

Occurrence of *Blastocystis*-subtypes in patients from Italy revealed association of ST3 with a healthy gut microbiota

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ABSTRACT

An epidemiological survey on *Blastocystis* was carried out enrolling a total of 2524 subjects referred to the Umberto I Academic Hospital in Rome, for the routine parasitological exams, during 2017–2018. The studied population included a sample of immunocompromised individuals ($N = 130$) followed at the same hospital.

DNA sequencing of the small subunit rRNA gene (SSU rDNA) locus was performed on samples positive to the copro-parasitological analysis to molecularly characterize the *Blastocystis*-subtypes. Microscopical analysis detected *Blastocystis* in 192/2524 (7.6%) of the enrolled subjects. It was the organism most frequently identified in the analysed faecal samples diagnosed in single infection (5.6%) or in co-infection with other enteric protozoa (2%). Furthermore, it was found mainly in immunocompromised patients (22.3%) compared to immunocompetent ones (6.8%). As expected, ST3 was the most occurring subtype identified in 40% of the subjects, followed by ST1 (29%), ST2 (16%), ST4 (12%), and ST7 (3%).

Next-generation sequencing (NGS) of the 16S rDNA was performed on a sub-sample of *Blastocystis*-ST3-carriers, homogenous by age and gender, as well as on *Blastocystis*-free subjects, to profile and compare their gut bacterial composition. A higher bacterial diversity was found in ST3-*Blastocystis*-carriers, which exhibited a high abundance of *Prevotella*, *Methanobrevibacter* and *Ruminococcus* while, a high percentage of *Bacteroides* was found in *Blastocystis*-free subjects.

This study evidenced the presence of *Blastocystis* in 7.6% of faecal samples in Italy and a high circulation of the protist among immunocompromised patients (22.3%). Molecular characterization of positive samples evidenced the occurrence of five different subtypes, including zoonotic ST such as the ST7, highlighting the risk of transmission from animals. Study of the gut microbiota composition confirms previous evidences according to which, the colonisation by *Blastocystis* would be linked with an eubiotic gut characterized by potentially beneficial species such as *Prevotella* and *Ruminococcus*, rather than with a dysbiotic state, with a high abundance of Enterobacteriaceae, and corroborated the role of the protist as "an old friend" of the human gut.

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1. Introduction

Blastocystis is currently known as belonging to the Stramenopiles, a complex and heterogeneous evolutionary assemblage of heterotrophic and photosynthetic protozoa (Silberman et al., 1996; Arisue et al., 2002; Riisberg et al., 2009). It colonises intestine of humans and wide range of animals. In humans, it exhibits a worldwide distribution, with a prevalence from 0.5%–30% and 30–76% in industrialized and developing countries, respectively (Alfellani et al., 2013). Molecular study based on the small subunit ribosomal RNA (SSU-rDNA) evidenced an extensive genetic heterogeneity allowing the identification in mammalian and avian hosts of at least 26 divergent lineages, termed subtypes (STs), which could be considered arguably separate species (Alfellani et al., 2013; Zhao et al., 2017; Maloney et al., 2019). Ten of the 26 subtypes, ST-1 to ST-9 and ST-12, have been identified in human samples, and all, except for ST-9, have also been reported in animals (Ramírez et al., 2016; Stensvold and Clark, 2016). Despite the pathogenetic role of *Blastocystis* has been the subject of many investigations (Sheehan et al., 1986; Dogruman-Al et al., 2009; Poirier et al., 2012; Wawrzyniak et al., 2013; Azizian et al., 2016; Mattiucci et al., 2016; Stensvold et al., 2009), it's so far remaining unclear. Several authors suggested that it could be linked to the impact of the protist on the gut microbiota. Analysis of the microbial communities in healthy individuals or affected by intestinal disorders evidenced three main microbiota patterns, named enterotypes, characterized by three dominant bacteria clusters: *Bacteroides* (enterotype I), *Prevotella* (enterotype II), or *Ruminococcus* (enterotype III) (Arumugam et al., 2011). Each enterotype harbours different bacteria genera, defines a distinctive way of generating energy and seem to be linked to dietary habits (Rinninella et al., 2019). Recent NGS-based studies associated *Blastocystis* with higher diversity of gut microbiota, low Body Mass Index (BMI), and the enterotype II and III (Andersen et al., 2015; Andersen and Stensvold, 2016). Furthermore *Blastocystis* was also linked to an eubiotic state, characterized by a preponderance of potentially beneficial species, belonging mainly to the phyla Firmicutes and Bacteroidetes, instead of those of the phylum Proteobacteria, since significantly higher *Faecalibacterium prausnitzii*-*Escherichia coli* ratio was found in *Blastocystis*-positive than in *Giardia*-positive individuals (Iebba et al., 2016a).

