UNIVERSITY OF SOUTH BOHEMIA in ČESKÉ BUDĚJOVICE
FACULTY OF SCIENCE

in cooperation with the public research institution:
Biology Centre of the Academy of Sciences of Czech Republic

Methods for studying gut parasites and their interaction with the host and the host microbiome

Bachelor Thesis

Jessica Drozdz

Institute of Parasitology
Supervisor: **Mgr. Martin Kolisko, PhD.**
Co-Supervisor: **Petr Táborský, MSc**

České Budějovice 2020

Drozdz J., 2020: Methods for studying gut parasites and their interaction with the host and the host microbiome. Bc.Thesis, in English 37 pages, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

ANNOTATION

Gastrointestinal issues and diseases have been falsely associated with *Blastocystis hominis* for decades and was treated mainly with metranidazole. The role of Blastocystis in the human gut is still unclear and has been found more frequently in healthy humans. The main focus of this thesis and study was to test isolation protocols for RNA extraction with the goal of obtaining sufficient *Blastocystis* data for genomic analyses.

DECLARATION

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography.
I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 20.05.2020

.....

Jessica Drozdz

ACKNOWLEDGEMENTS

I would like to express my sincerest and biggest thank you to Mgr. Martin Kolísko, Ph.D. for giving me the opportunity to work with him, treating me as an equal part of his team, his honest criticism, guidance, patience and constant support. Without him this study would have not been possible.

Furthermore I want to thank Petr Táborský for taking his time to guide me throughout the experimetns and his assistance whenever needed. Another thank you goes to RNDr. Zuzana Lhotská, for teaching me how to cultivate Blastocystis in vitro and keeping my cultures alive when needed.

Another special thank you goes to the whole team of Mgr. Martin Kolísko, Ph.D in the Institute of Parasitology, for making the work environment such an incredible and open place as well as for their help with the Bioinformatics analyses part.

Moreover, I want to mention Rachel Kolísko, MSc. for taking her time to comment on my thesis.

And last, I want to thank my family and friends for their love and support throughout my lab work and the thesis writing.

LIST of ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
BCP	(1-(1,3-Benzodioxol-5-ylcarbonyl))piperidine
CB	central body
DIC	differential interference contrast microscopy
ER	endoplasmatic reticulum
HIV	human immunodeficiency virus
HSP70c	cytosolic-type 70-kDa heat shock protein
IBD	inflammatory bowel disease
IL-8	interleukin 8
IBS	irritable bowel syndrome
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
ST	subtype
TEM	transmission electron microscopy
TRIzol	guanidinium thiocyanate

ABSTRACT

In this bachelor thesis, the possibility of transcriptomics of *Blastocystis* directly in the gut content of the rat host was tested. The caecum content samples were obtained six weeks post *Blastocystis* infection and several mutations of TRIzol protocol for RNA extraction were evaluated and resulting RNA was tested with NanoDrop for purity before sequencing. The sequencing data were then evaluated fort he presence of *Blastocystis* signal. The overall aim was to evaluate the possibility of larger experiment to clarify the role and impact of *Blastocystis* in the gut environment by understanding the changes in RNA expression pre- and post-infection.

Table of Contents

1. INTRODUCTION

1.1 *Blastocystis hominis*

Blastocystis sp. is an intestinal parasite or commensal residing in humans and various animals. The anaerobic unicellular eukaryote found specifically in the gastrointestinal tract of humans is called *Blastocystis hominis* (*B. hominis*, 1, 2). Taxonomically speaking, *B. hominis* belongs to the phylum Stramenopila, but it was mistaken for a yeast for several decades (3). The lack of pseudopodia, no corresponding proof of locomotion, immense variety in sizes, missing cyst form, as well as the overlooked amoeba form, indicated a yeast like single-celled organism (4). A series of phylogenetic studies (small subunit rRNA phylogeny, HSP70c phylogeny) revealed a close relationship between stramenopiles (oomycetes) and *Blastocystis hominis*, placing them within the closest relatives of the alveolates (5).

Further physiological and structural characteristics corroborate its non-fungal nature. These include: obligatory anaerobicity; optimal growth at 37°C; no growth on fungal or bacteriological media; optimal growth at neutral pH with presence of bacteria; no growth at pH 5.5; cell death at RT or overnight at 4°C; resistance to 400 μg/ml of amphotericin; capable of ingesting bacteria and other matter; no cell wall; anaerobic mitochondria; binary fission and no budding; Golgi apparatus; locomotion pseudopods; definite smooth and rough ER; membrane-bound CB (taking up 50-95% of total cell volume); possible edodyogeny and schizogeny (4, 6).

Blastocystis spp. is an incredibly diverse organism and at present, 17 subtypes, classified as ST1-17, of *B. hominis* have been officially recognized with the help of small subunit rDNA analysis (SSU rDNA), of which subtypes ST1-9 and ST12 have been identified in humans (7, 8, 9). Studies of the worldwide prevalence of *B. hominis* was found to be as low as 0.5-5% in industrialized countries and as high as 30-60% in developing countries with some exceptional cases (10, 11). Humans infected with this microbe may show a wide range of intestinal symptoms, with abdominal pain, diarrhoea, vomiting, nausea, fatigue and distention being the most common ones (12, 13, 14, 15). Furthermore, some studies implicated a direct link between the presence of *Blastocystis* and

intestinal/extraintestinal diseases, such as urticaria, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and gastroenteritis (16, 17, 18).

1.2 Blastocystis Morphology

The morphology of *B. hominis* occurs in four major forms: the central body (CB) form or central vacuole form; the granular form; the amoeboid form and the cyst form. Other variations have been described including the avacuolar form, the multivacuolar form or the schizont. Defining and categorising this parasite have been and is still challenging for cell biologists due to the large differences in sizes and forms found in vitro and in vivo (6, 16, 19, 20).

1.2.1. CB or central vacuole form

The CB form (Fig. 1), also known as the central vacuole form, is the most common *B. hominis* form found in human stool samples and in laboratory cultures. It is spherical in shape and its size ranges from 2 to 200 μm with an average of 4 to 15 μm (19). The central vacuole or central body restricts intracellular components, cytoplasm, and mitochondria to a limited space by taking up nearly the entire cell volume. Studies have now shown, that these previously-believed-to-be empty vacuoles are actually membrane bound bodies filled with flocculent material, carbohydrates, and lipids. Thus, suggesting their function as a storage organelle (21, 22, 23, 24).

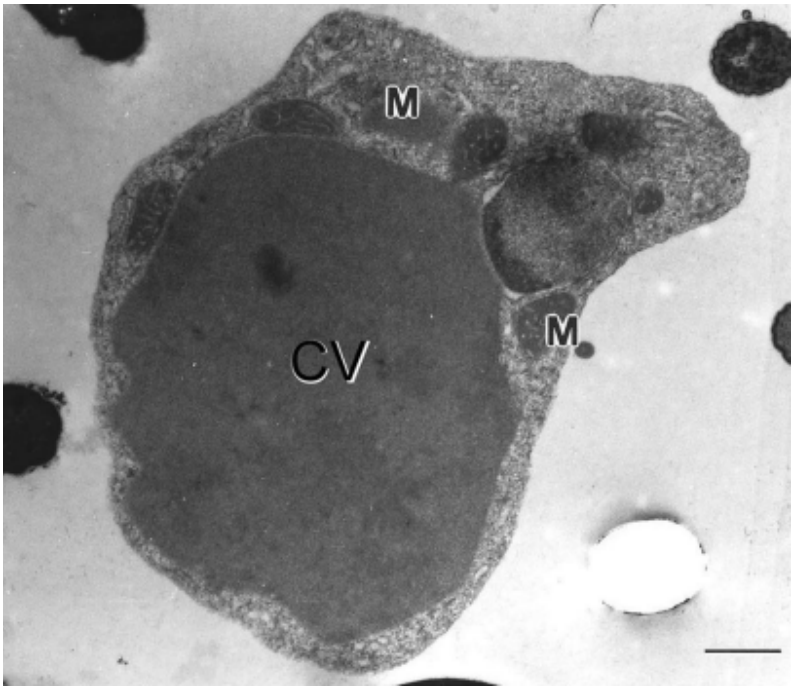

Figure 1: Transmission electron micrograph of a vacuolar form; CV: central vacuole; M: mitochondrion-like organelle; Taken from Tan, et al. (16).

The peripheral cytoplasmic rim contains one or two nuclei and thickened pods, which consist of mitochondria and other cell structures, appearing as rosettes (6, 25). A further characteristic of the CB form of *B. hominis* include a surface coat found in clinical samples, which protects it from osmotic shock and stores bacteria for nutritional purposes (26, 27).

1.2.2. Granular form

Under DIC and electron microscopy, the granular form (Fig. 2) morphologically resembles a spherical shape, purely filled with granules. A study using TEM identified and categorised the different morphological different types of granules into metabolic, reproductory, and lipid granules. Their average diameter is 10 μm. The CB in the granular form occupies most of the cell's volume and the reproductory granules are found exclusively in the CB. The metabolic granules, which play an important role in the metabolic pathways of the *B. hominis*, are only located in the cytoplasm. The lipid granules act as storage units and are the only form found in both the cytoplasm and the CB (6, 28, 29, 30). The electron-dense granules, shown in Fig. 2, represent mitochondria and are located in the central body and the cytoplasm. In addition, various other types of CB granules can be detected via light microscopy, including fine granular matter, electron-lucent empty, and electron-dense spherical granules. In comparison to the amoeboid form, the mitochondria appear smaller in the granular form. Further differences include multiple spherical nuclei and a smooth and rough ER that are only located in the peripheral sphere of the cytoplasm (6).

