Investigating the diversity, adaptations and potential roles of *Blastocystis* in the gut

Thesis submitted to the University of Kent for the Degree of Ph.D. in Microbiology

Emma Louise Betts

Declaration

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University of Institution of learning.

Emma Louise Betts

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Abstract

Blastocystis is ubiquitously distributed coloniser of the gastrointestinal tract. To date, 17 subtypes and other isolates have been characterised from a range of invertebrate and vertebrate hosts, including humans. Despite current knowledge regarding its prevalence, diversity and associations with the gut microbiota, there is still uncertainty about its role as a pathogen. Herein, I aimed to explore the diversity of *Blastocystis* and investigate its role(s) in the gut using a multiphasic approach, combining culturomics, with molecular biology, phylogenetics, metagenomics, transcriptomics, and metabolomics. This allowed for an investigation of not only the prevalence of *Blastocystis,* but aided in the exploration of its *in vivo* and *in vitro* function.

Results from my thesis demonstrate that *Blastocystis* had a high incidence in asymptomatic captive animals, with an excess of 40% in the species sampled. Additionally, numerous novel hosts were identified, while showing that co-infection with other microbial eukaryotes was relatively frequent. Bacterial community profiling of *Blastocystis* positive animals demonstrated no overall changes to bacterial diversity, but highlighted a number of taxa that were associated with *Blastocystis* colonisation. *In vitro* and *in vivo* NMR metabolomics further revealed a distinct metabolome in positive individuals. Here, a number of metabolites linked to eubiosis were identified including l-arginine, l-glutamine and propanoate. Lastly, treatment of *Blastocystis* with oxygen revealed a number of pathways possibly implicated in oxygen stress responses.

As a result, my Ph.D work provides a steppingstone to understand the prevalence of *Blastocystis* and the role of this questionable gut “parasite” in health and disease in both animals and humans.

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Abbreviations

AGP	American gut project
AOX	Alternative oxidase
ETC	Electron transport chain
Fe-S	Iron-sulphur
FECT	Formol ethyl acetate concentration technique
FGFP	Flemish Gut Flora Project
GI	Gastrointestinal
HIC	High income country
HMP	Human microbiome project
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LFC	Log fold change
LGT	Lateral gene transfer
LMIC	Low-to-Middle-income countries
MRO	Mitochondrion-related organelle
mtDNA	Mitochondrial DNA
NHP	Non-human primate
MDH	Malate dehydrogenase
NMR	Nuclear magnetic resonance
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase complex
PFO	Pyruvate:ferredoxin oxireductase
qPCR	Quantitative Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
RT	Room temperature
SSU rRNA	Small-subunit rRNA
ST	Subtype
STS	Sequence-tagged sites
TCA	Tricarboxylic acid
TOM70	Translocase of Outer Membrane

Chapter 1. Introduction

1 Introduction and History

Blastocystis spp. are anaerobic colonisers of the gastro-intestinal tract of many invertebrate and vertebrate hosts, including humans. The organism is placed within the Stramenopile lineage and is one of the only known members able to colonise humans. The Stramenopiles are a large and varied line of eukaryotes belonging to the kingdom Chromista. Members of this group are one of the richest and most diverse assemblages of unicellular and multicellular eukaryotes, varying in size, habitat and pathogenic potential. The group includes free-living unicellular flagellates and ciliates, fungus-like plant pathogens, microalgae and multicellular kelp as described in Figure 1.i (Clark *et al.* 2013; Silberman *et al.* 1996).

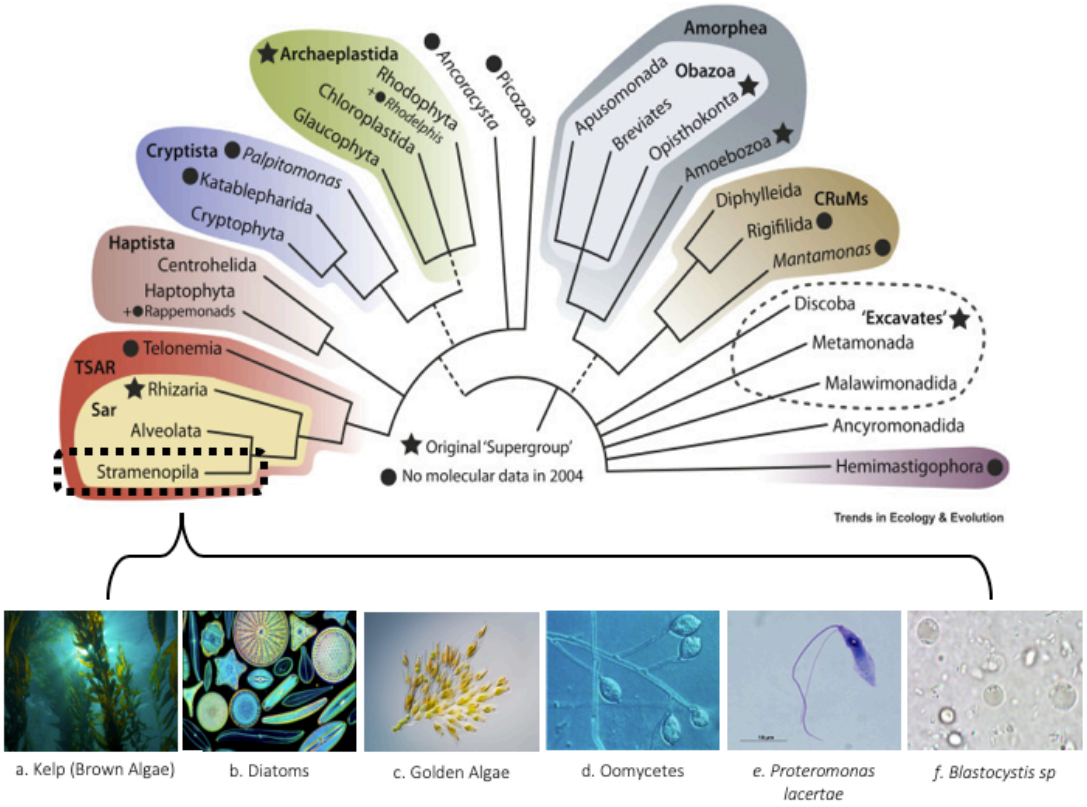

Figure 1.i Eukaryotic tree of life as proposed in 2019. The focus is directed towards the Stramenopila, **A-F** represent the diversity of the Strameonpile lineage, which range in size, morphology and habitat. **A.** (far left) is a large multicellular kelp of the order Laminariales, **B.** and **C.** represent other unicellular algae. **D.** show oomycetes which include parasitic members including the plant pathogen Phytophthora responsible for the Great Famine. **E.** is Proteromonas lacertae; this is the closest relative to *Blastocystis* and typically resides in the gut of reptiles. **F.** represents *Blastocystis* sp which are one of the only stramenopiles known to infect humans. Adapted from (Burki et al. 2019); A. (Gomez Suarez, 2017); B. (Berdan, 2017); C. (Kvarnström, 2018); D. (Bronkhorst, 2019); E. (Pérez-Brocal et al, 2010) and; F (CDC - DPDx - *Blastocystis Hominis*, 2019).

The official discovery of *Blastocystis* is often disputed. The first detailed description came from Alexeieff in 1911 (Alexeieff 1911), who identified an unknown microeukaryote in a range of vertebrate and invertebrate hosts which he named *Blastocystis enterocola* and classified as a yeast. There are a number of earlier reports that could describe *Blastocystis*, the earliest dating back to the 1840 London cholera epidemic where documentations by Brittan and Swayne (1849), Losch (1875) and Perroncito (1899) isolate an enteric microorganism that may have been *Blastocystis.* However, due to a lack of detailed accounts, it cannot be determined whether the microorganism was in fact *Blastocystis*. (Zierdt 1991).

The first documented isolation of *Blastocystis* from humans came just one year after its discovery by Brumpt (1912). *Blastocystis* was subsequently renamed *Blastocystis hominis* and is still sometimes referred to as this in present day literature alongside the updated, more appropriate subtyping system proposed in 2007 (Stensvold, Suresh, *et al.* 2007; Stenzel and Boreham 1996; Zierdt 1991).

Today, *Blastocystis* is becoming recognised as a widely prevalent protist and has been identified in human populations across the world, and in other taxa including artiodactyls, marsupials, perissodactyls, proboscideans, rodents, avian species, reptiles, fish and some insects (Boreham and Stenzel 1993; Stenzel and Boreham 1996; Cian *et al.* 2017; Tito *et al.* 2019; Valença-Barbosa *et al.* 2019). The 17 subtypes (STs) currently described are believed to have a relatively low host specificity and are generally considered to be ubiquitously distributed. Up-to-date estimates indicate the overall prevalence of *Blastocystis* in humans exceeds 1 billion (Andersen and Stensvold 2016) and colonisation rates supposedly range from 20% in Europe (Bart *et al.* 2013; El Safadi *et al.* 2016) to over 50% in some developing countries (Alfellani, Stensvold, *et al.* 2013; Ramírez *et al.* 2014), with one study even recording an incidence of 100% in a group of children in Senegal (El Safadi *et al.* 2014).

The protist's high and seemingly asymptomatic colonisation in humans and animals; relatively low host specificity and apparent worldwide distribution; questionable pathogenicity and pathogenic potential; position as a zoonotic agent and unknown roles within the microbiota are just some features that highlight its ambiguity and make it an organism of increasing interest.

2 Classification, Taxonomy and Identification

The taxonomic positioning of *Blastocystis* is relatively recent and was resolved using molecular methods, (see Figure 1.ii for schematic representation). The journey to its taxonomic position has been somewhat muddled and it underwent a number of re-classifications before being placed among the Stramenopile lineage. *Blastocystis* was first classified as a yeast, largely due to its appearance in faecal mounts and lack of pseudopodia and locomotion. Zierdt (1967) was the first to highlight that many of the other typical yeast characteristics were absent via his continual examination of *Blastocystis* physiology, culture and ultrastructural study. Documenting instead the major morphological and physiological characteristics, such as apparent lack of growth on fungal and mycoplasma media, which led to its first re-arrangement from yeast to protozoa in 1967 (Zierdt, Rude and Bull 1967). Since then, *Blastocystis* has been subject to further reclassification; including the subphylum Sporozoa and Sarcodina (Zierdt 1988; Zierdt 1991).

The advent of molecular techniques for phylogenetic investigation including genetic characterisation of the small-subunit rRNA (SSU rRNA) resulted in yet another alteration of the position of *Blastocystis* within the Eukaryote domain. In 1996, Silberman et al. resolved the phylogenetic position of *Blastocystis* using both the *Blastocystis* NandII strain and a Guinea pig isolate. Sequencing and alignment of the complete SSU rRNA with organisms from all main eukaryotic lineages placed *Blastocystis* within the Stramenopile lineage. This placement has since been further supported by molecular analysis of other genes, such as the cytosolic-type 70-kDa heat shock protein, translation elongation factor 2 and the non-catalytic 'B' subunit of vacuolar ATPase (Arisue *et al.* 2002; Silberman *et al.* 1996).

The Stramenopiles are a major lineage of complex heterogeneous single celled and multi-cellular organisms within the chromalveolates. Members of the Stramenopiles include: single-celled diatoms, which play a key role in carbon sequestration in the ocean; algae, including red, green and brown algae; oomycetes mainly comprised of water moulds; and plant pathogens.

Blastocystis is most closely related to *Proteromonas lacertae* (a flagellate found in the gut of amphibians and reptiles) (Silberman *et al.* 1996), yet it lacks the typical morphological features associated with many Stramenopiles. It may be one of the only Stramenopiles that lack these features and the reason for these absences is currently unknown.

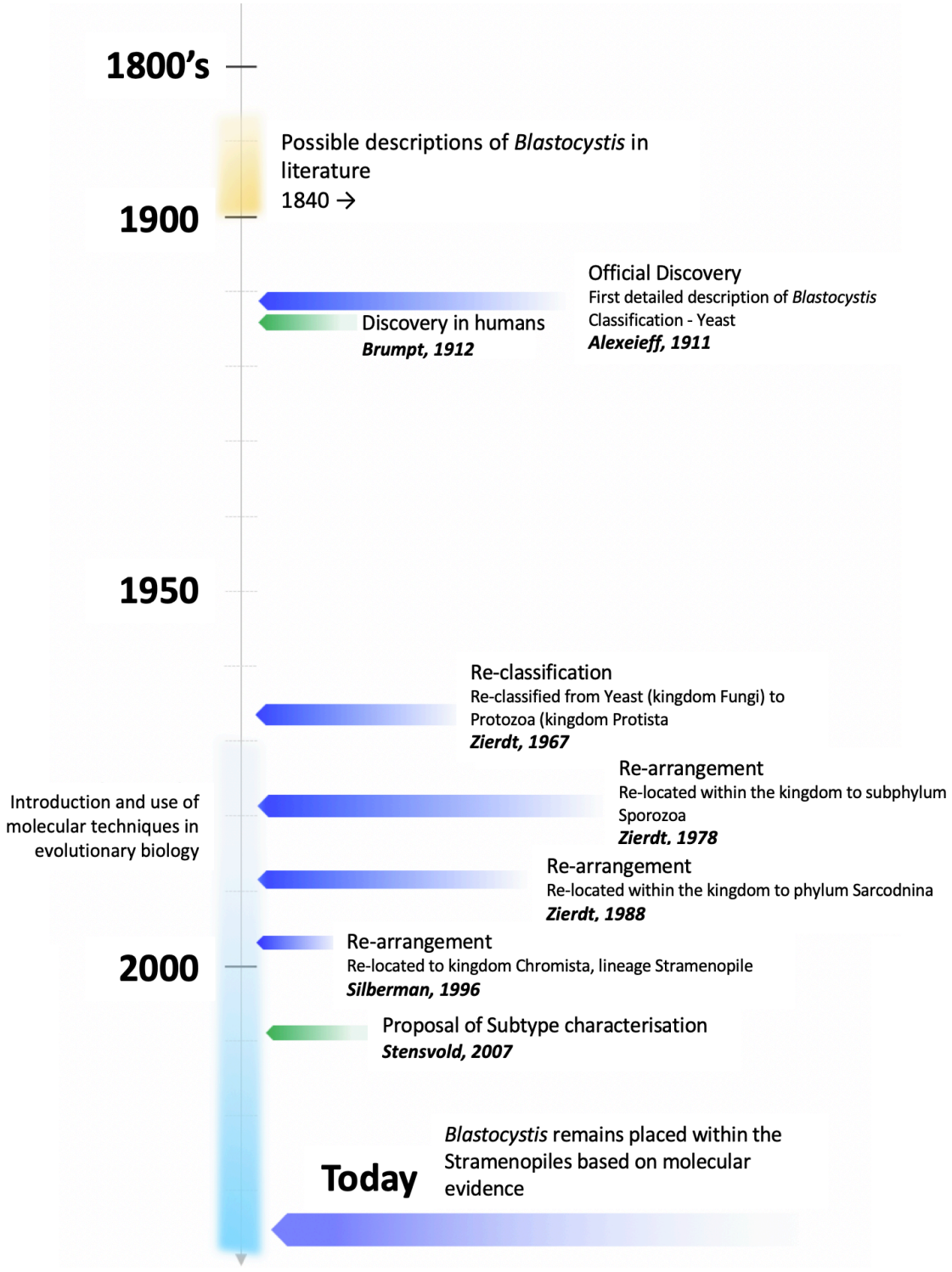

Figure 1.ii Timeline representing the re-classifications of *Blastocystis* upon its discovery in 1911 to present day. Blue arrows represent phylogenetic rearrangements, the Lilac arrow represents the stance of present day, Green arrows represent other notable time points in *Blastocystis* history

2.1 Genetic Profiling and Subtype Diversity

Molecular studies show that *Blastocystis* is a genus that encompasses a number of genetically distinct species. Traditionally, and from a taxonomic perspective, it became widely accepted that the species '*Blastocystis hominis*' denotes isolates from humans. Yet *B. hominis*-like isolates were identified in a range of animals, and most of which were morphologically indistinguishable from one another. Investigations of non-human *Blastocystis* isolates revealed separate species, for example, *Blastocystis lapemi* from the sea snake *(Lapemis hardwickii*) was proposed as a separate species due to different growth temperatures and chromosomal patterns (Parkar *et al.* 2010; Ramírez *et al.* 2014; Teow *et al.* 1991; Wawrzyniak *et al.* 2013). The heterogeneity among *Blastocystis* isolates led to early attempts to investigate this further. Published literature included the examination of protein and DNA by ultrastructure, which concluded that some distinct *Blastocystis* groups should be considered as demes: in line with the World Health Organisation definition (1978) (Boreham, Upcroft and Dunn 1992; Dunn, Boreham and Stenzel 1989). Kukoschke and Müller (Kukoschke and Muller 1991; Müller 1994) investigated protein profiles of *B. hominis* isolates; Mansour (Mansour *et al.* 1995) provided iso-enzyme and protein profiles proving the existence of at least two separate isolates and finally, through the use of molecular characterisation techniques, including the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), a detailed analysis could be made to identify separate species that could colonise different hosts (Clark 1997; Hoevers *et al.* 2000; Kaneda *et al.* 2001; Rivera and Tan 2005; Snowden *et al.* 2000; Thathaisong *et al.* 2003; Wong *et al.* 2008; Yan *et al.* 2007; Yoshikawa *et al.* 2000; Yoshikawa *et al.* 2003). PCR and phylogenetic analysis for characterisation of *Blastocystis* began being used in the 1990's and early 2000's (Abe 2004; Arisue, Hashimoto and Yoshikawa 2003; Böhm-Gloning, Knobloch and Walderich 1997; Clark 1997; Noël *et al.* 2003; Silberman *et al.* 1996; Stensvold, Arendrup, *et al.* 2007; Stensvold *et al.* 2006; Thathaisong *et al.* 2003). It is probably the most common method employed today for characterisation and molecular epidemiological studies, such advances in these techniques have played a pivotal role in understanding the genetic diversity and prevalence of *Blastocystis* to date (Betts *et al.* 2018; Rezaei Riabi *et al.* 2018; Udonsom *et al.* 2018; Valença-Barbosa *et al.* 2019).

2.2 Subtype Assignment and Characterisation Criteria

Traditionally, when novel *Blastocystis* species were first identified, they were named after the infected host, for example *B. hominis* in humans and *B. ratti* in rats. However the advent of molecular characterisation revealed that different species were able to infect a range of hosts, leading to this naming system causing confusion and inaccuracies (Clark 1997; Noël *et al.* 2005). Due to the growing confusion regarding *Blastocystis* terminology, in 2007, Stensvold et al. proposed a standardisation of *Blastocystis* nomenclature by introducing a naming system for different species using subtypes (ST) (Stensvold, Suresh, *et al.* 2007) which generally encompass isolates from mammalian hosts. The introduction of this categorisation method relied on published data including ribodeme and sequence information to collectively group genetically similar isolates within the proposed subtypes. At the time of publication, nine subtypes (ST1 to ST9) isolated from humans had been described from ribosomal DNA sequences. Since 2007, eight more subtypes have been identified, yet the understanding of subtype assignment is still somewhat ambiguous and lacks isolates from reptiles, amphibians and insects. Isolates from non-mammalian and avian hosts generally retain species names, for example, *B. lapemi*. From initial phylogenetic analysis of ST1 to ST9, the minimum genetic divergence between the subtype sequences was approximately 5%. However today, as more samples from an increasing range of host taxa are sampled, the genetic diversity within subtypes is increasing and a number of possible novel subtypes are being discovered that genetically differ by less than 5% (Clark *et al.* 2013; Stensvold 2013a).

Today 17 genetically distinct subtypes have been recognised and some recent publications propose novel subtypes ST18 – ST26, including reports by Maloney (Maloney *et al.* 2019) and Zhao (Zhao *et al.* 2017). However, there are a number of issues with these as described by Stensvold and Clark in a recent publication that aimed to debunk the main issues with identifying novel subtypes (Stensvold and Clark 2020). For example, a number of the proposed subtypes shared overwhelming similarity to established subtypes, including ST20 which is likely ST5, and is the result of chimeric DNA sequences created during PCR amplification with other subtypes (ST18, ST19) or unrelated organisms (Beghini *et al.* 2017; Kodio *et al.* 2019; Stensvold and Clark 2020).

Before novel subtypes should be accepted, it was suggested by Clark et al. (Clark *et al.* 2013) that additional sampling is required as it will either better resolve gaps in existing clades and

resolve positioning within existing subtypes, or confirm that sequences cluster separately. 'Novel' isolates should be scrutinized, and genome-level profiling by sequencing the entire small ribosomal subunit RNA gene (ideally from culture), and phylogenetic analysis with reference sequences using a range of estimation methods such as Maximum Likelihood and distance models need to be employed before an isolate can be considered a new subtype. Subtype 3 is one such example where molecular characterisation must be thorough so as to not confuse distinct isolates as separate subtypes due to the great genetic variation; which is up to 3% within supported clades (Boenigk *et al.* 2012; Clark *et al.* 2013; Stensvold 2013a; Yoshikawa, Koyama, *et al.* 2016).

Interestingly, proposed subtypes ST21, ST23 – ST26 present possible novel subtypes. They have numerous sequence deposits from different research groups, which decreases the chance of them being artefacts. However they lack full SSU rRNA sequences which means their phylogenetic position currently remains unsolved (Stensvold and Clark, 2020).

2.3 Genetic Characterisation

Molecular identification for characterisation and subtyping can be executed by various means. The first diagnostic PCR was developed in 2006 and was based on SSU rRNA gene sequences from Genbank, however there is some speculation that preferential amplification of ST3 occurs with these primers meaning they are often not the primer of choice nowadays (Stensvold 2013a). Arguably the most common target today is the 600-bp 'barcode region' developed by Scicluna (Scicluna, Tawari and Clark 2006). This region is generally considered a good choice due to large repositories in sequence databases; including the NCBI GenBank, and is popular within recent literature (Deng *et al.* 2019; Tito *et al.* 2019). There are a number of other regions that are also used diagnostically by researchers which range in sequence length and provide alternative methods for subtype screening (Parkar *et al.* 2010; Santín *et al.* 2011; Scicluna, Tawari and Clark 2006; Stensvold *et al.* 2006).

Other genome-based methods for subtype specific identification and characterisation include PCR-RFLP (often riboprinting), PCR with sequence-tagged sites (STS), and more recently, subtype-specific PCR primers. Their usefulness in subtyping is varied, for example, PCR-RFLP is not generally a suitable means for genotype classification mainly because the same RFLP profile may not show the same sequence, with ambiguous profiles possibly indicating mixed subtype infections. Furthermore, differences in restriction enzymes make comparisons between studies difficult (Arisue, Hashimoto and Yoshikawa 2003; Hoevers *et*

al. 2000; Scanlan, Stensvold and Cotter 2015; Snowden *et al.* 2000; Yoshikawa, Wu, *et al.* 2004).

PCR – STS amplifies sequence-tagged sites from subtypes and is often based on randomly amplified polymorphic DNA regions. Although good for detection of mixed *Blastocystis* infections which mitigates the need for sequencing for subtype identification, it is currently limited to ST1 - ST7 and has often failed to identify positive infections – likely due to them not amplifying key alleles (Abe, Wu and Yoshikawa 2003; Scanlan, Stensvold and Cotter 2015; Stensvold, Suresh, *et al.* 2007; Stensvold 2013b; Yoshikawa *et al.* 2000; Yoshikawa *et al.* 2003).

Lastly, subtype-specific PCR primers have been developed by some groups. (Scanlan, Stensvold and Cotter 2015; W. Wang *et al.* 2014; Yoshikawa and Iwamasa 2016) Although this method does not require sequencing, primers have only been developed for ST1 – ST9, with Scanlan et al. developing the first targeting ST1-ST4 for application in human centric studies of *Blastocystis* subtype prevalence in 2015. Yoshikawa et al. expanded this to encompass all human associated subtypes in 2016. However, non-specific PCR amplification has been observed from faecal samples, likely due to high concentrations of other organisms, meaning further modifications are necessary to optimise the method (Yoshikawa and Iwamasa 2016).

3 Morphology

Blastocystis is a polymorphic microeukaryote, currently possessing four described morphological forms. It is likely that the different morphologies are largely dependent on their external environment and stress, including, but not limited to; oxygen, osmosis, *in vitro* culture and drug treatment. All of which have been experimentally shown or proposed to impact *Blastocystis* physiology and may produce unusual morphological forms including the 'medusa head form' and 'chestnut barr cell' (Parija and Jeremiah 2013; Vdovenko 2000).

The vacuolar or central-body form is the most often described from culture and stool samples, it is generally spherical in shape and typically measures between 4 µm – 15 µm in diameter, but can range from anywhere between 3 µm - 120 µm (Parija and Jeremiah 2013; Stenzel and Boreham 1996). The majority of the cell is made up of a large membrane bound body which contains a scattered 'flocculent' material comprised of carbohydrates and lipids, linking its role to a storage organelle in addition to speculated roles in programmed cell death (Yoshikawa, Kuwayama and Enose 1995; Yoshikawa, Satoh and Enose 1995). The cells

usually have up to two nuclei in the surrounding cytoplasm and other organelles including the Golgi apparatus, microtubules, vacuoles and mitochondria-like organelles. A surface layer of varying thickness often coats the protozoan which may play roles in trapping bacteria (Zaman *et al.* 1999; Zaman, Howe and Ng 1997) or possibly acting as a protective layer (Cassidy, Stenzel and Boreham 1994).

The granular form typically shares many of the same morphological characteristics as the vacuolar form. The main observable difference is the granular appearance of the central body and cytoplasm which have been proposed as three separate granular types: metabolic, reproductive and lipid types. The lipid and granular are found both in the cytoplasm and central body and function as a storage bodies (Parija and Jeremiah 2013; Tan 2008). The metabolic granules are present in the cytoplasm and are most likely involved in metabolic pathways for maintenance of cell physiology and homeostasis. The reproductive granules found in the central body were thought to be involved in the asexual reproduction of *Blastocystis*, however all evidence for this is based on microscopy and warrants further investigation (Tan and Stenzel 2003).

The amoeboid form is observed less frequently than the abovementioned morphologies. The ultra-structure was first published in 2006 by Tan et al. and shows a non-motive, adhesive form of the protozoan which has been isolated in the stool of patients presenting symptomatic infection (Tan and Suresh 2006a; Zhang, Zhang, *et al.* 2012). When it has been isolated from symptomatic patients it presents different morphologies, causing some confusion when initially describing its physiology. These differences many represent different genotypes of the protozoan, however the amoeboid form generally has up to two pseudopodia and one large vacuole, or multiple vacuoles (Dunn, Boreham and Stenzel 1989; Tan 2008; Tan and Suresh 2006a).

The cyst form of *Blastocystis* is the form most recently discovered and has aided in our understanding of transmission and infection routes (Yoshikawa, Yoshida, *et al.* 2004). They are mostly spherical in shape and have an average diameter between 2 µm – 5 µm, with larger isolates found from animal hosts (Stenzel, Boreham and McDougall 1991; Stenzel, Lee and Boreham 1997). The thick, multi-layered cyst wall offers protection against harsh environmental conditions including water and extreme temperatures, although there is debate regarding longevity (Moe *et al.* 1996). Variations may be due to different subtypes. In this stage it lacks the cell body and the condensed cytoplasm contains storage vacuoles possibly containing glycogen and mitochondria. Multiple nuclei have also been observed

(Stenzel, Boreham and McDougall 1991; Tan and Suresh 2006a) and a surface coat has also been found surrounding newly developed cysts.

In addition to these morphological forms, others have been identified. Including an avacuolar form where the central body is absent and a multivacuolar form which, instead of a central body contains a number of smaller vacuoles, the sizes and arrangements of these morphologies may be subtype specific, or may be due to excystation or encystation processes (Chen *et al.* 1999; Moe *et al.* 1999; Stenzel, Boreham and McDougall 1991; Tan 2008).

3.1 Laboratory Identification Methods

Numerous identification techniques exist for identifying *Blastocystis* from faecal samples. Many reports compare their effectiveness and relative sensitivity and specificity with mixed outcomes. The majority of comparative studies favour PCR identification due to its relatively high sensitivity when compared to microscopy and culturing. However, as with all methods there are benefits and drawbacks to each, and the lack of standardised methodology in identification may be an accompanying factor contributing to the disparity in its prevalence across epidemiological studies worldwide. Identification is typically carried out by microscopy with staining, however in recent years *in vitro* culture and PCR methods of detection have become increasingly popular. Microscopy is often used in clinical settings as it carries the advantage of being inexpensive and fast, yet there is considerable difficulty in making correct identification. Its polymorphic nature means that it is inconsistently identified and often mistaken for other microeukaryotes, including yeast. The identifiable vacuolar form is less frequently isolated from faecal samples in comparison to the cyst form which has a prevalence of up to 28.5% in faecal samples (Poirier *et al.* 2011). Its small size makes this cyst form relatively inconspicuous and thus it is often missed from faecal samples, particularly when other morphologically noticeable parasites are present. Staining of faecal smears aids identification and numerous staining techniques have been documented with the most common including Lugol's iodine, acid fast, Giemsa and trichrome. Trichrome stain is possibly the most commonly used due to its reported higher sensitivity when compared to other stains (Dogruman-Al *et al.* 2010). Yet, detection rates with microscopy vary between studies from 0% (Suresh and Smith 2004), 5% (Termmathurapoj *et al.* 2004), 9% (Roberts *et al.* 2011), 13.98% (Padukone *et al.* 2018), and 36.7-50% (Dogruman-Al *et al.* 2010). Generally, microscopy alone may not be the most effective method for *Blastocystis*

identification, and it is often accompanied by short term *in vitro* culture from 24 to 72 hours. There are a number of reports that support the increased sensitivity short term culture has on detection rates (Leelayoova *et al.* 2002; Stensvold, Arendrup, *et al.* 2007; Termmathurapoj *et al.* 2004), but there is some dispute on whether culturing preferentially favours the growth of certain subtypes. Stensvold et al. investigated this and documented no difference in subtype distribution between cultures maintained for up to 28 days and DNA extracted straight from the source, and so whether there is preferential selectiveness and to what extent remains unclear (Parkar *et al.* 2007; Stensvold, Arendrup, *et al.* 2007). Another method reported in literature is the formol ethyl acetate concentration technique (FECT) which is a standard parasitological detection method. Its usefulness is disputed due to its underestimation of parasite load. In 2004, Suresh and Smith documented *Blastocystis* occurrence to be 0% using this method alone, yet with *in vitro* cultivation *Blastocystis* was detected in 39/1000 samples. The detectability of *Blastocystis* using this method may result in misidentification possibly influenced by the phenotypic properties of particular subtypes and aging faecal samples (Stensvold, Arendrup, *et al.* 2007; Stensvold *et al.* 2006; Suresh and Smith 2004).

Nowadays molecular methods for identification and characterisation are far more common. Although PCR is costlier and time consuming it does not rely on living and intact target organisms, meaning fresh faecal samples are not essential. It can detect parasite loads far lower than microscopy and is not influenced by parasite morphology and human error. This method can provide information on subtypes and genetic isolates either by sequencing or using STS primers which is not possible with microscopy alone. Published reports indicate that using PCR gives increased identification rates when compared to other methods (Padukone *et al.* 2018; Roberts *et al.* 2011; Stensvold, Arendrup, *et al.* 2007) which can be further amplified by prior short term culture.

The development of quantitative PCR (qPCR) provides a quantitative approach to *Blastocystis* identification which is generally absent from other described methods that are mainly qualitative or semi-quantitative at best. The ability to estimate *Blastocystis* load in a faecal samples can help uncover the roles played in both carrier load, and in symptomatic disease which is often disputed (Leder *et al.* 2005; Poirier *et al.* 2011). However, a genus-specific qPCR method is difficult to produce as there is large genetic diversity within the genus. An early record of qPCR came from Jones et al. in 2008. This protocol targeted an unknown gene and amplified ST1, ST3 and ST4 to a lower limit of 760 individual organisms per 100 mg of faecal sample. This study only sampled from three patients and the limited

subtype range meant that wider application was limited. (Jones, Ganac, *et al.* 2008). More recently Poirier et al. in 2011 developed qPCR for all known human subtypes and was able to detect 10^2 individual organisms per gram of faecal sample to a specificity of 95%, which is more sensitive when compared to microscopy accompanied with *in vitro* culture. The only limitations were the identification of false positives which were confirmed with sequencing and the restriction to human subtypes only (Poirier *et al.* 2011). In 2012, Stensvold et al. further developed the qPCR methodology and designed a sensitive assay able to detect the human subtypes without false positive results (Stensvold *et al.* 2012). qPCR in addition to PCR are now common practice for prevalence studies and are often used in conjunction for molecular epidemiological studies (Forsell *et al.* 2017; Tito *et al.* 2019).

3.2 Laboratory Culture

After first being cultured in 1921 (Barret 1921), several *Blastocystis* subtypes have successfully been cultured under axenic and xenic conditions in a range of medias. Common in recent literature include Jones' media, Boeck and Drbohlav's inspissated egg media, Iscove's Modified Dulbecco's Media, agar slant with Locke solution, Minimal Essential Media, and TYGM-9 media; which are commonly accompanied with inactivated horse or calf serum at approximately 10% (Boreham and Stenzel 1993; Roberts *et al.* 2011; Zhang, Qiao, *et al.* 2012). The generation time of cultures depends on isolate, temperature and growth media; typically axenic cultures double between 6 – 23 hours and usually reach confluence around day 4 post-inoculation (Boreham and Stenzel 1993; Dunn and Boreham 1991; Zierdt and Swan 1981; Tan 2008). Ideally, for use in molecular and biochemical studies, *Blastocystis* cultures should be axenic. Axenization was first carried out by Zierdt and Willians in 1974 (Zierdt and Williams 1974) using 1000 µg/ml streptomycin, 4000 µg/ml ampicillin and 50 µg/ml amphotericin B over the period of 6 – 10 transfers taking approximately 1 month. Another study by Lanuza recommended 0.4% ampicillin, 0.1% streptomycin and 0.0006% amphotericin B mixture in addition to a Ficoll-metrizoic acid gradient which produced axenic cultures in a number of isolates in 3 – 5 weeks (Lanuza *et al.* 1996). Within the literature, other successful methods have included colony growth in soft agar, followed by inoculation in media. This method has the benefit of two selection processes; antibiotics and colony selection (Ng and Tan 1999). Current axenic cultures have successfully been performed on a number of subtypes, including ST1-Nand II, ST1-NUH9, ST4-WR1, ST4-S1, ST7-ST7A-H. Other isolates have proven more difficult to grow and maintain, and it has been proposed

that the presence of bacteria is necessary for growth, either via nutritional benefit or providing favourable growth conditions (Boreham and Stenzel 1993; Zierdt 1991).

4 Life Cycle and Transmission

One of the first proposed life cycles of *Blastocystis* was by Zierdt in 1973 (Zierdt 1973) and was deduced from observations of the different life cycle stages in culture. Here cells were seen to divide by binary fission in the vacuolar form, which is the most often observed form of reproduction. Other modes of division have since been proposed in addition to binary fission, and include budding and plasmotomy (Tan and Suresh 2007; Zhang *et al.* 2007; Zhang, Zhang, *et al.* 2012). There are possible accounts of sexual reproduction (Basak, Rajurkar and Mallick 2014) however, as an animal model or organoid system is yet to be established that can reproduce the entire life cycle, the main mechanism of reproduction is still unclear (Lepczyńska *et al.* 2017). Today it is generally accepted that cysts are the only transmissible form. Experimental evidence from orally infected BALB/c mice and Wistar rats elucidates to faecal-oral transmission (Moe *et al.* 1996; Moe *et al.* 1997; Yoshikawa, Yoshida, *et al.* 2004). In humans, many cases of infection are linked to ingestion of contaminated water, food, and possibly soil (Figure 1.iii). The source of contamination is often linked to animals or human transmission, especially in Low-to-Middle-Income Countries (LMICs) where there is often inadequate drinking water and sanitation (Anuar *et al.* 2013; Lee, Chye, *et al.* 2012; Sánchez *et al.* 2017).

Once the cyst enters a suitable host and reaches the large intestine it is reported that excystation then occurs, giving rise to the commonly identified vacuolar form which can then further develop into the other morphologies. Observation of amoeboid, avacuolar and the multivacuolar forms in symptomatic patients link these to possible pathogenesis (Moe *et al.* 1997). The vacuolar form is able to encyst and be excreted in the faeces for further transmission or possibly remain as a thin walled cyst and re-infect the host (Singh *et al.* 1995; Tan 2008).

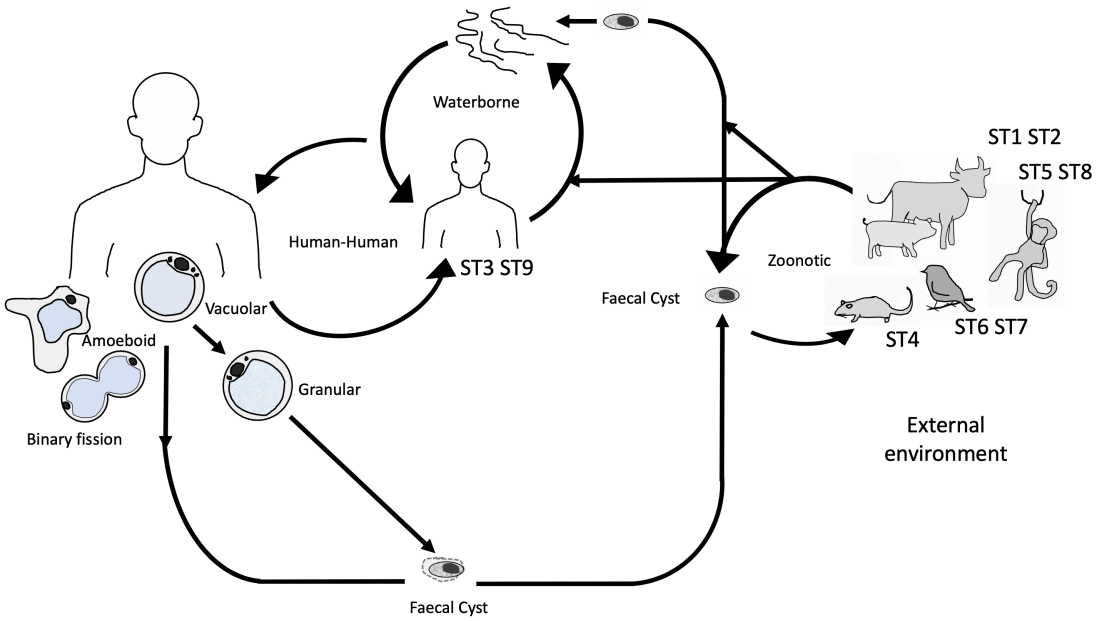

Figure 1.iii Proposed life cycle and possible transmission routes of *Blastocystis* from current literature. This suggests zoonotic transmission of animal associated STs (ST1, ST2, ST4-ST8) between human and animals and human-human transmission of ST3 and ST9 via faecal-oral and waterborne routes. Hosts are infected via the faecal cyst which is shed into the environment from an infected host. Upon entry into the host *Blastocystis* emerges at the vacuolar form which can interchangeably alter between the amoeboid and granular forms. Vacuolar and granular forms can undergo encystation to form the cyst which is excreted in the faeces *(*CDC - DPDx - Blastocystis Hominis *2019)*.

5 Epidemiology and Host Specificity

Blastocystis is one of the most commonly identified protozoa in epidemiological studies and in recent years there has been a large increase in the number of reports documenting it's distribution. The range and prevalence of the 17 described subtypes varies significantly depending on geography and host of interest. Figure 1.iv provides a schematical representation, denoting both the current and proposed subtypes, and the host ranges as summarised by Stensvold (Stensvold 2020). It is likely that the number of subtypes identified and their distribution patterns will increase as more efforts are made to sample from understudied populations and new animal hosts. Unsurprisingly, the majority of existing data regarding subtype prevalence and distribution exists largely within the scope of a few taxa: mainly humans and few animal species. Wild animals are probably the least studied group, but could represent the most interesting in regard to subtype diversity and our understanding of zoonotic potential. Such sampling bias limits our understanding of the

diversity of this microeukaryote with still much to discover among subtypes found in poorly sampled hosts.

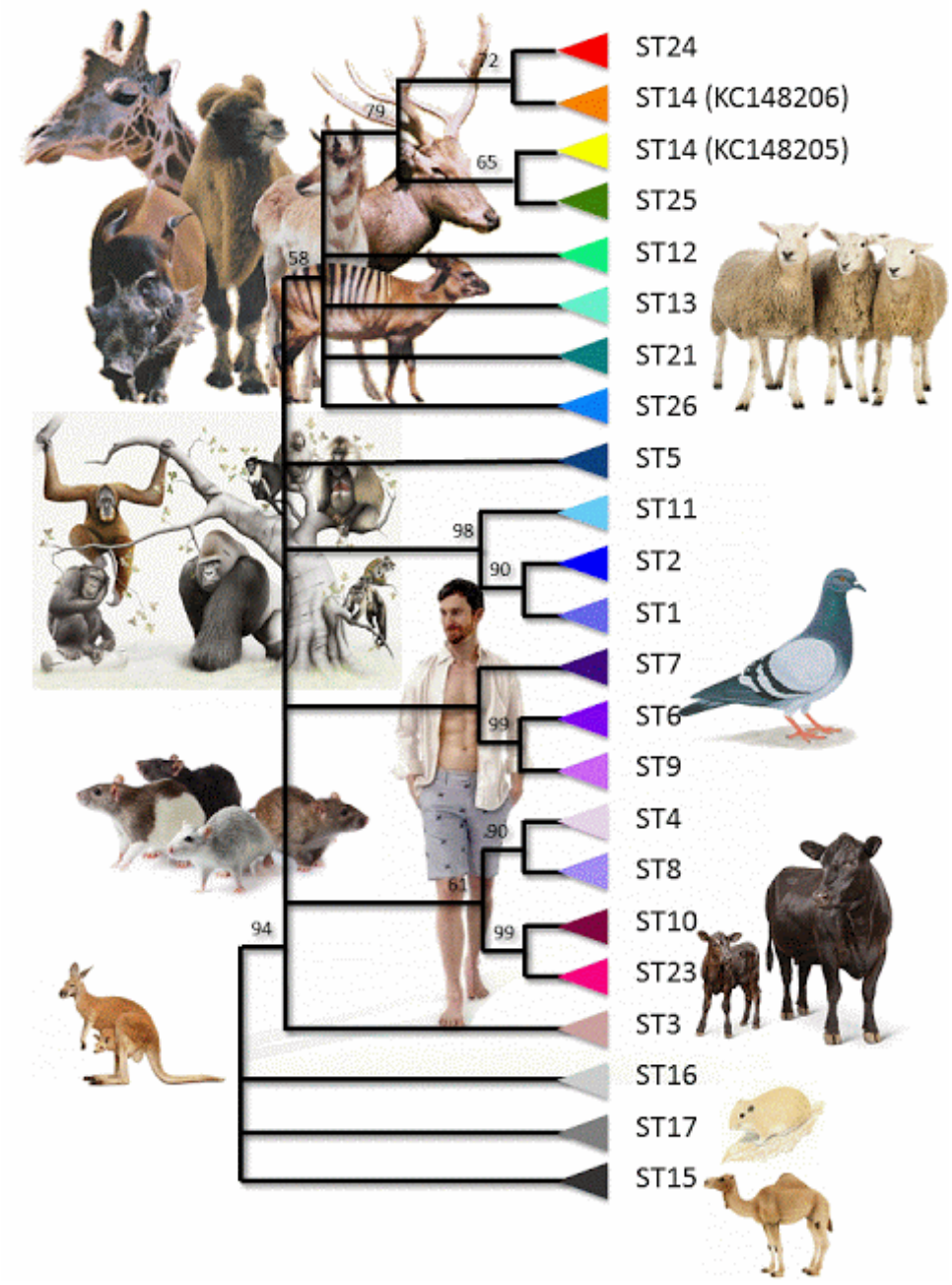

Figure 1.iv Simplified phylogenetic tree representing the typical subtype distribution of *Blastocystis* and associated host species. To date ST1-ST17 are accepted subtypes, ST21, ST23 - ST26 may also represent novel subtypes but currently lack full SSU rRNA sequences. ST 18- ST20 and ST22 should be rejected as they fail to meet the requirements of novel subtypes and are likely artefacts. Reprinted from (Stensvold 2020).

