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Research paper

Dientamoeba fragilis associated with microbiome diversity changes in acute gastroenteritis patients

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ABSTRACT

This study examined the correlation between intestinal protozoans and the bacterial microbiome in faecal samples collected from 463 patients in New Zealand who were diagnosed with gastroenteritis. In comparison to traditional microscopic diagnosis methods, Multiplexed-tandem PCR proved to be more effective in detecting intestinal parasites. Among the identified protozoans, *Blastocystis* sp. and *Dientamoeba fragilis* were the most prevalent. Notably, *D. fragilis* was significantly associated with an increase in the alpha-diversity of host prokaryotic microbes.

Although the exact role of *Blastocystis* sp. and *D. fragilis* as the primary cause of gastroenteritis remains debatable, our data indicates a substantial correlation between these protozoans and the prokaryote microbiome of their hosts, particularly when compared to other protists or patients with gastroenteritis but no detectable parasitic cause. These findings underscore the significance of comprehending the contributions of intestinal protozoans, specifically *D. fragilis*, to the development of gastroenteritis and their potential implications for disease management.

1. Introduction

The gut microbiota is a complex and diverse ecological community essential for normal gut function, homeostasis, and modulating host immunity [1]. The human gut microbiota includes bacteria, viruses, and eukaryotes such as fungi, helminths, and protozoa [2]. Gut microbiota composition is influenced by age, dietary factors, antibiotic use, and host genetic factors [3]. It is important to note that most studies relating to gut microbiota have focused on bacterial content, mostly due to the relative ease and tractability of 16S rRNA gene amplification and the established bioinformatic protocols for prokaryotes [4–6]. Despite the predominant bias towards the prokaryotic microbiome, a small number of studies have shown that intestinal protozoa such as *Entamoeba* spp. and *Blastocystis* sp. are associated with significant shifts in the composition of the microbiome [7–10]. None-the-less the role of the protozoan microbiome in shaping the gut microbiome remains neglected and its consequence on human health unrecognized. One of the many complications with understanding the role of the human 'protistome' (the protozoan component of the microbiome) is the limited number of protozoans genetically identified and of those even fewer have any associated biological information. Even in the case of well-known and ubiquitous protozoans such as *Blastocystis* sp. and *Dientamoeba fragilis* it is still not known if these species are pathogenic or commensal [11–15].

Prior to 2014, New Zealand diagnostics heavily relied on microscopy and antigen detection techniques. However, after the introduction of multiplex tandem PCR (MT-PCR) techniques post 2014, the sensitivity of the detection improved and gave insight to more accurate data. In this study, we first demonstrate the efficacy of these two microscopy/antigen detections versus MT-PCR methods in detecting intestinal parasites and evaluate the prevalence of detection of intestinal parasites in patients in New Zealand. Then, to improve our understanding of the pathophysiology observed by these common enteric infections, we investigated the

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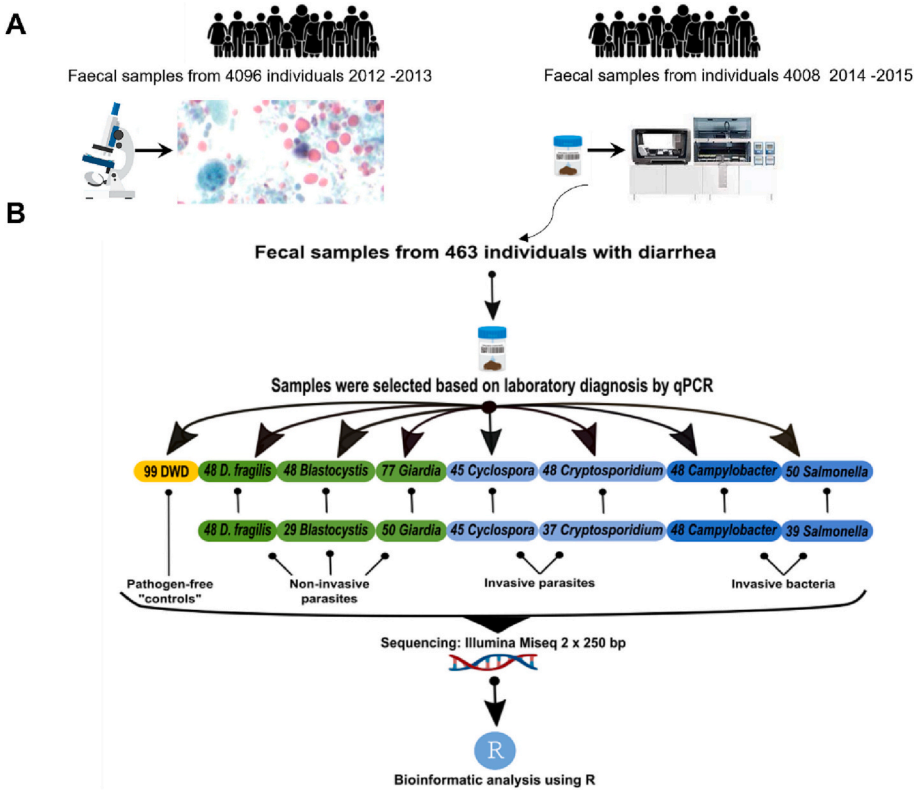