Conversely, Nourrisson and colleagues evidenced a decrease of faecal microbiota protective bacteria in *Blastocystis*-colonised individuals (Nourrisson et al., 2014). Moreover, it has been suggested that microbiota composition in *Blastocystis*-carriers may be dependent on the organism's ST and that certain isolates of *Blastocystis*, as the ST7, potentially lead to an imbalance of the gut microbiota (Yason et al., 2019; Tito et al., 2019). Finally, it has been proposed that some populations are more susceptible to *Blastocystis* colonisation (Wawrzyniak et al., 2013). Thus, this protozoon is frequently found in immunocompromised individuals, such as those with human immunodeficiency virus/acquired immunodeficiency syndrome or cancer (Piranshahi et al., 2018; Kurniawan et al., 2009; Tan et al., 2009). Studies of gut microbiota in HIV-infected patients evidenced a change in the *Bacteroides*:*Prevotella* ratio (Williams et al., 2016), which could also be linked to sexual practice and lifestyle rather than HIV infection and Highly Active AntiRetroviral Therapy (HAART)-treatment (Noguera-Julian et al., 2016).

However, no specific investigations have been so far conducted to investigate on the relationship between the presence of *Blastocystis* and the faecal microbiota composition in such group of patients.

Aim of this study was to: i) assess the circulation of *Blastocystis* among patients referred to the Academic Hospital "Policlinico Umberto I" for the routine parasitological exams; ii) molecular characterize the *Blastocystis*-STs from positive subjects; iii) analyse the faecal microbial composition from a cohort of patients, including immunocompromised subjects, found positive to *Blastocystis*-ST3.

2. Materials and methods

2.1. Faecal samples collection

Faecal samples were collected from subjects referred to the Diagnostic Parasitology laboratory and to the Department of Translational and Precision Medicine of the Academic Hospital "Policlinico Umberto I", during the years 2017–2018. This epidemiological survey was carried out after the previously study on *Blastocystis* STs from 189 isolates, collected during the years 2012–2014 among mildly symptomatic patients, or those affected by inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) or chronic diarrhoea, or otherwise immunosuppressed referred at the same hospital (Mattiucci et al., 2016). In such survey six subtypes (ST1, 2, 3, 4, 6, 8) were detected and significant occurrence of *Blastocystis* ST4 in patients suffering from IBS, IBD or chronic diarrhoea was observed (Mattiucci et al., 2016).

Demographic data were obtained from subjects included in the study, as well as their written informed consent. The study was approved with respect to the Helsinki Declaration by the Ethical Committee of the Academic Hospital "Policlinico Umberto I" (license number: n. 4836).

2.2. Laboratory procedures

From one to three faecal samples were collected from each subject and submitted to the microscopic observation of the wet smears stained with Lugol, directly and after Ridley concentration (Ridley and Hawgood, 1956). Genomic DNA was then extracted from samples positive to the microscopic observation and submitted to PCR amplification using primers previously described (Scicluna et al., 2006), which target a fragment of about 600 bp from the *Blastocystis*-SSU rDNA gene, following PCR protocol and conditions as previously described (Mattiucci et al., 2016). The resulting chromatograms were analysed and edited by

using the software Chromas version 2.33 (Technelysium Pty Ltd., Australia). The sequences obtained were compared to those of *Blastocystis* STs previously deposited in GenBank using the BLAST application (www.ncbi.nlm.nih.gov/BLAST). The STs were identified by determining the exact match or closest identity (99%), according to the classification given by Stensvold et al. 2007 (<https://pubmlst.org/blastocystis/>). NGS of the bacterial 16S rRNA was thus carried out on a subsample of subjects *Blastocystis*-free and *Blastocystis*-carriers homogeneous by age (<40 years-old), gender (male) and found positive for the same subtype (ST3). NGS and data analysis was supported by BMR Genomics (Padua, Italy). Briefly, V3-V4 regions of 16S rRNA gene were amplified using the primers Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3') (Takahashi et al., 2014). Primers were modified with forward overhang: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]-3' and with reverse overhang: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA-[locus-specific sequence]-3' necessary for dual index library preparation, following Illumina protocol (<https://web.uri.edu/gsc/files/16s-metagenomic-library-prep-guide-15044223-b.pdf>). PCR mix was composed as follows: 5 µl DNA, 6.2 µl of mix (Taq Platinum-Thermo Fisher, Primer and MgSO₄) and 13.8 µl DNA-se free water. Amplification conditions were: 1 cycle at 94 °C for 1 min, 25 cycles at 94 °C for 1 min, 55° for 30 s, 68° for 45 s and 1 final cycle at 68 °C for 7 min. Amplicons of expected size (about 476 bp) were purified by means Beads Ampure XP 0.8x, normalized, pooled and run on Illumina MiSeq with 2 × 300 bp approach in order to produce about 50000 reads (±20%).

