Food and Waterborne Parasitology 33 (2023) e00210

Contents lists available at ScienceDirect

Food and Waterborne Parasitology

journal homepage: www.elsevier.com/locate/fawpar

Monitoring potentially pathogenic protists in sewage sludge using Metataxonomics

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ARTICLE INFO

Keywords:
Sewage-sludge
Protists
Entamoeba
Blastocysts
Metataxonomics
Wastewater

ABSTRACT

Intestinal parasites continue to pose a significant threat to human health worldwide, particularly among children. Contaminated water and soil serve as major transmission vehicles for these parasites and intestinal protists are among the most prevalent parasites in both developed and developing nations. Traditionally, parasites have been studied using human or animal fecal samples, while studying them in environmental samples has been challenging due to technical limitations. However, advancements in Next-Generation Sequencing (NGS) and bioinformatic approaches now enable the detection of parasite DNA in environmental samples. In this study, we applied a metataxonomic and phylogenetic strategy to detect and classify DNA of protists present in sewage sludge from two major cities in Colombia: Medellin and Cali. We successfully detected several human pathogenic parasites including *Giardia intestinalis*, *Entamoeba histolytica,* and *Blastocystis* sp., among other protists, in all sludge samples examined. We also investigated the entry and exit of parasite DNA from the San Fernando wastewater treatment plant (WWTP). We observed a higher number of parasite DNA sequences in the plant's influent wastewater, but we also detected the discharge of DNA from pathogenic parasites in both effluent waters and biosolids.

1. Introduction

Protists are a diverse taxonomic group widely found in terrestrial and aquatic environments, where they perform several important functions in biogeochemical cycles and controlling microbial community composition, primarily through predation (Caron et al., 2017; Schulz-Bohm et al., 2017; Singer et al., 2021; Xiong et al., 2021). Moreover, they frequently interact with other microorganisms, acting as 'Trojan Horses' of bacterial pathogens (Henriquez et al., 2021; Rayamajhee et al., 2021, 2022). Many protists have established parasitic relationships with humans, colonizing the intestine, and are usually transmitted through the fecal-oral route, as well as in contaminated food, water, or soil (Nichols, 2000; Nieves-Ramírez et al., 2018; Dixon, 2021). Parasitic protists can infect both human and animal hosts, leading to diseases that impose a significant burden on public health (Fletcher et al., 2012; Li et al., 2020). In Colombia, human protist infections are common, with a high prevalence of *Giardia intestinalis*, *Entamoeba*, and *Blastocystis* spp. (Ministerio de Salud y Protección Social and Universidad de Antioquia, 2015).

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https://doi.org/10.1016/j.fawpar.2023.e00210
Received 28 July 2023; Received in revised form 21 September 2023; Accepted 22 September 2023
Available online 23 September 2023

In recent years, pathogenic protists have been reported in sewage sludge, which is a semi-solid waste produced during the separation of solid material from the wastewater through primary and secondary (activated sludge) treatment of domestic and industrial wastewater (Amorós et al., 2016). The sewage sludge that receives biological or thermal treatment to reduce the load of pathogens is sometimes referred to as biosolids and has a potential application as a fertilizer in agriculture and forestalling practices (Lu et al., 2012). However, there are public health concerns due to the potential contamination of agricultural land and water with heavy metals, organic pollutants, and pathogens that come in human or animal feces (Laura et al., 2020; Yakamercan et al., 2021).

National and international regulations have been implemented to monitor pathogen indicators, including enteric bacteria, nematodes, and viruses, in order to assess the safety of reusing sewage sludge or biosolids (Kacprzak et al., 2017). However, it is also crucial to control pathogenic protists due to their high prevalence in certain communities worldwide and their persistence in the environment for several months (Amorós et al., 2016). Cysts and oocysts possess a multilayer wall that is highly resistant to chemical and physical disturbances (Jain et al., 2019). Several studies have identified elevated levels of protists, such as *Cryptosporidium* spp. and *Giardia* cysts, in sewage sludge using conventional methods that involve concentration techniques and immunomagnetic separation (IMS). Nevertheless, the performance of these classical parasitological methods is a concern for the scientific community(Stensvold and Nielsen, 2012; Amorós et al., 2016).

High-throughput sequencing (HTS) technologies have proven to be useful in revealing the occurrence of multiple prokaryotic and eukaryotic pathogens within clinical and environmental samples (Moreno et al., 2018; Oluseyi Osunmakinde et al., 2019; Zahedi et al., 2019; Rusiñol et al., 2020; Garner et al., 2021; Gu et al., 2021; Henriquez et al., 2021; Sengupta et al., 2022). Several studies have reported that a metataxonomic approach, utilizing primers targeting the 18S rRNA, can detect protist pathogens at very low abundances in complex communities, such as fecal and wastewater samples (Zahedi et al., 2019; Chihi et al., 2022). However, there is a knowledge gap concerning the application of metataxonomic strategies to investigate the persistence of pathogenic protists in sewage sludge and biosolids. In this study, we aimed to address this gap by employing a metataxonomic/metabarcoding approach to assess the protist parasite load in sewage sludge from two of Colombia's largest cities, Medellin and Cali, known for their high prevalence of intestinal parasites.

2. Material and methods

2.1. Sewage sludge collection and DNA extraction

We selected four WWTP in the Andean region of Colombia which serves a population of around 5.5 million. The San Fernando and the Aguas Claras wastewater treatment plants (WWTP) are located in the metropolitan area of Medellin City and serve the population of Medellin and other neighboring municipalities. The Cañaveralejo WWTP serves the city of Cali. The El Retiro WWTP is a rural plant that serves a smaller rural community and is located in El Retiro, Antioquia. All the plants are located in the Andean region of Colombia and the altitude ranges from 1080 m (Cali) to 2175 m. San Fernando and Aguas Claras WWTP are located at 1550 m and 1300 m, respectively.

