Title: Associations between gut microbiota and common luminal intestinal parasites

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Abstract (100–120 words)

The development and integration of DNA-based methods in research and clinical microbiology laboratories have enabled standardised and comprehensive detection and differentiation of the microbes colonising our guts. For instance, the single-celled parasites *Blastocystis* and *Dientamoeba* appear to be much more common than previously thought, especially so in healthy individuals. While increasing evidence appears to suggest limited pathogenicity of these parasites, next-generation sequencing-based studies have helped us appreciate links between parasite colonisation and certain host phenotypical characteristics and gut microbial profiles. The fundamental question remains as to whether such parasites are merely indicators or active manipulators of gut microbiota structure and function. In this article, we collate existing evidence that these parasites are, as a minimum, indicators of intestinal microbiota structure.

Towards mapping of the human gut eukaryome

An increasing body of evidence suggests links between gut microbiota composition and various diseases [1-6]. Application of a holistic view of the structure and function of the gut microbiota

requires the inclusion of not only bacteria, but also parasites, fungi, archaea, viruses, and phages [7-12]. To some extent corroborating the "**Old Friends hypothesis**" (see Glossary), Parfrey *et al.* recently produced data exemplifying the "defaunation" of the human gut [13]. Here, diversity patterns of intestinal eukaryotes were compared between individuals with a westernized lifestyle (from the cities of Colorado and Philadelphia in USA) and individuals with an agrarian lifestyle (from rural communities in Malawi). The authors observed that individuals with non-western diets and lifestyles resemble non-human mammals in terms of micro-eukaryotic diversity, while individuals with a western lifestyle have low levels of micro-eukaryotic diversity.

The introduction of real-time PCR and other DNA-based technologies including next-generation sequencing in modern clinical microbiology laboratories and research laboratories for detection and differentiation of intestinal parasites has opened up new avenues for exploring how parasites impact our lives [14]. For instance, the high sensitivity of real-time PCR tests has helped us understand that some parasites are—on an overall basis—much more common than previously anticipated, with prevalence rates approaching 100% in some communities, even in developed countries. Examples include *Blastocystis* and *Dientamoeba*, which appear to be more or less obligate eukaryotic members of the gut microbiota in some populations [15-21], while less frequent in others, including individuals with functional and organic bowel diseases and metabolic disorders [13, 22-25]. For instance, we recently showed *Dientamoeba* to be a consistent finding in the stool from children in childcare in Denmark [20], and similarly that *Blastocystis* is a frequent finding in children in Nigeria, with prevalence increasing by age [18]. We have also shown that healthy individuals are more likely to host these parasites than patients with irritable bowel syndrome (IBS) [25] and, especially, inflammatory bowel disease (IBD) [22]. Moreover, it appears that *Blastocystis*, *Dientamoeba* and *Entamoeba* are capable of long-term colonization of the human gut [15, 16, 20, 26]. For these parasitic genera, tools to differentiate colonization from infection are not available

[27-29]. It is also not unlikely that different genotypes or strains display different levels of virulence [30-32]. More precise molecular diagnostics are therefore required to allow for better mapping of strains/genotypes. However, in order to be able to distinguish clinically relevant strains from mere colonizers, a better understanding of the microbial eukaryotic contribution to the human gut ecosystem is probably required.