The sequencing reads were filtered for average quality (Q > 30), and R1 and R2 were merged using FLASH with default parameters. QIIME software version 1.9.1 was used to perform the full analysis of OTUs selected for the statistical analysis using the `pick_closed_reference_otus.py` wrapper from the Greengenes reference database (13.8 version) (Caporaso et al., 2010).

2.3. Data analysis

To test the distribution of different *Blastocystis* subtypes among different age groups and gender, the Wilcoxon signed-rank non-parametric test was used. Data were analysed using IBM SPSS, version 21.0 (SPSS Inc., Chicago, USA). Alpha (within a community) and beta (between communities) diversity metrics, as well as taxonomic community assessments, were produced using QIIME 1.9.1 scripts on the normalized read counts. The following indexes were calculated for each sample: Chao1 bias-corrected and observed OTUs. Alpha-diversity rarefaction curves were produced by plotting these several diversity metrics against the number of sequences considered from a sample. Differences in diversity between *Blastocystis*-carriers (both immunocompetent and immunocompromised) and *Blastocystis*-free subjects were tested by Kruskal-Wallis test using the Stats Direct Statistical software v. 3.1.20 (<http://www.statsdirect.com>, England: StatsDirect Ltd. 2013). Multivariate analysis was done through the `compare_category.py` script of QIIME. Differences in beta diversity (weighted UniFrac distances) were identified using Analysis of Similarity (ANOSIM) and the effect of size indicated by an R-value (between -1 and +1), with a value of 0 representing the null hypothesis. Adonis (ANOVA tests group) was applied to verify variations within a category and between categories. Analyses were performed among the required groups and on different metrics: weighted and unweighted. MatagenomeSeq was used to calculate species differential abundance between the groups. The Mann-Whitney test was then applied to evaluate the significance of the comparison. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Study subjects and coproparasitological results

During the years 2017–2018 a total of 2524 faecal samples were screened for intestinal protozoa among the patients admitted at the Diagnostic Parasitology laboratory, "Policlinico Umberto I", Rome, Italy. The study population included 2394 subjects referred at the hospital for the routinely copro-parasitological analyses, without any indication about their clinical status, and 130 HIV-positive patients regularly followed at the Department of Translation and Precision Medicine of the same hospital.

Microscopical analysis detected *Blastocystis* in 192/2524 (7.6%) of the enrolled subjects. In detail, *Blastocystis* was frequently found in the group of immunocompromised patients (22.3%) compared to immunocompetent ones (6.8%).

As expected, it was the most frequent organism identified in the faecal samples diagnosed in single infection (5.6%) or in coinfection with other enteric protozoa (2%), followed by *Endolimax nana* (2.5%), *Entamoeba coli* (1.7%), *Giardia intestinalis* (1.2%), *Entamoeba complex* (0.5%), *Iodamoeba butschlii* (0.3%).

Blastocystis-carriers were mainly males (65%), mean age 47.35 (95% CI, 40.94–54.74), 62% were from Italy.

3.2. Molecular characterization of *Blastocystis* subtypes

A total of 142/192 stool samples positive to the microscopical analysis were successfully DNA extracted and subtyped by sequences analysis of the SSU-rDNA (barcode region). BLAST searches allowed the identification of 5 STs, with different percentage values.

The most common subtype was the ST3 identified in 40% of the subjects, followed by ST1 (29%), ST2 (16%), ST4 (12%), and ST7 (3%). No significant correlation was found between STs distribution and patients' gender. Regarding age, some significant difference was observed in the distribution of ST1 and ST4 between different age groups, being more prevalent in subjects <40 years than in the older ones (*p* = 0.0048 and *p* = 0.0068, respectively).