Wastewater treatment in Colombia is carried out mainly by aerobic and anaerobic open lagoons (55%), activated sludge (22%), and up-flow anaerobic reactor systems (9%). The WWTP San Fernando treats an influent of 1,3m^3 of municipal wastewater and serves a population of 700,000 inhabitants. The wastewater treatment is performed with activated sludge systems and anaerobic digestion is used to treat the solids. This WWTP generates 85 tons of biosolids per day. WWTP Aguas Claras treats an influent of 5m^3/S of municipal wastewater and serves inhabitants and serves 2,200,000 inhabitants. This WWTP generates 126 tons of biosolids per day. The wastewater treatment is performed with activated sludge systems. Anaerobic digestion and thermic drying are used to treat the solids. Cañaveralejo WWTP treats an influent of 4m^3/S of municipal wastewater and serves 2,600,000 inhabitants. Perform an advanced primary treatment to treat the wastewater. This WWTP generates 60 tons of biosolids per day. Anaerobic digestion is used to treat the solids. El Retiro treats an influent of 0.6m^3/S of municipal wastewater and serves 20,000 inhabitants. Perform an activated sludge system to treat the wastewater. This WWTP generates 2 tons of biosolids per day. Dry beds are used to treat the solids.

Sewage sludge samples were collected through the years 2021 and 2022. Nearly 40 g of sludge were collected on sterile Falcon 50 mL tubes and transported refrigerated to the lab where DNA extraction was carried out the same day. The sludge sample was homogenized with a sterile stick, and then 200 mg was processed with DNAEASY POWER soil DNA kit (QIAGEN). Inlet and outlet wastewater samples of the San Fernando WWTP were collected on sterile 1 L Schott bottles. Samples of 100 mL of water were centrifuged at 6000 RCF for 15 min and then 200 mg of the sediment was processed with the same DNA kit. Purified DNA was quantified using a UV light absorption method and then stored at −20 °C until its PCR amplification.

2.2. Ribosomal 18S—V4 gene metataxonomic experiment and informatic processing

We used primers targeting the eukaryotic 18S ribosomal gene, specifically the hypervariable region 4 (18S—V4). The forward primer was 18S-V4Fw: CCAGCAGCCGCGGTAATTCC (Choi and Park, 2020), and the reverse primer was 18S—V4 Rev.: RCYTTCGYYCTTGATTRA. PCR amplification, library construction, and NGS sequencing were outsourced to Macrogen (Seoul, Korea), where they used a MiSeq (Illumina) platform to generate paired-end reads of 300 bases each. Amplicon reads underwent processing using the MOTHUR pipeline version 1.44 (Schloss et al., 2009), involving steps such as forward and reverse read merging, removal of sequences with ambiguous bases, exclusion of sequences shorter than 300 bases, elimination of sequences with homopolymers longer than 8 bases, clustering, chimera removal, and construction of molecular operational taxonomic units (mOTUs) at 97% nucleotide identity. Taxonomic assignment of the generated mOTUs was conducted using the MOTHUR program's 'classifyseqs' routine, which involved

comparisons with the SILVA v138 ribosomal database (Quast et al., 2013). mOTUs identified as eukaryotes were retained for subsequent BLASTN (Altschul et al., 1990) selection and subsequent phylogenetic analysis. We calculated general statistics, including the number of high-quality sequences, the number of mOTUs, and coverage estimation, using MOTHUR's 'summary.single' command. The raw amplicon sequences have been deposited in the NCBI SRA database under the Bioproject accession PRJNA976754.

2.3. Protist sequence identification and phylogenetic analysis of the mOTUs

To detect the DNA of the protists of interest present in the sewage sludge samples, we selected a list of potentially pathogenic species that are commonly found in human intestines and that could, therefore, be found in wastewater treatment plant sludges. The selected reference protists were: *Acanthamoeba castellanii, Acanthamoeba griffini, Acanthamoeba hatchetii, Acanthamoeba pearcei, Acanthamoeba polyphaga, Balamuthia mandrillaris, Balantidium ctenopharyngodoni, Balantidium duodeni, Balantidium entozoon, Balantidium grimi, Balantidium polyvacuolum, Balantioides coli, Blastocystis* subtypes 1 to 17 (STs 1–17), *Chilomastix mesnil, Cryptosporidium parvum, Cyclospora cayetanensis, Cystoisospora belli, Dientamoeba fragilis, Endolimax nana, Entamoeba bangladeshi, Entamoeba bovis, Entamoeba chattoni, Entamoeba chiangraiensis, Entamoeba coli, Entamoeba dispar, Entamoeba ecuadoriensis, Entamoeba gingivalis, Entamoeba hartmanni, Entamoeba histolytica, Entamoeba invadens, Entamoeba moshkovskii, Entamoeba muris, Entamoeba nuttalli, Entamoeba polecki, Entamoeba ranarum, Entamoeba struthionis, Entamoeba suis, Giardia ardeae, Giardia intestinalis, Giardia microti, Iodamoeba sp, Naegleria fowleri, Pentatrichomonas hominis.*

The sequences corresponding to the 18 s rRNA gene of the above-mentioned protists were searched in the nucleotide database of the NCBI, and we selected those with a length $\geq$ 1000 bp. Sequences carrying ambiguous bases were excluded from the reference database. The accession number for these sequences can be found in Supplementary Table 1.

To identify the putative mOTUs that correspond to the protist of interest in this work, we performed a BLASTN search strategy. To do so, MOTHUR-generated mOTUs were compared with the custom 18S ribosomal gene reference database of the above-mentioned parasites and considered valid candidates for subsequent phylogenetic analysis those that showed a bit score $\geq$ 400.

The selected and filtered reference sequences, along with the mOTUs identified by BLASTN comparisons as putative protist 18S-V4 sequences, were loaded into MAFFT v7.215 (Katoh and Standley, 2013) software for global nucleotide sequence alignment. Subsequently, a manual inspection of the alignment was carried out to identify possible conflictive sequences. Then, using the IQ-TREE2 software (Minh et al., 2020), a phylogenetic tree of maximum likelihood was constructed with the aligned sequences. Outgroup taxa were included in all trees for root assignment. ModelFinder was used to automatically select the best substitution model (Kalyaanamoorthy et al., 2017). In addition, ultrafast boot approximation (UFBoot) (Hoang et al., 2018) was used with a value of 1000 replicas to test the topology of the trees.

2.4. Data handling and graphical analysis

The trees were visualized using FigTree v1.44 (MacOS) software. The topology of each tree was inspected, and relevant clades were collapsed. Coloring and other graphical edits were carried out afterward with generic graphic editors. Heatmaps and stacked graphs were generated in R using the libraries ggplot2, and data.table, dplyr, and polychrome.

