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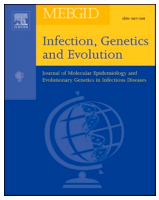

Metataxomics reveals *Blastocystis* subtypes mixed infections in Colombian children

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ABSTRACT

Blastocystis sp., is an intestinal protist with a broad host range and a high prevalence in human populations worldwide, even in developed Western countries. The publication of conflicting evidence has divided the scientific community about the pathogenic role of this parasite. Even though, genetic studies on *Blastocystis* sp. revealed associations between genotypes and different pathogenic profiles. Conventionally, the detection of this parasite is based on microscopic or PCR methods, which offer meager or null performance in detecting mixed infections. In this work, we applied a metataxonomic NGS approach targeting the V4 region of the eukaryotic *SSU-rRNA* gene and classical phylogenetic methods. This approach allowed us to detect *Blastocystis* sp. in stool samples from infected children living in an urban setting in the city of Medellin attending the same daycare center. Phylogenetic analysis identified the subtypes present in the children as ST1, ST2, and ST3. Besides, mixed infections of subtypes ST1 + ST3 were spotted in 16% of the analyzed stool samples.

1. Introduction

Blastocystis sp. is an anaerobic intestinal protist with a broad host range, including humans (Skotarczak, 2018). It is one of the most prevalent intestinal microbial eukaryotes worldwide, with more than a billion people infected, with rates ranging from 0.5 to 100% (Popruk et al., 2021). *Blastocystis* sp., has been shown to be transmitted through the fecal–oral route as well as through contaminated water and soil (Jinatham et al., 2022; Ma et al., 2022). High prevalence data are reported mainly in developing countries, which is associated with deficient hygiene, poor sanitation, and zoonotic transmission (Nemati et al., 2021; Popruk et al., 2021). *Blastocystis*'s clinical significance remains unclear, mainly because it is common in both healthy people and patients suffering from gastrointestinal and systemic symptoms (Andersen and Stensvold, 2016; Coyle et al., 2012; Leder et al., 2005). In addition to intestinal symptoms, *Blastocystis* sp., has been associated with chronic inflammatory diseases, such as irritable bowel syndrome – IBS (Abedi et al., 2022), autoimmune processes such as reactive arthritis, and allergic skin disorders such as urticaria (Alamlih et al., 2020; Aykur et al., 2022; Bahrami et al., 2019). Furthermore, *Blastocystis* sp., has been observed in ulcers in the large intestine of one immunocompetent adult (Janarthanan et al., 2011).

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A growing interest in this protist has prompted studies on its genetic diversity and zoonotic potential. Currently, *Blastocystis* sp. is genotyped into subtypes (STs) with 38 described so far (Maloney et al., 2022). ST1–ST8, ST10, ST12, ST14, and ST16 are described as associated with zoonotic transmission; ST9 has so far been reported with the anthroponotic transmission, and the remaining STs are transmitted only between animals (Popruk et al., 2021; Skotarczak, 2018). Subtypes ST1, ST2, and ST3 are the most frequent STs in humans worldwide, although ST4 seems to be the most common in European human populations (Alfellani et al., 2013). There are several studies that suggest an association between the pathogenic potential of *Blastocystis* sp., and the intra and inter STs variability (Ertug et al., 2016; Mirza and Tan, 2009; Osman et al., 2013; Rezaei Riabi et al., 2018). Reports of *Blastocystis* sp. mixed subtype infections.

in human samples describe a prevalence between 1.1 and 22% (Maloney et al., 2019; Scanlan et al., 2015). Therefore, the subtyping of *Blastocystis* sp. is important because it helps to recognize transmission routes and potential sources of a specific subtype and can help to broaden knowledge about its pathogenicity. Regarding the relationship between the parasite subtypes and pathogenicity in humans, part of the scientific community describes subtypes ST1, ST3, and ST4 as the most pathogenic, while ST2 is associated with mild symptoms (Kosik-Bogacka et al., 2021). In a recent publication, Deng et al. associated *Blastocystis* subtype 7 in diarrheal patients with changes in the bacterial community structure and reduced bacterial diversity (Deng et al., 2022). Moreover, *Blastocystis* sp. intestinal colonization has been associated with augmented bacterial diversity in Latin American (Nieves-Ramírez et al., 2018) and western European subjects (Forsell et al., 2017; Tito et al., 2019). In Egypt, El-Badry et al. found that *Blastocystis* sp., was the predominant parasite present in IBS patients, being the subtype ST3 the most common (El-Badry et al., 2018). In the same country, Ali et al. found subtypes ST1, ST2, and ST3 in both control and colorectal cancer patients, while subtype ST7 was only found in the cancer patients (Ali et al., 2022).

Blastocystis genotype subtyping is regularly based on PCR and direct amplicon sequencing of partial fragments of the *SSU-rRNA* gene (Parkar et al., 2010; Santín et al., 2011). With this methodology, the capillary sequencing chromatograms will show only the nucleotide signal of the predominant subtype, in the case where different STs are present in one individual. On the other hand, if two equally abundant different STs were extant in the same sample, mixed-peak chromatograms would be present, limiting the interpretation of results. For this reason, PCR and direct amplicon sequencing are not suitable procedures to detect mixed subtype *Blastocystis* sp. infections. In brief, using only conventional PCR to detect *Blastocystis* sp. might underestimate the effect of mixed inflections and can mislead clinical and pathogenic inferences due to the possible synergistic effects that different *Blastocystis* sp., subtypes can display. Additionally, it can limit the definition of adequate prevention and treatment strategies and hinders the concealment of biological events related to transmission dynamics (Maloney et al., 2019; Skotarczak, 2018).