3.3. Bacterial microbiota composition of *Blastocystis*-ST3 faecal samples

The gut microbiota composition of *Blastocystis*-colonised patients was investigated by a cross-sectional study on subsample of patients, as above described, homogeneous by age (<40 years-old) and gender (male) and found positive for the same subtype (ST3). Thus, 16S rDNA sequencing was performed on a total of 27 male subjects, <40 years old, divided in *Blastocystis*-ST3-carriers immunocompetent and immunocompromised ($N = 18$) and *Blastocystis*-free subjects ($N = 9$, control group). A higher bacterial diversity in faecal microbiota was evidenced in the *Blastocystis*-ST3 carriers. Indeed, the Chao1 richness index and the observed OTUs in colonised patients resulted significantly higher than in *Blastocystis*-free subjects ($p = 0.040$ and $p = 0.038$, respectively) (Table 1 and Fig. 1). The Principal Component Analysis (PCA) plot obtained from the beta-diversity calculation in QIIME demonstrated a relative clustering of samples, whereby the scores for Factor1 and Factor2 account for 22.33% of the variance in the data. This difference between the bacterial communities resulted significant, as determined using the ANOSIM nonparametric statistical test analysis of similarity, where $R = 0.18$ ($p = 0.025$) (Fig. 2A).

The composition of gut microbial of *Blastocystis*-ST3-carriers and *Blastocystis*-free subjects was further examined evidencing a relative clustering of the group of subjects (*Blastocystis*-ST3-carriers and *Blastocystis*-free) linked to the bacterial communities (Fig. 2B).

In detail, at the phylum level, Firmicutes, Bacteroidetes and Proteobacteria were the most predominant phyla in all patients' faecal samples with different prevalence values in colonised and not-colonised patients with *Blastocystis* (53.1% vs 38.2%; 28.3% vs 45.4%; 8.1% vs 14.3%, respectively). The differences in the relative abundances of OTUs were further analysed using the Mann-Whitney test at different taxa levels. When comparing *Blastocystis*-ST3-carriers with free ones, at the class level, a higher abundance of Bacteroidia was found in *Blastocystis*-free subjects in comparison with the colonised ones ($p = 0.013$); while, at the family level, the presence of *Blastocystis* is significantly associated with an increased relative abundance of Prevotellaceae, Methanobacteriaceae, Clostridiaceae, Lachnospiraceae, Erysipelotrichaceae and Pasteurellaceae ($p = 0.044$; $p = 0.022$; $p = 0.005$; $p = 0.04$; $p = 0.04$; $p < 0.001$; $p = 0.03$, respectively), and low level of Bacteroidaceae and Veillonellaceae ($p < 0.001$; $p = 0.04$, respectively) (Fig. 3).

At the genus level, *Prevotella*, *Methanobrevibacter*, *Ruminococcus* were significantly more abundant in *Blastocystis*-carriers ($p = 0.04$; $p = 0.02$; $p = 0.01$, respectively), while *Bacteroides* showed higher abundance in those *Blastocystis*-free subjects ($p < 0.001$) (Fig. 4). As expected, all *Blastocystis*-ST3-carriers, both immunocompetent and immunocompromised subjects, showed similar faecal microbiota composition, despite a significantly higher relative abundance of Bifidobacteriaceae ($p = 0.03$) was detected in immunocompetent individuals compared to those immunocompromised patients, here analysed.

4. Discussion

This study aimed to describe the occurrence of *Blastocystis* in patients, mainly Italians, admitted at the Academic Hospital "Policlinico Umberto I", Rome, Italy and submitted to microscopical analysis of faecal samples. The protist was detected in about 8% of the subjects and, as expected, resulted the organism more frequently diagnosed in faecal samples. Despite the microscopy is the classical procedure for diagnosing parasitic infections and is still considered the primary and cheapest test offered by most routine diagnostic services, it exhibits well-known limits regarding the skills and accuracy of the microscopist and the sensitivity (85.9% vs PCR = 100%) (Formenti et al., 2017). In this study the stool microscopy was performed on one to three sample/patient and a double blinded analysis of each smear was done to assure the diagnostic reliability. Despite a higher prevalence should be expected using molecular diagnostic tools, the epidemiological scenario described in this study resulted in line with that from a previously survey (prevalence = 7.1%) on Italian population (Masucci et al., 2011). Molecular methods, herein applied to 142 out of 192 (74%) microscopy-positive samples, confirmed the copro-parasitological analysis and allowed the subtype identification. In this study, in addition to the four most common STs identified in humans such as ST1, ST2, ST3, ST4, already identified in our previous survey (Mattiucci et al., 2016), the ST7 was also detected. This subtype, as the ST5, ST6, ST8 is rarely identified in humans and therefore, it has been suggested that these rare subtypes in humans are of zoonotic origin (Stensvold and Clark, 2016).

The occurrence of such STs in Italy was expected, as ST3 was already identified in symptomatic patients (Mattiucci et al., 2016; Meloni et al., 2011) as well as the ST7 has been preliminarily identified in farmed and edible animals (Gabrielli et al., 2018), highlighting the risk of transmission of *Blastocystis*, through animal handling.