The above-mentioned types of advances have spurred an interest in mapping and exploring the gut eukaryome [7-10], as well as investigating the interplay between gut parasites and gut bacteria. Interestingly, parasitic genera such as *Blastocystis*, *Dientamoeba* and *Entamoeba*, which are all considered luminal (i.e. non-invasive) intestinal parasites (except for *Entamoeba histolytica*, colonization by which may be invasive), appear more commonly in healthy individuals than in patients with metabolic, organic and functional gastrointestinal disorders [21, 23, 25, 33-36], which has promoted the idea of some parasites being beneficial to the host rather than culprits of disease [37]. Since vast differences in colonization rate are seen across age groups [17], health status [25], and geographical regions [13], which might be the factors driving these differences? While exposure to faecally contaminated matter is most likely one of these factors, another crucial factor might be individual susceptibility to colonization. In this regard, susceptibility to parasite infection may be linked to specific ecological conditions in the gut, including those that have to do with gut microbiota. This line of thinking is supported by several animal experimental studies that have provided evidence of **probiotics** preventing or modulating parasite infection [38]. In fact, the gut microbiota may not only be driving the susceptibility to, but also the outcome of, parasite infection, as suggested by Berrilli *et al.* [39]. Moreover, differences in microbiota signatures, i.e., differences in microbiota taxa, be it on the species, genus, family or even phylum level, may reflect the severity of parasite infections.

With the current opportunities for exhaustive gut microbiota profiling using next-generation sequencing, an important step towards fine-tuning our clinical and public health understanding of colonization by intestinal parasites is to study these parasites in relation to their ecological niche, including relationships with gut microbiota. Such steps are already being taken [40]; however, it is also important to develop hypotheses that might explain these relationships.

Evidence of links between common intestinal parasites and gut bacterial communities

Over the past few years, specific gut microbiota patterns have been shown to be linked to colonization with common parasitic protists (Table 1). Especially, the relationship between *Blastocystis* and gut bacteria has been a popular research focus [15, 23, 35, 41-47]. In 2011, Arumugam *et al.* launched the concept of **enterotypes** of the human gut microbiome [48]. Analysing the gut microbiota of healthy and diseased individuals across nations, they observed a clustering of individuals into one of three microbiota patterns, the so-called "enterotypes". Each of these three enterotypes were identifiable by the variation in the levels of one of three bacterial genera: *Bacteroides*, *Prevotella*, and *Ruminococcus*. In the study by Arumugam *et al.*, only bacterial data were communicated; no breakdown of the eukaryotic components of the gut microbiota was provided. To mitigate this and taking a retrospective approach to studying the data produced by Arumugam and colleagues, Andersen *et al.* [35] not only identified the prevalence and subtype distribution of *Blastocystis* in various cohorts of healthy and diseased individuals, but also explored links between *Blastocystis* and gut bacteria. They found that *Blastocystis* carriage was significantly less common in individuals with a *Bacteroides*-driven enterotype than in those with a *Ruminococcus*- or *Prevotella*-driven enterotype. They also found that *Blastocystis* colonization was associated with higher bacterial richness (number of individual bacterial taxa) and lower body mass

index (BMI) (findings summarized in [28]). A somewhat similar approach was recently taken by Beghini *et al.* [23], who found a strong link between *Blastocystis*, the Archaean *Methanobrevibacter smithii* and several bacterial species across 12 metagenomic datasets. Moreover, similar to observations made by Andersen *et al.* (2015), an inverse relationship was identified between *Blastocystis* carriage and BMI, showing that *Blastocystis* colonization is inversely associated with increasing BMI.

To validate the findings by Andersen *et al.* in 2015 [35], members of the same team took to analyzing another set of faecal samples using real-time PCR technology [43]. Again, *Blastocystis* was investigated in relation to major groups of bacteria using a modified **GUt Low-Density Array (GULDA)**-approach and *Dientamoeba* was included in the study as well as a "control parasite". Both *Dientamoeba* and *Blastocystis* were studied in relation to the six bacterial taxa *Bacteroides*, *Prevotella*, the butyrate-producing clostridial clusters IV and XIVa, the mucin-degrading *Akkermansia muciniphila*, and the indigenous group of *Bifidobacterium*, and it was observed that carriers of *Blastocystis* alone or along with *Dientamoeba fragilis* typically had gut microbiota characterized by low relative abundances of *Bacteroides* and clostridial cluster XIVa and high levels of *Prevotella*. Hence, colonization with *Blastocystis* was again linked to a low relative abundance of *Bacteroides*. In fact, when comparing parasite-negative with parasite-positive samples, the relative abundance of *Bacteroides* in the parasite-negative samples was significantly higher compared with parasite-positive samples ($P < 0.001$) [43].