Fig. 1. Overview of study design and gut microbiome analysis. **A.** Samples were collected from patients suffering from acute gastroenteritis. Samples were analysed for pathogens by microscopy or multiplex PCR (MP-PCR) pre-2014 and post-2014 respectively. **B.** Out of the 4008 samples analysed with MT-PCR, 463 randomly selected samples were further analysed for extracellular parasites, intracellular parasites, and pathogenic bacteria. Samples with no detectable diarrhoeagenic parasites or bacteria were designated as the pathogen-free control (DWD). The 16S rRNA gene of all samples was sequenced using an Illumina MiSeq.

associations between extracellular protists (*Dientamoeba fragilis Blastocystis* sp. and *Giardia* spp.) and intracellular protists (*Cryptosporidium* spp. and *Cyclospora* spp.) and the gut microbiome (prokaryotic). The strengths of this study include variation of the sampled population, sample sizes for each parasite infection and control group, and comprehensive microbiome profiling using 16S rRNA sequencing. To the best of our knowledge, this is the largest study comparing the associations between intracellular and extracellular parasite infection and the gut microbiome in the southern hemisphere to date.

2. Methods

2.1. Patients' faecal samples

All sampling and diagnostic tests have been performed by Southern Community Laboratories (SCL). Faecal samples were conventionally collected from patients with acute gastrointestinal symptoms (vomiting and/or diarrhoea (three or more loose stools per day)) from various areas in New Zealand between 2012 and 2015, mostly from the South Island through general practitioners' requests. Equal numbers female and male samples were chosen for this study. This research was approved by the University of Otago Ethics committee (reference code: H17/028).

2.2. Definition of intracellular and extracellular protozoan parasites

Parasites in this study have been grouped into two biologically distinct entities, invasive and extracellular parasites. Invasive parasites such as *Cryptosporidium* spp. and *Cyclospora* spp. require sexual and asexual reproduction to occur within the epithelial cells of the small intestine of the host. In contrast, extracellular parasites such as *Dientamoeba fragilis, Blastocystis* sp. and *Giardia* spp., multiply in the extracellular environment (in the intestinal lumen) and do not have an obligate intracellular stage. While *D. fragilis, Blastocystis* sp. and Giardia spp. may cause damage to the intestinal epithelia, none invade individual cells and grow in an intracellular manner.

2.3. Conventional laboratory methods

For the detection of *Cryptosporidium* spp. and *Giardia* spp. the Prospect Giardia/Cryptosporidium ELISA Microplate kit was used (Thermofisher #R2458496) as per the manufacturer's instructions. Faecal samples were either visualised directly under microscopy as wet mounts or smears were stained either with Trichrome (Ngaio #SDL782) for microsporidia or modified acid-fast stain (BD 212518) for coccidia oocysts (Fig. 1A).

2.4. DNA extraction

Faecal samples (0.5–1 g) were supplemented with 1.2 mL of Stool Transport and Recover buffer (S.T.A.R; Roche #03335208001) and vigorously vortexed until samples were homogenised. Samples were then heated at 95 °C for 15 min, left to cool before they were centrifuged at 6400 ×*g* for five minutes and 500 μL of the supernatant was processed through Abbott m2000sp using the Abbott mSample Preparation System^{DNA} (Abbott #06 K12–24). The open mode 'RNADNA Plasma for 96 samples_deep' protocol was used, and nucleic acids were eluted in 50 uL. Extracted nucleic acids (10 μL / sample) were processed through the High-Plex 24 system (AusDiagnostics #91501) for MT-PCR (Fig. 1A). Immediately after extraction from the faecal samples, DNA was aliquoted into five cryotubes and stored at -80oC until needed (thus avoiding freeze thaw degradation).

2.5. MT-PCR

The High-Plex 24 system (AusDiagnostics #91501) employs a two-step nested PCR. First a short-multiplexed pre-amplification reaction using the Parasite 8Plex primers' kit (AusDiagnostics #25021) was used using the preprogramed cycling option (AusDiagnositc 8 Plex) targeting *Giardia, Cryptosporidium, Cyclospora, Blastocystis, E. histolytica* and