Furthermore, in the recent years, several studies in order to address the issue of *Blastocystis* pathogenicity, suggested that it could be related to genetic differences on the subtype- or strain-level (Stensvold et al., 2009; Wu et al., 2014), correlating the

Table 1

Comparison of the bacterial richness and diversity in the group of ST3-carriers both immunocompromised and immunocompetent ones and *Blastocystis*-free subjects. Statistical analysis was performed using the Kruskal-Wallis test. Significant results were marked with*.

Group of patients (N)	Richness estimator			
	Observed OTUs	p-value K-W	Chao 1	p-value K-W
<i>Blastocystis</i> -free (9)	340.37	0.038*	365.95	0.040*
<i>Blastocystis</i> -ST3 carriers immunocompromised (9)	402.54		432.33	
<i>Blastocystis</i> -ST3 carriers immunocompetent (9)	454.38		479.82	

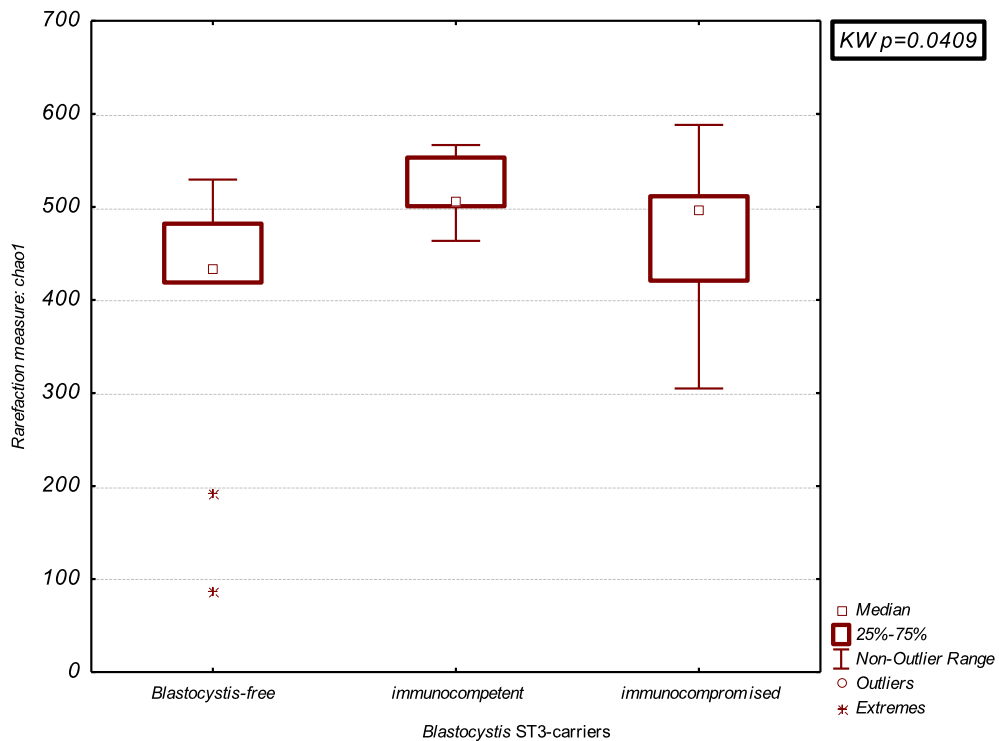


Fig. 1. Boxplot of Chao1 diversity index between *Blastocystis*-free and *Blastocystis*-ST3-carriers divided in immunocompromised and immunocompetent subjects. Statistical analysis was performed using the Kruskal-Wallis (K-W) test.

ST-1, 4, and ST-7 with pathological alterations in humans, while ST-2 and ST-3 has been identified as non-pathogenic (Stensvold et al., 2009; Domínguez-Márquez et al., 2009; Eroglu et al., 2009). Also, the presence of both pathogenic and apathogenic strains within one subtype has been reported (Hussein et al., 2008). A recent multi-locus sequence typing analysis of *Blastocystis* ST-3 and ST-4 has provided valuable insight into genetic variation within and between the two subtypes, evidencing high or low level of genetic diversity for ST-3 and ST4, respectively (Stensvold et al., 2012). Similar results have been obtained in our previous survey where 3 haplotypes (H1, H3, H7) have been identified in ST3 isolates, while a single haplotype (H2) was observed in ST4 symptomatic patients (Mattiucci et al., 2016). Thus, we suggested that the intra-subtype diversity showed by ST3 and ST4 could be linked to the evolutionary history of *Blastocystis* subtypes and ST3 may have co-evolved with human hosts over a longer period than ST4, which may have extended its range to humans more recently. Furthermore, it has been demonstrated that *Blastocystis* ST7 caused a decrease in beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* (Yason et al., 2019).