3. Results

3.1. Raw read results, sequence filtering, and mOTU construction

The bioinformatics strategy followed in this work is presented in a flowchart (Supplementary Fig. 1, panel A), and the geographical localization of the studied wastewater treatment plants-WWTP in Colombia is presented on a map (Supplementary Fig. 1, panel B). For each amplicon library, we generated at least 50,000 raw read pairs, with the maximum throughput per sample reaching 99,600 (Supplementary Table 2). Following read merging and cleaning steps, the number of high-quality filtered sequences obtained varied from 31,648 to 76,120. The observed number of molecular operational taxonomic units (mOTUs) varied between 525 and 1591 in the tested samples. The coverage index demonstrated a value of $\geq$99 for all samples, indicating satisfactory sampling efforts. While most amplified amplicons were of eukaryotic origin (98%), a minor fraction of prokaryote ribosomal sequences was also amplified. These contaminant sequences were excluded from the analysis.

3.2. Protist mOTU taxonomic assignment

To accurately identify mOTUs related to pathogenic protists, we employed a combined strategy involving BLASTN searches and phylogenetic analysis. In the initial stage, we compared the mOTUs with a custom protist 18S sequence database (see methods for details) using the BLASTN algorithm. Twenty-seven mOTUs successfully passed our BLASTN filters and were then subjected to the second stage, which involved phylogenetic confirmatory analysis. Our phylogenetic analysis effectively clustered the reference sequences into well-supported monophyletic groups (UFB support $\geq$95) for all the protists relevant to this study. In several cases, we achieved well-supported branches at the species level.

The phylogenetic results presented for the *Entamoeba* species show the reference species clustered into well-supported monophyletic groups (UFB support $\geq$97) for most of the relevant species, confirming that four mOTUs belong to this genus. Furthermore, DNA from the species *E. histolytica*, *E. coli*, *E. hartmanni*, and *E. moshkovskii* were confirmed to be present in the sewage sludge samples

(Fig. 1). Other amoebas seem to be absent in the analyzed sludge samples, as the BLASTN results did not yield hits for *Endolimax nana* or *Iodameba*.

DNA from the Fornicata *Giardia Intestinalis* was also detected and confirmed through phylogenetic analysis. Two mOTUs were grouped with the reference sequences of *Giardia intestinales* a UFB support of 98 (Fig. 2, panel A).

The search for *Balantidium\Balantioides* DNA revealed the presence of *Balantidium* in the sludge dataset but not *Balantioides coli*. The *Balantidium* mOTU was grouped with 100 UFB support with the species *B. ctenopharyngodoni* and

B. polyvacuolum. However, it was not possible to definitively determine the species to which it belongs. Therefore, we classified this mOTU as *Balantidium* sp. (Fig. 2, panel B).

Blastocystis was the protist represented by the largest number of mOTUs, with a count of 13. Most of these mOTUs were confidently assigned to the human subtypes ST1, ST2, ST3, and ST4 (95–100 UFB support). Additionally, two mOTUs were grouped within a well-supported clade with the zoonotic subtype ST14, confirming its taxonomic assignment (Fig. 3).

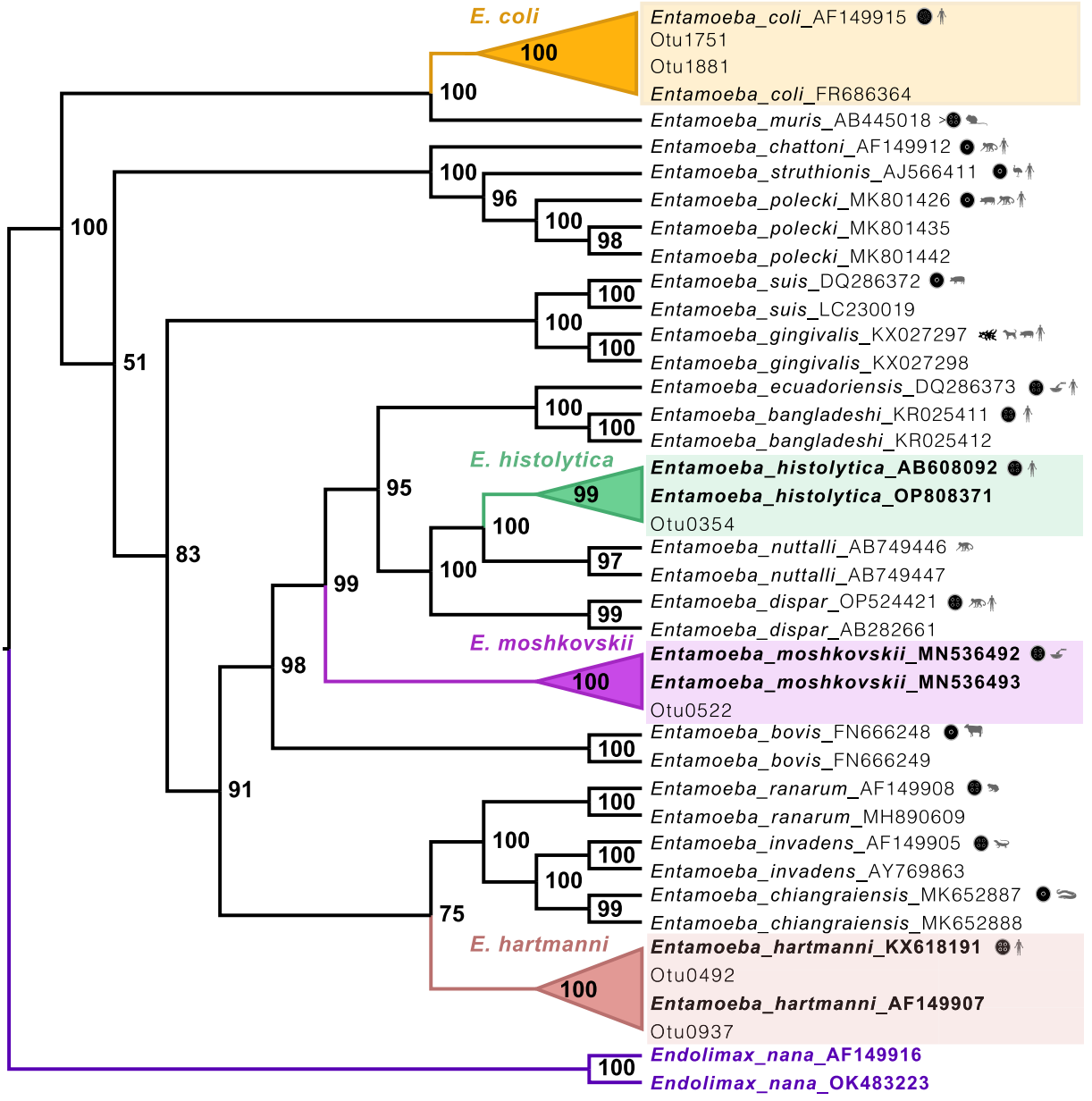

Fig. 1. Phylogenetic tree of genus *Entamoeba*. Maximum likelihood consensus phylogenetic tree constructed using sequences of the 18S ribosomal gene from the genus *Entamoeba*. The phylogenetic analysis was performed using UFBoot with 1000 replicates to evaluate the reliability of the resulting tree. Each taxon is identified with its corresponding scientific name, and the accession codes of the reference sequences used are provided. Additionally, an illustration is presented showing the hosts and/or locations where isolates of the mentioned species have been described.

