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Brief Original Article

Gut microbiota related to *Giardia duodenalis*, *Entamoeba* spp. and *Blastocystis hominis* infections in humans from Côte d'Ivoire

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Abstract

Introduction: Literature data provide little information about protozoa infections and gut microbiota compositional shifts in humans. This preliminary study aimed to describe the fecal bacterial community composition of people from Côte d'Ivoire harboring *Giardia duodenalis*, *Entamoeba* spp., and *Blastocystis hominis*, in trying to discover possible alterations in their fecal microbiota structure related to the presence of such parasites.

Methodology: Twenty fecal samples were collected from people inhabiting three different localities of Côte d'Ivoire for copromicroscopic analysis and molecular identification of *G. duodenalis, Entamoeba* spp., and *B. hominis*. Temporal temperature gradient gel electrophoresis (TTGE) was used to obtain a fingerprint of the overall bacterial community; quantitative polymerase chain reaction (qPCR) was used to define the relative abundances of selected bacterial species/group, and multivariate statistical analyses were employed to correlate all data.

Results: Cluster analysis revealed a significant separation of TTGE profiles into four clusters ($p < 0.0001$), with a marked difference for *G. duodenalis*-positive samples in relation to the others ($p = 5.4\times10^{-6}$). Interestingly, qPCR data showed how *G. duodenalis*-positive samples were related to a dysbiotic condition that favors potentially harmful species (such as *Escherichia coli*), while *Entamoeba* spp./*B. hominis*-positive subjects were linked to a eubiotic condition, as shown by a significantly higher *Faecalibacterium prausnitzii-Escherichia coli* ratio.

Conclusions: This preliminary investigation demonstrates a differential fecal microbiota structure in subjects infected with *G. duodenalis* or *Entamoeba* spp./*B. hominis*, paving the way for using further next-generation DNA technologies to better understand host-parasite-bacteria interactions, aimed at identifying potential indicators of microbiota changes.

Key words: microbiota; *Giardia duodenalis*; *Entamoeba* spp.; *Blastocystis hominis*; dysbiosis; Côte d'Ivoire.

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Introduction

The human intestinal microbiota contains a complex combination of microorganisms, including bacterial and viral taxa [1]. Microbiota is also characterized by the eukaryotic component encompassing helminths and protists [2,3]. Parasites react with the microbial community and with the host, greatly impacting the host/gut microbiota balance and influencing microbiota protective and immunomodulatory functions [1,3,5-8]. Among protozoa, *Giardia duodenalis* (syn. *G. intestinalis, G. lamblia*), *Entamoeba histolytica/E. dispar*, and *Blastocystis hominis* are common gastrointestinal parasites widespread in developing countries, where the lack of safe water and sanitation leads to increased exposure to infectious agents. It is now well known that these protozoa are constantly interacting with the intestinal microbiota, but their possible relation to imbalances in microbiota composition, termed dysbiosis, is still unclear. *Giardia* infection may trigger long-lasting changes in the spatial distribution of commensal microorganisms, enhancing bacterial invasiveness and inflammatory responses in the gut mucosa during the post-clearance phase [9]. Regarding *E. histolytica*, significant alterations of predominant gut bacteria and a depletion of some predominant genera (*e.g.*, *Bacteroides*, *Eubacterium*, *Lactobacillus*) in the gut of infected patients has been described [10]. The

hypothesis that *Blastocystis* might also be linked to intestinal flora imbalance has been recently supported in a study involving patients with irritable bowel syndrome [11].

The main purpose of this preliminary study was to highlight the relation of *G. duodenalis*, *Entamoeba* spp., and *B. hominis* infections with fecal microbiota composition in subjects living in southern Côte d'Ivoire. In addition, a dysbiosis evaluation was done to ascertain the potential alteration of the bacterial community associated with the protozoa infections.