In this study, focused on the ST3, *Blastocystis* was associated to an eubiotic state characterized by a preponderance of potentially beneficial species, belonging to the phyla Firmicutes and Bacteroidetes, such as those of the genera *Ruminococcus* and *Prevotella*, respectively (Iebba et al., 2016b). Furthermore, our results are in line with that reported in other studies (Andersen et al., 2015; Nieves-Ramírez et al., 2018), which showed the protist significantly more abundant in subjects with *Ruminococcus* or *Prevotella*-driven enterotype and less common in individuals with *Bacteroides*-driven enterotype (Fig. 4). They also evidenced that *Blastocystis* colonisation was linked with higher bacterial richness and lower BMI (Andersen et al., 2015).

Our study confirms and supports this hypothesis evidencing a higher bacterial diversity in *Blastocystis*-ST3 carriers compared to that identified in *Blastocystis*-free individuals (Fig. 1). Moreover, at the family level, Prevotellaceae, Methanobacteriaceae, Clostridiaceae were also more abundant, whereas Bacteroidaceae were enriched in *Blastocystis*-free subjects (Figs. 2–3). As expected, several taxa detected in *Blastocystis*-carriers included bacteria strictly anaerobes and known to produce butyrate, which is considered one of the most important metabolite for maintaining health of colon tract in humans, as it serves as the major energy source of colonocytes, possesses anti-inflammatory properties, and it regulates gene expression, differentiation and apoptosis in host cells. Butyrate is also required to activate oxidative metabolism and, therefore, to provide low level of oxygen in the gut lumen. Such eubiotic environment supports continued colonisation by *Blastocystis*, as was recently elucidated (Stensvold and van der Giezen, 2018).

Similarly, to that reported for *Blastocystis* and confirmed also in our survey, several studies evidenced an increase of bacterial diversity in patients infected by helminths as hookworms and a shift in the composition of the human gut microbiota towards a 'healthy' phenotype, as well as increased levels of metabolites with anti-inflammatory properties. According to the Hygiene or Old Friends hypothesis' (Rook, 2010), certain STs of *Blastocystis*, such as ST3, would be considered as 'old friends' (Mattiucci et al., 2016), as in the case hookworms. They may be required for maintaining the intestinal homeostasis and/or play a role as a marker of intestinal homeostasis (Stensvold and van der Giezen, 2018).