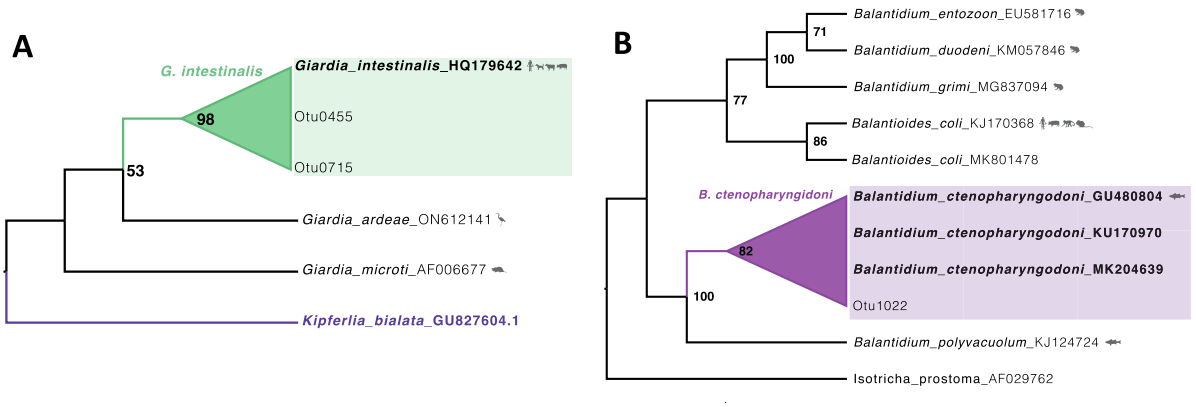

Fig. 2. A. Phylogentic tree of genus *Giardia*. Maximum likelihood consensus phylogenetic tree constructed using sequences of the 18S ribosomal gene from the genus *Giardia*. The phylogenetic analysis was performed using UFBoot with 1000 replicates to evaluate the reliability of the resulting tree. Each taxon is identified with its corresponding scientific name, and the accession codes of the reference sequences used are provided. Additionally, an illustration is presented showing the hosts and/or locations where isolates of the mentioned species have been described. **B.** Phylogentic tree of genus *Balantidium*. Maximum likelihood consensus phylogenetic tree constructed using sequences of the 18S ribosomal gene from the genus *Balantidium*. The phylogenetic analysis was performed using UFBoot with 1000 replicates to evaluate the reliability of the resulting tree. Each taxon is identified with its corresponding scientific name, and the accession codes of the reference sequences used are provided. Additionally, an illustration is presented showing the hosts and/or locations where isolates of the mentioned species have been described.

Finally, seven mOTUs were assigned to the genus *Acanthamoeba* with a UFB support of 100, of which two can be assigned to the species *A. hatchetii* with 100 UFB support. The remaining couldn't be assigned confidently to one species and were classified as *Acanthamoeba* sp. (Fig. 4). It is also important to mention that we searched for other environmental amoebas relevant to human health, such as *Balamuthia* and *Naegleria*, without positive results.

3.3. Protist parasite DNA present in sewage sludge

After the taxonomic assignment of the protist mOTUs, we moved to the analysis of the presence of DNA of each protist within the sewage sludge samples. We generated a binary heatmap graph that allows a comparative observation of the presence\absence of DNA of the mentioned parasites (Fig. 5, panel A). As can be seen in Fig. 5, *Blastocystis* and *Acanthamoeba* are present in all sludge samples. *Giardia intestinalis* is present in all sludge samples of the largest cities, Medellin (San Fernando) and Cali (Canaveralejo), but is absent in the sludge of the rural WWTP El Retiro. *E. histolytica* was only present in the Canaveralejo-Cali WWTP. This sludge also carries the DNA of the other entamoebas *E. coli.* and *E. moshkovskii*. *Acanthamoeba hatchetii* was present in the sludge of all the largest urban WWTP of Cali and Medellin while absent in the rural WWTP sludge of El Retiro WWTP. *Blastocystis* ST1 subtype was present in all four sludge samples, while ST2 was present in San Fernando-Medellin, Canaveralejo-Cali and El Retiro. ST3 DNA was detected in El Retiro and Canaveralejo-Cali. *Blastocystis* ST4 was only present in the El Retiro sludge. In general, all sludges carry the DNA of at least one pathogenic protist, but the Canaveralejo-Cali sludge is the one with more diverse spectrum of potential pathogens. The experiment design followed in this work wasn't conceived to relatively quantify and compare the abundance of taxa between samples (we didn't normalize the number of sequences obtained per library). Even though, we wanted to complement this analysis with a graphic representation of the number of high-quality sequences that we retrieved for each protist in the four sludge samples. As can be seen in Fig. 5, panel B (Supplementary Table 3), the largest number of sequences of protist were obtained in the Canaveralejo-Cali sludge reaching 225 sequences and followed by El Retiro with 82 sequences. San Fernando-Medellin and Aguas claras-Medellin showed lower counts with 9 and 14, respectively. Most of the amplified sequences correspond to *Blastocystis* ($n = 174$), 74% of them subtype ST1, and *Acanthamoeba* ($n = 142$).