Methodology

Sample collection and management

Fecal samples were randomly collected in three localities in the Grand-Bassam department of southern Côte d'Ivoire. The study was based on 20 subjects, 6 males and 14 females, between 1 and 74 years of age (average 21.7 years), with or without symptoms (Table 1). All other relevant clinical information, including having other diseases and antibiotic therapy, were noted. One fecal sample was collected in a sterile stool container from each patient. After registration, the sample was divided into two aliquots. The first aliquot was immediately examined for intestinal parasites in the Clinical Laboratory of the Don Orione Centre in Bonoua by wet mount Lugol's iodine staining method and formol ethyl-acetate concentration technique, and analyzed by light microscope. The second aliquot was preserved for microbial community analysis by adding the Qiagen Allprotect Tissue Reagent (QIAGEN, Hilden, Germany), frozen at -20°C, and transported to the University of Rome Tor Vergata, Italy, where it was kept at -80°C until DNA extraction and molecular identification of the parasites. The microbial community analysis was conducted at the Sapienza University of Rome, Italy. Informed consent was obtained from all individual participants included in the study; for minors, informed consent was obtained from their guardians. All procedures performed in this study involving human participants were approved by the medical committee of the Don Orione Centre and were in accordance with the 1964 Helsinki Declaration and its later amendments.

Fecal DNA extraction

Total DNA was extracted by QIAmp Stool Mini Kit (QIAGEN, Hilden, Germany). The manufacturer's protocol was modified by incubating samples with proteinase K overnight at 56°C, followed by incubating for 4 hours at 37°C with 2 mg/mL (final concentration) of lysozyme (Sigma-Aldrich, St. Louis, USA). Eluted DNA concentration was quantified by an Eppendorf spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm, and its quality was checked through the 260/280 nm ratio and 1% agarose gel electrophoresis.

Table 1. Patients' characteristics and intestinal protozoa detected

Subject	Sampling area	Age (years)	Sex	Symptoms of infection	Protozoa			HAC cluster[a]
					G. duodenalis	*Entamoeba* spp.	*B. hominis*	
M1	Bonoua	8	F	Abdominal pains	Negative	Negative	+	B+
M2	Bonoua	21	F	Abdominal pains	+	Negative	+	G+
M3	Bonoua	74	M	Asymptomatic	Negative	*E. hartmanni*	+	E/B+
M4	Bonoua	5	F	Frequent abdominal pains	+	Negative	Negative	G+
M5	Kimoukro	8	F	Frequent abdominal pains	+	Negative	+	G+
M6	Kimoukro	6	M	Frequent abdominal pains	+	*E. hartmanni*	+	All
M7	Kimoukro	10	F	Frequent abdominal pains	+	*E. coli*	+	All
M8	Bonoua	5	F	Frequent abdominal pains	+	*E. dispar*	Negative	G+
M9	Bonoua	3	M	Asymptomatic	+	Negative	+	G+
M10	Bonoua	1	F	Fever	Negative	Negative	Negative	NaN
M11	Bonoua	9	M	Asymptomatic	Negative	Negative	Negative	NaN
M12	Bonoua	40	F	Asymptomatic	Negative	Negative	+	B+
M13	Bonoua	30	M	Asymptomatic	Negative	Negative	Negative	NaN
M14	Assouindé	12	F	Asymptomatic	+	*E. coli*	+	All
M15	Assouindé	12	M	Asymptomatic	Negative	*E. hartmanni*	+	E/B+
M17	Bonoua	50	F	Asymptomatic	+	*E. hartmanni*	+	All
M18	Bonoua	41	F	Asymptomatic	Negative	Negative	+	B+
M19	Bonoua	67	F	Asymptomatic	Negative	Negative	Negative	NaN
M20	Bonoua	9	F	Asymptomatic	+	Negative	+	G+
M21	Bonoua	24	F	Asymptomatic	Negative	Negative	+	B+

[a] Cluster belonging after hierarchical ascendant clusterization analysis on temporal temperature gradient gel electrophoresis profiles.

Parasite identification

Molecular identification of *G. duodenalis*, *Entamoeba* spp., and *B. hominis* was done by end-point PCR and sequence analysis of the SSU rDNA region. For *G. duodenalis*, a conventional nested PCR was performed as described [12]; *B. hominis* was detected by nested-PCR analysis for accurate subtyping [13,14]. Finally, the identification of *Entamoeba* spp. was done as previously reported [15]. All PCRs were carried out in a 25 µL volume containing 12.5 µL of PCR master mix 2X (Promega Italia, Milan, Italy), 100–200 ng of template DNA, and 0.6 mM of each primer. PCR products were visualized by electrophoresis on 1% agarose gel stained by SYBR Safe DNA gel stain (Invitrogen, Life Technologies, Monza, Italy). Amplicons were purified using the mi-PCR Purification Kit (*Meta*bio*n* International AG, Planneg, Germany), sequenced by the Bio-Fab Research (Rome, Italy). Species identification was determined by comparing the obtained sequences in GenBank database by using the standard nucleotide BLAST search.