5.1 Subtype Distribution in Humans

Humans are one of, if not, the most, extensively studied host of *Blastocystis* and it is commonly reported as the most frequently identified intestinal parasite (Piubelli *et al.* 2019; Tan 2008). To date, nine human associated subtypes (ST1 to ST9) have been identified, all of which have a varying degree of prevalence and geographic distribution. As the proposed mode of transmission is the ingestion of cysts from faecal-oral routes, it is unsurprising that places with lower sanitation and poor infrastructure would have higher case numbers (Yoshikawa 2012). The remaining subtypes (ST10 - ST17) are associated with animals. In 2016, ST12 was described in the human gastro-intestinal tract for the first time in South American populations, nevertheless as noted by the author, this finding warrants further investigation to rule out the possibility of transient infection from a zoonotic source (Ramírez *et al.* 2016). Furthermore in 2020, ST10 and ST14 were described from humans for the first time with a prevalence of 0.45% in a Senegalese cohort and may have been the result of livestock or waterborne transmission (Khaled *et al.* 2020).

ST1 – ST4 are the main colonisers of humans and account for up to 95% of infection with ST3 making up the majority followed by ST1. Presently ST9 is the only subtype to be described exclusively in humans (Alfellani, Stensvold, *et al.* 2013; El Safadi *et al.* 2016; Tito *et al.* 2019; Yoshikawa, Wu, *et al.* 2004). The human-associated subtypes cover a worldwide and varied geographic distribution, their prevalence may be influenced by a number of external factors. Examples of such factors include: animal interactions, sanitation and waste/water management, human diet and health and low host specificity (Anuar *et al.* 2013; El Safadi *et al.* 2014; Ithoi *et al.* 2011; Tan 2008).

5.1.1 Geographic Distribution in Humans

Although fewer in number when compared to works in developed countries, epidemiological subtyping studies of *Blastocystis* from Low-to-Middle-Income Countries (LMICs) reveal a wider distribution of *Blastocystis*; many of which are diagnosed via microscopy in parasitological surveys (Figure 1.v) (Tsaousis *et al.* 2020). In LMICs, reports document *Blastocystis* in exceedance of 50% (Pegelow *et al.* 1997; Ramírez *et al.* 2014) in comparison to a maximum to 20% in Europe and High Income Countries (HICs) (Bart *et al.* 2013; Roberts *et al.* 2013). Likely explanations for this include increased exposure to animals and less stringent sanitation and waste management practices leading to water and food

contaminated with *Blastocystis* cysts from human or animal faeces (Eroglu and Koltas 2010; Leelayoova *et al.* 2008; Li, Zhou, *et al.* 2007).

Not only does the incidence of *Blastocystis* vary worldwide, but the distribution of subtypes have notable geographical patterns and ranges. Numerous studies document differences between the distribution of the human associated subtypes across the world and mixed subtype colonisation is not uncommon. As more epidemiological studies are undertaken, clearer patterns are emerging regarding *Blastocystis* distribution across different countries and within local populations variance is witnessed, and sociodemographic themes are thought to play a pivotal role in the subtype distribution.

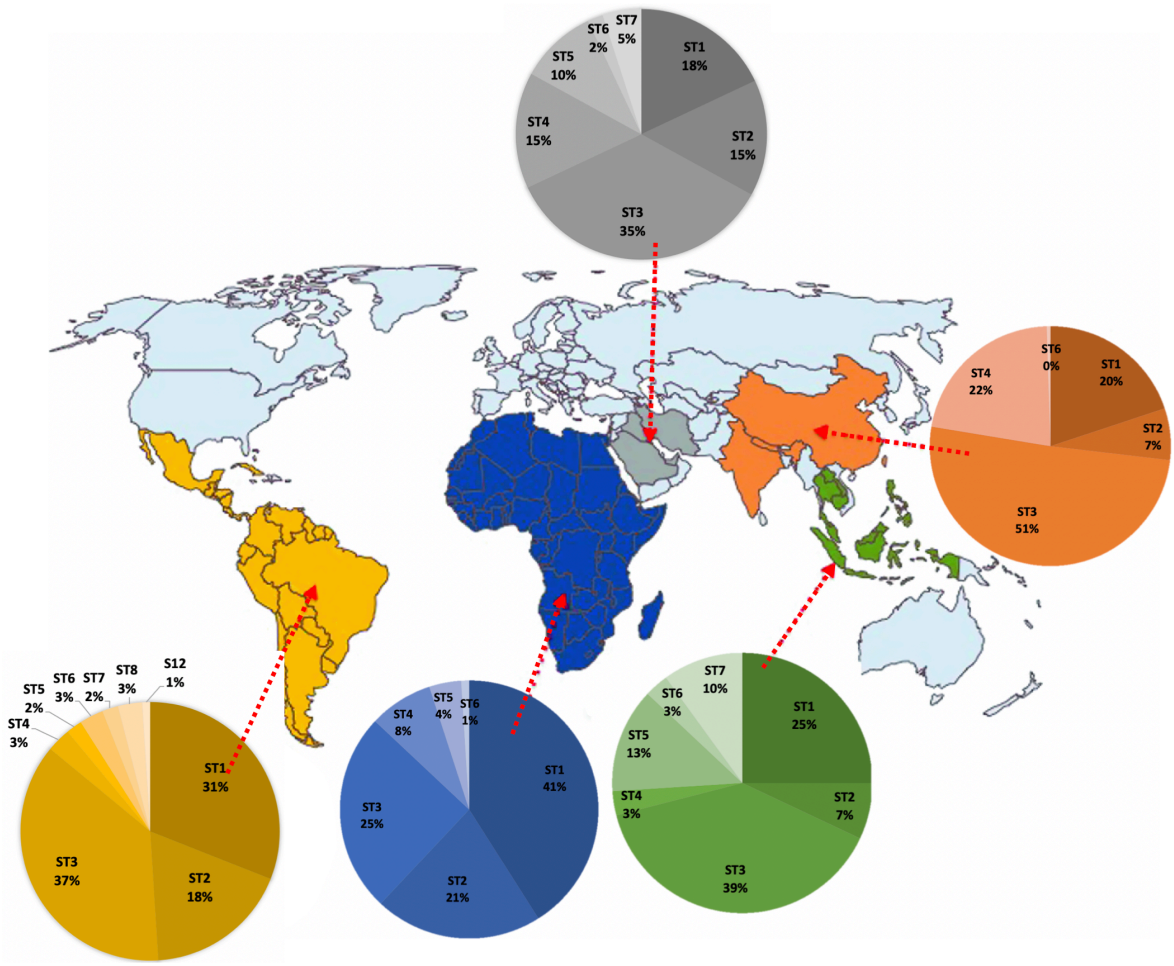

Figure 1.v Prevalence and biogeographical distribution of human *Blastocystis* subtypes in low and middle income countries. Adapted from Eukaryome impact on human intestine homeostasis and mucosal immunology (61-74), by A. Tsaousis, 2020, Springer, Copyright 2020 Springer Nature Switzerland AG 2020, reprinted with permission.

In Europe the average prevalence is thought to lie between 10%-25% which is generally lower than other regional averages (Forsell *et al.* 2012; Tan 2008). Whilst ST3 is the most commonly isolated worldwide, ST4 is infrequently identified in studies outside of Europe. Within Europe ST4 had an average prevalence of 16.5% across 10 European countries in 2012 (Forsell *et al.* 2012) and has been reported to exceed 60% within some study populations; examples include incidences of 94.1%, 63% and 74% in Spain, France and Denmark respectively (Domínguez-Márquez *et al.* 2009; Poirier *et al.* 2011; Stensvold *et al.* 2011). The relatively high prevalence and restricted geography could possibly be explained by the proposal of its emergence into the human population in Europe (Clark *et al.* 2013; Forsell *et al.* 2012; Stensvold *et al.* 2011; Tito *et al.* 2019).

Across South America *Blastocystis* is prevalent in both humans and animals. The majority of molecular studies are parasitological investigations aiming to obtain details on the geographical distribution and burden of intestinal parasites across populations with different sociodemographic variables (some studies also include indigenous populations). Most studies that provide subtyping data are centred around Colombia, Argentina, Bolivia, Peru, Brazil and Ecuador, where prevalence typically ranges between 40% - 70% (David *et al.* 2015; Macchioni *et al.* 2015; Macchioni *et al.* 2016; Malheiros *et al.* 2011; Melo *et al.* 2017; Oliveira-Arbex, David and Guimarães 2018; Ramírez *et al.* 2016; Valença-Barbosa *et al.* 2017). Many of these reports focus on the incidence of protozoan and helminth infections in human populations; pets, livestock, other animals, and influent and effluent water are often screened alongside humans to assess possible modes of transmission and infection reservoirs. Most published works investigating South American populations identify a high incidence of ST1, ST2 and ST3 in humans. The percentage at which these subtypes are identified often vary between countries and subpopulations but ST1 and ST3 are generally the most often characterised followed by ST2. Low incidences of ST6 and ST8, possibly from avian and animal sources, have been identified. In contrast to European studies, there is an overall absence of ST4 (David *et al.* 2015; Forsell *et al.* 2017; Jiménez, Jaimes and Ramírez 2019; Malheiros *et al.* 2011; Melo *et al.* 2019; Oliveira-Arbex, David and Guimarães 2018; Ramírez *et al.* 2014; Ramírez *et al.* 2017; Rojas-Velázquez *et al.* 2018; Sánchez *et al.* 2017; Seguí *et al.* 2018; Stensvold and Clark 2016; Villamizar *et al.* 2019). ST9 has been recorded in only one South American country to date by Macchioni et al. in 2015, where an investigation of soil-transmitted helminths and intestinal protozoa in children was being undertaken in Bolivia (Macchioni *et al.* 2015).

Prevalence studies of *Blastocystis* in North America are few in number. A study by Jones et al. in 2008 on a symptomatic cohort is one of the only subtyping studies conducted to date. ST3 was the most commonly identified subtype, however the sample population was small (n=21) (Jones, Whipps, *et al.* 2008). A larger study by Amin et al. in 2010 described a prevalence of *Blastocystis* from 5,291 patients to be approximately 16%, with seasonal variation. Subtyping was not performed in this study (Amin 2010). The majority of remaining information surrounding subtype distribution lies within studies such as that by Nash et al. in 2017 who identified *Blastocystis* ST1-ST3 from samples in the human microbiome project (HMP) whilst investigating the mycobiome (Nash *et al.* 2017). One similar study used data from the American gut project (AGP) (n=7,567) for detection of *Blastocystis* subtypes. The subtypes identified here match corresponding data for the Flemish Gut Flora Project (FGFP) and the TwinsUK study, demonstrating a high prevalence of ST1-ST4 with ST4 being the most prevalent subtype; demonstrating similarities between North America and Europe and possibly linking ST4 to High-income countries (HICS) (Tito *et al.* 2019).

Epidemiological studies of *Blastocystis* across the African continent are increasing in number and results often differ greatly between the countries and within subpopulations. Prevalence can range between 2.5% - 58% (Abdulsalam *et al.* 2013; Alfellani, Stensvold, *et al.* 2013; D'Alfonso *et al.* 2017) and more detailed distribution surveys highlight local patterns. Examples include ranges of 26% -28% in Libya (Alfellani *et al.* 2007; Alfellani, Stensvold, *et al.* 2013) and 1% in Tanzania (Forsell *et al.* 2016), with one report of 100% prevalence in Senegal (El Safadi *et al.* 2014). Across the majority of surveys, ST3 and/or ST1 are the most abundantly identified in countries including Angola, Qatar, Senegal. Tanzania, Côte d'Ivoire, Egypt, Ghana, Nigeria, Libya and Liberia also report high incidences of these subtypes (Abdulsalam *et al.* 2013; Abu-Madi *et al.* 2015; Alfellani, Stensvold, *et al.* 2013; Dacal *et al.* 2018; D'Alfonso *et al.* 2017; Di Cristanziano *et al.* 2019; El Deeb and Khodeer 2013; El Safadi *et al.* 2014; Forsell *et al.* 2016; Hussein *et al.* 2008; Souppart *et al.* 2010). A study by Alfellani and colleagues in 2013 included a comprehensive subtyping study of the previously unsampled African countries: Liberia, Libya and Nigeria. As mentioned prior, ST3 had a high prevalence across all three countries (30-60%), as did ST1, which was detected at rates of 45.5% and 50% in Libya and Nigeria respectively (Alfellani, Stensvold, *et al.* 2013). The incidence of the other subtypes varies between reports. ST4 for example, has been described in Egypt (18.2%), Senegal (1.9%), Liberia (12.0%) and Nigeria (13.6%) (Alfellani, Stensvold, *et al.* 2013; El Safadi *et al.* 2014; Hussein *et al.* 2008) yet not detected in others

(Abaza *et al.* 2014; El Deeb and Khodeer 2013; Poulsen *et al.* 2016). ST6 and ST7 are usually recorded in low percentages across Africa and ST5 was identified in one report from school children in Angola which may be the result of zoonotic transmission (Dacal *et al.* 2018). ST8 has not been detected in human studies from this region, likely due to its rarity among humans and/ or geographical restriction.

Prevalence studies in Australia are few in number. Published works generally portray similar patterns as described in Europe with ST1-ST4 are generally the most commonly identified, (Nagel *et al.* 2012; Roberts *et al.* 2013; W. Wang *et al.* 2014). ST6, ST7 and ST8 were also identified from one study cohort from Sydney, possibly the result of zoonotic transmission (Roberts *et al.* 2013).

Across Asia and the Middle-East the number of epidemiological surveys regarding *Blastocystis* and other intestinal protozoa are increasing. A great many of these studies focus on communities within Thailand, Iran, Turkey and Malaysia, as well as in HIV/AIDS patients. Although many of the investigative studies are microscopy based, in recent years the number of subtyping reports has increased, allowing for distribution to be determined. The prevalence of *Blastocystis* across these regions ranges drastically from 0.4% and 0.7% in some microscopy based studies (Kyaw *et al.* 2018; Suntaravitun and Dokmaikaw 2018), to 82.9% in a molecular based study (Adao *et al.* 2016). There is a high incidence of ST1-ST3, with a particularly high rate of ST3 colonisation across the majority with one report documenting 100% in India (AbuOdeh *et al.* 2016; Badparva *et al.* 2014; Cakir, Cicek and Yildirim 2019; Das *et al.* 2016; Dogruman-Al *et al.* 2008; Gong *et al.* 2019; Jantermtor *et al.* 2013; Khademvatan *et al.* 2017; Li, Zhou, *et al.* 2007; Mardani Kataki, Tavalla and Beiromvand 2019; Mingmongkol *et al.* 2015; Mohammad, Al-Mekhlafi and Anuar 2018; Pandey *et al.* 2015; Pipatsatitpong *et al.* 2015). The prevalence of the remaining subtypes follows a similar incidence to that of Africa and South America; ST4 is generally reported in low percentages or is absent (Adao *et al.* 2016; Gong *et al.* 2019; Li, Zhang, *et al.* 2007; Noradilah *et al.* 2017; Popruk *et al.* 2015; Yowang *et al.* 2018; Zulfa, Sari and Kurniawan 2017) and to date there are only two studies which identify a higher prevalence of 40.9% and 84.1% in Iran and Nepal respectively (Khoshnood *et al.* 2015; Lee, Tan, *et al.* 2012). ST5 is reported infrequently and to a varying degree, ranging from 1.4% - 33% (Badparva *et al.* 2014; Moosavi *et al.* 2012). One record of up to 78.5% in pig handlers was reported but this is unsurprising as swine are often considered the reservoir host for ST5 (Pintong *et al.* 2018).

Likewise, ST6 and ST7 are reported in low percentages and are possibly linked to contact with probable reservoir hosts (Jantermtor *et al.* 2013; Palasuwan *et al.* 2016; Rezaei Riabi *et al.* 2018; Thathaisong *et al.* 2013; Vargas-Sanchez *et al.* 2015; Yowang *et al.* 2018).

There are numerous factors possibly influencing the distribution and prevalence of *Blastocystis* subtypes observed across the world; within the literature some have investigated factors that possibly contribute to observed distributions. Environmental factors such as seasonality (Alfellani *et al.* 2007; Noradilah *et al.* 2017) and drinking water (Anuar *et al.* 2013; Diarthini *et al.* 2018; Gong *et al.* 2019; Kyaw *et al.* 2018; Leelayoova *et al.* 2008; Poulsen *et al.* 2016) are seen to impact prevalence among study populations. Working and living alongside animals is another likely cause for subtype disparity among populations (Parkar *et al.* 2010; Schär *et al.* 2014; Shaker *et al.* 2019). This includes livestock (W. Wang *et al.* 2014), pets (Belleza *et al.* 2015) and wild animals (Yoshikawa *et al.* 2009). This shared subtype colonisation, in addition to increased incidence of certain subtypes among animal handlers working in zoos or on farms, accounts for a notable proportion of the more sporadic accounts of rarer subtypes isolated from humans – particularly in LMICS.

5.2 Subtype Distribution in Animals

Epidemiological surveys regarding *Blastocystis* in animals have expanded in recent years alongside livestock and pet surveys. The discovery and establishment of reservoir hosts of *Blastocystis* from a range of taxa has led to the discovery of more subtypes. ST10, ST11 and ST13 to ST17 are found exclusively in non-human hosts, displaying some variability in their distribution among host genera. Current animal based subtyping surveys display patterns of subtype distribution across different taxa as described in Figure 1.vi, yet many genera remain largely under sampled; including reptilian and other ectothermic hosts, insects and fish. The most well resolved animal hosts include primates, artiodactyls and rodent species (Yoshikawa, Koyama, *et al.* 2016).

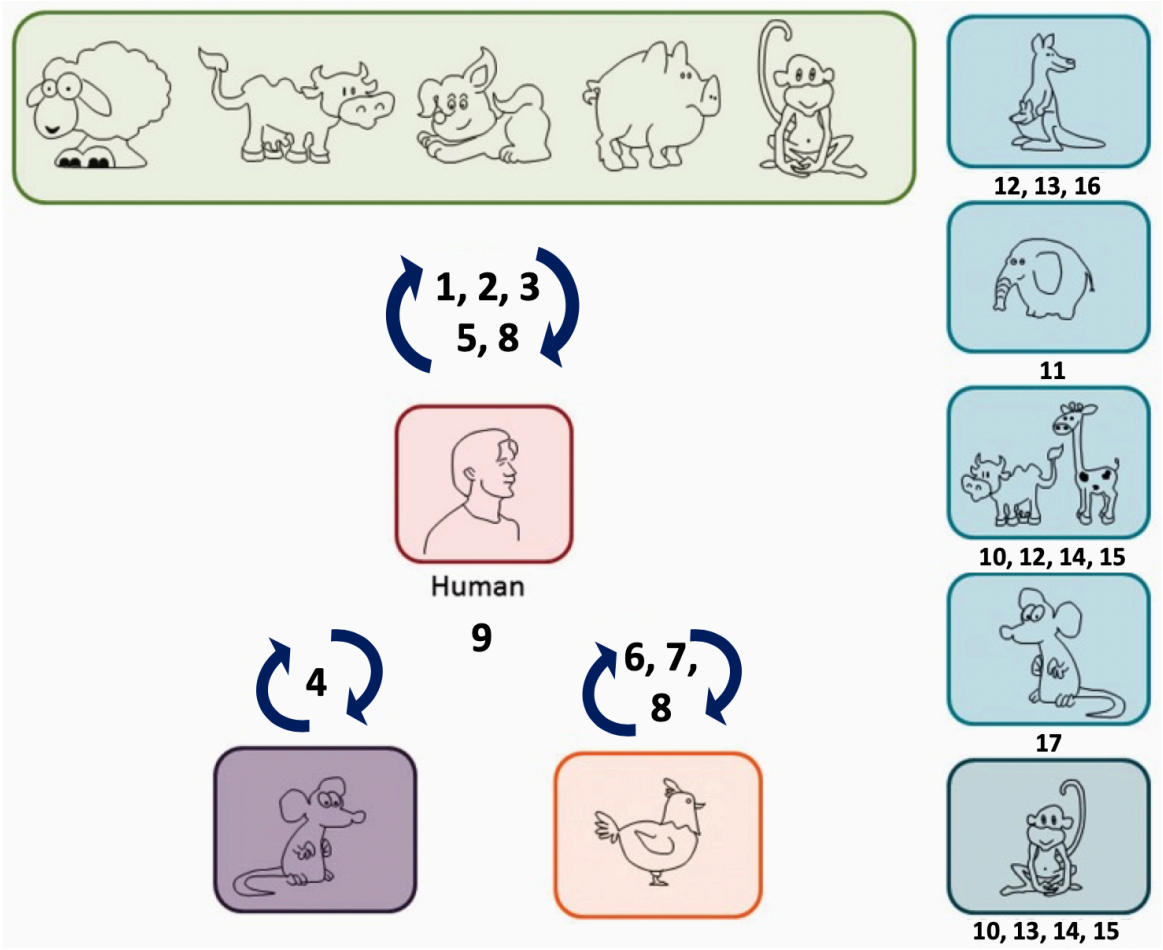

Figure 1.vi Updated depiction of the distribution and relative host specificity of *Blastocystis* subtypes in humans and animals as described by Wawrzyniak and colleagues (2013). ST1-ST9 can infect humans. ST1, ST2, ST5 and ST8 can infect a number of mammals whereas ST4 seems to be restricted to rodents and humans. ST6-ST8 are associated with avian hosts. ST10-ST17 have a range of host species and are almost exclusively found in animals including Artiodactyls, non-human primates, rodents and marsupials. Adapted from "*Blastocystis*, an unrecognized parasite: an overview of pathogenesis and diagnosis" by I. Wawrzyniak, 2013, Therapeutic Advances in Infectious Disease 1(5), p. 167-178. *Copyright 2013 by Authors 2013.*

Few subtypes have a limited host range, the rest have been recorded in a number of hosts. ST1, ST2 and ST3 are commonly isolated from non-human primates (NHPs), which often display different haplotypes than those identified in humans (Alfellani, Jacob, *et al.* 2013; Helenbrook, Shields and Whipps 2015; J. Li *et al.* 2019; Parkar *et al.* 2007; Parkar *et al.* 2010; Petrášová *et al.* 2011; Santín *et al.* 2011; Valença-Barbosa *et al.* 2019; Yoshikawa *et al.* 2009). ST1 and ST3 are widely distributed across the animal kingdom and are also commonly identified in captive animals and members of the *Suidae* family along with other artiodactyla including ruminants. ST1 is also frequently found in marsupials, birds and carnivores (Cian

et al. 2017; Parkar *et al.* 2007; Stensvold, Alfellani, *et al.* 2009; Valença-Barbosa *et al.* 2019; Wang *et al.* 2018). ST4 is most often found in rodents which are postulated to be a reservoir host (Yoshikawa, Tokoro, *et al.* 2016). ST5 is primarily isolated from hooved animals and is largely associated with pigs. Additionally, ST5 is also often found in African apes, although it is generally absent from new world primates (Alfellani, Jacob, *et al.* 2013; Cian *et al.* 2017; Santín *et al.* 2011; Wang *et al.* 2018). ST6-ST7 have a narrow host range and are generally restricted to avian hosts, with occasional sporadic reports from livestock and NHPs (Stensvold, Alfellani, *et al.* 2009; Yoshikawa, Abe and Wu 2004; Zanzani *et al.* 2016). Infrequently these subtypes have been reported in humans (Greige *et al.* 2018). *Blastocystis* has a relatively low prevalence in carnivorous animals (Cian *et al.* 2017; Paulos *et al.* 2018), possibly due to diet as its prevalence may be associated with plant based diets as speculated in humans (Forsell *et al.* 2017). Some reports of wild/stray dogs recorded a higher prevalence, the reason for this is unknown, but may relate to diet and / or sanitation (Ramírez *et al.* 2014; Wang *et al.* 2013). ST8 has a relatively low incidence and narrow host range. It is most often found among arboreal primates (Alfellani, Jacob, *et al.* 2013; Cian *et al.* 2017), but is also identified in marsupials (Ramírez *et al.* 2014), sporadically in rodents (Valença-Barbosa *et al.* 2019), once in an Armadillo, and in the same study it was also isolated from swine reared for livestock for the first time (Valença-Barbosa *et al.* 2019). ST10 and ST14 are the most prevalent subtypes identified in artiodactyls. The majority of surveys focus on cattle, but these subtypes have also been identified in sheep, goat, deer and camel (Alfellani, Taner-Mulla, *et al.* 2013; Fayer, Santin and Macarisin 2012; Lee *et al.* 2018; Masuda *et al.* 2018; Ren *et al.* 2019). Many of the subtypes between ST13 to ST17 include full length or almost full-length SSU rDNA sequences. ST11 has been reported only sporadically in animals, it was first described by Parkar et al. in 2010 in Asian elephants from Australia (Parkar *et al.* 2010), since then is has also been detected in Elephants in a separate study (J. Li *et al.* 2019) and the full SSU rRNA was recently sequenced (Maloney, Molokin and Santin 2020). ST12 was first detected in giraffes and kangaroos from an Australian zoo (Parkar *et al.* 2010) and has since been recorded again in giraffes in addition to waterbuck, both of which were from China (Zhao *et al.* 2017). More recently it was recorded in cattle, goats and other bovids in epidemiological surveys (Ren *et al.* 2019; Udonsom *et al.* 2018). ST13 has a broad host range, but is rarely found in characterisation studies. It was first identified in the Australian marsupial: the quokka (*Setonix brachyurus*) (Parkar *et al.* 2010), and has also been isolated from primates (J. Li *et al.* 2019; Petrášová *et al.* 2011; Zhao *et al.* 2017) and artiodactyls (Alfellani, Taner-Mulla, *et al.* 2013; Wang *et al.* 2018). Upon their

characterisation, ST15, ST16 and ST17 typically form basal branches which group with ectothermic isolates (Alfellani, Taner-Mulla, *et al.* 2013; Yoshikawa, Koyama, *et al.* 2016; Yowang *et al.* 2018). These have been isolated from few hosts, including camels, cattle and primates (ST15) (Alfellani, Jacob, *et al.* 2013; Alfellani, Taner-Mulla, *et al.* 2013), kangaroos (ST16) (Yoshikawa, unpublished) and gundi (ST17) (Alfellani, Taner-Mulla, *et al.* 2013). Other species have also been identified but not given subtypes, these include ectothermic host species of which there are 14 currently identified (Yoshikawa, Koyama, *et al.* 2016), such clades are generally unstable due to the limited data available in sequence databases.

5.2.1 Importance of Animal Study

Although the majority of subtyping and prevalence studies focus on human populations, there is an increasing interest in broadening our understanding of the microbial diversity across other host taxa. There is a particular interest in regard to animals that may serve as disease reservoirs, and numerous reports exemplify the transmission of *Blastocystis* between animals and humans in close contact. Examples include ST2, ST5 and ST6 (Greige *et al.* 2018; Schär *et al.* 2014; Yoshikawa *et al.* 2009). An understanding of subtype distribution in animals is also fundamental for conservation efforts, which is of growing importance in today's society. The monitoring of the microbiota has recently been linked to the success of re-wilding and translocation projects, where disease transmission can be monitored between captive and wild populations and establishing the constitution of a 'healthy' microbiota may support animal fitness and adaptability (Chong *et al.* 2019; Kohl and Dearing 2014).

5.2.2 Reservoir Hosts and Zoonotic Potential

Nöel et al. in 2005 first proposed that *Blastocystis* subtypes have possible reservoir hosts and using recent epidemiological surveys some subtypes can with some confidence be associated with a particular host species. As documented above, the subtypes exhibit certain prevalence patterns among hosts. These patterns could account for possible reservoir hosts which are likely able to provide sources of human infection and can account for many of the cases of human infections that lie outside the human associated subtypes. Many transmission occurrences between animal and human are likely due to close contact, for example animal handlers in zoos (Alfellani, Jacob, *et al.* 2013; Parkar *et al.* 2007; Rivera

2008). Subtypes with supposedly narrower host ranges have been associated with zoonotic transmission. ST6 - ST7 account for approximately 10% of human isolates identified outside of Europe and are often isolated from humans who are in close, prolonged contact with avian species. There are a number of reports that document ST6 and ST7 in poultry workers, and examples include a recent report by Greige et al. in 2018 who investigated subtype prevalence between workers at three poultry slaughterhouses in Lebanon where poultry slaughterhouse workers and a control group were all tested for *Blastocystis*. Results showed an apparent lack of ST6 and ST7 in individuals who had no contact with poultry in comparison to the workers who had contact, and two people were identified to have ST6 that closely related to the ST6 genotype of study birds.

ST8 has a narrow host range and is usually confined to NHPs but has been isolated from humans in some cases, this includes where humans had been in contact with primates (howler monkeys (*Alouatta palliata aequatorialis*)) (Helenbrook, Shields and Whipps 2015; Scicluna, Tawari and Clark 2006). This subtype has also been noted to cause symptomatic disease in few human infections (Roberts *et al.* 2013; Stensvold *et al.* 2008).

ST5 is commonly associated with pigs and is often isolated in asyptomatic pig handlers (Pintong *et al.* 2018; W. Wang *et al.* 2014).

It is important to note that in many instances the subtypes isolated from human and animal require further analysis in order to unequivocally determine the origin of infection, and/or whether genetic isolates are related. Currently, genomic data for many of these studies reveals considerable overlap between in infecting subtypes, but, as noted by Alfellani et al. and Stensvold et al. in 2013 and 2012 respectively: studying allelic differences between infecting subtypes reveal more concise conclusions about zoonotic occurrences (Alfellani, Jacob, *et al.* 2013; Greige *et al.* 2018; Sanpool *et al.* 2015; Stensvold, Alfellani and Clark 2012; Stensvold and Clark 2016; Yowang *et al.* 2018). Determining the source of *Blastocystis* infection in animal handlers and animals, and establishing which subtypes are possible commensal colonisers of animals; especially those in intensive farming where disease spread is facilitated by living conditions and prophylactic antimicrobial drug use is common-practice, needs to be considered in order to help support healthy animals. (Valença-Barbosa *et al.* 2019; Wang *et al.* 2018).

5.2.3 Animal Conservation

When undertaking epidemiological surveys in animals it is important to try and sample from a range of sources, countries, zoos, wild and captive animals in order to best establish the true inter- and intra-species diversity. Furthering our understanding of the typical prevalence in animals will not only help establish the host range of *Blastocystis* subtypes and possible reservoir hosts, but can aid understanding of the impact of captivity on animals. For example, transmission events between human and animal, and between animals of different species in captivity is likely (Parkar *et al.* 2010). The acquisition of different subtypes may have an unknown impact on host health and poses the risk of introducing *Blastocystis* to novel hosts. Furthermore, even if *Blastocystis* poses no health risks – it may act as a marker for assessing transmission of other (possibly pathogenic) gut microbes, such as other intestinal parasites and bacteria.

Additionally, captive animals may have altered diets and are likely treated with antimicrobial drugs. The impact of such aspects on the gut microbiota are important to consider when addressing animal health. In terms of *Blastocystis,* being able to establish what constitutes part of a supposedly healthy microbiota can prove useful when assessing animal health and possible release into the wild or movement between wildlife parks – this is especially important when considering the possibility of introducing captivity-acquired subtypes into wild host populations. The subtypes found in captive animals have been reported to predominantly match that of their wild counterparts. Possibly indicating that the microbiota is resistant to change, and captive lifestyles are likely similar to that of the wild (Parkar *et al.* 2007; Parkar *et al.* 2010).

5.3 Limitations of Epidemiological Studies

For all of the usefullness of epidemiological studies focusing on *Blastocystis*, there are obvious limitations which will affect the validity and accuracy of data. One key contributor to disparity in the literature is the different detection techniques, and relative sensitivities used in surveys can impact the results documented. Many parasitological reports from LMICs are surveys of numerous enteroparasites including protozoa and helminths with the aim to assess potential pathogenicity in symptomatic vs asymptomiatic cohorts. There is no particular focus on *Blastocystis,* and such surveys are often done via microscopy and staining

which is less sensitive yet inexpensive and time efficient. It does however mean that no subtyping data is collected and thus means that there is often less subtyping data than in HICs (Belleza *et al.* 2015; Kyaw *et al.* 2018; Liao *et al.* 2017; Sangaré *et al.* 2015; Suntaravitun and Dokmaikaw 2018). Amongst epidemiological reports which do include subtypes, there is no standardised molecular approach to identification. The use of STS primers gives a fast diagnostic approach for subtyping, and is less labour intensive, inexpensive and can identify mixed infections. However, it is only possible for ST1-ST7 and can miss genetic variants leading to false negative results. Stensvold et al. noted that an STS based method introduced by Yoshikawa et al. (1998, 2000, 2003) may selectively amplify select clades within target subtypes (Stensvold, Alfellani and Clark 2012). The other main identification method involves the sequencing of small subunit rRNA gene, and although costlier and more time consuming, allows for phylogenetic analysis of isolates. This allows for intra-subtype analysis and can help determine transmission and zoonotic potential. However, mixed infections and dominant subtypes may cause inaccuracies with this method which is often used in HICs (Stensvold 2013b).

Moreover, additional influences on epidemiological surveys include the sample size and target cohort. As mentioned above, many of the studies in LMICS are parasitological surveys and are often carried out on symptomatic hospital patients, inflammatory bowel disease (IBD) sufferers and those with HIV/AIDS. Sampling from select subpopulations can skew data and give an inaccurate portrayal of subtype distribution i.e. through the possibility of pathogenic subtypes and gut dysbiosis influencing the subtypes isolated and not truly representing the population prevalence (Mirjalali *et al.* 2017; Roberts *et al.* 2013).

6 Symptoms, Pathogenicity and Treatment

Blastocystis is one of the most commonly isolated microeukaryotes from stool samples of healthy individuals and patients experiencing gastrointestinal symptoms. Its role as a pathogen has been the subject of numerous investigations, yet it remains controversial. A defined link between *Blastocystis* and disease has yet to be explicitly defined (Ajjampur *et al.* 2016; Clark *et al.* 2013; Gentekaki *et al.* 2017; Stensvold and van der Giezen 2018). When isolated from a host as the only possible causative agent of disease, *Blastocystis* is associated with a range of nonspecific symptoms, the most common being acute or chronic diarrhoea and abdominal pain, other symptoms often noted in literature include nausea, abdominal discomfort and bloating (Coyle *et al.* 2012; Stensvold, Nielsen, *et al.* 2009). Interestingly

symptomatic infection is often documented in immunocompromised individuals, including HIV patients, however whether this is due to sampling bias needs to be thoroughly investigated before conclusions can be drawn (Kurniawan *et al.* 2009). Clark et al. (Clark 1997) was the first to propose that symptomatic infection may be attributed to particular genetic isolates, with additional research indicating that the uncertainty in pathogenicity is possibly linked to its vast genetic diversity, life cycle stages and parasite load (Gentekaki *et al.* 2017; Kaneda *et al.* 2001; Noël *et al.* 2005; Seguí *et al.* 2017; Stensvold, Suresh, *et al.* 2007). Although there is some evidence of pathogenicity linked to ST1-ST3, ST5 and ST7 it must be taken into consideration that these subtypes, excluding ST5 and ST7 (which could be the result of zoonosis) are most abundant from all human prevalence studies regardless of symptoms. Thus, their associations with pathogenicity may be because they are the most often described in humans in general, and not just in symptomatic patients. Table 1.i represents pathogenicity outcomes from a number of epidemiological studies, including IBD cohorts, and includes other eukaryotic parasites that were found in the study. It demonstrates the lack of conclusive evidence for either condition and it should be noted that identified subtypes rarely gave rise to one single outcome. Where possible, the statistically significant findings are presented.

Table 1.i Identification of *Blastocystis* (and other intestinal parasites) in patients experiencing symptomatic disease or asymptomatic human cohorts across a number of epidemiological studies. It includes studies which tested for and identified co-parasitism via microscopic and molecular detection methods. The numbers in parenthesis represents the count for other parasites as reported by the investigating group regardless of the disease state. The number may be out of the total cohort or from polyparasitism cases only depending on the study. Co-parasitism with *Blastocystis* represents intestinal eukaryotes that were found in the cohort overall, and are not linked to particular subtypes here.

***Blastocystis* Subtype**	**Cohort Size**	***Blastocystis* positive**	**Co-parasitism with *Blastocystis***	**Reference**
ST1 (Asymptomatic) **ST2 ST3** (Symptomatic)	277	125	*Entamoeba* sp., *Endolimax* sp. *Trichuris sp,*. *Iodamoeba* sp. (20) *Giardia* sp. (17)	(Ramírez *et al.* 2014)
ST2 ST7 ST8 (Symptomatic) **ST1 ST3 ST4 ST6** (Symptomatic, Asymptomatic)	513	98	*Giardia* sp. (5) *Dientamoeba* sp. (2) *Endolimax* sp. (4) *Cryptosporidium* sp. (1) *Entamoeba* sp. (2)	(Roberts *et al.* 2013)

ST3 ST4 ST7 (Symptomatic, Asymptomatic) **ST1 ST2** (Asymptomatic)	186	27	*Giardia* sp. (2)	(Poirier *et al.* 2011)
ST1 ST4 (Symptomatic, Asymptomatic) **ST2 ST3** (Asymptomatic)	723	186		(Tito *et al.* 2019)
ST1 ST2 ST3 ST7 (Asymptomatic)	181	76		(Oliveira-Arbex, David and Guimarães 2018)
ST1 ST2 ST3 ST ST7 ST5 ST12 (Asymptomatic) **ST1 ST2 ST3 ST6** (Symptomatic)	346	NA		(Ramírez *et al.* 2016)
ST1 ST2 ST3 ST6 ST7 (Asymptomatic)	126	10	*Giardia* sp. (19) *Dientamoeba* sp. (19) *Cryptosporidium* sp. (0) *Entamoeba* sp. (31) *Iodamoeba* sp. (1) *Endolimax* sp. (1) Hookworm (3)	(David *et al.* 2015)
ST1 ST2 ST3 ST4 ST5 (Symptomatic) **ST6** (Asymptomatic)	58	31	*Endolimax* sp. (2) *Entamoeba* sp. (4) *Taenia* sp. (1) *Giardia* sp. (1)	(Melo *et al.* 2019)
ST1 ST2 ST3 ST4 ST6 ST7 (Symptomatic, Asymptomatic)	2,026	256	*Ascaris* sp. (42) *Trichuris* sp. (21) Hookworm (18) *Giardia* sp. (102) *Entamoeba dispar, E. histolytica,* *E. moshkovskii.* (210) *Entamoeba coli* (141) *Hymenolepis* sp. (2) *Endolimax* sp. (85)	(Ramírez *et al.* 2017)
ST1 ST2 ST3 (Asymptomatic)	182	109	*Chilomastix* sp. *Entamoeba* sp. *Endolimax* sp. *Hymenolepis* sp. *Giardia* sp. *Iodamoeba* sp.	(Rojas-Velázquez *et al.* 2018)

ST1 ST2ST3 ST4 ST6 ST8 (Symptomatic, Asymptomatic) **ST4** (Symptomatic)	766	216	*Giardia* sp. (84) *Endolimax* sp. (114) *Entamoeba* sp. (77) *Chilomastix* sp. (3) *Retortamonas* sp. (2) *Ascaris* sp. (38) *Trichuris* sp. (35) Ancylostomatidae (8) *Strongyloides* sp. (2) *Enterobius* sp. (3)	(Seguí *et al.* 2018)
ST1 ST2 ST3 ST4 (Asymptomatic)	258	100	*Giardia* sp. (27) *Cryptosporidium* sp. (25) *Entamoeba* sp. (31) *Chilomastix* sp. (4) *Endolimax* sp. (16)	(Villamizar *et al.* 2019)
ST1 ST2 ST4 (Symptomatic)	51			(Domínguez-Márquez *et al.* 2009)
ST1 ST2 ST3 ST4 (Symptomatic)	444	25		(Stensvold *et al.* 2011)
Blastocystis (Asymptomatic)	268	43	*Entamoeba* sp. (115) *Giardia* sp. (101) *Hymenolepis* sp. (15) *Chilomastix* sp. (3) *Endolimax* sp. (16) *Iodamoeba* sp. (10) *Taenia* sp. (3) *Ascaris* sp. (4) Hookworm (1)	(Macchioni *et al.* 2015)
ST1 ST2 ST3 (Symptomatic, Asymptomatic)	382	80		(Malheiros *et al.* 2011)
Blastocystis **(ST3)** (Asymptomatic)	156	102	*Entamoeba* sp. (5)	(Nieves-Ramírez *et al.* 2018)
ST1 ST2 ST3 ST4 ST5 ST6 ST7 ST8 (Symptomatic, Asymptomatic)	421	356		(Alfellani, Stensvold, *et al.* 2013)
ST1 ST2 ST3 ST6 (Symptomatic, Asymptomatic)	270	67	*Endolimax* sp. (5) *Dientamoeba* sp. (5) *Giardia* sp. (5) *Entamoeba* sp. (9) *Enterobius* sp. (3) *Cystoisospora* sp. (1)	(Casero *et al.* 2015)

			Chilomastix sp. (1) *Iodamoeba* sp. (1)	
Blastocystis (Symptomatic)	163	49	*Dientamoeba* sp. (101) *Giardia* sp. (10) *Cryptosporidium* sp. (0) *Entamoeba* sp. (0)	(Maas *et al.* 2014)
Blastocystis Amoeboid (Symptomatic) ***Blastocystis*** (Asymptomatic)	20	NA		(Tan and Suresh, 2006a)
Blastocystis (Symptomatic, Asymptomatic)	64	NA		(Kaneda *et al.* 2001)
ST1 ST2 ST3 (Symptomatic, Asymptomatic)	1,262	133		(Mohamed *et al.* 2017)
ST1 ST3 (Symptomatic, Asymptomatic) ST2 (Symptomatic)	57	NA		(Abaza *et al.* 2014)
ST1 ST3 (Symptomatic, Asymptomatic)	2,134	66		(Yoshikawa, Wu, *et al.* 2004)
ST1 ST3 (Symptomatic, Asymptomatic) ST2 (Asymptomatic)	35	NA		(Yan *et al.* 2006)
Blastocystis (ST3) (Symptomatic, Asymptomatic)	NA	NA		(Rajamanikam *et al.* 2019)
Blastocystis (Symptomatic, Asymptomatic)	307	37	*Entamoeba* sp. (3) *Giardia* sp. (2)	(Udkow and Markell 1993)
Blastocystis (Symptomatic, Asymptomatic)	1,000	203	*Cryptosporidium* sp. (0) *Giardia* sp. (15) *Dientamoeba* sp. (1) *Enterobius* sp. (5) *Hymenolepis* sp. (2) *Ascaris* sp. (5) *Entamoeba* sp.	(Nimri and Batchoun 1994)

ST1 (Symptomatic) **ST2 ST3** (Asymptomatic)	32	NA	(Eroglu *et al.* 2009)
ST1 ST2 ST3 (Symptomatic, Asymptomatic)	286	92	(Dogruman-Al *et al.* 2008)
ST1 ST2 ST6 ST7 (Asymptomatic) **ST4** (Symptomatic) **ST3** (Symptomatic, Asymptomatic)	51	NA	(Vassalos *et al.* 2010)
ST1 ST2 ST3 ST7 (Symptomatic, Asymptomatic)	100	97	(Vargas-Sanchez *et al.* 2015)

Although pathogenicity is controversial, when identified from symptomatic patients treatment is usually prescribed. There are several drugs available, however within the literature there is variation and contradiction within reports regarding alleviation of infection. Probably the most commonly prescribed medication is metronidazole; this antiprotozoal and antibiotic is often prescribed as a first line treatment either alone or as combination therapy and has been demonstrated to eradicate *Blastocystis in vitro* and in approximately 80% of human cases. On the other hand, numerous reports also document *Blastocystis* resistance to metronidazole. This may possibly due to a lack of genes responsible for the activation/inactivation of this drug into its active state and the fact that the concentration of metronidazole that reaches the lumen is very low (Coyle *et al.* 2012; Nasirudeen *et al.* 2004; Nigro *et al.* 2003; Rajamanikam *et al.* 2019; Stensvold *et al.* 2008; Tan 2008). Other treatments include: nitazoxanide, trimethoprim-sulfamethoxazole, paramomycin, iodoquinol, ketoconazole, secnidazole, emetine and tinidazole. There is some discrepancy with regards to the efficacy of some of these drugs, and resistance to metronidazole and ketoconazole amongst others has been observed (Yakoob *et al.* 2004). Plausible reasons for drug resistance and subsequent infection relapse include; *Blastocystis* forming cytotoxic resistant cysts, increased reproduction rates, and re-infection (Nigro *et al.* 2003; Rajamanikam *et al.* 2019; Tan 2008; Haresh *et al.* 1999; Zaman and Zaki 1996). Another possibility is that different subtypes have varying degrees of susceptibility to these treatments (Dhurga *et al.* 2012; Stensvold *et al.* 2010). For example Mirza and colleagues

demonstrated metronidazole resistance in ST7, emetine resistance in ST4 and mixed resistance for both with tinidazole (Mirza, Teo, *et al.* 2011).