3.4. Parasite DNA dynamics in the San Fernando WWTP

In the last experiment, we wanted to study the dynamics of the protist in one WWTP, analyzing the influent wastewater and comparing the protist DNA profiles with the effluent water and sewage sludge that comes out of the plant. For this purpose, we selected the San Fernando-Medellin WWTP. The binary heatmap shows, as expected, that the influent wastewater carries DNA of a higher diversity of pathogenic protists (Fig. 6, panel A). The entamoebas *E. histolytica*, *E. moshkovskii*, *E. coli*, as well as *Giardia intestinalis*, *Blastocystis* subtypes ST to ST3 and ST14, and *A. hatchetti* were found in influent wastewater. As mentioned above, in the biosolid of the San Fernando-Medellin WWTP, we can detect DNA of *G. intestinalis*, *Blastocystis* ST1 and ST2, and *Acanthamoeba*. On the other hand, in the effluent wastewater, we detected DNA of *G. intestinalis, E. histolytica, Blastocystis* ST2 and ST3, and *Balantidium* sp. These results might indicate that primary pathogens such as *G. intestinalis* and *E. histolytica* enter the WWTP plant and could escape the treatment process and pass to the city river where the WWTP discharges the treated water. Again, as mentioned above, even though the read counts were not normalized and we cannot compare the relative abundance of the different taxa between samples, we wanted to

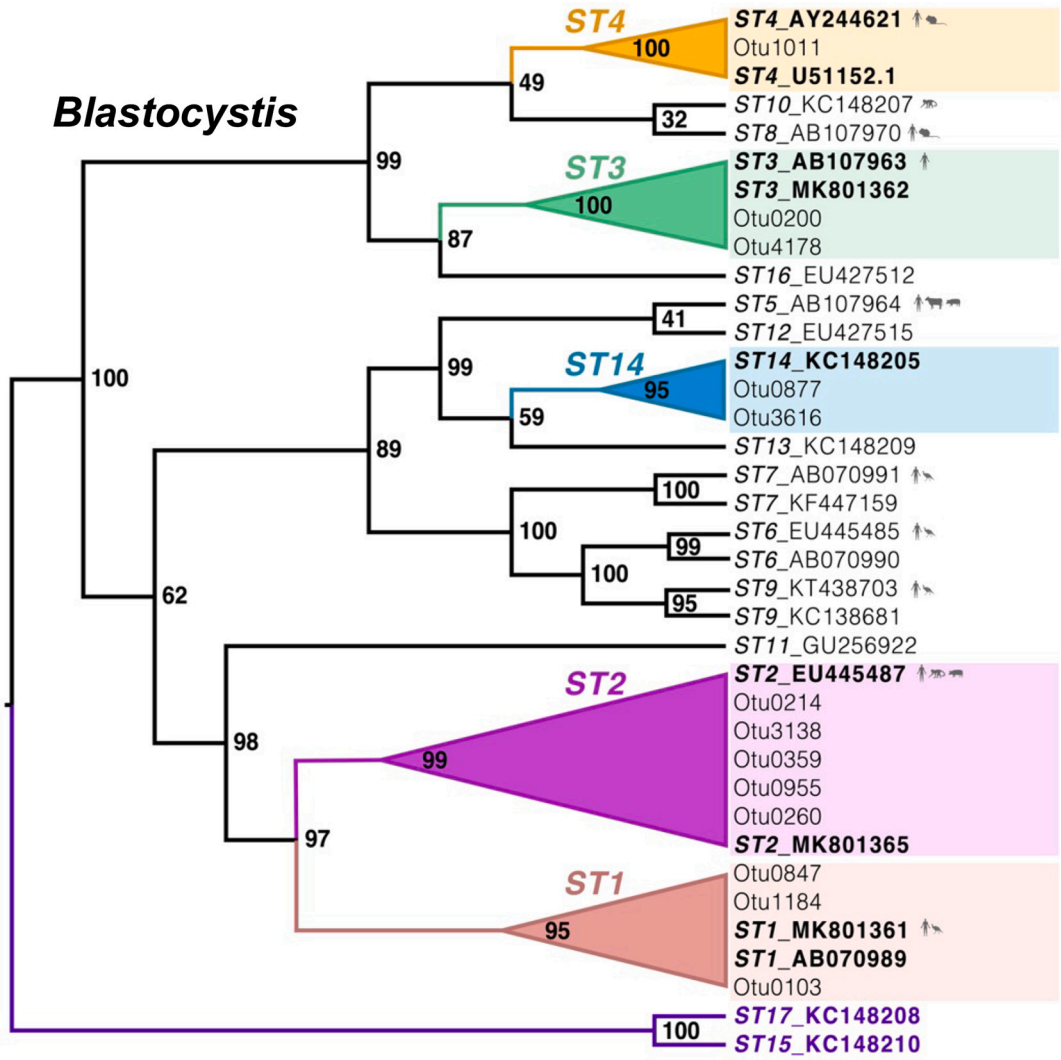

Fig. 3. Phylogentic tree of genus *Blastocystis*. Maximum likelihood consensus phylogenetic tree constructed using sequences of the 18S ribosomal gene from the genus *Blastocystis*. The phylogenetic analysis was performed using UFBoot with 1000 replicates to evaluate the reliability of the resulting tree. Each taxon is identified with its corresponding scientific name, and the accession codes of the reference sequences used are provided. Additionally, an illustration is presented showing the hosts and/or locations where isolates of the mentioned species have been described.

represent the absolute number of high-quality sequences observed in these three samples. The influent wastewater has the highest counts of protist parasites ($n = 776$), of which most correspond to *Blastocystis* (77%) (Fig. 6, panel B) (Supplementary Table 4). *Giardia intestinalis* and *E. histolytica* shared the second position in the rank of abundances in the influent water with 51 high-quality sequences each. The effluent water and biosolid had lower counts, with 21 and 9, respectively. In the San Fernando-Medellin effluent wastewater, the highest counts were for *Balantidium* sp. ($n = 8$), followed by *Blastocystis* ST3(n = 7) and ST2($n = 3$), and *E. histolytica* ($n = 2$).