PCR amplification of bacterial 16S rDNA gene and TTGE analysis

In order to amplify the V6–V8 region of bacterial 16S rDNA, PCR reactions including the universal primers GCclamp-U968 and L1401 (Bio-Fab Research, Rome, Italy) were used, and 500 ng of DNA of PCR product from each sample was used to perform the subsequent TTGE experiments, as previously described [16].

Quantitative PCR (qPCR)

Quantitative analysis of the fecal microbiota composition was determined with (qPCR) using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, USA). Each PCR reaction was carried out in a final volume of 2 5µL containing 12.5 µL Mix (SensiMixTM SYBR Hi-ROX Kit, Bioline, London, UK), 1.25 µL of primers (forward and reverse), 2.5 µL of BSA (bovine serum albumin), and 2 µL of template DNA (approximately 100 ng). PCR amplification was performed in 40 cycles using the following conditions: 95°C for 10 minutes, then 40 cycles of 95°C for 10 seconds, annealing temperature as in Table 2 for 20 seconds, and 72°C for 27 seconds. Species-specific

Table 2. Primers used in this study for SYBR Green qPCR reactions

Species	Primers	Sequence	Annealing temperature (°C)	Amplified fragment (bp)	Number of 16S rDNA operons	Reference
Bacteroides fragilis	B_fra_F B_fra_R	GAAAGCATTAAGTATTCCACCTG CGGTGATTGGTCACTGACA	56	176	6	[17]
Peptostreptococcus productus	P_prod_F P_prod_R	GGTGGCAAAGCCATTCGGT GTTACGGGACGGTCAGAG	56.5	182	1	[17]
Enterococcus spp.	E_spp_F E_spp_R	CCCTTAATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	56.5	144	4	[18]
Faecalibacterium prausnitzii	F_prau_F F_prau_R	CCCTTCAGTGCCGCAGT GTCGCAGGATGTCAAGAC	54.5	158	1	[19]
Clostridium leptum group	C_leptum_group_F C_leptum_group_R	GTTGACAAAACGGAGGAAGG GACGGGCGGTGTGTACAA	56	244	2	[20]
Clostridium coccoides group	C_coccoides_group_F C_coccoides_group_R	ACTCCTACGGGAGGCAGC GCTTCTTAGTCAGGTACCGTCAT	60	139	3	[20]
Lactobacillus/Enterococcus group	Lac_Enter_F Lac_Enter_R	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	53	341	7	[20]
Bacteroides group	Bact_group_F Bact_group_R	GGTTCTGAGAGGAGGTCCC GCTGCCTCCCGTAGGAGT	59.5	64	6	[20]
Prevotella group	Prevo_group_F Prevo_group_R	CAGCAGCCGCGGTAATA GGCATCCATCGTTTACCGT	54	309	6	[20]
Escherichia coli	E_coli_F E_coli_R	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	56.5	340	7	[18]
Bifidobacterium group	Bifido_group_F Bifido_group_R	CTCCTGGAAACGGGTGGT GCTGCCTCCCGTAGGAGT	57.5	204	4	[20]
Eubacterial universal primers	Tot_bact_F	ACTCCTACGGGAGGCAGCAGT	61	172	-	[20]

primers used, chosen from literature, are reported in Table 2 [17-20]. In order to ensure a suitable inter-species data normalization and interpretation, a correction for number of 16S rRNA operons was conducted and extrapolated from PATRIC (Pathosystems Resource Integration Center) website (https://www.patricbrc.org) (Table 2). Standard curves for each bacterial species/group were done using a range of 10^9–10^{10} copies of the plasmid (pUC57) containing one copy of the target [21]. The qPCR runs were analyzed using the 7300 System SDS Software (Applied Biosystems, Foster City, USA), and results expressed followed the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines [22,23].

Statistical analysis

TTGE profiles were analyzed using Totallab 1D software (Totallab, Newcastle upon Tyne, UK), while Prism 5 (Graph Pad, LaJolla, USA) and SPSS (SPSS, Chicago, USA) statistical software were used to analyze qPCR data and to perform Pearson's correlation. The Mann-Whitney U test by Prism 5 was used for comparing qPCR results, and a p value ≤ 0.05 was considered significant. Orthogonal projection onto latent structure (OPLS-DA) analysis was performed with SIMCA-P+ software (Umetrics, Umeå, Sweden).