An added dilemma is that there is often uncertainty regarding dosages and treatment regimens for patients and it is often difficult to determine whether treatment success is due to drug intervention (Sekar and Shanthi 2013; Haresh *et al.* 1999). A study in Taiwan found that of 100 patients infected with *Blastocystis,* 91.2% of then had undetectable infection a year later despite no specific treatment (Kuo *et al.* 2008). Although there are discrepancies and uncertainties, it is generally accepted that patients with gastro-intestinal symptoms seek medical attention as the therapeutic improvement in the majority of patients who are treated thus warrants the need to prescribe medication.

6.1 Association with Inflammatory Bowel Related Diseases

Inflammatory bowel disease (IBD) is a term used to describe two inflammatory bowel disorders: Ulcerative colitis and Crohn's disease, which are both currently incurable. These disorders are of increasing importance, particularly in Europe and the Middle East (Dogruman-Al *et al.* 2010; Nourrisson *et al.* 2014), and are generally characterised by relapsing episodes of abdominal discomfort, bloating, diarrhoea and weight loss. The pathophysiology of IBD and Irritable bowel syndrome (IBS) is poorly understood and there is no single cause however genetic factors, gender, environment, immune system and the microbiota all thought to be involved (Nourrisson *et al.* 2014; Poirier *et al.* 2012; Rigottier-Gois 2013; Xavier and Podolsky 2007).

The first link between *Blastocystis* and IBD was made by Giacomettu in 1999 (Giacometti *et al.* 1999), observations from this work documented a relatively high incidence of *Blastocystis* among IBD patients. Since then, the link between *Blastocystis* and IBD, and *Blastocystis* and IBS, has been made by others (Cekin *et al.* 2012; Kök *et al.* 2019; Tai *et al.* 2011; Yakoob *et al.* 2004; Yamamoto-Furusho and Torijano-Carrera 2010). Although this has been contradicted (Coskun *et al.* 2016; Krogsgaard *et al.* 2015; Petersen *et al.* 2013; Rossen *et al.* 2015; Tito *et al.* 2019) leading to much dispute about the roles of *Blastocystis* in inflammatory bowel diseases. Reasons for the disparity in pathogenesis range widely within the literature. However, when considering the complexity of relationships and feedback loops between microbiota and host, it is unreasonable to investigate the role of *Blastocystis* in IBD independently but instead consider its role within the microbial/host community. The

literature highlights possible influencers of its role in disease; some reports indicate the importance of subtype, and some studies have demonstrated links between particular isolates and disease. For example one cohort of IBD patients from Pakistan had a high prevalence of ST1 (Yakoob *et al.* 2010). Another study in Mexico also documented high rates of ST1 and also ST3 in IBS patients; although it should be noted that that these subtypes are common in asymptomatic humans (Jimenez-Gonzalez *et al.* 2012). Parasite load, host factors such as immunological response to infection, sampling from active or remised patients, and considering the classification of disease, are other important factors to consider when investigating *Blastocystis* in IBD as they will impact associated symptoms and may have different underlying causes (Cekin *et al.* 2012; Dogruman-Al *et al.* 2009; Nourrisson *et al.* 2014).

Interactions of *Blastocystis* with the surrounding microbiota is an increasingly studied element relating to pathogenicity in IBD. Microbiota shifts and disturbances are observed in IBD (Nishikawa *et al.* 2009; Öhman and Simrén 2013) and it could be reasonable to suggest that bacterial communities can contribute to *Blastocystis* pathogenicity or proliferation under certain conditions (i.e. by providing favourable conditions for them to flourish and alter conditions in the gut).

Conversely *Blastocystis* is often found in asymptomatic, seemingly healthy individuals and is sometimes described as an indicator of positive gut health. Oxygen levels are speculated to be increased in IBD patients (the Oxygen hypothesis) which challenges the strict anaerobic nature of *Blastocystis* and other anaerobic microbes, but promotes colonisation with facultative anaerobes (Rigottier-Gois 2013; Rivera-Chávez, Lopez and Bäumler 2017). Krogsgaard et al. reported that *Blastocystis* was higher in asymptomatic individuals from a Danish cohort: 22% compared to 15% prevalence in patients, indicating no role in disease (Krogsgaard *et al.* 2015). Another study by Coskun et al. demonstrated a low colonisation in ulcerative colitis patients (Coskun *et al.* 2016), and a large study conducted on a Flemish cohort identified a 4% prevalence in active IBD patients compared with 30% in healthy individuals (Tito *et al.* 2019).

Altered and / or reduced microbial diversity may be another reason why *Blastocystis* is absent in some IBD patients which links to the roles of *Blastocystis* as an indicator for monitoring gut instability. Some reports even speculate *Blastocystis* may play protective role in the gut, and can be used as a biomarker for eubiosis. Beneficial bacteria including *Clostridia*, *Ruminococcaceae* and *Prevotellaceae* are often correlated to the presence of *Blastocystis*. Whereas pro-inflammatory *Enterbacteriaceae* is often found in individuals

lacking *Blastocystis, Enterbacteriaceae* is a facultative anaerobe and acts as a marker for dysbiosis. Such shifts in microbial communities could promote inflammation, which happens to be a hallmark of IBD (Audebert *et al.* 2016; Byndloss *et al.* 2017; Iebba *et al.* 2016; Kök *et al.* 2019). However, the fact that observable differences between human populations is observed leads to the plausibility that the presence of *Blastocystis* is not a direct cause of IBD but plays an unclear role within the microbiota regarding disease. Inconsistent data caused by a lack of uniformity among samples sizes, diagnostic methods, lifestyle and geography will all no doubt contribute to the uncertainty.

7 Roles and Interactions of *Blastocystis* with the Microbiota

Within the gastrointestinal tract of humans there is a varied, dynamic and complex relationship between the host and microbiota. Established shortly after birth and maintained throughout life, the microbiota has likely co-evolved with mankind over thousands of years to provide a mutually beneficial relationship (Rinninella *et al.* 2019). The complex community makeup is compiled of trillions of microbes which include bacteria, microeukaryotes, archaea and viruses, and are influenced by a number of factors, such as diet, antibiotic use, disease, age and genetics (Nieves-Ramírez *et al.* 2018). The microbiota is involved in numerous key physiological roles including immunomodulation, nutrient acquisition, and short chain fatty acid synthesis. Additionally it has been referred to as a "forgotten organ" (O'Hara and Shanahan 2006) as well as being implicated in a range of diseases and disorders that have been directly or indirectly linked to the GI (gastrointestinal) tract. There are many gaps in what is known about the impact of bacterial communities on parasite prevalence and vice versa. Some interesting links have been identified, for example the presence of *Lactobacillus* may reduce the proliferation of *Giardia* (Müller and von Allmen 2005). Few publications however focus on interactions between *Blastocystis* and the GI microbiota.

7.1 *Blastocystis* and the Bacterial Component of the Microbiota

Although there is no certain composition of a 'normal' gut microbiota, general patterns of difference are observed between those suffering from a range of diseases. Dysbiosis of the GI tract is often characterised by inflammation as seen in IBD, where altered and reduced

communities of bacteria are often seen. There are many drivers for the bacterial makeup of the GI tract and within the bacterial component 12 phyla have been identified thus far with the Bacteroides, Firmicutes, Proteobacteria and Actinobacteria representing over 90% (Donaldson, Lee and Mazmanian 2016; Rinninella *et al.* 2019) within three possible stratified enterotypes: *Bacteroides*, *Prevotella* or *Ruminococcus* (Arumugam *et al.* 2011). The eukaryotic component makes up a smaller proportion of the microbiota by number and has only recently been examined in depth. Investigation of the roles of *Blastocystis* as an influencer in the gut is a relatively recent area of study and many studies demonstrate a rich microbial diversity in its presence; such reports focus on bacterial richness at different taxonomic levels. A study by Audebert et al. in 2016 applied next generation sequencing to assess bacterial diversity in patients with *Blastocystis* against those without. The report documents differences in bacterial communities from the phylum level - which are relatively consistent, down to the family and genus levels where bacterial community differences are observed between colonised and un-infected hosts. Overall, *Blastocystis* was associated with an increase in bacterial diversity; this included *Roseburia* and *Faecalibacterium,* which can be considered hallmarks of eubiosis and associated with the production of the short chain fatty acid Butyrate which is a key metabolite for maintaining GI tract homeostasis (Audebert *et al.* 2016). A number of other studies also support increased bacterial richness and diversity in the presence of *Blastocystis* colonisation (Andersen *et al.* 2015; Andersen *et al.* 2016; Beghini *et al.* 2017; Forsell *et al.* 2017; Kodio *et al.* 2019; Nash *et al.* 2017; Tito *et al.* 2019).

Reports linking *Blastocystis* to reduced microbial diversity includes that by Nourrisson et al. 2014, who focused on bacterial communities in IBS patients. It was found that *Blastocystis* was associated with a decrease in bacterial diversity including *Bifidobacterium* spp. and *Faecalibacterium prausnitzii*, which are associated with eubiosis (Nourrisson *et al.* 2014). Others, including a report by Yason et al. in 2019, proposed that certain *Blastocystis* isolates may be causing bacterial community disruptions, and the confusion between dysbiosis in the presence of *Blastocystis* may be due to reports not identifying the colonising subtypes. In this study, ST7 isolates were linked to an increase in the facultative anaerobe *E.coli* and a decrease in *B. longum*: an obligate anaerobe which helps maintain the epithelial barrier and is considered a probiotic. Nonetheless, other *Blastocystis* subtypes were linked to increased bacterial diversity (Yason *et al.* 2019). Another study noted a decrease in *Prevotella copri* in patients with ST3 and it was postulated that *Blastocystis* may be exerting predatory effects on certain bacteria (Nieves-Ramírez *et al.* 2018). Opposite effects were observed in both ST3

and ST4 on the mucin utilising bacteria *Akkermansia,* which was positively correlated with ST4 and negatively with ST3, this was observed in a large cohort study by Tito et al. in 2019 (Tito *et al.* 2019). Recently, this approach has been expanded to include NHPs, and results indicated a decline in bacterial diversity in the presence of *Blastocystis* (Renelies-Hamilton *et al.* 2019).

Competition between *Blastocystis* and some bacteria for ecological niches and nutrients are plausible explanations for the notable absence of some bacteria in the presence of *Blastocystis* in the gut (Forsell *et al.* 2017). The presence of *Blastocystis* may also be dependent on the presence and activity of certain bacterial communities; If there is low microbial richness, for example in a state of dysbiosis, then *Blastocystis* may not be able to establish itself within the GI tract (Andersen *et al.* 2015; Andersen *et al.* 2016; Audebert *et al.* 2016; Forsell *et al.* 2017).

Many reports note a link between an absence of *Bacteroides*-dominated enterotypes with *Blastocystis* colonisation (Andersen *et al.* 2015; Andersen *et al.* 2016; Forsell *et al.* 2017; Tito *et al.* 2019). Although cause and effect cannot be proven, a possible reason for this correlation may be due to a reduction in microbes associated with butyrate production. This causes an increase in oxygen levels from reduced oxygen metabolism by epithelial colonocytes, thus leading to unfavourable conditions for *Blastocystis* proliferation (Stensvold and van der Giezen, 2018). It should also be considered that general associations with higher bacterial taxonomic levels need to be loosely considered as *Blastocystis* may interact differently between bacterial genus and species level members as described by Beghini et al. in 2017. This study found that although there was a general positive correlation between *Blastocystis* and Clostridiales and Firmicutes, some members were notably absent at the species level (Beghini *et al.* 2017). Observing the effects of *Blastocystis* on particular bacterial species *in vitro* such as that done by Yason et al. in 2019 (Yason *et al.* 2019), is one method which can be used to investigate the complex roles of *Blastocystis* in the gut on individual bacterial species- for example those coined hallmarks for eubiosis or dysbiosis. Few reports document no associations between *Blastocystis* and the gut microbiota, this may be due to small cohort or methodology (Nagel *et al.* 2016; Ramirez-Miranda *et al.* 2010; Surangsrirat *et al.* 2010). Table 1.ii summarises some recent prevalence studies carried out investigating bacterial communities and *Blastocystis* colonisation in humans with the publications including statistically significant findings ($p<0.05$), which are summarised (Table 1.ii).

Table 1.ii Statistically significant results of bacterial community prevalence in correlation to *Blastocystis* colonisation

OTU	Taxonomic level	Association with *Blastocystis*	Reference
Firmicutes	Phylum	No association Positive	(Audebert *et al.* 2016) (Beghini *et al.* 2017) (Kodio *et al.* 2019)
Elusimicrobia	Phylum	Positive	(Kodio *et al.* 2019)
Lentisphaerae	Phylum	Positive	(Kodio *et al.* 2019)
Euryarchaeota	Phylum	Positive	(Kodio *et al.* 2019)
Bacteroides	Phylum	Negative	(Forsell *et al.* 2017) (Andersen *et al.* 2015) (Beghini *et al.* 2017) (Andersen *et al.* 2016)
Proteobacteria	Phylum	No association Negative	(Audebert *et al.* 2016) (Beghini *et al.* 2017) (Kodio *et al.* 2019)
Actinobacteria		No association Negative	(Beghini *et al.* 2017) (Kodio *et al.* 2019)
Clostridia	Class	Positive	(Audebert *et al.* 2016) (Kodio *et al.* 2019)
Elisimicrobia	Class	Positive	(Kodio *et al.* 2019)
Lentisphaeria	Class	Positive	(Kodio *et al.* 2019)
Metanobacteria	Class	Positive	(Kodio *et al.* 2019)
Deltaproteobacteria	Class	Positive	(Kodio *et al.* 2019)
Mollicutes	Class	Positive	(Audebert *et al.* 2016)
Planctomycetacia	Class	Negative	(Kodio *et al.* 2019)
Rubrobacteria	Class	Negative	(Kodio *et al.* 2019)
Deinococci	Class	Negative	(Kodio *et al.* 2019)
Gammaproteobacteria	Class	Negative	(Kodio *et al.* 2019)
Bacilli	Class	Negative	(Audebert *et al.* 2016) (Kodio *et al.* 2019)
Bacteroidia	Class	Negative	(Gabrielli *et al.* 2020)
Clostridiales	Order	Positive	(Audebert *et al.* 2016) (Beghini *et al.* 2017) (Kodio *et al.* 2019)
Victivallales	Order	Positive	(Kodio *et al.* 2019)

Methanobacteriales	Order	Positive	(Kodio *et al.* 2019)
Elusimicrobiales	Order	Positive	(Kodio *et al.* 2019)
Aeromonadales	Order	Positive	(Kodio *et al.* 2019)
Acidaminococcales	Order	Positive	(Kodio *et al.* 2019)
Desulfovibrionales	Order	Positive	(Kodio *et al.* 2019)
Planctomycetales	Order	Negative	(Kodio *et al.* 2019)
Rhodobacterales	Order	Negative	(Kodio *et al.* 2019)
Sphingomonadales	Order	Negative	(Kodio *et al.* 2019)
Rubrobacterales	Order	Negative	(Kodio *et al.* 2019)
Veillonellales	Order	Negative	(Kodio *et al.* 2019)
Pasteurellales	Order	Negative	(Kodio *et al.* 2019)
Micrococcales	Order	Negative	(Kodio *et al.* 2019)
Pseudonocardiales	Order	Negative	(Kodio *et al.* 2019)
Enterobacteriales	Order	Negative	(Kodio *et al.* 2019)
Myxococcales	Order	Negative	(Kodio *et al.* 2019)
Bifidobacteriales	Order	Negative	(Kodio *et al.* 2019)
Lactobacillales	Order	Negative	(Audebert *et al.* 2016) (Kodio *et al.* 2019)
Ruminococcaceae	Family	Positive Negative	(Audebert *et al.* 2016) (Kodio *et al.* 2019) (Alzate *et al.* 2020)
Prevotellaceae	Family	Positive	(Audebert *et al.* 2016) (Gabrielli *et al.* 2020) (Alzate *et al.* 2020)
Methanobacteriaceae	Family	Positive	(Gabrielli *et al.* 2020)
Clostridiaceae	Family	Positive	(Kodio *et al.* 2019) (Gabrielli *et al.* 2020)
Lachnospiraceae	Family	Positive	(Kodio *et al.* 2019) (Gabrielli *et al.* 2020)
Erysipelotrichaceae	Family	Positive	(Gabrielli *et al.* 2020)
Pasteurellaceae	Family	Positive	(Gabrielli *et al.* 2020)
Enterobacteriaceae	Family	Negative	(Audebert *et al.* 2016) (Kodio *et al.* 2019)
Streptococcaceae	Family	Negative	(Audebert *et al.* 2016) (Kodio *et al.* 2019)
Lactobacillaceae	Family	Negative	(Audebert *et al.* 2016)
Enterobacteria	Family	Negative	(Audebert *et al.* 2016)
Bifidobacteriaceae	Family	Negative	(Kodio *et al.* 2019)

Leuconostocaceae	Family	Negative	(Kodio *et al.* 2019)
Bacteroidaceae	Family	Negative	(Gabrielli *et al.* 2020)
Veillonecellaceae	Family	Negative	(Gabrielli *et al.* 2020)
Acetanaerobacterium	Genus	Positive	(Audebert *et al.* 2016)
Acetivibrio	Genus	Positive	(Audebert *et al.* 2016)
Coprococcus	Genus	Positive	(Audebert *et al.* 2016)
Hespellia	Genus	Positive	(Audebert *et al.* 2016)
Oscillibacter	Genus	Positive	(Audebert *et al.* 2016)
Papillibacter	Genus	Positive	(Audebert *et al.* 2016)
Sporobacter	Genus	Positive	(Audebert *et al.* 2016)
Ruminococcus	Genus	Positive	(Audebert *et al.* 2016) (Andersen *et al.* 2015) (Kodio *et al.* 2019) (Gabrielli *et al.* 2020)
Prevotella	Genus	Positive	(Audebert *et al.* 2016) (Andersen *et al.* 2015) (Gabrielli *et al.* 2020) (Alzate *et al.* 2020)
Methanobrevibacter	Genus	Positive	(Gabrielli *et al.* 2020)
Clostridium	Genus	Positive	(Kodio *et al.* 2019)
Roseburia	Genus	Positive	(Audebert *et al.* 2016)
Faecalibacterium	Genus	Positive	(Audebert *et al.* 2016)
Sprorolactobacillus	Genus	Positive	(Forsell *et al.* 2017)
Bifidobacterium	Genus	Negative	(Nourrisson *et al.* 2014) (Nieves-Ramírez *et al.* 2018) (Kodio *et al.* 2019)
Streptococcus	Genus	Negative	(Kodio *et al.* 2019)
Shigella	Genus	Negative	(Kodio *et al.* 2019)
Bacteroides	Genus	Negative	(Gabrielli *et al.* 2020)
Clostridial cluster XlVa		Negative	(Andersen *et al.* 2016)
Clostridial cluster IV		Negative	(Andersen *et al.* 2016)
Candidatus *Carsonella*	Species	Positive	(Audebert *et al.* 2016)
Butyrivibrio crossotus	Species	Positive	(Beghini *et al.* 2017)
Eubacterium siraeum	Species	Positive	(Beghini *et al.* 2017)
Clostridium saudii	Species	Positive	(Kodio *et al.* 2019)
Methanobrevibacter smithii	Species	Positive	(Kodio *et al.* 2019)
Coprococcus catus	Species	Positive	(Beghini *et al.* 2017)
Oxalobacter formigenes	Species	Positive	(Beghini *et al.* 2017)

Eggerthella		Negative	(Beghini *et al.* 2017)
Faecalibacterium prausnitzii	Species	Negative	(Nourrisson *et al.* 2014)
		Positive	(Iebba *et al.* 2016)
Escherichia coli	Species	Negative	(Iebba *et al.* 2016)
		Increase	(Yason *et al.* 2019)
Prevotella copri	Species	Negative	(Nieves-Ramírez *et al.* 2018)
Ruminococcus bromii	Species	Positive	(Nieves-Ramírez *et al.* 2018)
Streptococcus sp.	Species	Negative	(Kodio *et al.* 2019)
Bifidobacterium sp.	Species	Negative	(Kodio *et al.* 2019)
Shigella sp.	Species	Negative	(Kodio *et al.* 2019)

7.2 *Blastocystis* and the Eukaryotic Residents of the Microbiota

There are few studies that investigate the impact of mixed colonisation between *Blastocystis* and other eukaryotic microbes within the gut, current literature mainly focusses on assessing the prevalence of intestinal parasites and mixed infection with other intestinal parasites. A study by Nieves-Ramírez et al. in 2018 investigated both the bacterial diversity and eukaryotic diversity but focused solely on yeast and fungal communities. The results show that *Blastocystis* colonisation is generally associated with an increase in yeast and fungal communities, with a notable absence of *Hymenolepis nana*. This is of some interest as it is associated with gut pathology but does not alter bacterial communities. It was noted by the authors however, that the small sample size meant that this may not be a generally observed trend and thus the results warrant further investigation (Nieves-Ramírez *et al.* 2018). Another study by Piubelli et al. in 2019 identified frequent co-infection of *Blastocystis* with other intestinal parasites of up to 46%. *Dientamoeba fragilis* presented a statistically significant association with *Blastocystis*, and they also documented mixed and triple subtype infections between ST1-ST3, ST1-ST2-ST3 and ST1-ST3-ST4 (Piubelli *et al.* 2019). Mixed subtype infections are documented intermittently across human populations and are often identified in animals (Tito *et al.* 2019), however interactions between different subtypes and other intestinal protozoa remain unknown.

The different subtypes exhibit a varying degree of gene variation marking adaptive capabilities to the gut. For example, genome based studies have found that ST1 possess genes involved in antibiotic synthesis, ST3 is more sensitive to oxidative stress compared to other subtypes and certain isolates of ST7 possess pathogenic properties and metronidazole resistance. Such findings possibly suggest that different subtypes occupy different ecological

niches within the GI tract (Eme *et al.* 2017; Tito *et al.* 2019; Yason *et al.* 2019). The absence of studies identifying subtypes when investigating the roles of *Blastocystis* in the microbiome greatly limits our understanding of subtype specific roles and pathogenicity in the GI tract.

Co-parasitism prevalence studies of *Blastocystis* and other possible disease causing protozoa including *Cryptosporidium, Entamoeba, Eimeria* and *Giardia* are often recorded in epidemiological studies across human populations (Dacal *et al.* 2018; Osman *et al.* 2016; Perea *et al.* 2020; Reh *et al.* 2019; Sánchez *et al.* 2017) and in animals (Fayer, Santin and Macarisin 2012; W. Li *et al.* 2019; Osman *et al.* 2015; Sá *et al.* 2013; Udonsom *et al.* 2018) with *Blastocystis* often being the most commonly identified and mixed infections are relatively low in humans but higher in animals. Clinical symptoms and the impact of co-infection is largely unknown, most studies fail to investigate the interactions of mixed parasite infections i.e. competition for ecological niches, predation, effects on microbial communities and pathogenic potential, but understandably such studies would be difficult to execute as the gut is exceedingly complex and diverse and standardisation of methodologies currently lack. The same is true for mixed *Blastocystis* infection; when investigating the impacts of *Blastocystis* on the microbiota, mixed infections and multiple *Blastocystis* subtypes is not always considered.

7.3 *Blastocystis* and Interactions with the Host

It has been observed that *Blastocystis* likely has an impact in shaping bacterial communities and may have unexplored interactions with microbial eukaryotes in the gut microbiota. The question thus arises of the impact of colonisation that *Blastocystis* has on the host; either through mediating responses in bacterial communities, or by direct interaction with the host and possibly pathogenesis. It is speculated that perhaps the subtypes associated with symptomatic carriage, such as ST1, ST2, ST4 and ST7 elicit more profound negative interactions upon the host. This may occur through shifting bacterial communities away from an eubiotic state, generating unfavourable metabolites, or eliciting pro-inflammatory responses. Whereas others prove to be non-harmful and may be involved in host immune system modulation and stimulation of mucus which is favourable for gut health (Lepczyńska *et al.* 2017).

Experimental evidence has since provided some indication of pathogenicity, yet as many early reports lack genotype information, no definitive conclusions can be drawn regarding

isolate or subtype specific pathogenicity. These early reports monitored serologic responses to infection (Kaneda *et al.* 2000), in addition to demonstrating damage to mucosa microvilli and invasion of the gastrointestinal wall which caused weight loss and diarrhoea in infected mice (Elwakil and Hewedi 2010; Zhang *et al.* 2006) and increased intestinal permeability in humans (Dagci *et al.* 2002).

Persistent and symptomatic infection has also been linked to the release of secretory products both *in vitro* and *in vivo*. Examples of this include production of possible virulence factors such as cysteine proteases which are utilised by parasitic protozoa to aid development and pathogenicity (McKerrow *et al.* 1993; Sio *et al.* 2006). Observations of IL-8 modulation may implicate this cytokine in inflammatory responses related to *Blastocystis*. IL-8 is known to recruit inflammatory cells to the gut mucosa resulting in tissue damage and disruption demonstrating the ability of *Blastocystis* to cause dysbiosis in the gut (Long *et al.* 2001).

Subtype targeted studies have demonstrated the production of IgA proteases in ST4 and ST7 (Puthia, Vaithilingam, Lu and Kevin S. W. Tan 2005; Tan 2008) and hyaluronidase enzyme (Chandramathi *et al.* 2010). *Blastocystis* subtypes including the WR1 isolate (ST4) and ST1 are linked to proinflammatory immune responses via protease activity and modulation of IL-8 responses in the gut (Long *et al.* 2001; Puthia, Lu and Tan 2008; Sio *et al.* 2006; Tan 2008). WR1 isolates can also cause physical disruption to the gastrointestinal epithelium, increasing permeability (Puthia, Selena W. S. Sio, *et al.* 2006). A study by Hussein et al. described varying degrees of pathogenicity in human isolates of ST1-ST4 which were used to infect Wistar rats (Hussein *et al.* 2008). ST1 was found to be the most relevant to pathogenicity and ST2 had no effect on morbidity, ST3 and ST4 had both pathogenic and non-pathogenic strains. It should be considered however, that the bacterial component of the gut microbiota was not controlled in these rats meaning pathogenic responses caused by the bacteria cannot be ruled out. Many epidemiological surveys often lead to inconclusive results and thus the nature of pathogenic potential is difficult to discern. This can be observed in ST3 and ST1 which are commonly isolated in symptomatic disease (Byndloss *et al.* 2017; Elwakil and Talaat 2009; Tan 2008), and the different morphological forms could be an attribute of pathogenicity. The amoeboid form has been associated with symptomatic disease and proinflammatory responses, with a reported correlation between the amoeboid form and increased levels of protease activity; this possibly exacerbates intestinal symptoms (Rajamanikam and Govind 2013) and it is often isolated from symptomatic patients (Tan and

Suresh 2006a; Tan and Suresh 2006b). However, the mechanisms relating the amoeboid form to protease activity warrants further investigation.

There are also some reports of *Blastocystis* being linked to skin manifestations (urticaria) such as that from a case report in 2008 of a patient infected with amoeboid forms of ST3 (Katsarou-Katsari *et al.* 2008). Bálint et al. found 11.25% of 80 *Blastocystis* positive patients experienced skin manifestations but lacked subtyping data and found no correlation with the amoeboid form (Bálint *et al.* 2014). Verma et al. also reported a case study of a *Blastocystis* positive patient with skin lesions thought to be due to the release of interleukins and pro-inflammatory responses (Verma and Delfanian 2013).

8 'Omics Studies in *Blastocystis*

Literature searches of *Blastocystis* related 'Omics studies are relatively few in number. The most well characterised area are genomic studies of *Blastocystis* mitochondrial genomes ST1-ST4, ST6-ST9 (Jacob *et al.* 2016; Pérez-Brocal and Clark 2008; Stechmann *et al.* 2008; Wawrzyniak *et al.* 2008) and nuclear genomes for ST4 and ST7 (Denoeud *et al.* 2011; Wawrzyniak *et al.* 2015). Draft assemblies for ST2, ST3, ST6, ST8 and ST9 can also be found on genome databases yet currently lack annotation (Andersen *et al.* 2015). In 2017, a draft genome and transcriptome for the NandII ST1 strain was published and compared against the published genomes of ST4 and ST7. The strain was found to be genetically heterogenous with variation between gene number and structure and each subtype possessed a number of unique genes, demonstrating the large genetic diversity with exists within the *Blastocystis* genus. The annotated genomes of ST1, ST4 and ST7 also include data for predicted proteomes and secretomes which all provide insight into the adaptations of *Blastocystis* to life in the GI tract. It should however be noted that some differences are likely influenced by annotation methods (Denoeud *et al.* 2011; Gentekaki *et al.* 2017). In-depth characterisation of ST1 has revealed mixed genomes with approximately 2.5% of genes originating from other eukaryotes or prokaryotes.

Investigation of lateral gene transfer (LGT) in *Blastocystis* has revealed a number of acquired genes and resulting proteins that have possible roles in colonisation and adaptation to the GI tract. Adaptations include oxygen-stress responses, host-immune evasion, carbohydrate-scavenging, nitrogen metabolism and pathogenicity. Most are of bacterial origin and include genes acquired from Firmicutes, Proteobacteria and Bacteroidetes. In some instances the acquired genes are directly linked to pre-existing pathways in *Blastocystis* as well as

providing novel functionalities which likely help *Blastocystis* successfully colonise the gut and likely plays an important role in the evolution of *Blastocystis*'s adaptation to the gut (Eme *et al.* 2017). Thus, further 'omics-based studies can be used to aid mapping of pathways of *Blastocystis* and may shed light on roles and interactions within the GI tract. To date, literature searches show no metabolomic studies of *Blastocystis*. However, others have applied these methods to other intestinal parasites yielding interesting findings (Vermathen *et al.* 2018).

8.1 Mitochondrion-Related Organelles

Although *Blastocystis* is strictly anaerobic, mitochondrion-related organelles (MROs) are observed in the cytoplasm at different morphological stages. This organelle is the most extensively studied feature of *Blastocystis* due to its unusual metabolic properties that 'blur' the distinctions between the previously established mitochondrial-related organelles as defined by Embley and Martin in 2006 (Embley and Martin 2006). To date the mitochondrial DNA (mtDNA) from ST1-ST4 and ST6-ST9 have been sequenced thus far and are identical in gene content and gene order (Jacob *et al.* 2016). Current *in silico* predictions and localisation studies provide evidence for the presence of a mitochondrial genome which has both anaerobic and aerobic components, with the anaerobic metabolism capabilities likely shaped via lateral gene transfer acquisitions and seems to exist as an intermediate between anaerobic mitochondria and hydrogenosomes (Tsaousis, Yarlett and Tan 2019). Iron-sulphur (Fe-S) cluster assembly machinery (Tsaousis *et al.* 2012; Tsaousis *et al.* 2014), parts of the glycolytic pathway (Río Bártulos *et al.* 2018), urea cycle enzymes, transporters for metabolite exchange, and quinone metabolism have all been identified (Gentekaki *et al.* 2017; Stechmann *et al.* 2008). Mitochondrial protein import complexes, for instance, the Translocase of Outer Membrane (TOM70) have been localised to the MROs (Tsaousis *et al.* 2011). Stechmann et al. in 2008 was the first to predict both the anaerobic pyruvate:ferredoxin oxireductase (PFO); which is typical in anaerobic organisms, and the pyruvate dehydrogenase complex (PDH); which is found in aerobic mitochondria. This means *Blastocystis* has two possible mechanisms for pyruvate decarboxylation in cellular respiration (Stechmann *et al.* 2008). A partial tricarboxylic acid (TCA) cycle has also been localised to the MRO, where this TCA cycle is incomplete and likely functions in reverse to the canonical cycle. The electron transport chain (ETC) also functions in reverse and lacks complexes III and IV in addition to the ATP synthase. It is possible that the function of the

ETC is to produce the proton gradient, which it has been found to do so in the unrelated ciliate: *Nyctotherus ovalis,* and is possibly a result of convergent evolution to adapt to anaerobic conditions as schematically represented in Figure 1.vii (Denoeud *et al.* 2011; Gentekaki *et al.* 2017; Stechmann *et al.* 2008; Tsaousis *et al.* 2020).

Blastocystis has recently been found to respire oxygen via a mitochondrial alternative oxidase (AOX) protein, thus challenging its strict anaerobic nature. It is possible that the AOX may serve as a terminal electron acceptor or function as a protective mechanism in oxidative stress which may occur in the GI tract if oxygen levels were to fluctuate during states of dysbiosis (Standley and van der Giezen 2012; Tsaousis *et al.* 2018).

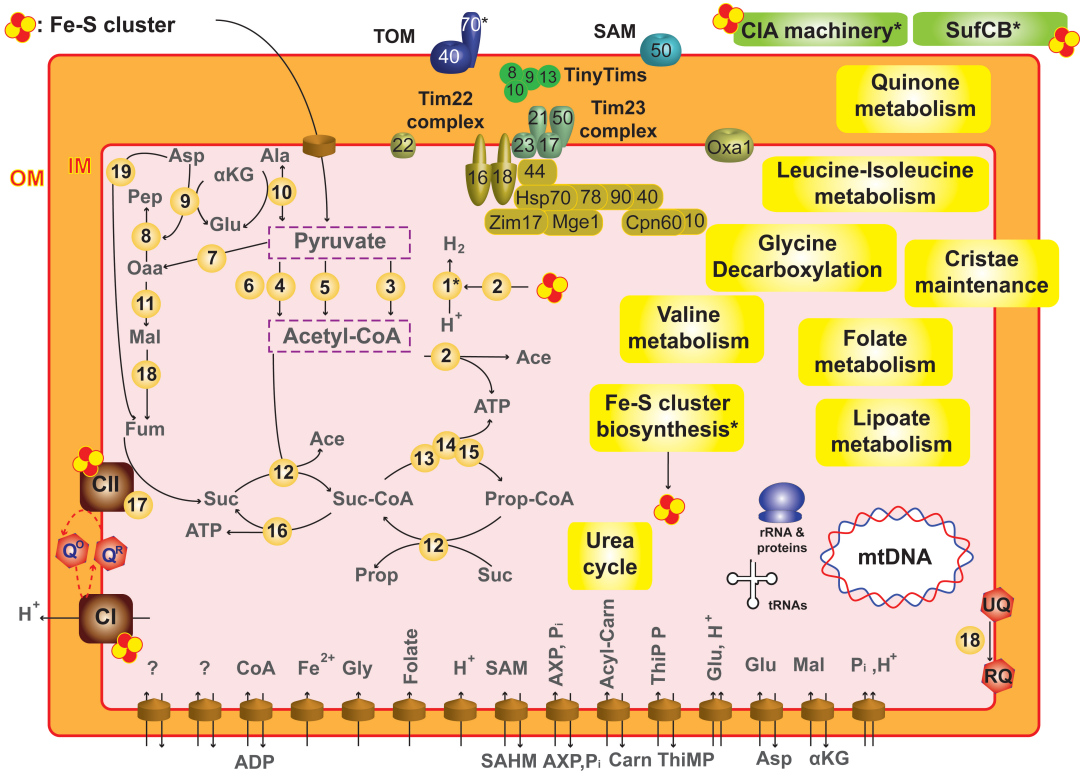

Figure 1.vii Schematic representation of the *Blastocystis* mitochondrion-related organelle. It includes the proposed metabolic map denoting roles in energy generation and amino acid and lipid metabolism based upon current predictions. Proteins are numbered as (1) FeFe-hydrogenase, Flavodoxin; (2) iron-only hydrogenase maturation rSAM protein HydE; (3) por, nifJ; pyruvate-ferredoxin/flavodoxin oxidoreductase; (4) dihydrolipoamide succinyltransferase; (5) PDK2_3_4; pyruvate dehydrogenase kinase 2/3/4; (6) 2-oxoglutarate dehydrogenase; (7) Pyruvate carboxylase, alpha subunit; (8) phosphoenolpyruvate carboxykinase (ATP; (9) Aspartate aminotransferase; (10) Alanine aminotransferase; (11) Malate dehydrogenase; (12) Acetate:Succinate CoA transferase; (13) Methylmalonyl-CoA mutase; (14) Methylmalonyl-CoA epimerase; (15) Propionyl-CoA carboxylase alpha subunit; (16) Succinyl-CoA Synthetase; (17) Succinate dehydrogenase subunit 5; (18) Rhodoquinone Biosynthesis enzyme RquA; (19) Aspartate ammonia lyase. Reprinted from Eukaryome impact on human intestine homeostasis and mucosal immunology (61-74), by A. Tsaousis, 2020, Springer, Copyright 2020 Springer Nature Switzerland AG 2020, reprinted with permission.

9 Thesis Goals

Blastocystis is a globally distributed micro-eukaryote that has been of increasing interest since its first discovery over a century ago. Despite the current knowledge of its prevalence, diversity, and roles within the gut community, there is much to still uncover. Particular interests have revolved around elucidating its roles as a pathogen or asymptomatic carrier in humans. This is of particular clinical importance nowadays as the gut microbiome is unveiling as a hallmark of health and disease. The research carried out during this Ph.D will aim to explicate the question regarding the roles of *Blastocystis* in the gut and in health and disease (Figure 1.viii). This question will be tackled by first adding to current research; investigating the prevalence of *Blastocystis* subtypes and other intestinal microeukaryotes with pathogenic potential in animal hosts, including non-human primates. This will increase understanding of the subtype diversity across a range of taxa, aiding understanding of subtype-host specificity and help draw inferences of subtypes of zoonotic origin. The presence of *Blastocystis* will be investigated in co-abundance studies with other intestinal parasites, including *Cryptosporidium* sp. and *Giardia sp*.. *Blastocystis* will also be investigated in attempt to assess its influence on the bacterial component of the gut microbiota.

Next, the roles of *Blastocystis* in the gut microbiome will be explored using NMR metabolomics, which is yet to be applied to *Blastocystis* research. Comparisons of metabolic profiles between uninfected and infected/colonised human cohorts can help establish whether or not the presence of *Blastocystis* causes shifts in the GI tract metabolome. By considering metabolic shifts in the gut opposed to studying *Blastocystis* independently, this work provides a dynamic approach to the overall affect colonisation has on microbial communities within the gut. This will be conducted via the investigation of the resulting metabolic profiles, and examining biomarkers for eubiotic or dysbiotic states.

To further explore *Blastocystis* and roles in disease, additional 'omics-based studies will be applied to investigate the pathways involved in elevated oxygen levels in ST3 and ST4, both of which are identified in human hosts. The aim here is to explore changes in pathways associated with oxygen stress and accompany this with metabolite profiles. This work is linked to the Oxygen Hypothesis where oxygen levels are increased in patients with certain gastrointestinal diseases; in particular with IBD where the role of *Blastocystis* is largely disputed. By investigating *Blastocystis* under oxygen stress and in anaerobic conditions, possible associations between the protist and IBD can be further explored.

Overall, this work aims to help clarify possible roles of *Blastocystis* in the gut and its involvement in health and disease – either as a possible pathogen or commensal.

9.1 Thesis Organisation

The results presented in this Ph.D thesis takes the form of two publications in peer reviewed journals, and three results chapter. The thesis organisation will follow the general order described in Figure 1.viii and in total will be comprised of 8 chapters as follows:

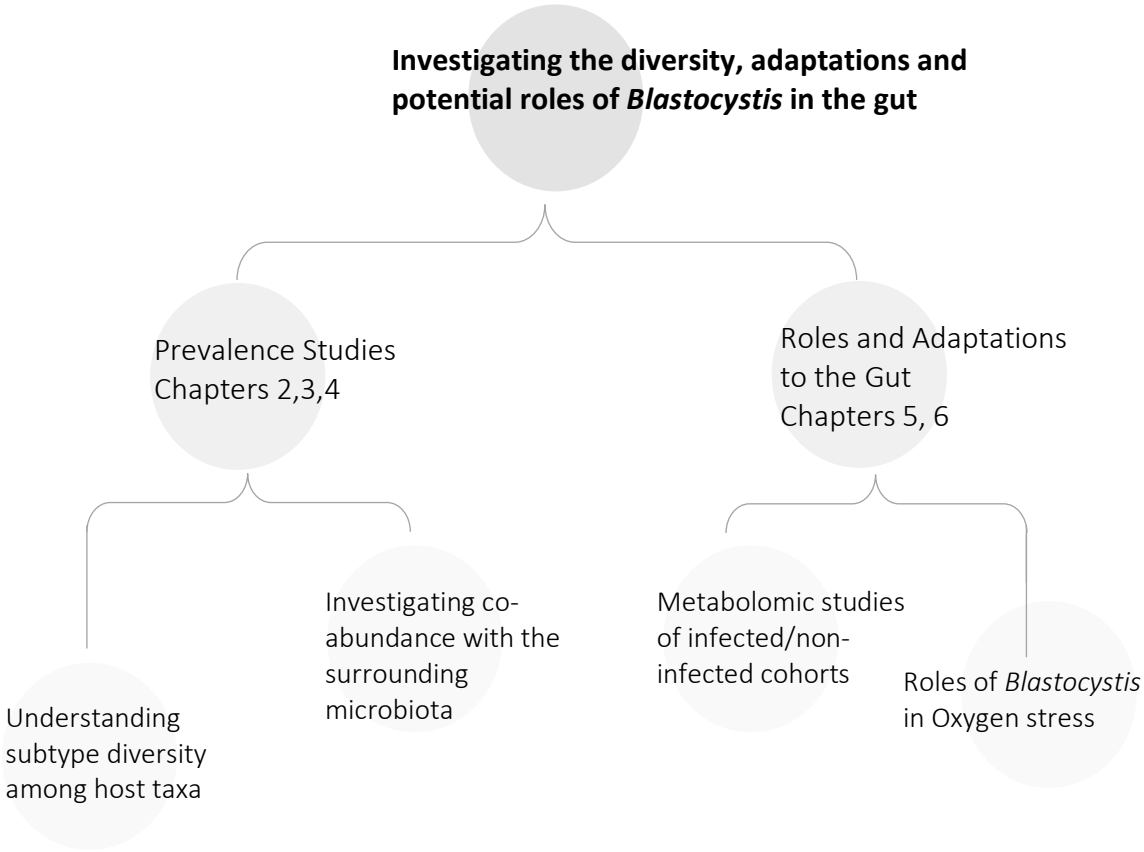

Figure 1.viii Diagram representing the structural overview of this Ph.D thesis with the research being split into two main sections and the corresponding chapters investigating the research questions.

Chapter 1: Introduction. This illustrates key background information regarding the protozoa *Blastocystis*, including its prevalence and known host range, diversity and current portrayal in health and disease.

Chapter 2: Molecular epidemiology of *Blastocystis* in captive animals. It contains the article: Betts, E.L., Gentekaki, E., Thomasz, A., Breakell, V., Carpenter, A.I. and Tsaousis, A.D., 2018. Genetic diversity of *Blastocystis* in non-primate animals. *Parasitology*, *145*(9), pp.1228-1234.