Audebert and colleagues performed a cross-sectional study including 48 *Blastocystis*-positive and 48 *Blastocystis*-negative patients and performed 16S rDNA sequencing to map *Blastocystis*-associated gut microbiota, identifying higher bacterial diversity in the faecal microbiota of *Blastocystis*-colonized patients, a higher abundance of clostridia, and a lower abundance of Enterobacteriaceae [41]. Earlier on, however, Nourrisson and colleagues had suggested that

Blastocystis might be linked to microbiota imbalance, observing that, compared with controls, levels of "gut-protective" *Faecalibacterium prausnitzii* were decreased in *Blastocystis*-colonised males and that levels of *Bifidobacterium* sp. were decreased in males with irritable bowel syndrome (IBS) type C [44].

In a study comparing the microbiota in individuals with *Giardia*, *Blastocystis*, and *Entamoeba*, **dysbiosis**, as evidenced by a low *F. prausnitzii-Escherichia coli* ratio, was identified in individuals with *Giardia*, while those with *Blastocystis* and *Entamoeba* appeared to be characterized by **eubiosis**, characterized primarily by a high *F. prausnitzii-E. coli* ratio [47].

Studying the gut microbiota of African rural populations, Morton and colleagues showed that across populations, intestinal colonisation by the genus *Entamoeba* could be predicted with 79% accuracy based on the composition of an individual's gut microbiota [49]. They moreover observed that several of the taxa critical to distinguishing the absence or presence of *Entamoeba* are in fact signature taxa for autoimmune disorders such as Crohn's Disease. While this appears to suggest that *Entamoeba* is a surrogate marker for gut microbial communities protecting against gut inflammatory diseases, it is not clear whether the parasite *per se* might also play a role in the protection against this and other autoimmune conditions, bringing into memory the idea of the Old Friends hypothesis.

Microbial profiling studies have indicated that the gut microbiota of patients with IBD differ from that of healthy individuals [50]. In patients with IBD, a decrease in strict anaerobic bacteria and a shift towards facultative anaerobes such as members of the family Enterobacteriaceae have been suggested to reflect disruption of anaerobiosis, indicating a role for oxygen in intestinal dysbiosis [53, 54]. Although it was known that oxygen concentrations increase in a disturbed gut ecosystem, it was only recently that the molecular mechanism was elucidated [55]. In a healthy gut, bacteria

produce butyrate [56], which is the preferred metabolic substrate of colonocytes [57]. Butyrate is used by colonic epithelial cells in the beta-oxidation pathway and yields ATP. This pathway uses molecular oxygen and thereby reduces the oxygen concentration in the intestine. In addition, the produced butyrate is sensed via a human nuclear receptor and subsequently represses the inducible nitric oxide synthase [55], which leads to a reduction of nitrate [58]. This all leads to a reduction in the proliferation of facultative anaerobes [55]. Butyrate is actually required to activate oxidative metabolism [59]. A shift away, caused by antibiotics for example, from obligate anaerobic bacteria from the Firmicutes and Bacteroides phyla towards members of the Enterobacteriaceae disrupts the host's butyrate-linked control mechanism [55] and represses oxidative metabolism [59], increasing luminal oxygen in the human gut. This might partly explain why parasites such as *Blastocystis* are rare in patients with intestinal dysbiosis-linked diseases: *Blastocystis* being a strict anaerobe [60], it is expected that its ability to establish or maintain itself in a micro-aerophilic environment is limited (Figure 1).