The use of next-generation sequencing (NGS) and metagenomic/metataxonomic strategies in the study of *Blastocystis* sp. has grown in recent years. These methodologies should be preferred for a variety of reasons, but mainly because of their ability to detect *Blastocystis* sp. subtypes and mixed infections, achieving a more detailed study of the genetic diversity of the parasite (Stensvold, 2013). Recent studies that have analyzed the genetic diversity of *Blastocystis* sp. through a metataxonomic approach and the *SSU-rRNA* gene as the target marker were able to detect *Blastocystis* sp. subtypes in different samples, including wastewater, surface water, and feces (Calero-Bernal et al., 2020; Maloney et al., 2019; Stensvold et al., 2020; Zahedi et al., 2019).

In Colombia, data from the National Survey of Intestinal Parasitism reveal *Blastocystis* sp. as the most prevalent intestinal protist in children, with 52.1% of positive individuals (Ministerio de Salud y Protección Social. Universidad de Antioquia., 2015). Additionally, studies conducted between 2009 and 2021, report a prevalence of *Blastocystis* sp. between 12.6 and 87.1% (Osorio-Pulgarin et al., 2021; Ramírez et al., 2017; Sánchez et al., 2017; Villamizar et al., 2019). Most of these studies were based on microscopic detection, although several used molecular evaluation techniques that allowed *Blastocystis* sp. subtyping. Few of them used methodological strategies that allowed the evaluation of mixed infections in humans (Higuera et al., 2020; Potes-Morales et al., 2020; Ramírez et al., 2017, 2014; Sánchez et al., 2017; Villamizar et al., 2019).

This work aims to identify the *Blastocystis* sp. subtypes circulating in children from Medellin (Antioquia, Colombia) and to assess the presence of subtypes of mixed infections. To do so, we applied a combined metataxonomic and phylogenetic strategy. Our results provide genotype information that is useful to better understand *Blastocystis* sp. biology. Besides, the NGS-based approach targeting the V4 region of the *SSU-rRNA* gene seems to be a valuable resource for intestinal parasite detection, and it has the potential to assess the genetic diversity of eukaryotes such as *Blastocystis* sp. in clinical samples.

2. Methods

2.1. Population of study and DNA collection

Twenty-five stool samples from children <5 years from daycare centers in Medellin (Colombia). Stool samples were collected in a previous study, and for the present analysis, we used the stored (frozen) DNA samples. DNA extraction processing was performed as described elsewhere by the authors of this manuscript (Alzate et al., 2020). Briefly, DNA extraction was carried out using the STOOL DNA purification kit NORGEN (Canada) following the manufacturer's standard protocol. Genomic DNA concentration was estimated using the Picogreen fluorescent method. The children lived in the city of Medellin, the second largest city in Colombia, and all had access to potable treated tap water. All children were positive for *Blastocystis* sp. using the microscopic smear test. Most of them also carried protozoa, mainly *Entamoeba* spp., or *Giardia* sp. Eight of the 25 children had diarrhea at the moment of the stool sample collection. We performed an additional control on the DNA samples amplifying the bacterial *16S rRNA* gene with the goal to rule out the presence of PCR inhibitors. This information, including purified DNA concentrations, is summarized in Supplementary Table 1. An example of the microscopic images of the observed *Blastocystis* sp. parasites is presented in Supplementary Fig. 1.

2.2. Wastewater environmental DNA collection and processing

Influent wastewater was collected at the wastewater treatment plant – WWTP San Fernando in Medellín, Antioquia, Colombia. In one visit to the WWTP, 2 L of wastewater were collected in sterile bottles and immediately transported to the lab. Wastewater was allowed to settle for 2 h in the lab at room temperature. Afterward, the sediment was collected with a 10 mL pipette collecting and transferred to 50 mL polypropylene falcon tubes. In total 100 mL of the wastewater and sediment were collected at this step. Next, the two 50 mL tubes were centrifuged at 6000 RCF. Upon the form pellet total, DNA was extracted using the QIAGEN POWERSOIL DNA extraction kit following the company's standard protocol. DNA was quantified and stored at −20 °C until its amplification.

2.3. Ethics statement

The ethical clearance of this study followed the ethics declaration of Helsinki and the directive of the Colombian Ministry of Health No. 008430 of 1993. The study was approved by the Ethics Committee from the Nursing Faculty of the University of Antioquia, under the official document No. CEI-FE 2021-01. Parents or legal guardians of all the enrolled individuals signed informed consent.

2.4. 18S-V4 rRNA gene metataxonomic analysis

For the metataxonomic experiment, we used primers that targeted the hypervariable region 4 (V4) of the eukaryotic *SSU-rRNA* gene (18S-V4). Forward: 18S-V4F: CCAGCAGCCGCGGTAATTCC (Choi and Park, 2020) and Reverse: 18S-V4R: RCYTTCGYYCTTGATTRA (designed in this work). Amplicon libraries were sequenced using Macrogen (Seoul, Korea) in a MiSeq (Illumina) platform, reading paired-end reads of 300 bases. The sequences obtained were analyzed using the MOTHUR software v1.44. The taxonomic assignment of the constructed molecular OTUs (mOTUs) (97%) was carried out using the classifyseqs command of mothur with the SILVA v138 ribosomal DNA database. Stack bar plots were generated with the R Phyloseq library using normalized read counts (median value normalization).