Chapter 3: Molecular epidemiology of *Blastocystis* and other intestinal protozoa in captive animals. It contains the article: Betts, E.L., Gentekaki, E. and Tsaousis, A.D., 2020. Exploring Micro-Eukaryotic Diversity in the Gut: Co-occurrence of *Blastocystis* Subtypes and Other Protists in Zoo Animals. *Frontiers in Microbiology*, *11*, p.288.

Chapter 4: Investigation into community-level differences in the bacterial microbiota in water voles with *Blastocystis* colonisation.

Chapter 5: ^{1}H NMR metabolomics studies of *Blastocystis* colonising the human gut.

Chapter 6: *Blastocystis* 'omics responses to oxygen stress.

Chapter 7: Discussion. This contains an overview of the findings of this work and provides critical analysis of the results.

Chapter 8: Methods and Materials. This includes a detailed description of the methods used.

Chapter 2. Molecular epidemiology of *Blastocystis* in captive animals

This chapter contains the article: Betts, E.L., Gentekaki, E., Thomasz, A., Breakell, V., Carpenter, A.I. and Tsaousis, A.D., 2018. Genetic diversity of *Blastocystis* in non-primate animals. *Parasitology*, *145*(9), pp.1228-1234.
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Author Contributions:

EB carried out the sample collections, culturing, data analysis, and wrote a first draft of the manuscript. AT and EG directed research, planned experiments, analysed data, and wrote the manuscript.

1 Introduction

Blastocystis is a ubiquitous protozoa commonly isolated from a range of host taxa. To date, there are 17 characterised subtypes and a number of other uncharacterised isolates. There is some host specificity documented across these subtypes but the extent of their host range is unknown.

In this chapter, *Blastocystis* prevalence is assessed in a cohort of captive animals housed in a conservation park in the United Kingdom. The main goal of this study was to contribute to the current knowledge regarding subtype diversity and distribution across different animal species and assess the possibility of asymptomatic carriage. The subtypes identified in this study were in general agreement with the typical distribution and host range. None of the animals sampled from were symptomatic for gastrointestinal disease, possibly indicating that the presence of these subtypes may represent a normal component of the intestinal flora. Four novel hosts were identified and included the pine marten (Order: Carnivora), adding to the limited knowledge of *Blastocystis* in carnivorous animals.

In conclusion this study demonstrated a high prevalence of *Blastocystis* in asymptomatic animals held in captivity and confirmed co-colonisation with multiple subtypes in a number of animals.

2 Main Article

Parasitology

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Genetic diversity of *Blastocystis* in non-primate animals

Emma L. Betts[1], Eleni Gentekaki[2], Adele Thomasz[3], Vicki Breakell[3], Angus I. Carpenter[3] and Anastasios D. Tsaousis[1]

[1]Laboratory of Molecular and Evolutionary Parasitology, RAPID group, School of Biosciences, University of Kent, Canterbury, Kent, UK; [2]School of Science and Human Gut Microbiome for Health Research Unit, Mae Fah Luang University, Chiang Rai, Thailand and [3]Wildwood Trust, Herne Common, Herne Bay, Kent, UK

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Author for correspondence:
Anastasios D. Tsaousis, E-mail: A.Tsaousis@kent.ac.uk and Eleni Gentekaki, E-mail: gentekaki.ele@mfu.ac.th

Abstract

Blastocystis is an anaerobic protist, commonly inhabiting the intestinal tract of both humans and other animals. *Blastocystis* is extremely diverse comprising 17 genetically distinct subtypes in mammals and birds. Pathogenicity of this enteric microbe is currently disputed and knowledge regarding its distribution, diversity and zoonotic potential is fragmentary. Most research has focused on *Blastocystis* from primates, while sampling from other animals remains limited. Herein, we investigated the prevalence and distribution of *Blastocystis* in animals held within a conservation park in South East England. A total of 118 samples were collected from 27 vertebrate species. The barcoding region of the small-subunit ribosomal RNA was used for molecular identification and subtyping. Forty one per cent of the species were sequence positive for *Blastocystis* indicating a high prevalence and wide distribution among the animals in the park. Six subtypes were identified, one of which is potentially novel. Moreover, the majority of animals were asymptomatic carriers, suggesting that *Blastocystis* is not pathogenic in animals. This study provides a thorough investigation of *Blastocystis* prevalence within a wildlife park in the UK and can be used as a platform for further investigations on the distribution of other eukaryotic gut microbes.

Introduction

Blastocystis is a microbial eukaryote that inhabits the gastrointestinal tract of a variety of animals including humans, other primates, amphibians, reptiles and even insects (Abe, 2004; Stensvold *et al.* 2009; Parkar *et al.* 2010; Roberts *et al.* 2013; Yoshikawa *et al.* 2016). After fungi, *Blastocystis* is one of the most prevalent microbial eukaryotes in metazoans (Scanlan *et al.* 2014).

Until recently, *Blastocystis* was overlooked due to its small size and lack of distinct morphological features. Nonetheless, the advent of molecular methods has revealed an astounding genetic heterogeneity of *Blastocystis*. To date, 17 genetically diverse lineages have been reported in mammals and birds (subtypes; ST), based on the differences of the small subunit ribosomal RNA (SSU rRNA) (Stensvold and Clark, 2016). *Blastocystis* has wide host range, with the same subtype found in several animal genera. Emerging data, however, suggests that host specificity should be assessed based on lower than genus level taxonomy (Alfellani *et al.* 2013*c*). Of the 17 STs, only the first nine (ST1–ST9) and recently, ST12 have been found in humans (Ramirez *et al.* 2016; Stensvold and Clark, 2016). *Blastocystis* has been reported in wild animals, pets and domesticated animals and those that are housed in zoos (Parkar *et al.* 2010; Ruaux and Stang, 2014; Schar *et al.* 2014; Wang *et al.* 2014; Amenu *et al.* 2015; Figueroa, 2015; Puebla *et al.* 2017). Nonetheless, the comprehensive range of non-primate hosts of the various STs remains unclear, since only a limited number of studies focus on screening such animals (Abe *et al.* 2002; Lim *et al.* 2008; Perez Cordon *et al.* 2008; Parkar *et al.* 2010; Roberts *et al.* 2013).

The presence in various animals of *Blastocystis* isolates that belong to the same STs as those in humans has led to the speculation that the organism has zoonotic potential (Rajah Salim *et al.* 1999; Parkar *et al.* 2010; Ramirez *et al.* 2016). Nonetheless, this scenario has come under scrutiny in recent years, since cases where the direction of transmission has been established conclusively are absent. Moreover, most molecular investigations of *Blastocystis* isolates from domesticated animals and their keepers have not revealed any shared subtypes, though there are notable exceptions (Alfellani *et al.* 2013*b*; Wang *et al.* 2014). Due to this controversy, there is an urgent need for further investigations on the distribution of *Blastocystis* in animals in captivity, since prevalence data and molecular characterization of *Blastocystis* in such animals remain sparse.

Herein, we examine *Blastocystis* isolates from Wildwood Trust, a wildlife park in East Kent, UK. The park's collection consists mostly of UK native and previously native wildlife, meaning that the chance of the identified isolates being local is very high. The aim of this study was to investigate the prevalence, distribution, genetic diversity and host range of *Blastocystis* STs in animals at Wildwood Trust.

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Materials and methods

Study site – source of specimens

A total of 118 faecal samples were collected from 27 different host species (Table 1) located at Wildwood Trust (Herne Bay, Kent, UK). Sampling covered a range of mammalian species, four bird species and one reptile species (Table 1).

Sample collection and storage

A licensed veterinarian visits the park on a monthly basis to monitor the animals' health, during the week of sampling no animals exhibit diarrhoea. Faecal samples were collected between the months of July 2016 to January 2017. Wildwood Trust staff collected samples under the guidance of laboratory members; A minimum of one sample was collected per animal species, where possible (Table 1). In the cases where multiple animals of the same species were enclosed together, several samples were collected.

Once collected, samples were placed in sealed, sterile falcon tubes and stored at 4 °C until DNA extraction. The faecal samples were subdivided shortly after collection to be used for microscopy, culturing and DNA extraction. Heat-fixed slides were made from all samples collected within an hour of collection.

Table 1. Animal samples collected from study hosts

Host	Scientific name	Location	Sample number	PCR positive (clone number)
Carnivora				
Badger	*Meles meles*	Wildwood	2	–
European Brown Bear	*Ursus arctos arctos*	Wildwood	2	–
Lynx	*Lynx lynx*	Wildwood	3	–
Otter	*Lutra lutra*	Wildwood	7	–
Pine Marten	*Martes martes*	Wildwood	2	1 [1]
Polecat	*Mustela putorius*	Wildwood	1	–
Red Fox	*Vulpes vulpes*	Wildwood	3	–
Scottish Wild Cat	*Felis silvestris*	Wildwood	11	–
Stoat	*Mustela ermine*	Wildwood	3	–
Anseriformes				
Barnacle Goose	*Branta leucopsis*	Wildwood	1	–
Pink Footed Goose	*Anser brachyrhynchus*	Wildwood	1	–
Artiodactyla				
Muntjac Deer	*Muntiacus reevesi*	Wildwood	1	1 [1]
European Bison	*Bison bonasus*	Wildwood	3	3 [11]
Eurasian Elk	*Alces alces*	Wildwood	2	1 [4]
Pygmy Goat	*Capra aegagrus hircus*	Wildwood	2	2 [3]
Red Deer	*Cervus elaphus*	Wildwood	1	1 [8]
Reindeer	*Rangifer tarandus*	Wildwood	1	–
Soay Sheep	*Ovis aries*	Wildwood	1	1 [1]
Wild Boar	*Sus scrofa*	Wildwood	2	1 [1]
Squamata				
Four-lined Snake	*Elaphe quatuorlineata*	Wildwood	1	–
Eulipotyphla				
Hedgehog	*Erinaceus europaeus*	Wildwood	1	–
Water Shrew	*Neomys fodiens*	Wildwood	6	–
Passeriformes				
Raven	*Corvus corax*	Wildwood	3	–
Red Billed Chough	*Pyrrhocorax pyrrhocorax*	Wildwood	1	–
Rodentia				
Red Squirrel	*Sciurus vulgaris*	Wildwood	3	2 [1]
Water Vole	*Arvicola amphibious*	Wildwood	3	2 [12]
Water Vole	*Arvicola amphibious*	Bulphan	(5) 30[a]	5 [17]
Water Vole	*Arvicola amphibious*	Tilbury	(3) 18[a]	3 [9]
Diprotodontia Wallaby	*Macropus rufogriseus*	Wildwood	3	2 [2]

[a]High sample number due to repetitive sampling from a small population. Numbers in parentheses denote number of water vole individuals.

Culturing

Within half an hour of sampling, a small amount of faecal sample from randomly selected animals were separately inoculated in sterile falcon tubes containing the following media: two tubes containing modified LYSGM [16·07 mM potassium phosphate dibasic, 2·94 mM potassium phosphate monobasic, 128·34 mM sodium chloride, 2·5 g L^{-1} yeast extract, 0·5 g L^{-1} liver extract, 5% adult bovine (Sigma)/horse serum (Gibco); modified TYSGM-9, without mucin (Diamond, 1982), http://entamoeba.lshtm.ac.uk/xenic.htm], two tubes of TYM (22·2 g L^{-1} trypticase peptone, 11·1 g L^{-1} yeast extract, 16·23 mM maltose, 9·17 mM L-cysteine, 1·26 mM L-ascorbic acid, 5·1 mM potassium phosphate dibasic, 6·53 mM potassium phosphate monobasic) (Diamond, 1957, 1983) enriched with 5% fetal bovine serum (FBS; Sigma) and 2 tubes with 0·5% Liver Digest (LD) medium (0·5 g L^{-1} Oxoid liver extract). One tube of each medium type was incubated at 35 °C and the rest were left at room temperature. Samples were examined every 3 days under light microscope with neutral red staining (see below). Cultures positive for *Blastocystis*, were subcultured every 10 days.

Staining and microscopy

For the identification of live cells within cultures, a neutral red staining technique was employed (DeRenzis and Schechtman, 1973). Ninety-four μL of re-suspended cultured samples were mixed with equal volumes of freshly prepared 0·04% neutral red staining (Sigma, N2889) in 0·5 mL tubes and incubated for 10 min at the temperature in which samples were cultured. The samples were then centrifuged at 5000 ***g*** for 30 s. The supernatant was removed and the pellet was re-suspended in 20 μL of 1 × PBS (pH 7·2) by vortexing. Ten μL of the mixture was placed on a glass slide under a 22-mm square coverslip and individual cells were observed under 200× and 400× magnification.

DNA extraction, PCR, cloning and sequencing

DNA from feces and cultures were extracted using the Microbiome DNA Purification Kit Purelink (Fisher, UK), following the manufacturer's specifications and protocols. The extracted DNA was stored at −20 °C for long-term usage. To amplify the fragment of interest, polymerase chain reaction (PCR) was carried out using the extracted DNA. DNA extracted from an axenic *Blastocystis* NandII culture was used as positive control in every PCR application. The conditions of amplification were as follows: 2 μL of the extracted DNA was used for amplification of a *Blastocystis* sp SSUrRNA product. 10 μL 5× buffer (Promega), 1 mM $MgCl_2$, 0·4 μM forward primer, 0·4 μM reverse primer, 0·2 mM dNTPs (Promega), 0·25 μL Taq polymerase, 30·75 μL HPLC grade water 2 μL DNA. The fragment was amplified in a total of 50 μL reaction, according to the standard conditions of for HiFI Taq polymerase (Promega). The broad specificity primers RD3 5′-GGGATCCTGATCCTTCCGCAGGTTCACCTAC-3′ and RD5 5′-GGAAGCTTATCTGGTTGATCCTGCCAGTA-3′ (Clark, 1997) were used for the first PCR. Cycling conditions were as follows: 95 °C 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing 55 °C for 30 s, extension at 72 °C for 1 min 40 s and final extension at 72 °C for 5 min.

A second nested PCR was performed using the forward RD5F 5′-ATCTGGTTGATCCTGCCAGT-3′ and reverse BhRDr 5′-GAGCTTTTTAACTGCAACAACG-3′ (Scicluna *et al.* 2006) primers giving a fragment at approximately 650 bp. This fragment is considered the barcoding region for *Blastocystis* identification. Concentration of reagents in each reaction and PCR conditions were the same as above. One μL from the PCR mentioned above was used as template.

Positive PCR reactions from the nested-PCR were gel-extracted using the Thermo Scientific GeneJET Gel Extraction Kit (following manufacturer's instructions) and subsequently cloned in the pGEM-T vector (Promega) using the manufacturer's protocol. Five to ten colonies from each transformation were selected for sub culturing and plasmid purification using the GeneJET Plasmid Miniprep Kit. Positive plasmids were screened by digestion with EcoRI restriction enzyme, to confirm presence of the fragment of interest. Positive plasmids were bidirectionally sequenced using T7 and SP6 universal primers by Eurofins, UK.

Genetic distance and phylogenetic analysis

The obtained sequences were trimmed to eliminate vector fragments and forward and reverse sequences of each sample were joined using Sequencher. Blast searches against GenBank using the sequences obtained were performed to exclude bacterial contamination. A dataset including all new sequences identified as *Blastocystis* along with sequences spanning the breadth of diversity of *Blastocystis* subtypes was build and aligned using MAFFT v.7 (Katoh and Toh, 2010). The alignment was further improved by visual check where necessary. Genetic distance was calculated using the Kimura2 parameter criterion. Gaps were considered as complete deletions. For this calculation, only the barcoding region of *Blastocystis* was used.

For the phylogenetic analysis, four additional outgroup taxa were included to the alignment and the entire sequence of SSUrRNA was used. The alignment contained a total of 90 taxa. Several sequences were represented only by their barcoding region in which case, the missing part of the sequence was considered as missing data. Following alignment with MAFFT, ambiguous positions were removed using trimAL (Capella-Gutierrez *et al.* 2009). After trimming the alignment contained 1163 sites. Phylogenetic trees were constructed by using maximum likelihood and Bayesian inference methods. Maximum likelihood trees were computed using the RAxML software (Stamatakis, 2006). For each dataset bootstrap support was evaluated from 1000 bootstrap replicates. Bayesian inference tree was computed using MrBayes (Ronquist and Huelsenbeck, 2003). Posterior probabilities were computed by running four chains with sampling occurring every 100th generation, whilst 25% of trees were discarded as burn-in. Trees were run for 1 500 000 generations at which point all parameters converged at 0·01.

Results

Culturing

Blastocystis grew in the tubes containing LYSGM and TYM + FBS at both 35 °C and room temperature. There was no *Blastocystis* growth in the 0·5% LD medium.

Screening of faecal samples

A total of 118 samples from 27 species were examined of which 71 clones were sequence positive belonging to 11 species (41%) (Table 1). Nonetheless, there was a notable difference in the presence of *Blastocystis* across hosts. With the exception of a single case, all sequence positive samples came from non-carnivorous animals. This was despite repeated sampling and sequencing attempts (Table 1). Specifically, 7/8 (87·5%) of artiodactyls, 2/2 (100%) of rodents and 1/9 (11%) of carnivores were sequence positive for *Blastocystis*. No sequence positive samples were found in birds, snakes and insectivores (Table 1).

Subtype identification and distribution in various hosts

Among the 71 *Blastocystis*-positive samples, six STs were detected (Table 2, Fig. 1): ST1, ST4, ST5, ST10, ST14 and a potentially new subtype. Subtypes 4 and 10 colonized the most species (seven and six respectively) followed by ST14 (three), ST1 (two), ST5 (one) and a novel subtype (one). We provide the first molecular data and subtyipng of *Blastocystis* from elk, water voles, pine martens and red squirrels. The Eurasian elk (artiodactyl) were the hosts harbouring the widest range of subtypes, followed by pygmy goat (artiodactyl) and water vole (rodent). Most notably, four subtypes were found in the elk (ST4, ST10, ST14, novel), while goat and water vole harboured three (ST1, ST10 and ST14 in goat and ST1, ST4 and ST10 in water vole). The hosting of multiple subtypes within elk is of no doubt, as there is just a single elk in the park. The same cannot be verified for the goats and voles as the park houses several of them. Nonetheless, only two faecal samples were collected, which means that there are at least two subtypes present in a single goat. The three subtypes in water vole were identified only in the captive population of which three were sampled. The presence of all subtypes can be confirmed here, due to cloning being used rather than PCR purification of a single product.

Several samples were collected from two rodent species; the red squirrel and water vole. Subtype 4 was commonly detected in both species, while the range of subtypes previously reported within rodents can be expanded to include ST1. Several colonies were also screened from wallabies, diprotodontid marsupials. All samples from wallabies harboured ST10, which had not been reported previously from these marsupials.

Phylogenetic analysis

Though 71 clones were sequenced, only 20 of them were used in the phylogenetic analyses. In the cases where clusters contained identical clones, only a few representative sequences were kept. In total, the new sequences were subtyped as follows: ST4 (n = 41); ST10 (n = 22); ST14 (n = 4); ST1 (n = 2); ST5 (n = 1) novel ST (n = 1). All *Blastocystis* sequences formed a strongly supported cluster (100BS/1·00BI). Most newly sequenced isolates grouped within clades formed by previously published subtypes (Fig. 1). The most basal sequences belonged to *Blastocystis* isolates from reptiles and cockroaches along with those from ST15, ST16 and ST17 in agreement with previous studies (Alfellani *et al.* 2013*c*; Yoshikawa *et al.* 2016). Subtype 3 sequences grouped together and sister to a clustered formed by ST10, ST8 and ST4. Subtypes 7, 9 and 6 clustered together, while ST11, ST2 and ST1 formed a separate clade. Subtypes 12 and 5 also grouped together. Subtypes 13 and 14 were not well resolved even when a subtree was constructed (data not shown). The ELB_WW Elk 1 clone 1 did not fall within any of the 17 STs and its position remains unresolved.

Discussion

Approximately 61 animals from 27 species were examined. Forty one per cent of all animals were sequence positive for *Blastocystis*. In select cases, we attempted to establish cultures of *Blastocystis*. The organism has been previously cultured in a wide range of media including egg slant medium with Locke's solution, Iscove's modified Dulbecco's medium, Robinson's medium and Jones' medium (Clark and Diamond, 2002; Tan, 2008). The latter is a widely used formulation ideal for short term culturing of multiple subtypes (i.e. a few days). *Blastocystis* isolates originating from endothermic hosts are customarily cultured at 35 °C. Reported here was cultivation of *Blastocystis* from a water vole (*Arvicola amphibius*) in TYM medium enriched with FBS. The culture had been maintained in the laboratory for at least 11 months. Although the origin of the isolate is an endothermic animal, it grew over abundantly at room temperature. This indicates that some isolates of *Blastocystis* can grow at lower temperatures given certain types of media. Whether all isolates of *Blastocystis* or only some can grow in TYM + FBS at room temperature needs further study.

Most of the animals that we examined harboured a single subtype of *Blastocystis*. Nonetheless, some animals carried more than one subtype. Mixed colonization was confirmed, because we employed cloning and screened multiple colonies from each sample, while previous studies only used direct sequencing from PCR products (Stensvold *et al.* 2012; Roberts *et al.* 2013; Alfellani *et al.*

Table 2. Subtype results from sequencing positive samples

					Blastocystis ST					
Host	Name	Location	PCR positive samples	Sequence positive clones	ST1	ST4	ST5	ST10	ST14	ST?
European Bison	*Bison bonasus*	Wildwood	3	11	–	–	–	11/11	–	–
Eurasian Elk	*Alces alces*	Wildwood	1	4	–	1/4	–	1/4	1/4	1/4
Muntjac Deer	*Muntiacus reevesi*	Wildwood	1	1	–	–	–	–	1/1	–
Pine Marten	*Martes martes*	Wildwood	1	1	–	1/1	–	–	–	–
Pygmy Goat	*Capra aegagrus hircus*	Wildwood	2	3	1/3	–	–	1/3	1/3	–
Red Deer	*Cervus elaphus*	Wildwood	1	8	–	2/8	–	6/8	–	–
Red Squirrel	*Sciurus vulgaris*	Wildwood	2	1	–	1/1	–	–	–	–
Soay Sheep	*Ovis aries*	Wildwood	1	1	–	–	–	–	1/1	–
Wallaby	*Macropus rufogriseus*	Wildwood	2	2	–	–	–	2/2	–	–
Water Vole	*Arvicola amphibius*	Wildwood	2	12	1/12	10/12	–	1/12	–	–
Water Vole PP	*Arvicola amphibius*	Bulphan	5	17	–	17/17	–	–	–	–
Water Vole TB	*Arvicola amphibius*	Tilbury	3	9	–	9/9	–	–	–	–
Wild Boar	*Sus scrofa*	Wildwood	1	1	–	–	1/1	–	–	–

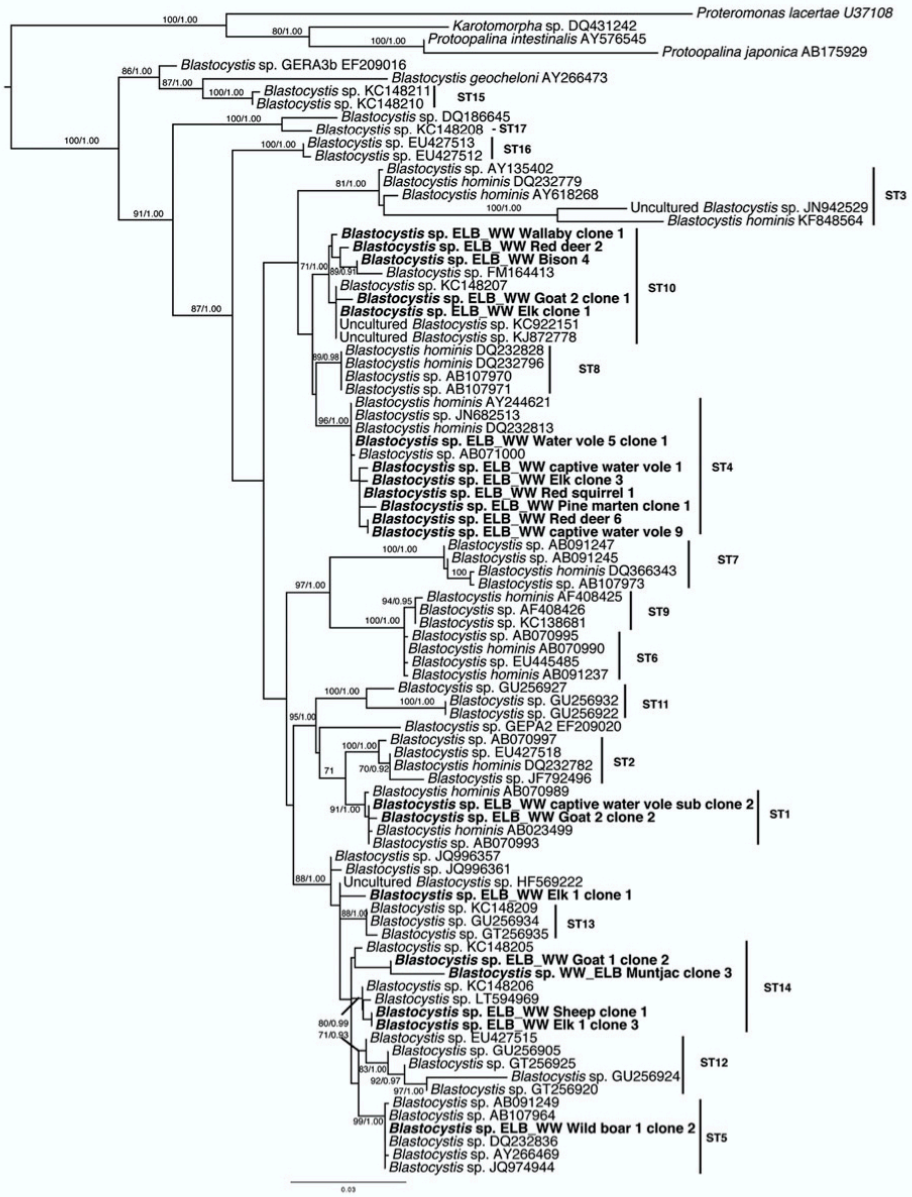

Fig. 1. Maximum likelihood phylogenetic tree inferred from 90 SSUrRNA sequences and 1163 sites. Newly generated sequences are shown in bold. Numerical values on the branches indicate bootstrap percentages and posterior probabilities in this order. Only bootstrap support values greater than 70 are shown. The accession numbers of all newly generated sequences are presented in online Supplementary Table S1.

2013*b*, *c*; Stensvold, 2013). Using this strategy, it was found that elk (*Alces alces*) harboured four subtypes, with this being the first time *Blastocystis* has been reported in this mammal. In cases where multiple subtypes are found within a single host, it is important to exclude contamination from other sources. The park has a single elk, which is housed in an isolated enclosure on its own. Moreover, the faecal sample was collected at the moment of defecation precluding contamination from small, non-resident animals. More than one subtype was also detected in pygmy goats (ST = 3), red deer (ST = 2) and water voles (ST = 3). Unlike in the case of the elk, we cannot definitively conclude that the detected subtypes in goats originated from a single individual *per se*, since enclosures housed multiple animals of the same species. While colonization with multiple subtypes is rare in humans, not much information is available for other animal species (Meloni *et al.* 2012). In light of our findings, it is tempting to speculate that the microbiota of at least some animals includes *Blastocystis* subtypes. Sampling from more animals and use of

methodologies similar to ours will shed further light as to whether presence of multiple subtypes is the norm within these and other animals.

Water voles also constitute an interesting case. There are two, temporary populations of water vole being held within the park, together with permanent residents. These two groupings of water vole are temporarily brought in to captivity as part of a licensed, development mitigation programme and are subsequently to be introduced back into their natural environment locations; two separate sites in Essex, UK. This study can report that 'wild' water vole harboured ST4 only, whereas those in permanent captivity also harboured ST1. Wild water voles were sampled multiple times, while captive ones provided only a limited number of samples. Despite considerable effort (PCR, cloning and screening of clones) we were unable to detect ST1 in wild water voles. It is tempting to speculate that the 'captive' water vole acquired ST1 after their introduction in the park and that this is one of the many microbiota-related alterations associated with life in captivity (Waite and Taylor, 2014; Kohl *et al.* 2017). However, since captive voles originated from two additional locations other than Essex, this hypothesis needs further testing involving surveys of all populations of origin.

ST10 and ST4 were the most widely distributed subtypes, each isolated from five species. As previously described, artiodactyls carried mostly ST10 (Alfellani *et al.* 2013*c*). It has been speculated that rodents are reservoirs of ST4 for human infection, though not all rodent species carry this specific ST (Alfellani *et al.* 2013*b*). Subtypes 3 and 17 were also found in rodents in previous investigations (Stensvold *et al.* 2009; Alfellani *et al.* 2013*a*). Herein, this study detected ST4 in all *Blastocystis* positive samples of rodents. Nonetheless, other subtypes were also found in the screened rodents: ST10 in red squirrels and ST1, ST5 and ST10 in water voles. Therefore, the study has been able to expand the number of subtypes recorded in rodents by identifying ST1 and ST10. It was also possible to expand the range of subtypes identified in goats to include ST14, along with the previously identified ST10, ST1, ST3, ST6 and ST7 (Alfellani *et al.* 2013*b*). The study also detected ST14 in four hosts, all of which belong to the artiodactyls.

To determine the monophyly and relationships among STs, phylogenetic analyses were performed. Traditionally, sampling of *Blastocystis* had focused on primates, especially humans. As a result, STs that were present in non-primates were reported infrequently and the clades of these STs remained sparsely populated. For instance, the resolution of the ST13 and ST14 has been problematic. Previously, Alfellani *et al.* (2013*c*) speculated that ST14 should be split into two subtypes, but refrained from doing so pending further sampling. The current study has shown that, when our isolates were added to the tree, ST14 splits into two distinct clades, with our samples populating both of these clades, hence, supporting the idea that it should be considered as two STs. Moreover, one isolate from elk grouped independently of all other STs, suggesting that this might be a novel ST. Genetic divergence analysis of the barcode region indicated that the genetic distance between our isolate and all other STs is over 5%, with the exception of ST13, with which it differed by 4·4%. The recommended threshold to define a new sequence is 5% divergence (Clark *et al.* 2013). Nonetheless, the full sequence and further samples are needed to confirm this finding since this is an individual partial sequence.

In summary, we present here a comprehensive study of *Blastocystis* prevalence focusing exclusively on non-primate animals in a zoo setting in the UK. Presented here has been the presence of six subtypes, with one potentially being novel. Through the use of cloning, it has been possible to conclusively record the presence of multiple STs within an individual animal. The sequences generated from this study have populated STs that were considered rare and for which not many sequences exist. Collectively, these highlight the need for sampling from a wide range of hosts.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017002347.

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Conflicts of interest. The authors declare no conflict of interest.

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Chapter 3. Molecular epidemiology of *Blastocystis* and other intestinal protozoa in captive animals

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Author Contributions:

EB carried out the collections, culturing, collected and analysed all the data, and wrote a first draft of the manuscript. AT and EG directed research, planned experiments, analysed data, and wrote the manuscript.

1 Introduction

Building upon the results obtained in Chapter 2, this chapter investigated the co-occurrence of *Blastocystis* with other intestinal protozoa in a range of host animals.

In this chapter, the prevalence of *Blastocystis*, *Cryptosporidium* sp., *Eimeria* sp., *Entamoeba* sp., and *Giardia* sp. were assessed across captive animals housed in a large zoo and conservation park in the United Kingdom.

The aim of this study was to assess these common intestinal protists, most of which are associated with gastrointestinal symptoms in humans, in asymptomatic captive animals. This was done to allow zoonotic potential of these animals to be considered and demonstrate the co-colonisation of multiple protist species within a single host. From this work, a large group of non-human primates (NHPs) were sampled from and a high prevalence of *Blastocystis* was recorded which correlated with that of wild counterparts. Thus, indicating that captivity may not alter microbial communities to a large degree, at least in the case of these primates. Novel hosts for *Blastocystis* were documented in carnivores, further expanding its range in group of animals.

In conclusion, this study reported a high prevalence of *Blastocystis* and other potentially pathogenic protozoa in asymptomatic animals living in captivity, the high co-colonisation rates documented here indicate that the traditional definition of these microbes as 'parasites' may need to be re-considered for some hosts.

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Exploring Micro-Eukaryotic Diversity in the Gut: Co-occurrence of *Blastocystis* Subtypes and Other Protists in Zoo Animals

Emma L. Betts[1], Eleni Gentekaki[2,3] and Anastasios D. Tsaousis[1]**

[1] Laboratory of Molecular and Evolutionary Parasitology, RAPID Group, School of Biosciences, University of Kent, Canterbury, United Kingdom, [2] School of Science, Mae Fah Luang University, Chiang Rai, Thailand, [3] Gut Microbiome Research Group, Mae Fah Luang University, Chiang Rai, Thailand

Blastocystis is a genetically diverse microbial eukaryote thriving in the gut of humans and other animals. While *Blastocystis* has been linked with gastrointestinal disorders, its pathogenicity remains controversial. Previous reports have suggested that one out of six humans could be carrying *Blastocystis* in their gut, while the numbers could be even higher in animals. Most studies on *Blastocystis* are either exclusively targeting the organism itself and/or the associated prokaryotic microbiome, while co-occurrence of other microbial eukaryotes has been mainly ignored. Herein, we aimed to explore presence and genetic diversity of *Blastocystis* along with the commonly occurring eukaryotes *Cryptosporidium*, *Eimeria*, *Entamoeba* and *Giardia* in the gut of asymptomatic animals from two conservation parks in the United Kingdom. Building upon a previous study, a total of 231 fecal samples were collected from 38 vertebrates, which included 12 carnivorous and 26 non-carnivorous species. None of the animals examined herein showed gastrointestinal symptoms. The barcoding region of the small subunit ribosomal RNA was used for subtyping of *Blastocystis*. Overall, 47% of animal species were positive for *Blastocystis*. Twenty six percent of samples carried more than one subtypes, including the newly identified hosts Scottish wildcat, bongo and lynx. Fifty three percent of samples carried at least another microbial eukaryote. Herewith, we discuss potential implications of these findings and the increasingly blurred definition of microbial parasites.

Keywords: *Blastocystis*, genetic diversity, subtyping, co-occurrence, phylogeny, micro-eukaryome

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****Correspondence:***
Eleni Gentekaki
gentekaki.ele@mfu.ac.th
Anastasios D. Tsaousis
A.Tsaousis@kent.ac.uk;
tsaousis.anastasios@gmail.com

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INTRODUCTION

The gut microbiome comprises the collective genomes of microbial symbionts and is composed of bacteria, fungi, viruses and protists within the gastrointestinal (GI) tract of a host (Blaser, 2014). Though literature associated with bacterial microbiota is increasing, studies on the rest of the microbiome components are just beginning to surface. Historically, presence of protists in the gut has been considered as parasitism, thus these microbial eukaryotes have been subject to rigorous elimination in both humans and other animals (Parfrey et al., 2011). Despite this, current data demonstrates that some protists are more common than previously thought, raising the possibility

of commensalistic or even mutualistic roles in the gut ecosystem (Lukes et al., 2015; Chudnovskiy et al., 2016). In this regard, no other protist has been studied more extensively than the anaerobic stramenopile *Blastocystis*. Its prevalence in humans has been estimated to a staggering one billion (Stensvold, 2012). Though a similar estimation for animals is not available, data from numerous animal studies covering broad range of hosts strongly suggest that colonization rate in animals is likely higher than in humans.

Blastocystis is extremely heterogeneous genetically (Gentekaki et al., 2017). Based on the SSU rRNA gene, *Blastocystis* from avian and mammalian hosts is divided into 17 subtypes, which are considered separate species (Stensvold and Clark, 2016b). Nonetheless, there are many sequences originating from ectothermic hosts that do not belong to any of the designated subtypes (Yoshikawa et al., 2016). The various subtypes of *Blastocystis* do not seem to be host-specific. For example, ST1 to ST9 have been identified in humans, but also in other hosts (Stensvold and Clark, 2016b). The exception seems to be ST9, which has yet to be identified in a non-human host (Stensvold and Clark, 2016a). ST10 to ST17 have been found only in animals so far, with the exception of ST12, which has also been identified in humans (Ramirez et al., 2016).

Though *Blastocystis* has been found in individuals with gastrointestinal symptoms, asymptomatic carriage is also common (Scanlan et al., 2014; AbuOdeh et al., 2016; Nieves-Ramirez et al., 2018; Yowang et al., 2018; Mardani Kataki et al., 2019). *In vitro* experiments using cell lines have shown the invasion potential of some strains/subtypes of *Blastocystis* (Puthia et al., 2008; Wawrzyniak et al., 2012), with no evidence to date that this also occurs *in vivo* (Clark et al., 2013). Experimental infections in mouse models have been achieved only after an inoculum of considerable size (up to 4×10^7) is administered (Moe et al., 1997; Elwakil and Hewedi, 2010). Recent studies on animals have shown that *Blastocystis* exists asymptomatically in a broad array of hosts (Betts et al., 2018; Wang et al., 2018b). Collectively, these findings highlight the uncertainty surrounding pathogenicity status of *Blastocystis* in both humans and other animals.

Presence of multiple *Blastocystis* subtypes in humans is not often reported (Whipps et al., 2010; Scanlan and Stensvold, 2013). To our knowledge, only a few reports have demonstrated mixed colonization in animals (AbuOdeh et al., 2016; Cian et al., 2017; Betts et al., 2018). In our previous work, Betts et al. (2018) examined *Blastocystis* distribution in a wildlife park in the United Kingdom, and identified various genetic isolates in a number of different animals across the park. Importantly, we also demonstrated presence of up to four subtypes in healthy captive animals (Betts et al., 2018). At that time, while microscopically screening the fecal samples, we noted presence of other protists as well. Most previous studies have been focused on identifying single target protist species, but only a few have focused on co-occurrence of multiple microbial eukaryotes in the gut. Herein, we have expanded the study area to include an additional wildlife park. We aimed to further characterize presence of *Blastocystis* isolates along with additional microbial eukaryotes across a broad range of taxa in the two parks.

MATERIALS AND METHODS

Study Sites

Two zoos situated in the Southeast, United Kingdom were sampled in this study: 1) Wildwood Conservation Park, Herne Bay, Kent, United Kingdom (51°19′54.1″N 1°07′10.1″E). This is a small conservation park housing native vertebrate and invertebrate species from the United Kingdom and mainland Europe with the exception of the red-necked wallaby (*Macropus rufogriseus*). The park is actively involved in breeding and re-introduction programs for native animals including the European water vole (*Arvicola amphibious*) and Scottish wildcat (*Felis silvestris silvestris*), and 2) Howletts Wild Animal park, Canterbury, Kent, United Kingdom (51°16′11.8″N 1°09′25.0″E). This is a large zoo with over 400 animals from 50 vertebrate and invertebrate species from across the globe. The zoo has a large primate collection, including one of the largest family groups of western lowland gorilla (*Gorilla gorilla gorilla*) in the world. The zoo is involved in a number of re-introductory schemes, mainly into national parks. Both zoos closely monitor animal health, through licensed veterinarians once a month. To our knowledge none of the animals in this study presented symptomatic gastrointestinal diseases or diarrhea.

Sample Collection

A total of 231 fresh fecal samples have been collected from 38 vertebrate species between July 2016 and March 2019 (**Supplementary Table S1**). One hundred and eighteen samples were from a previous collection (Accession numbers of *Blastocystis* positive samples: MF186640-MF186709; Betts et al., 2018) and the rest were newly collected. Sixty-seven of these samples were from nine vertebrate species collected from Howletts Zoo between November 2017 and February 2019 and the remaining samples were collected form 31 species at Wildwood. Samples from gray wolf (*Canis lupus*) and European bison (*Bison bonasus*) were collected from both zoos. Sampling covered a total of 33 mammalian species, four bird species and one reptile (**Supplementary Table S1**). In both zoos, a minimum of one fecal sample was collected from each enclosure. In enclosures where more than one animal resided, between two and five samples were collected, each of which was considered as individual sample. For some water voles (*Arvicola amphibious*), a number of repeat collections were carried out over the course of 12 months (**Supplementary Table S1**). Fresh fecal samples were collected in the morning either before or shortly after enclosures were cleaned. For some animals, including avian species and the reptile; where age of fecal sample is difficult to determine, multiple samples were collected. Zookeepers supervised all collections.

Once collected, fecal samples were stored at 4°C in sterile falcon tubes within 1 h of collection until DNA extraction. In some instances, heat fixed slides were prepared. Within an hour of sampling, a small amount of fecal sample from the water voles and other randomly selected animals were separately inoculated in four sterile falcon tubes containing the following media: two tubes containing modified LYSGM [16 · 07 mM potassium

phosphate dibasic, 2 · 94 mM potassium phosphate monobasic, 128 · 34 mM sodium chloride, 2 · 5 g L^{-1} yeast extract, 0 · 5 g L^{-1} liver extract, 5% adult bovine (Sigma)/horse serum (Gibco); modified TYSGM-9, without mucin (Diamond, 1982)[1], two tubes of TYM (22 · 2 g L^{-1} trypticase peptone, 11 · 1 g L^{-1} yeast extract, 16 · 23 mM maltose, 9 · 17 mML-cysteine, 1 · 26 mM L-ascorbic acid, 5 · 1 mM potassium phosphate dibasic, 6 · 53 mM potassium phosphate monobasic) (Diamond, 1957, 1983) enriched with 5% fetal bovine serum (FBS; Sigma) and 2 tubes with 0 · 5% Liver Digest (LD) medium (0 · 5 g L^{-1} Oxoid liver extract). The tubes were incubated at 35°C. samples were examined for *Blastocystis* under the microscope every 3–5 days. After initially leaving the cultures for 2 weeks, they were subcultured every 10 days.

DNA Extraction, Amplification of Target Gene and Molecular Characterization

Genomic DNA was extracted directly from a minimum of 250 mg of fresh fecal sample or culture pellet using the Microbiome DNA Purification Kit Purelink (Fisher, United Kingdom) to the manufacturer's instructions. DNA was eluted in 100 µl elution buffer and aliquotted. The working stock was stored at −20°C, while the rest was placed at −80°C for long-term storage. Extracted DNA was used for the polymerase chain reaction (PCR) with specific primers targeting regions of interest (**Supplementary Table S2**). PCR was carried out using the 2X PCRBIO Taq DNA Polymerase (PCRBIOSYSTEMS). Reagents per 25 µl reaction were as follows: PCRBIO Taq mix, 0.4 µM forward primer, 0.4 µM reverse primer, 19 µl nuclease free water and 2 µl DNA (ranging in concentration 10–50 ng/µl). Details of amplification conditions for all species in this study are provided in **Table 1**.

Fragments amplified to the correct size were excised and extracted using the Thermo Scientific GeneJET Gel Extraction Kit (following manufacturer's instructions) purified gel extracts were eluted in 30–50 µl of elution buffer. If PCR reactions were left for 7 days before ligation, a polyadenylation reaction was carried out with the following protocol: per reaction 0.25 µl GoTaq DNA Polymerase (Promega), 7 µl Gel extraction, 2 µl 5X GoTaq Buffer (Promega), 0.5 mM MgCl2, 2.5 mM dATP (Promega) and 0.3 µl nuclease water at 72°C for 30 min. 1.5 µl of polyadenylation product or gel extract was cloned using the pGEM-T easy vector system I (Promega) following manufacturer's protocol. Between 3 and 10 colonies per transformation were grown in 5 ml overnight cultures. Plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (following manufacturer's instructions). Before sequencing, a restriction digest using *Eco*RI (Promega) was carried out to confirm fragment insertion, per 10 µl reaction, 0.25 µl *Eco*RI, 5 µl miniprep elution, 1 µl 10X buffer H and 3.75 µl dH20 was incubated at 37°C for 2 h and visualized on a 1.5% agarose gel. Positive samples were sequenced using both the T7 or SP6 universal primers by Eurofins, United Kingdom.

