter jejuni* and *Vibrio cholerae* are examples of important food- and water borne illnesses. However, the bacterial family that is particularly associated with infections of the gut are *Enterobacteriaceae*. They include species of genera such as *Salmonella*, *Escherichia*, *Enterobacter*, *Citrobacter*, *Yersinia*, and *Shigella*. Many of these pathogens cause infections that are restricted to the gut and are self-limiting, such as non-typhoidal serovars of *Salmonella enterica* (e.g. Typhimurium),

CONTACT Judith Behnsen  jbehnsen@uic.edu  Department of Microbiology and Immunology, University of Illinois Chicago, 835 S Wolcott Ave, Chicago, IL MSB E807, USA

*These two authors are contributed equally to this work.

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Citrobacter rodentium, different pathotypes of *E. coli*, and *Shigella* species. Others, such as *Salmonella enterica* serovar Typhi can also cause systemic infections.⁹ In order to colonize the gut environment, enteric bacterial pathogens must effectively compete with the members of the gut microbiome.^{10–13}

Bacterial microbiome and enteric pathogens

The role of the gut microbiota in the pathogenesis of enteric pathogens has been well documented in numerous research articles^{14–19} and summarized in many review articles.^{20–24} However, these publications focus on only one component of the microbiome: bacteria. *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia*²⁵ are important members of the human gut microbiome. These gut commensal bacteria have numerous functions in the intestines and are integral for gut metabolism and immune priming during development. Importantly, they provide colonization resistance to pathogens. Relevant to interactions discussed in this review is for example the breakdown of carbohydrates from the diet by fermentative bacteria into short-chain fatty acids (SCFAs). SCFAs are consumed by epithelial cells but also serve to inhibit bacterial virulence factors. Gut commensal bacterial metabolism thus provides protection against pathogens.²⁶ Other mechanisms of how gut bacteria inhibit enteric pathogens

include competition for space and for specific nutrients.²⁷

Other microbiome members

The bacteria-centric view of the microbiome has broadened in recent years to include other organisms that inhabit the gastrointestinal tract. We now recognize that the microbiome consists of not just bacteria, but also of other prokaryotes (archaea), eukaryotes (fungi, protozoa, parasitic worms), and viruses (archaeal, bacterial and eukaryotic) (Figure 1). While we have learned much about these additional microbiota members, they are still generally considered “neglected”, as research efforts are severely lacking behind the study of the bacteriome. The diverse components of the neglected microbiome not only vary in terms of their average estimated physical size but also in terms of their absolute numbers in the gut (Figure 1). They contribute significantly to general gut metabolism, gut homeostasis, and provide protection from pathogenic infections.^{28,29} Additionally, many inflammatory diseases result from or induce imbalances in the composition of the microbiome.^{27,30} In the following sections we will introduce the gut virome, archaeome, mycobiome and parasitome. We

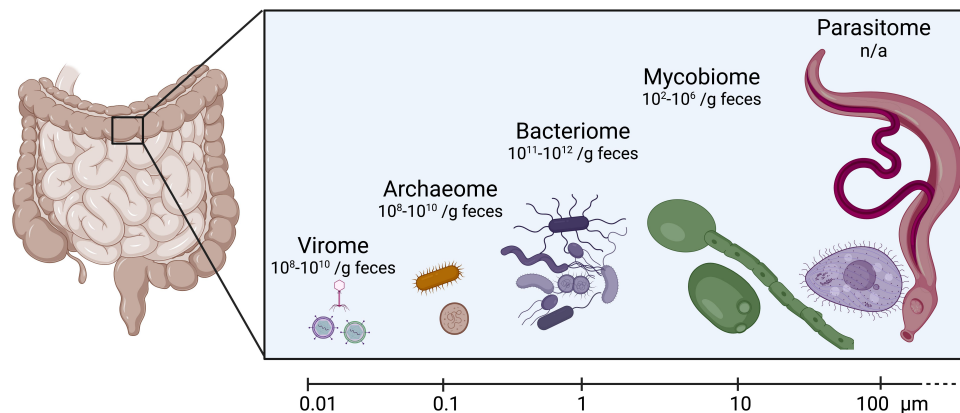


Figure 1. Components of gut microbiota, their approximate size and abundance. The gut microbiota includes viruses, archaea, bacteria, fungi, and parasites. These diverse microorganisms vary in size and abundance in the gut. Only approximate numbers are given for the colon; absolute abundance can vary by orders of magnitude based on location in the gut and applied analysis methods.^{31,225}

will describe their composition and development, but focus specifically on how these neglected microbiome members modulate the pathogenesis of enteric pathogens.

Gut virome

Composition and development

By physical size, the smallest members of the gut microbiome are viruses. However, in overall abundance, their number is close to that of bacteria (Figure 1). The gut virome is defined as viruses and their genomes that are present in the gastrointestinal tract, which includes bacteriophages, eukaryotic phages, endogenous retroviruses, and archaeal viruses.^{32,33} Mycoviruses could potentially be part of the gut virome, but this has not been studied to date. Interestingly, plant viruses that are primarily derived from the environment and diet are also found in the gut.³⁴

During the development of the human gut microbiome, prophages are the pioneers of the gut virome. Analysis of the gut microbiome by shotgun metagenomics in neonates showed rapid colonization by bacteria immediately after birth. Within a month, prophages were induced, leading to the development of a gut virome dominated by bacteriophages. Eukaryotic viruses were detected later during development, at around four months of age.³⁵ Once established, the gut virome remains largely unchanged in healthy individuals. A metagenomics study showed a stable gut virome in healthy adults for up to 26 months, with a high prevalence of temperate bacteriophages (phages that integrate into the host chromosome and reside as prophages). The most abundant phages detected were crAssphages, a group of temperate phages present in *Bacteroides*, *Faecalibacterium*, *Eubacterium*, *Prevotella*, and *Parabacteroides*. Stable colonizers also included *Caudovirales* and *Microviridae* bacteriophages as well as eukaryotic adenoviruses and herpesviruses.^{36,37} According to the human Gut Virome Database, based on 2,697 human gut metagenomes, bacteriophages dominate 97.7% of the human gut virome, with eukaryotic viruses constituting 2.1%, and archaeal viruses making up 0.1% of the human gut virome.^{32,38}

Role of the gut virome during enteric bacterial infections

Even though gut viruses are a substantial part of the gut microbiome, the functions of most viruses in the gut virome have yet to be fully established.³¹ Nevertheless, insightful research has been performed on the role of the gut virome during enteric infections. Studies showed that bacteriophages can direct bacterial evolution and diversity by horizontal gene transfer and can influence bacterial populations, their functions in the gut, and the gut metabolome by interacting with their bacterial hosts.^{39,40} Recent research highlights the importance of phages in maintaining the colonization resistance to enteric pathogens that is mediated by the gut bacteriome. Administration of targeted phage cocktails against *E. coli* and *E. faecalis* had a lasting impact on colonization resistance against *S. Typhimurium*. Phage-mediated reduced abundance of *E. coli* and *E. faecalis* resulted in decreased colonization resistance to *S. Typhimurium*. Unexpectedly, colonization resistance remained low even after *E. coli* and *E. faecalis* abundance recovered, hinting at phage-mediated effects that are not limited to reduction of target bacteria abundance.⁴¹ Similar to the gut bacteriome, the gut virome can aid in the maintenance of gut homeostasis but can also induce an immune response and modulate the host response to enteric infections (Figure 2).²²⁵

Direct inter-species interactions

The prophages present in bacterial hosts directly influence bacterial virulence by several methods (Figure 2). Prophages can encode for toxins and other virulence factors, which consequently increases the coding potential of their hosts. Examples of phage-derived toxins are Shiga toxin in Shiga toxin-producing *Escherichia coli* (STEC),⁴² Pantan-Valentine leukocidin (PVL), a toxin with activity against phagocytes, toxic shock syndrome toxin (TSST) in *Staphylococcus aureus*,^{43,44} and accessory cholera exotoxin (ace) in *Vibrio cholerae*.^{45,46} Prophages also encode for other virulence factors. Efficient entry of *S. Typhimurium* into intestinal epithelium for example requires the effector SopE, which is