4. Discussion

Studying the presence of parasites in WWTP effluent water and sewage sludge is essential to public health regulators (Diamond et al., 2022). This information allows them to ensure the effectiveness of the treatment systems and justify demanding appropriate corrective measures to be taken by the industry in order to prevent the spread of parasitic diseases and ensure community safety. Another interesting point of view is that by analyzing the influent wastewater of the WWTP, you can monitor the circulating parasites in its respective community. This new field of epidemiology has been coined as wastewater-based epidemiology or sewage-based epidemiology (Choi et al., 2018; Lorenzo and Picó, 2019; OKeeffe, 2021). Here, wastewater serves as an industrial-scale pooled sample of the community feces that helps to monitor the presence of pathogens at a population level. In this case, by analyzing the DNA present in the influent wastewater of a community, we can have a glimpse of the circulating infectious diseases at a certain time point (OKeeffe, 2021). Wastewater-based epidemiology has gained significant attention and utility, particularly during public health crises,

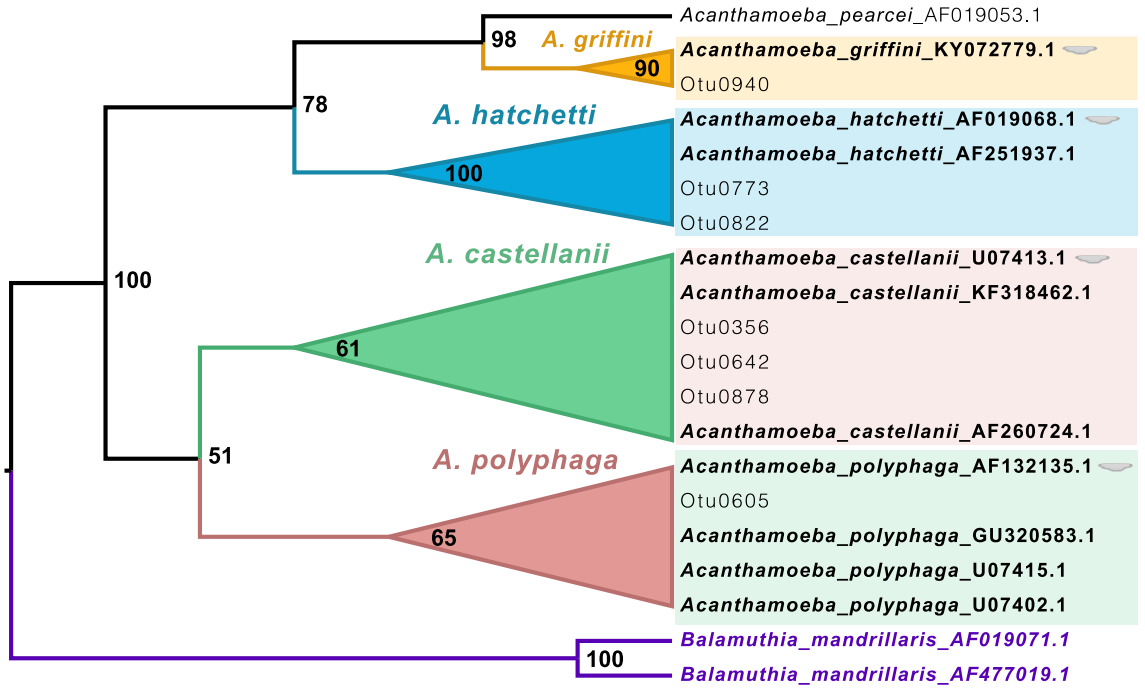

Fig. 4. Phylogentic tree of genus *Acanthamoeba*. Maximum likelihood consensus phylogenetic tree constructed using sequences of the 18S ribosomal gene from the genus *Acanthamoeba*. The phylogenetic analysis was performed using UFBoot with 1000 replicates to evaluate the reliability of the resulting tree. Each taxon is identified with its corresponding scientific name, and the accession codes of the reference sequences used are provided. Additionally, an illustration is presented showing the hosts and/or locations where isolates of the mentioned species have been described.

such as the COVID-19 pandemic. Analyzing SARS-CoV-2 RNA in wastewater was useful to provide an early warning system for tracking the spread and prevalence of the virus in certain communities (Betancourt et al., 2021).

Metataxonomic approaches have proven to be a valuable tool in the study of bacterial (Bedoya et al., 2019, 2020; Xiong et al., 2021) and fungal diversity (Carbonero-Pacheco et al., 2022; Peñuela-Martínez et al., 2023), in some cases even displacing classical microbiological techniques such as culture and direct visualization. Nonetheless, the application of this technology to protists has been slow but steady, and recent works have shown its effectiveness and advantages over traditional microbiological analysis (Garner et al., 2021; Gu et al., 2021; Chihi et al., 2022; Sengupta et al., 2022). The metataxonomic approach applied in this work was shown to be successful in detecting both Chromist and Protozoa in the sludge and wastewater samples. Additionally, the use of phylogenetic analysis allowed us to more precisely classify entamoebas that are indistinguishable using microscopic methods such as *E. histolytica*, *E. dispar*, and *E. moshkovskii*. In the case of *Blastocystis* sp., the only available method to discriminate among subtypes is by using 18S ribosomal gene sequences. In the case of this Chromist, is of particular importance to discriminate among them since we now know that their subtypes that can be transmitted, depending on the STs, following anthropogenic or zoonotic transmission routes. Additionally, the pathogenicity of *Blastocystis* seems to be related to its subtype, so determining its ST group allows us to better assess the risk to humans and determine the possible natural hosts (Jeremiah and Parija, 2013; Ramírez et al., 2014; Stensvold and Clark, 2016; Betts et al., 2018; Hublin, Maloney and Santin, 2021; Popruk et al., 2021; Moreno-Mesonero et al., 2022).

Monitoring a wide spectrum of protist species in environmental samples is a challenging task (Sagova-Mareckova et al., 2021). To accomplish this goal, you need to have standardized specific PCRs for every pathogen, in this case, dozens of species, and there is always a risk of false positive results due to the high microbial diversity present in wastewater and sewage sludge samples. The use of metataxonomic approaches circumvents some of these problems thanks to the availability of sequences of the target gene, i.e. the 18S—V4 (DeMone et al., 2020). The nucleotide sequence data, in combination with phylogenetic analysis, allow to tackle both limitations. Firstly, based on high-quality filtered sequences, phylogenetic trees will elucidate between DNA sequences of the closely related species of pathogens and discern among false positive amplicons. Secondly, the use of wide-spectrum degenerate primers allows the amplification of a broad spectrum of parasite taxa in one single experiment.