Results

Parasites prevalence

No helminth infections were detected by microscopic examination. Overall, 16 of 20 subjects were positive for intestinal protozoa. Microscopic and molecular analysis allowed identification of *Giardia duodenalis* (n = 10), *Blastocystis hominis* (n = 14), *Entamoeba coli* (n = 2), *E. dispar* (n = 1), and *E. hartmanni* (n =3). *E. histolytica* was not detected. Among 16 patients who resulted positive for parasites, multiple infections were detected in 11 subjects: *B. hominis* plus *G. duodenalis* (n = 4), *B. hominis* plus *E. hartmanni* (n = 2), *E. dispar* plus *G. duodenalis* (n = 1), and four patients were infected with three parasite species (Table 1).

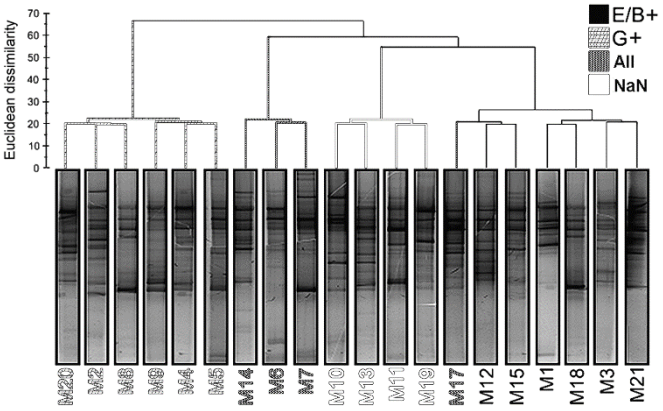
Bacterial microbiota characterization

Based on a hierarchical ascendant clusterization (HAC) analysis, TTGE profiles clustered into four different groups: G+ (*G. duodenalis* positive); E/B+ (*Entamoeba* spp. and *Blastocystis hominis* positive); NaN (negative for protozoa); and All (positive for all three parasites) (Figure 1). As shown in the dendrogram, the separation into four main clusters was highly significant ($\chi^2 = 52.86$, $p < 0.0001$). G+ fecal profiles were significantly different compared to the other three clusters, as assessed by orthogonal projection onto latent structure (OPLS-DA) analysis (Fisher's $p = 5.4\times10^{-6}$).

Quantitative PCR of selected bacterial species or groups

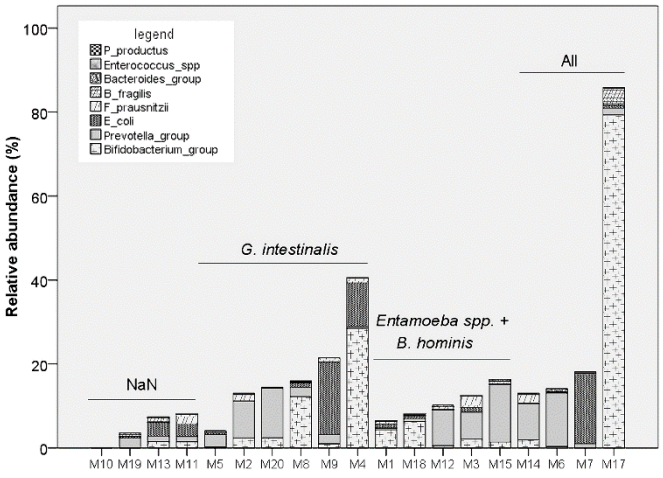
For each subject enrolled, a quantitative representation of the relative abundance of the bacterial species is reported (Figure 2). With respect to the NaN group, G+ significantly increased the mean sum of the relative abundances as a whole (18.2% ± 5.0% versus 4.8% ± 1.9%, p = 0.019), while a similar increase was

Figure 1. Temporal temperature gradient gel electrophoresis profiles dendrogram based on fecal samples of subjects from Côte d'Ivoire grouped by means of Euclidean dissimilarity and agglomeration method of Ward.