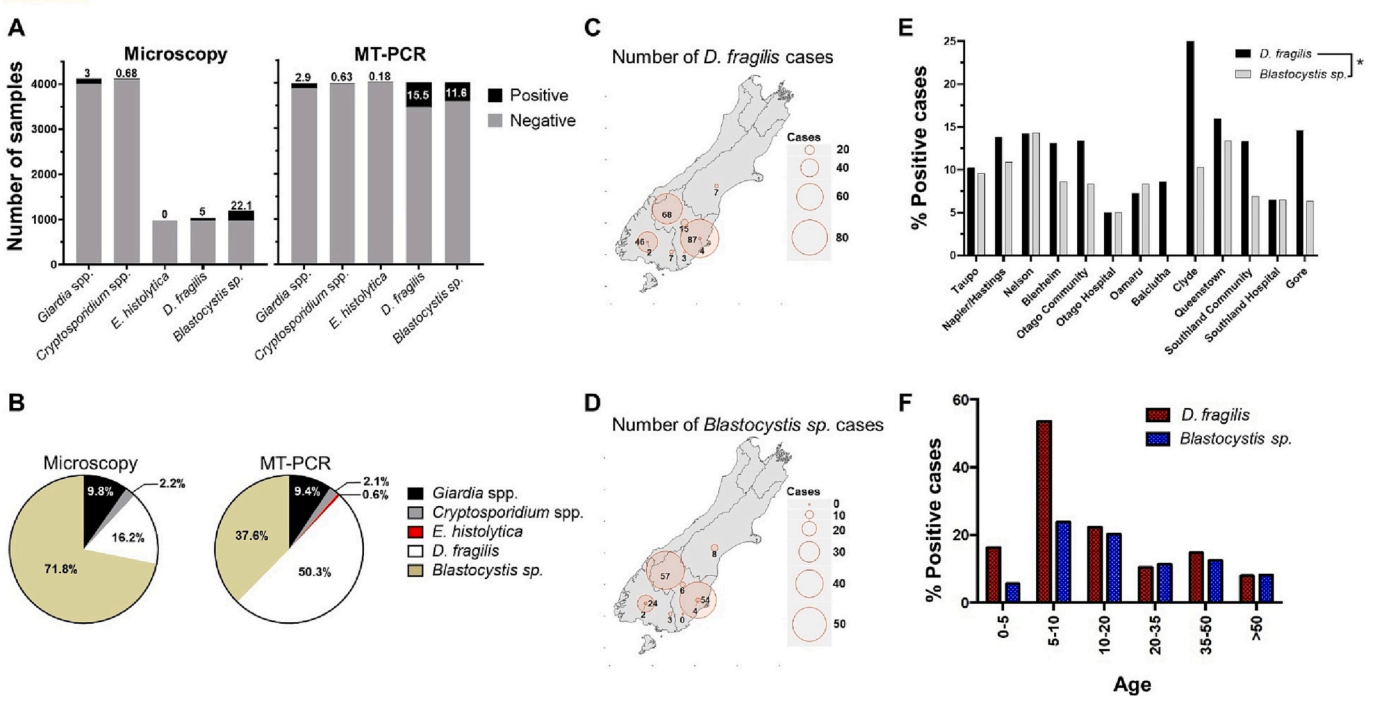

Fig. 2. Detection and proportion of intestinal parasites. Faecal samples of patients suffering from unexplained diarrhoea were collected to be tested for pathogens through the Southern Community Laboratory (SCL) in New Zealand, through general practitioner requests. At SCL, DNA was extracted from the samples and High-Plex 24 system (AusDiagnostics #91501) MT-PCR was used to detect diarrhoea-causing pathogens. **A.** Detection of selected intestinal parasites and percentages of positive cases before ($n = 4096$) and after the introduction of multiplex PCR (MT-PCR) ($n = 4008$). **B.** Percentage of each selected parasite with respect to all positive cases before and after the introduction of MT-PCR. **C, D,** and **E.** Geographical distribution of positive cases for *D. fragilis* and Blastocystis in New Zealand. Except Taupo and Napier/Hastings (both towns from the North Island of NZ) the remaining 11 locations (Hospitals or Towns) are from the South Island of NZ. Two-tailed parametric unpaired *t*-test pooled from all regions: $^*p < 0.0332$. **F.** Age distribution of positive cases for *D. fragilis* and Blastocystis in the South Island of New Zealand.

D. fragilis). The PCR products were then aliquoted into individual wells of a kit-specific 384-well plate (AusDiagnostics #25021) and amplification of the first PCR products is performed and detected using the High-Plex 384-well analyser and detection software. For the gut microbiota interaction analysis (Fig. 1), samples from 463 patients with *Giardia* spp., *D. fragilis*, *Blastocystis* sp., *Salmonella*, *Campylobacter, Cyclospora* and *Cryptosporidium*, and specific pathogen free controls ($n =$ 77, 48, 48, 45, 48, and 99 respectively) were selected for 16S rRNA sequencing. *Salmonella* and *Campylobacter* ($n = 50$, 48 respectively).