Taking all these studies into account, it appears that colonization by some intestinal parasites can be predicted with quite a high degree of accuracy merely by studying the composition of gut bacteria [23, 49], and the negative association between *Blastocystis* colonization and levels of *Bacteroides* shown by independent research groups [23, 35, 43] adds further support to this hypothesis. A possible explanation for the negative correlation between *Blastocystis* and *Bacteroides* might be that the latter, unlike the *Firmicutes*, generally are not the main butyrate producers in the gut [56] and therefore contribute to a lesser extent to an environment where *Blastocystis* thrives.

Associations between microbial signatures and parasite pathogenicity

A few examples of links between microbes and parasite pathogenicity have been published. One example is the study by Gilchrist and colleagues, who observed that high parasite burden coupled with increased levels of *Prevotella copri* was linked to symptomatic infection with *Entamoeba histolytica* in Bangladeshi children [61]. Recently, members of the same group noticed that dysbiosis induced by antibiotic treatment increased the severity of amebic colitis and delayed clearance of *E. histolytica* in an amoebic colitis mouse model [62]. Other examples of links between *E. histolytica* virulence and gut microbiota were recently reviewed by Burgess and Petri [63]. Similarly, *Giardia intestinalis*, the most common waterborne cause of diarrhea, was also found to be associated with a perturbed intestinal microbiota in a mouse model system. *Giardia* infection was linked to an increase of facultatively and strictly aerobic bacteria [64]. However, an increase of Enterobacteriaceae normally seen in dysbiosis [54] was not seen in infections with *Giardia*, and strict aerobes belonging to the beta-proteobacteria increased instead suggesting that parasite-linked dysbiosis can lead to different microbiota compostions. Another example demonstrating differences in gut microbial diversity is found in helminth infections [65, 66]. Individuals infected with helminths showed a greater microbial diversity compared to individuals who were not infected. As the populations studied were indigenous Malaysians, their gut flora was not immediately comparable to other intestinal microbial diversity studies that generally focus on individuals consuming Western diets [13, 66]. Interestingly, in an experimental system, introduction of a benign tapeworm into a rat model did not lead to an increase of bacterial alpha diversity [67]. On the other hand, the gut microbiota of mosquitos did contribute to killing of the host when infected with a pathogenic fungus [68], demonstrating that the presence of gut microbiota does not automatically protect its host from a disastrous outcome after an infectious challenge.

In many other environments, microbial eukaryotes play an important ecological role as bacterial grazers [69-71]. Nutrient cycling and bacterial protein turnover are important ecological roles

played by microbial eukaryotes, observed in e.g. intestinal systems such as the rumen of large herbivores [72]. Intestinal pathogens such as *Entamoeba* are also known to feed on bacteria, and perhaps where evidence of a causative link between disease and the presence of a given parasite is weak, microbial eukaryotes found in the intestine might have roles in the local food web by providing or recycling nutrients. Especially in cases where long-term colonization has been observed [15, 16, 20, 26], it might be prudent to consider alternative roles for microbial eukaryotes in the human gut other than causing disease; eradication of organisms traditionally considered pathogens might not always be advisable, bringing into mind again the "Old Friends" hypothesis [73].

How can these links be explored in further detail?

There are several ways in which to explore the above-mentioned links further. Some could involve studies of the parasite itself (including studies of the ecological requirements of the parasites [nutrition, oxygen tension, etc.] through genomic, transcriptomic and metabolomic analyses). Others could involve exhaustive microbiota surveys and experimental studies. Conclusions based on cross-sectional data, which are the data mostly available at present, could be confirmed by using longitudinal data (cohort studies) on individuals losing or gaining parasites to look for simultaneous changes in gut microbiota structure or function, by **16S analysis** and **PICRUSt** analysis, respectively, or by the analysis of metagenomics data. Moreover, *in vitro* and *in vivo* models could be developed for testing the susceptibility to infection in similar hosts with different gut microbiota established by selective use of, for instance, pre- and probiotics, antibiotics, and/or co-infections. *In vitro* studies could take advantage of the fact that *Blastocystis* is one of the few parasites that are easily grown in the lab and could therefore make use of cultures where culture-negative stool is

grown in culture medium (e.g., Jones' medium [74]) and later spiked with *Blastocystis*, keeping some cultures unspiked as controls. Longitudinal sampling before and after the spiking event could be used for studies of the relative stability of the faecal microbiota during *Blastocystis* colonization, and to explore changes in microbiota due to *Blastocystis* colonisation.