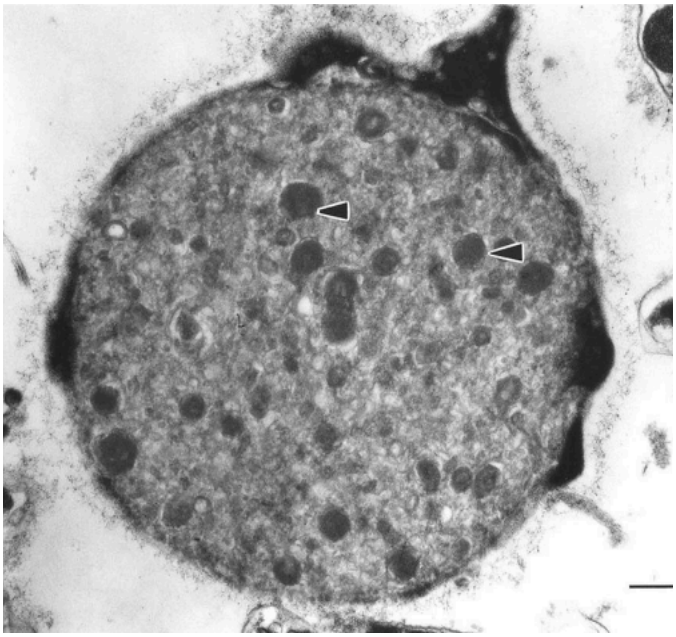

Figure 2: Granules occupying the central vacuole of a granular form of B. hominis; arrows: electron-dense granules; Taken from Tan, et al. (16).

1.2.3. Amoeboid form

The amoeboid form (Fig. 3) is a rather challenging form due to its ability to revert to the CB form and its irregularity in shapes. Typical characteristics include: one or two pseudopods, nonmotile, a finely filamentous layer of 0.2 to 0.25 μm in diameter covering the cell surface, pockets (90 nm in diameter) dispersed throughout the cell membrane, and lacking or underdeveloped smooth/rough ER (6). Additionally, the cytoplasm may contain a single large vacuole or multiple smaller vacuoles (31). Possible ways to identify the amoeboid form in wet mounts include TEM, light microscopy, and DIC (6). Because the amoeboid form is usually found in diarrhoeal fluid of patients, the possibility that it is the pathogenic form is under investigation (32, 33). As the amoeboid forms mimics leukocytes in fluid samples, a gram stain test of air-dried smears is applied to diagnose *B. hominis.* The amoeboid form of *B. hominis* will form lyses, whereas the leukocytes will stain (6).

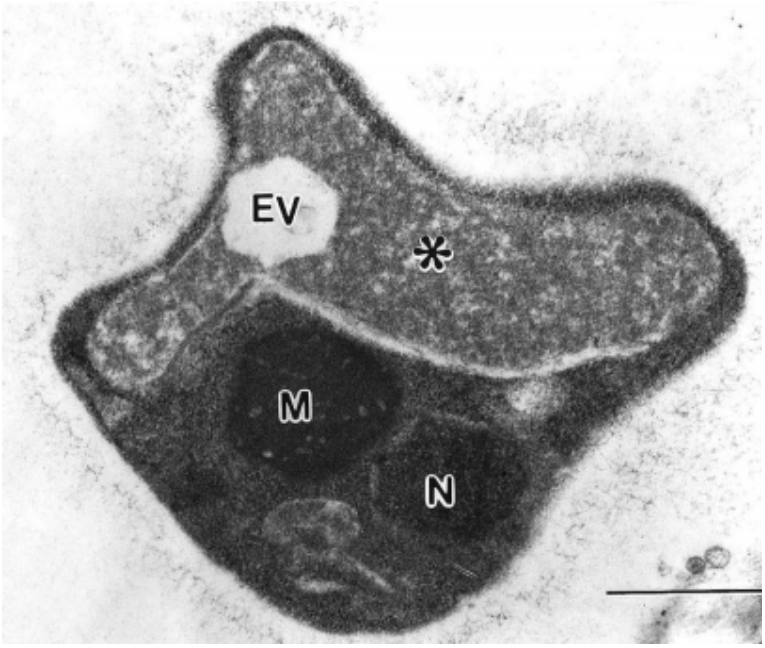

Figure 3: Type of amoeboid form shown by Tan, et al.; EV: empty vacuole; asterisk: central vacuole; M: mitochondrion-like organelle; N: nucleus (16).

1.2.4. Cyst form

The cyst forms (Fig. 4 and Fig. 5) have been overlooked for years and are easily mistakable for faecal debris. Being 2 to 5 μm in size, they are the smallest forms of *B. hominis* found in humans. Larger sizes can be isolated from animal hosts. The cytoplasm contains various small vacuoles, mitochondria, and glycogen storages, and can have up to four nuclei. The ovoid, spherical cyst is covered in a thick multi-layered cyst wall, and an optional surface coat. Due to its protective layers, the cyst can survive one month at 25°C and even up to two months at 4°C outside the host. This ability, amongst others, suggests that the cyst form must be the transmissible, infective form of *B. hominis*. The transformation of cysts into vacuolar forms occurs during excystation in the large intestine. TEM and SEM are suitable for the examination of cysts (6, 34, 35, 36, 37, 38, 39, 40, 41, 42).

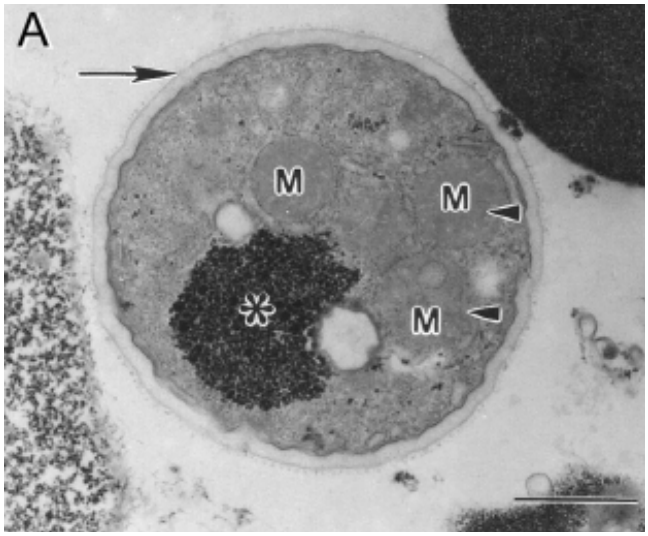

Figure 4: Mature cyst form of Blastocystis ST 4; M: mitochondrion-like organelle; asterisk: glycogen mass; arrows: faint saccate cristae; Taken from Tan, et al., (16).

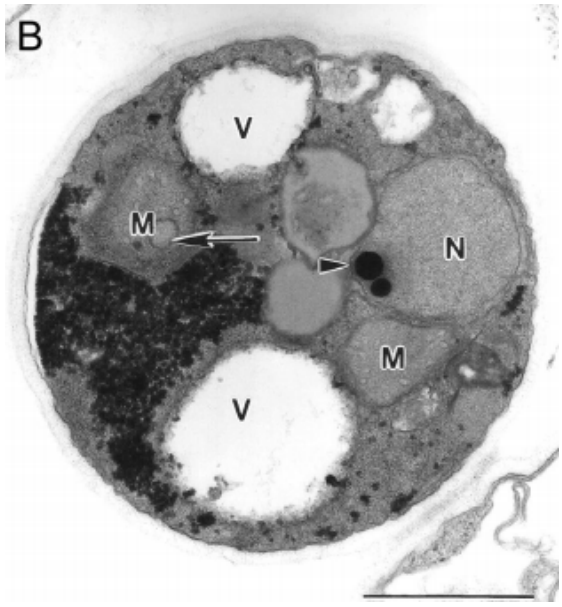

Figure 5: Mature cyst form of Blastocystis ST 4; V: vacuoles; N: nucleus; M: mitochondrion-like organelle; arrow (right): chromatin mass; arrow (left): circular cristae; Taken from Tan, et al. (16).

1.2.5. Other forms

Various studies have described further unusually shaped *B. hominis* forms, most likely being the predominant forms in schizogony, encystation, or excystation. These include the avacuolar form lacking a visible central vacuole and the multivacuolar form, which contains multiple small vacuoles. The avacuolar and multivacuolar forms measure 5 to 8 μm in diameter, are commonly uninucleate (although sometimes binucleate) and occur in vivo. Noticeably increased nuclei size in comparison to all *B. hominis* types is characteristic for the avacuolar form. Furthermore, TEM and light microscopic analyses found additional unidentified structures, occurring as golden cells or cells with no cytoplasm (6, 19, 20, 35, 36).

1.3 Life cycle - Division

B. hominis has been classified into 17 subtypes, of which 10 have been found in human samples (7, 8). As visualised in Fig. 8, the proposed transmission of the cyst can occur through different animal hosts, such as poultry, rats, pigs and chimpanzees. Importantly, compatibility between the host and the various subtypes is thought to play an important role (43, 44, 45, 46). Once inside the large intestine of the host, the cyst undergoes excystation into the vacuolar form (42). This protozoan only reproduces in asexual ways, such as binary fission, plasmotomy, budding, multiple fission, schizogony and endodygony (Fig. 6 and Fig. 7). Binary fission is the most frequently observed form of division found in vacuolar forms (6, 47). The vacuolar form possesses the ability to convert to the amoeboid and granular forms, but there remains no proof of direct cyst formation in the host. Furthermore, no direct evidence exists of changes from amoeboid to vacuolar forms (16, 48). Plasmotomy occurs in amoeba by cutting off their nuclei-carrying progeny without a central

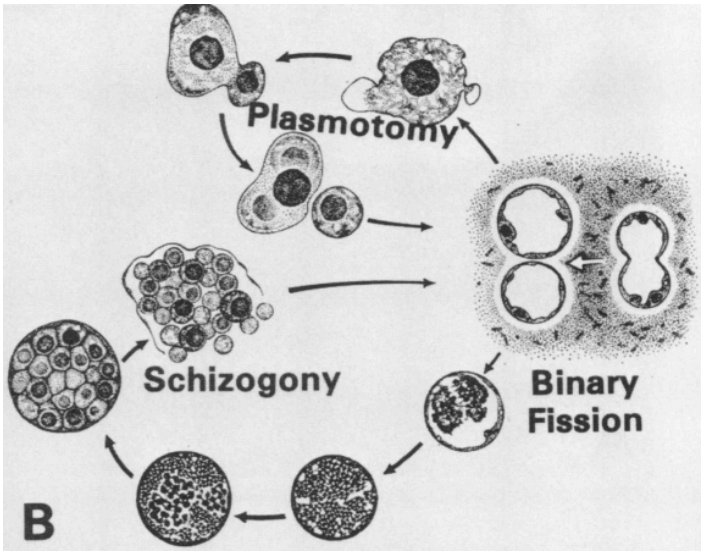

Figure 6: Various types of asexual reproduction; Taken from Zierdt, et al. (6)

body. In schizogony the progeny transfers to the schizont (parent cell) in the CB until the filled-up cell releases its inner matter. In this process, it loses its central body membrane, which leads to an intermingling between the cytoplasm and internal matter. Another reproduction type is endodyogony, where two nuclei (progeny) originate within one parent cell. In the case of the CB form, the average generation time for a single cell to undergo full division is 11.7 hours (6, 49).