G+: *G. duodenalis*; All: all three parasites; NaN: none of the parasites; E/B+: *Entamoeba* spp. and *B. hominis.*

Figure 2. Relative abundance (%) of fecal bacteria species/groups (y axis) reported as the percentage on overall total bacteria. Samples were grouped on x axis (NaN, G+, E/B+, All).



observed for the E/B+ group, although to a lesser extent (10.7% ± 1.7%, p = 0.032). Even if a slight increasing trend for *E. coli* levels in the G+ group was observed in the NaN group, it was not significant (p = 0.381), while the increase of the *Bifidobacterium* group in G+ was statistically significant (p = 0.047). No differences in bacterial/group-relative abundances were observed in E/B+ samples with respect to NaN.

Dysbiosis evaluation

The ratio between the relative abundances of *F. prausnitzii* (a beneficial species) and *E. coli* (a potentially harmful species), called the F/E ratio, was used as a dysbiosis index [24,25]. A significant difference in F/E ratio was observed in the E/B+ group of subjects with respect to NaN (4.02 ± 2.02 versus 0.26 ± 0.19, p = 0.032). No statistical difference in F/E ratio was shown by G+ subjects with respect to NaN (p = 0.129), meaning that, even if a substantial difference was found in G+ fecal microbiota composition, an alleged dysbiosis could not be revealed by F/E ratio.

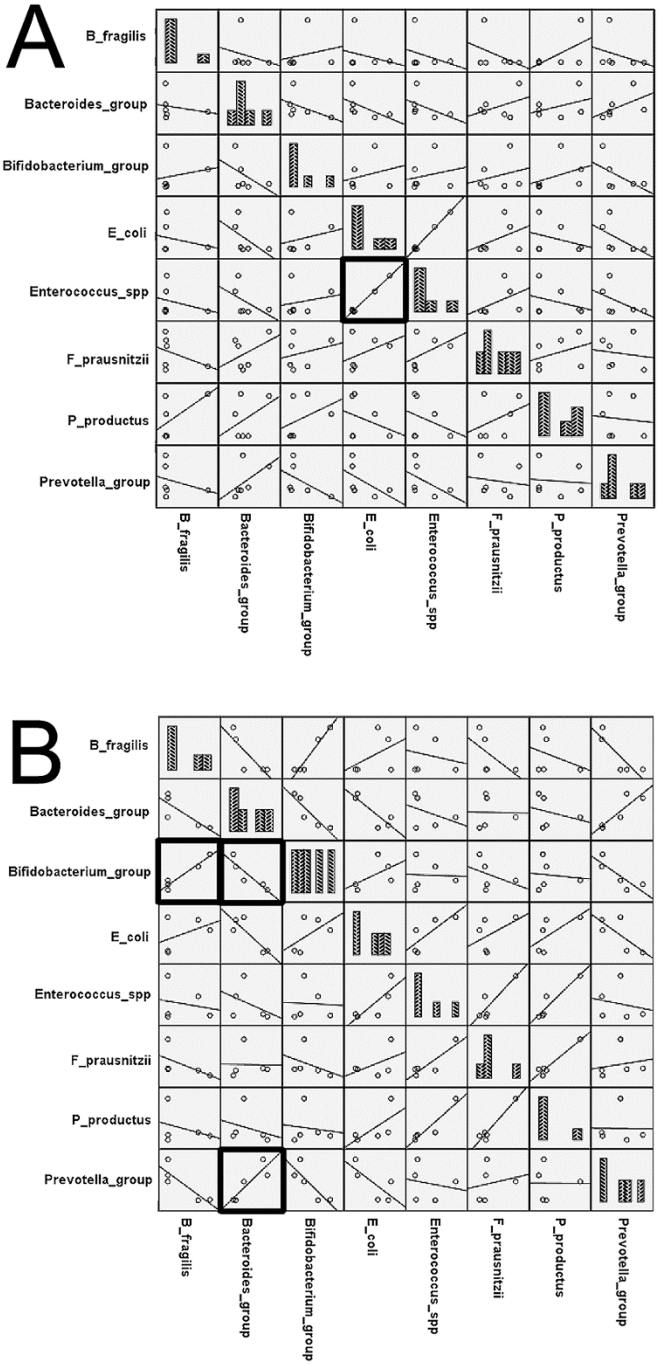
In order to determine putative correlations among the different levels of the selected bacterial species/groups, Pearson's correlation index was computed within G+ (panel A) and E/B+ (panel B) classes for all qPCR data (Figure 3). Within G+ subjects, a positive correlation was found among *E. coli* and *Enterococcus* spp. (Pearson's correlation coefficient = 0.996). Regarding the E/B+ subjects, positive correlations were found among the *Bifidobacterium* and *B. fragilis* group (Pearson's correlation coefficient = 0.966), and among the *Prevotella* and *Bacteroides* group (Pearson's correlation coefficient = 0.882), while a negative correlation was found among the *Bifidobacterium* group and *Bacteroides* group (Pearson's correlation coefficient = -0.898).