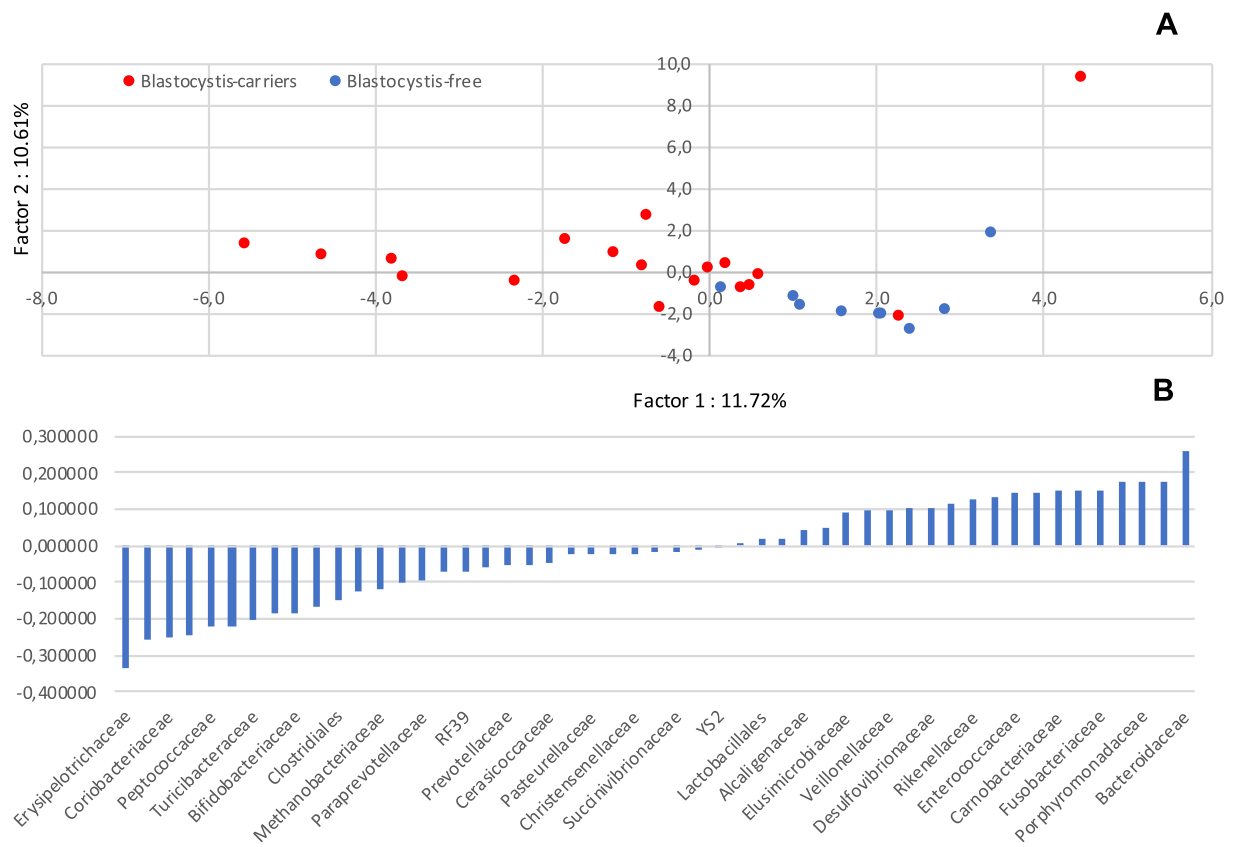


Fig. 2. Analyses of the variance in bacterial microbiota composition at the family taxonomic rank observed in *Blastocystis*-ST3 carriers and *Blastocystis*-free subjects. A) PCA plot of microbial communities in *Blastocystis*-ST3 carriers (red dots) and *Blastocystis*-free subjects (blue dots); B) eigenvectors of correlation matrix of microbial communities in the 2 groups of subjects. RF39: unclassified Enterococcaceae; YS2: Cyanobacteria.

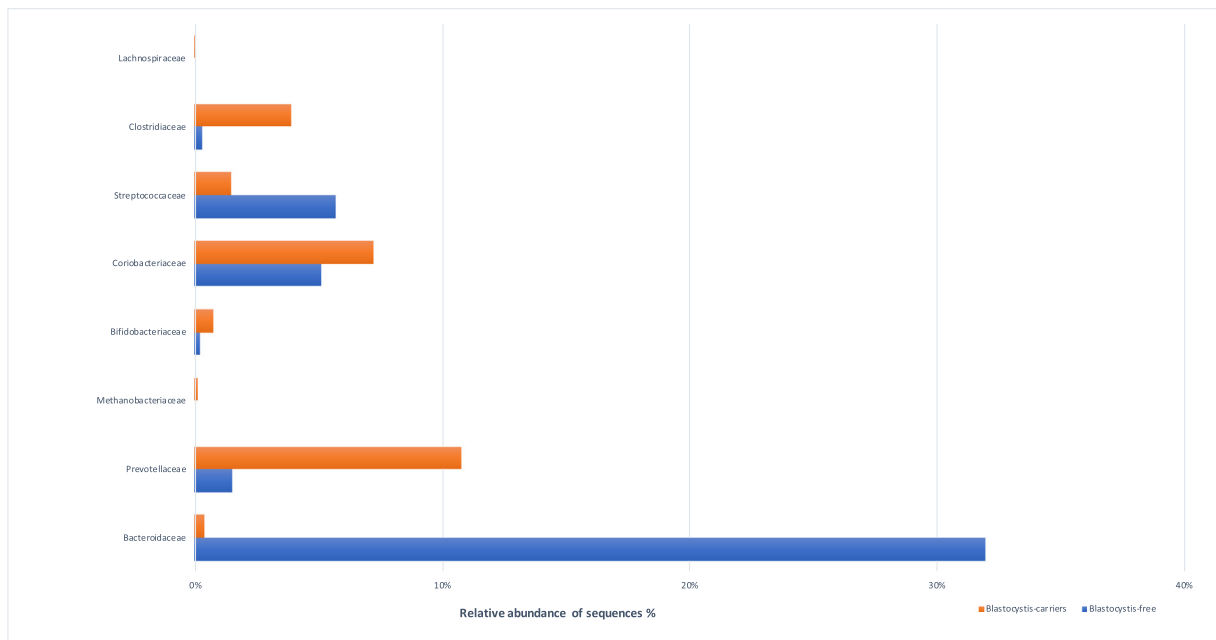


Fig. 3. Comparisons of the median values of the relative abundance at the level of bacterial family (level 5) for Bacteroidaceae, Prevotellaceae, Methanobacteriaceae, Clostridiaceae, Lachnospiraceae, Veillonellaceae, Erysipelotrichaceae, Pasteurellaceae in *Blastocystis*-carriers and *Blastocystis*-free subjects (orange and blue bars, respectively). The Mann-Whitney test was used to evaluate the 2 groups.