2.5. Phylogenetic analysis and parasite relative abundance analysis

mOTUs (molecular OTUs) assigned to *Blastocystis* sp. by the mothur *classifyseqs* algorithm genus were extracted and analyzed using traditional phylogenetic methods. Reference sequences of *Blastocystis* sp. subtypes were downloaded from the NCBI GenBank database and then aligned by the MAFFT software with the selected *Blastocystis* sp. mOTUs. *Blastocystis* sp. *SSU-rRNA* gene references accessions: AB070989.1, GU256922.1, EU445487.1, AB107964.1, EU427515.1, KC148209.1, KC148205.1, AY244621.1, U51152.1, AB107970.1, KC148207.1, AB107963.1, EU427512.1, EU445485.1, KT438703.1, KC148210.1, and KC148208.1. Next, a maximum-likelihood phylogenetic tree was constructed using IQTREE2 software (Minh et al., 2020). We performed 5000 ultrafast bootstrap tests (-B 1000) (Hoang et al., 2018), and the best model was selected according to BIC (Schwarz, 1978) with the flag -m MFP. The best-fit model selected for the phylogeny according to BIC was TPM3 + F + R4. The Log-likelihood of the consensus tree was −11,442.909593. The phylogeny was graphically edited using FigTree program v1.4.

For relative abundance analysis of the observed parasites or the *Blastocystis* sp. subtypes, a staked bar plot figure was generated in R with the GGPLOT2 library. The observed read count for each parasite taxon within each stool sample was used to estimate its relative abundance. In the case of *Blastocystis* sp. subtypes, we followed a similar strategy. We initially defined the ST of each *Blastocystis* sp. mOTUs and then counted the number of reads assigned to each subtype and then plotted it with the R program.

2.6. Data analysis and graph preparation

Data processing and graphical analysis were performed in R environment (R version 4.0.4 64, x86_64-apple-darwin17.0 (64-bit)) and R studio (Version 1.2.1335) using packages PHYLOSEQ, VEGAN and GGPLOT2.

3. Results

3.1. Metataxomic view of the eukaryotic intestinal DNA of the parasitized children

Twenty-five stool samples from parasitized children were included in the present eukaryotic metataxonomic analysis. All children were found to be positive for *Blastocystis* infection using classical parasitological microscopic stool analysis. In several children, other protozoa were also observed within the stool samples, like *Entamoeba* spp., *Giardia* sp., *Endolimax* sp., and *Chilomastix* sp. (Supplementary Table 1). The Metataxonomic experiment yielded between 39 k and 110 k raw reads per sample. After QC filtering, the read count for all samples ranged between 11,451 and 83,420. The estimated coverage for the detection of the eukaryotes present in the stool sample was above 99% for all samples. The number of detected eukaryotic mOTUs ranged from 113 to 435. The metataxonomic experiment showed the presence of DNA in the stool samples of a broad spectrum of eukaryotes, from unicellular species to metazoans and plants (Supplementary Table 2). The most relevant higher rank Taxa The most relevant higher-ranking taxa were Vertebrata, Ascomycota, Basidiomycota, Arthropoda, Phragmoplastophyta, Fornicata, Nematozoa, and Ciliophora. One of the most abundant categories observed was Incertae Sedis. Interestingly, most of the sequences assigned to this category correspond to *Blastocystis* sp. (99.999%) (Supplementary Table 2) (Fig. 1, Panel A). Going deeper into the genus category, the topmost frequent eukaryotes observed were *Blastocystis*, *Saccharomyces*, *Pichia*, and *Musa*. Other eukaryotic groups were also present, but the classifying algorithm failed to assign a genus for several of them as its classification remained at higher taxonomic ranks, such as Mammalia, Embryophyta, Clavispora-Candida clade, and Ascaridida (Fig. 1, panel B). These results indicate that with this methodology, we can detect not only the DNA of the eukaryotic intestinal microbiota but also the DNA of consumed food since we observed plant DNA such as *Musa* sp. (Banana), *Oryza* sp. (Rice), *Triticum* sp. (wheat), and *Zea* sp. (Corn) (Supplementary Table 2). We wanted to have an insight into the parasites detected with this strategy. With this aim, we filtered the taxonomy table generated by the *Mothur* program and found the presence of parasites of the genus: *Cryptosporidium*, *Giardia*, *Blastocystis*, *Dientamoeba*, and nematodes of the orders Ascaridida and Trichocephalida. Additionally, we also detect *Rhogostoma*, an amoeba that is commonly found in wastewater. It is noteworthy to mention that 40% of the children harbored at least another parasite, besides *Blastocystis*. Several of these parasites were overlooked with the classical stool microscopic analysis (Fig. 2).

3.2. Blastocystis detection based on a metataxonomic approach

The metataxonomic approach allowed us to quantify the *Blastocystis* 18S-V4 amplicon relative abundances within each stool sample. This relative abundance analysis indicated that this protist could be extremely abundant or hardly detectable, depending on the sample (Fig. 1, panel B).