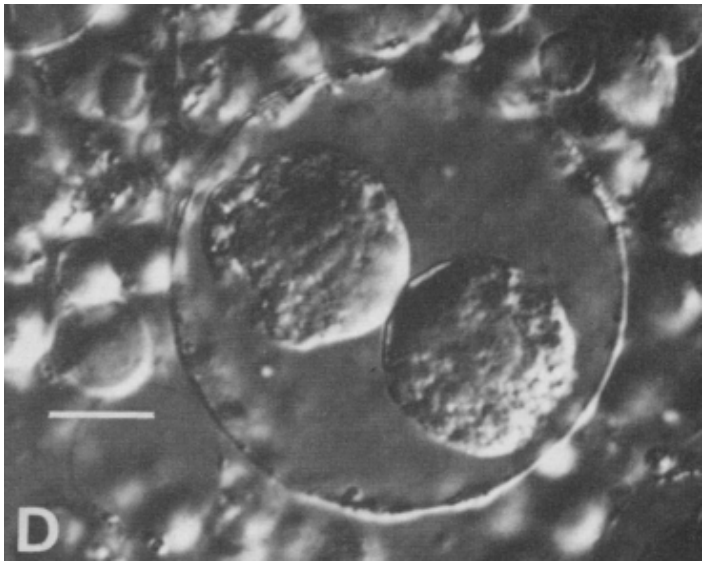

Figure 7: Cell in endodyogony; Taken from Zierdt, et al. (6).

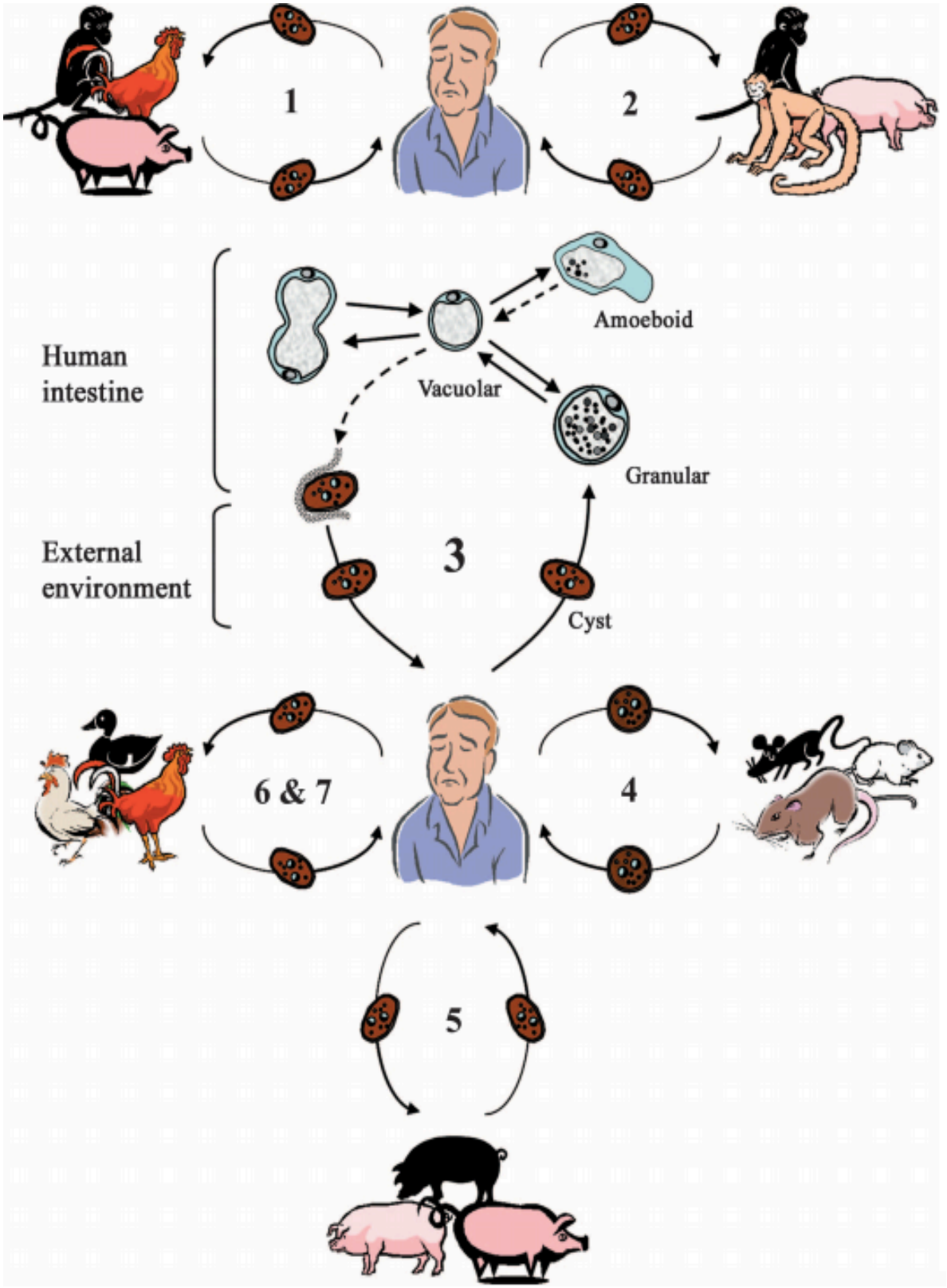

Figure 8: Putative life cycle and zoonotic transmission of various host specific STs (1-7) of B. hominis; Modified from Tan et al. (16).

1.4 *Blastocystis hominis* infection

As the granular, vacuolar, and amoeboid forms show weak survival rates outside the host, it is the cyst form with its thick protective walls that is the infective form of *B. hominis*. Studies suggest that the faecal-oral route is the most common transmission mode (6, 50, 51, 52, 53, 54, 55, 56, 57, 58). Humans living closely with animals were thought to be more likely infected, but no studies have strongly supported this theory (13, 57). Nevertheless, a link between cockroaches and higher infection rates of humans has been demonstrated (59).

Another proven way of *B. hominis* transmission is through water systems. Sewage influent and effluent samples from Scotland and Malaysia show evidence of *Blastocystis* cysts. Further studies have shown that conventional disinfection and cleaning methods for our drinking water are ineffective at killing *Blastocystis* cysts. Higher chlorination dosages and longer exposure times are recommended for increasing the effectiveness of methods to destroy the waterborne transmission of *Blastocystis* (60, 61).

Once infected with *B. hominis*, nausea, abdominal pain, bloating, flatulence, chronic diarrhoea, distention, anorexia, watery diarrhoea, mucus diarrhoea, vomiting, dehydration and sleeplessness were described as possible symptoms. In various cases, patients have shown blood in stools, increased leukocytes and mucus in stools, as well as severe eosinophilia (12, 14, 15, 62, 63). However, there has been little to no evidence that *B. hominis* was the causative agent for these occurrences. A Turkish study suggested *B. hominis* as the cause of some of these symptoms by examining patients who tested negative for other known pathogens and who showed at least five *B. hominis* cells per high-power field in their stool samples (14).

1.4.1. Correlation between *Blastocystis hominis* infection and its various subtypes

There are 17 subtypes of *B. hominis* that reside in animal and human hosts. *Blastocystis* isolates from around the world were compared and screened, showing that ST1-9, ST 12, Novel ST, or a mix of these subtypes are found in human samples (7, 8, 64).

Isolates of ST1, ST2 and ST4 are identical in humans and animals. These identical isolates are evidence of human-animal transmission. ST3 is said to have high host specificity, as its isolates cannot be clustered due to differences between human-based ST3 and animal-based ST3 (44). Considering all subtypes, ST1, ST2, ST3 and ST4 make up 90% of all subtype isolates diagnosed in humans around the world (7, 65). In Europe, ST3 is the most frequently found subtype, followed by ST4. For no obvious reasons, ST4 occurs seldomly in Asia, South America and Africa (7, 8, 66). ST1, ST2, ST4 and ST8 have been suggested to be potentially pathogenic due to their occurrence in patients with severe symptoms. Nevertheless, several animal studies support pathogenic and non-pathogenic behaviour of ST3 and ST4 (67, 68, 69, 70, 71, 72). In cases of urticaria the most abundant subtypes occurring in the host are ST3 and ST2. This disease could be caused by or made worse due to an inflammatory response of the host trying to fight off the parasite (73). ST5 and ST9 are common within animal hosts, but are rare in human hosts. This implies a zoonotic transmission path between humans and animals (74). ST15 and ST17 are found almost exclusively in reptiles and insects (75). How subtypes spread around the world, why certain subtypes are only found in specific hosts, and why there is an absence of some subtypes in specific areas remain unclear and these questions need further investigation.