One of the major limitations to developing integrative studies of the entire gut microbiota and the effect it exerts on human health and disease is the lack of genomic data on gut parasites. No matter whether metagenomics or amplicon-based methods be used for exhaustive detection and differentiation of gut parasites, problems arise when DNA is annotated to the species—or even genus—level. Some parasites exhibit remarkable genetic diversity, and while genomic data for e.g. *Blastocystis* and *Dientamoeba* is appearing in publicly available databases [75-80], a couple of nuclear ribosomal rDNA sequences is the only available genome data for other intestinal parasites such as *Iodamoeba* and *Endolimax* [81-83] and also for even more common parasites such as *Entamoeba coli* [84]. Therefore, in order to optimize mapping of sequenced parasite DNA, genomic data on such parasites should be made available. Moreover, several common intestinal parasites exhibit remarkably extensive genetic diversity, and the research looking into associations between various genotypes/subtypes/strains and eubiosis/dysbiosis remains limited. Finally, few studies have taken into account colonization by fungi such as yeasts (*Candida*, *Saccharomyces*, etc.), and there is hardly any qualitative or quantitative information on inter-fungal relationships and relationships between fungi and other microbial inhabitants of the gut.

Perspectives

Probiotics have been used to prevent but also treat protozoal infections, exemplified in a recent review by Vitetta *et al.* [38], and so future investigations may benefit from looking deeper into how

administration of probiotics might interfere with parasite colonization. In this context, it would also appear relevant to mention **faecal microbiota transplantation (FMT)**, which has gained a lot in popularity over the recent years, especially for the treatment of recurrent *Clostridium difficile* infection [85, 86]. Future use of FMT might include alleviation and/or treatment of other diseases, including IBD, type 2 diabetes, metabolic disease, and maybe even neuropsychiatric diseases [87, 88]. The presence and effect of e.g. *Blastocystis* and *Dientamoeba* in donor stool is largely overlooked. There seems to be lack of consensus as to which organisms should rule out donors; examples of *Blastocystis* and *Dientamoeba* colonization being obstacles to accepting FMT donors exist [89, 90]; meanwhile, other recommendations do not include these parasites in the panel of pathogens disqualifying donors [91]. Nevertheless, if i) high microbiota diversity is linked to parasite colonization, ii) high microbiota diversity is an attractive asset of FMT donor stool, and iii), parasite colonization is linked to gut health-promoting bacterial communities, future investigations should look into the effect of co-administering common intestinal parasites to patients with conditions for which FMT is used, and also into whether in fact these parasites can be transplanted from a donor to a recipient using FMT.

Concluding Remarks

While we still know little about the potential pathogenicity of common luminal intestinal parasites, epidemiological data strongly suggest that at least some of these parasites are linked to gastrointestinal health rather than disease. Based on profiling of gut bacterial communities, we are learning to predict whether or not an individual is colonized with intestinal parasites. This article has provided some hypotheses and ideas for studies that could help us detail and strengthen our knowledge regarding associations between parasites and gut microbiota, with a view to exploring

the importance of these parasites in public health and clinical settings and their potential as gut microbiota and overall health indicators and regulators.