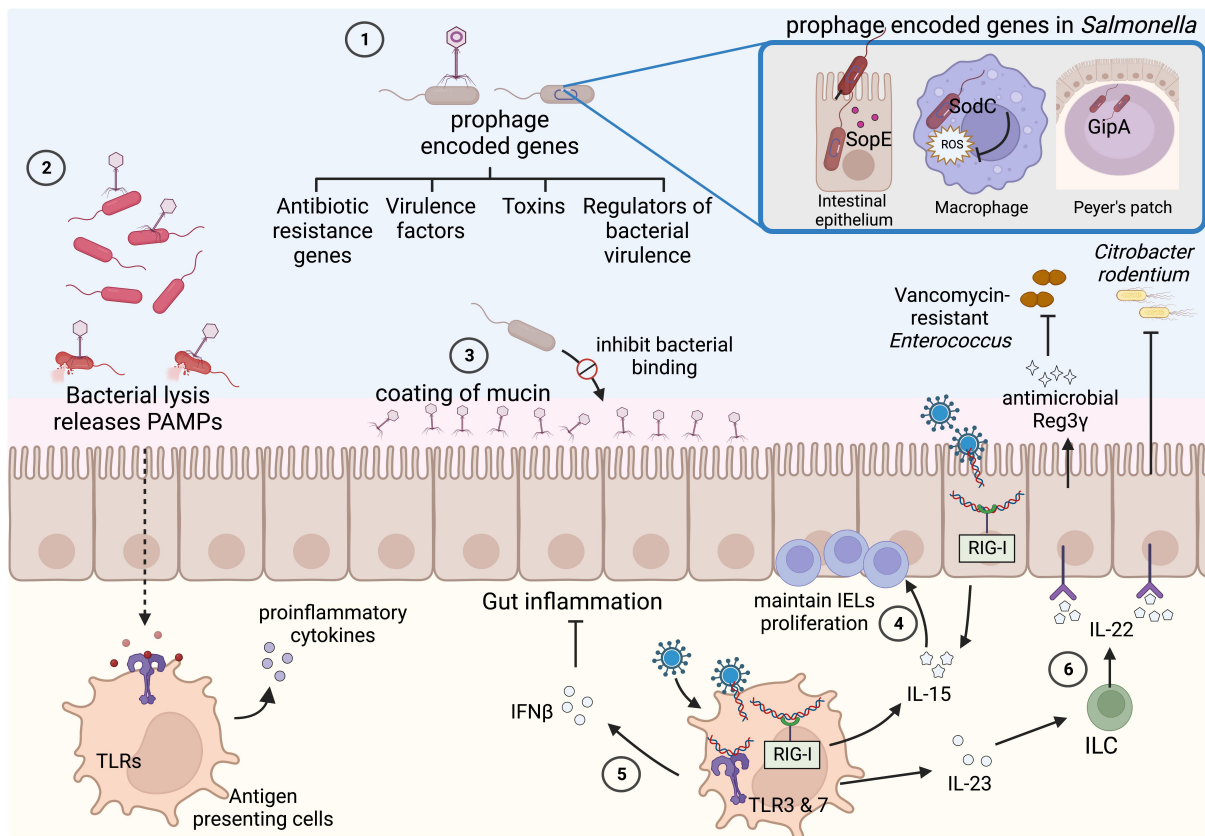


Figure 2. Effect of the gut virome on enteric bacterial infections. Viruses in the gut are primarily bacteriophages and eukaryotic viruses. (1) Prophages alter bacterial phenotypes by encoding genes involved in antibiotic resistance, virulence factors, toxins and regulation of virulence factors. For example, the *S. Typhimurium* prophage Sopφ encodes for SopE, which is required for invading the intestinal epithelium. Prophage Gifsy-1 encodes SodC and GipA, which are required for survival in macrophages and Peyer's patches, respectively. (2) Bacteriophages can induce bacterial lysis, releasing pathogen-associated molecular patterns (PAMPs). These bacterial PAMPs are recognized by antigen-presenting cells (APCs) via pathogen recognition receptors, such as TLRs, leading to the release of proinflammatory cytokines. (3) Bacteriophages can also coat the mucus layer of the gut, preventing bacterial binding to the epithelium. (4) Eukaryotic RNA viruses are detected by cytosolic viral RNA-sensing receptor, RIG-I, resulting in the release of IL-15. IL-15 helps to maintain the number of intestinal intraepithelial lymphocytes (IELs), strengthening the intestinal defense against bacterial infection. (5) Nucleic acids of gut eukaryotic viruses are sensed by TLR3 and TLR7 on APCs, which induces secretion of anti-inflammatory interferon β (IFN-β). (6) Eukaryotic viruses such as murine norovirus induce an interleukin-23 (IL-23)-dependent burst of IL-22 secretion by innate lymphoid cells. The resulting enhanced production of the antimicrobial peptide Reg3γ by epithelial cells can protect against infection by *Citrobacter rodentium* and Vancomycin-resistant *Enterococcus*.

produced by the temperate phage Sopφ (Figure 2).^{47,48} After crossing of the epithelial barrier, *Salmonella* preferentially localizes to Peyer's patches, secondary lymphoid tissues predominantly found in the small intestine. Survival in Peyer's patches requires another prophage, Gifsy-1, which encodes for the *gipA* gene (Figure 2).⁴⁹ The Gifsy-1 prophage also encodes for a superoxide dismutase (SodC), which provides protection against superoxide generated by phagocytes, therefore increasing *S. Typhimurium* survival inside phagocytic cells (Figure 2).⁵⁰ The platelet adherence of *Streptococcus mitis* is mediated by surface proteins present in

a prophage, SM1,⁵¹ and resistance to the neutrophil-mediated killing of *S. aureus* is mediated by a set of genes called chemotaxis inhibitory protein (CHIPS), which are also encoded by a prophage.⁵² In addition to toxins and effectors, temperate phages can encode resistance to many different antibiotics. Such phages were found in many species, such as *S. Typhimurium*,⁵³ *E. coli*,⁵⁴ or *S. aureus*.⁵⁵ While antibiotic resistance is mainly transferred to other bacteria by conjugation or transformation, phage transduction is increasingly being recognized as an additional effective means to transfer antibiotic resistance. For this, prophages have to be induced (see below).

Prophages can not only increase coding potential, they can also regulate bacterial gene expression and modulate the mammalian immune system's response to their bacterial host. Bacterial pathogens can be rendered more virulent through the induction of prophages. Induction of the prophage CTX ϕ , encoding cholera toxin, can result in the transfer of lytic phage to an avirulent strain of *V. cholerae*, rendering it virulent.⁵⁶ In *Corynebacterium diphtheriae*, phage induction can increase the synthesis of diphtheria toxin.⁵⁷ However, even without induction, prophages can regulate virulence. The phage transcription factor Cro has been shown to activate the type III secretion system (T3SS) in enterohemorrhagic *E. coli*.⁵⁸ Similarly, production of the M protein on the surface of *Streptococci* is regulated by a prophage.^{59–61}

Phage-induced bacterial lysis is also an important immune response modulator and can even protect the human host. Phage-induced bacterial lysis can increase the presence of pathogen-associated molecular patterns (PAMPs) in the mammalian host. These bacterial PAMPs are recognized by antigen-presenting cells and activate an immune response (Figure 2). Elevated levels of cell-free DNA in the systemic circulation were for example detected within 24 h following bacteriophage oral administration.⁶² However, whether bacteriophages also increase gut permeability facilitating the translocation of PAMPs to the systemic circulation is currently not known. Phages also coat mucin glycoproteins in the gut by binding via IgA-like protein domains present on phage capsids.⁶³ This strategic position allows phages to lyse bacteria traversing the mucus layer, thereby protecting the underlying epithelial membrane from potentially harmful bacteria (Figure 2).

Maintaining gut homeostasis and immune modulation

The gut virome plays a crucial role in maintaining the health of the gastrointestinal tract. This is highlighted by a study showing the protective role of the virome against the development of colitis. The pretreatment of mice with an antiviral cocktail led to more severe dextran sulfate sodium (DSS)-induced colitis, when compared with mice that were not pretreated. This protective effect was found to be

mediated by Toll-like receptor (TLR) 3 and TLR7, which are sensors for double-stranded and single stranded RNA viruses, and the subsequent induction of Interferon β (IFN- β) secretion (Figure 2).⁶⁴ Recently, gut viruses have also been shown to aid in maintaining the number of intestinal intraepithelial lymphocytes (IELs), which are crucial components of an effective mucosal barrier. The cytosolic viral RNA-sensing receptor in antigen-presenting cells, RIG-I, recognizes commensal viruses and maintains IELs in an Interleukin (IL)-15 axis-dependent manner (Figure 2). Treatment with antiviral cocktails reduced the number of IELs in the intestine.⁶⁵

The composition of the gut virome is considered to be stable for years, but the expansion of certain species has been observed in the case of intestinal inflammation. Phages of *Caudovirales* species, including *Escherichia*, and *Enterobacter* phages, were more abundant in patients with ulcerative colitis (UC), as compared with healthy controls.⁶⁶ UC patients also showed an expansion of *Enterobacteriaceae*,⁶⁷ which could be the reason for the observed increase in phages that infect this bacterial family. Multiple viral functions, particularly associated with increased host bacteria fitness and pathogenicity, were also increased in UC patients.⁶⁶ The important role of the gut virome during inflammatory diseases was further highlighted by a study in mice, which showed that the presence of an enteric virus, murine norovirus (MNV), increased the susceptibility of Atg16L1 (an autophagy gene) hypomorphs to develop Crohn's disease-like pathologies.⁶⁸