Acanthamoebas are free-living amoebas that are commonly found in aquatic environments, including sewage systems. Most species of *Acanthamoeba* do not pose a health risk to humans, but *A. castellani* and *A. polyphaga* have been described as etiological agents of rare infections like keratitis and amoebic granulomatous encephalitis (Khan, 2006; Siddiqui and Khan, 2012). In this work, we found that DNA of *Acanthamoeba* is present in all biosolid samples, as well as in the influent wastewater of the San Fernando WWTP, a finding concordant with similar studies published in other countries (Schroeder et al., 2001; Magnet et al., 2012; Muchesa et al., 2014). Our results confirmed the presence of *A. griffin*, but another acanthamoeba was also spotted, an *Acanthamoeba* species closer to *A. castellani*

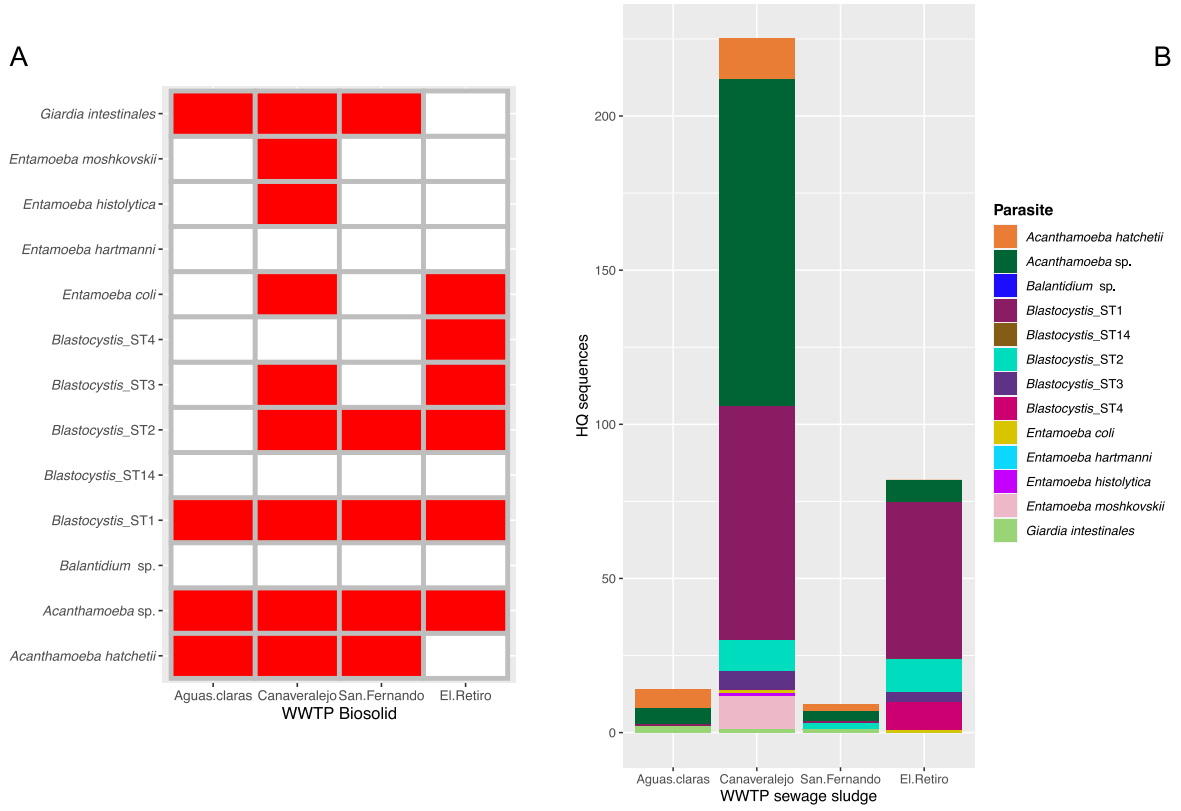

Fig. 5. Panel A. Binary heat map showing the protist taxa found in the sewage sludge of the four wastewater treatment plants studied. The name of the wastewater treatment plants is denoted on the x-axis, while on the y- axis the protist taxa. The red color indicates presence, while the white color represents the absence of the respective taxa. Panel B. Stacked bar plot depicting the number of high-quality (HQ) sequences observed in each wastewater treatment plant. Parasite taxa are represented by colors according to the legend. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and *A. polyphaga*, but we couldn't assign its taxonomic rank to the species level accurately. Despite the low risk of human infection by this environmental amoeba, it should be mentioned that another indirect but meaningful pathogenic role has been assigned to this cercozoan, since it has been demonstrated that they can behave as carriers of pathogenic bacteria such as *E. coli*, *Mycobacterium* spp., *P. aeruginosa*, *Shigella*, *Salmonella* and *Legionella* (Borecka et al., 2020; Henriquez et al., 2021; Rayamajhee et al., 2021, 2022).

The sewage sludge analyzed here showed the presence of DNA of several human pathogens like *E. histolytica*, *G. intestinalis*, and *Blastocystis* subtypes ST1, ST2, and ST3. *Blastocystis* ST1 was present in all sludge samples. This subtype is among the most prevalent in human populations worldwide and has been associated with pathogenicity (Alfellani et al., 2013; Beghini et al., 2017). The subtype ST4 was only present in the rural El Retiro WWTP sludge. This subtype has been associated frequently with European populations (Stensvold et al., 2011; Jiménez, Jaimes and Ramírez, 2019), although it has been reported in Colombia as well (Jiménez, Jaimes and Ramírez, 2019). The human-associated protist load seems different in the tested sludges, the WWTP Cañaveralejo is the one carrying more DNA of them simultaneously. This sludge sample was positive for *G. intestinalis*, *E. histolytica*, *E. moshkovskii*, *E. coli*, and *Blastocystis* subtypes ST1, ST2, and ST3. These results agree with the number of high-quality sequences observed in this plant, >15 times the number observed in the Medellin WWTPs San Fernando and Aguas Claras. It must be stressed that this metataxonomic experiment is not quantitative and cannot be directly related to absolute concentrations or relative abundance of the protists in the sludge or waters analyzed. Other methods must be applied to measure absolute concentration or compare relative abundances.

All the sewage sludge samples of the large cities Medellin and Cali showed to be positive for *G. intestinalis*, and *Blastocystis* subtypes ST1. These three WWTPs generate, combined, 271 tons of sewage sludge per day, and these results should encourage the regulatory authorities to evaluate the risk of the presence of infective stages of these parasites.