The *SSU-rRNA* gene V4 hypervariable region has been shown to have the discriminatory potential to assess the genetic diversity of *Blastocystis* subtypes (Tito et al., 2019), but the *Mothur* automatic classification algorithm is programmed to classify up to the genus category for this parasite. To gain insights into the subtypes of *Blastocystis* present in the samples, we applied complementary phylogenetic exploration. We used reference sequences of the *SSU-rRNA* gene of the *Blastocystis* subtypes ST1 to ST17. The computed tree depicts a topology where ST15 and ST17 form a well-supported monophyletic clade (100% UFBoot), and the remaining subtypes are grouped into well-supported branches with also 100% UFBoot support. All the *Blastocystis* molecular OTUs (mOTUs) found on the children's stool samples, in total 38, were grouped with either ST1, ST2, or ST3 clades (Fig. 3). Most of the mOTUs were classified as ST1 (37%) and ST2 (53%). The ST3 subtype accounted for 10% of the mOTUs (Supplementary Table 3). These three clades, ST1, ST2, and ST3; showed robust support with UFBootstrap values of 100%, 100%, and 99%, respectively.

On the other hand, regarding *Blastocystis* subtypes infection per child, *Blastocystis* ST1 was the most frequently found in the children, reaching 68% of the cases (17/25), followed by ST3 in 32% of the samples (8/25). The subtype ST2 was found in 16% of children (4/25) (Fig. 4) (Supplementary Table 3). It must be mentioned that some children carried more than one *Blastocystis* subtype. This situation explains why the sum of the percentages is above 100%. This will be considered in detail below.

3.3. Blastocystis subtypes mixed infections

Blastocystis sp. mixed subtypes infections were assessed by identifying the *Blastocystis* mOTUs that belong to the different subtypes.

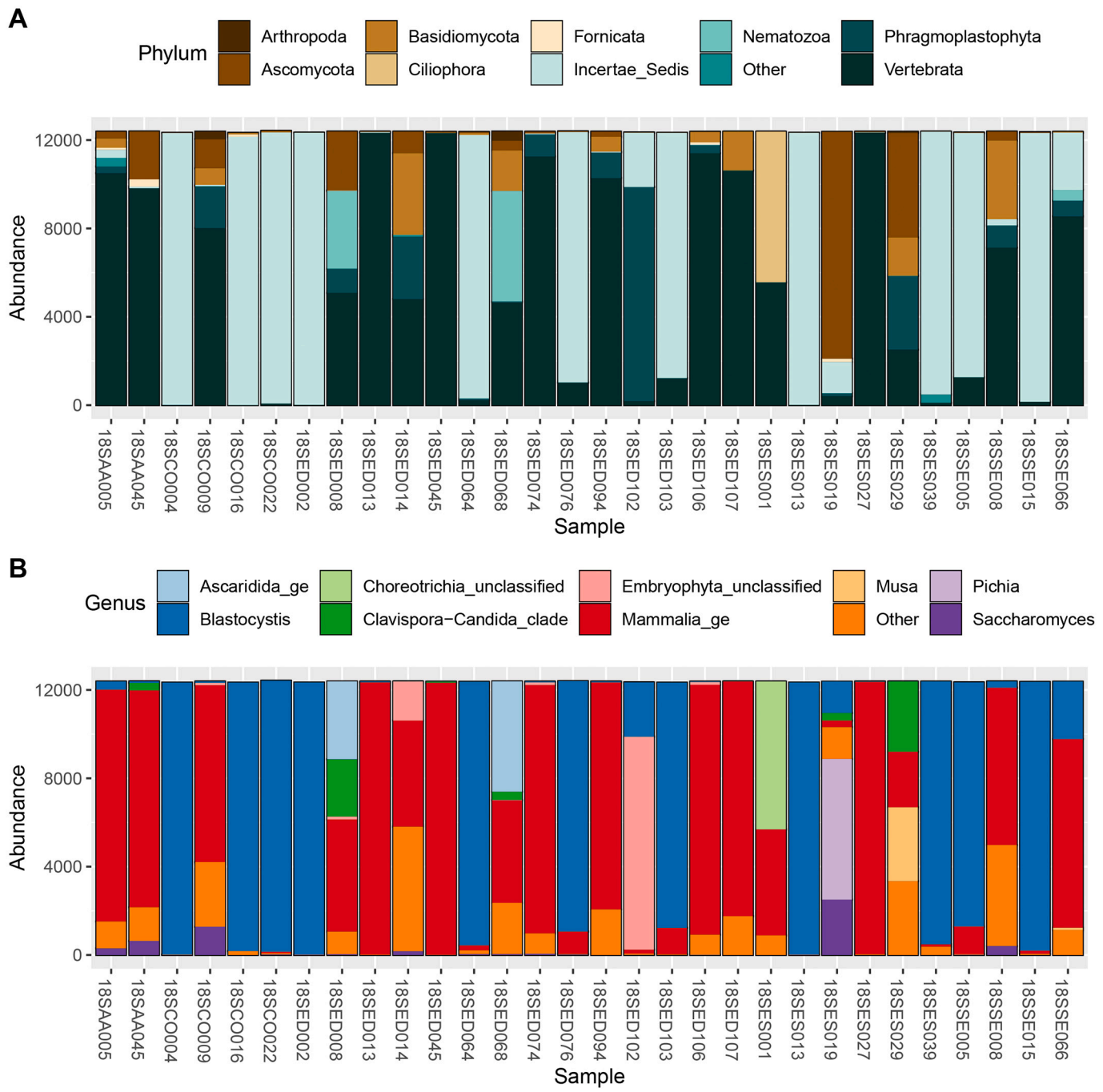

Fig. 1. Top 10 most abundant eukaryotes (DNA).
Stacked bar plot depicting the classification and relative abundances of the eukaryotic DNA detected in the 25 children's stool samples. Only the top 10 most abundant taxa are presented. Phylum category is presented on Panel A and genus on Panel B. Samples codes are presented on the X-axis.