Products from commensal bacteria can induce immune signaling pathways of the host and thereby enhance barrier functions in the intestine.⁶⁹ Remarkably, the presence of viruses is sufficient to provide similar cues to the host. Infection by MNV reversed the intestinal abnormalities caused by depletion of bacteria. Intestinal morphology and lymphocyte differentiation, which are altered in germ-free mice due to the absence of commensal bacteria, were restored when germ-free mice were infected with MNV.⁷⁰ The ability of gut viruses to maintain gut homeostasis therefore prompted the question of whether they also prime the gut immune system for protection against bacterial infections. MNV indeed

provided an IL-22-dependent protection against lethal infection by *Citrobacter rodentium* and Vancomycin-resistant *Enterococcus* (VRE) (Figure 2).^{71,72} These beneficial effects could not only be effectively promoted by live virus but also by a resiquimod (R848), a synthetic ligand of TLR-7. Engagement of TLR-7 on dendritic cells resulted in antiviral responses, but also in the production of IL-23. The resulting immune response ultimately culminated in the production of the antimicrobial peptide Reg3γ (Figure 2).⁷² These examples of inter-kingdom interactions between viruses, bacteria and host highlight that viruses need to be considered when attempting to detangle the complex web of interactions with enteric bacterial pathogens in the gut.

Gut archaeome

Composition and development

The domain Archaea consists of anaerobic prokaryotes that share traits with bacteria, but also resemble eukaryotes in some ways. Morphologically, archaea look like bacteria; they do not have a nucleus and can be rod or round shaped (Figure 1). However, cellular processes like transcription and translation are more similar to eukaryotes. Archaea display unique metabolic and biochemical pathways that separate them from the other domains of life, such as the ability to respire H₂ into methane.⁷³ We normally think of archaea as extremophiles; resistant organisms that live in almost uninhabitable environments with high temperatures (45–80°C), high salt (>0.8 M), and high acidity (pH < 3) or alkalinity (pH > 9).⁷⁴ However, archaea are also found to reside within humans and other animals. The first discovery of archaea in the human gut was made in the 1960's, but identification methods were not specific enough to determine the species.^{73,75} Direct culturing of archaea from intestinal samples is difficult due to their specific growth requirements. Molecular methods, such as 16S rRNA sequencing and metagenomic analysis, have improved the identification of archaea, but full genome sequence databases that form the basis for such analyses are far behind those available for bacteria. Additionally, archaea-specific primers are not routinely used in microbiota sequencing, which limits the ability to detect archaeal constituents.^{75,76}

The archaeome is thought to appear shortly after birth, with transmission from the mother to the infant. However, infant microbiomes are more variable than adults and may have transient colonization by archaea.^{77,78} By adulthood, the archaeome is stable, but can be disrupted by diet, gastrointestinal stress, and changes in the host immune system.^{76,78} Studies have identified certain archaeal species (*Methanobrevibacter smithii* and *Methanosphaera stadtmanae*) that are almost universally present throughout human populations, and have even been proposed as identifiers of human fecal contamination of waste-water, as they are highly specific to humans and not found in the environment.^{79,80} The majority of these human-associated archaea are classified as methanogens. These microbes are able to take byproducts from bacterial fermentation and further metabolize them into methane, exemplifying a cross-feeding behavior between microbiome members.

Maintaining gut homeostasis and immune modulation

The most commonly identified archaeon in the human gut is the methanogen *Methanobrevibacter smithii*, which is found in 30–95% of individuals studied and comprises up to 10% of all anaerobes.^{28,80,81} *M. smithii* is able to combine excess H₂ with CO₂ to produce methane (CH₄), acting as a hydrogen sink (Figure 3). High levels of hydrogen in the intestines can cause alterations in the redox potential, which inhibits bacterial metabolism rates.^{82–84} It is hypothesized that *M. smithii* can activate host immune cells, although the specific microbe-associated molecular patterns (MAMPs) have not been identified. One investigation found that intestinal epithelial cells do not react to *Methanobrevibacter* species, but that dendritic cells are stimulated and able to phagocytose the commensal archaeal species.⁸¹

Interactions with enteric bacterial pathogens

In a human clinical study investigating the role of microbiota composition and functionality in vaccine efficacy and infection, higher abundance of *Methanobrevibacter* was associated with less severe

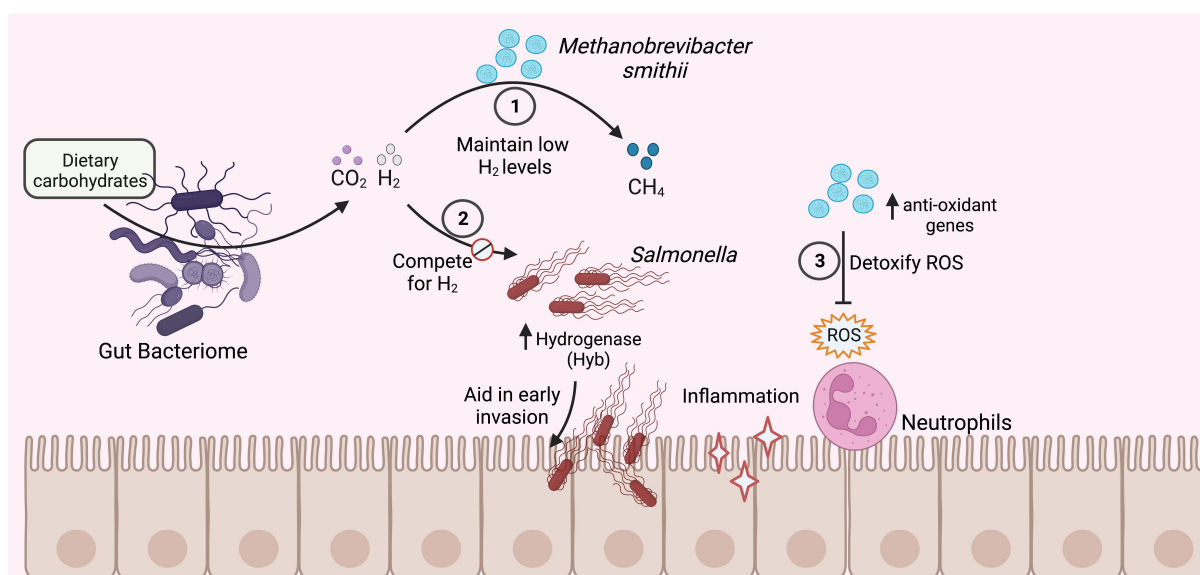


Figure 3. Effect of the gut archaeome on enteric bacterial infections. Methanogenic archaea dominate the archaeome in the human gut. Bacteria ferment dietary carbohydrates into short-chain fatty acids, with CO_2 and H_2 as byproducts. (1) Organisms like *Methanobrevibacter smithii* convert hydrogen into methane, maintaining low levels of hydrogen in the intestines. (2) *S. Typhimurium* can utilize hydrogen as an energy source to increase invasion into intestinal epithelial cells. *M. smithii* sequesters hydrogen and competes with *S. Typhimurium*, limiting its virulence. (3) *S. Typhimurium*, and to a limited degree *S. Typhi*, causes intestinal inflammation and ROS production by immune cells. Anti-oxidant encoding-genes in *M. smithii* are upregulated in response to oxidative stress during *S. Typhi* human infection, potentially detoxifying and balancing the redox environment and limiting *S. Typhi* disease progression.

symptoms following *Salmonella* Typhi challenge.⁸⁵ The researchers further observed that *Methanobrevibacter* antioxidant-associated genes were upregulated during *S. Typhi* infection. They propose this could be due to *S. Typhi*-induced reactive oxygen species (ROS) production by phagocytes. ROS production is an immune mechanism used to combat pathogens, but it can also lead to intestinal inflammation and an altered redox potential, potentially causing harm to the host and commensal organisms.⁸⁶ *Salmonella* Typhimurium has a number of superoxide dismutases that allow for detoxification of superoxides and enable it to survive the oxidative stress.⁸⁷ *S. Typhimurium* has been shown to take advantage of an inflammatory environment to outcompete commensal bacteria, reducing colonization resistance and enhancing infection.^{22,87–89} It is possible that antioxidative effectors produced by resident archaea could combat the ROS response, limiting inflammation and damage, thereby providing protection from *S. Typhimurium* infection.^{85,86} It has also been suggested that *M. smithii*, in addition to hydrogenotrophic commensal bacteria, can compete with *S. Typhimurium* for hydrogen during metabolic

processes (Figure 3). *S. Typhimurium* encodes for hydrogenases that allow the bacterium to use hydrogen as a source of energy during early infection.^{90–92} The *S. Typhimurium* *hyb* hydrogenase has been shown to be required for the initial (first 24 h of infection) invasion of the gut epithelium in low complex microbiota (LCM) mice, presumably before high levels of inflammation in the gut are reached.⁹⁰ Interestingly, addition of an H_2 -consumer during *S. Typhimurium* infection reduced the colonization of *Salmonella*. An avirulent *S. Typhimurium* strain was used as the competing consumer in these experiments, but it is possible that addition of a methanogenic archaeon would result in the same phenotype.⁹⁰ These data suggest that initial invasion by *S. Typhimurium* could be inhibited by the presence of a competitor, such as *M. smithii*.^{76,90} (Figure 3).