Phylogenetic Analysis

Raw reads were trimmed to remove remaining vector fragments and unambiguous bases at the ends of the reads. BLAST search using the newly obtained sequences against the non-redundant (nr) database was used to identify sequence positive clones. A dataset was assembled including all new sequences in addition to reference sequences encompassing the breadth of diversity of *Blastocystis* and an alignment was carried out using MAFFT v.7 (Katoh and Toh, 2010). Alignment contained four outgroup taxa for a total of 171 taxa. After aligning with MAFFT, ambiguous positions were masked with trimAl (Capella-Gutierrez et al., 2009). Following trimming, the alignment contained 1326 positions. A maximum likelihood tree was constructed using the RAxML software version 8 (Stamatakis, 2014, 2015) on the online platform CIPRES (Miller et al., 2010).[2] For each dataset bootstrap support was calculated from 1000 replicates.

[1]http://entamoeba.lshtm.ac.uk/xenic.htm

[2]http://www.phylo.org/

TABLE 1 | Summary of amplification conditions from this study.

Target Organism	Primer Pair	Primer Type	Initial Denaturation Conditions		Denaturation Conditions		Annealing Conditions		Extension Conditions		Cycle Number	Final Extension Conditions	
			Temp °C	Time min/s	Temp °C	Time min/s	Temp °C	Time min/s	Temp °C	Time min/s		Temp °C	Time min/s
Blastocystis	RD3/RD5	External	95	5 min	95	30 s	55	30 s	72	1 min 40 s	35	72	5 min
Blastocystis	RD5F/BhRDr	Internal	95	5 min	95	30 s	55	30 s	72	1 min 40 s	35	72	5 min
Cryptosporidium	CRY F1/CRY R1	External	94	2 min	94	50 s	53	50 s	72	1 min	24	72	10 min
Cryptosporidium	CRY F2/CRY R2	Internal	94	2 min	94	50	56	30 s	72	1 min	30	72	10 min
Giardia	RH11/RH4	-	96	2 min	96	45 s	58	30 s	72	45 s	30	72	4 min
Eimeria	EIF1/EIR3	External	94	5 min	94	30 s	57	30 s	72	2 mins	30	72	10 min
Eimeria	EIF3/EIR3	Internal	94	3 min	94	30 s	60	30 s	72	1 min 30 s	40	72	7 min
Entamoeba	542/543	-	94	5 min	94	30 s	55	30 s	72	30 s	35	72	2 min

RESULTS

Culturing

Blastocystis was cultured in tubes containing both types of media. Isolates from fox, lynx, wallaby, elk and otter grew at 35°C, while the ones from water voles grew at room temperature (**Supplementary Figure S1**). We were unable to establish cultures from other hosts.

Screening of Fecal Samples

Building upon sampling from a previous study, a total of 231 fecal samples from 38 vertebrate species were examined. It should be noted that the percent positive percentages herein are the minimum since PCR amplification rather than qPCR was used. *Blastocystis* was detected in 18/38 species (47%). A total of 255 clones were sequence positive for *Blastocystis*; 184 of these clones were from the current study. Of the 12-carnivorous species only three (pine marten, lynx and Scottish wild cat) were sequence positive for *Blastocystis* (25%, **Table 2**). There were no sequence positives for badger, European brown bear, otter, polecat, red and arctic foxes, stoat, gray and Iberian wolves, despite having multiple samples from different time points from these species. For non-carnivorous species, 15/26 (58%) were sequence positive, while barnacle and pink footed geese, four lined snake, hedgehog, water shrew, raven, red billed chough, black and brown rats, pied tamarind and black rhinoceros were negative (**Table 2**). *Blastocystis* was found in all artiodactyl species examined, but not all fecal samples were sequence positive. Sequence positive results for samples were as follows: Carnivora 3/50 (6%); Artiodactyla 20/36 (56%); Anseriformes 0/2 (0%); Squamata 0/1 (0%); Eulopotyphia 0/0 (0%); Passeriformes 0/4 (0%); Rodentia 29/81 (36%); Diprotodontia 2/5 (40%); Primates 27/43 (63%); Perissodactyla 0/0 (0%).

Regarding subtypes from cultures, we only looked at water voles as their cultures were numerous. We found only ST1 and ST4, while the rest of the STs found in the faces were not recovered.

Diversity and Distribution of Subtypes

In total, 10 known subtypes were detected: ST1, ST2, ST3, ST4, ST5, ST8, ST10, ST13, ST14, and ST15 (**Table 3**). Of those, ST2, ST3, ST8 and ST15 were not found in our previous collection. Subtype 4 was the most commonly isolated, found in 83/255 (33%) clones across 11 species. This was followed by ST2, isolated from 80/255 samples (31%); ST10 27/255 (11%); ST1 26/255 (10%); ST14 17/255 (7%); ST5 13/255 (5%); ST3 and ST15 4/255 (2%); ST13 1/255 (0.4%). Three sequences grouped with the *B. lapemi* clade.

All artiodactyls, except for the European Bison (*Bison bonasus*) housed at Howletts, had at least one positive ST identification. The subtypes found in this group coincided with published data with most isolates belonging to ST5, ST10 and ST14. ST5 was present in 6/36 (17%) samples; ST10 in 10/36 (28%) samples; ST14 in 7/36 (19%); ST4 in 2/36 (6%) samples; ST1 and ST13 both 1/36 (3%). 5/36 samples exhibited co-occurrence with two or more STs. The bongo calf (*Tragelaphus eurycerus*) –shared the same STs (10 and 14) with its mother as opposed to the father, who is housed separately and in whom we only detected ST14.

Eighty-one samples from four species belonging to the order Rodentia are presented in this study. Brown rat (*Rattus norvegicus*) and black rat (*Rattus rattus*) yielded no *Blastocystis* positive isolates. ST2 and ST4 were detected in three samples were from Red squirrel (*Sciurus vulgaris*). Water vole (*Arvicola amphibious*) samples accounted for a total of 26/81 (32%) positive *Blastocystis* samples and 88 positive clones. a total of 74 water vole samples have been taken to date, 26/74 (35%) are sequence positive for one or more STs. The large sample number is due to the sizable cohort in the study, which included repeat sampling over an extended period of time. Three groups of water vole were sampled: captive voles from Wildwood (22 samples) and wild caught voles from two areas in Essex, United Kingdom; Tilbury (17 samples) and Bulphan (35 samples). The wild caught voles were routinely screened over the course of 10–12 months. Amongst sequence positive samples the captive voles had a total of 30 positive clones obtained from 9/22 (41%) positive samples; Tilbury voles had 28 positive clones from 5/17 (29%) samples, while Bulphan voles yielded 29 clones from 11/35 (31%) positive samples. ST4 was the most commonly identified across both captive and wild voles, representing 76/88 (86%) of the clones and 23/26 (88%) samples. ST1, ST15 and a subtype placing with *B. lapemi* were all identified in two samples, ST1 and *B. lapemi* clade ST were isolated in captive voles, whereas ST15 was found in one wild vole across repeat sample time points. ST10 and ST14 were identified in one sample each from captive voles. Co-occurrence of two or more STs was identified in four voles, all of which were captive. ST4 was present in all of these co-occurrence instances along with ST1, ST10, ST14, and *B. lapemi* clade ST.

A total of 43 non-human primate (NHP) samples were collected from Howletts zoo as follows: 25 gorillas (*Gorilla gorilla gorilla*) samples from four family groups ranging in size (G1, G3 G4 and G5) and one individual were collected across two collection times, 13 Javan gibbon (*Hylobates moloch*) samples from individuals across seven groups ranging in size (A-G) and five pied tamarin (*Saguinus bicolor*) samples from group enclosures. Of these samples, 16/25 gorillas (64%); 11/13 (85%) Javan gibbons were sequence positive for at least one ST, while no *Blastocystis* was detected in any of the pied tamarins (**Table 4**). In terms of clones, for the gorillas, 64 positive clones were sequenced, of which 45/64 (70%) were ST2; 9/64 (14%) ST1; 8/64 (13%) ST3; and 2/64 (3%) were ST5. There were no notable differences observed among family groups. Specifically, all family groups had a relatively high incidence of ST2, while ST5 was only reported from family group 5. Co-colonization with two STs was seen in four of the gorilla samples (**Table 4**). The Javan gibbons represent one of the highest proportions of sequence positive clones for *Blastocystis* STs, from the 11 positive samples, 45 clones were sequenced. ST1 represented 18/45 (40%) of these clones; ST2 17/45 (38%); ST3 and ST5 both 4/45 (9%); ST8 1/45 (2%); ST15 1/45 (2%). Of the gibbon groups, Group F was the only one to not have any sequence positive data across two sample collections. Of all the groups, Group G was only sampled from once as its members were released to the wild between collections. Differences were observed among groups between the sample

TABLE 2 | Prevalence of *Blastocystis*, *Giardia*, *Cryptosporidium*, *Entamoeba,* and *Eimeria* in study animals.

Host	Scientific Name	Location	No. faecal samples collected	*Blastocystis* No. positive (% Positive)	*Giardia* No. Positive (% Positive)	*Cryptosporidium* No. Positive (% Positive)	*Entamoeba* No. Positive (% Positive)	*Eimeria* No. Positive (% Positive)
Carnivora (*T* = 50)								
Badger	*Meles meles*	Wildwood	2	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)
European Brown Bear	*Ursus arctos arctos*	Wildwood	4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Lynx	*Lynx lynx*	Wildwood	5	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)
Otter	*Lutra lutra*	Wildwood	7	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pine Marten	*Martes martes*	Wildwood	2	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)
Polecat	*Mustela putarius*	Wildwood	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Red Fox	*Vulpes vulpes*	Wildwood	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Arctic Fox	*Vulpes lagopus*	Wildwood	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Scottish Wild Cat	*Felis silvestris*	Wildwood	13	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)
Stoat	*Mustela ermine*	Wildwood	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gray Wolf	*Canis lupus*	Howletts	3	0 (0)	0 (0)	0 (0)	0 (0)	1 (33)
Gray Wolf	*Canis lupus*	Wildwood	2	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)
Iberian Wolf	*Canis lupus signatus*	Howletts	3	0 (0)	0 (0)	0 (0)	0 (0)	1 (33)
Anseriformes (*T* = 2)								
Barnacle Goose	*Branta leucopsis*	Wildwood	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pink Footed Goose	*Anser brachyrhynchus*	Wildwood	1	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
Artiodactyla (*T* = 36)								
Muntjac	*Muntiacus reevesi*	Wildwood	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
European Bison	*Bison bonasus*	Wildwood	5	3 (60)	0 (0)	0 (0)	2 (40)	1 (20)
European Bison	*Bison bonasus*	Howletts	4	0 (0)	0 (0)	0 (0)	2 (50)	2 (50)
Eurasian Elk	*Alces alces*	Wildwood	3	1 (33)	0 (0)	0 (0)	0 (0)	0 (0)
Pygmy Goat	*Capra aegagrus hircus*	Wildwood	2	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)
Red Deer	*Cervus elaphus*	Wildwood	3	1 (33)	0 (0)	0 (0)	1 (33)	0 (0)
Reindeer	*Rangifer tarandus*	Wildwood	1	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Soay Sheep	*Ovis aries*	Wildwood	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)
Wild Boar	*Sus scrofa*	Wildwood	4	2 (50)	0 (0)	0 (0)	1 (25)	0 (0)
Red River Hog	*Potamochoerus porcus*	Howletts	6	3 (50)	0 (0)	1 (17)	1 (17)	0 (0)
Bongo	*Tragelaphus eurycerus*	Howletts	6	1 (17)	0 (0)	0 (0)	2 (33)	1 (17)
Squamata (*T* = 1)								
Four-lined Snake	*Elaphe quatuorlineata*	Wildwood	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Eulopotyphla (*T* = 7)								
Hedgehog	*Erinaceus quatuorlineata*	Wildwood	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Water Shrew	*Neomys fodiens*	Wildwood	6	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)
Passeriformes (*T* = 4)								
Raven	*Corvus corax*	Wildwood	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Red Billed Chough	*Pyrrhocorax pyrrhocorax*	Wildwood	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

(Continued)

TABLE 2 | Continued

Host	Scientific Name	Location	No. faecal samples collected	*Blastocystis* No. positive (% Positive)	*Giardia* No. Positive (% Positive)	*Cryptosporidium* No. Positive (% Positive)	*Entamoeba* No. Positive (% Positive)	*Eimeria* No. Positive (% Positive)
Rodentia (*T* = 81)								
Black Rat	*Rattus rattus*	Wildwood	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Brown Rat	*Rattus norvegicus*	Wildwood	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Red Squirrel	*Sciurus vulgaris*	Wildwood	5	3 (60)	0 (0)	0 (0)	0 (0)	0 (0)
Water Vole	*Arvicola amphibious*	Wildwood	22	10 (45)	4 (18)	15 (68)	0 (0)	0 (0)
Water Vole	*Arvicola amphibious*	Tilbury	17	5 (29)	7 (41)	2 (12)	1 (6)	1 (6)
Water Vole	*Arvicola amphibious*	Bulphan	35	12 (34)	17 (49)	4 (11)	2 (6)	4 (11)
Diprotodontia (*T* = 5)								
Wallaby	*Macropus rufogriseus*	Wildwood	5	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)
Primates (*T* = 43)								
Western Lowland Gorilla	*Gorilla gorilla gorilla*	Howletts	25	16 (64)	0 (0)	1 (4)	2 (8)	2 (8)
Javan Gibbon	*Hylobates moloch*	Howletts	13	11 (85)	1 (8)	6 (46)	1 (8)	0 (0)
Pied Tamarin	*Saguinus bicolor*	Howletts	5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Perissodactyla (*T* = 2)								
Black Rhinoceros	*Diceros bicornis*	Howletts	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

collections. For example, ST5 and ST15 were detected in Group B upon first collection, yet in the second ST1 and ST2 were found.

In general, differences in ST distribution and prevalence are seen between the two zoos, the most obvious attribute to this is the differences in sampled taxa. Samples from Wildwood were comprised largely of members from the orders Rodentia, Artiodactyla and Carnivora, with Water voles and Scottish wild cats being sampled several times. Samples from Howletts were mainly from NHPs and other members of the Artiodactyla. The European bison and gray wolf were the only species sampled across both parks. Notably, *Blastocystis* was not isolated from any wolf or bison samples from Howletts, even though the bison housed at Wildwood and Howletts are related. The differences in ST distribution among the parks reflect the taxa housed within. Wildwood comprises largely of ST4 and ST10, STs commonly associated with rodents and hooved animals, whereas ST2, ST1 and ST5 are isolated on Howletts and are commonly associated with NHPs.

In total, 25 of the *Blastocystis* positive samples harbored more than one subtype; specifically, two subtypes were detected in 22 samples, three subtypes in two samples, while one sample contained four subtypes.

Newly generated sequences have been submitted to GenBank (MN526748- MN526930).

Co-occurrence of *Blastocystis* and Other Protists

Fecal samples were screened for *Cryptosporidium*, *Eimeria*, *Entamoeba*, *Giardia* and *Isospora*. Of the 81 *Blastocystis* positive samples, 43 (53%) harbored at least one of the above-mentioned protists in addition to *Blastocystis* (**Table 5**). Of those, 35 samples had one additional protist as follows: 14 cases from samples of Rodentia (all water voles), 13 from Artiodactyla (three from European bisons, three from bongos, two from Red river hogs, two from pygmy goats, one from wild boar, one from soay sheep, and one from red deer) and eight from NHPs (four from gorillas and three from Javan gibbons). Seven samples carried *Blastocystis* and two other protists: four Rodentia (all from water voles), two NHPs (both from Javan gibbons), and one from Artiodactyla (reindeer). A single sample from water vole was found with three other protists. The widest range of host species where co-occurrence was noted in the Artiodactyla. *Cryptosporidium* was detected in 31 (13%) samples and co-occurred with *Blastocystis* in 19 cases (61%); 22 (9%) samples were positive *Entamoeba*, 14 of which (64%) were found with *Blastocystis*; 29 (12%) samples harbored *Giardia* which co-occurred with *Blastocystis* in 10 cases (35%); 17 (7%) samples were positive for *Eimeria*, while nine were found with *Blastocystis*. Of the three (1%) *Isospora* positive samples, none co-occurred with *Blastocystis*.

Phylogenetic Analysis

All *Blastocystis* sequences grouped together with maximum support (100BS) (**Figure 1**). Newly acquired sequences belong to ST1, ST2, ST3, ST4, ST5, ST8, ST10, ST14, ST15, and the *B. lapemi* clade. In agreement with previous studies, ST15, ST16 and ST17 along with sequences originating from ectotherms placed in the

TABLE 3 | *Blastocystis* subtypes and co-occurrence with other microbial eukaryotes.

Host	Location	No. sequence positive clones	*Blastocystis* ST											Co-occurrence with other protists
			ST1	ST2	ST3	ST4	ST5	ST8	ST10	ST13	ST14	ST15	ST?	
Carnivora														
Pine Marten	Wildwood	1	–	–	–	1/1	–	–	–	–	–	–		–
Lynx	Wildwood	2	–	1/2	–	–	–	–	–	–	1/2	–		–
Scottish Wild Cat	Wildwood	2	–	–	–	1/2	–	–	–	–	1/2			–
Artiodactyla														
Muntjac	Wildwood	1	–	–	–	–	–	–	–	1/1	–	–		–
European Bison	Wildwood	11	–	–	–	–	–	–	11/11		–	–		*Entamoeba, Eimeria*
Eurasian Elk	Wildwood	6	–	–	–	1/6	–	–	1/6	–	4/6	–		–
Pygmy Goat	Wildwood	3	1/3	–	–	–	–	–	1/3	–	1/3	–		*Entamoeba*
Red Deer	Wildwood	8	–	–	–	3/8	–	–	5/8	–	–	–		*Entamoeba*
Reindeer	Wildwood	1	–	–	–	–	–	–	1/1	–	–	–		*Entamoeba, Eimeria*
Soay Sheep	Wildwood	1	–	–	–	–	–	–	–	–	1/1	–		*Entamoeba*
Wild Boar	Wildwood	2	–	–	–	–	2/2	–	–	–	–	–		*Entamoeba*
Red River Hog	Howletts	5	–	–	–	–	5/5	–	–	–	–	–		*Cryptosporidium, Entamoeba*
Bongo	Howletts	10	–	–	–	–	–	–	5/10	–	5/10	–		*Entamoeba, Eimeria*
Rodentia														
Red Squirrel	Wildwood	4	–	3/4	–	1/4	–	–	–	–	–	–		–
Water Vole	Wildwood	30	3/30	–	–	24/30	–		1/30		–	–	2/30	*Cryptosporidium, Giardia*
Water Vole	Tilbury	28	–	–	–	25/28	–	–	–	–	–	3/28		*Cryptosporidium, Entamoeba, Giardia, Eimeria*
Water Vole	Bulphan	29	–	–	–	29/29	–	–	–	–	–	–		*Cryptosporidium, Entamoeba, Giardia, Eimeria*
Diprotodontia														
Wallaby	Wildwood	2	–	–	–	–	–	–	2/2	–	–	–		–
Primates														
Western Lowland Gorilla	Howletts	64	9/64	45/64	8/64	–	2/64	–	–	–	–	–		*Cryptosporidium, Entamoeba, Eimeria*
Javan Gibbon	Howletts	45	18/45	17/45	4/45	–	4/45	1/45	–	–	–	1/45		*Cryptosporidium, Giardia, Entamoeba*

most basal positions (Alfellani et al., 2013; Yowang et al., 2018). Subtypes 3, 4, 8, and 10 grouped together, while subtypes 7, 9 and 6 formed a clade. Two of the water vole sequences grouped within the clade formed by *B. lapemi* and *B. pythoni*. Subtypes 1, 2 and 11 grouped together and sister to the clade formed by subtypes 5, 12, 13, and 14.

DISCUSSION

Animals from 38 species from two animal parks in the United Kingdom were sampled over a period of 3 years. Eighty-two samples from 47% of all animal species were sequence positive for *Blastocystis*. Of those 82, (21/82) 26% were found to harbor more than one ST, while 53% also harbored other protists. *Blastocystis* was present in animals from both parks. As expected, ST4 was dominant in rodents, whereas ST10 and ST14 dominated in artiodactyls. In primates, ST1 and ST2 were dominant. We reported *Blastocystis* presence in the Lynx and the Scottish wild cat for the first time. Both of these animals are carnivorous. Our study confirms previous findings on reduced presence and often absence of *Blastocystis* in carnivores and high prevalence in artiodactyls (Alfellani et al., 2013; Cian et al., 2017, Zhao et al., 2017). It is well known that dietary, behavioral and environmental factors shape bacterial communities, though this has yet to be shown for microbial eukaryotes. In that vein, a possible explanation for the above observation could be that captive carnivores consume a diet consisting of almost exclusively refrigerated meat, which is devoid of other eukaryotes. This considerably reduces contamination. Nonetheless, a recent study on free-living carnivorous animals confirmed presence of *Blastocystis* in only 1.6% of hosts (Calero-Bernal et al., 2019), suggesting that additional factors might account for the low prevalence. Artiodactyls are herbivorous animals that consume exclusively fiber, while carnivores consume only animal protein. Thus the two also differ considerably in the overall

TABLE 4 | *Blastocystis* subtyping in captive Javan gibbons (*Hylobates moloch*) and West Lowland gorillas (*Gorilla gorilla gorilla*) from two sample collections with co-occurrence of other protists within sampled groups.

Host	Collection Number	Family Group	No. Positive Sequences	*Blastocystis* ST						Co-occurrence with other protists
				ST1	ST2	ST3	ST5	ST8	ST15	
Javan Gibbon A	1	A	6	3			2	1		*Cryptosporidium*
Javan Gibbon B	1	B	3				2		1	–
Javan Gibbon C	1	C	3	2	1					–
Javan Gibbon D	1	D	3		3					*Giardia*
Javan Gibbon E	1	E	3		3					–
Javan Gibbon F	1	F	0							–
Javan Gibbon G	1	G	3		3					*Cryptosporidium*
Javan Gibbon A	2	A	4	1		3				–
Javan Gibbon B	2	B	2	1	1					*Cryptosporidium, Entamoeba*
Javan Gibbon C	2	C	12	11	1					–
Javan Gibbon D	2	D	2			2				–
Javan Gibbon E	2	E	4		4					–
Javan Gibbon F	2	F	0							–
Javan Gibbon G	2	G	0							N/A

Host	Collection Number	Family Group	No. Positive Sequences	*Blastocystis* ST				Co-occurrence with other protists
				ST1	ST2	ST3	ST5	
West Lowland Gorilla 1	1	5	4		4			*Entamoeba*
West Lowland Gorilla 2	1	5	5		5			*Eimeria*
West Lowland Gorilla 3	1	4	6		6			–
West Lowland Gorilla 4	1	4	5		5			–
West Lowland Gorilla 5	1	3	4			4		–
West Lowland Gorilla 6	1	3	6		6			–
West Lowland Gorilla 7	1	3	6		5	1		–
West Lowland Gorilla 8	1	3	4		4			–
West Lowland Gorilla 9	1	3	1	1				–
West Lowland Gorilla 10	1	3	3	3				–
West Lowland Gorilla 1	2	1	2	2				–
West Lowland Gorilla 8	2	3	5		4	1		*Cryptosporidium*
West Lowland Gorilla 4	2	3	4		2	2		*Eimeria*
West Lowland Gorilla 10	2	4	3		3			–
West Lowland Gorilla 11	2	5	3	3				–
West Lowland Gorilla 12	2	5	3		1		2	–

structure and physiology of their respective gastrointestinal tracts. Both diet and physiology likely contribute to microbiota composition, and as a result, the microbial communities of artiodactyls and carnivores differ considerably (Sanders et al., 2015; Nishida and Ochman, 2018). In general, herbivores, to which artiodactyls belong, harbor high microbial diversity, while carnivores encompass the least diverse microbial communities amongst mammals (Nishida and Ochman, 2018). High microbial diversity and specific microbial profiles are linked to presence of *Blastocystis* in human studies though a causative link has yet to be established (Andersen et al., 2015; Audebert et al., 2016; Iebba et al., 2016; O'Brien Andersen et al., 2016; Beghini et al., 2017; Forsell et al., 2017; Nieves-Ramirez et al., 2018; Tito et al., 2019). A similar result has also been obtained from a study focusing on wild chimpanzees (Renelies-Hamilton et al., 2019). Given the high prevalence of *Blastocystis* in artiodactyls it would be interesting to explore whether such specific profiles exist in these animals as well.

As in our previous study (Betts et al., 2018), we identified multiple subtypes of *Blastocystis* in the same host. In addition to the elk, pygmy goat, red deer and water vole hosts bearing multiple subtypes, we add the Scottish wildcat (ST4 and ST14), bongo (ST10, ST14), and lynx (ST2, ST14). Previous reports also noticed presence of multiple STs in animals (Fayer et al., 2012; Badparva et al., 2015; AbuOdeh et al., 2016). Cian et al., documented several instances of mixed colonization of subtypes (11%), especially in primates and artiodactyls (Cian et al., 2017), while Wang et al., reported mixed colonization in 58% of a pig population (Wang et al., 2014). Collectively these data strengthen previously raised hypotheses that occurrence of multiple subtypes in animals is not unusual, but rather common (Fayer et al., 2012; Betts et al., 2018). Thus, a logical extension of this study would

TABLE 5 | Co-occurrence of *Blastocystis* with other microbial eukaryotes.

Sample	Order	Location	*Blastocystis* ST	*Cryptosporidium*	*Giardia*	*Eimeria*	*Entamoeba*	*Isospora*
Water Vole TB30.1	Rodentia	Tilbury	4	yes	yes	yes		
Javan Gibbon Group D	Primate	Howletts	2	yes	yes			
Water Vole R22	Rodentia	Wildwood	4	yes	yes			
Water Vole TB32.1	Rodentia	Tilbury	4	yes	yes			
Reindeer	Artiodactyla	Wildwood	10			yes	yes	
Water Vole TB29.1	Rodentia	Tilbury	15			yes	yes	
Javan Gibbon Group B	Primate	Howletts	1, 2	yes			yes	
Water Vole Q52	Rodentia	Wildwood	unknown	yes	yes			
Javan Gibbon Group G	Primate	Howletts	2	yes				
Western Lowland Gorilla 1 G5	Primate	Howletts	2				yes	
Western Lowland Gorilla 2 G5	Primate	Howletts	2			yes		
Water Vole C3	Rodentia	Wildwood	4	yes				
Water Vole C3	Rodentia	Wildwood	4	yes				
Water Vole C4	Rodentia	Wildwood	4	yes				
Water Vole C4	Rodentia	Wildwood	4	yes				
Water Vole PP01.2	Rodentia	Bulphan	4	yes				
Water Vole PP03.1	Rodentia	Bulphan	4		yes			
Water Vole PP03.2	Rodentia	Bulphan	4		yes			
Water Vole PP03.3	Rodentia	Bulphan	4		yes			
Water Vole PP03.4	Rodentia	Bulphan	4		yes			
Water Vole PP04.1	Rodentia	Bulphan	4		yes			
Water Vole PP05.2	Rodentia	Bulphan	4			yes		
Water Vole PP05.3	Rodentia	Bulphan	4			yes		
Red River Hog 2	Artiodactyla	Howletts	5	yes				
Red River Hog 3	Artiodactyla	Howletts	5				yes	
Wild Boar 1	Artiodactyla	Wildwood	5				yes	
European Bison 1	Artiodactyla	Wildwood	10				yes	
European Bison 1	Artiodactyla	Wildwood	10			yes		
European Bison 2	Artiodactyla	Wildwood	10				yes	
Bongo M	Artiodactyla	Howletts	14				yes	
Pygmy Goat 1	Artiodactyla	Wildwood	14				yes	
Soay Sheep	Artiodactyla	Wildwood	14				yes	
Water Vole TB29.2	Rodentia	Tilbury	15		yes			
Pygmy Goat 2	Artiodactyla	Wildwood	1, 10				yes	
Javan Gibbon Group C	Primate	Howletts	1, 2	yes				
Water Vole R12	Rodentia	Wildwood	1, 4	yes				
Javan Gibbon Group A	Primate	Howletts	1, 5, 8	yes				
Bongo Calf	Artiodactyla	Howletts	10 14				yes	
Bongo F	Artiodactyla	Howletts	10, 14			yes		
Western Lowland Gorilla 8 G3	Primate	Howletts	2, 3	yes				
Western Lowland Gorilla 4 G3	Primate	Howletts	2,3			yes		
Red Deer 1	Artiodactyla	Wildwood	4, 10				yes	
Water Vole Q99	Rodentia	Wildwood	4, unknown	yes				
Javan Gibbon Group B	Primate	Howletts	5, 15	yes				

be to disentangle whether co-occurring subtypes occupy distinct functional niches in the complex gut ecosystem, a direction that has also been suggested by Beghini et al. (2017).

Co-occurrence of *Blastocystis* with *Entamoeba*, *Giardia*, *Cryptosporidium* and *Eimeria* in multiple animal species across the two parks was also examined. Most previous studies have either looked for multiple parasites from single animal species or have targeted one microbial eukaryote in various hosts (Fayer et al., 2012; Parsons et al., 2015; Enriquez et al., 2016, 2019; Jacob et al., 2016). Herein, *Blastocystis* did not co-occur with other protists in any of the carnivores, even though we did observe co-occurrence of *Cryptosporidium* and *Eimeria* in gray and Iberian wolves. The case of artiodactyls is particularly notable. Eight out of ten artiodactyls that were *Blastocystis* positives co-occurred with an *Entamoeba* species. Out of these, three co-occurred with *Blastocystis*, *Entamoeba* and

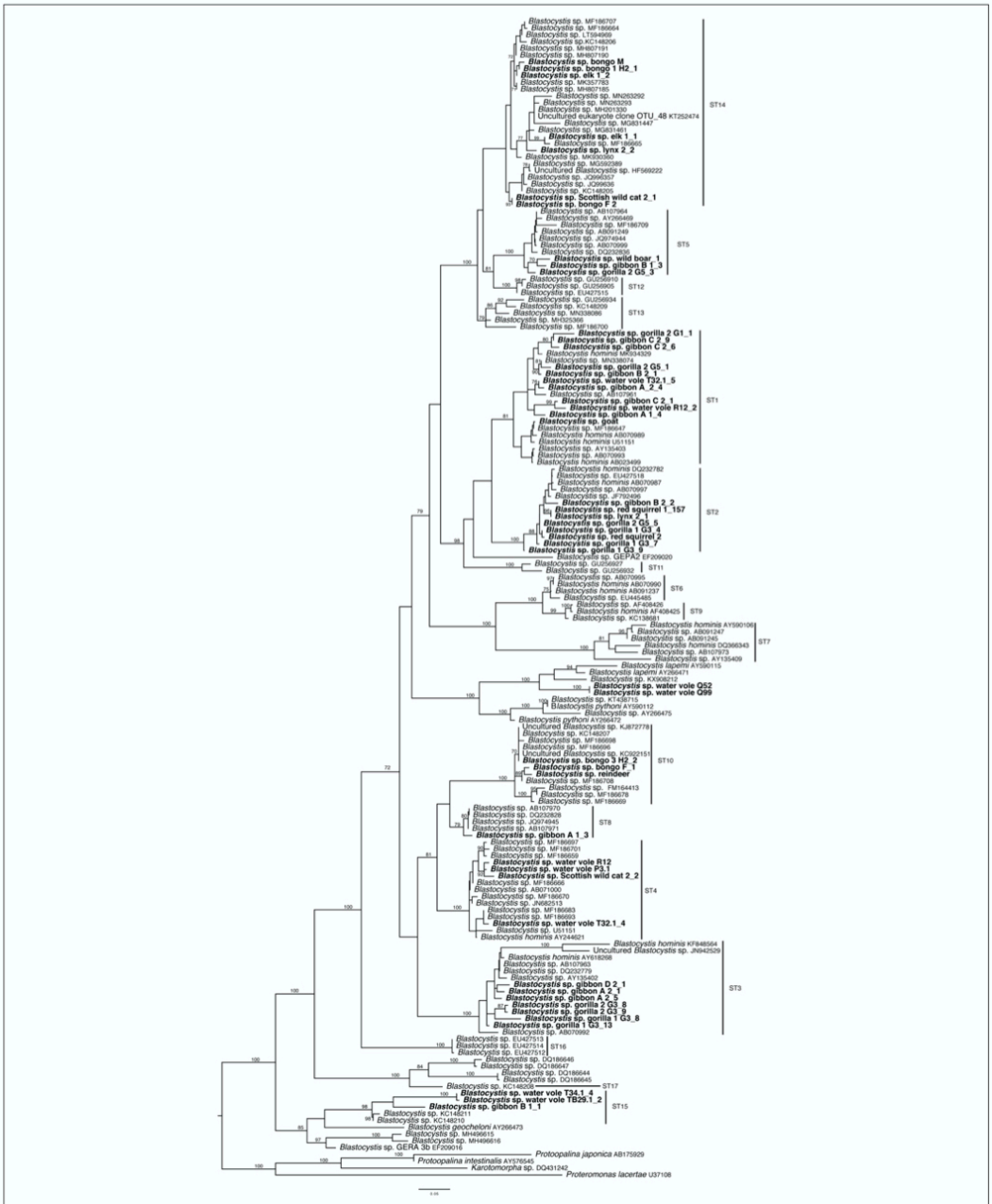

FIGURE 1 | Maximum likelihood phylogenetic tree inferred from 171 sequences and 1326 sites using RAxML v. 8. New sequences are in bold lettering. Numerical values indicate bootstrap support values and only those of over 70 are shown.

Eimeria (European bison, reindeer, bongo), while one animal had *Blastocystis*, *Entamoeba* and *Cryptosporidium* (red river hog). Significantly, none of the animals exhibited diarrheal episodes or other obvious gastrointestinal symptoms as confirmed by zookeepers and licensed veterinarians. Typically, microbial eukaryotes in animals are identified and reported upon onset of gastrointestinal symptoms. Herein, we sampled and detected gut protists before presentation of symptoms, though the possibility that some of the animals might have had symptoms before they were brought into the parks cannot be excluded. Asymptomatic carriage of a single or multiple protists in animals is not uncommon and the concern of zoonotic transmission has often been articulated (Fayer et al., 2012; Cian et al., 2017; Desoubeaux et al., 2018; Udonsom et al., 2018; Wang et al., 2018a; Enriquez et al., 2019). In case of zoonosis, detecting the reservoir is difficult as there is no reason to check the original host for presence of pathogens. The level and type of interaction among *Blastocystis*, other microbial eukaryotes (including fungi) and the rest of the host microbiome is unclear. Future animal studies should focus on exploring the eukaryotic component of the gut microbiome rather than targeting individual microbial species, in order to shed light on the role of eukaryome as a whole in the gut ecosystem. Combination of *in vitro* and *in vivo* targeted metagenomics and metabolomics approaches along with network analysis will greatly increase our understanding of these issues.

The case of *Blastocystis* is of interest. In the past, co-occurrence of *Blastocystis* with pathogens in stool samples of humans with gastrointestinal symptoms was likely one of the reasons for its controversial pathogenicity. Since adaptation of the subtyping system, the argument has been framed around specific subtypes or strains being pathogenic. Nonetheless, in a rather anthropocentric approach, assessment of the pathogenic potential of *Blastocystis* has focused primarily on humans and the "human" subtypes ST1 to ST9, while non-human metazoans and the rest of the subtypes have been largely overlooked. Moreover, the health status of animal subjects in many studies is not reported. When animals happen to have diarrhea the subtype present in these animals is often not mentioned, rather percent overall occurrence of individual subtypes is emphasized. Consequently, *Blastocystis* pathogenicity in animals is not well understood. It would be interesting to see whether any of the animals sampled herein will present any symptoms in the future. To that end, we have communicated with the zoo stuff to inform us in case symptoms develop in any of these animals.

To determine to which subtype the new sequences belonged, phylogenetic analysis was performed. Two of the newly generated sequences, both of which come from water voles, did not group with any of the known subtypes, but as sister to *Blastocystis lapemi*. There are two sequences designated as *B. lapemi* in the database, both of which originated from sea snakes (Yoshikawa et al., 2004; Noel et al., 2005). A third sequence that also groups within the clade and is genetically distinct comes from a monitor lizard. Therefore, either *B. lapemi* is not limited to sea snakes or all these sequences represent different species. In the absence of a culture and a full SSU rRNA sequence we designate those three sequences as *Blastocystis* sp. Four sequences – one coming from gibbon and three from water voles – group with ST15. Water vole is a newly reported host for ST15. Previously, Betts et al. (2018) had reported a potentially novel subtype, but had refrained from establishing it as such since the whole sequence was not available. Since then, several studies focusing on animals have contributed significantly toward populating previously isolate-sparse subtypes. As a result, the phylogenetic landscape of *Blastocystis* is changing. Expanded taxon sampling including several additional ST14 isolates from the database and from the current study has shown that ST14 is now divided into three distinct subclades, with new isolates populating all three. The previously suspected novel sequence (Betts et al., 2018) groups in one of the three. Thus, either ST14 has high intra-subtype divergence or it must be separated to at least two maybe even three subtypes. Nonetheless several subtypes harbor a high degree of genetic diversity except for ST4, which is the least genetically diverse (Stensvold and Clark, 2016b; Beghini et al., 2017). Given the variable degree of intra-subtype diversity, caution should be taken when establishing new subtypes. Genetic diversity within subtypes should be properly assessed. Commonly, closely related sequences from specific subtypes are included in the analysis, while more divergent representatives are not, leading to establishment of erroneous STs. Finally, the whole SSU rRNA region should be sequenced and phylogenies should include the breadth of *Blastocystis* diversity. Consistent approaches to subtyping *Blastocystis* will further elucidate the variety of subtypes that exist and their associations with specific hosts (El Safadi et al., 2016; Betts et al., 2018; Robertson et al., 2019).

In the current study, we employed cloning and demonstrated the presence of multiple subtypes within a single host and also presence of multiple eukaryotes within a host. We would like to emphasize that DNA was mainly extracted directly from fresh fecal samples without culturing in Jones media. Even though we still cannot guarantee that all subtypes present in the stool samples were amplified, selective pressures and constraints that culturing imposes were circumvented. In working with fecal samples other issues came to light. One of them is primer specificity. Eukaryotic microbe primers amplify the microbe of interest provided it is there. Our screening showed that all pairs of specific primers and most especially those of *Blastocystis* and *Entamoeba* also amplified several other eukaryotes. For example, approximately ∼40% of the sequenced clones did not correspond to *Blastocystis* specific sequences. Development of new *Blastocystis*-specific primers that will amplify a large fragment of the SSU rRNA gene are urgently needed, since this will reduce the costs of cloning and sequencing.

CONCLUSION

Herein we have identified asymptomatic carriage of multiple microbial eukaryotes in a number of animal species. This is defined as presence of multiple *Blastocystis* subtypes in single hosts and in many cases these co-occur with up to three other microbial eukaryotes. Given the higher prevalence of overlap of microbial eukaryotes in animals and especially in artiodactyls, the latter might provide a model not only for studying the spectrum

of parasitism (Rueckert et al., 2019), but also the associated microbial communities and how those relate with the different parts of this spectrum.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

EB carried out the collections, culturing, collected and analyzed all the data, and wrote a first draft of the manuscript. AT and EG directed research, planned experiments, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00288/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 4. Investigation into community-level differences in the bacterial microbiota in water voles with *Blastocystis* colonisation

1 Introduction

In this chapter the impact of *Blastocystis* colonisation on bacterial communities in the gut was investigated. The gastrointestinal tract is a dynamic and varied ecosystem made up of trillions of bacteria, viruses, protozoa, fungi and archaea that likely co-evolved with the host. As a result, mutually beneficial relations have developed over a prolonged period of time. Most studies aiming to understand the relationship between the microbiome and host are largely human centric and focus mainly on relations with the bacterial microbiota. Microbiome based research of non-human vertebrates mainly encompasses livestock (Henderson *et al.* 2015; Jami, White and Mizrahi 2014; Jami *et al.* 2013), companion animals (Coelho *et al.* 2018; Jha *et al.* 2020) and other notable species, such as those at risk of extinction (Cheng *et al.* 2015; Chong *et al.* 2019). A common objective of animal studies has been to explore the extent of bacterial community perturbations in the gut caused by anthropogenic intervention and investigate resulting ramifications on animal fitness and longevity (Chong *et al.* 2019; Clayton *et al.* 2016; Kohl and Dearing 2014; Mathews *et al.* 2005; McKenzie *et al.* 2017; Zhang *et al.* 2019). Recent investigations using gut microbial profiling have focused on assessing the implications of captivity compared to wild counterparts across a range of taxa (Clayton *et al.* 2016; Gibson *et al.* 2019; Oliveira *et al.* 2020; Tang *et al.* 2020). Roles of microbiota on host survival upon release have also been examined. Collectively, these studies support monitoring of the microbiota of animals involved in re-introduction and/or translocation projects. Regrettably, the majority of studies fail to include intestinal protozoa, even though emerging evidence suggests that some species persist as asymptomatic colonisers of the intestinal tract (Betts *et al.* 2018; Betts, Gentekaki and Tsaousis 2020; Chong *et al.* 2019; Gelling *et al.* 2015; Leighton 2002; Mathews *et al.* 2006; Redford *et al.* 2012; Seddon, Armstrong and Maloney 2007). Recent studies have showcased that rodents constitute an attractive model for examining these questions (Betts *et al.* 2018; Betts, Gentekaki and Tsaousis 2020).

The European water vole (*Arvicola amphibius*) is a semi-aquatic rodent that was widespread across Britain in the early 1900's. However, in the past few decades its population has dropped drastically, disappearing from over 89% of previously occupied sites. This has been attributed mainly to habitat destruction and the invasion of the non-native American mink (*Neovison vison*) (MacPherson and Bright 2011). In the UK attempts are being made to re-introduce this mammal into protected areas of marshland and repair fractured populations (Gow, Holder and Jeffrey 2004.; Gow 2007). An emerging factor in achieving both of these

objectives is the gut microbiota. Previous studies have primarily looked at prokaryotic microbiota, while the eukaryotes have been mostly overlooked (Bahrndorff *et al.* 2016; Parfrey *et al.* 2014). Nonetheless, a few studies have demonstrated high prevalence and co-occurrence of several eukaryotic microbes in the stool of many animals, hinting at potentially important roles as well (Betts, Gentekaki and Tsaousis 2020).

Specifically, the prevalence and role of protozoan parasites in captive and wild water voles has been poorly studied and the consequences of their colonisation on host fitness and impact on the natural gut flora and perturbations to homeostasis is unknown. *Cryptosporidium* sp.*, Giardia* sp. and *Blastocystis* have all been linked to water-borne transmission and have been isolated from various hosts including voles both in captivity and the wild. Nevertheless, due to a lack of research studies investigating the roles of aforementioned protozoa on the gut microbiome, the resulting impact on the host is debated (Betts *et al.* 2018; Betts, Gentekaki and Tsaousis 2020; Gelling *et al.* 2012; Leighton 2002).

Herein, we investigated the gut microbiota of captive voles, some of which are involved in re-introductory and breeding schemes. Annotation and characterisation of the gut microbiota encompassed not only the bacterial component but also included the questionable pathogen *Blastocystis.*

The results presented here provide the first investigation exploring the association of *Blastocystis* with bacterial communities in the gut of captive water voles and builds upon sequencing data obtained for *Blastocystis* in Chapter 3. The results demonstrated that the overall diversity of the gut microbiota was not significantly altered by the presence of *Blastocystis*. Nonetheless the abundances of a number of taxa were associated with *Blastocystis* colonisation.

2 Results

Twenty-nine faecal samples were collected from 16 voles. Twenty-eight of these were successfully screened and characterised; sample R95 (20.02.19) was not used due to low DNA recovery. Twelve voles were sampled twice, while four of them were sampled a single time. All samples were screened for *Blastocystis*, *Cryptosporidium* sp., and *Giardia* sp.. The sequence data from *Blastocystis* was detailed in Chapter 3, and in summary: 28 faecal samples from 16 water voles were examined and *Blastocystis* was detected in 7/16 voles (44%) and in 9/28 samples (32%). A total of 39 clones were sent for sequencing and 16/39 were positive (41%).