Furthermore, the relative abundance of the different STs was quantified based on the number of sequences assigned to each *Blastocystis* mOTU. A stacked bar analysis of the different STs found in each child is presented in Fig. 4. Most of the infected children harbored one single ST (84%), whereas 4 (16%) showed mixed infections of ST1 and ST3 subtypes. The ST2-infected individuals showed no mixed subtype infections (Supplementary Table 3). For samples 18SES039 and 18SED076, the ratio of ST3 amplicons was so low that it was hardly visible in the graphic; it is easier to see the result in Supplementary Table 3.

3.4. *Blastocystis* detection in the municipal wastewater

Our metataxonomic approach was also able to detect *Blastocystis* sp. in the Medellin San Fernando WWTP. The phylogenetic analysis confirmed the *Mothur* pipeline results and allowed us to determine that the subtypes ST1 and ST2, present in the children's stool samples, are also present at the San Fernando WWTP. One additional *Blastocystis* STs were detected in the wastewater sample was not spotted on the stool samples, the ST14 (Fig. 3).

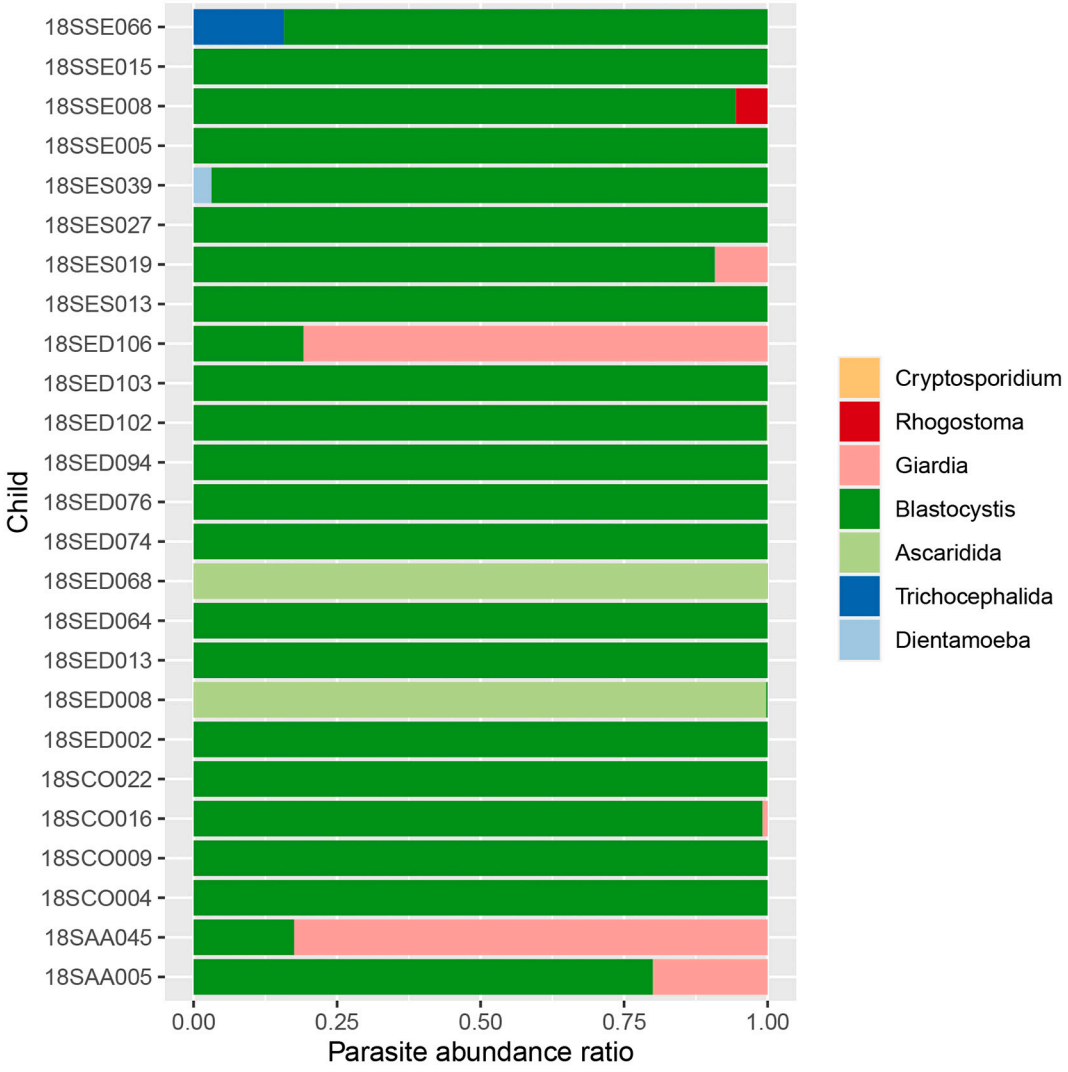

Fig. 2. Stacked bar plot analysis of the parasites detected with the metataxonomic approach.
Relative abundance of the different eukaryotic parasites detected using the metataxonomic approach (targeting the V4 hypervariable region of the *SSU-rRNA* gene). Parasite abundance is presented as a normalized number of reads. The stool sample code is presented on the Y-axis. The relative read abundance of each parasite is represented in the X-axis.