The protist DNA dynamics analysis of the San Fernando WWTP showed somehow an expected trend. Almost all the detected parasites in this study were observed in the influent water of this WWTP: *G. intestinalis*, *E. histolytica*, *E. moshkovskii*, *E. hartmanni*, *E. coli*, and *Blastocystis* subtypes ST1, ST2, ST3, and ST14. The latter subtype has been associated with cattle, like pigs (Udonsom et al., 2018), and in the area of influence of the San Fernando WWTP, there are pig farms. The number of sequences detected in the influent water was strikingly high compared to the biosolid and the effluent water, >36× fold. This result can be interpreted as a suggestive finding since the methodology followed in this work does not allow for a comparison of relative abundance between samples. The treated wastewater and the biosolid not only showed a considerably lower number of protist sequences but also a reduced number of

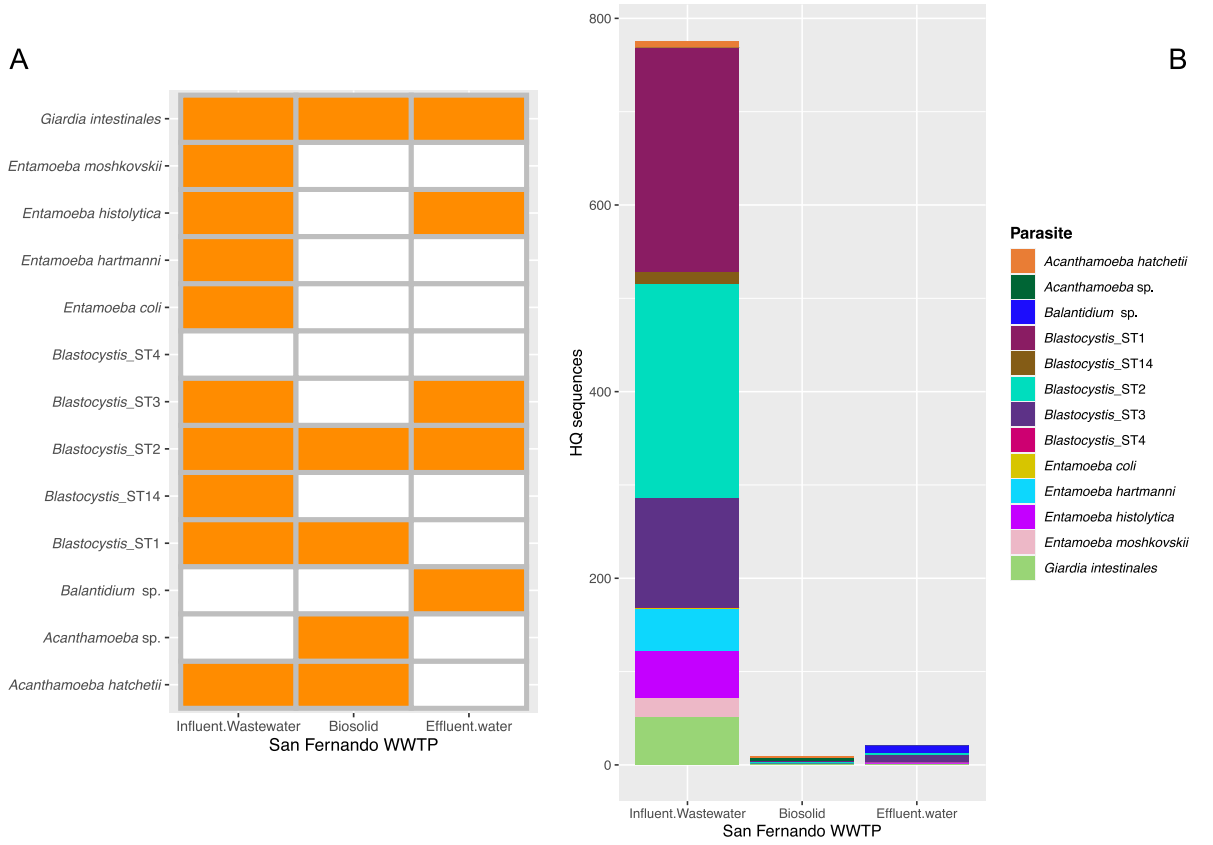

Fig. 6. Panel A. Binary heat map showing the protist taxa found in the samples of the San Fernando wastewater treatment plant: Influent wastewater, biosolid, and effluent water. The protist taxa are denoted in the y-axis. The orange color indicates presence, while the white color represents the absence of the respective taxa. Panel B. Stacked bar plot depicting the number of high-quality (HQ) sequences observed in the respective sample of the San Fernando wastewater treatment plant. Parasite taxa are represented by colors according to the legend.

protist taxa, especially in the biosolid. It is noteworthy to mention that our results point to the hypothesis that *G. intestinalis* might be entering the WWTP but continues to be present in the effluent water and the sludge. The efficiency of parasite removal and inactivation in the secondary treatment (activated sludge) remains largely unexplored in Colombia. Previous studies in Spain have documented the elimination of *Giardia* sp. from wastewater through their adsorption onto flocs, followed by sedimentation within the reactor. However, the efficacy of cyst removal fluctuates within a range of 0.54–2.85 log units, depending on operational conditions. To mitigate health risks associated with wastewater reuse, it becomes imperative to employ tertiary technologies such as filtration, UV radiation, or chemical agents (Suarez et al., 2022).

One additional observation regarding the influent wastewater is that the protist pathogens observed correspond to the most prevalent pathogens circulating in the region (Ministerio de Salud y Protección Social and Universidad de Antioquia, 2015; Jiménez, Jaimes and Ramírez, 2019; Osorio-Pulgarin et al., 2021). Furthermore, it is estimated that *Blastocystis* is one of the most prevalent human parasites in the Colombian population, and this protist accounted for the higher number of sequences of the human-associated protists observed in influent water of San Fernando WWTP and all the sludge samples. This result reassures the use of metataxonomic strategies as a powerful tool for wastewater-based epidemiology, in this case, of intestinal parasites.

There has long been a debate about the notion that the mere presence of DNA does not necessarily indicate the presence of viable infective stages. This argument hinges on the stability of DNA in non-viable cells, as noted by Cangelosi et al. in 2014 (Cangelosi and Meschke, 2014). This argument must be acknowledged as a limitation of the present work. While the detection of pathogenic protist DNA in both effluent water and sewage sludge, as presented in this study, may be initially interpreted as an alert for Latin American wastewater treatment systems, it also underscores the importance of expanding the roster of parasite protist indicators. This expansion is essential for a more comprehensive evaluation of the risks associated with wastewater and sludge generated within these industrial systems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We extend our gratitude to the staff of the wastewater treatment plants—San Fernando, El Retiro, Aguas Claras, and Cañaveralejo—for granting us access to the samples and for their valuable assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2023.e00210.

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