2.6. 16S rRNA sequencing

Cryopreserved DNA from 463 randomly selected (using an R-package random number generator and MS-Excel listed samples) patient samples (sampling was stratified in an effort to select a maximum of 60 isolates for each pathogen group and 100 pathogen free DNA samples) was sent to Argonne National Laboratory (ESPSF ANL) in Lemont, Illinois, USA for 16S rRNA sequencing. The sequenced samples also included two laboratory positive controls (zymoBIOMICS Microbial Community DNA (Lot no. ZRC19811, concentration of 0.1 μg/μL) and two negative controls (Invitrogen Thermo Fisher Scientific RT-PCR Grade water (Lot no. 1804029)). The V4 hypervariable region of the 16S rRNA gene (primers 515f and 806R) was sequenced using Earth Microbiome Project protocols [16] on an Illumina Miseq, using 2x250bp read chemistry.

2.7. Bioinformatic and statistical analysis

R and R studio were used to analyze and visualize data obtained from all 16S rRNA gene sequenced samples. Quality filtering and the construction of ASVs (Amplicon sequence variants) was done by DADA2 (Divisive Amplicon Denoising Algorithm) and analysed using PhylosEq. [17].

Packages "vegan" and "deseq" [18] were used for microbiome analysis. Using the "rstatix" package normality was tested, and non-parametric data was then analysed using a Dunn test for multiple pairwise comparisons and false discovery rate (FDR) corrected. Odds ratios were calculated using the fisher test function in the "stats" R package. All R-based bioinformatic scripts used in this project are available at: https://gitlab.com/abdla136/parasites. Sequence data are available from SRA BioProject PRJNA801778.

For the co-infection studies the likelihood of infection with one parasite given the presence or absence of another parasite was assessed by calculating odds ratios (ORs) and their 95% confidence intervals using the Monte Carlo method. For example, the odds of being infected with *Blastocystis* given the presence of *D. fragilis* was compared with the odds of being infected with *Blastocystis* in the absence of *D. fragilis.* These are referred to as the ORs for co-infection and a two-sided test Fishers Exact statistical test was used to test for significant differences from the null value (i.e. OR = 1).

3. Results

3.1. MT-PCR reveals a high proportion of D. fragilis parasite in the intestinal tract of acute gastroenteritis

Prior to and following the introduction of MT-PCR, 4096 and 4008 faecal samples were collected, respectively (Fig. 2A). The proportion of *D. fragilis*, *Blastocystis* sp., *Cryptosporidium* spp., *Giardia* spp., and *E. histolytica* were 16.2%, 71.8%, 2.2%, 9.8%, and 0% respectively for the pre-MT-PCR (Fig. 2B). Following the introduction of MT-PCR, *D. fragilis* made up the highest proportion of intestinal parasites found in the faecal samples (50.3%) compared to other (37.7% *Blastocystis*, 2.1% *Cryptosporidium* spp., 9.4% *Giardia* spp., and 0.6% *E. histolytica;*