4. Discussion

The metataxonomic experiment proved successful in granting the detection of *Blastocystis* sp., genomic DNA in human stool samples. Nonetheless, the *Blastocystis* amplicon yield showed an extreme range of read counts. While in some individuals *Blastocystis* was the most abundant eukaryotic taxon, reaching up to tens of thousands of reads in one stool sample, in two subjects only one read was detected. Moreover, this experiment also exhibited a wide eukaryote taxonomic range detection since the DNA of protists was amplified as well as the DNA of metazoans and plants. This is an interesting finding since it shows that metataxonomics can be applied to characterize intestinal resident parasitic microbiota (eukaryotic parasites), but it is also possible to detect transient DNA that might come from the meals. This situation should be considered at the moment of result interpretation because the detection of amplified DNA by this means cannot be directly inferred as intestinal colonization and active replication of the eukaryote in question.

Our findings add evidence to the vast scientific literature that points to *Blastocystis* subtypes ST1, ST2, and ST3 as the most common subtypes present in human intestines (Osorio-Pulgarin et al., 2021; Popruk et al., 2021). In this study, we focused our analysis on little kids (up to 5 years old), and the observed trend coincides with what is observed in adults around the globe. Subtypes ST1 to ST4 have been described as the most common colonizing the intestines of human western populations (Forsell et al., 2017; Jiménez et al., 2019; Muñoz-Sánchez et al., 2021; Potes-Morales et al., 2020; Scanlan et al., 2015). By contrast, subtype ST4 was not detected within the studied children group, although it has been described by other authors in the country (Higuera et al., 2020; Osorio-Pulgarin et al., 2021; Sánchez et al., 2017; Villamizar et al., 2019). Several studies about *Blastocystis* genetic diversity in Colombia have also shown similar behavior related to the circulating subtypes in the human population worldwide (Higuera et al., 2020; Osorio-Pulgarin et al., 2021; Potes-Morales et al., 2020; Ramírez et al., 2017, 2014; Villamizar et al., 2019). Our study has a limited number of samples, only 25 children and only one sample of 2 L of wastewater were analyzed. This situation limits the possibility of extrapolate the conclusions of this study into a wider view of the situation of *Blastocystis* sp. in the country.

The metataxonomic approach proved valuable not only in the stool samples but in environmental samples too. The successful detection of different *Blastocystis* subtypes in the wastewater system opens the door to using this technology to study parasite dynamics in environmental settings. The same subtypes found in the infected children were detected in the wastewater samples. The subtype ST14, only detected in the wastewater, is rarely reported in humans ("The 3rd International