Figure legend

Figure 1. Dynamic interplay between gut flora and human colonocytes. In a healthy gut, fermentation by obligate anaerobic bacteria and *Blastocystis* results in increased short chain fatty acid production. Butyrate is the preferred metabolic substrate of colonocytes and used in mitochondrial β-oxidation producing ATP, resulting in a decrease of available oxygen. In addition, butyrate is sensed by the nuclear PPAKγ receptor, which suppresses iNOS and thereby nitrate in the intestinal lumen. Butyrate enhances mitochondrial oxidative phosphorylation. Overall, this results in a hypoxic intestinal lumen, which supports a microbiota dominated by health-enhancing microbes and which favours continued colonisation by *Blastocystis*. In dysbiosis, the increase in facultative anaerobes such as Enterobacteriaceae results in a decrease of available butyrate. As a result, colonocyte metabolism shifts away from β-oxidation and oxidative phosphorylation to increased glycolysis and therefore does not reduce the oxygen concentration. The nuclear PPAKγ receptor no longer suppresses iNOS, resulting ultimately in increased nitrate in the gut that can be used by the dysbiotic flora as an electron acceptor to support their growth.

GLOSSARY

Probiotics: Live micro-organisms, which, when administered in adequate amounts, confer a health benefit on the host.

Dysbiosis/Eubiosis: The human gut is colonized primarily be species belonging to the following phyla: Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria and Fusobacteria [92, 93]. To give a distinct definition of dysbiosis and eubiosis is not straightforward, but has nevertheless been attempted: A gut microbiota in a eubiotic state is characterized by a preponderance of potentially beneficial species, belonging mainly to the two bacterial phylum Firmicutes and Bacteroides, while potentially pathogenic species, such as those belonging to the phylum Proteobacteria (Enterobacteriaceae) are present, but in a very low percentage. In the case of dysbiosis, "good bacteria" no longer control the "bad bacteria", which take over [92]. While the use of this definition might be pragmatic in some situations, it should be noted that a clear dichotomy between beneficial and pathogenic species is unlikely to exist. Secondly, this definition considers beneficial taxa to be mostly Firmicutes and Bacteroides, while pathogenic species would be mostly Proteobacteria; obviously, the situation is more complex. For instance, changes in the Firmicutes/Bacteroides ratio, as for example observed in obese individuals [94], also appears to be associated with dysbiosis. Less concrete and more holistic definitions are given by Miniello *et al.*, "Beyond the microbial richness and diversity, a 'eubiotic' gut microbiota is characterized by the presence of the microbes that enhance metabolism, resilience to inflammation, resistance to autoimmunity. Dysbiosis is considered as an alteration in microbiota community structure and/or function, capable of causing/driving a detrimental distortion of microbe-host homeostasis." [95]

16S analysis: Also referred to by some as "metagenomic" analysis. In fact, 16S analysis usually refers to the procedure where genomic DNA from a given sample is subject to broad-specificity

amplification of bacterial small subunit ribosomal DNA, sequenced by a next-generation sequencing method, and annotated to a taxonomic level by the use of online databases.

Enterotype: In a study by Arumugam *et al.* [48], application of multidimensional cluster analysis and principal component analysis to fecal metagenomes revealed three distinct clusters, the so-called enterotypes, each of which is characterized by a dominance in abundance of one of three bacterial genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3).

GUt Low-Density Array (GULDA): is a validated, high-throughput real-time quantitative PCR-based analysis platform developed for simultaneous analysis of differences in abundance of 31 different microbial 16S gene targets in faecal samples.

'Old Friends' hypothesis: Development of the immune system requires input from at least three sources collectively referred to as the 'old friends': (i) the commensal microbiotas transmitted by mothers and other family members; (ii) organisms from the natural environment that modulate and diversify the commensal microbiotas; and (iii) the 'old' infections that could persist in small isolated hunter-gatherer groups as relatively harmless subclinical infections or carrier states. These categories of organisms have to be tolerated and hence play a role in the development and regulation of the immune system [96].

PICRUSt analysis: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) is a bioinformatics software package designed to predict metagenome functional content from marker gene (e.g., 16S rRNA) surveys and full genomes (http://picrust.github.io/picrust/).

Faecal microbiota transplantation (FMT): is a procedure in which faecal matter, or stool, is collected from a donor, mixed with a saline or other solution, strained, and infused in a patient

(recipient) by, for instance, colonoscopy or an orogastric tube with a view to restoring a healthy intestinal microbiota.