1.4.2. *Blastocystis* in HIV positive patients

HIV sero-positive patients with diarrhoea show a higher prevalence of *B. hominis* (76) than HIV-negative patients. Hence, colonization by *Blastocystis* might be a possible explanation of some cases of diarrhoea in the HIV-positive patient's. People with AIDS, and HIV both tend to develop chronic diarrhoea (77, 78). A possible explanation is that *B. hominis* suppresses immunological responses, preventing non-specific intestinal defence mechanisms from working (79, 80). In a study of 318 HIV-positive patients with diarrhoea (> 4 weeks) in Indonesia, 73.6% tested positive for the parasite (79). Another study of HIV/AIDS patients found a lower percentage of patients were positive for *B. hominis* (19.8%) (80). The isolates were identified as ST3 (55%), ST4 (25%), ST1 (15%), and ST2 (5%). Although the prevalence of the parasite in the general population of people with HIV does not differ from non HIV/AIDS patients, it could nevertheless be a pathogen causing diarrhoea in immunosuppressed patients (80). Further investigation is needed with comparisons to healthy individuals to define the role of *B. hominis* in immunosuppressed patients.

1.4.3. *Blastocystis hominis* in IBS and IBD patients

Similar symptoms, including abdominal pain, nausea, and diarrhoea, occur in both *B. hominis* infection and in irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) patients. Several recent studies have tried to prove a link between these chronic gastrointestinal illnesses and the parasite, including the possibility of a role for IL-8 and IL-10 gene polymorphisms. The weakened and altered microbial environment in the intestine of IBS patients might promote a favourable surrounding for *B. hominis* to thrive (17, 81). A study (82) consisting of 27 patients with IBD & IBS (6 IBD & 21 IBS patients) reported that 67% were positive for *B. hominis* infection: 33% (2/6) were IBD patients and 76% (16/21) were IBS patients. The IBS *B. hominis*-positive cases proved to be ST2 and ST3 isolates. Another study from Pakistan (83) identified 53% of their IBS patients to be positive with *Blastocystis* infection. Nevertheless, no direct proof of *B. hominis* as the causative agent for the symptoms in IBS and IBD patients has yet been obtained and this requires further study (82, 83, 84). Moreover, comparison of *Blastocystis* prevalence in healthy individuals in the corresponding population would be necessary.

1.5 *Blastocystis* pathogen, commensal or beneficial symbiont

Years of studying *Blastocystis* have brought up new insights and cast doubts about its so-called pathogenic nature. *B. hominis* has been associated with gastrointestinal issues, but recent studies have found this protists more and more frequently in healthy individuals. This indicates that *B. hominis* may have been falsely described as a pathogen because of the lack of comparison studies of healthy individuals. This calls into question whether or not *B. hominis* is a beneficial symbiont of our microbiome (85, 86).

Another key reason that reinforced doubt in regards to the status of *B. hominis* as pathogen or symbiont is the missing specific pathology typical for parasites (87). Studies of the intestinal microbial fauna affirm that the eukaryote *B. hominis* is a common part of the human intestinal microbiome (85, 88). Furthermore, many eukaryotic organisms have been found to colonize the human gut as commensals over a long period of time, including *Entamoeba coli*, *Dientamoeba*, *Enteromonas hominis,* and *Retortamonas intestinalis*. Nevertheless, depending on the state of the host and gut ecosystem, the eukaryotic organisms could show parasitic behaviour. Nutritional and physical aspects of the host play an important role in this transition. Malnourishment and immunodeficiency increase the chances of parasitic diseases (89).

Recent studies have shown associations between intestinal microbial communities and *B. hominis*, forming enterotypes. These enterotypes are patterns in the microbiota with varying presence of different bacterial genera - *Bacteroides*, *Prevotella,* and *Ruminococcus*. In an environment of *Bacteroides* enterotype, the presence of *Blastocystis* is significantly reduced in comparison to *Ruminococcus* or *Prevotella* enterorypes. *Bacteroides* are not the main butyrate producers, which is needed for fermentation and an anaerobic environment in the gut. Disruption of butyrate production due to antibiotics and *Enterobacteriaceae,* increases luminal oxygen and nitrogen, creating an environment where *B. hominis* cannot thrive. Therefore, it is likely that the gut, whose microflora dominated by *Bacteroides*, is less likely to be colonized by *Blastocystis*. Further studies have noted a connection between low BMI and increased *Blastocystis*, as well as rich bacterial flora and a higher occurrence of *B. hominis* (89, 90, 91, 92, 93, 94, 95, 96).

Industrialized regions are directly linked with lower bacterial and eukaryotic microbiome in human intestines as a consequence of improved hygiene, sanitary conditions, and medications. Lower diversity can lead to increased gastrointestinal issues once microbial eukaryotes, for example *Blastocystis*, enter the host (86). Colonization of host intestines varies with health inequalities, age differences, and geographical regions. This indicates a more complex relationship between the host and the gut microbiome (86, 97, 98). Considering all the facts, the term parasite might not be suitable for all eukaryotes residing in the gut, and should be replaced with symbiont, which encompasses all types of organism-host interactions – parasitism, commensalism and mutualism (89).

Medication and industrially-produced food play an important role in the disturbances and consequently the potentially parasitic behaviour of eukaryomes (the eukaryotic members of the microbiome community). Dysbiosis of the sensitive human gut could lead to severe issues. The human gut is one of the least explored and understood organs and further studies are needed in order to gain a better understanding of this complex subject. The role of *Blastocystis* as a parasite, commensal or symbiont, could be defined by the current state of the microbiome and by the genetic or cultural background of the host. There might also be specific strains of *Blastocystis* that are pathogenic and others that are not. More information regarding specific interactions between *Blastocystis* and the host is necessary to elucidate these questions.

1.6 Treatment

Due to the various *B. hominis* subtypes and patient specific issues, finding the right treatment is challenging. Nevertheless, the most commonly used antimicrobial drug is metronidazole. Several distinctly different regimes, including amounts of 250 to 750 mg three times a day for 10 days and 1.5 mg/day for 10 days have been applied with mixed results (12, 99, 100, 101, 102, 103). Furthermore, combined and non-combined dosages with paromomycin, TMP-SMX, nitazoxanide, emetine, iodochlorhydroxyquin, pentamidine, furazolidone, and secnidazolek have been used without proving 100% efficacy in all cases (99, 104, 105, 106, 107, 108). Treatment with only paramomycin, an antimicrobial for acute and chronic intestinal amoebiasis, was shown to be highly effective in all studies (16, 101, 109, 110, 111) yet, metronidazole remains the preferred treatment due to the fact that paramomycin does not attack the parasite itself, but destroys the bacterial flora in the intestine of the patient, making it impossible for the parasite to reside. The controversial cotrimoxazole has shown mixed results in different studies: in one, it had high efficacy, with over 90% diminished symptoms, but in another, its efficacy was as low as 22% (99, 112). Drug resistance raises another challenge, as *B. hominis* cysts have exhibited resistance to up to 5mg/ml of metronidazole (113, 114, 115). In addition, drug efficacy is highly dependent on the specific subtype of *B. hominis* (116).

1.7 Cysteine protease

Protease activity and IL-8 gene expression are linked to pathogenic behaviour in various parasites including *Giardia*, *Trichomonas*, *Trypanosoma*, *Entamoeba*, *Leishmania,* and *Plasmodium*, *Helicobactor pylori*, *Escherichia coli,* and *Bacteroides fagilis* (117, 118, 119, 120, 121). Cysteine proteases promote the degradation of IkBs, which releases NF-kB transcription factors. These transcription factors translocate to the nucleus and activate IL-8 gene expression in colonic epithelial T84 cells (producing CXC chemokine). Once the IL-8 genes are activated, they guide polymorphonuclear leukocytes to inflamed areas of the intestine and activate monocytes. To prove the link between *B. hominis* infections and IL-8 gene expression, a study was performed with lysates of *B. ratti* WR1. In comparison to the control group, the WR1 lysate showed high protease activity. Further testing showed that inhibition of IL-8 mRNA was possible by using the cysteine protease inhibitors iodoacetamide (50 μM) and E-64 (10 μM) and antiprotozoal drug treatment with metronidazole (10 μg/ml) (122). Furthermore, cysteine as well as aspartic proteinases are responsible for degradation of the immunoglobulin (including IgA) defence. Human secretory IgA plays a role in the eradication of pathogens and if it is lacking, it is easier for the parasites to thrive in the host (123, 124).

1.8 Blastocystis genome

It has already been established that the known subtypes of *Blastocystis hominis* vary between their genomes and they show differences even within the same classification. STs diversity comes from their genome size, the number of introns, the gene content and their guanine-cytosine (GC) content. Each subtype has around 6.2% to 20.5% unique proteins with which they can be identified. It has been suggested that the significant variations in distribution and sizes within gene families of protein kinase and protease might play a role in their virulence factor (125). Another feature of *Blastocystis*, is its potential to generate stop codons by polyadenylation (126).

2. AIM

In-depth study of the interactions between *B. hominis* and the intestinal environment are necessary in order to gain a better understanding of the role of *Blastocystis* in the gut ecosystem and its status as pathogen, commensal, or beneficial symbiont. For such study, we need a traceable experimental infection system of *B. hominis*. Our collaborator, Katerina Jirku, has developed an infection model system of *B. hominis* in rats. The main goal of my thesis is to establish a method for examining the gene expression patterns of *Blastocystis* in the host environment. The biggest problems to overcome include successful isolation of RNA from faecal matter to be used for RNAseq sequencing and the recovery of a strong enough *Blastocystis* signal in the data. This will allow for future studies conducting in-vivo analyses of *B. hominis* to determine changes in the RNA expression patterns and the associated alterations in the metabolism of the rat gut microbiome, as well as to pinpoint putative interaction points.

Specific aims:

1. Test isolation protocols for RNA extraction of rat gut content
2. Test whether an efficient amount of *Blastocystis* data can be recovered from gut content of *Blastocystis*-infected rats

3. MATERIALS & METHODS

3.1. Cultures

Four rats were infected with *Blastocystis hominis* ST3 using the oral-faecal route. Three weeks post infection, stool samples were obtained for cultivation and RNA extraction. The cultures were passaged for a period of two weeks, while continuously checked for signs of *Blastocystis* under a light microscope, in order to confirm *B. hominis* infection. The medium used for cultivation was Jones' Medium. Caecum gut content was collected from sacrificed infected rats for RNA extraction and sequencing.

3.2. Cultivation

For the cultivation of *B. hominis*, Jones' Medium was used.