Archaea are an important component of the healthy gut microbiota. They participate in syntrophic interactions with fermentative bacteria and help maintain the redox potential with the gastrointestinal tract.⁸⁴ These organisms also aid in providing colonization resistance and metabolite

competition to enteric pathogens.^{85,90} Further research into the functions of the human gut archaeome can identify their role in gut homeostasis and provide an avenue for treatment options to combat dysbiosis and enteric diseases.

Gut mycobiome

Composition and development

Not only prokaryotes and viruses but also microeukaryotes can be found in the gut environment. The gut mycobiome is the collection of fungi present in the gastrointestinal tract. In humans, fungi are detected as early as 10 days after birth.⁹³ The presence of fungi in the meconium (the first stool) raises the possibility that a gut mycobiota exists even prior to birth.⁹⁴ However, this notion is contested, given that contamination by environmental fungi during sample acquisition and processing cannot be excluded.^{94,95} Fungal diversity and the fungal species present in the infant gut mycobiota initially share a lot of similarities with the maternal mycobiota and seem to vary by mode of child birth. Infants that were delivered vaginally showed an enrichment in *Candida* spp., and infants born through emergency C-section were predominantly colonized with species of the skin commensal fungal genus, *Malassezia*.^{96,97} Overall, the most abundant fungal genera identified in infant gut mycobiota studies were *Candida*, *Rhodotorula*, *Malassezia*, *Saccharomyces*, and *Debaryomyces*.^{93,98–101} In particular, *Debaryomyces hansenii* was one of the most abundant fungal species present in the infant gut during breastfeeding.⁹³ It is only after weaning that fungi acquired through the diet, such as *Saccharomyces cerevisiae*, become highly abundant.⁹³

The adult gut is predominantly composed of Ascomycota, Basidiomycota, and Zygomycota at the phylum level and *Candida*, *Paecilomyces*, *Penicillium*, *Aspergillus*, *Trichosporon*, *Rhodotorula*, *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, *Fusarium*, and *Cryptococcus* at the genus level.^{102–105} To date, more than 200 species of fungi have been identified in the adult gut. This seems to indicate a diverse fungal microbiome in the human gut. It is important to note though that the composition of the gut mycobiota can vary drastically between individuals^{106,107} and a majority of species were only detected in a single sample.^{108,109} The definition of

core mycobiome members that are present in most individuals has therefore been challenging. One of the mycobiome members that has been frequently found in fecal samples of healthy adults and that might constitute a core genus is *Malassezia*.^{107,110,111} However, whether *Malassezia* is a true gut resident requires further investigation using more invasive sampling techniques, as the contamination of the feces by skin flora cannot be excluded.¹⁰⁸ Geographical location might additionally influence the composition of the mycobiome. *Malassezia* was present in volunteers recruited from Houston, Texas, but not found in healthy adults from Pennsylvania.^{107,112}

Role of gut mycobiome during enteric bacterial infections

Generally, low fungal abundance is considered to be a characteristic of a healthy gut. Conversely, higher abundance of fungi, and particularly an increase in the yeast *Candida albicans*, have been associated with disease. An imbalance in the gut mycobiota composition has been associated with enteric diseases, such as irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD), and even diseases affecting other distal organs, such as Alzheimer's disease, cancer, and alcohol-induced liver diseases.^{113–119} Many studies have also examined the interaction of bacteria with the gut mycobiota. For example, gut commensal bacteria, such as *Bacteroidetes* and *Firmicutes*, provided colonization resistance against *C. albicans*.¹²⁰ Herman et al. reported a positive association between the presence of *Candida* and species of the genus *Enterobacter* among 68,000 clinical fecal samples.¹²¹ Similarly, recurrence of *S. Typhi* and *S. Paratyphi* infection increased when patients were colonized with *Candida* spp.¹²² *C. albicans* also reduced the necessary infectious dose and increased the dissemination of *Staphylococcus aureus*, *Serratia marcescens*, and *Streptococcus faecalis* in mice.¹²³ Importantly, *C. albicans* can increase *S. aureus* toxin production during *in vitro* and *in vivo* experiments, synergistically increasing bacterial virulence.^{126,127} Although inoculations were performed intraperitoneally in this particular set of experiments, similar interactions could occur in the gut, where fungal and bacterial partners can co-

occur during enteric infections. Conversely, *Saccharomyces boulardii* is used as a probiotic to alleviate the intestinal colonization of *Clostridium difficile* following broad-spectrum antibiotic therapy.^{128–130} Thus, the effect of the gut mycobiota during enteric bacterial infections should not be neglected. In this section, we will discuss how the gut mycobiota affects enteric bacterial infections, either due to direct interkingdom interactions or indirectly by maintaining immune homeostasis.

Direct inter-species interactions

Direct interactions between the gut mycobiota and certain enteric bacterial pathogens can lead to either synergistic or antagonistic effects during disease progression.¹³¹ Most of these direct interactions were elucidated in *in vitro* systems or at body sites, such as the oral cavity, vagina, or the lungs, but could similarly occur in the gut.^{124,125,132–136} The direct binding of the probiotic yeast

Saccharomyces boulardii to Gram negative bacterial pathogens *E. coli* and *S. Typhimurium* is suggested to decrease bacterial virulence. The yeast is therefore frequently studied as an additive to livestock feed.¹³⁷ The underlying mechanisms could be either that binding to the yeast decreases the pathogens' binding to intestinal epithelium or that binding decreases the transit time through the gut and therefore facilitates pathogen elimination¹³⁸ (Figure 4). The binding is inhibited in the presence of mannose, indicating the association of these bacteria to the surface mannose residues present on *S. boulardii*.^{138–140} Fungal-bacterial mixed biofilms have been observed at different body sites, including the gut. These biofilms provide protection against antimicrobial drugs and immune cell recognition (Figure 4).^{141,142} Using an *in vitro* biofilm model, *C. tropicalis*, *E. coli*, and *Serratia marcescens* were observed to form more robust biofilms when all partners were present, compared to single or double species biofilms. The lipopolysaccharides produced by *S. marcescens* and *E. coli* significantly