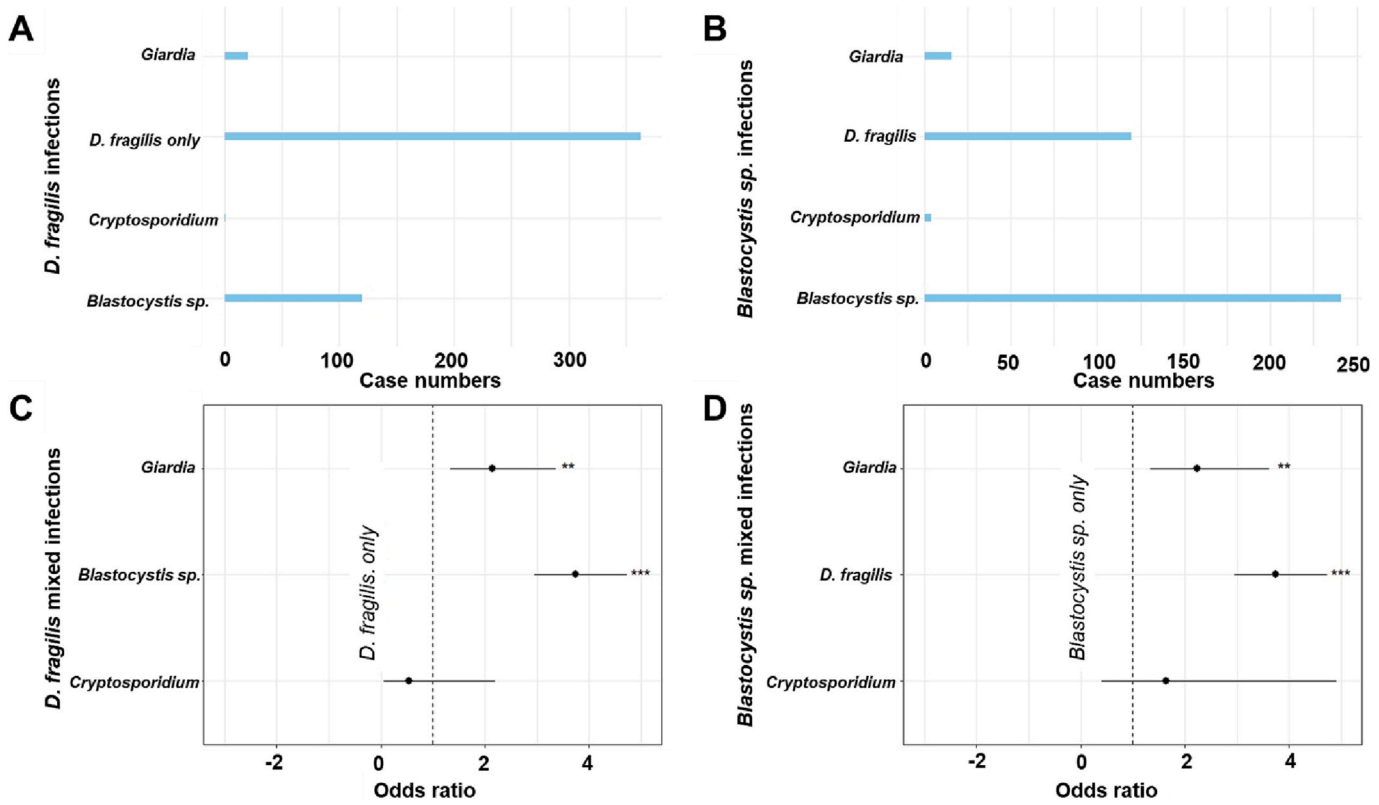

Fig. 3. The likelihood of co-infection of *Blastocystis* sp. / *D fragilis* with other intestinal parasites. Odds ratios (OR) for parasite co-infection were calculated, and a two-sided Fishers Exact test was used to test whether co-infections cases with **(A & C)** *D. fragilis* or **(B & D)** Blastocystis were associated. *, **, *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Fig. 2B and C). Both *D. fragilis* and *Blastocystis* were the most detected parasites within the panel in all select regions (except for Balclutha; Fig. 2C-1D). The proportion of *D. fragilis* was significantly higher than *Blastocystis* overall ($p < 0.0332$; two-tailed parametric unpaired *t*-test) with samples from Clyde most likely to contain *D. fragilis*.

The proportion of gut infections was not dependent on the sex of the patient ($p > 0.1$, chi-squared test, data not presented). In terms of age distribution (Median age 40.05 years), the presence of both *D. fragilis* and *Blastocystis* was highest in children aged between 5 and 10 (Fig. 2F). Although no statistical significance was observed, two fold more *D. fragilis* was found in 5–10-year-old children compared to *Blastocystis*.

3.2. Blastocystis and D. fragilis co-infection is common

We analysed the samples that were positive for *Blastocystis* and *D. fragilis* and determined their likelihood of co-infection with other intestinal pathogens by estimating ORs and confidence intervals (CIs) (Fig. 3). We calculated the OR of individuals testing positive for other common parasite infections when infected with *D. fragilis* or *Blastocystis*. Of the five parasitic coinfection combinations, significant ORs were obtained for all except those containing *Cryptosporidium* (OR < 1). The ORs associated with *Giardia* coinfection with a *D. fragilis* or *Blastocystis* infection were 2.14 (95% CI, 1.33 to 3.36) and, 2.24 (95% CI, 1.34 to 3.62), respectively. The point estimate of the ORs associated with co-infection with *D. fragilis* and *Blastocystis* was the largest of all the tested coinfections with a value 3.74 (95% CI, 2.94 to 4.73); however, the difference is not statistically significant. Thus, we cannot confidently conclude that those infected with either *Blastocystis* or *D. fragilis* are more likely to also be infected with the other, compared to alternative coinfection combinations. The knitted form of code used to calculate.

3.3. Extracellular and intracellular infections have contrasting effects on ecosystem alpha diversity

Alpha diversity refers to the diversity of species within a single sample, while beta diversity refers to the differences in microbial community composition between samples. We studied the relationship between a variety of parasitic infections and the microbiome in a large population of individuals in New Zealand. We used 16S rRNA sequencing to profile the microbiomes of 463 subjects with diarrhoea and a laboratory diagnosis by MT-PCR. Cases ($n = 364$) tested positive for one or more diarrhoeagenic parasites or bacteria, and controls ($n = 99$) tested negative for all pathogens (Fig. 1). This study focused on the effects of mono-parasitic infections; however, samples with co-infections (positive results for two or more parasites) were also analysed for comparison. Faecal samples were collected for laboratory diagnosis prior to the initiation of this study.