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524

525 Table 1. Examples of studies on associations between intestinal parasites (protists only) and gut microbial communities.

Method	**Parasite**	**Association(s) observed**	**Reference**
Metagenomics	*Blastocystis*	Inverse association between body mass index and *Blastocystis* and strong co-occurrence with archaeal organisms (*Methanobrevibacter smithii*) and several bacterial species. Negative association between *Blastocystis* and levels of *Bacteroides*.	[23]
Real-time PCR	*Blastocystis*	Linked to eubiosis; significantly higher *Faecalibacterium prausnitzii-Escherichia coli* ratio in *Blastocystis*-positive than in *Giardia*-positive individuals.	[47]
Real-time PCR	*Entamoeba*	Linked to eubiosis; significantly higher *Faecalibacterium prausnitzii-Escherichia coli* ratio in *Entamoeba*-positive than in *Giardia*-positive individuals.	[47]
Real-time PCR	*Giardia*	Linked to dysbiosis; significantly lower *Faecalibacterium prausnitzii-Escherichia coli* ratio in *Giardia*-positive than in *Entamoeba*- and *Blastocystis*-positive individuals.	[47]
Metagenomics	*Blastocystis*	*Blastocystis* found in individuals with *Prevotella* and *Ruminococcus* enterotypes and not in those with *Bacteroides* enterotype; *Blastocystis* colonisation linked to higher bacterial richness.	[35]
Real-time PCR	*Blastocystis*	Low relative abundances of *Bacteroides* and clostridial cluster XIVa and high levels of *Prevotella* in *Blastocystis*-positive individuals.	[43]
Amplicon-based next-generation sequencing	*Entamoeba*	Relative abundance of the phyla Cyanobacteria, Elusimicrobia, Euryarchaeota, Firmicutes, Spirochaetes, Tenericutes all higher in *Entamoeba*-positive individuals. The relative abundance of the phylum Bacteroidetes lower in *Entamoeba*-positive individuals.	[49]
Amplicon-based next-generation sequencing	*Blastocystis*	Higher bacterial diversity in the faecal microbiota of *Blastocystis*-positive patients, a higher abundance of Clostridia, and a lower abundance of Enterobacteriaceae.	[41]
Real-time PCR	*Blastocystis*	Levels of "gut protective" *Faecalibacterium prausnitzii* were decreased in *Blastocystis*-colonised males and levels of *Bifidobacterium* sp. were decreased in males with IBS type C.	[44]
Amplicon-based next-generation sequencing	*Blastocystis*	*Blastocystis*-positive individuals had increased bacterial diversity, but no significant difference in fungal diversity was observed.	[12]

Metagenomics	*Blastocystis*	The relative abundance of bacteria belonging to *Bacteroides* was lower in *Blastocystis* carriers compared with non-carriers, and *Blastocystis* carriage was also associated with significantly higher bacterial richness.	[97]

526

OUTSTANDING QUESTIONS BOX

1. Can current evidence of links between intestinal parasites and microbiota diversity from cross-sectional studies be confirmed and further investigated by data from longitudinal studies?
2. What is the relationship between common parasites and fungi colonizing the intestinal tract in terms of abundance and diversity? Similarly, are intestinal eukaryotic microbial communities found in people consuming non-western diets a potential avenue to exploring how to prevent western diseases?
3. Can hypotheses be generated on links between parasites and certain bacterial taxa, and can *in vivo* and *in vitro* models be developed and included in prospective studies to provide supportive evidence of such links?
4. Are intestinal parasites merely indicators of particular gut microbiota profiles or are they modulators, actively impacting the structure and function of gut microbiota?
5. The answer to 4) will also answer the question as to whether intestinal protists are interesting mainly from a public health view point or also from a clinical/therapeutic view point.
6. Are bacteria driving not only the ability of parasites to establish but also their virulence?

Figure