Discussion

Few data are available on the gut microbiota composition in subjects from sub-Saharan Africa [19,26]. Moreover, to the best of our knowledge, only a recent study examined *Blastocystis* spp. prevalence and gut microbiota structure in a shotgun metagenomic dataset of human fecal samples [27], but no subjects from sub-Saharan Africa were involved.

The present pilot study aimed to shed light on possible differences in the fecal microbiota of 20 subjects living in Côte d'Ivoire associated with the presence or absence of *Giardia duodenalis*, *Entamoeba* spp., and *Blastocystis hominis*. Upon HAC analysis, fecal TTGE profiles were significantly grouped into four clusters based on the presence of parasites: *Giardia* positive (G+), *Entamoeba* spp. and *Blastocystis* positive (E/B+), positive for all three parasites (All), and negative for any parasite (NaN). From these data, a

Figure 3. Correlation between fecal bacterial species. The graph on the dot matrix of dispersion refers to the bacteria fecal species or groups selected, within G+ (panel A), or within E/B+ (panel B). Black squares define significant correlation ($p \leq 0.05$) found with a correlation coefficient of Pearson. Bar plots of diagonal define the distribution of data within each bacteria species/groups.



noticeable separation of G+ subjects from the others seems to be evident (Figure 1). These results could indicate that the microbial communities are differently shaped in the presence of specific parasites, and that *G. duodenalis* could reflect a particular behavior in the fecal microbiota ecology during gut colonization. In addition, in G+ subjects, a positive correlation among *Escherichia coli* versus *Enterococcus* spp. (Figure 3, panel A) was observed together with a significant increase ($p < 0.05$) of bifidobacteria; also, a trend of increase of the species *Escherichia coli* was evidenced when they were compared with NaN group (Figure 3, panel A). It could be argued that *G. duodenalis* might favor facultative anaerobic and potentially pathogenic species, such as *E. coli* and *Enterococcus* spp. The increase of bifidobacteria in G+ subjects could represent the microbiota attempt to respond to the inflammation induced by *G. duodenalis*. It is known that bifidobacteria might exert a variety of beneficial health effects, including modulation of local and systemic immune responses [28,29]. Additionally, *Bifidobacterium* strains may discourage the growth of some Gram-negative diarrheagenic bacteria from adults, as evidenced also in infants [29].

In E/B+ subjects, a significant positive correlation was found among *Bifidobacterium* versus *B. fragilis*, and *Prevotella* versus *Bacteroides*, and a negative correlation was found between *Bifidobacterium* versus *Bacteroides* (Figure 3, panel B). The genera *Prevotella* and *Bacteroides* belong to the phylum Bacteroidetes, and are all species/genera considered beneficial. The results observed in E/B+ subjects seem to indicate that the presence of the parasites *Entamoeba* spp. and *B. hominis* would alter the microbiota balance towards a eubiotic condition, as shown by the high F/E ratio in respect to NaN. This finding is in contrast to that found for *G. duodenalis*, and the comparison of *E. coli* relative abundances, higher in G+ and lower in E/B+ (Figure 3), seems to reinforce such a hypothesis.

Conclusions

In conclusion these preliminary baseline data encourage us to pursue further research using next-generation DNA technologies for a prospective extension of the potential role of micro-eukaryotes in microbiota composition [30]. Gut microbiota studies in endemic countries could represent a useful approach to understand the complex scenario during multiple parasitic infections and to identify potential indicators of microbiota changes [11] and tropical enteropathy [31]. Finally, these studies could also provide potentially critical information for protozoa-related dysbiosis in asymptomatic subjects that could be involved in low-grade systemic inflammation of non-communicable chronic degenerative diseases such as obesity, diabetes, and atherosclerosis, all with increasing prevalence in developing countries.

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Authors' contributions

SS, FB, and RD contributed equally to this work.

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