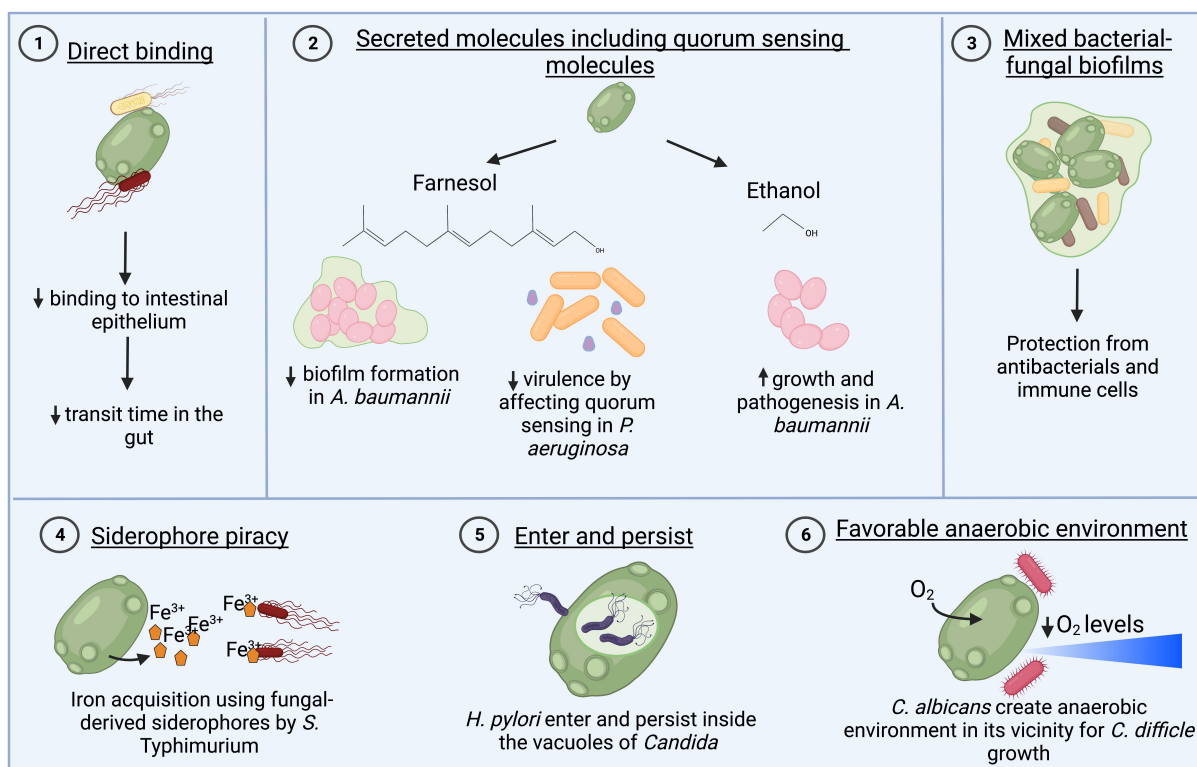


Figure 4. Direct interactions of gut fungi with enteric bacterial pathogens. Fungi within the gut microbiome interact and influence enteric bacterial pathogens by different mechanisms, potentially impacting their virulence. (1) Fungi can directly bind bacteria and reduce their binding to the gut epithelium. (2) Secreted molecules including quorum sensing molecules, such as farnesol and ethanol, can affect bacterial biofilm formation, virulence, and growth. (3) Fungi and bacteria can form mixed bacterial-fungal biofilms, which protect the bacterial pathogen against antibacterial drugs and immune cells. (4) *Salmonella Typhimurium* can utilize siderophores produced by fungi to acquire iron in iron-limited environments. (5) *Helicobacter pylori* can enter and grow inside the vacuole of *Candida*. (6) *C. albicans* provides a favorable environment for the growth of the anaerobic bacterium *Clostridium difficile* by utilizing oxygen in its vicinity.

enhanced fungal biofilm maturation.¹⁴³ These polymicrobial biofilms are highly relevant to enteric infections, as *E. coli*, *S. marcescens*, and *C. albicans* are abundant in fecal samples from IBD patients.¹⁴⁴ Similarly, mixed-species biofilms of *C. albicans* and *Citrobacter freundii* were shown to form more rapidly than single-species biofilms, with fungal cells forming the main core of biofilm and bacteria associating with the periphery.¹⁴⁵

Members of the gut mycobiota can also directly interact with enteric bacterial pathogens via secreted molecules such as quorum sensing molecules. The quorum-sensing molecule farnesol is produced by *C. albicans*, which colonizes the gut and can cause systemic disease when breaching the intestinal barrier. Farnesol increased resistance to vancomycin in *Staphylococcus aureus* during systemic co-infections with both pathogens. Higher resistance was in part mediated by an increase in the expression of drug efflux pumps.¹⁴⁶ Conversely, farnesol can also have detrimental effects on *S. aureus*. It can synergize with antimicrobial compounds and impair *S. aureus* biofilm formation and membrane integrity.¹⁴⁷ Farnesol also affected quorum sensing regulation in *Pseudomonas aeruginosa*, decreased *P. aeruginosa* virulence,¹⁴⁸ and reduced biofilm formation and viability of *A. baumannii* (Figure 4).^{149,150} Conversely, other secreted factors such as ethanol from *Saccharomyces cerevisiae* stimulated the growth and pathogenicity of several *Acinetobacter* species including *Acinetobacter baumannii* in a nematode model with *Caenorhabditis elegans* (Figure 4).¹⁵¹ A protease secreted by *S. boulardii* degraded toxins A and B of *C. difficile* and provided protection against *C. difficile* infection.^{128,152} In addition, *S. boulardii* can also detoxify cholera toxin by internalizing and therefore neutralizing its A subunit.¹⁵³ There are other examples of direct interactions between a specific enteric bacterial pathogen and the mycobiota. One of these interactions is “enter and persist”, as shown for *Helicobacter pylori*, which can enter and grow inside of the vacuole of *Candida* spp. as a survival strategy (Figure 4).¹⁵⁴ Another interaction is based on the creation of a favorable anaerobic environment. *C. albicans* enhanced the growth of *C. difficile*, an anaerobic bacterium, by consuming oxygen and thereby reducing oxygen tension for *C. difficile* in the vicinity of the yeast (Figure 4).^{155,156} On the

other hand, *C. albicans* colonization changes lipid species levels in the cecal lumen, which limits *C. difficile* colonization.¹⁵⁷ In a recently published study, our lab has shown another example of a direct interaction mechanism: siderophore piracy. *S. Typhimurium* has receptors for fungal siderophores and can acquire iron in the gut using xenosiderophores produced by fungi (Figure 4). This fungal siderophore mediated acquisition of iron by *S. Typhimurium* suggests an inter-kingdom cross-feeding between gut mycobiota and enteric bacteria.¹⁵⁸

Maintaining gut homeostasis and immune modulation

Recent studies have suggested that the gut mycobiota aids in the maintenance of gut immune homeostasis. Fungal dysbiosis is highly associated with inflammatory diseases, especially in immunocompromised patients. The host immune response can be modulated by the cell wall components (β -glucan, mannans, and chitin) present in commensal fungi, such as *S. cerevisiae* and *C. albicans*. These pathogen-associated molecular patterns (PAMPs) are recognized by pathogen recognition receptors (PRRs), such as TLR2 and Dectin-1 (recognizes β -glucans and chitin), and mannose receptor, TLR4, Dectin-2, Mincle, and DC-SIGN (recognize mannans and mannoproteins) present on the surface of dendritic cells and macrophages (Figure 5).^{159–164} The recognition of such PAMPs primes immune cells to protect against enteric infections (Figure 5). This is exemplified by the finding that pre-exposure of human monocytes to *S. cerevisiae* chitin promotes proinflammatory cytokines production and increases intracellular killing of fungi and bacteria.¹⁶⁵

Several studies evidently showed how gut commensal fungi are involved in maintaining mucosal immunity and thus indirectly help to combat enteric infections. Colonization with intestinal fungi, for example, resulted in the accumulation of circulating Th17 CD4⁺ T cells and granulocytes.^{166–168} These cells are protective against enteric infections that require the mounting of an effective Th17 response for pathogen clearance (Figure 5). The maturation of murine lymph