3.4. Extracellular and intracellular infections have contrasting relationships with microbiome alpha diversity

Prior studies suggest parasites may have differing effects on the gut microbial ecology based on their method of infection (Chabé et al., 2017). We compared the relationship between intracellular and extracellular infections and the total number of gut species (observed richness) and how species were distributed (Pielou evenness). Apart from *Cyclospora*, all intracellular infections had lower median observed richness than DWD controls, and all extracellular parasites had higher median richness. However, this difference was only significant for *D. fragilis* and *Salmonella.* Samples testing positive for the extracellular parasite *D. fragilis* had significantly higher richness ($p < 0.0001$, Kruskal-Wallis test) than DWD controls, while *Salmonella*-positive samples had significantly lower richness ($p < 0.0001$, Kruskal-Wallis test) than

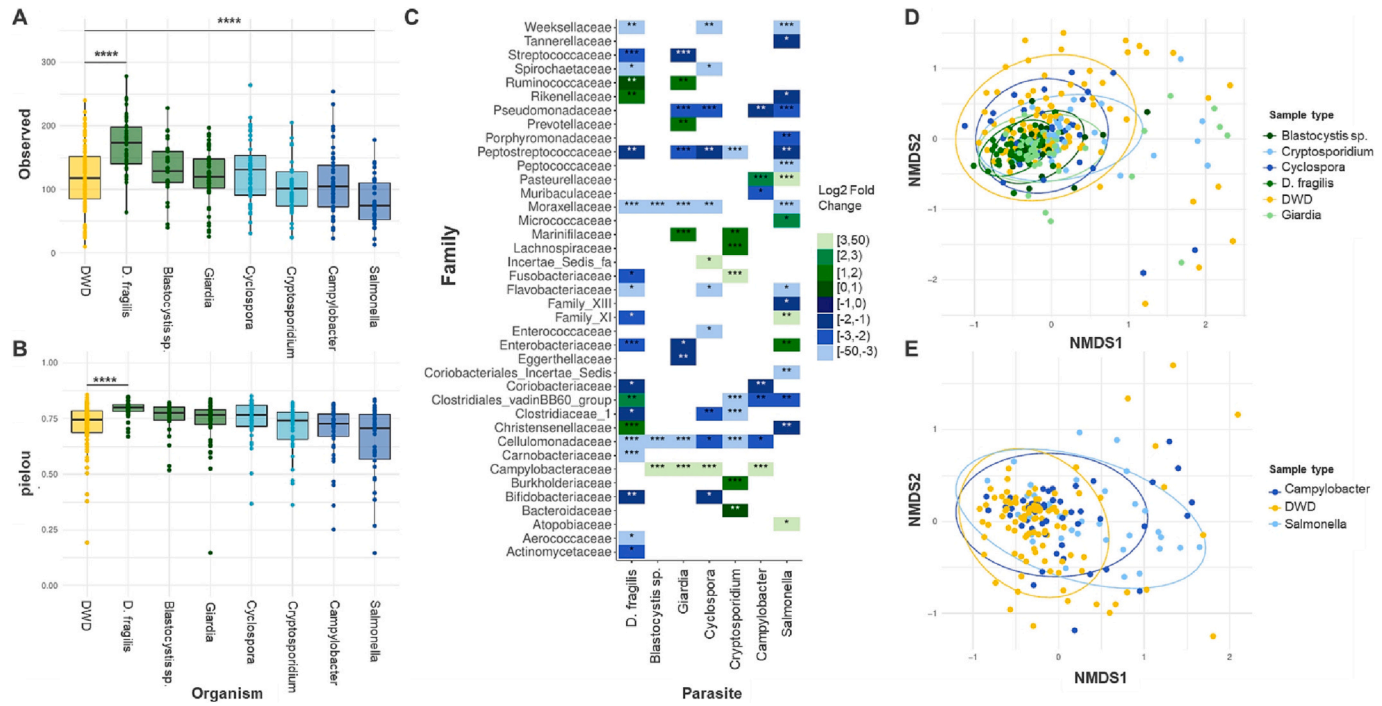

Fig. 4. Alpha diversity and changes in gut community composition due to infection of extracellular and invasive parasite infected samples. **A.** Observed richness and **B.** Pielou evenness of infections tested using two-sided t-test. Controls are labelled DWD (Diarrhoea without diagnosis). Green represents extracellular infections. Blue represents intracellular protozoan (*Cryptosporidium* spp. and *Cyclospora* spp.) and bacterial infections. **** denotes $p < 0.0001$**C.** Log2 fold changes in taxa of parasite-infected samples compared to DWD (control) samples at the family level. **D.** NMDS ordination plot of protozoan parasite infected samples compared to each other and controls, denoted as DWD ("Diarrhoea without diagnosis). Stress = 0.188 **E.** NMDS ordination plot of bacteria infected samples compared to each other and controls. Stress = 0.196. Note: NMDS stands for non-metric multidimensional scaling, which is a statistical method used to analyze and visualize complex microbiome data. An NMDS ordination plot is a graphical representation of the similarities and differences between samples in a microbiome study based on their microbial composition; helping to identify potential relationships between the microbiome and various environmental or host factors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

controls (Fig. 4A). Similarly, extracellular parasite-positive samples had higher median evenness than controls, but this difference was only significant for *D. fragilis*-positive samples ($p < 0.0001$, Kruskal-Wallis test) than controls (Fig. 4B). These results show that *D. fragilis* is significantly associated with microbiome alpha-diversity.