The 28 faecal samples from 16 voles were screened for *Giardia* sp. 5/28 (18%) samples and 4/16 (25%) voles were sequence positive for *Giardia* sp. and a total of 95 clones were sent for sequencing; 11/95 (12%) were sequence positive for the organism of interest, the remainder were faecal sample contaminants, such as vegetation.

5/28 (18%) samples and 5/16 voles (31%) were sequence positive for *Cryptosporidium* sp. and generated 11/68 positive clones (16%). Among the sequence positive samples all were identified in the first collection, this is summarised in Table 4.i.

Table 4.i Protozoa screening results from the study cohort. Screened parasites included *Blastocystis,* which was the main focus of this study, in addition to *Cryptosporidium* sp. and *Giardia intestinalis*. *G. intestinalis* was being monitored in the vole population prior to this study and a number of the voles were undergoing treatment for *Giardia* infection

Water Vole	Collection Date	Microbiome profile ID	Prior drug Treatment	Vet diagnosis for *Giardia*	*Giardia* screening result	*Cryptosporidium* Screening result	*Blastocystis* subtype/isolate screening result
C3	18.01.19	C3	Fenbendazole	Positive			4
C4	18.01.19	C4	Fenbendazole	Positive		*Cryptosporidium*	4
Q49	18.01.19	Q49	None	Positive	*G. intestinalis*	*Cryptosporidium*	
Q51	18.01.19	Q51	None	Positive			
Q52	18.01.19	Q52	None	Negative	*G. intestinalis*		
Q75A	18.01.19	Q75A	None	Negative			
Q84	18.01.19	Q84	Metronidazole	Positive			
Q85A	18.01.19	Q85A	None	Negative			
Q88	18.01.19	Q88	None	Negative			
Q99	18.01.19	Q99	Metronidazole	Positive			
R12	18.01.19	R12	None	Negative			
R13	18.01.19	R13	None	Positive		*Cryptosporidium*	
R22	18.01.19	R22	Fenbendazole	Positive		*Cryptosporidium*	4
R34	18.01.19	R34	Metronidazole	Positive		*Cryptosporidium*	
R95	18.01.19	R95	None	Negative			
C3	20.02.19	C3.1	None	Positive	*G. intestinalis*		4
C4	20.02.19	C4.1	None	Positive			4
Q51	20.02.19	Q51.1	None	Positive			
Q52	20.02.19	Q52.1	None	Negative	*G. intestinalis*		*B. lapemi*

Q75A	20.02.19	Q75A.1	None	Negative		
Q84	20.02.19	Q84.1	Fenbendazole	Positive		1
Q85A	20.02.19	Q85A.1	None	Negative	*G. intestinalis*	
Q88	20.02.19	Q88.1	None	Negative		
Q99	20.02.19	Q99.1	Fenbendazole	Positive		4, *B.lapemi*
R12	20.02.19	R12.1	None	Negative		1,4
R13	20.02.19	R13.1	None	Positive		
R22	20.02.19	R22.1	None	Positive		
R4	20.02.19	R4.1	None	Negative		

2.1 Characterisation of bacterial communities in the stool

A total of 2,469,175 reads were obtained from 16S rRNA amplicon sequencing. After quality-filtering and processing, the total read count measured at 1,509,628 with an average of 53,915 reads per sample. The maximum and minimum counts per sample were 76,169 and 34,050 respectively. The final operational taxonomic unit (OTU) number was 778. Low count filtering was applied with a minimum count of four reads at a 20% prevalence across the samples (meaning at least 20% of its values contained at least four counts). Data normalisation was used to account for the large variability of total read counts between samples. The library was not rarefied in order to reduce loss of possibly significant data from high sequence counts due to the relatively small difference in library sizes (<10x). Variance filtering screened out features that were close to constant and was measured using the inter-quartile range, which was set to a 10% threshold. A total of 162 low abundance features were removed based on low read count and 20 low variance features were removed based on the inter-quartile range; 171 features remained. The data was scaled via total sum scaling to address uneven sequencing depth.

2.2 Taxonomic composition, diversity and community profiling

There are few overall observable differences in the taxonomic composition of the samples in the present study. OTUs spanned seven phyla and all but one sample was dominated by Bacteroidetes (63% relative abundance across all samples) and secondly Firmicutes (31% relative abundance across all samples). Q88.1, R34, R4 and R95 all had dominating levels of Firmicutes, other phyla identified included Actinobacteria, Cyanobacteria, Proteobacteria and Tenericutes. Excluding Bacteroidetes and Firmicutes, the remaining phyla accounted for less than 10% of overall abundance (Figure 4.i).

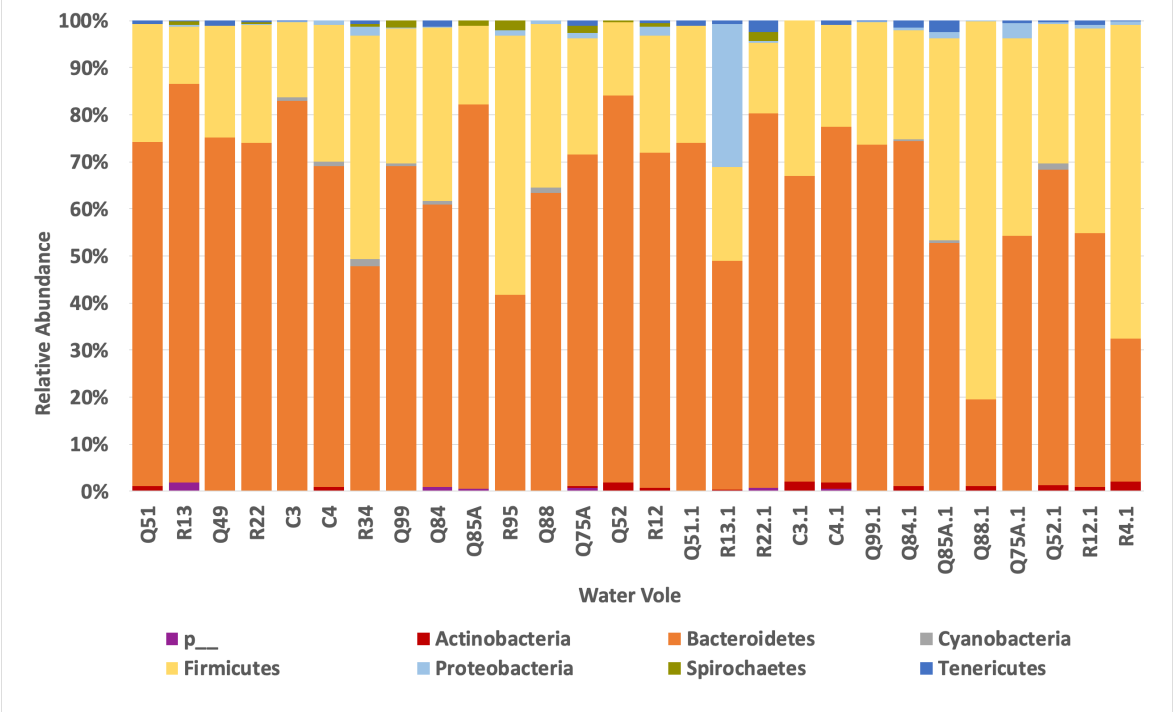

Figure 4.i Relative taxa abundance of the sampled water voles at the phylum level. Across the majority of voles, Bacteroidetes (orange) dominate. This is followed by the Firmicutes (yellow) which collectively account for over 90% of the OTU abundance at the phylum level. R13.1 shows a significant increase in Proteobacteria (light blue). p_ signifies all merged OTU phylum data that had a relative abundance that was below 1% across samples.

Overall, less than 2% of reads were not assigned at the phylum level. At the family and genus level, the number of unassigned OTUs increased from approximately 50% at the family level to over 60% at the genus level. These are likely made up of bacteria comprised of many different genera that, to date, lack extensive annotation.

At the genus level, approximately 38% of the remaining (relative) abundance is composed of members of *Duncaniella*, followed by *Ruminococcus* (7%), *Alistipes* (5%), *Allobaculum* (5%), *Muribaculum* (4%), *Christensenella* (4%), *Prevotella* (3%), *Bacteroides* (3%), *Clostridium* (2%), *Coprococcus* (2%), *Anaeromassilibacillus* (2%), *Flavonifractor* (2%), *Alloprevotella* (1%), *Anaerotignum* (1%), *Dubosiella* (1%), *Eisenbergiella* (1%), *Eubacterium* (1%), *Lactobacillus* (1%), *Prarprevotella* (1%), *Pedobacter* (1%) (Figure 4.ii).

Abundances did not differ in terms of sampling time points, with the following exceptions: at the phylum level, the relative abundance of Proteobacteria in vole R13 in the first collection (R13) was <1%, while this increased to approximately 32% in the second collection

(R13.1). At the genus level, the vole Q88 had an average abundance of *Christensenella* of 7% in the first collection (Q88), yet its abundance was increased to over 40% in the second collection (Q88.1, Coloured in dark grey) (Figure 4.ii)

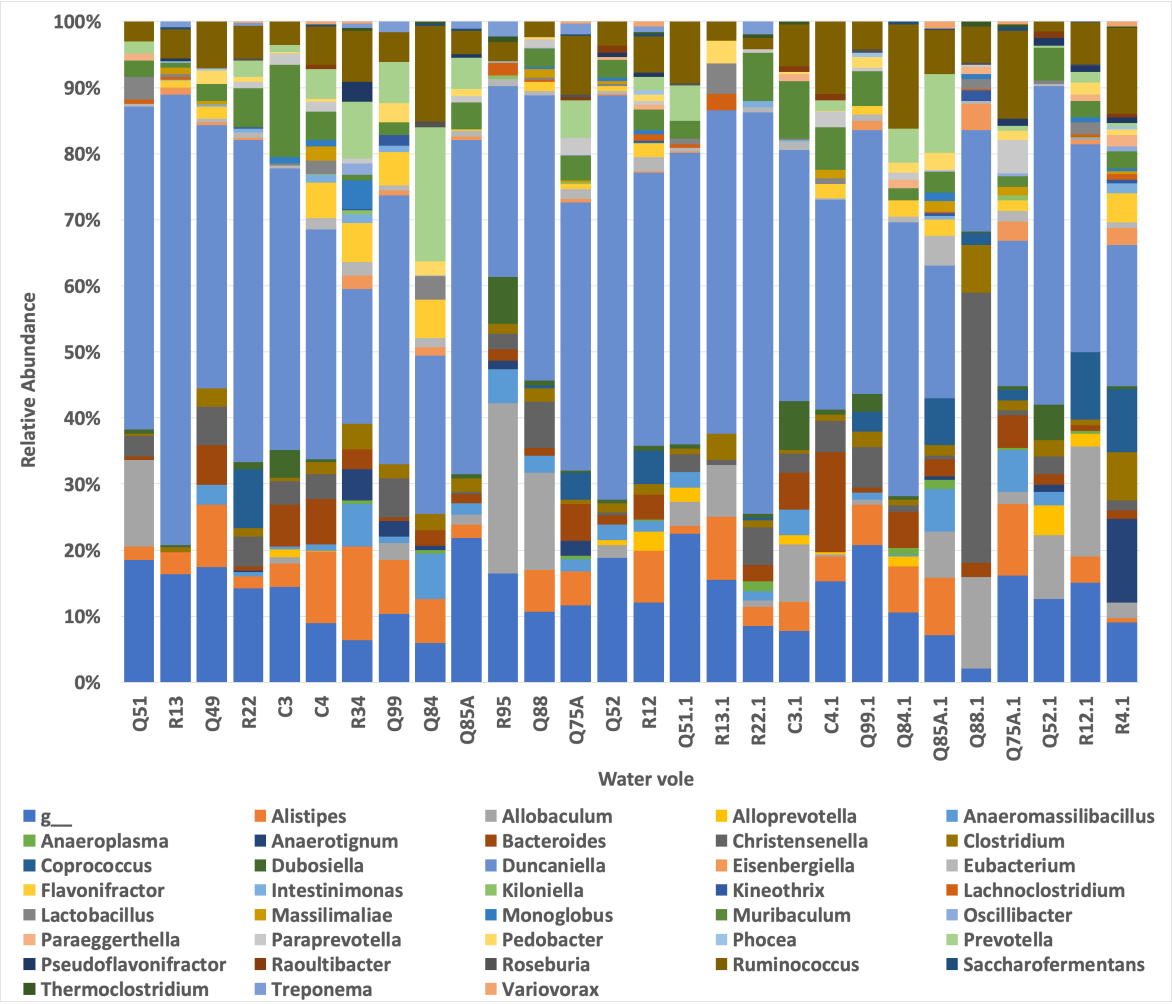

Figure 4.ii Relative taxa abundance of the sampled water voles at the genus level. Across the majority of voles, the relative abundance of OTUs at the genus level was consistent. g_ signifies all merged OTU genus data that had a relative abundance that was below 1% across samples.

Relative abundances of OTUs between *Blastocystis* positive samples, which were negative for other protozoa (n=5) were also compared against the samples that were uninfected with *Blastocystis*, *Cryptosporidium* sp. and *Giardia* sp. (n=14) Summarised in Table 4.ii. All subsequent results herein for *Blastocystis* are based on comparisons made between these two groups. This was done to minimise the impact of co-parasitism on the results. However, drug treated voles were kept in the cohort, as at the time of sampling all treatment had ceased a minimum of 10 days prior to sampling.

Table 4.ii Summary of the voles that will be included in subsequent analysis for *Blastocystis*-related investigation. Previous drug treatment (ending 10+ days prior to collection) was also recorded.

Water vole ID	Collection date	*Blastocystis* positive	Prior drug treatment
C3	18.01.19	Yes	Yes
C4	20.02.19	Yes	No
R12	20.02.19	Yes	No
Q99	20.02.19	Yes	Yes
Q84	20.02.19	Yes	Yes
Q51	18.01.19	No	No
Q51	20.02.19	No	No
Q75A	18.01.19	No	No
Q75A	20.02.19	No	No
R22	20.02.19	No	No
Q85A	18.01.19	No	No
Q88	18.01.19	No	No
Q88	20.02.19	No	No
R12	18.01.19	No	No
R13	20.02.19	No	No
R4	20.02.19	No	No
R95	18.01.19	No	No
Q84	18.01.19	No	Yes
Q99	18.01.19	No	Yes

The Relative abundances for *Blastocystis* positive and *Blastocystis* negative voles at the phylum level (Figure 4.iii) and the genus level (Figure 4.iv) are displayed below, using the selected data detailed in Table 4.ii.

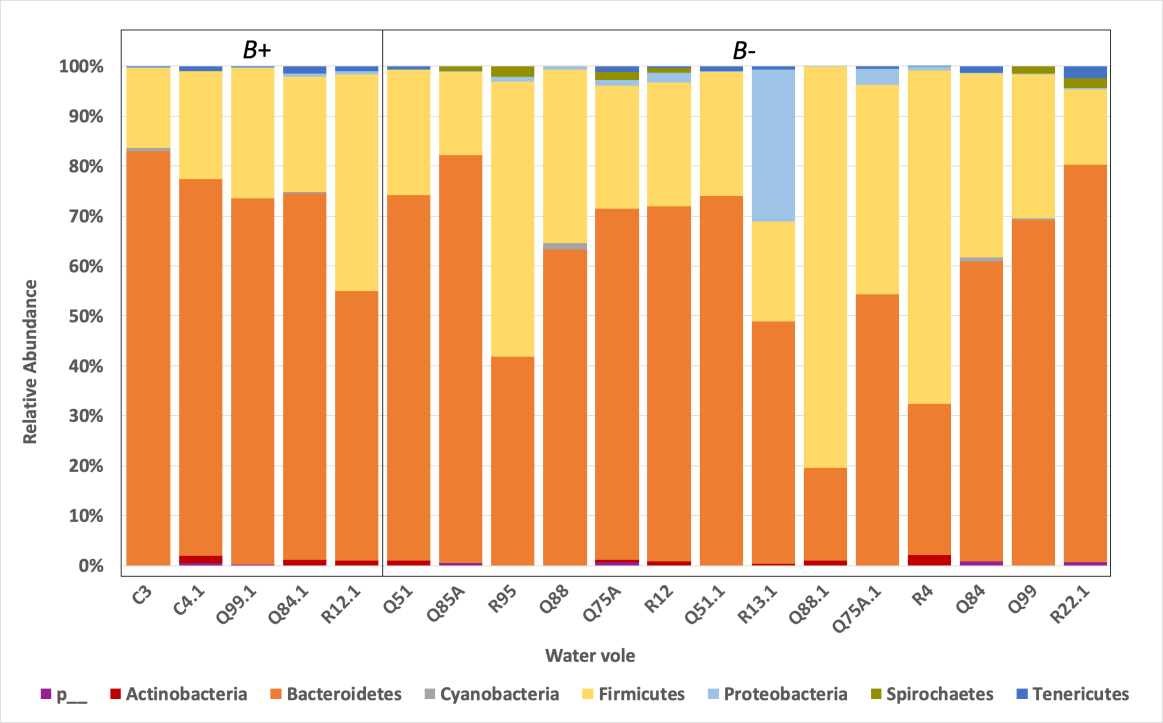

Figure 4.iii Relative taxa abundance of *Blastocystis* positive voles (*B*+) and *Blastocystis* negative voles (*B*-) at the phylum level. Between the two groups the relative abundance of OTUs at the phylum level are consistent. p_ signifies all merged OTU phylum data that had a relative abundance that was below 1% across samples.

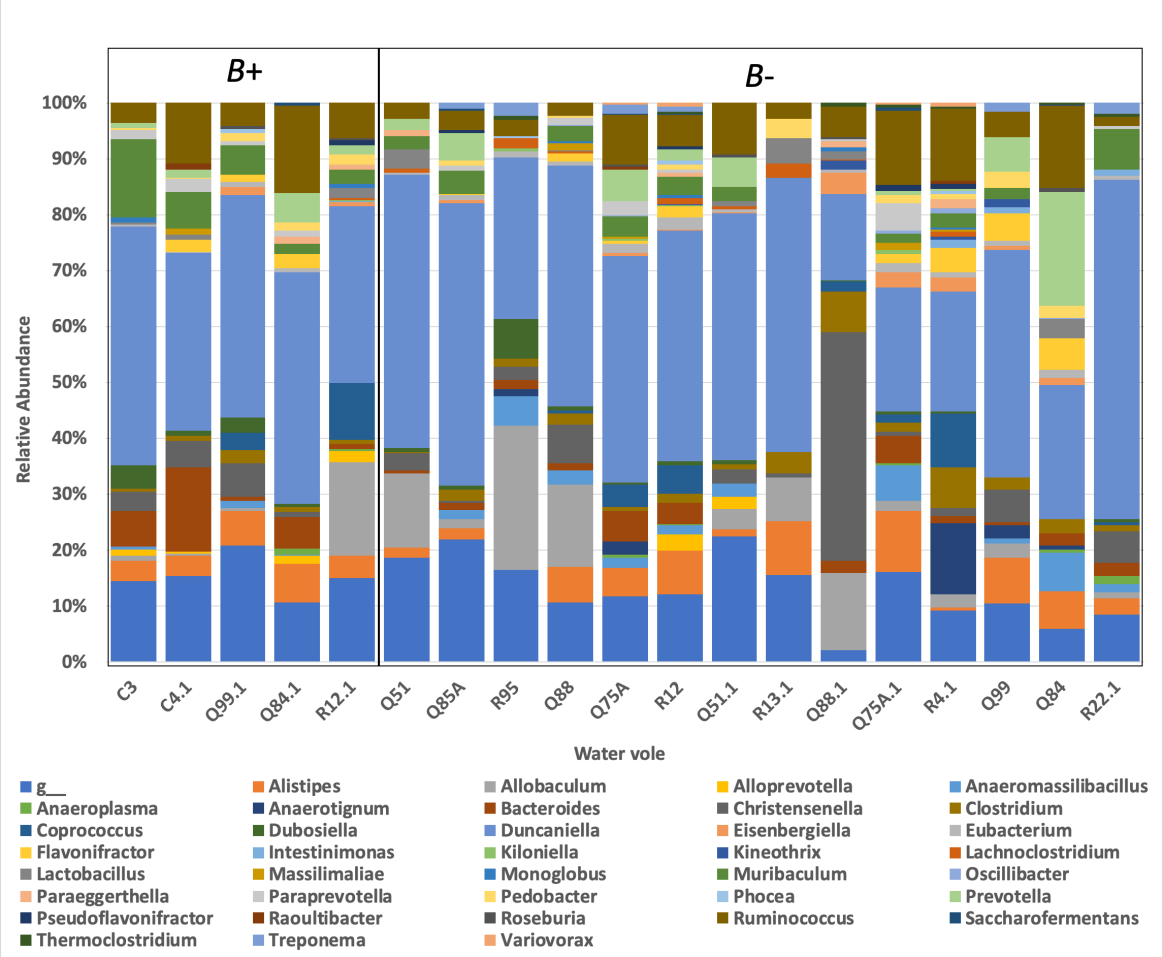

Figure 4.iv Relative taxa abundance of *Blastocystis* positive voles *(B+)* and *Blastocystis* negative voles *(B-)* at the genus level. Between the two groups the relative abundance of OTUs at the phylum level are consistent. g_ signifies all merged OTU genus data that had a relative abundance that was below 1% across samples.

2.3 Microbial diversity measures

We carried out diversity measures at all taxonomic levels for the sample cohort. Alpha diversity (variability within samples) was quantified using three methods: Observed species (measurement of taxa richness and provides no weight to the evenness of the communities), Chao1 (measurement of taxa richness which accounts for low abundance species) and Shannon indices (equal measurement of taxa richness and evenness).

The Shapiro-Wilk test for normality classified the data as non-normally distributed thus non-parametric tests were used for all statistical analysis.

For each result, between-group variations were measured using Mann-Whitney U test. The recorded *p*-values for each alpha diversity measure was above the cut off threshold (p=0.05). Overall, no significant difference in OTU richness was observed between positive

and negative voles, results are summarised in Table 4.iii and alpha diversity plots for the phylum and genus are shown in Figure 4.v.which are visually displayed in boxplots.

Table 4.iii Alpha diversity results for *Blastocystis* positive (*B*+) voles compared with negative (*B*-) voles. Diversity was measured using three methods: Observed, Chao1 and Shannon indices at each taxonomic level. No statistically significant results were identified ($p<0.05$)

Experimental Factor	Taxonomic Level	Diversity measure	*p*-value	Mann-Whitney statistic
Blastocystis	Phylum	Observed	0.55111	41.5
		Chao1	0.55111	41.5
		Shannon	0.3913	25
	Class	Observed	0.34373	45.5
		Chao1	0.34373	45.5
		Shannon	0.2193	21
	Order	Observed	0.45552	43.5
		Chao1	0.45552	43.5
		Shannon	0.2193	21
	Family	Observed	0.67615	40
		Chao1	0.67615	40
		Shannon	0.34262	24
	Genus	Observed	0.88941	37
		Chao1	0.88941	37
		Shannon	0.68679	30

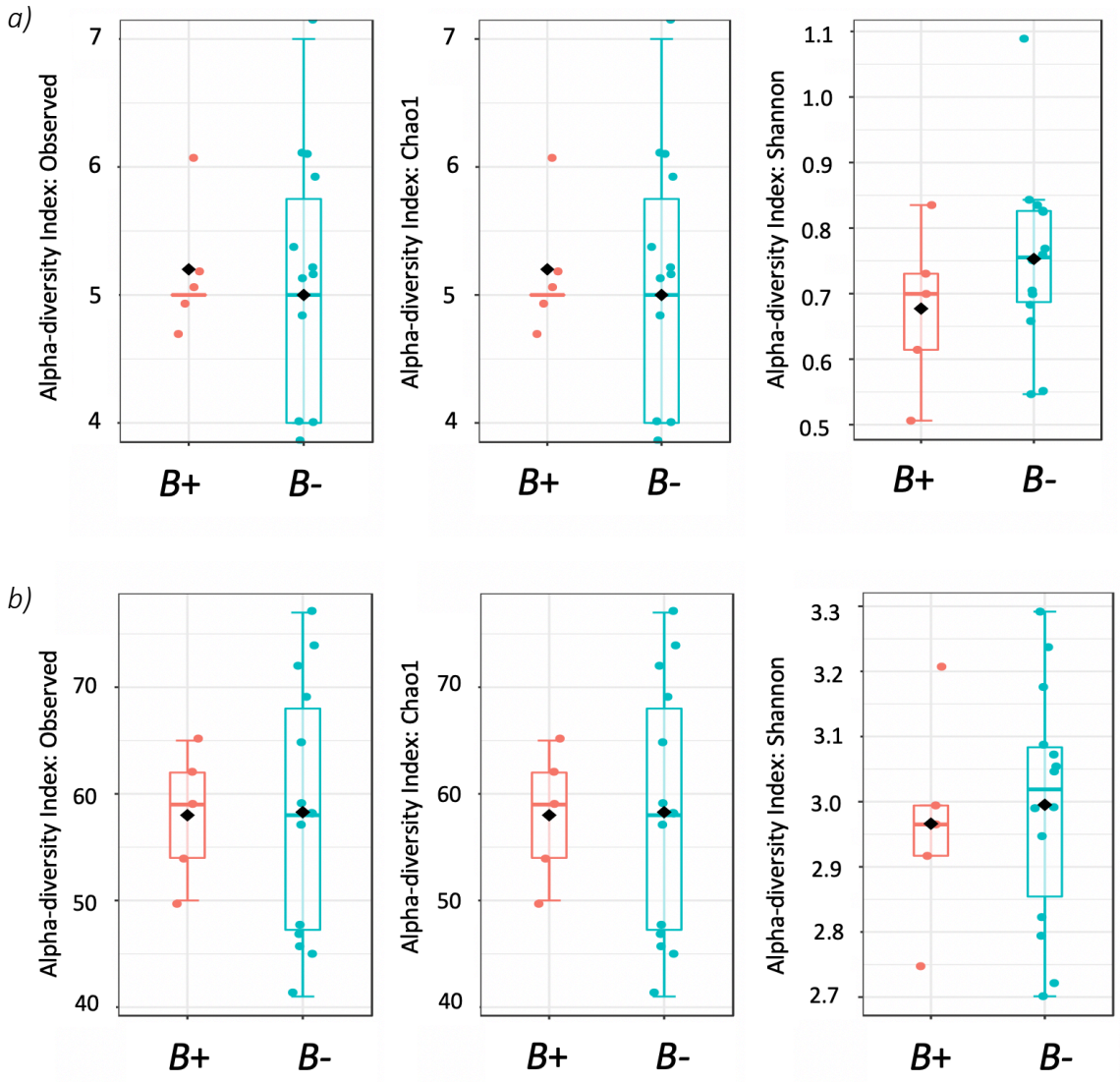

Figure 4.v Box plots showing alpha diversity (Observed, Chao1 and Shannon indices). Blue plots represent the negative (*B-*) samples and plots in red represent positive (*B+*) voles. *a)* shows alpha diversity box plots at the phylum level, *b)* shows results from the genus level. Boxes represent the interquartile range (IQR) between the 1st and 3rd quartiles. The horizontal line inside the box represents the median. Whiskers represent the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively. Solid coloured 'dots' represent data points that were greater than 1.5 times and less than three times the IQR.

Beta diversity measures were implemented using Bray-Curtis dissimilarity index to establish variability estimates between samples. This was accompanied by a PERMANOVA test to determine if centroids differed between variables of interest. Analysis was visualized with 2D ordination plots based on principal coordinate analysis (PCoA) Figure 4.vi shows plots for the phylum and genus level. Results showed no significant difference between the microbial communities of positive voles against negative voles ($p<0.05$).

Table 4.iv Beta diversity results for *Blastocystis* positive (*B*+) voles compared with negative (*B*-) voles. Diversity was measured using Bray-Curtis dissimilarity index with Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA). No significant results were identified (p<0.05)

Experimental Factor	Taxonomic Level	F- value	R-squared vale	*p*-value
Blastocystis	Phylum	1.1208	0.061854	< 0.311
	Class	0.82066	0.046051	< 0.44
	Order	0.81818	0.045918	< 0.444
	Family	0.72235	0.040759	< 0.597
	Genus	0.82372	0.046215	< 0.519

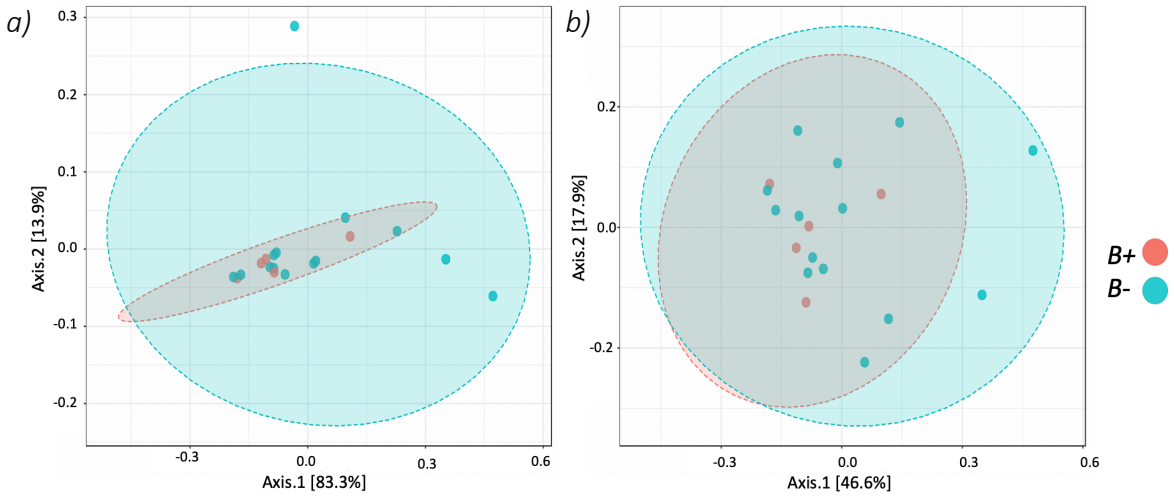

Figure 4.vi Principal Coordinate Analysis (PCoA) plots of the beta diversity of *B*+ voles (red) and *B*- voles (blue) based on Bray-Curtis dissimilarity index *a)* shows the PCoA at the phylum level and *b)* shows the PCoA at the genus level

Alpha and beta diversity was also assessed for the different drug treatments across the entire sample cohort to determine if there was a significant impact the bacterial diversity. All results demonstrated no significant impact of drug treatment on diversity measures (Supplementary table 4.i and Supplementary table 4.ii).

2.4 Microbial community comparison and classification

To calculate the microbial community abundance, we used two different abundance analysis methods. Firstly, classical univariate analysis was used to assess microbial community abundance differences, and this revealed no significant differences between *B*+ and *B*- voles (p>0.05). The second method used was MetagenomeSeq. This identified a total of 19 discriminant results when *B*+ was compared against *B*-. Results are summarised in Table 4.v and community abundance across different OTUs is displayed in Table 4.vi. Figure 4.vii displays box-plots for the log transformed count of notable OTUs which included bacteria belonging to the same taxonomic lineages, box-plots for all other significant OTUs are in Supplementary figure 4.i. In summary, all identified OTUs were significantly decreased in *Blastocystis* positive voles, only the genus *Anaerocella* was significantly increased (Figure 4.viii). At the phylum level Spirochaetes was significantly decreased, this decline in abundance was observed through the rest of this lineage and included the genus *Treponema.* Betaproteobacteria was also significantly decreased, and following members of this lineage including Burkholderiales, *Comamonadaceae* and the genus *Variovorax* were all significantly decreased too. A number of other genera were significantly associated with the presence of *Blastocystis*: a total of nine genera decreased and the one genus *Anaerocella* increased, as described above. These tests were also carried out to determine the significance of drug treatment on bacterial communities (Supplementary table 4.iii and Supplementary table 4.iv)

Table 4.v Significant OTUs identified by MetagenomeSeq as differentially abundant between *B*+ and *B*- samples (p<0.05). A total of 19 significant results were identified here, the observed difference in community abundance summarises the observed change between *B*+ and *B*- for each OTU

OTU	Name	Observed difference in community abundance	*p*-value	FDR
Phylum	Spirochaetes	Decrease in infected	5.43E-09	4.35E-08
Class	Spirochaetia	Decrease in infected	1.15E-09	1.49E-08
Class	Betaproteobacteria	Decrease in infected	3.48E-07	2.26E-06
Class	Epsilonproteobacteria	Decrease in infected	4.43E-05	1.92E-04
Order	Spirochaetales	Decrease in infected	3.86E-08	5.78E-07
Order	Burkholderiales	Decrease in infected	1.37E-06	1.03E-05
Family	*Spirochaetaceae*	Decrease in infected	1.26E-08	3.03E-07
Family	*Oscillospiraceae*	Decrease in infected	8.01E-07	9.61E-06
Family	*Comamonadaceae*	Decrease in infected	4.38E-06	3.50E-05
Genus	*Treponema*	Decrease in infected	1.90E-08	8.35E-07
Genus	*Variovorax*	Decrease in infected	2.17E-06	4.77E-05
Genus	*Kineothrix*	Decrease in infected	5.33E-05	6.99E-04

Genus	*Oscillibacter*	Decrease in infected	6.36E-05	6.99E-04
Genus	*Robinsoniella*	Decrease in infected	4.30E-04	3.79E-03
Genus	*Thermoclostridium*	Decrease in infected	1.40E-03	1.03E-02
Genus	*Kiloniella*	Decrease in infected	3.06E-03	1.92E-02
Genus	*Anaeromassilibacillus*	Decrease in infected	5.77E-03	2.99E-02
Genus	*Anaerotignum*	Decrease in infected	6.11E-03	2.99E-02
Genus	*Anaerocella*	Increase in infected	7.99E-03	3.52E-02

Phylum	Class	Order	Family	Genus	Result
Spirochaetes	Spirochaetia	Spirochaetales	*Spirochaetaceae*	*Treponema*	Decrease
(Proteobacteria)	Betaproteobacteria	Burkholderiales	*Comamonadaceae*	*Variovorax*	Decrease
(Proteobacteria)	Epsilonproteobacteria				Decrease
(Firmicutes)	(Clostridia)	(Eubacteriales)	*Oscillospiraceae*	*Oscillibacter*	Decrease
(Firmicutes)	(Clostridia)	(Eubacteriales)	*(Lachnospiraceae)*	*Kineothrix*	Decrease
(Firmicutes)	(Clostridia)	(Eubacteriales)	*(Lachnospiraceae)*	*Robinsoniella*	Decrease
(Firmicutes)	(Clostridia)	(Eubacteriales)	*(Oscillospiraceae)*	*Thermoclostridium*	Decrease
(Proteobacteria)	(Alphaproteobacteria)	(Kiloniellales)	*(Kiloniellaceae)*	*Kiloniella*	Decrease
(Firmicutes)	(Clostridia)	(Eubacteriales)	*(Oscillospiraceae)*	*Anaeromassilibacillus*	Decrease
(Firmicutes)	(Clostridia)	(Eubacteriales)	*(Lachnospiraceae)*	*Anaerotignum*	Decrease
(Bacteroidetes)	(Bacteroidia)	(Bacteroidales)	*(Rikenellaceae)*	*Anaerocella*	Increase

Table 4.vi Taxa table showing the relationships between identified OTUs via MetagenomeSeq. The observed change in OTU abundance in *B+* voles compared with *B-* voles is summarised in the 'Result' column. The taxa in parentheses represent bacterial lineages but were not identified in MetagenomeSeq analysis. Taxa not in parentheses are significant OTUs identified in MetagenomeSeq analysis.

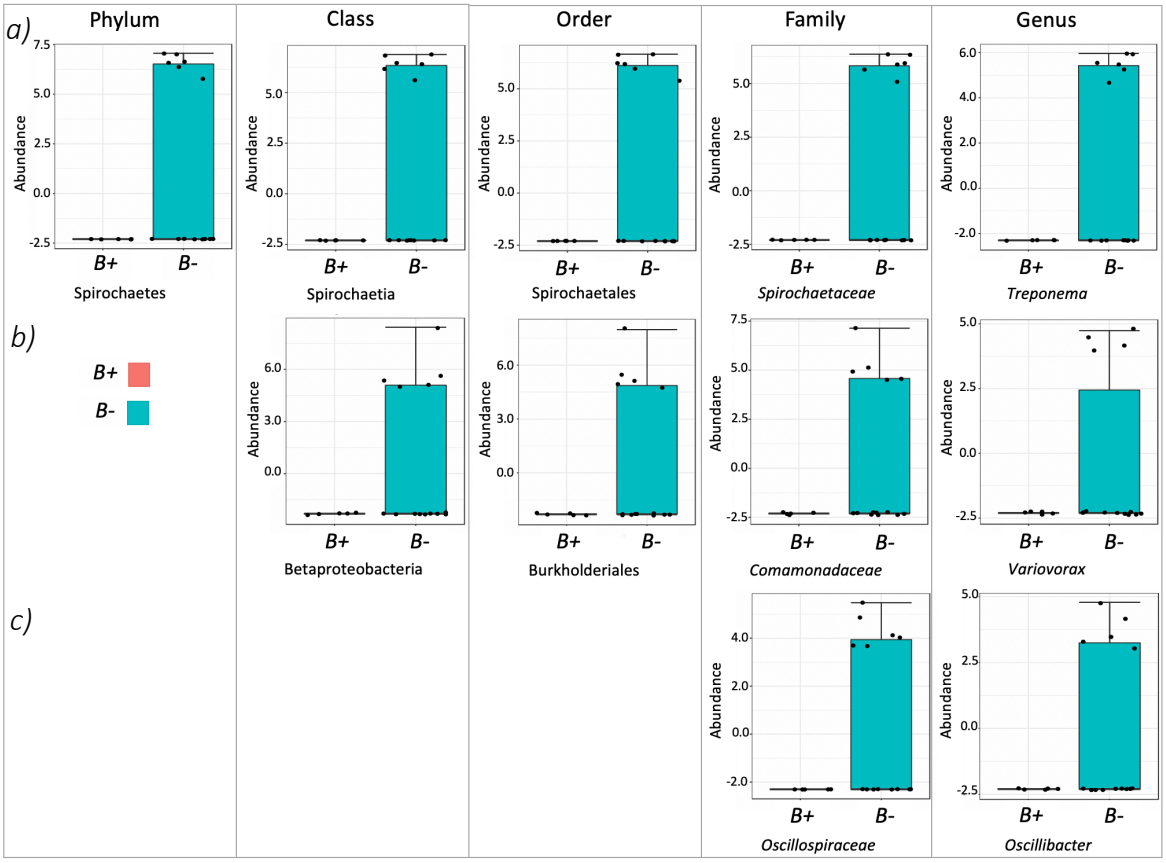

Figure 4.vii Box-plots showing the log transformed counts of distinguished OTUs identified by MetagenomeSeq analysis (p<0.05). This includes multiple taxa belonging to the same lineage *a)* OTUs belonging to the Spirochaetes lineage *b)* OTUs belonging to the Betaproteobacteria lineage *c)* OTUs belonging to the *Oscillospiraceae* lineage. Plots in blue represent the data from *B-* voles, red plots are from *B+* voles.

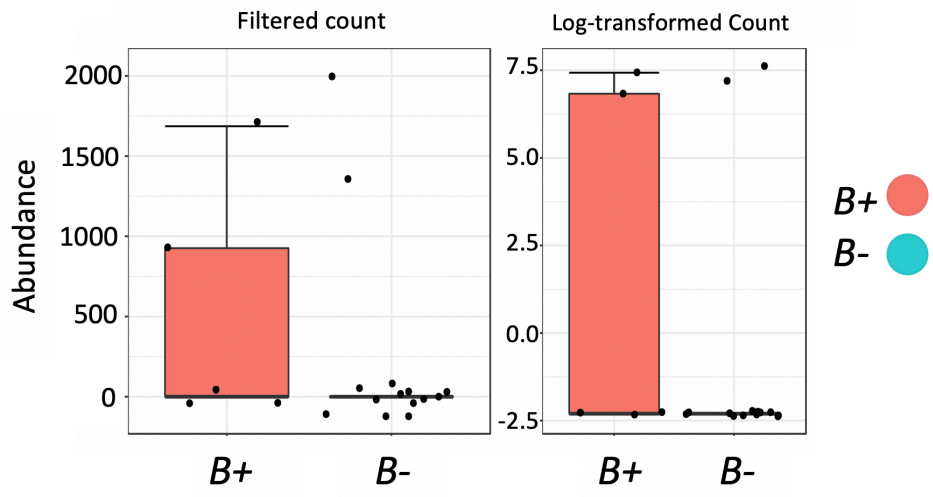

Figure 4.viii Box-plots showing the filtered count and log transformed counts from MetagenomeSeq data of the genus *Anaerocella.* This was the only bacterial taxon positively associated with *Blastocystis* colonisation in the study cohort (p<0.05). Red plots reflect *B+* data, and blue plots represent *B-* data.

Lastly, Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was implemented to investigate community comparisons. This method determines which OTU is most likely to explain the differences between classes by using standard statistical significance tests with additional tests which consider biological consistency and effect relevance. LEfSe using FDR-adjusted data (*p*value cutoff = 0.1) demonstrated that no significant taxa were observed when *B*+ voles were compared against *B*- voles.

3 Discussion

In this study, we investigated the association of *Blastocystis* with faecal bacterial communities from water voles (*Arvicola amphibius)* presenting no gastrointestinal symptoms. Twenty-eight samples were successfully characterised, of which 7/16 voles (44%) were *Blastocystis* positive. Half of the voles were also infected with another protist and were excluded from the cohort to increase our confidence that changes in bacterial abundance were associated with *Blastocystis* colonisation rather than other protozoa or a synergistic effect.

In the majority of the voles, Bacteroidetes and Firmicutes were the two dominant phyla regardless of infection status, reflecting results across other mammalian studies (Ley *et al.* 2008). The Firmicutes/Bacteroidetes percentage here displayed a higher abundance of Bacteroidetes (65%) in comparison to Firmicutes. This abundance generally fits with other vole based studies of captive and wild-captured cohorts (Allan *et al.* 2018), but differs greatly from rats, which have been noted to be distinct from other murine animals as the majority of taxa belong to the Firmicutes and Bacteroidetes have a relatively prevalence of below 10% (Li *et al.* 2017). In general, studies in humans and rodents have shown that Bacteroidetes driven microbiota may be the result of low fat / high fibre diet (Čoklo, Maslov and Kraljević Pavelić 2020; Evans *et al.* 2014; Ferrario *et al.* 2017; Simões *et al.* 2013). The voles in this study were fed a diet rich in fibrous material including fruits, legumes, willow leaves and bark, which likely accounts towards the high abundance of Bacteroidetes here (Curtis *et al.* 2018).

Microbiome data regarding water voles (*Arvicola amphibious*) is non-existent. Nonetheless, a number of studies on other rodents exist including those of the subfamily Arvicolinae. Generally, the core vole microbiota was made up predominantly of Firmicutes and Bacteroidetes with communities diversifying further at the lower taxonomic levels, in accordance with numerous microbiome-based studies on eukaryotic organisms (Allan *et al.* 2018; Bo *et al.* 2019; Curtis *et al.* 2018; Parfrey *et al.* 2014). For example, an increase in Bacilli was observed in wild Amargosa voles, whereas the abundance of Erysipelotrichaceae was higher in those kept in captivity. Comparison of the microbiota of Brandt's voles in cold versus warm environments revealed a change in the Firmicutes to Bacteroidetes ratio. More research investigating the bacterial community abundance and composition in similar species will help establish the community makeup of these small rodents.

Interestingly, one vole (R13, collection 2) displayed an abundance profile that was markedly different from the rest, including the abundance data from first collection a month prior.