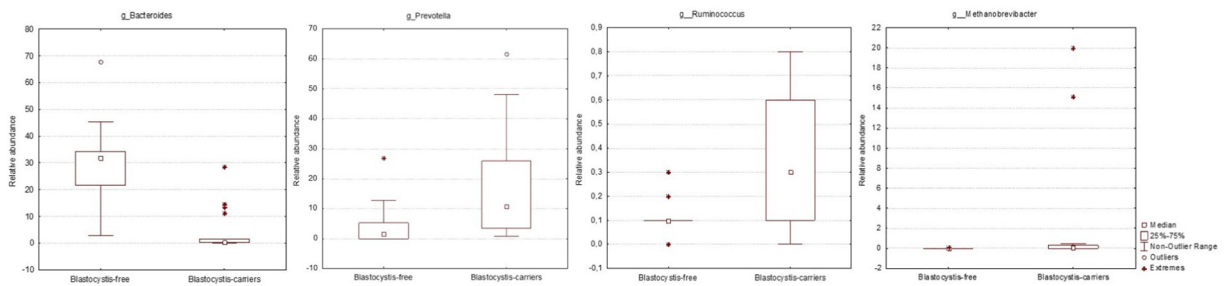


Fig. 4. Relative abundances of bacterial genera that significantly differ between *Blastocystis*-carriers and *Blastocystis*-free subjects. The Mann-Whitney test was used to evaluate the 2 groups.

Although *Blastocystis* was never considered as an opportunistic organism, it has also been frequently found in immunocompromised individuals presenting diarrhoea, with prevalence value ranging from 15% to 72.4% (Tan et al., 2009). In this study, *Blastocystis* was more frequently identified in faecal samples from HIV-positive patients (22%) than in immunocompetent subjects (7%) reporting values in line with previously surveys (Piranshahi et al., 2018; Tan et al., 2009). However, no significant differences in faecal microbiota composition was found between HIV-positive and immunocompetent subjects, despite immunocompromised individuals showed low level of bacterial richness (Fig. 1). Several Authors have evaluated the intestinal microbiome in HIV-positive subjects, with somewhat inconsistent or controversial results (Williams et al., 2016). This may be because of small sample sizes, lack of appropriate controls, and/or regional differences in dietary and environmental factors. Regardless, many authors evidenced a change in the ratio *Bacteroides:Prevotella*; this finding has been suggested to be linked to the antiretroviral therapy (Vesterbacka et al., 2017; Ling et al., 2016), and/or to the sexual practice and lifestyle (Noguera-Julian et al., 2016).

Faecal samples from our HIV-positive patients exhibited the so-called *Prevotella* enterotype (or enterotype II), which characterizes the HIV-positive treated patients (Lozupone et al., 2013; Vujkovic-Cvijin et al., 2013) and, as above described, favours the *Blastocystis* colonisation. Taking these observations into account, it appears that the high prevalence of *Blastocystis* observed in these patients is a consequence of the microbiota composition which highly supports the colonisation by the protist.

In conclusion, despite large-scale surveys using molecular approaches would be needed to assess the *Blastocystis* epidemiology in Italy, the present study characterized *Blastocystis*-subtypes from several faecal samples collected from subjects with different immunological status, evidencing a high circulation of the protist in immunocompromised individuals. Zoonotic STs as ST7 were identified in humans highlighting the risk of *Blastocystis* transmission from animals. Despite the study of the microbiota composition was performed on few subjects mainly Italians colonised by the ST3, the results so far obtained confirm the association between the presence of the protist, and a greater bacterial diversity with the high occurrence of *Prevotella* and *Ruminococcus*-enterotype and with a eubiotic gut. Surely, more investigations on the relationship between the intra-subtype variability or haplotypes and the gut microbiota are needed to confirm this positive association.

As the subtype identification and intra-subtype variability seems to be essential for assessing the relationship between *Blastocystis*, microbiota profile and human disease, further studies on the gut microbiota in patients colonised by different STs at the inter- and intra-subtype level should be enhanced, as well as affected by other immunological disorders in order to add knowledge on the potential role played by this protist in the human gut microbiota composition.

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Declaration of competing interest

The authors declare that they have no competing interests.

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