3.5. Taxa are differentially abundant between bacterial parasites and other parasite infections

To determine which community species were associated with the observed differences in beta diversity, we used DESeq2 to measure which taxa had significant differences in abundance between controls and each parasitic infection. Bacteria from the families Weeksellacae, Pseudomonadaceae, and Flavobacteriaceae were significantly less abundant in infections from all three 'invasion type' groups (pathogenic bacteria, intracellular and extracellular parasites), while Pasteurellaceae increased in all groups. Cellulomonadaceae and Bifidobacteriaceae were less abundant in parasite-infected samples. Enterobacteriaceae increased in bacterial and *Giardia* infections, but decreased in all other parasite infections, while Lachnospiracae showed the opposite behavior. The Ruminococcaeae behaved similarly to the Lachnospiraceae, decreasing in bacterial and *Cyclospora* infections while increasing in all other parasite infections (Fig. 4C $q < 0.1$ and Fig. 5).

3.6. The gut microbial population between individuals is more alike during extracellular parasitic infections

We used ordination to visualize whether infection made microbial communities more similar. Apart from pathogenic bacterial infections, the gut microbiota of parasitic-infected individuals appeared less heterogeneous (greater clustering and less dispersion) than that of controls (Fig. 4D and E). Infection accounted for a small but significant portion of community variance (1–3%) for all tested pathogens except *Cyclospora* (Table 1, Adonis $p < 0.01$).

All intracellular infections except *Cyclospora* had significant differences in community composition (i.e., presence and abundance of community taxa) relative to controls (Table 1, Adonis $p < 0.05$. Beta dispersion testing showed that communities infected with intracellular parasites had the same level of within-group variance as controls (Table 1).

4. Discussion

The introduction of molecular diagnostics to investigate acute gastroenteritis has significantly enhanced our understanding of aetiologies. This is particularly the case when changing from microscopic to molecular detection of protozoal parasites [19]. Only after the introduction of MT-PCR was it revealed that *D. fragilis* was the most common intestinal protist found in acute gastroenteritis samples. The prevalence of, *D. fragilis* varies between 0.4% and 71% globally, depending on cohort and diagnostic method [20]. Certainly, our data shows a three-fold increase in the proportion of *D. fragilis* detected using a PCR-based diagnostic technique compared to microscopy (assuming major temporal changes are unlikely). Several past studies show that microscopic methods are inferior to PCR due to the 'fragile' nature of *D. fragilis* when shed in faeces [21–24]. The high prevalence in symptomatic children aged 5–10 years of age has also been shown in European studies [25,26].

While we found the presence *D. fragilis* was significantly associated with *Blastocystis* in symptomatic patients, there is evidence that both species can reside in the gut microbiome of healthy individuals for an