Procedure:

First, separate solutions of 0.946 g Na2HPO4 and 0.908 g KH2PO4 in 100 ml of distilled water was produced. Furthermore, 1.8 g of NaCl was dissolved in 200 ml of distilled water. The medium was prepared by mixing appropriate amounts of each solution (31.2 ml Na2HPO4, 10.4 ml KH2PO4 and 187.5 ml NaCl). Next, 0.23 g of yeast extract was added to the mixture, and the solution and was sterilized by autoclaving at 121°C (pressure at 101.5 kPA). After cooling, inactivated horse serum was added to a concentration of 10% of the final volume. The medium was then distributed in 4 ml amounts into sterile culture tubes and stored at 40°C.

3.3. Gut samples

Seven weeks post infection, samples from the gut (specifically the caecum, which has previously been shown to contain the large amounts of active *Blastocystis* cells) were collected. The rats were sacrificed and caecum gut content was immediately pushed out into a tube pre-filled with 5 ml of TRIzol reagent. Samples were immediately stored at -80°C until RNA extraction could be performed. A detailed description of the RNA extraction procedures can be found in section 3.5.

3.4. Collection and storage of the samples

All stool samples were collected by hand, put into Jones' Medium (see 3.2) and stored in an incubator at 37.5°C. The content of the rat gut was collected and stored in 12 ml tubes and frozen in liquid TRIzol at -80°C in collaboration with K. Jirku's Laboratory.

3.5. RNA extraction

The *in vitro* cultures from the stool samples were used as a pre-evaluation test to confirm *Blastocystis* infection and were not important for further investigation. The RNA extraction was focused on the gut content of the rats. We tested two protocols: one that follows regular RNA extraction using TRIzol reagent and a second where the TRIzol protocol was stopped after the protein extraction step and the RNA-containing supernatant was used as input into a Qiagen RNA mini prep kit (Qiagen, USA). For both protocols, we have used Chloroform and BCP for protein extraction.

Sample preparation

a. Fill up tube (containing the sample) to 10 ml with TRIzol.
b. Shake vigorously 15-30 s and split in half – 5 ml in each of 2 new 10 ml tubes.
c. Fill up again to 10 ml with TRIzol.
d. Centrifuge at 4200 rpm for 2 min at 4°C.
e. Transfer clean supernatant into fresh tube.

RNA isolation

1. **RNA extraction using Trizol Reagent Protocol only**

a. Add 0.2 ml of BCP/Chloroform per 1ml of Trizol and vigorously shake for 15-30 s.
b. Leave sample for 5 min at room temperature.
c. Centrifuge at 11 000 x g for 25 min at 4°C.
d. Transfer supernatant to a fresh tube.

e. Add 750 µl isopropyl alcohol.
f. Invert each sample carefully a few times and leave for 10 min at room temperature.
g. Centrifuge at 11 000 x g for 25 min at 4°C and discard the supernatant. The pellet contains the purified RNA.
h. Add 1 ml of ethanol (75%) to wash the pellet.
i. Centrifuge at 11 000 x g for 5 min at 4°C and discard the supernatant.
j. Repeat steps h. and i. 3 times.

The resulting pellet was left to dry from the leftover ethanol. For further analysis, 65 µl of RNase-free water was added.

2. RNA isolation of gut samples via TRIzol and BCP (or Chloroform) and an RNA Kit

f. Add 0.2 ml of BCP/Chloroform per 1ml of Trizol and vigorously shake for 15-30 s.
g. Shake vigorously again 15-30 s and leave it 5 min at room temperature.
h. Centrifuge the sample at 11 000 x g for 25 min at 4°C.
i. Transfer the supernatant containing the RNA to a fresh tube.
j. Continue with the protocol according to the Qiagen RNA Kit.

Extracted RNA was then evaluated for purity by NanoDrop, and for quantity measurements by Qubit. Selected samples were sent to a commercial company (Macrogen, Korea) for sequencing library construction (mRNA TruSeq) and sequencing on the HiSeq Illumina platform with 2x100bp reads.

3.6. Processing of data

The sequencing library construction and sequencing itself on the Illumina HiSeq platform (2x 100bp) were outsourced to Macrogen (Korea). The quality of raw reads was first investigated using the Fast program (127). Software Trimmomatic (128) with default settings was used to quality and adapter trim all reads. Reads were then assembled into transcripts using Trinity software with default settings (129). Each transcript was then "blasted" against the NCBI NR database (current as of September 2019) using the program

Diamond ("blastx" search mode with maximum e-value threshold set to e-5) (130). Taxonomic information for the top hit of each transcript was recovered using a python script: in short, accession numbers of all top hits are "translated" into NCBI taxonomic IDs with the blastdbcmd program (131). The taxonomic ID is then translated into taxonomy via an in-house script using a locally-installed NCBI taxonomic mysql database. The hits were considered to be of *Blastocystis* origin if the top hit matched any *Blastocystis* sequence, or host-derived if the top hit matched any *Rattus* sequence.

Reads were mapped to transcripts of *Blastocystis* origin and *Rattus* origin using the program Bowtie2 (132) with default settings. Indexes for Bowtie mapping were also prepared using default settings. Reads were also mapped to *Rattus norvegicus* genome (NCBI Refseq database) using the splicing/intron aware mapper HiSat2 (133) with default settings (here the *Rattus* genome index was downloaded from the HiSat website: https://daehwankimlab.github.io/hisat2/download/). It was not possible to map reads to the available *Blastocystis* genome, as the isolate used for the infection experiment is too divergent for efficient mapping.

4. RESULTS

4.1. RNA extraction success rate

Caecum content was collected from four rats, kept in Trizol, and RNA was extracted according to section 3.5. The extraction via Trizol and Chloroform/BCP for gut content samples showed unreliable results and failed repeatedly for some samples. The protocol where TRIzol protocol was interrupted and followed by usage of Quiagen RNA extraction kit failed completely. However, we have obtained two total RNA samples for Illumina sequencing that were sent for library construction and sequencing – RBA-0007 (250 ng/µl) and RBA-0019 (265 ng/µl).

4.2. Sequencing & assembling results

Reads were quality and adapter processed prior to assembly, and trimmed by 9bp at the beginning, as the first nine base pairs showed strong compositional bias. Overall, we have obtained ~24 million and ~19 million reads for sample RBA-0007 and RBA-0019, respectively. Assembly of each dataset by Trinity produced ~30 000 and ~28 000 thousand putative transcripts. Of those, ~23 000 and 19 000 produced hits against the NCBI NR database using the Diamond "blastx" search (e-value threshold 0.00001, current as of September 2019). See table 1 for a detailed description of the assembly statistics.

Table 1: *overview of assembly and blast analyses*

	RBA-0007	**RBA-0019**
Total reads	24115109	19186273
Total contigs	30486	27931
Contigs with hits	23090	19994
***Blastocystis* contigs**	11516	11606
Rat contigs	2522	2994

No hit contigs	7396	7937
Other species hits	9052	5394

Parsing of the Diamond blastx search identified contigs that most likely originated from *Blastocystis* (both datasets ~11 000 transcripts), the host (*Rattus norvegicus*; ~2 500 and ~2 900 transcripts) and contaminants (~9 000 and ~5 000 contigs). The "contaminants" were mostly of prokaryotic origin, likely contamination from the sample itself, or human origin, which is either contamination originating from the sequencing centre or the experimental worker.

In order to gain a better understanding of the proportion of reads originating from *Blastocystis* and the host, we mapped reads from both sequencing datasets against each set of transcripts (i.e. of *Blastocystis* and *Rattus* origin). This resulted in 86.5 % and 84.2 % of reads (for RBA-0007 and RBA-0019, respectively) which were mapped to *Blastocystis*, while only 1.16 % and 1.6 % of the reads (for RBA-0007 and RBA-0019, respectively) mapped to contigs originating from the host (see Table 2).

Table 2: Mapping of reads to contigs of Blastocystis and Rattus origin

	RBA-0007 #reads	**RBA-0007 %reads**	**RBA-0019 #reads**	**RBA-0019 %reads**
***Blastocystis* mapped**	20864714	86.52%	16166853	84.26%
***Rattus* mapped**	280110	1.16%	317732	1.6%

We also mapped the reads directly to the *Rattus norvegicus* genome, which resulted in a mapping of 1.9 % and 2.3 % of the reads (for RBA-0007 and RBA-0019, respectively). The Diamond analyses have also shown that our *Blastocystis* isolate is quite divergent from the publicly available genomes with as low as 85% similarity score to the closest sequenced species.

Table 3: Percentages of assembled data to contigs and genome of Rattus novegicus

	Assembled *Rattus* data	***Rattus novegicus***
RBA-0007	1.16%	1.9%
RBA-0019	1.6%	2.3%

Comparing only the contig hits, the amount of *Blastocystis* contigs are relatively high in comparison to the host contigs with 49.87% and 58.05% for RBA-0007 and RBA-0019, respectively. The distribution is not equal in both samples and reinforces the observed sample specificity in this experiment.

Table 4: Distribution of contig hits

	RBA-0007 #	**RBA-0007 %**	**RBA-0019 #**	**RBA-0019 %**
Contigs with hits	23090	100%	19994	100%
***Blastocystis* contigs**	11516	49,87%	11606	58.05%
***Rattus* contigs**	2522	10.92%	2994	14.97%
Other species hits	9052	39.20%	5394	26.97%

5. DISCUSSION

Research on the composition of bacterial microflora – the gut microbiome – has recently become a mainstream topic in microbial ecology and has now basically formed its own field (95) with hundreds of thousands of published studies. However, understanding the role of microbial eukaryotes (eukaryome) in gut ecosystems remains completely elusive. As previously mentioned, one of these microbial eukaryotes is *Blastocystis hominis*, whose role as a commensal, parasite, or beneficial symbiont in the human gut remains entirely unclear with results that are commonly conflicting (85, 86). This has become very apparent recently, as many studies of the human gut microbiome have revealed the presence of *Blastocystis* in individuals with no gastrointestinal distress (85). Understanding the role of *Blastocystis* is important for the proper treatment of potential patients with gastrointestinal distress that are colonized by *Blastocystis*. The goal of my study was to test whether it is possible to study the functional genomics of *Blastocystis in-vivo*. This is a crucial step for future larger *in-vivo* studies of *Blastocystis* to develop a better understanding of its role in the gut ecosystem.