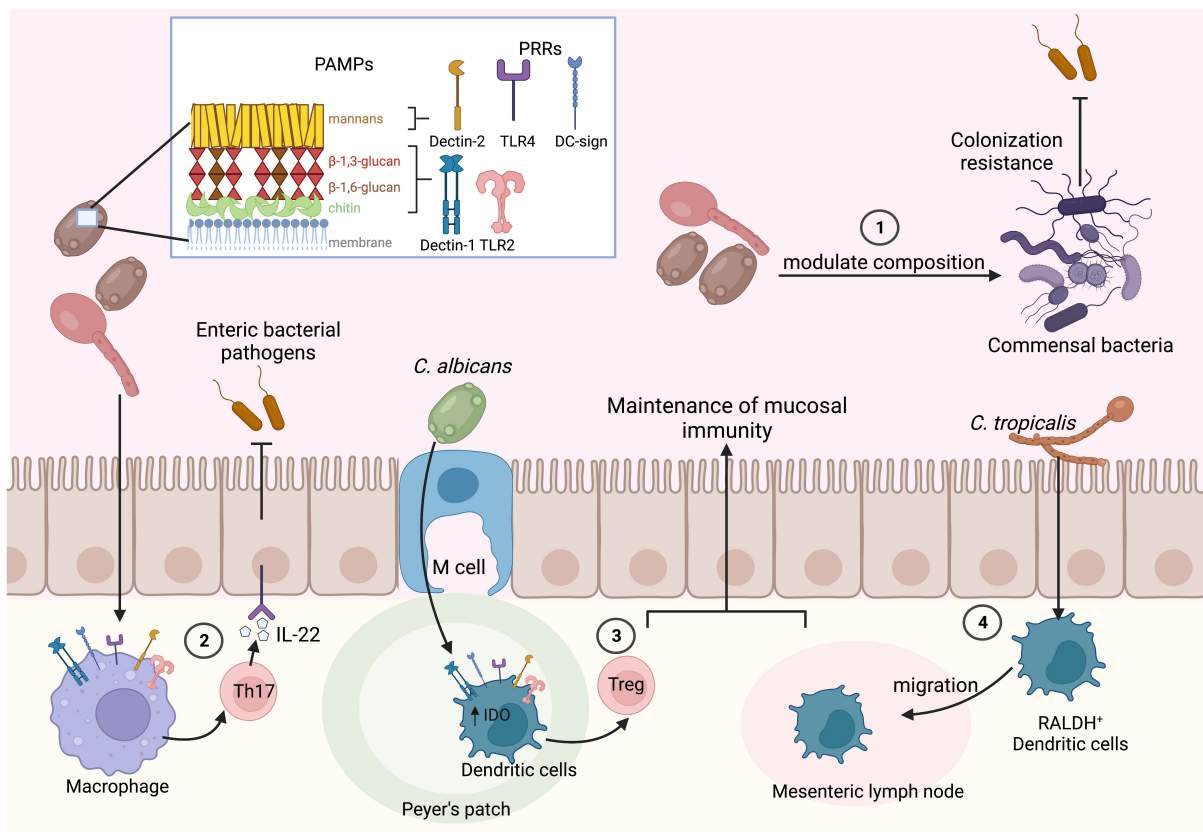


Figure 5. Indirect effects of the gut mycobiome on enteric bacterial infections. (1) Gut fungi are involved in modulating the composition and colonization of commensal gut bacteria, which can in turn affect the colonization resistance to bacterial pathogens. (2) The fungal cell wall is composed of a chitin skeleton linked to a network of β -1,3-glucans and β -1,6-glucans, anchoring proteins and mannans toward the cell surface. Fungal cell wall components act as pathogen-associated molecular patterns (PAMPs) and are recognized by the immune cells via different pathogen recognition receptors (PRRs). The activation of immune cells such as macrophages by these PAMPs induce the release of IL-22 from Th17 cells. IL-22 plays a key role in maintaining gut barrier function against enteric pathogens. (3) Dendritic cells associated with Peyer's patches recognize *C. albicans* and signal to promote the accumulation of regulatory T cells. The accumulation is dependent on the induction of 2,3-indoleamine dioxygenase (IDO) by dendritic cells. (4) *C. tropicalis* induces the migration of gut RALDH⁺ dendritic cells to the peripheral lymph nodes and helps in the maturation of the lymph nodes. These suggested mechanisms help to maintain mucosal immunity.

nodes involves the migration of dendritic cells expressing retinol dehydrogenase (RALDH⁺) from the intestinal lamina propria to the peripheral and mesenteric lymph nodes. Germ-free mice did not accumulate these RALDH⁺ dendritic cells in the lymph nodes, which negatively affected lymph nodes structure and cellularity. The decrease in RALDH⁺ dendritic cells was also observed in wild type mice treated with an antifungal cocktail, but not in antibiotic treated mice. Interestingly, oral gavage with *C. tropicalis* regained the migration of gut RALDH⁺ dendritic cells to the peripheral lymph nodes (Figure 5). This effect was found to be specific to the gut commensal fungus *C. tropicalis*, as other commensal fungi, such as *Trichosporon asahii* or *S. cerevisiae* did not induce

this migration.^{169,170} During inflammatory conditions, the presence of gut mycobiota members also provided protection to the gastrointestinal tract. Colonization with *C. albicans* helped to maintain immune homeostasis and to limit inflammation by promoting the expansion of regulatory T cells during DSS induced colitis. Peyer's patch dendritic cells were shown to recognize the yeast, which induced 2,3-indoleamine dioxygenase, which in turn promoted regulatory T cell generation (Figure 5).^{171,172} Pre-colonization with *C. albicans* also enhanced the protective immune response and survival after *C. difficile* challenge.¹⁷³ Mucosa-associated fungi, including *C. albicans*, were additionally shown to strengthen intestinal epithelial function and protect against enteric bacterial

infections with a mechanism dependent on IL-22 production by CD4+ T helper cells.¹⁶⁶

The gut mycobiome can also indirectly affect colonization resistance provided by the gut bacteriome (Figure 5). Long-term treatment with antifungal drugs altered the composition of the bacterial community with a decrease in the relative detection of *Bacteroides*, *Allobaculum*, *Clostridium*, *Desulfovibrio*, and *Lactobacillus* spp., and an increase in the relative detection of *Anaerostipes*, *Coprococcus*, and *Streptococcus*.¹⁷⁴ Presence of fungal species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *I. orientalis*, and *R. mucilaginosa*) were shown to induce marked changes in the composition of a defined bacterial microbiome in gnotobiotic mice and significantly reduced colonization of *Bifidobacterium longum* and *Lactobacillus reuteri*.¹⁷⁵ Furthermore, introduction of *C. albicans* to the gut environment altered how bacterial communities recover after antibiotic treatment. Administration of antibiotics facilitated the colonization of *C. albicans*, which subsequently hindered the long-term colonization by commensal *Lactobacillus* strains and instead facilitated the colonization of the pathobiont *E. faecalis*. Presence of *C. albicans* also changed the abundance of bacteria involved in colonization resistance to bacterial infection, such as *Bacteroidetes*, *Lactobacillaceae*, and *Lachnospiraceae*.^{176,177} In addition, fungal dysbiosis has been shown to reduce efficacy of fecal microbiota transplantation for *C. difficile* infection. A high abundance of *C. albicans* in donors reduced the efficacy, whereas a high abundance of *Saccharomyces* and *Aspergillus* in donors increased the efficacy of fecal microbiota transplant in controlling *C. difficile* infection.¹¹⁴

The described interactions of mycobiota members with bacterial pathogens highlight that also microbiome members with low abundance can greatly influence gut homeostasis and bacterial pathogenesis.

Gut parasitome

In contrast to the obvious commensal nature of most components of the microbiome, there may be a role for parasites in the healthy gut. The term parasitism is defined as the interaction between organisms in which one (the parasite) reaps benefits

to the detriment of the other (the host). Although most pathogens can be described as parasites, this term is used medically to describe protozoa and helminths.^{178,179} Protozoa are single-celled, simple eukaryotic organisms that are typically less than 50 µm in size (Figure 1). Examples of protozoan parasites are *Plasmodium* species, which cause malaria; *Entamoeba histolytica*, a causative agent of intestinal amebiasis; and *Giardia duodenalis*, a common enteric parasite that causes the diarrheal disease giardiasis.^{178,180} These organisms go through complex life stages; cysts are shed through feces into the environment and contaminate water and food. Once ingested, the protozoa encyst and progress into the trophozoite stage, considered to be the infectious form. Many protozoa cause diarrheal diseases by directly invading and damaging the intestinal tissues, but they can also produce toxic molecules that aid in disrupting host defenses.^{86,178}

Helminths are multicellular organisms with worm-like morphologies that can range from a few millimeters to several centimeters in size (Figure 1).^{178,180} Common helminths are tapeworms (cestodes), flukes (schistosomes and trematodes), and roundworms (nematodes). These parasites are the main targets of deworming medications given to domestic pets and livestock and can be transmitted to humans. Helminths also cycle through different life stages, similar to protozoa, in which the eggs are shed in feces and then hatch into larvae in intermediate hosts (i.e. snails), which mature into the pathogenic adult form. However, some helminths, such as schistosomes, penetrate the skin and disseminate through the blood to target organs. These large, multicellular worms can form blockages in different organs, leading to tissue damage.^{178,181} Despite this, many enteric parasitic infections are asymptomatic and can persist for years. Parasitic infections are more common in tropical regions, developing countries, and areas with inadequate water and food sanitation, but can also affect developed countries.

Composition and development

Although the name parasite implies harm to the host, some parasitic organisms may exhibit asymptomatic colonization of the host and engage in

a commensal relationship, which has led to the inclusion of the parasitome as a component of the microbiota.^{182,183} *Blastocystis*, *Dientamoeba*, and certain *Entamoeba* species are parasites that have been found to colonize the human gastrointestinal tract and may provide beneficial impacts on the bacterial richness and diversity.^{77,182,184,185} In fact, *Blastocystis* prevalence can range from less than 10% up to 100% in fecal samples from healthy human subjects, depending on the location of the surveyed population.^{186–190} One retrospective metagenomic analysis found *Blastocystis* in 18.4% of all Danish and Spanish individuals involved in the study, with a higher prevalence in healthy versus ulcerative colitis patients (20.3% vs 14.9%, respectively).¹⁹¹ In contrast, a study of Senegalese children identified *Blastocystis* in 100% of study participants, regardless of health status (defined as asymptomatic or symptomatic gastrointestinal disease).¹⁹² It is important to note that although the commensal status of *Blastocystis* and other gut-associated protozoa is controversial, the presence of *Blastocystis* has been correlated with an increase in bacterial diversity and lower abundance in *Enterobacteriaceae*.^{77,182,185} Higher bacterial diversity is commonly seen as an attribute of a healthy gut microbiota.^{77,193} The studies mentioned above are sequencing-based and do not provide any mechanism for how or why protozoa can impact the diversity of intestinal bacteria. Further research is needed to determine the commensal versus pathogenic nature of *Blastocystis* within the human gastrointestinal tract.