These results were consistent throughout the cohort with few exceptions: R13 experienced a dramatic increase in the abundance of Proteobacteria which was below 1% in the first collection yet exceeded 32% of the relative abundance in the second. This was accompanied by a decrease in the average relative Bacteroidetes between collections (relative abundance decrease from approximately 80% to 40%). This vole died from unknown causes on the morning of the second collection, but high abundances of Proteobacteria are often associated with dysbiosis in murine studies, possibly indicating some sort of disruption to homeostasis that could be linked to its death (Carvalho *et al.* 2012; Maharshak *et al.* 2013). This may indicate a use for assessing Proteobacteria as a biomarker for intestinal dysbiosis in captive voles.

The investigation of microbial community abundance differences between *Blastocystis* positive and negative voles was the main focus of this work and results revealed that the overall diversity of the microbiota was not significantly altered in the voles with *Blastocystis* (or in those that underwent drug treatment (Supplementary table 4.i)). Diversity measures were assessed through alpha and beta diversity. The lack of any significant change is surprising as numerous other reports have detailed shifts in microbial diversity following protozoal infection, including *Blastocystis* (McKenney *et al.* 2017; Ras *et al.* 2015; Renelies-Hamilton *et al.* 2019). A plausible explanation for the overall lack of significant data may be due to the small sample size (five *Blastocystis* positive voles).

Shifts in microbiota communities upon anti-microbial drug administration is a well-documented phenomenon (Cho *et al.* 2012; Dudek-Wicher, Junka and Bartoszewicz 2018; Schubert, Sinani and Schloss 2015). In this study fenbendazole and metronidazole were used as antiprotozoal treatments against *Giardia* and treatments had ended by the time of sampling (Metronidazole 14 days prior, Fenbendazole 10 days prior). The administration of these drugs had no overall significant impact on community profiles when compared to untreated voles. Similar recordings have also been made in fenbendazole treated mice and dogs and may be a result of the drug being effective against eukaryotes only (Fujishiro *et al.* 2020; Korte *et al.* 2018). Metronidazole is both an antibiotic and antiprotozoal drug and is cytotoxic to anaerobes (Weir and Le 2020). One could thus speculate that this drug would have a more profound effect on the microbiota, as demonstrated in a number of reports in a range of hosts, including rats (Haak *et al.* 2019; Igarashi *et al.* 2014; Pélissier *et al.* 2010; Pilla *et al.* 2020). However, there was no overall change noted in alpha or beta diversity in

metronidazole treated voles. A possible reason for this is the duration of time since treatment and sampling. The metronidazole treated voles had finished their treatment approximately 10 days before the first sampling meaning that community richness and evenness was possibly restored to a profile indistinguishable to that of the untreated voles, similar events have been recently reported in metronidazole-treated dogs (Pilla *et al.* 2020). It should however be kept in mind that numerous reports document altered gut microbiota after antibiotic treatment that persists for up to a year after administration (Croswell *et al.* 2009; Jernberg *et al.* 2010).

Although the overall microbial community richness was unaltered in voles with *Blastocystis*, closer inspection of community comparisons between positive and negative animals indicate that certain bacterial taxa were associated with positive voles. Numerous reports have documented bacteria induced changes resulting from protozoa / parasite infection, however few focus on overall community richness and evenness and instead investigate changes in specific bacterial taxa (Mammeri *et al.* 2019; Mejia *et al.* 2020; Walk *et al.* 2010; Yason *et al.* 2019). Our results show no association of alpha or beta diversity alterations with *Blastocystis*, but in line with other reports, demonstrate alterations in specific taxa.

Abundance of bacteria belonging to the Spirochaetes phylum differed significantly between *Blastocystis* positive and negative animals. This phylum was observed as part of the core microbiota of the bank vole (*Myodes glareolus*) (Lavrinienko, Tukalenko, Mousseau, *et al.* 2020; Lavrinienko, Tukalenko, Kesäniemi, *et al.* 2020) and is possibly a common constituent of certain vole species (Knowles *et al.* 2019). Within Spirochaetes, *Treponema* was significantly decreased. This genus has been associated with roles in plant material degradation in the rumen (Bekele, Koike and Kobayashi 2011) and is enriched in the gut of humans inhabiting rural areas and wild apes, while it is typically absent in humans with urban diets (Angelakis *et al.* 2018; Bittar *et al.* 2014). Interestingly *Treponema* has also been associated with the vole microbiota (Bo *et al.* 2019). The relative decline in Spirochaetes across *Blastocystis* positive voles may indicate an impaired ability of these voles to degrade fibrous foods, however other common degraders such as *Prevotella*, *Ruminococcus* and *Oscillospira* were not identified as decreased in this dataset, meaning that perhaps a decline in this taxon may not significantly impact the animal. Betaproteobacteria (phylum Proteobacteria) and subsequent related taxa were also significantly decreased in positive voles. Bacteria belonging to the Proteobacteria phylum are generally associated with

dysbiosis and pathogenesis in the gut (Rizzatti *et al.* 2017), the significant decrease in this taxon could therefore not be of detriment to the vole and may in fact aid in the removal of potentially pathogenic or opportunistic bacteria. However, the roles of Betaproteobacteria in the murine gut have not been well characterised, therefore the outcome of this decline warrants further investigation.

A number of genera were significantly decreased in the presence of *Blastocystis*, this included *Kineothrix*, which is a butyrate producer belonging to the family *Lachnospiraceae* (phylum Firmicutes) (Haas and Blanchard 2017). Butyrate is an essential metabolite produced by gut bacteria with notable anti-inflammatory and immunomodulation properties that has been correlated with gut homeostasis (Canani *et al.* 2011). Perhaps the decline of this may contribute to a state of dysbiosis in the gut. Similarly, *Robinsoniella,* which is another member of the *Lachnospiraceae* has been associated with fibre digestion in ruminants, indicating a possible benefit of the taxon in the gut on herbivorous animals like voles (Niu *et al.* 2019). Interestingly, a number of other genera of the Firmicutes lineage were decreased and included *Thermoclostridium, Anaeromassilibacillus* and *Anaerotignum*. Currently most of these have uncharacterised roles in the murine microbiota. Nonetheless, Firmicutes are generally associated with fermentation of dietary fibre and production of short-chain fatty acids, which are a marker of eubiosis in the gut (Simpson and Campbell 2015). A reduction in a number such bacteria may lead to dysbiosis, moreover dysbiosis in the gut may also facilitate their decline. The only significantly increased bacteria was *Anaerocella,* which belongs to the Bacteroidetes phylum. Although the role of this bacteria is currently unknown in the microbiota, the family Rickenellaceae to which it belongs is associated with high-fat diets and aging in humans and mice (Kim *et al.* 2012; Langille *et al.* 2014).

Conflicting reports exist regarding microbiota richness and diversity associated with the presence of *Blastocystis* (Audebert *et al.* 2016; Kodio *et al.* 2019). In mice, its presence was associated with a decrease in *Lactobacillus* and *Bifidobacterium*; both of which are beneficial microbes and are used as probiotics. However, an increase in bacterial richness and decrease in the Firmicutes/Bacteroidetes percentage, in addition to an increase in *Proteobacteria* and *Tenericutes* that was observed in one study conducted on rats (Defaye *et al.* 2020; Yason *et al.* 2019).

Although our results demonstrated no alteration in richness within the gut, the relative shifts in abundance of certain bacterial species may present opportunities for opportunistic

pathogens to proliferate in the altered environment (Bidu *et al.* 2018; Lobionda *et al.* 2019), meaning that although *Blastocystis* likely has no direct pathogenic role here, the indirect associations it may have on other bacterial communities could lead to dysbiosis and disease.

There are several inherent limitations to investigations of the microbiome, especially in a captive sample cohort. The lack of prior knowledge regarding microbial community composition in water voles makes comparisons between wild and captive populations impossible at this time. Therefore, establishing the possibility of assessing differences in bacterial community profile differences with wild voles could not be determined, although similar studies have been carried out in other vole species (Allan *et al.* 2018).

Repeat sampling was carried out one month apart, both in the winter months, meaning that seasonality likely had little impact on the microbiota and this was further confirmed by diversity measures (results not shown). It would be interesting to investigate the impact of seasonal variations on the microbiota which has been observed in other species (Maurice *et al.* 2015). Ideally repeat sampling over a prolonged period would allow for fluctuations in community profiles to be better established, it would also help mitigate the impact of intermittent parasite shedding as well as establish if *Blastocystis* colonisation is long-term and remained asymptomatic.

Molecular characterisation was the primary method of *Blastocystis* detection here (Padukone *et al.* 2018; Roberts *et al.* 2011; Stensvold, Arendrup, *et al.* 2007), however a hallmark of such characterisation studies is that it cannot definitively prove absence – it can only demonstrate presence, and factors such as PCR inhibitors are possible candidates for misidentification. Another commonly encountered problem was the lack of informative data revolving around the differentially abundant bacteria identified herein, meaning their roles in the gut ecosystem and responses to protozoal colonisation cannot be deciphered at this time.

In conclusion, these results provide an insight into the prevalence of *Blastocystis* and its association with bacterial communities present in the gut of captive water voles. We have demonstrated that *Blastocystis* colonisation is associated with shifts in certain bacterial communities; this may possibly be in attempt to establish colonisation or may be the result of the bacterial community response to an invading parasite. Furthermore, it cannot be

discounted that shifts in these communities may lead to a favourable/unfavourable environment for *Blastocystis* colonisation.

The apparent lack of symptoms in the cohort and lack of overall shift in community richness and diversity of positive voles indicates that *Blastocystis* may not be causing a detrimental effect on the microbiota. One could also postulate that the antiprotozoal treatments may be unnecessary if there is no symptomatic infection and no risk of zoonotic transmission. Unfortunately, the anthropogenic effects on the microbiome cannot be fully understood presently. Further investigation into the effects *Blastocystis* and associated microbial profiles across a range of host taxa in captivity and wild populations will hopefully shed light on the roles and implications of protozoal colonisation and resulting impacts this may pose for conservation efforts.

Chapter 5. ^{1}H NMR metabolomic studies of *Blastocystis* colonising the human gut

1 Introduction

A healthy gastrointestinal (GI) tract is composed of trillions of microorganisms including bacteria, viruses, fungi and protozoa that play complex roles in gut homeostasis and host health. These include immune system modulation, protection against disease and aiding in nutrient absorption and synthesis (Basolo *et al.* 2020; Kho and Lal 2018; Rowland *et al.* 2018; Wu and Wu 2012). When the balance is disrupted, states of dysbiosis can lead to gastrointestinal and systemic disease with evidence supporting roles in obesity, diabetes, inflammatory bowel disease (IBD) and autoimmune disease (Kho and Lal 2018).

The development in high-throughput characterisation techniques has accounted for a greater understanding of the composition of the gut microbiota, yet there is still uncertainty regarding the roles of protozoal colonisers in the gut. When intestinal protozoa are identified they are usually considered detrimental to homeostasis and eliminated. It has been demonstrated that some microbial eukaryotes can act as 'ecosystem engineers' and have the ability to modulate host immune responses and the gut environment in addition to disrupting bacterial communities which results in a disturbance in gut homeostasis (Burgess *et al.* 2017; Hicks *et al.* 2000; Jenkins *et al.* 2018; Leung, Graham and Knowles 2018). There is evidence of the contrary; where colonisation with certain 'pathogens' has been linked to increased microbial diversity in the gut and positive gut health (Burgess *et al.* 2017; Chabé, Lokmer and Ségurel 2017). One such questionable parasite is *Blastocystis,* once in the host it can persist in the large intestine for prolonged periods of time and has been linked to gastrointestinal disorders including Irritable Bowel Syndrome (IBS) and IBD (Boorom *et al.* 2008; Mohamed *et al.* 2017; Stark *et al.* 2007; Taşova *et al.* 2000; Yakoob *et al.* 2004). However, asymptomatic colonisation is also often reported and some recent reports have even linked it to increased bacterial diversity and gut health, contradicting its role as a pathogen (Audebert *et al.* 2016; Nieves-Ramírez *et al.* 2018; Burgess *et al.* 2017). Furthermore, *Blastocystis* lacks many mechanisms commonly associated with pathogenicity, such as lectins and rhoptries (Gentekaki *et al.* 2017). Yet has been found to illicit immunomodulatory effects; including the degradation of the IgA antibody (Puthia, Vaithilingam, Lu and Kevin S.W. Tan 2005), inhibition of nitric oxide synthase (Mirza, Wu, *et al.* 2011) and the degradation of tight junction proteins, thereby increasing intestinal permeability (Mirza *et al.* 2012; Puthia, Selena W.S. Sio, *et al.* 2006). This has resulted in its pathogenicity being hotly disputed with some believing *Blastocystis* to be a mutualist

member of the gut microbiota (Andersen *et al.* 2015; Stensvold and Clark 2016; Yason *et al.* 2019).

While numerous reports document the co-habitation of *Blastocystis* with other members of the microbiota, investigations of functional roles and phenotypic products associated with *Blastocystis* are currently limited. 1D ^{1}H Nuclear magnetic resonance (NMR) is a quantitative and reproducible metabolite detection method which has been used to analyse the metabolomes of many bacterial (Li *et al.* 2005), mammalian (Bruno *et al.* 2018), plant (Turbitt *et al.* 2019), fungal (Peng *et al.* 2018) and protozoan (Miller *et al.* 2019; Vermathen *et al.* 2018) cells. NMR based metabolomics of faecal samples provides a non-invasive, non-targeted, high-throughput approach to examine the gastrointestinal tract (GI) via metabolite profiles that reflect the physiological processes occurring in the gut. This can provide indications of gut homeostasis by monitoring pathway intermediates, amino acids and disease biomarkers (Deda *et al.* 2017; Kang *et al.* 2018; Zhgun and Ilina 2020). In recent years, there has been a marked increase in the use of metabolomic based studies for exploring host parasite interactions (Bond *et al.* 2015; Hublin *et al.* 2012; Hublin *et al.* 2013; Miller *et al.* 2019), as well as aiding research into chemotherapeutics (Allman *et al.* 2016; Jeelani *et al.* 2012) and linking molecular compositions of stool with the microbiota to further our understanding of the gut ecosystem (Baldassarre 2018).

Herein we used NMR metabolomics to investigate the metabolic profiling of *Blastocystis* colonisation in humans compared from two different countries (Turkey and South Korea).

Results demonstrated *Blastocystis* colonisation causes significant alterations in the metabolome of infected humans. Here, numerous metabolites were associated with infection and pathway analysis further revealed metabolic pathways that may too be related with infection. Taken together, this metabolomics based study of *Blastocystis* highlights the importance of investigating this protozoan within the gut ecosystem and can help unravel the mystery of its questionable pathogenicity.

2 Results

In order to assess variations in metabolic profiles from *Blastocystis* infected and uninfected individuals, we prepared and analysed a total of 111 faecal samples from two countries. 71 faecal samples from Turkey (kindly provided by Prof. Funda Dogruman AL) and 40 samples from South Korea (kindly provided by Dr. Eun Jeong Won) were submitted for metabolite extraction and profiling (Supplementary table 5.i). Of the 71 samples from Turkey [55 *Blastocystis* positive (*B*+), 16 *Blastocystis* negative (*B*-)], 69 were successfully extracted and screened for metabolites (53 *B*+, 16 *B*-). Additionally, 40/40 South Korea samples were successfully analysed (20 *B*+, 20 *B*-).

2.1 Metabolite profiles from *B+* and *B*- individuals

^{1}H NMR metabolomics of *Blastocystis* positive and *Blastocystis* negative humans were compared against a reference database of 338 compounds from the Chenomx database which identified a total of 317 and 283 metabolites from the Turkish and South Korean cohort respectively. Supplementary figure 5.i shows an example of the NMR spectra from *B*+ and *B*- individuals from South Korea.

Initial analysis revealed ethanol to be a possible outlier in the Turkish cohort (Supplementary figure 5.ii), the compound concentrations reflected a high concentration in the majority of faecal samples and was likely an artefact of inadequate sample drying during the extraction protocol. This metabolite was subsequently removed from further analysis to reduce skewing of data that represents the metabolites excreted from the gut. All subsequent analysis included the modified dataset which excluded ethanol from the Turkey cohort.

Univariate analysis highlighted a total of 98 significant metabolites from the Turkey cohort that were above the fold change threshold of 2 (Supplementary Table 5.ii); 66 metabolites were significantly increased in *B*+ samples and 32 were decreased in the *B*+ samples compared against *B*- controls.

Univariate analysis from the South Korean cohort revealed a total of 57 significant metabolites (Supplementary Table 5.iii); here 44 were significantly increased in *B*+ samples and the remaining 13 were significantly decreased in *B*+ samples compared against *B*- controls.

Of the 98 metabolites from Turkey and 66 metabolites from South Korea, a total of 24 of these were identified across both cohorts and are listed below (Table 5.i). Here 83% (20/24)

followed the same pattern of distinction across both countries. The remaining 17% (4/24) significant metabolites had opposing results between the cohorts. These differentially identified metabolites are highlighted in orange and to summarise: Benzoate, Methylamine and Pyroglutamate were increased in the Turkey samples but significantly decreased in South Korean samples, trans-Aconitate was significantly increased in the South Korean samples but decreased in those from Turkey.

Table 5.i. Significant metabolites identified across both sample cohorts. Those in green represent metabolites that were significantly increased in *B+* samples as identified by univariate analysis. Those in orange were significantly decreased in the *B+* samples when compared against *B-*. 4/24 metabolites had different expression results between cohorts.

Turkey	South Korea
2-Oxocaproate	2-Oxocaproate
3-Hydroxykynurenine	3-Hydroxykynurenine
3-Phenylpropionate	3-Phenylpropionate
Benzoate	Benzoate
Caprate	Caprate
Caprylate	Caprylate
Dimethylamine	Dimethylamine
Ethylene glycol	Ethylene glycol
Glycerol	Glycerol
Isopropanol	Isopropanol
Isovalerate	Isovalerate
Mandelate	Mandelate
Methanol	Methanol
Methylamine	Methylamine
Methylsuccinate	Methylsuccinate
Niacinamide	Niacinamide
Pyroglutamate	Pyroglutamate
Sebacate	Sebacate
Suberate	Suberate
Sucrose	Sucrose
trans-Aconitate	trans-Aconitate
Trimethylamine N-oxide	Trimethylamine N-oxide
Valerate	Valerate
Valine	Valine

Unsupervised principal component analysis (PCA) of the normalized metabolite concentrations indicated distinct clustering of *B*+ against *B*- samples. Analysis revealed that *B*+ samples demonstrated a degree of separation from *B*- (Figure 5.i) for Turkey and South Korea (Figure 5.ii).

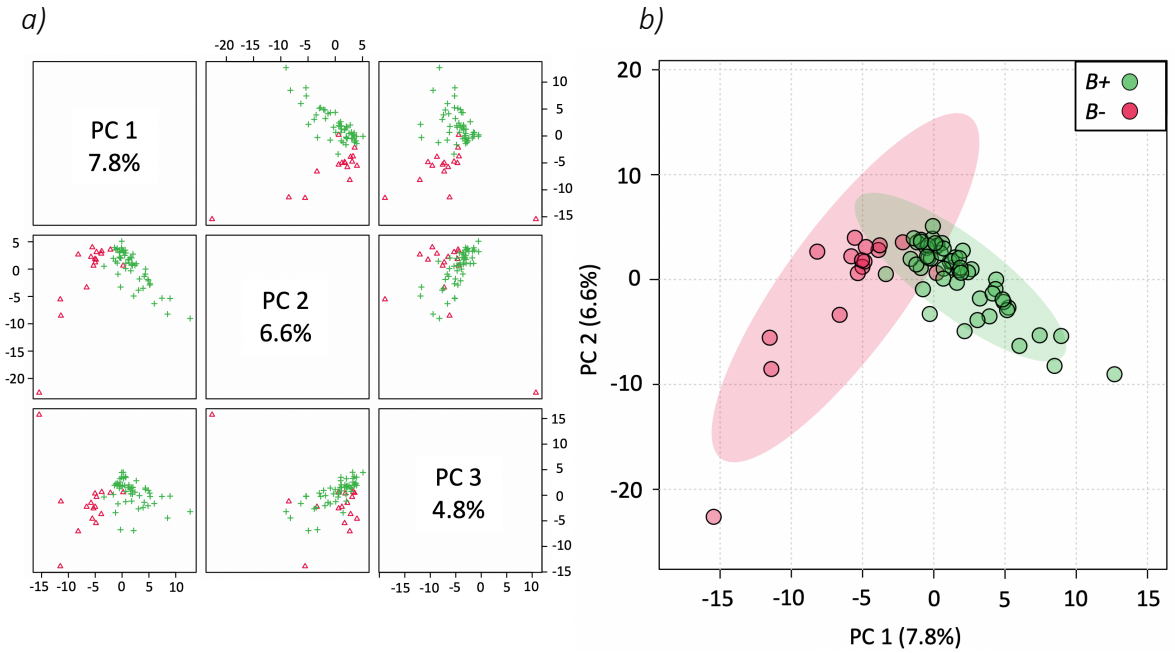

Figure 5.i Principal component analysis and 2Dscores plot of *B*+ and *B*- faecal metabolite profiles from the Turkey cohort. *a)* Pairwise score plots between selected principal components (PC) with the variance explained in each diagonal cell. The pairwise score plot for the top three PCs are displayed and *B*+ samples are represented as green crosses, *B*- samples are red triangles. *b)* shows the 2D score scatter plot based on components 1 and 2. Variances are shown in parenthesis on the axis. The shaded areas indicate the 95% confidence ellipse regions based on the data points for groups. *B*+ are shown in green and *B*- in red.

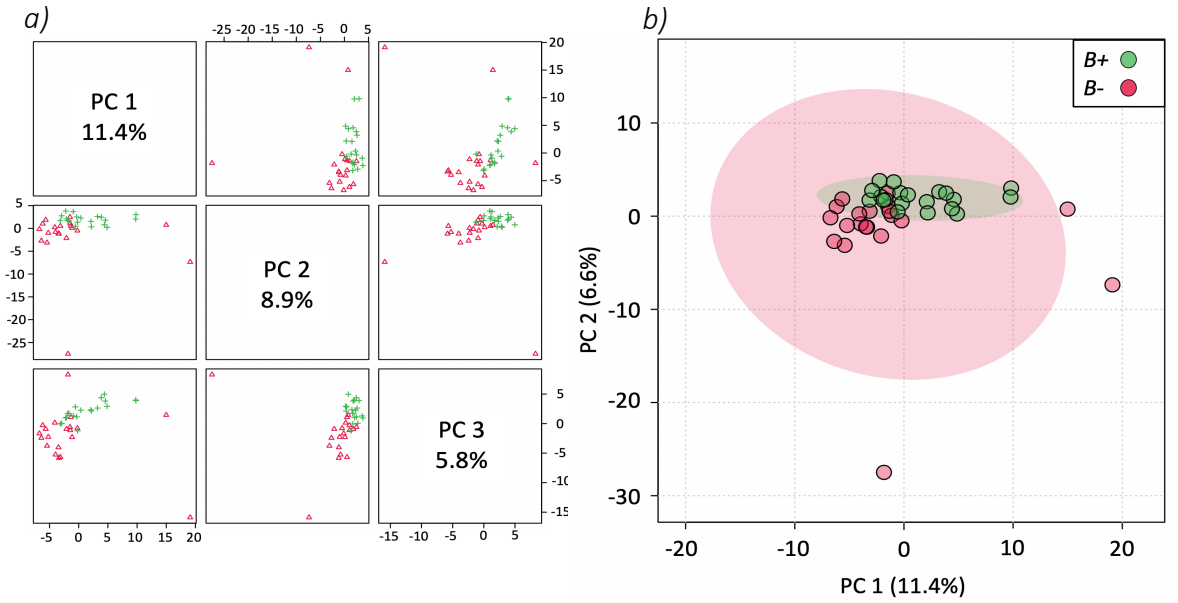

Figure 5.ii Principal component analysis and 2Dscores plot of *B*+ and *B*- faecal metabolite profiles from the South Korea cohort. *a)* Pairwise score plots between selected principal components (PC) with the variance explained in each diagonal cell. The pairwise score plot for the top three PCs are displayed and *B*+ samples are represented as green crosses, *B*- samples are red triangles. *b)* shows the 2D score scatter plot based on components 1 and 2. Variances are shown in parenthesis on the axis. The shaded areas indicate the 95% confidence ellipse regions based on the data points for groups. *B*+ are shown in green and *B*- in red.

The variance between *B*+ and *B*- samples was further examined by way of supervised analysis with Partial least squares discriminant analysis (PLS-DA) which divided groups into infected (*B*+) and uninfected (*B*-). Results were cross-validated using Leave-One-Out Cross-Validation (LOOCV) (Supplementary figure 5.iii). PLS-DA maximises the covariance between the data and group and the variance is explained in Figure 5.iii and Figure 5.iv, which represent Turkey and South Korea respectively.

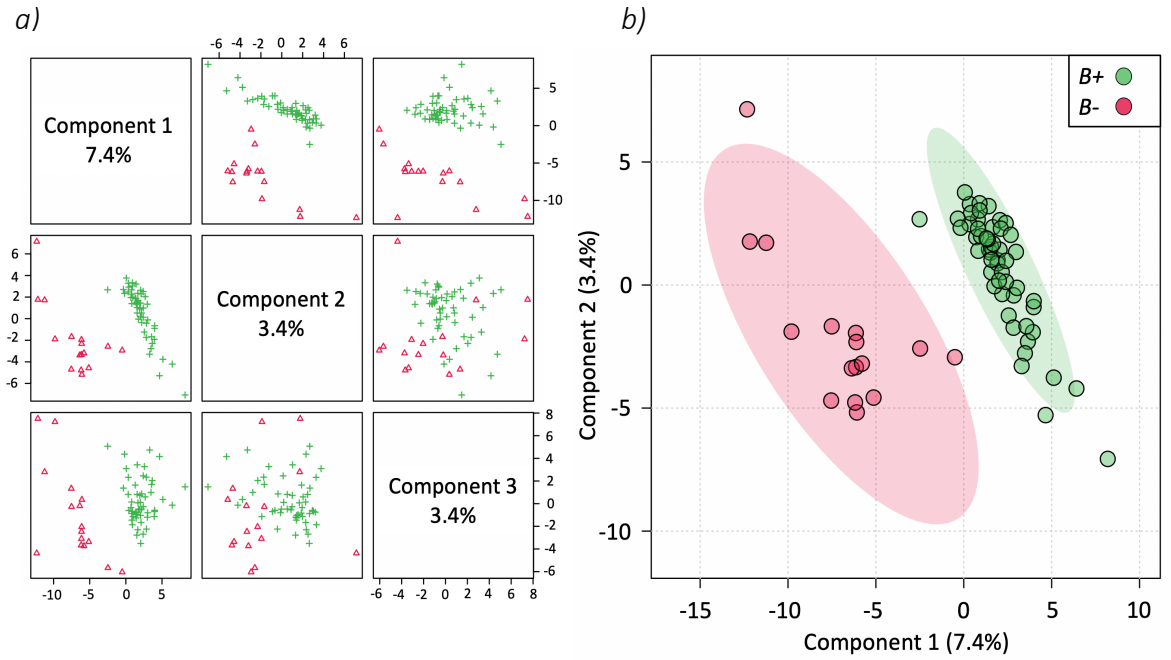

Figure 5.iii Partial Least Squares Discriminant Analysis (PLS-DA) plot and 2D scores plot of *B*+ and *B*- faecal metabolite profiles from the Turkey cohort. *a)* PLS-DA scores plot showing the pairwise score plot for the top three components. *B*+ samples are represented as green crosses, *B*- samples are red triangles. *b)* shows the 2D score scatter plot based on components 1 and 2 from the PLS-DA plot. Variances are shown in parenthesis on the axis. The shaded areas indicate the 95% confidence ellipse regions based on the data points for groups. *B*+ are shown in green and *B*- in red. B+ are shown in green and B- in red.

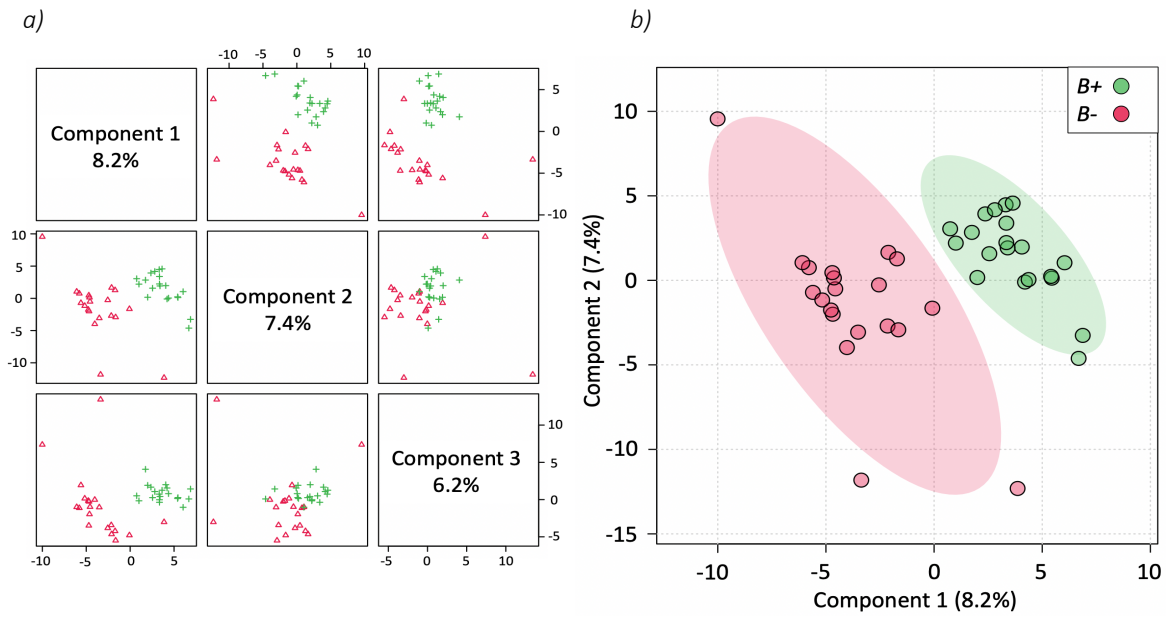

Figure 5.iv Partial Least Squares Discriminant Analysis (PLS-DA) plot and 2D scores plot of *B*+ and *B*- faecal metabolite profiles from the South Korean cohort. *a)* PLS-DA scores plot showing the pairwise score plot for the top three components. *B*+ samples are represented as green crosses, *B*- samples are red triangles. *b)* shows the 2D score scatter plot based on components 1 and 2 from the PLS-DA plot. Variances are shown in parenthesis on the axis. The shaded areas indicate the 95% confidence ellipse regions based on the data points for groups. *B*+ are shown in green and *B*- in red.

Identified metabolites were further ranked by the PLS-DA Variable Importance in Projection (VIP) score (cut off at a score of 2). Here up to and including the top 15 ranked significant metabolites were identified and their relative concentrations in *B*+ and *B*- samples indicated (Figure 5.v).

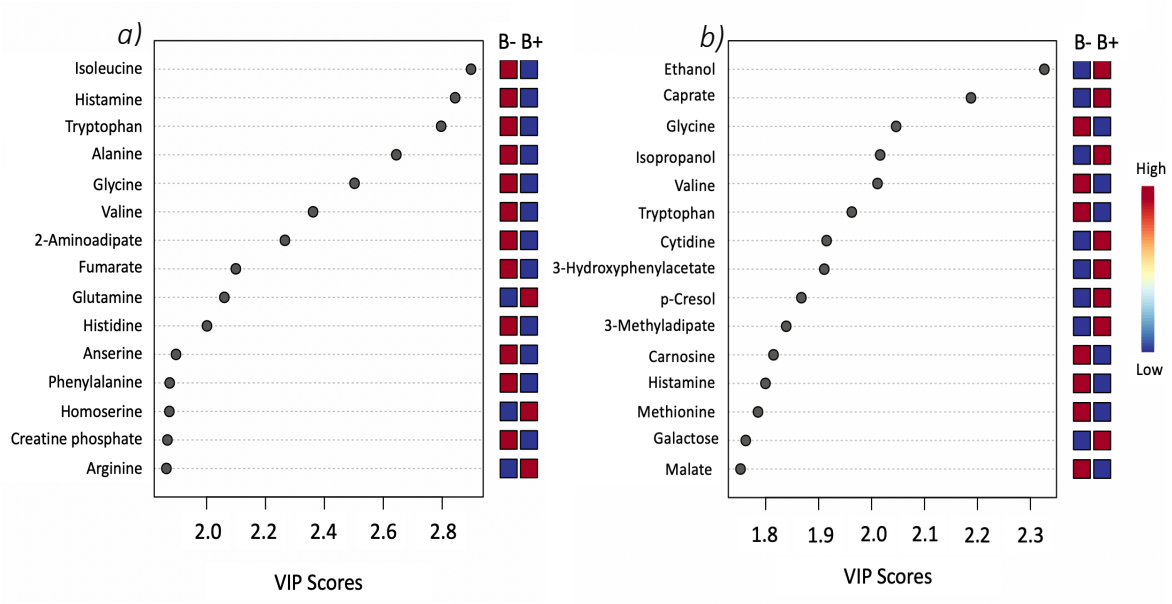

Figure 5.v A VIP score plot summarizing the 15 most important metabolites determined by the PLS-DA plot. The x-axis indicates the VIP score corresponding to significant metabolites on the y-axis. The coloured boxes on the right represent the relative concentration of the metabolite for *B*+ (infected) or *B*-(control) samples. *a)* VIP score plot comparison *B*+ and *B*- from the Turkey cohort. *b)* VIP score plot comparison *B*+ and *B*- from the South Korean cohort.

2.2 Metabolic profile and pathway analysis of *Blastocystis* infected individuals

Significant metabolites represent compounds that were altered in the presence or absence of *Blastocystis* in the gut (it should be noted that the alteration may not necessarily be directly due to its presence). These metabolites were subjected to further analysis by way of pathway analysis. This was performed to identify possible pathways that were significantly altered in *B*+ and *B*- data and are based on adjusted *p*-values from pathway enrichment analysis procedures. A total of 53 metabolic pathways for Turkey were relevant in distinguishing *B*+ and *B*- samples, of these 32 had an adjusted *p*-value <0.1. These are listed in Table 5.ii and correspond to Figure 5.vi, which provides an illustration of the metabolome with pathways that matched to metabolites from *B*+ and *B*- as circles. All pathways were incomplete except the Neomycin, kanamycin and gentamicin biosynthesis pathway which consisted of two metabolite hits (D- Glucose and D-Glucose 6-Phosphate). Pathway analysis was also carried out for South Korean samples, a total of 55 pathways were identified, and of these six had an adjusted *p*-value <0.1 (Table 5.iii and Figure 5.vii).

Table 5.ii Details of pathway analysis for the Turkey cohort, only values with *p*-adjust <0.1 are included. The 'Total' column equates to the total number of compounds in the given pathway and the 'Hits' are compounds from this dataset that match to a given pathway. The *p*-adjust values are adjusted Raw *p*-values following the Holm-Bonferroni method. FDR is false discovery rate adjusted *p*-value. The 'Impact' is a value calculated from pathway topology analysis and is a combination of centrality and enrichment results calculated by the addition of importance measures for metabolites which are divided by the sum of importance measures of all metabolites in a pathway. Pathway impact analysis is calculated using MetPa (Xia and Wishart 2010).

Pathway Name	Total	Hits	Raw p	-LOG10(p)	p- adjust	FDR	Impact
Aminoacyl-tRNA biosynthesis	48	20	1.75E-10	9.7564	9.29E-09	9.29E-09	0.167
Glycine, serine and threonine metabolism	33	16	2.03E-09	8.6916	1.06E-07	5.00E-08	0.775
Alanine, aspartate and glutamate metabolism	28	11	2.83E-09	8.5479	1.44E-07	5.00E-08	0.623
Histidine metabolism	16	8	2.83E-08	7.5486	1.41E-06	3.30E-07	0.672
Selenocompound metabolism	20	1	3.11E-08	7.5068	1.53E-06	3.30E-07	0.000
Primary bile acid biosynthesis	46	4	8.35E-08	7.0781	4.01E-06	7.38E-07	0.023
Pantothenate and CoA biosynthesis	19	6	2.84E-07	6.5461	1.34E-05	1.97E-06	0.029
Lysine degradation	25	5	3.30E-07	6.4821	1.52E-05	1.97E-06	0.188
Glyoxylate and dicarboxylate metabolism	32	13	3.35E-07	6.4755	1.52E-05	1.97E-06	0.362
Arginine biosynthesis	14	10	4.37E-07	6.3599	1.92E-05	2.31E-06	0.482
beta-Alanine metabolism	21	7	4.82E-07	6.3169	2.07E-05	2.32E-06	0.455
Tyrosine metabolism	42	11	1.01E-06	5.9951	4.25E-05	4.40E-06	0.204
Arginine and proline metabolism	38	11	1.08E-06	5.9671	4.42E-05	4.40E-06	0.443
Valine, leucine and isoleucine biosynthesis	8	6	2.44E-06	5.6118	9.78E-05	9.25E-06	0.000
Glutathione metabolism	28	6	3.60E-06	5.4433	1.41E-04	1.27E-05	0.375
Pyrimidine metabolism	39	7	4.26E-06	5.371	1.62E-04	1.41E-05	0.210
Valine, leucine and isoleucine degradation	40	7	4.93E-06	5.3068	1.83E-04	1.54E-05	0.061
Porphyrin and chlorophyll metabolism	30	3	5.31E-06	5.2748	1.91E-04	1.56E-05	0.028
Sphingolipid metabolism	21	2	1.60E-05	4.7971	5.58E-04	4.45E-05	0.014
Tryptophan metabolism	41	9	2.95E-05	4.5309	1.00E-03	7.80E-05	0.555
Pyruvate metabolism	22	5	5.34E-05	4.2726	1.76E-03	1.35E-04	0.383
D-Glutamine and D-glutamate metabolism	6	2	1.37E-04	3.8626	4.39E-03	3.16E-04	0.500
Nitrogen metabolism	6	2	1.37E-04	3.8626	4.39E-03	3.16E-04	0.000
Cysteine and methionine metabolism	33	9	1.44E-04	3.8426	4.39E-03	3.17E-04	0.502
Phenylalanine metabolism	10	5	2.86E-04	3.5438	8.29E-03	6.00E-04	0.357
Biotin metabolism	10	2	2.94E-04	3.5312	8.29E-03	6.00E-04	0.200
Propanoate metabolism	23	5	4.79E-04	3.3195	1.29E-02	9.41E-04	0.041
Glycerophospholipid metabolism	36	5	6.01E-04	3.2215	1.56E-02	1.14E-03	0.073
Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	7.60E-04	3.119	1.90E-02	1.39E-03	1.000

Citrate cycle (TCA cycle)	20	7	9.00E-04	3.046	2.16E-02	1.59E-03	0.338
Neomycin, kanamycin and gentamicin biosynthesis	2	2	3.44E-03	2.463	7.92E-02	5.71E-03	0.000
Purine metabolism	65	11	3.45E-03	2.4622	7.92E-02	5.71E-03	0.055

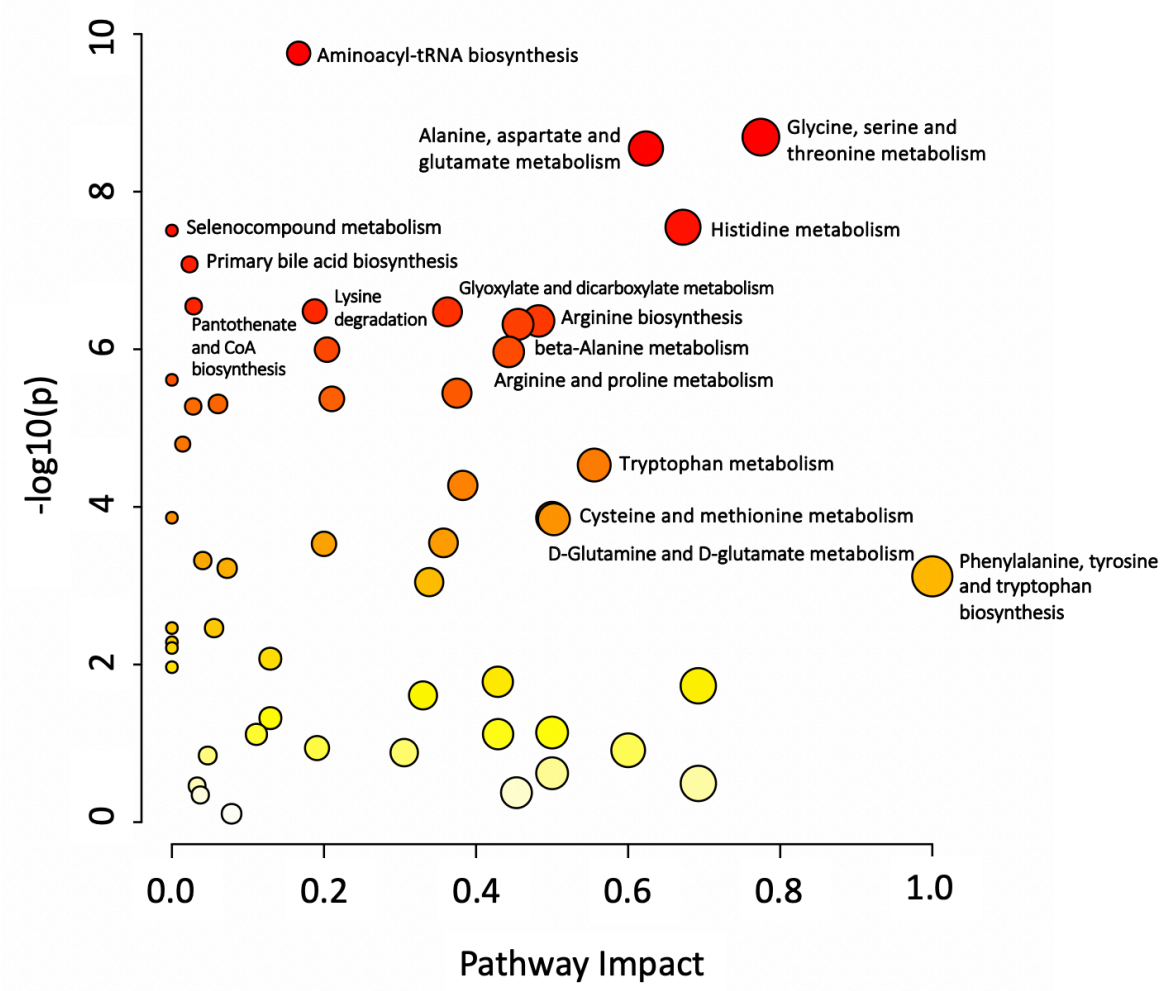

Figure 5.vi Metabolome view of pathway analysis based on pathways that involve significant metabolites from *B*+ and *B*-samples from Turkey. Pathway and library information was supplied from KEGG based on *Homo sapiens* with pathway information up to date from October 2019. The colour and size of circles are a representation of the *p*-value (colour) and the pathway impact value (circle size). Darker coloured circles (Red) represent more statistically significant findings and the transition to yellow follows the decline in significance. Larger circles have an increased pathway impact. Metabolites in the top right-hand corner are significantly changed and are likely to have an impact on the pathway. Labelled pathways include the top 10 with a *p*-adjusted <0.1 and the top ten pathway impact values.

Table 5.iii Details of pathway analysis for the South Korean cohort, only values with *p*-adjust <0.1 are included. The 'Total' column equates to the total number of compounds in the given pathway and the 'Hits' are compounds from this dataset that match to a given pathway. The *p*-adjust values are adjusted Raw *p*-values following the Holm-Bonferroni method. FDR is false discovery rate adjusted *p*-value and the Impact is the impact value was calculated from pathway topology analysis.