We collected caecum content from four rats infected by *Blastocystis* (infection was confirmed by culturing from the faecal samples), and stored the samples in TRIzol for future RNA extraction. Storage in TRIzol has been demonstrated to be a somewhat unfortunate choice, as only two samples produced amounts of pure total RNA high enough for further analysis. This cannot be simply explained by insufficient laboratory practice, as we have persistently repeated the failed samples only to fail again, while other samples repeatedly produced decent results. Retrospectively, this is not surprising as faecal matter is known to contain many compounds impeding RNA isolation and specialized kits are commonly used. The two successful samples produced 250 ng/µl (total ~5µg) and 265 ng/µl (total ~5.2 µg) of total RNA.

The main reason to perform this study was to establish, whether RNA extracted from caecum content will contain enough mRNA from *Blastocystis* for us to be able to perform downstream bioinformatics analyses of gene expression levels. We expected two basic issues: the sample could have been overpowered by bacterial contamination or by host data. The bacterial contamination is less of a concern as polyA-selected RNA is used for library construction and most bacterial RNAs do not possess polyA tails or stretches. An

overwhelming presence of data originating from host cells would be a much bigger concern as, for example in the case of a similar experiment with *Giardia intestinalis* only about 6% of reads originated from the parasite and the rest were mostly from the host (mouse model) (134, 135). Fortunately, neither bacterial nor host contamination was an issue in this study.

Given that our *Blastocystis* isolate is highly divergent from the published genomes, we could not map the reads to the genome. This is not surprising since *Blastocystis* isolates, even with the same subtype, tend to differ greatly from each other (125). Instead, we assembled transcriptomes, identified *Blastocystis* transcripts and mapped the reads against them. About 50-58% of assembled transcripts were of *Blastocystis* origin and mapping of reads showed that over 80% of reads came from *Blastocystis*. Therefore, the majority of the reads are from *Blastocystis*. This also suggests, that this eukaryotic microbe resides very commonly in the caecum content and is likely present in larger quantities. It also suggests that it is not attached to the host tissue, or that not many cells are attached to the gut tissue, as we have only analysed the content and not the tissue itself. Even though we only analysed data from two independent individuals, the high percentage of *Blastocystis*-originating reads shows that larger experiments following rats for a period of several months post-colonization by *Blastocystis* and analysing the differential expression patterns of *Blastocystis* is possible.

After finishing the wet lab part of my thesis work two years ago, the team has since established a reliable extraction protocol using a commercial kit (ZymoBiomics DNA/RNA Miniprep Kit Zymo Research, USA), and have further continued their research on *Blastocystis*.

6. CONCLUSION

Our work has shown that RNA extraction of *Blastocystis* from rat gut content is possible. The procedure and extraction protocol with TRIzol worked, but it is not reliable, resulting in repeated failures to extract a sufficient amount of RNA. On the other hand, adequate amounts of *Blastocystis*-originating reads were obtained from two samples by mRNA sequencing. Furthermore, we have shown that relatively low host and bacterial contamination can be achieved. This knowledge has allowed the execution of larger experiments with two rat cohorts (*Blastocystis* negative and colonized). The transcriptomic studies of *Blastocystis* and the gut microflora will help us understand the role of *Blastocystis* in the gut microbial ecosystem of human beings.

7. REFRENCES

1) Alfellani, Mohammed A., et al. "Genetic diversity of *Blastocystis* in livestock and zoo animals." *Protist* 164.4 (2013): 497-509.
2) Forsell, Joakim, et al. "The relation between *Blastocystis* and the intestinal microbiota in Swedish travellers." BMC microbiology 17.1 (2017): 231.
3) Silberman, Jeffrey D., et al. "Human parasite finds taxonomic home." *Nature* 380.6573 (1996): 398-398.
4) Zierdt, C. H. "*Blastocystis hominis*, a long-misunderstood intestinal parasite." *Parasitology Today* 4.1 (1988): 15-17.
5) ARISUE, NOBUKO, et al. "Phylogenetic position of *Blastocystis hominis* and of stramenopiles inferred from multiple molecular sequence data." *Journal of Eukaryotic Microbiology* 49.1 (2002): 42-53.
6) Zierdt, Charles H. "*Blastocystis hominis*--past and future." Clinical Microbiology Reviews 4.1 (1991): 61-79.
7) Alfellani, Mohammed A., et al. "Variable geographic distribution of *Blastocystis* subtypes and its potential implications." *Acta tropica* 126.1 (2013): 11-18.
8) Ramírez, Juan David, et al. "Geographic distribution of human *Blastocystis* subtypes in South America." *Infection, Genetics and Evolution* 41 (2016): 32-35.
9) Popruk, Supaluk, et al. "Subtype distribution of *Blastocystis* in Thai-Myanmar border, Thailand." *The Korean journal of parasitology* 53.1 (2015): 13.
10) Wawrzyniak, Ivan, et al. "*Blastocystis*, an unrecognized parasite: an overview of pathogenesis and diagnosis." *Therapeutic advances in infectious disease* 1.5 (2013): 167-178.
11) Horiki, Noriyuki, et al. "Epidemiologic survey of *Blastocystis hominis* infection in Japan." *The American journal of tropical medicine and hygiene* 56.4 (1997): 370-374.
12) Tasova, Yesim, et al. "Clinical significance and frequency of *Blastocystis hominis* in Turkish patients with hematological malignancy." *Acta Medica Okayama* 54.3 (2000): 133-136.
13) Doyle, P. W., et al. "Epidemiology and pathogenicity of *Blastocystis hominis*." *Journal of Clinical Microbiology* 28.1 (1990): 116-121.
14) Kaya, Selçuk, et al. "Pathogenicity of *Blastocystis hominis*, a clinical reevaluation." *Turkiye Parazitol Derg* 31.3 (2007): 184-7.

15) El-Shazly, A. M., et al. "*Blastocystis hominis* among symptomatic and asymptomatic individuals in Talkha Center, Dakahlia Governorate, Egypt." *Journal of the Egyptian Society of Parasitology* 35.2 (2005): 653-666.
16) Tan, Kevin SW. "New insights on classification, identification, and clinical relevance of *Blastocystis spp*." *Clinical microbiology reviews* 21.4 (2008): 639-665.
17) Stark, D., et al. "Irritable bowel syndrome: a review on the role of intestinal protozoa and the importance of their detection and diagnosis." International journal for parasitology 37.1 (2007): 11-20.
18) Yakoob, Javed, et al. "Irritable bowel syndrome: is it associated with genotypes of *Blastocystis hominis*." Parasitology research 106.5 (2010): 1033-1038.
19) Stenzel, D. J., and P. F. Boreham. 1996. *Blastocystis hominis* revisited. Clin. Microbiol. Rev. 9:563–584.
20) Stenzel, D. J., P. F. Boreham, and R. McDougall. 1991. Ultrastructure of *Blastocystis hominis* in human stool samples. Int. J. Parasitol. 21:807–812.
21) Lee, M. G., and D. J. Stenzel. "A survey of *Blastocystis* in domestic chickens." *Parasitology research* 85.2 (1999): 109-117. 12
22) YOSHIKAWA, HISAO, NOBUKO KUWAYAMA, and YOSHIMI ENOSE. "Histochemical detection of carbohydrates of *Blastocystis hominis*." *Journal of Eukaryotic Microbiology* 42.1 (1995): 70-74.
23) Yoshikawa, Hisao, Junko Satoh, and Yoshimi Enose. "Light and electron microscopic localization of lipids in *Blastocystis hominis*." *Microscopy* 44.2 (1995): 100-103.
24) Stenzel, D. J., L. A. Dunn, and P. F. L. Boreham. "Endocytosis in cultures of *Blastocystis hominis*." *International journal for parasitology* 19.7 (1989): 787-791.
25) MacPherson, D. W., and W. M. MacQueen. "Morphological diversity of *Blastocystis hominis* in sodium acetate-acetic acid-formalin-preserved stool samples stained with iron hematoxylin." Journal of clinical microbiology 32.1 (1994): 267-268.
26) Cassidy, M. F., D. J. Stenzel, and P. F. L. Boreham. "Electron microscopy of surface structures of *Blastocystis sp*. from different hosts." Parasitology research 80.6 (1994): 505-511.
27) Zaman, Viqar, Josephine Howe, and M. Ng. "Observations on the surface coat of *Blastocystis hominis*." Parasitology research 83.7 (1997): 731-733.
28) Matsumoto, Yoshitsugu, Minoru Yamada, and Yukio Yoshida. "Light-microscopical appearance and ultrastructure of *Blastocystis hominis*, an intestinal parasite of

man." *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. Series A: Medical Microbiology, Infectious Diseases, Virology, Parasitology* 264.3-4 (1987): 379-385.

29) Suresh, K., et al. "A multiple fission-like mode of asexual reproduction in *Blastocystis hominis*." Parasitology research 80.6 (1994): 523-527.

30) Tan, Henry K., and Charles H. Zierdt. "Ultrastructure of *Blastocystis hominis*." Zeitschrift für Parasitenkunde 42.4 (1973): 315-324.

31) Tan, T. C., and K. G. Suresh. "Amoeboid form of *Blastocystis hominis*—a detailed ultrastructural insight." Parasitology research 99.6 (2006): 737-742.

32) Zhang, Xu, et al. "Ultrastructural insights into morphology and reproductive mode of *Blastocystis hominis*." Parasitology research 110.3 (2012): 1165-1172.

33) Tan, T. C., and K. G. Suresh. "Predominance of amoeboid forms of *Blastocystis hominis* in isolates from symptomatic patients." Parasitology research 98.3 (2006): 189-193.

34) Yoshikawa, Hisao, et al. "Fecal-oral transmission of the cyst form of *Blastocystis hominis* in rats." Parasitology research 94.6 (2004): 391-396.

35) Chen, X. Q., et al. "In vitro encystation and excystation of *Blastocystis ratti*." Parasitology 118.2 (1999): 151-160.