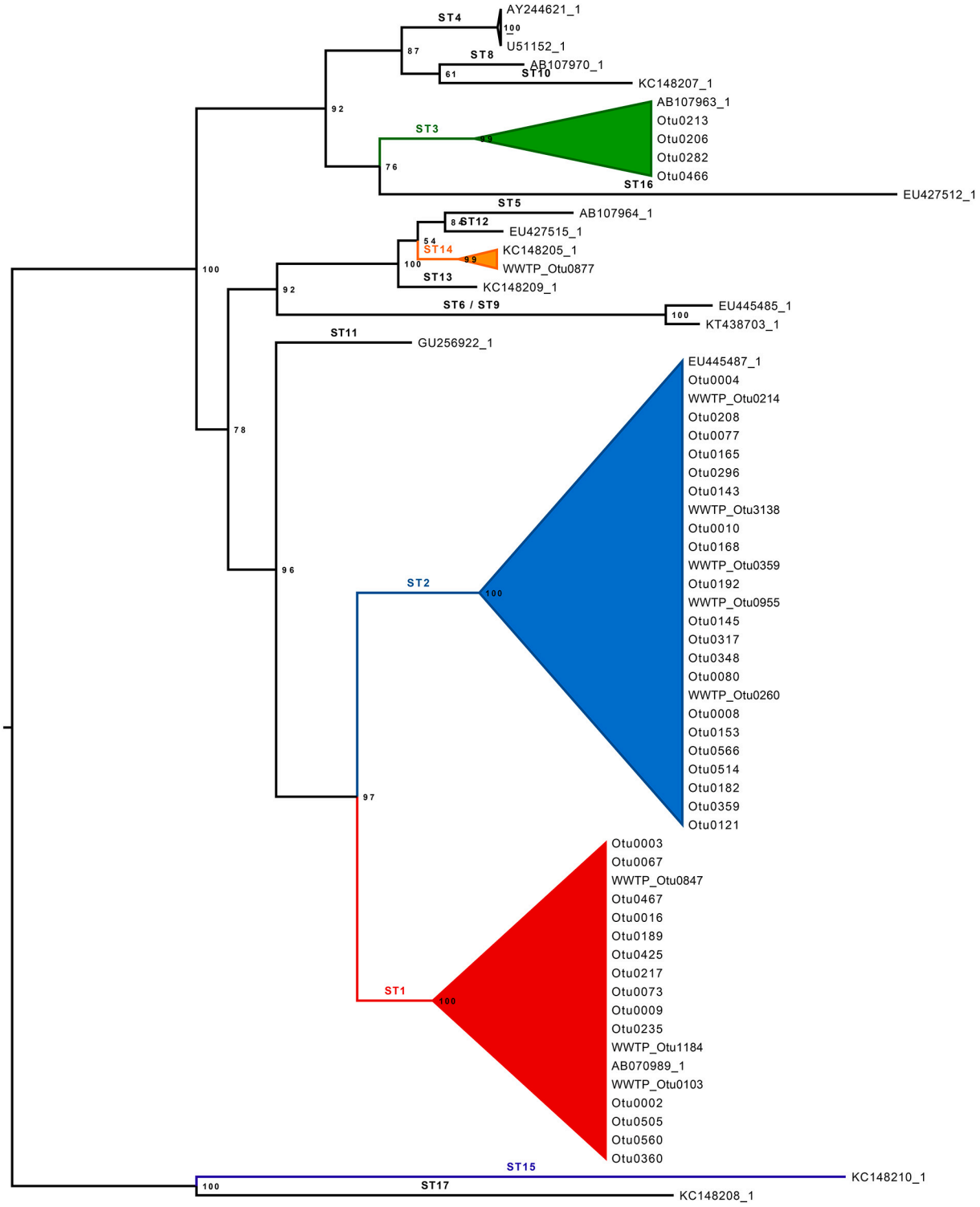

Fig. 3. Phylogenetic tree depicting the *Blastocystis* mOTUs subtype assignation.
Maximum-likelihood phylogenetic tree computed using the *Blastocystis SSU-rRNA* gene V4 hypervariable region with 1000 UFBootstrap pseudo-replicates. The *Blastocystis* mOTUs derived from children's stool samples are labeled starting with the letters "Otu", while the mOTUs derived from wastewater samples start with the acronym WWTP. Subtype lineages are labeled above their respective branch and support UFB values are indicated and colored at their respective branch nodes.

Blastocystis Conference: Coming Together Virtually," 2021) but is widely distributed in cattle (Hublin et al., 2021), pigs (Udonsom et al., 2018), and domestic animals in Colombia (Higuera et al., 2021). In the geographical region that serves this WWTP pig farming is common, which could explain this finding. Other studies based on NGS approaches and the *SSU-rRNA* gene have explored the microbial community present in wastewater samples, confirming its usefulness to fulfill this purpose. Moreno et al. (Moreno et al., 2018) were able to detect the presence of *Blastocystis, Giardia intestinalis*, *Acanthamoeba castellanii*, *Toxoplasma gondii*, *Entamoeba histolytica* at species and genotype levels in irrigation water samples. Zahedi et al. (Zahedi et al., 2019) using this strategy detected 49 eukaryotic Phyla in samples from