Parasitic infections have been shown to impact the makeup of the normal bacterial flora within the gut. Mice infected with *Trichuris muris*, the mouse whipworm, underwent significant but transitory alterations in the diversity and abundance of *Bacteroidetes*, as well as functional differences in metabolomic activity. However, incomplete restoration of the microbiota was seen after parasitic infection was cleared, indicating that there may be an active crosstalk between the microbiota and parasites.¹⁹⁴ Interestingly, another set of studies found that *T. muris* requires the presence of a bacterial microbiota in order to establish an infection. *T. muris* larvae were unable to develop and colonize germ-free mice but this ability was restored when mice were colonized with a single bacterial

species.¹⁹⁵ These experiments illustrate the evolutionary relationship between different members of the gut microbiota.

Immune modulation

Parasitic organisms have evolved alongside humans for most of our existence and have developed strategies to evade or modulate the host immune system. Recognition begins with parasitic invasion of the mucosa and cytokine responses of the intestinal epithelial cells. Some protozoans trigger a Th1 response, while helminths tend to elicit a Th2 response.¹⁸¹ However, immunosuppression is a common method parasites use to enhance their survival in a human host.^{179,181,196} Protozoans have been shown to secrete virulence factors that can directly downregulate pro-inflammatory cytokines, inducing an anti-inflammatory environment.¹⁸¹ Colonization with *Trichuris muris* has been shown to be protective against intestinal damage and to promote normal goblet cell development in mice lacking Nod2. *Nod2*^{-/-} mice colonized with *Bacteroides vulgatus*, a common member of the gut microbiota, develop spontaneous intestinal abnormalities, such as lack of functional goblet cells, that mimic human Crohn's disease. *T. muris* infection limited *B. vulgatus* colonization via the induction of a strong Th2 immune response.¹⁹⁷ The lack of parasitic exposure has been credited with increasing allergy and autoinflammatory conditions, commonly referred to as the Hygiene Hypothesis.¹⁹⁸ This theory posits that greater sanitation and cleanliness reduces exposure to common microbes that are important to immune system development and priming of responses. Allergies induce robust IgE antibody production against specific agents, and extreme allergic responses are due to hyperactive immune activity. Parasitic colonization can induce similar IgE expansion, although the antibodies are nonspecific.¹⁹⁹ Nonspecific IgE antibodies can suppress the production of more specific antibodies, thereby suppressing an immune response to an allergen.¹⁹⁹ Along these lines, the use of helminths or parasite-associated metabolites have begun to be investigated as anti-inflammatory

therapeutics, although this idea is still controversial.^{200–202} Regardless, commensal parasites may be important for overall immune system functioning and their role in homeostasis and infection should not be overlooked.

Interactions with enteric bacterial pathogens

Very few studies have directly investigated the role the parasitome plays in microbiota homeostasis, and even fewer have focused on the contribution intestinal parasites have on enhancing or inhibiting enteric bacterial infections. However, this neglected component of the microbiome has been shown to have clinical relevance that necessitates further investigation.

Co-infections of parasites and enteric bacterial pathogens have been reported in areas where both pathogens are endemic.^{191,203,204} *Entamoeba* species have been found along with enteropathogenic *E. coli* (EPEC) or *Shigella dysenteriae* in children with diarrheal disease.^{205–207} *In vitro* studies have found that co-infection of intestinal epithelial cells by the amoeba *E. histolytica* and enteropathogenic bacteria led to increased immune activation, epithelial cell damage, and virulence, compared to co-infection with the avirulent species *E. dispar*.²⁰⁸ This is possibly due to enhanced *E. histolytica* virulence upon phagocytosis of bacteria. *E. histolytica* displayed increased adhesion to host cells and increased production of cysteine proteinases after phagocytosis of EPEC or *Shigella*, which the researchers hypothesized was responsible for increased host cell damage.²⁰⁸

Similarly, helminth and trematode co-infections with Non-Typhoidal *Salmonella* are common in certain populations.^{209,210} Helminth worms, such as *Heligmosomoides polygyrus*, have been shown to facilitate high levels of *S. Typhimurium* colonization of the small intestine (Figure 6).^{209,211} This could be due to alterations in metabolites that confer colonization resistance to bacterial pathogens and/or due to modulation of the immune response that prevents bacterial clearance, but the researchers note that this effect is not due to increased host cell invasion by *S. Typhimurium*.^{190,209} Interestingly, deworming of mice prior to *S. Typhimurium* infection abrogated this enhanced colonization phenotype, indicating

that the presence of helminth worms is necessary to promote *S. Typhimurium* growth *in vivo*.²¹¹ Prophylactic deworming in endemic regions may be a way to reduce the severity of enteric diseases.

Commensal protists are likely present in many other species and are not exclusive to humans. One study has identified a rodent parasite that exists as a commensal member of the microbiota and provides protection against invasive intestinal bacterial infections. *Tritrichomonas musculus*, a close ortholog to *Dientamoeba fragilis*, can stably colonize the mouse gastrointestinal tract.²¹² Mice colonized with *T. musculus* showed an expansion of Th1 and Th17 cells in the mucosa of the colon. Upon *S. Typhimurium* challenge, *T. musculus*-infected mice did not develop cecal inflammation and had significantly less dissemination of *S. Typhimurium* to peripheral organs, as compared to mice without protists.²¹² This protection was dependent on inflammasome activation and IL-18 release from epithelial cells and demonstrates how parasites can modulate the immune system in a way that prevents bacterial invasion and infection²¹² (Figure 6).

There are several examples of non-gut parasitic infections that enhance *S. Typhimurium* infection via the modulation of the host immune response. Blood-associated parasites, like Schistosomes and *Plasmodium* species (causative agents of schistosomiasis and malaria, respectively), have been shown to enhance *S. Typhimurium* pathogenicity by impairing the host immune response.^{210,213,214} Blood flukes, like *Schistosoma mansoni*, have complex life cycles in which different stages of development are associated with infection of various parts of the body. These organisms penetrate the skin and circulate in the blood to the lungs and heart, eventually settling in the liver and undergoing maturation into the adult worm form.²¹⁵ Adult worms lay eggs that progress to the lumen of the intestines, bladder, and ureter, and these eggs are shed in feces or urine back into the environment. Schistosome eggs have strong immunomodulatory effects, inducing a robust Th2 response with high IL-4 and IL-3 levels and suppressing pro-inflammatory cytokines.²¹⁵ Co-infection of mice with *Schistosoma mansoni* and *Salmonella Typhimurium* resulted in significantly higher *Salmonella* colonization of the ileum, cecum, and colon, compared to single infection controls.²¹³