36) Moe, K. T., et al. "Development of *Blastocystis hominis* cysts into vacuolar forms in vitro." Parasitology research 85.2 (1999): 103-108.

37) Moe, K. T., et al. "Observations on the ultrastructure and viability of the cystic stage of *Blastocystis hominis* from human feces." Parasitology research 82.5 (1996): 439-444.

38) Stenzel, D. J., and P. F. L. Boreham. "A cyst-like stage of *Blastocystis hominis*." International journal for parasitology 21.5 (1991): 613-615.

39) Zaman, V., J. Howe, and M. Ng. "Ultrastructure of *Blastocystis hominis* cysts." Parasitology Research 81.6 (1995): 465-469.

40) Zaman, V., J. Howe, and M. Ng. "Scanning electron microscopy of *Blastocystis hominis* cysts." Parasitology research 84.6 (1998): 476-477.

41) Zhang, Xu, et al. "Study on morphology of *Blastocystis hominis* in culture and from diarrhea patients." Zhongguo ji sheng chong xue yu ji sheng chong bing za zhi= Chinese journal of parasitology & parasitic diseases 21.2 (2003): 116-118.

42) Moe, K. T., et al. "Experimental *Blastocystis hominis* infection in laboratory mice." Parasitology research 83.4 (1997): 319-325.

43) Navarro, C., et al. "High prevalence of *Blastocystis sp*. in pigs reared under intensive growing systems: frequency of ribotypes and associated risk factors." Veterinary parasitology 153.3-4 (2008): 347-358.

44) Noël, Christophe, et al. "Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis." Journal of clinical microbiology 43.1 (2005): 348-355.

45) Rivera, Windell L. "Phylogenetic analysis of *Blastocystis* isolates from animal and human hosts in the Philippines." Veterinary parasitology 156.3-4 (2008): 178-182.

46) Yan, Yiming, et al. "*Blastocystis sp*. subtype 5: a possibly zoonotic genotype." Parasitology research 101.6 (2007): 1527-1532.

47) Zhang, X., et al. "Morphology and reproductive mode of *Blastocystis hominis* in diarrhea and in vitro." Parasitology research 101.1 (2007): 43-51.

48) Khan, Naveed Ahmed, ed. Emerging protozoan pathogens. Taylor & Francis, 2008.

49) ZIERDT, CHARLES H., and JUDITH C. SWAN. "Generation time and growth rate of the human intestinal parasite *Blastocystis hominis*." The Journal of protozoology 28.4 (1981): 483-485.

50) Ash, L. R., and T. C. Orihel. "*Blastocystis hominis* and fecal elements." *ln L. R Ash and TC Orihel Atlas of Human Parasitology. 3rd cd. American Socisty of Clinical Pathologists Press, Chicago* (1990): 88-89.

51) Boreham, Peter FL, and Deborah J. Stenzel. "*Blastocystis* in humans and animals: morphology, biology, and epizootiology." Advances in parasitology. Vol. 32. Academic Press, 1993. 1-70.

52) Casemore, D. P. "Foodborne protozoal infection." *Lancet (British edition)* 336.8728 (1990): 1427-1432.

53) Garcia, Lynne Shore. Diagnostic medical parasitology. Vol. 49. John Wiley & Sons, 2016.

54) Kain, Kevin C., et al. "Epidemiology and clinical features associated with Blastocystis hominis infection." *Diagnostic microbiology and infectious disease* 8.4 (1987): 235-244.

55) Lambert, Michel, J. Gigi, and C. Bughin. "Persistent diarrhoea and *Blastocystis hominis*." *Acta Clinica Belgica* 47.2 (1992): 129-130.

56) Miller, Richard A., and Barbara H. Minshew. "*Blastocystis hominis*: an organism in search of a disease." *Reviews of infectious diseases* 10.5 (1988): 930-938.

57) Nimri, Laila, and Raymound Batchoun. "Intestinal colonization of symptomatic and asymptomatic schoolchildren with *Blastocystis hominis*." *Journal of Clinical Microbiology* 32.11 (1994): 2865-2866.

58) Singh, M., et al. "Elucidation of the life cycle of the intestinal protozoan *Blastocystis hominis*." *Parasitology research* 81.5 (1995): 446-450.

59) Zaman, V., et al. "Isolation of *Blastocystis* from the cockroach (Dictyoptera: Blattidae)." *Parasitology research (1987)* 79.1 (1993): 73-74.

60) Suresh, K., H. V. Smith, and T. C. Tan. "Viable *Blastocystis* cysts in Scottish and Malaysian sewage samples." Appl. Environ. Microbiol. 71.9 (2005): 5619-5620.

61) Zaki, Mehreen, Viqar Zaman, and Nazar Ali Sheikh. "Resistance of *Blastocystis hominis* cysts to chlorine." *Journal of Pakistan Medical Association* 46.8 (1996): 178.

62) Suresh, K., and H. Smith. "Comparison of methods for detecting *Blastocystis hominis*." European Journal of Clinical Microbiology and Infectious Diseases 23.6 (2004): 509-511.

63) Sheehan, DANIEL J., B. G. Raucher, and JOHN C. McKITRICK. "Association of *Blastocystis hominis* with signs and symptoms of human disease." *Journal of clinical microbiology* 24.4 (1986): 548-550.

64) Jiménez, Paula Andrea, Jesús Eduardo Jaimes, and Juan David Ramírez. "A summary of *Blastocystis* subtypes in North and South America." Parasites & vectors 12.1 (2019): 376.

65) Poirier, Philippe, et al. "Development and evaluation of a real-time PCR assay for detection and quantification of *Blastocystis* parasites in human stool samples: prospective study of patients with hematological malignancies." *Journal of clinical microbiology* 49.3 (2011): 975-983.

66) Forsell, Joakim, et al. "High occurrence of *Blastocystis sp*. subtypes 1–3 and *Giardia intestinalis* assemblage B among patients in Zanzibar, Tanzania." *Parasites & vectors* 9.1 (2016): 370.

67) Hussein, Eman M., et al. "Pathophysiological variability of different genotypes of human *Blastocystis hominis* Egyptian isolates in experimentally infected rats." *Parasitology research* 102.5 (2008): 853-860.

68) Roberts, T., et al. "Subtype distribution of *Blastocystis* isolates identified in a Sydney population and pathogenic potential of *Blastocystis*." *European journal of clinical microbiology & infectious diseases* 32.3 (2013): 335-343.

69) Stensvold, C. R., et al. "Symptomatic infection with *Blastocystis sp*. subtype 8 successfully treated with trimethoprim–sulfamethoxazole." Annals of Tropical Medicine & Parasitology 102.3 (2008): 271-274.

70) Ramírez, Juan David, et al. "*Blastocystis* subtypes detected in humans and animals from Colombia." *Infection, Genetics and Evolution* 22 (2014): 223-228.

71) Domínguez-Márquez, M. Victoria, et al. "High prevalence of subtype 4 among isolates of *Blastocystis hominis* from symptomatic patients of a health district of Valencia (Spain)." *Parasitology research* 105.4 (2009): 949.

72) Stensvold, Christen Rune, et al. "*Blastocystis sp*. subtype 4 is common in Danish *Blastocystis*-positive patients presenting with acute diarrhea." *The American journal of tropical medicine and hygiene* 84.6 (2011): 883-885.

73) Katsarou-Katsari, Alexandra, et al. "Acute urticaria associated with amoeboid forms of *Blastocystis sp*. subtype 3." *Acta dermato-venereologica* 88.1 (2008): 80-81.

74) El Safadi, Dima, et al. "Children of Senegal River Basin show the highest prevalence of *Blastocystis sp*. ever observed worldwide." *BMC infectious diseases* 14.1 (2014): 164.

75) Teow, W. L., et al. "A *Blastocystis* species from the sea-snake, Lapemis hardwickii (Serpentes: Hydrophiidae)." *International journal for parasitology* 21.6 (1991): 723-726.

76) Gassama, Amy, et al. "Ordinary and opportunistic enteropathogens associated with diarrhea in Senegalese adults in relation to human immunodeficiency virus serostatus." International journal of infectious diseases 5.4 (2001): 192-198.

77) Stark, Damien, et al. "Prevalence of enteric protozoa in human immunodeficiency virus (HIV)–positive and HIV-negative men who have sex with men from Sydney, Australia." *The American journal of tropical medicine and hygiene* 76.3 (2007): 549-552.

78) Cimerman, Sérgio, Benjamin Cimerman, and David Salomão Lewi. "Prevalence of intestinal parasitic infections in patients with acquired immunodeficiency syndrome in Brazil." International Journal of Infectious Diseases 3.4 (1999): 203-206.

79) Kurniawan, Agnes, et al. "Intestinal parasitic infections in HIV/AIDS patients presenting with diarrhoea in Jakarta, Indonesia." *Transactions of the Royal Society of Tropical Medicine and Hygiene* 103.9 (2009): 892-898.

80) Roberts, Tamalee, et al. "Update on the pathogenic potential and treatment options for *Blastocystis sp*." *Gut pathogens* 6.1 (2014): 17.

81) Olivo-Diaz, Angelica, et al. "Findings related to IL-8 and IL-10 gene polymorphisms in a Mexican patient population with irritable bowel syndrome infected with Blastocystis." *Parasitology research* 111.1 (2012): 487-491.

82) Dogruman-Al, Funda, et al. "Comparison of methods for detection of *Blastocystis* infection in routinely submitted stool samples, and also in IBS/IBD Patients in Ankara, Turkey." *PloS one* 5.11 (2010).

83) Yakoob, Javed, et al. "*Blastocystis hominis* and *Dientamoeba fragilis* in patients fulfilling irritable bowel syndrome criteria." *Parasitology research* 107.3 (2010): 679-684.

84) Dogruman-Al, Funda, et al. "*Blastocystis* subtypes in irritable bowel syndrome and inflammatory bowel disease in Ankara, Turkey." *Memórias do Instituto Oswaldo Cruz* 104.5 (2009): 724-727.