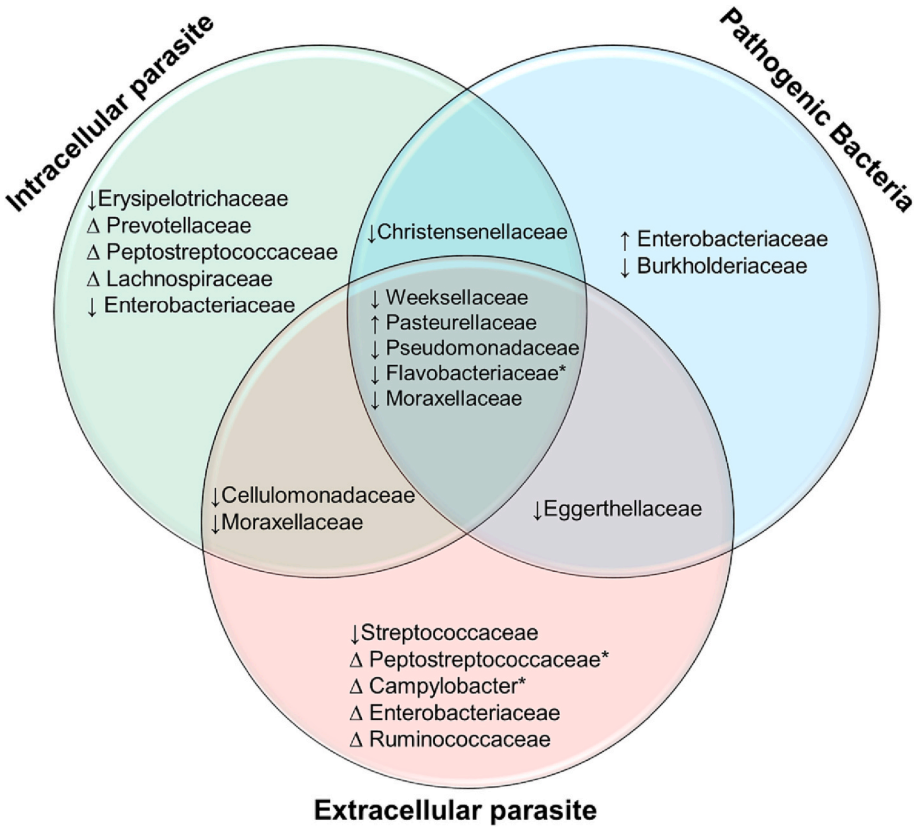

Fig. 5. A Venn diagram of taxa found in pathogenic samples tested by Deseq2. A summary of Fig. 4C. Each circle represents a parasite group: Intracellular parasite (the intracellular parasites, *Cryptosporidium* spp. and *Cyclospora* spp.), invasive bacteria, and extracellular parasites (i.e. *Giardia* spp. *Dientamoeba fragilis* and *Blastocystis* sp.). The arrows denote increase or decrease in log2 fold changes in parasite positive samples compared to controls. To be considered a result of the group, there must be a significant result from at least one parasite member without a contrasting result in the other members. If results contrast within a group a Δ is used.

Table 1
The effect of parasite mono-infection on community beta diversity. Permutation-based ANOVA and beta dispersion tests were used for community comparison.

Organism	Adonis R^2	Adonis *P* Value	Beta-Disper P Value	Intracellular Parasite
Giardia	0.019	0.001***	0.08	No
Blastocystis sp.	0.018	0.001***	0.006**	No
D. fragilis	0.037	0.001***	0.001***	No
Cryptosporidium	0.014	0.003**	0.186	Yes
Cyclospora	0.008	0.253	0.09	Yes
Salmonella	0.025	0.001***	0.228	$Bacteria_{(pathogenic)}$
Campylobacter	0.011	0.03*	0.464	$Bacteria_{(pathogenic)}$

extended period without provoking any detectable pathological symptoms [3,27–29]. Future studies should investigate strain variation in *Blastocystis* sp. and include case matched healthy controls or longitudinal samples [30].

Recent studies showed that *Blastocystis* spp. may display its pathogenicity indirectly via disrupting the gut microbiota, contributing to irritable bowel syndrome (IBS; [30,31]). While some studies indicate that *D. fragilis* is not significantly associated with IBS [32,33], murine infection with *D. fragilis* results in significant gastrointestinal disturbance, diarrhoea and weight loss [34,35]. Furthermore, levels of calprotectin (a marker of inflammatory disease in the lower gastrointestinal tract) were significantly elevated in infected animals [34]. If indeed *D. fragilis* and *Blastocystis* spp. plays a role in intestinal inflammation, it may explain in part the significant perturbation of the prokaryote microbiome observed in this study. Unfortunately, given the limitations of this study it is not possible to further speculate why *D. fragilis* infections in our study were strongly associated with increased species richness (alpha diversity) especially when compared to individuals infected with well-defined intracellular pathogens such as *Cryptosporidium* spp. [36].

It is interesting to note that the presence of *Cryptosporidium* spp. is negatively correlated with *D. fragilis* and *Blastocystis* spp. especially given that the latter parasites (both extracellular) inhabit a completely different niche (large intestine) compared to *Cryptosporidium* spp. which solely infects the epithelia of the small intestine [37].

While only *D. fragilis* had a significant effect on both the alpha and beta diversity of intestinal bacteria (detected in faeces), no overwhelming perturbation of microbiome was observed in acute gastroenteritis patients infected with protozoa. Importantly, our finding that *D. fragilis* significantly increased alpha diversity is supported independently by several studies which show that parasite colonization was associated to a rich and diverse bacterial microbiome [38]. Specifically, a study of symptomatic and asymptomatic individuals found that those infected with *D. fragilis* had a higher alpha diversity of gut bacteria compared to uninfected individuals [39,40]. However, despite the potential benefits of increased alpha diversity, the presence of *D. fragilis* has also been associated with alterations in beta diversity, or the overall composition of the gut microbiome. One study found that the presence of *D. fragilis* was associated with significant changes in the gut microbiome composition, including an increase in the relative abundance of the bacterial phylum Bacteroidetes and a decrease in the phylum Firmicutes (Stark et al., 2016). Another study found that *D. fragilis*-positive individuals had significantly different microbial community structures compared to uninfected individuals (Scanlan et al., 2014a).

Interestingly, the effect of *D. fragilis* on the gut microbiome may be strain dependent (in a similar manner to *Blastocystis* sp.). A study by Stark et al. (2016) found that different strains of *D. fragilis* had distinct effects on the gut microbiome, with some strains associated with significant changes in beta diversity and others not. This highlights the importance of characterizing the genetic diversity of *D. fragilis* when studying its impact on the gut microbiome.

Overall, while the presence of *D. fragilis* has been associated with increased alpha diversity of the gut microbiome, it has also been linked to significant alterations in the overall composition of the microbiome.

Future studies are needed to better understand the mechanisms underlying these effects and to determine the long-term impact of *D. fragilis* on gut health and dysbiosis, requiring case matched longitudinal studies into the role of the intestinal 'protistome' in human health and dysbiosis (especially using case matched healthy controls). We would particularly encourage future studies focusing on the longitudinal pathobiology of *D. fragilis* which remains unduly neglected.

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