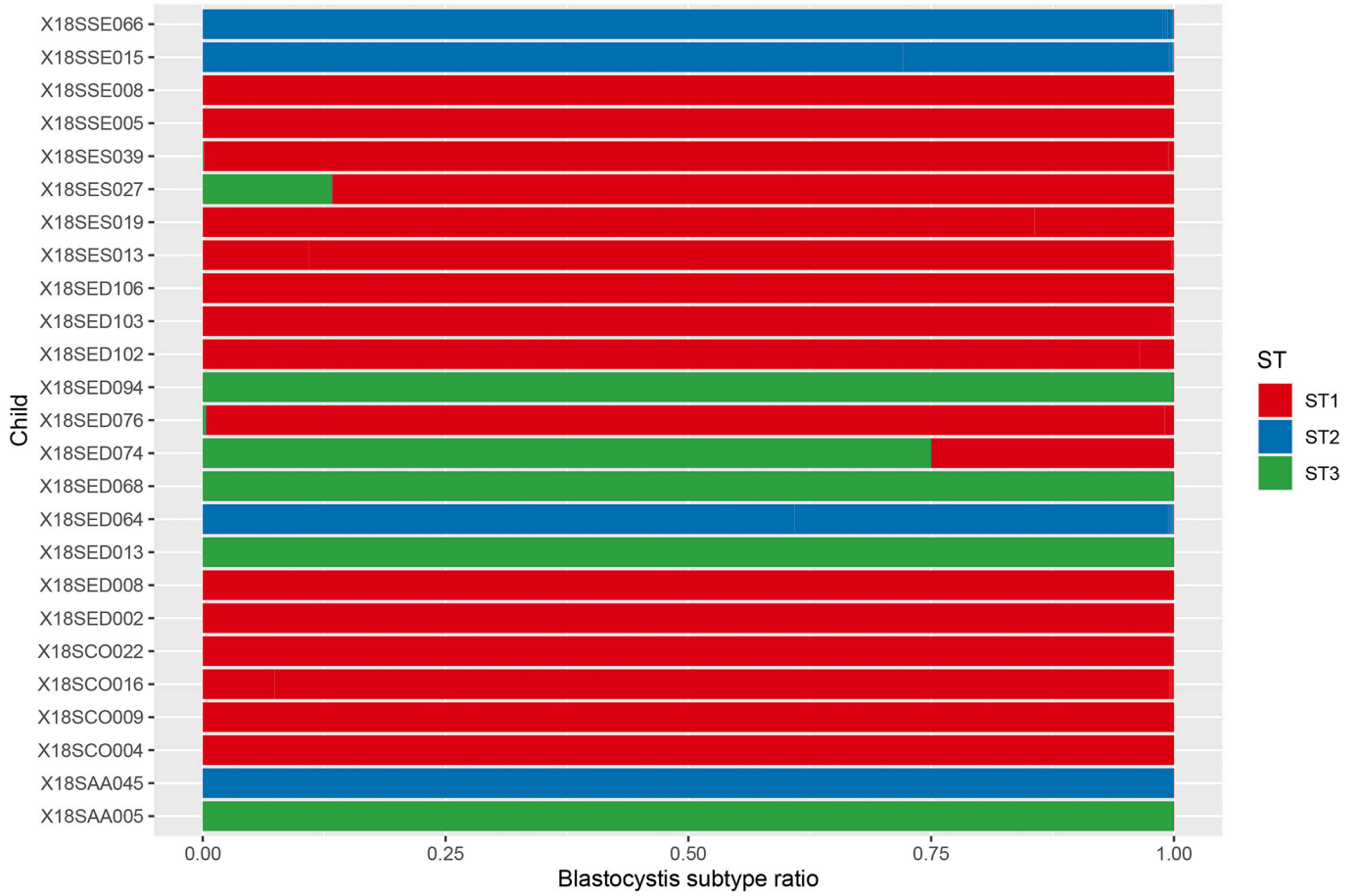

Fig. 4. Stacked bar plot analysis of the *Blastocystis* subtypes present within each sample.
The relative abundance of the different *Blastocystis* STs present in each stool sample was quantified based on the number of sequences assigned to each subtype and depicted in different colors. The code of the samples is presented on the Y-axis. For samples 18SES039 and 18SED076, the ratio of ST3 amplicons was so low that hardly visible in the graphic. For easier visualization, please refer to Supplementary Table 3.

WWTPs. Stensvold et al. (Stensvold et al., 2020) studied the genetic diversity of protists found in untreated wastewater, and were able to detect *Blastocystis* sp. (ST1–4 and ST8 subtypes were identified). Additionally, several archamoebid species were detected, including *Entamoeba histolytica*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba dispar*, *Entamoeba hartmanni*, *Endolimax nana*, and *Iodamoeba bütschlii*.

Blastocystis history has been cumbersome since its formal description back at the begging of the XX century (Zierdt, 1991). Initially, it was described as a primary pathogen, but by the end of the same century, the scientific community started to consider it not harmful (Coyle et al., 2012; Leder et al., 2005). However, in the last three decades, the pathogenic behavior of this Stramenopile has been placed in the spotlight again (Alzate et al., 2020; Skotarczak, 2018). Conflictive evidence has been published, nourishing the discussion groups on both sides. Our results might shed light, helping to understand the apparently chaotic pathogenic profile of *Blastocystis*. To start, the metataxonomic experiment showed that parasite abundance can be extremely variable among individuals, according to the amplicon relative abundance result. Remarkably, in some samples, based on the relative read counts, it seems to be one of the dominant intestinal eukaryotes. By contrast, in other individuals, it was hardly detectable. The relative abundance in a metataxonomic experiment should be interpreted with caution since it cannot be directly related to the absolute concentration of the parasite within the intestinal content. Complementary qPCR tests should be carried out to validate the parasite's relative abundance. Nevertheless, the overwhelming *Blastocystis* amplicon abundance differences observed here might indicate variations in the natural protist abundance in the infected individuals. Another interesting finding is the demonstration of mixed *Blastocystis* subtype infections, a phenomenon that has been described by other authors that have used classical cloning techniques (Betts et al., 2018) or NGS approaches in the study of human (Chihi et al., 2022) and animal stool samples (Abarca et al., 2021; Calero-Bernal et al., 2020; Higuera et al., 2021; Maloney et al., 2020, 2019; Russini et al., 2020), confirming that *Blastocystis* mixed-ST infections are much more common than previously thought. Maloney et al. found that NGS/metataxonomics is the best-suited technique that should be used to detect *Blastocystis* subtype mixed infections, outperforming the traditional PCR, plasmid library, or Sanger sequencing strategies (Maloney et al., 2019).

Our results demonstrated coinfection compatibility between ST1 and ST3 subtypes, which was also observed by Chihi et al. (Chihi et al., 2022), who evaluated human samples through NGS and found that coinfection with these two subtypes was also the most frequent. Higuera et al. also found coinfection compatibility of these two subtypes in Colombian pigs (Higuera et al., 2021). Results from investigations based on Sanger sequencing of the *SSU-rRNA* gene amplicon have also described this coinfection (Skotarczak, 2018). Future research based on NGS will help to elucidate the significance of mixed *Blastocystis* infections and the coinfection compatibility patterns.

One of the situations that hinder the understanding of the pathogenic behavior of *Blastocystis* is the broad spectrum of clinical manifestations reported on colonized individuals. So far, these variations are mainly attributed to the genetic diversity of the protist. However, our results support new variables that should be incorporated into the equation.

Blastocystis abundance variations and subtypes mixture can take place in some individuals. Additionally, although it was not addressed in this work, coinfection with other parasites, like intestinal protozoa, might play a role in the advent of clinical manifestations.

In conclusion, we found *Blastocystis* subtype mixed infections in the studied Colombian children. This might reflect a global phenomenon that should be assessed when studying this protist infection in human populations. To date, most of the works that describe *Blastocystis* subtyping are based on conventional PCR strategies and Sanger sequencing. This technology might render the results biased due to its limitation in detecting *Blastocystis* subtypes of mixed infections. *Blastocystis* sp. was detected in the regional WWTP-influent wastewater, and the subtyping analysis showed that the most frequent subtypes found in the children, ST1 and ST2, were also detected in these wastewaters.

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Author statement

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Declaration of Competing Interest

None.

Data availability

Raw read data generated for this work was deposited at the NCBI-SRA database under the bioproject accession PRJNA882890. The reviewers can privately access the reads with the following link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA882890?reviewer=d5eniogf8495amaonsel1p9mao.

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