85) Scanlan, Pauline D., et al. "The microbial eukaryote *Blastocystis* is a prevalent and diverse member of the healthy human gut microbiota." *FEMS microbiology ecology* 90.1 (2014): 326-330.

86) Parfrey, Laura Wegener, et al. "Communities of microbial eukaryotes in the mammalian gut within the context of environmental eukaryotic diversity." *Frontiers in microbiology* 5 (2014): 298.

87) Dunn, L. A., P. F. L. Boreham, and D. J. Stenzel. "Ultrastructural variation of *Blastocystis hominis* stocks in culture." *International journal for parasitology* 19.1 (1989): 43-56.

88) Scanlan, Pauline D., and Julian R. Marchesi. "Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and-independent analysis of faeces." *The ISME journal* 2.12 (2008): 1183-1193.

89) Lukeš, Julius, et al. "Are human intestinal eukaryotes beneficial or commensals?." *PLoS pathogens* 11.8 (2015).

90) Yeo, Soyoung, et al. "Influence of gastrointestinal stress on autoinducer-2 activity of two *Lactobacillus* species." *FEMS microbiology ecology* 91.7 (2015).

91) Nourrisson, Céline, et al. "*Blastocystis* is associated with decrease of fecal microbiota protective bacteria: comparative analysis between patients with irritable bowel syndrome and control subjects." *PloS one* 9.11 (2014).

92) Vital, Marius, Adina Chuang Howe, and James M. Tiedje. "Revealing the bacterial butyrate synthesis pathways by analyzing (meta) genomic data." *MBio* 5.2 (2014): e00889-14.

93) ZIERDT, CHARLES H. "Cytochrome‐Free Mitochondria of an Anaerobic Protozoan—*Blastocystis hominis*." *The Journal of protozoology* 33.1 (1986): 67-69.

94) Byndloss, M.X. et al. (2017) Microbiota-activated PPAR-gamma signaling inhibits dysbiotic Enterobacteriaceae expansion. Sci- ence 357, 570–575.

95) Stensvold, Christen Rune, and Mark van der Giezen. "Associations between gut microbiota and common luminal intestinal parasites." *Trends in parasitology* 34.5 (2018): 369-377.

96) Arumugam, Manimozhiyan, et al. "Enterotypes of the human gut microbiome." *nature* 473.7346 (2011): 174-180.

97) Röser, D., et al. "Dientamoeba fragilis in Denmark: epidemiological experience derived from four years of routine real-time PCR." *European journal of clinical microbiology & infectious diseases* 32.10 (2013): 1303-1310.

98) Krogsgaard, Laura Rindom, et al. "The prevalence of intestinal parasites is not greater among individuals with irritable bowel syndrome: a population-based case-control study." *Clinical Gastroenterology and Hepatology* 13.3 (2015): 507-513.

99) Moghaddam, Davood Dorostkar, E. Ghadirian, and M. Azami. "*Blastocystis hominis* and the evaluation of efficacy of metronidazole and trimethoprim/sulfamethoxazole." Parasitology research 96.4 (2005): 273-275.

100) Nassir, E., et al. "*Blastocystis hominis* as a cause of hypoalbuminemia and anasarca." *European Journal of Clinical Microbiology and Infectious Diseases* 23.5 (2004): 399-402.

101) Valsecchi, Rossano, Paolo Leghissa, and Vincenzo Greco. "Cutaneous lesions in *Blastocystis hominis* infection." *ACTA DERMATOVENEREOLOGICA-STOCKHOLM-*. 84.4 (2004): 322-323.

102) Cassano, Nicoletta, et al. "Remission of delayed pressure urticaria after eradication of *Blastocystis hominis*." ACTA DERMATOVENEREOLOGICA-STOCKHOLM- 85.4 (2005): 357.

103) Nigro, Luciano, et al. "A placebo-controlled treatment trial of *Blastocystis hominis* infection with metronidazole." *Journal of travel medicine* 10.2 (2003): 128-130.

104) Pasqui, A. L., et al. "Chronic urticaria and *Blastocystis hominis* infection. A case report." *European review for medical and pharmacological sciences* 8 (2004): 117-120.

105) Andiran, Nesibe, et al. "*Blastocystis hominis*—an emerging and imitating cause of acute abdomen in children." Journal of pediatric surgery 41.8 (2006): 1489-1491.

106) Cimerman, Sérgio, Maria Cecília Teixeira Ladeira, and Wilma Assunção Iuliano. "Blastocystosis: nitazoxanide as a new therapeutic option." *Revista da Sociedade Brasileira de Medicina Tropical* 36.3 (2003).

107) Diaz, Elvia, et al. "Epidemiology and control of intestinal parasites with nitazoxanide in children in Mexico." *The American journal of tropical medicine and hygiene* 68.4 (2003): 384-385.

108) Rossignol, Jean–FranÇois, et al. "Effect of nitazoxanide in persistent diarrhea and enteritis associated with *Blastocystis hominis*." *Clinical Gastroenterology and Hepatology* 3.10 (2005): 987-991.

109) Armentia, A., et al. "Urticaria by *Blastocystis hominis*. Successful treatment with paromomycin." *Allergologia et immunopathologia* 21.4 (1993): 149-151.

110) Kick, Gerold, F. Rueff, and Bernhard Przybilla. "Palmoplantar pruritus subsiding after *Blastocystis hominis* eradication." *Acta dermato-venereologica* 82.1 (2002): 60-60.

111) Pegelow, K., et al. "Parasitological and nutritional situation of school children in the Sukaraja district, West Java, Indonesia." The Southeast Asian journal of tropical medicine and public health 28.1 (1997): 173-190.

112) Ok, Ülgen Z., et al. "Effect of trimethoprim-sulfamethaxazole in *Blastocystis hominis* infection." *The American journal of gastroenterology* 94.11 (1999): 3245-3247.

113) Yakoob, Javed, et al. "In vitro susceptibility of Blastocystis hominis isolated from patients with irritable bowel syndrome." British journal of biomedical science 61.2 (2004): 75-77.

114) Haresh, K., et al. "Isolate resistance of *Blastocystis hominis* to metronidazole." *Tropical Medicine & International Health* 4.4 (1999): 274-277.

115) Zaman, V., and M. Zaki. "Resistance of *Blastocystis hominis* cysts to metronidazole." Tropical Medicine & International Health 1.5 (1996): 677-678.

116) Stensvold, Christen R., et al. "Eradication of *Blastocystis* carriage with antimicrobials: reality or delusion?." *Journal of clinical gastroenterology* 44.2 (2010): 85-90.

117) McKerrow, James H., et al. "The proteases and pathogenicity of parasitic protozoa." *Annual review of microbiology* 47.1 (1993): 821-853.

118) North, Michael J. "Comparative biochemistry of the proteinases of eucaryotic microorganisms." *Microbiological Reviews* 46.3 (1982): 308.

119) Beswick, Ellen J., et al. "The Helicobacter pylori urease B subunit binds to CD74 on gastric epithelial cells and induces NF-κB activation and interleukin-8 production." *Infection and immunity* 74.2 (2006): 1148-1155.

120) Dahan, Stephanie, et al. "Enterohemorrhagic Escherichia coli infection induces interleukin-8 production via activation of mitogen-activated protein kinases and the transcription factors NF-κB and AP-1 in T84 cells." *Infection and immunity* 70.5 (2002): 2304-2310.

121) Kim, Jung Mogg, et al. "Nuclear factor‐kappa B activation pathway in intestinal epithelial cells is a major regulator of chemokine gene expression and neutrophil migration induced by Bacteroides fragilis enterotoxin." *Clinical & Experimental Immunology* 130.1 (2002): 59-66.

122) Puthia, Manoj K., Jia Lu, and Kevin SW Tan. "*Blastocystis ratti* contains cysteine proteases that mediate interleukin-8 response from human intestinal epithelial cells in an NF-κB-dependent manner." *Eukaryotic cell* 7.3 (2008): 435-443.

123) Puthia, Manoj K., et al. "Degradation of human secretory immunoglobulin A by *Blastocystis*." *Parasitology research* 97.5 (2005): 386-389.

124) Huang, David B., and A. Clinton White. "An updated review on *Cryptosporidium* and *Giardia*." Gastroenterology Clinics 35.2 (2006): 291-314.

125) Gentekaki, Eleni, et al. "Extreme genome diversity in the hyper-prevalent parasitic eukaryote *Blastocystis*." *PLoS biology* 15.9 (2017): e2003769.

126) Klimeš, Vladimír, et al. "A large number of nuclear genes in the human parasite blastocystis require mRNA polyadenylation to create functional termination codons." *Genome biology and evolution* 6.8 (2014): 1956-1961.

127) Andrews, S. "A quality control tool for high throughput sequence data. FastQC." (2014).

128) Bolger, Anthony M., Marc Lohse, and Bjoern Usadel. "Trimmomatic: a flexible trimmer for Illumina sequence data." *Bioinformatics* 30.15 (2014): 2114-2120.)

129) Grabherr, Manfred G., et al. "Full-length transcriptome assembly from RNA-Seq data without a reference genome." *Nature biotechnology* 29.7 (2011): 644.).

130) Buchfink, Benjamin, Chao Xie, and Daniel H. Huson. "Fast and sensitive protein alignment using DIAMOND." *Nature methods* 12.1 (2015): 59.).

131) Altschul, Stephen F., et al. "Basic local alignment search tool." *Journal of molecular biology* 215.3 (1990): 403-410.

132) Langmead, Ben, and Steven L. Salzberg. "Fast gapped-read alignment with Bowtie 2." *Nature methods* 9.4 (2012): 357.)

133) Kim, Daehwan, Ben Langmead, and Steven L. Salzberg. "HISAT: a fast spliced aligner with low memory requirements." *Nature methods* 12.4 (2015): 357-360.)

134) Barash, Nanelle R., et al. "*Giardia* alters commensal microbial diversity throughout the murine gut." *Infection and immunity* 85.6 (2017): e00948-16.

135) Pham, Jonathan K., et al. "Transcriptomic profiling of high-density *Giardia* foci encysting in the murine proximal intestine." *Frontiers in cellular and infection microbiology* 7 (2017): 227.