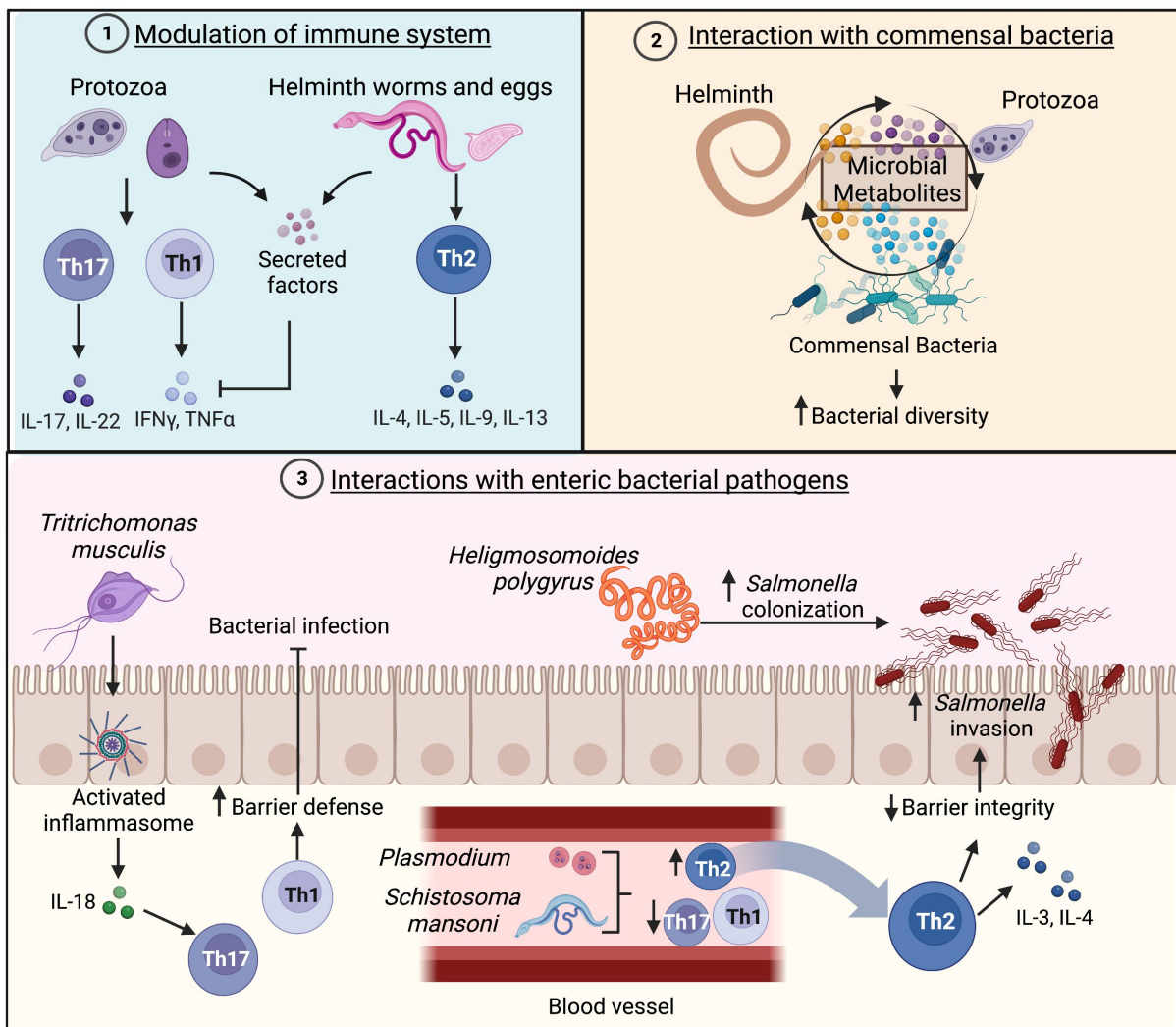


Figure 6. Effects of the gut parasitome on enteric bacterial infections. (1) Protozoa parasites induce Th1 and Th17 responses while helminths induce Th2 response. Secreted parasitic effectors inhibit pro-inflammatory cytokines. (2) Members of the gut parasitome interact with commensal bacteria and secrete metabolites that may serve to increase bacterial diversity. (3) The parasite *Tritrichomonas musculis* activates the epithelial cell inflammasome to induce production of IL-18, which leads to a Th1 and Th17 response that increases barrier defense and limits bacterial infection. The presence of *Heligmosomoides polygyrus* enhances *S. Typhimurium* colonization, although the mechanism is currently unknown. Blood-associated parasites, like *Plasmodium* species and *Schistosoma mansoni*, activate a Th2 response, which leads to IL-3 and IL-4 production, disrupting the epithelial barrier in the gut. *S. Typhimurium* takes advantage of this environment and shows increased epithelial cell invasion.

Interestingly, higher levels of inflammatory cells were present in the gut lumen of co-infected mice, but there was less inflammation of the mucosa. The presence of schistosome eggs induced a robust Th2 immune response and dampened the Th1/Th17 response that is critical for clearance of *S. Typhimurium*.²¹³ Similarly, *Plasmodium* species induce IL-10 production that, during coinfection with *S. Typhimurium* in mice, leads to diminished phagocyte recruitment to the liver.²¹⁴ Liver resident macrophages were skewed toward the anti-inflammatory M2 state in response to the malaria

pathogen, which leads to enhanced dissemination of *S. Typhimurium*.²¹⁴ *Plasmodium*-induced TNF α -signaling also resulted in reduced stomach acidity, which directly aided *S. Typhimurium* gastrointestinal colonization.²¹⁶

Although the notion of commensal parasites as members of the normal human gut microbiota may be controversial, their presence and influence should not be neglected in studies of the microbiome. Protozoa may interact with commensal bacteria to maintain gut homeostasis. Helminths may induce an anti-inflammatory environment,

potentially aiding enteric bacteria during infection. These underappreciated members of the gut microbiome play important roles that should not be excluded from future research.

Summary and outlook

Microbiome research output in general and especially research focused on the gut microbiome has seen exponential growth over the last decades. While most research focuses on bacteria, the most abundant members of the gut microbiome, significant advancements have been made also on neglected members, such as viruses, archaea, fungi, and parasites. In this review, we summarized current findings on their role during infections with enteric pathogens, such as members of the *Enterobacteriaceae*. Despite significant research advancements, many challenges remain. Most research focuses only on one microbiome component while ignoring the others. Consequently, research on the microbiome's influence on enteric infections is mostly restricted to studying one of its components. However, viruses, archaea, fungi, and to some extent also parasites integrate to influence mucosal immunity and colonization resistance toward enteric bacterial pathogens. Thus, to fully understand the gut microbiome's role during enteric infections, more inclusive future studies are needed that address more than one microbiome component at a time. One important step into this direction is the now more frequent assessment of fungal communities with Internally Transcribed Spacer (ITS) sequencing alongside routine 16S-sequencing. The recent technological advancements employing whole genome shotgun sequencing for gut microbiome research instead of bacteria-centric 16S sequencing also has tremendous potential for assessing neglected microbiome members. While still more expensive and requiring more extensive bioinformatic analysis, this less biased sequencing approach will help to broaden scientist's view of the role of different gut microbiome communities during health and disease conditions. In addition, crucial tools to determine functional roles include a) the strategic employment of defined microbial communities that include these neglected members, as well as b)

application of metabolomics to assess their function in the context of gut metabolic networks.

Over the past decades, colonization of germ-free mice with defined bacterial communities has enabled the discovery of important interactions that would have been missed in more complex systems. The first widely used bacterial community, the Altered Schaedler Flora (ASF), was highly reductionist in scope and contained only eight anaerobic bacterial species.^{217,218} Nevertheless, this model has been incredibly useful to study specific host-commensal-pathogen interactions and the influence of the gut bacteriome on various other body systems and functions.^{217,219} Efforts have been undertaken since to render defined microbiomes more reminiscent of an actual gut bacterial community that is connected in an efficient metabolic network. To that extent, communities that also include facultative anaerobes and other species have been developed, such as the GM15 and the Oligo-Mouse-Microbiota 12 (OMM12) communities.²²⁰ An important improvement of the OMM12 over ASF is for example that it confers colonization resistance to *Salmonella* Typhimurium.²²¹ Even larger collections have also been constructed, such as the 100 strain-containing Mouse Intestinal Bacterial Collection (miBC).²²² However, none of these communities include non-bacterial microbiota components, such as methane-producing archaea. The only notable exception are prophages that are likely present in their bacterial genomes. Future research would greatly benefit from the establishment of defined microbial communities that include components such as archaea, fungi, and commensal protists.

Non-bacterial members of the microbiota also contribute to the overall gut metabolome. However, the extent of their contribution is currently unknown. Some metabolic functions appear straightforward to deduce, e.g. the production of methane by methanogens. Other functions are less obvious and require additional study. Metabolites produced by non-bacterial microbiome members might influence enteric bacterial infection a) directly by competitive exclusion and b) indirectly by modulating the host immune system. One study showed that the addition of yeast species to a defined bacterial community in mice only minimally affected the overall gut

metabolome.¹⁷⁵ Conversely, the presence of *Saccharomyces cerevisiae* in humans enhanced host purine metabolism, resulting in higher levels of uric acid, which negatively influenced the pathologies associated with IBD.²²³ Protozoa and enteric helminths also contribute to the gut metabolome. Introduction of the protist *Blastocystis* via fecal material transfer (FMT) increased the production of short-chain fatty acids (SCFAs) that ameliorated colitis, whereas presence of enteric helminths changed the gut metabolic profile in a way that promoted *Salmonella* Typhimurium intracellular invasion.^{209,224} Future studies will determine to which extent non-bacterial members of the microbiota contribute directly or indirectly to the gut metabolome.

Other key factors, such as age and diet, also influence microbiome composition and richness of the neglected microbiomes. Thus, it will be essential to consider these factors while analyzing the contribution of neglected microbiome members on enteric infections. For example, age can affect the gut virome. Overall viral richness is highest in infants and adults and decreases in elderly individuals. Bacteriophages mirror this trend, but richness in eukaryotic viruses is highest during infancy.³⁸ Similarly, diet is an important factor influencing the composition of gut microbes. Dairy consumption is positively associated with *Saccharomyces* abundance, and carbohydrate intake is positively associated with *Candida* and *Methanobrevibacter* abundance.^{106,112} These changes also signify the importance of individual variations in the microbiome composition. However, studies have yet to be done to determine if age and diet also affect the gut parasitome.

Neglected microbiome members have been slowly moving into the focus of researchers. This largely under-explored research field will likely greatly expand in the future and reveal more fascinating roles of viruses, archaea, fungi, and parasites for gut biology in general and for the pathogenesis of enteric bacteria in particular.

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ORCID

Judith Behnsen  <http://orcid.org/0000-0003-0109-1090>

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