Pathway Name	Total	Hits	Raw p	-LOG10(p)	p- adjust	FDR	Impact
Nicotinate and nicotinamide metabolism	15	5	3.93E-04	3.4060	2.12E-02	1.07E-02	0.332
Histidine metabolism	16	8	5.91E-04	3.2281	3.13E-02	1.07E-02	0.672
Glycerophospholipid metabolism	36	4	7.11E-04	3.1484	3.70E-02	1.07E-02	0.083
Valine, leucine and isoleucine biosynthesis	8	4	7.89E-04	3.1030	4.02E-02	1.07E-02	0.000
Aminoacyl-tRNA biosynthesis	48	20	1.11E-03	2.9541	5.56E-02	1.20E-02	0.167
Glycolysis / Gluconeogenesis	26	4	1.84E-03	2.7345	9.03E-02	1.66E-02	0.215

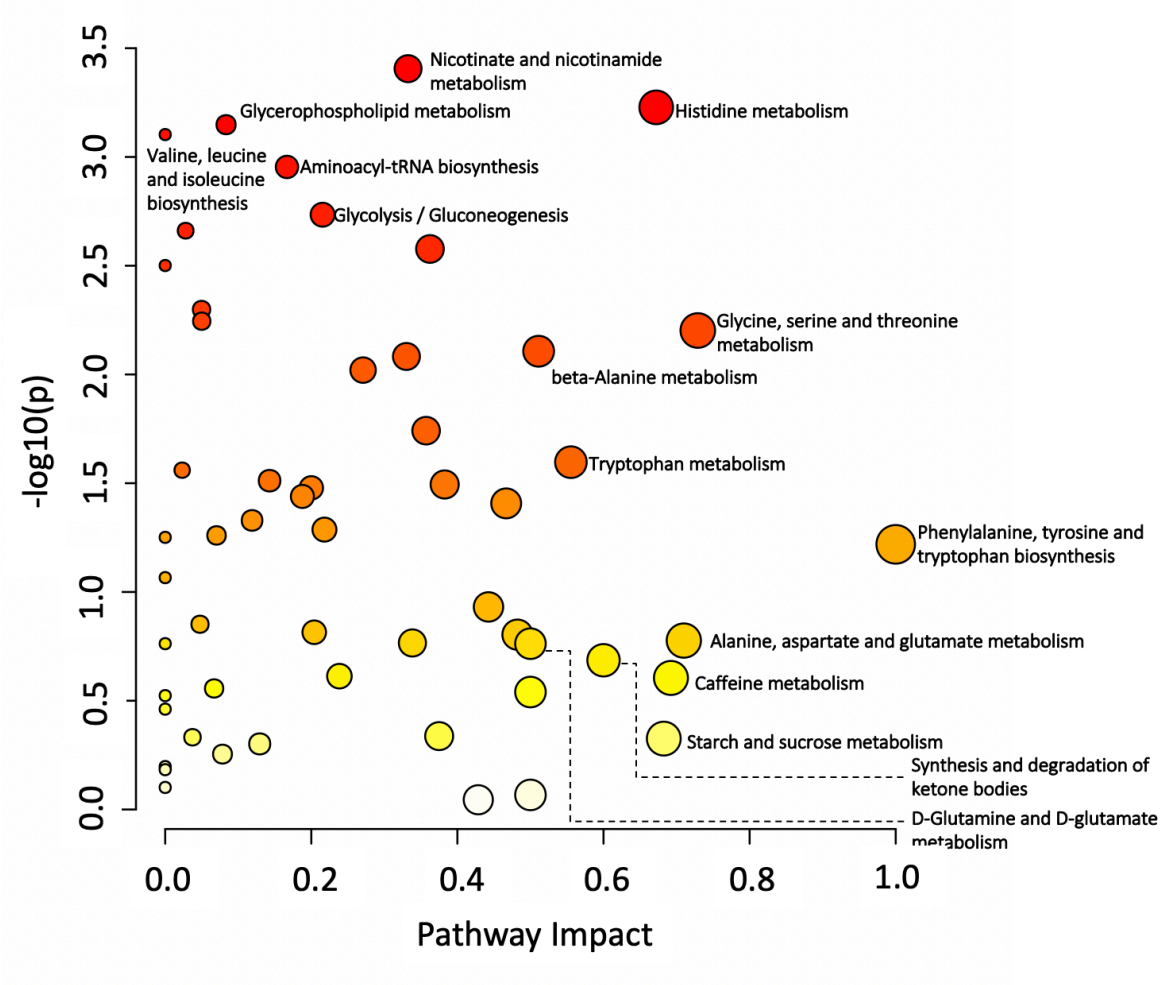

Figure 5.vii Metabolome view of pathway analysis based on pathways that involve significant metabolites from *B*+ and *B*-samples from South Korea. Pathway and library information was supplied from KEGG based on *Homo sapiens* with pathway information up to date from October 2019. The colour and size of circles are a representation of the *p*-value (colour) and the pathway impact value (circle size). Darker coloured circles (Red) represent more statistically significant findings and the transition to yellow follows the decline in significance. Larger circles have an increased pathway impact. Metabolites in the top right-hand corner are significantly changed and are likely to have an impact on the pathway. Labelled pathways include the top 10 with a *p*-adjusted <0.1 and the top ten pathway impact values.

A comparison of distinguished pathways between countries revealed two that were common across both sets of data (*p*-adjust <0.1), and included Histidine metabolism and Aminoacyl-tRNA biosynthesis.

Like most other identified pathways, these too were incomplete, however both displayed similar patterns in significant compounds. Figure 5.viii provides an illustrated view of the histidine metabolism pathway, all identified compounds are coloured according to their association with this dataset.

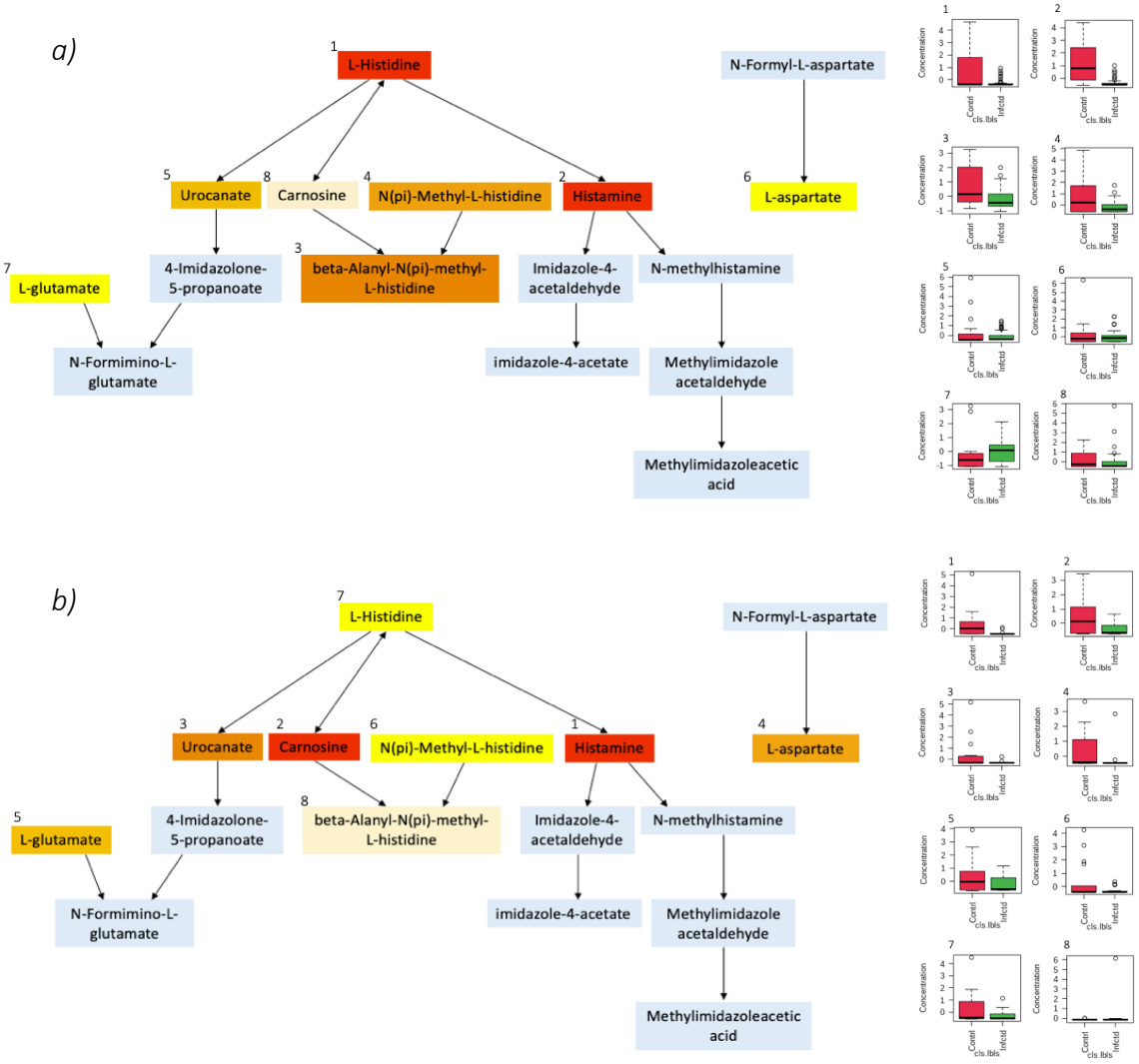

Figure 5.viii A schematic view of the histidine pathway which was one of two distinguished pathways between both study cohorts. *a)* shows the pathway map for the significant metabolites identified form the Turkey cohort, the box plots on the top right represent the statistical findings correlating to this pathway; plots 1-4 had a *p*-value of <0.05, the remaining 5-8 had a *p*-value of <0.1. *b)* shows the pathway map for the significant metabolites identified from the South Korea sample set, the box plots on the bottom right represent the statistical findings correlations to the compounds in this pathway. Box plots 1-2 had a *p*-value of <0.05, the remaining 3-8 had a *p*-value of <0.1.

Light blue squares represents the metabolites were not detected in the data, the remaining colour scale of red - orange - yellow represents the identified metabolites and their statistical significance. With red being metabolites with the lowest *p*-values, as the value gets closer to and above 0.05 the colour shifts to orange and yellow.

Similarly, the Aminoacyl-tRNA biosynthesis pathway was identified in both sample sets, the incomplete pathway consisted only of amino acids and no other pathway components were identified. From the Turkey cohort, most amino acids were significantly decreased in the infected group (11/17) and included L-histidine, L-phenylalanine, glycine, L-methionine, L-valine, L-alanine, L-leucine, L-tyrosine, L-threonine, L-tryptophan and L-proline, the remaining 6 were increased in infected samples (L-asparagine, L-arginine, L-glutamine, L-cysteine, L-serine and L-lysine) (p<0.05). Likewise, from the South Korean cohort 5/6 amino acids were decreased in the *B*+ group and included glycine, L-methionine, L-valine, L-tryptophan, L-threonine, only L-leucine was increased in *B*+ samples (p<0.05).

3 Discussion

To our knowledge, this is the first report of a metabolomics based study being carried out on *Blastocystis* in humans. The focus here was to assess metabolite profiles from infected and non-infected individuals from two different countries and explore possible roles *Blastocystis* may have on influencing the metabolome by using ^{1}H NMR spectroscopy (Emwas *et al.* 2019). Findings revealed a distinct variation in the metabolome of infected individuals compared against uninfected controls, with a number of distinguishable metabolites found between the groups. In addition to investigating metabolites individually, metabolites were mapped to associated metabolic pathways, this revealed several pathways that are possibly associated with *Blastocystis* infection.

3.1 Metabolite profiles of infected humans

Overall, a total of 98 and 57 significant metabolites were associated with *Blastocystis* infection in the Turkey and South Korean sample sets respectively. Of these, a number were positively correlated with the presence of *Blastocystis,* whilst a smaller proportion were decreased in its presence. Between the two countries, a total of 24 compounds were common to both, the majority of which were associated with diet intake and bacteria metabolism. A number of these compounds are poorly studied and their roles in the gut are currently unknown, making associations between their relative change in *Blastocystis* difficult to determine (Zhgun and Ilina 2020).

Of the identified metabolites that were increased, a number of these are usually obtained through diet and absorbed via the intestine (Oliphant and Allen-Vercoe 2019; Yang *et al.* 2012). Therefore, perhaps an increase in the concentration of these compounds in infected individuals is a result of them not being efficiently absorbed; *Blastocystis* has been previously linked to increased intestinal permutability and inflammation (Poirier *et al.* 2012) and this may impact intestinal function and the absorption of metabolites (Lennernäs 2007). However, it should be noted that there was an overall lack of other metabolites typically associated with malabsorption including an increase in bile acids, amino acids and sphingolipids, as well as a significant decrease in Short-Chain Fatty Acids (Franzosa *et al.* 2019; Lavelle and Sokol 2020; Marchesi *et al.* 2007). Furthermore, other dysbiosis-associated compounds such as volatile organic compounds (Zhgun and Ilina 2020; Ahmed *et al.* 2013) and inflammatory metabolites like histamine, where absent or decreased in

infected individuals (Smolinska *et al.* 2014), however some of these may be impacted by the extraction protocol.

Another possible explanation is the association of *Blastocystis* with bacterial communities that contribute to the production of these metabolites, for example 3-Phenylpropionate, isovalerate and Trimethylamine N-oxide. (Oliphant and Allen-Vercoe 2019; Pallister *et al.* 2017; Rios-Covian *et al.* 2020).

Trimethylamine N-oxide presents an interesting case. This metabolite is derived from trimethylamine, which is synthesised by bacteria in the gut and then taken up by the intestine, where it is subsequently oxidised in the liver forming Trimethylamine N-oxide. Trimethylamine N-oxide has been implicated in cardiovascular disease pathogenesis in a number of studies (Brown and Hazen 2015; Chen *et al.* 2017; Liu and Dai 2020). Trimethylamine was identified in low concentrations across both cohorts but no statistical significance between uninfected and infected individuals was found; however, Trimethylamine N-oxide was significantly decreased in both cohorts. Trimethylamine N-oxide excretion in the urine is often associated with diets high in protein. By taking this into account, a significant decline of this metabolite in *Blastocystis* positive individuals may actually be a result of a diet low in protein; which in turn may favour *Blastocystis* colonisation (Forsell *et al.* 2017; Rasmussen *et al.* 2012). This emphasises the complexity of the gut, and demonstrates the importance of considering the wider context of the gut environment (e.g. the metabolome). Unfortunately, the diet of study cohorts was not obtained and thus these interpretations are hypothetical.

Comparing the metabolome of infected and uninfected individuals by unsupervised methods demonstrated that variance exists between the two groups (Figure 5.i and 5.ii). Within the infected individuals, there was an observed loose 'clustering' of these samples possibly indicating some overall influence on the metabolome of infected individuals that creates a degree of homogeneity between samples which is not so much observed in the uninfected.

An inherent issue with faecal metabolomics is the non-homogeneity of faecal samples, unlike other bodily fluids such as blood and Cerebrospinal fluid. The faecal metabolome is influenced by numerous factors including diet and demographic, and the impact of sampling from different countries was minimised by keeping the two countries separate (Smith *et al.* 2020). Even with such disparity in samples, there was still a distinction between infected and

noninfected individuals indicating some response to infection that is similar through all infected samples.

This observation was further demonstrated by the South Korean cohort results which indicates a large range of variability in uninfected samples, and clustering of the infected samples (Figure 5.ii). It should be noted here that the infected group falls into the 95% confidence ellipse of the uninfected samples. However, all of the uninfected individuals used in this cohort had been admitted to hospital for a range of pathological conditions (Kim *et al.* 2020) meaning the scope of variance within the *B*- control group was likely very large.

The supervised PLS-DA method aided in separation of the groups and demonstrated a further significant degree of dissimilarity between the two groups, here the 95% confidence ellipse regions did not overlap, and the possibility of overfitting was eliminated by using LOOCV analysis (Supplementary figure 5.iii) meaning this data can be interpreted with confidence.

3.1.1 Variable Importance in Projection

Variable Importance in Projection (VIP) was used to identify distinguished metabolites between *B*+ and *B*- and the results here matched significant metabolites identified by univariate analysis. Among those identified from the Turkey cohort, the majority were low in *B*+ compared to *B*-. Figure 5.v shows metabolites with a VIP score of 2 and above. Interestingly, essential and non-essential amino acids contributed to the majority of variance between the groups. Isoleucine, tryptophan, anserine, alanine, glycine, histidine, phenylalanine and valine were all significantly higher in uninfected (*B*-) individuals, similar findings were also observed in mice infected with *Cryptosporidium* (Hublin *et al.* 2013; Miller *et al.* 2019) and *Entamoeba invadens* during encystation (Jeelani *et al.* 2012). Differences in amino acid concentrations were also found in a study conducted on *Cryptosporidium* in humans but this study reported the converse, with a higher abundance of amino acids in infected individuals, which was likely due to malabsorption in the intestine (Hublin *et al.* 2012). Only a few amino acids were increased in infected samples and included glutamine, L-arginine and homoserine. These may be unaffected by the presence of *Blastocystis* due to its potential ability to synthesise such amino acids, pathways for *de novo* amino acid biosynthesis have been reported for some metabolites and it is proposed that glutamine can be produced from conversion of other amino acids. However, the presence of all of these pathways need further clarification (Gentekaki *et al.* 2017). The lack of identified pathways for the *de novo* biosynthesis of other amino acids may account for the decline in their

concentration in infected individuals as *Blastocystis* may be scavenging them from the gut environment, or may be the result of gut bacteria scavenging (Eme *et al.* 2017).

Other metabolites that contributed to the most variance included creatine phosphate, fumarate, histamine and 2-aminoadipate, which were all elevated in the *B*- individuals. Interestingly, histamine was significantly increased in the *B*- group across both countries. This metabolite is influenced by numerous factors including the host, diet and the microbiota. Its increase in the gut has also been associated with irritable bowel syndrome and inflammatory bowel disease depending on active histamine receptors (Barcik *et al.* 2017; Fabisiak *et al.* 2017; Smolinska *et al.* 2014). Its decrease in *B+* samples may link *Blastocystis* to anti-inflammatory associations in the gut. The mechanism for this is unknown, but perhaps the presence of *Blastocystis* is associated with bacteria that possess the ability to degrade histamine or alternatively, *Blastocystis* colonisation is associated with reduction in bacteria that produce histamine. The pathway analysis revealed a decrease in histamine and some associated metabolites (Figure 5.viii), however metabolic products of histamine were not included in the metabolite profiling so an increase in histamine degradation cannot be determined here (Pugin *et al.* 2017; Smolinska *et al.* 2014). Nonetheless, these results demonstrate an apparent negative relationship between increased levels of histamine in the gut and the presence of *Blastocystis* which in turn supports the notion of *Blastocystis* colonisation in individuals without IBS.

Significant metabolites identified by VIP from the South Korean study cohort mostly differ to those found in Turkey. Here, ethanol was significantly increased in *B*+, unlike the Turkey cohort where ethanol was removed due to its likelihood of being an artefact left over from extraction; the concentrations of ethanol here were lower than that of the Turkey data set and did not skew the data (Supplementary figure 5.ii).

The reason for the marked increase of ethanol in *B*+ samples is unknown as published reports do not document the ability of *Blastocystis* to produce ethanol. In fact, ethanol extracts were identified as an inhibitor of *Blastocystis* growth in one report (Vital and Rivera 2009). Therefore, perhaps the increase is due to an increase in gut colonisers that produce ethanol (Elshaghabee *et al.* 2016). However, the results for ethanol here must be approached with caution as a systematic error cannot be ruled out for certain.

Caprate was also significantly increased in *B*+ individuals from South Korea, this metabolite is associated with diet, especially dairy products, and has been seen to modulate intestinal barrier function (Radloff *et al.* 2019), however, the reason for its increase in infected samples is unknown.

Other increased metabolites in South Korean *B*+ samples included isopropanol, cytidine, 3-hydroxyphenylacetate, 3-methyladipate galactose and p-Cresol. p-Cresol is a metabolite produced by numerous colonisers of the gut (Kawakami *et al.* 2007) and has demonstrated bacteriostatic effects that possibly provide a competitive advantage to some bacteria (Passmore *et al.* 2018). To our knowledge, p-Cresol production has not been documented in intestinal protozoa (Mohammed, Onodera and Or-Rashid 2003), indicating that diet or bacteria are the most likely contributors to its increase.

Additionally, all amino acids identified using VIP from South Korea were decreased in *B*+ samples and some of which followed a similar pattern to metabolite profiles form Turkey (histamine, tryptophan, glycine, valine, and methionine). Carnosine was also increased in the uninfected group, this is made up of beta-alanine and L-histidine which were either absent or decreased in infected individuals. The decrease of amino acids supports the notion mentioned above; that *Blastocystis* is not causing inflammation in the gut, as an increase of these metabolites have been identified as biomarkers for inflammation (Bosch *et al.* 2018; Marchesi *et al.* 2007). Although it cannot be ruled out that their decrease isn't solely due to the possibility of scavenging, a lack of other inflammatory biomarkers indicates that *Blastocystis* may not be promoting inflammatory responses in the gut.

A number of factors influence the composition of the metabolome, this includes but is not limited to diet, genetic makeup of the individual, demographics, obesity, age, gender, disease and extraction protocol (Jain, Li and Chen 2019; Sinha *et al.* 2016; Tang *et al.* 2019; Zierer *et al.* 2018). Such factors likely influence the results documented here and thus associations between *Blastocystis* and the metabolome can only be inferred. Moreover, the infecting *Blastocystis* subtype and infection load may elicit different responses on the metabolome. Numerous reports have previously questioned the pathogenic potential of *Blastocystis* and hypothesize that the infecting subtype possibly influences pathogenicity (Kaneda *et al.* 2001; Poirier *et al.* 2012; Tito *et al.* 2019). It would be interesting here to further investigate metabolite profiles associated with different subtypes in order to assess possible host-microbiota-subtype differences. Unfortunately, this was not possible herein due to the absence of subtype data for the samples used, however the PCA analysis did not reveal any distinct clusters within the positive samples which may have been the result of particular subtypes. Perhaps an investigation of different subtypes would have to focus on positive samples independently to draw away from the impact of negative vs positive within the cohort.

3.2 Pathway analysis

Pathway analysis revealed a number of potential pathways impacted by *Blastocystis* infection. Here 32 pathways were identified for the Turkish cohort and six pathways for South Korean samples (*p*-adj <0.1). The majority of identified pathways were incomplete and thus interpreted with caution. Most shared common significant metabolites that were also identified by univariate analysis and the majority of metabolites linking *Blastocystis* to these pathways were the amino acids.

Of the common pathways identified across both countries, Aminoacyl-tRNA biosynthesis and Histidine metabolism presented the two most significant findings. The results for Aminoacyl-tRNA biosynthesis, identified only first step of the pathway and consisted of amino acids involved in the biosynthesis pathway. The significant decrease of many of the amino acids herein has been examined above, and there is a possibility that this is due to *Blastocystis* scavenging metabolites it cannot synthesise itself, as seen in other intestinal protozoan infections (Hublin *et al.* 2013). Yet, it should be considered that the *Blastocystis* carriage load is unknown and is likely not at a high enough density to significantly impact metabolite levels in this way. Future studies could remedy this by including a quantitative method for *Blastocystis* detection, such as qPCR which can help determine infection load.

The other common pathway – Histidine metabolism was increased in the *B*- samples. As discussed above, histidine metabolism results in the production of the proinflammatory compound histamine, which was decreased in infected individuals, possibly indicating an absence of inflammation in the gut. Other pathway intermediates including urocanate, L-aspartate carnosine and N(pi)-Methyl-L-histidine were also increased in the *B*- group. However, the roles of these metabolites in the intestine are poorly understood. The remaining compounds were not included in the initial analysis and thus no interpretations cannot be made.

Additional key pathways that may impact gastrointestinal tract homeostasis included the arginine biosynthesis pathway. Within this pathway, a number of metabolites including l-arginine were significantly increased in infected individuals from the Turkey cohort. This may be of importance as l-arginine has been identified in the intestine as a modulator for maintaining immunophysiological functions, with a decrease associated with inflammation and immunopathology (Fritz 2013). Therefore, the increase in l-arginine in infected individuals may offer some protection against inflammation in the intestine.

Another pathway often linked to intestinal health was the tryptophan pathway. Tryptophan was identified in all methods of comparison and plays a vital role in intestinal health (Taleb 2019). This pathway included a number of components that were decreased in *B*+ samples, with tryptophan being the first step in the metabolism to synthesise other metabolites including melatonin and 5-Hydroxyindoleacetate, both of which were significantly decreased in *B*+ samples in the Turkey cohort. In the South Korean samples, tryptophan and melatonin were also decreased in *B*+ individuals, the decrease of tryptophan may be linked to a decline in gut health, possibly indicating a link to dysbiosis via altered bacterial communities involved in its synthesis (Nieves-Ramírez *et al.* 2018).

Nicotinate and nicotinamide metabolism was another identified pathway from the South Korean cohort, the significant metabolites here included quinolinate, nicotinate and nicotinamide, all of which were increased in *B*+ individuals. Quinolinate has been shown to be produced by bacterial colonisers of the gut from the metabolism of tryptophan, however the impact on the microbiota is not clearly understood; yet there is evidence of it being a possible neurotoxic metabolite (Kaur, Bose and Mande 2019).

D-glutamine and D-glutamate metabolism was another common pathway to both sample cohorts but was only significantly altered in the Turkey cohort ($p<0.1$). Here, l-glutamine was significantly increased in the *B*+ samples. This amino acid has been linked to maintaining gut physiology and anti-inflammatory regulation, in addition to being an important source of energy within the gut (Ameho *et al.* 1997; Kim and Kim 2017; Rao and Samak 2012). An increase in infected individuals may be a result of diet as this amino acid is generally not associated with production by bacteria in the gut, however, its increased presence in the gut indicates a state of eubiosis (Ma and Ma 2019).

Interestingly, the propanoate metabolism pathway was significantly increased in the Turkey cohort. Propanoate is a Short-Chain Fatty Acid that's presence indicates gut health and eubiosis (Hosseini *et al.* 2011). The pathway was identified in *B*+ and *B*- individuals with an increase in infected individuals ($p<0.1$). It's synthesis is associated with main gut colonisers including members of the Bacteroidetes and Firmicutes. Interestingly, a proposed pathway for propanoate biosynthesis via succinate degradation has been proposed in *Blastocystis,* but not yet experimentally proven (Stechmann *et al.* 2008). Therefore, it could be theorised that the increase of this metabolite may possibly be a direct result of its formation by *Blastocystis* however, further experimental exploration of this is necessary before

conclusions can be drawn, and it is likely the increase is the result of production by certain communities of bacteria which *Blastocystis* colonisation may be associated with.

The work presented here is the first to employ ^{1}H NMR metabolomics to study the possible impact of *Blastocystis* infection in the gut of humans. The results demonstrate that although there are characteristic shifts in the metabolome of infected individuals. There was a lack of identified metabolites that can be significantly linked to dysbiosis in the gut, and in fact the apparent increase in metabolites like propanoate may indicate a positive association of *Blastocystis* on the gut. It should be noted that the two cohorts used in this study differed in their symptomology, with those from Turkey all having diarrhoea opposed to the infected South Korean samples presenting no gastrointestinal symptoms. This difference may be a result of a number of factors in addition to *Blastocystis* infection, which has questionable pathogenic potential (Tan *et al.* 2010). As a result, all analysis was carried out separately in order to minimise the variance, not only between symptoms but also between the numerous variables associated with human metabolomic studies. The metabolite extractions also took place in the local laboratories where the samples were collected and although the same protocol was followed, some differences between the extracted samples was to be expected; therefore, analysing them separately accounted for this.
Still, the results highlighted a number of common elements throughout *Blastocystis* infection, including distinguished metabolites and possible associated pathways.

Overall, no conclusive evidence can be produced regarding definitive roles of *Blastocystis* in the host-microbiota relationship, however an overall lack of significant dysbiotic-associated biomarkers and increase in some metabolites associated with eubiosis such as propanoate, and l-glutamine may offer a tentative reasoning for non-pathogenic associations in the gut. A plausible reason for this is the co-colonisation of *Blastocystis* with certain bacterial communities that are linked to positive gut health.
Future studies investigating particular subtypes in symptomatic and asymptomatic cohorts in addition to metabolomics coupled with transcriptomics as demonstrated by Jeelani et al. on *Entamoeba invadens* (Jeelani *et al.* 2012) may further help uncover the extent of influence this protozoan has on the microbiota and its role as a pathogen.

Chapter 6. *Blastocystis* ‘omics responses to oxygen stress

1 Introduction

Current investigations of 'omics related studies on *Blastocystis* are relatively limited. Of those that do exist, the majority are genomic based with particular emphasis on mitochondrial genomes, and to date mitochondrial genomes: ST1-ST4, ST6-ST9 (Jacob *et al.* 2016; Pérez-Brocal and Clark 2008; Stechmann *et al.* 2008; Wawrzyniak *et al.* 2008) and nuclear genomes of ST1, ST4 and ST7 (Denoeud *et al.* 2011; Eme *et al.* 2017; Gentekaki *et al.* 2017; Wawrzyniak *et al.* 2015) have been characterised. Additionally, unannotated draft genome assemblies of ST2, ST3, ST6, ST8 and ST9 can also be found on genome databases (Andersen *et al.* 2015).

Predicted proteome and secretome based studies of *Blastocystis* encompass the annotated genomes of ST1, ST4 and ST7 (Denoeud *et al.* 2011) and current transcriptomic based studies are limited to one currently published study on the NandII isolate (ST1) published in 2017 which compared data to genomes of ST4 and ST7 (Gentekaki *et al.* 2017). These results have provided important information regarding the adaptation of *Blastocystis* to the intestine and further investigation of *Blastocystis* lateral gene transfer (LGT) has shed light on acquired genes associated with oxygen-stress responses, host-immune evasion and pathogenicity (Eme *et al.* 2017). Currently there are no published reports focusing on the metabolome and the overall small number of other 'omics-based studies highlights the need for further investigation into this area of *Blastocystis* based research.

The present understanding of the roles of *Blastocystis* in the gut, its interactions with the microbiota and implications in diseases such as inflammatory bowel disease (IBD) remains largely unknown; this in part could be remedied by more 'omics-based studies. Consequently, the predicament of whether *Blastocystis* should be considered a parasite or not is hotly debated. Intestinal dysbiosis and inflammatory diseases such as IBD and irritable bowel syndrome (IBS) are often associated with a proliferation of facultative anaerobes and decrease in strict anaerobes in the gut (Lloyd-Price *et al.* 2019; Distrutti *et al.* 2016). Therefore, it has been speculated that oxygen levels are increased in these patients and this provides unfavourable conditions for strict anaerobic colonisers like *Lactobacilli* and *Bifidobacteria* and also *Blastocystis*.

The disputed position of *Blastocystis* as a pathogen is further exacerbated by its unknown role in IBD and IBS. For instance, numerous reports exist that associate *Blastocystis* infection with IBD (Cekin *et al.* 2012; Kök *et al.* 2019; Tai *et al.* 2011; Yakoob *et al.* 2004; Yamamoto-Furusho and Torijano-Carrera 2010), yet, in many instances these results are contradicted

and *Blastocystis* is found to be absent in such diseases (Coskun *et al.* 2016; Krogsgaard *et al.* 2015; Petersen *et al.* 2013; Rossen *et al.* 2015; Tito *et al.* 2019). Furthermore, a positive association of *Blastocystis* and IBD would challenge its strict anaerobic nature (Rigottier-Gois 2013; Kök *et al.* 2019; Cekin *et al.* 2012).

In this chapter we aim to expand the limited range of 'omics-based studies on *Blastocystis* by investigating the differential gene expression of human associated *Blastocystis* subtypes: ST3 and ST4, under anaerobic conditions and in increased oxygen levels *in vitro*. This will be achieved by recreating conditions reflecting that of the shifting oxygen levels in the gut, which may occur in some gastrointestinal disorders. By focusing on the transcriptome of these subtypes we hope to identify differentially expressed pathways, some of which may linked stress responses to increased oxygen and reactive oxygen species (ROS). Possible phenotypic outcomes of oxygen stress responses will be further characterised by analysing the metabolome of the culture environment. Figure 6.i provides an illustrative summary of the experimental overview.

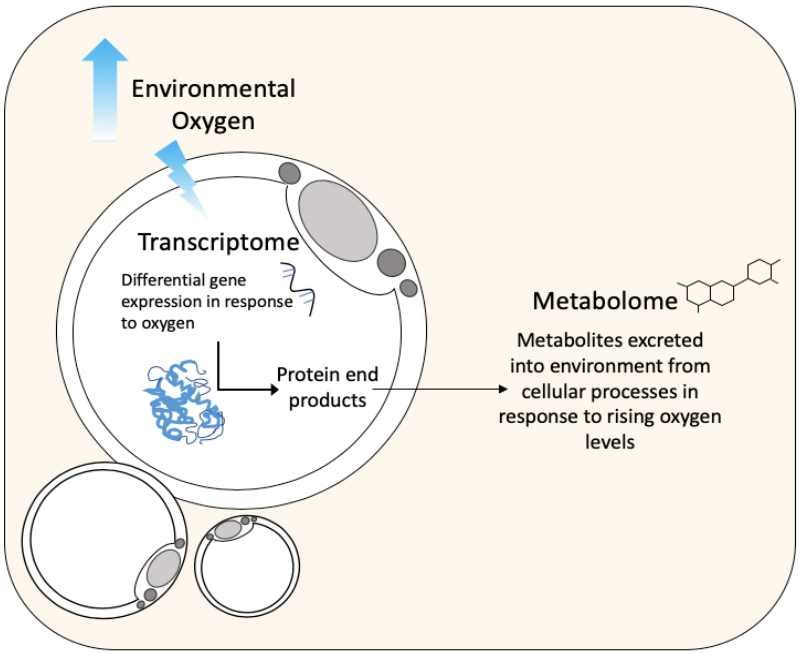

Figure 6.i Illustrative overview of the outcome of oxygen treatment on *Blastocystis* cultures. Within this experiment both the transcriptome response to oxygen stress and produced metabolites will be investigated against *Blastocystis* maintained in anaerobic 'control' conditions.

Results from this chapter demonstrate a number of differentially expressed pathways between anaerobic and oxygen-stress induced *Blastocystis,* some of which may possibly be linked to stress responses to oxygen. These responses support the possibility of *Blastocystis* being able to cope with some degree of oxygen in the gut and may support the idea of its continued colonisation in the gut of IBD patients.

2 Results

To investigate changes in gene expression levels caused by elevated oxygen, xenic *Blastocystis* cultures of ST3 and ST4 were either grown anaerobically or flushed with 5% oxygen for 30 minutes before RNA extraction. A total of 24 RNA samples were extracted for downstream analysis; 12 samples per subtype were collected with six anaerobic and six 5% oxygen for each. From the collected samples, the five with the highest RNA concentration and best 260/280 and 260/230 ratios were sent for RNA sequencing (RNA-Seq). TruSeq mRNA stranded sequencing libraries were prepared and sequenced on NovaSeq Illumina Platform (Outsourced to Macrogen, Korea), and of the 20 submitted samples, one sample failed. The Trinity pipeline was used to generate *de novo* transcriptome assemblies and DESeq2 was used to carry out differential expression analysis; Figure 6.ii provides a visual representation of the results. Isoforms with +/- 1 and 5 log fold change (LFC) data for each subtype compared against corresponding anaerobic data were used in annotation and mapping. Transcripts with the longest open reading frame were used for differential gene expression analysis.

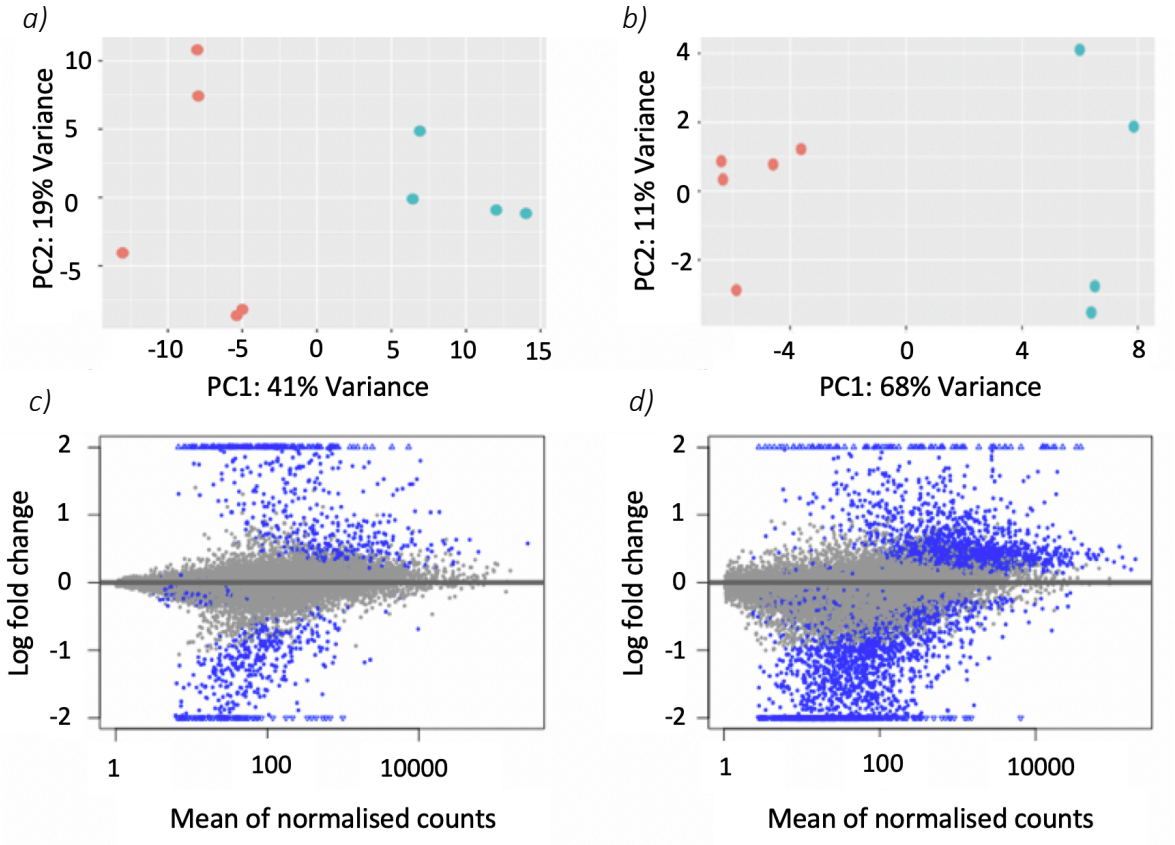

Figure 6.ii Differential expression analysis of *Blastocystis* subtypes when exposed to oxygen and under anaerobic conditions.

a-b) five anaerobic biological replicates and four oxygen stress replicates were successfully analysed for each subtype, each condition clustered within each samples as displayed in the principal components plots (PCA) for each subtype; *a)* PCA plot for ST3, *b)* PCA plot for ST4

c-d) MA-plot depicting the shrunken log2 fold changes (FC) of oxygen stress over the mean of normalized counts for all of the transcripts input into the DESeq2 dataset created in R. Coloured points represent differentially regulated genes (p<0.1). Points with fall outside the window are presented as upward or downward facing triangles. *c)* ST3 log2 FC MA plot. *d)* ST4 log2 FC MA plot.

2.1 Identification of differentially expressed transcripts

BlastKOALA was used to generate K values from LFC subtype transcript data; annotation of the transcriptome varied between samples. Positively annotated transcripts were analysed via the KEGG (Kyoto Encyclopedia of Genes and Genomes) module mapper for pathway reconstruction, with modules representing characteristic sets of genes that are linked to specific metabolic pathways (Table 6.i), Figure 6.iii offers a visual representation of the annotation within each subtype and condition.

Table 6.i Summary of entries and annotation outputs from BLASTKOALA and KEGG mapper. Here assigned pathways include those which are complete or are missing one module, as described by KEGG module mapper

Sample	Entries input	Entries output	% Annotation	Total pathways	Assigned pathways	Complete pathways
ST3 -1 LFC	1505	464	30.8%	213	8	2
ST3 -5 LFC	519	159	30.6%	111	0	0
ST3 +1 LFC	971	342	35.2%	138	5	1
ST3 +5 LFC	256	112	42.8%	70	0	0
ST4 -1 LFC	5380	1360	25.3%	315	16	2
ST4 -5 LFC	1337	326	24.4%	172	0	0
ST4 +1 LFC	1170	357	30.5%	226	6	1
ST4 +5 LFC	249	79	31.7%	74	0	0

KEGG module reconstruction yielded several differentially expressed pathways. Only complete pathways and those with one module missing as identified in the module mapper were included in subsequent analysis. Pathway reconstruction maps were created for samples with suitable protein-coding genes, all -/+1 LFC transcripts yielded some form of pathway, yet none of the +/- 5 LFC transcripts contained annotated genes that formed complete or near complete pathways. The following results depict the observed differentially expressed changes from the selected transcripts that matched genes from the KEGG mapper, the 'M' number represents the modules identified and 'KO' identifiers denote the experimentally characterised genes/proteins in KEGG which our transcripts were assigned to.

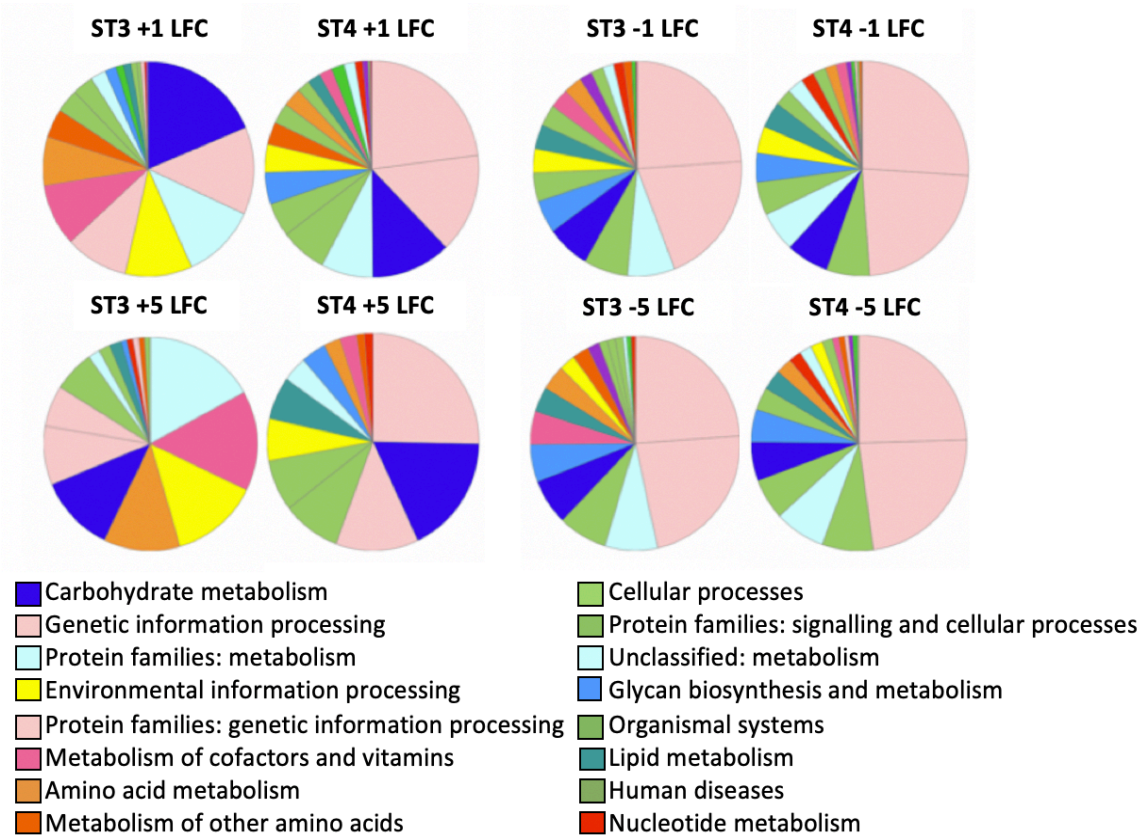

Figure 6.iii Pie charts representing the annotation data carried out using KEGG module mapper for each subtype under different conditions at +/-1 and 5 LFC.

2.2 Complete pathways

Analysis revealed a total of three complete pathways with differential expression under oxygen stress compared to anaerobic conditions. Of these, two belonged to ST4 and one to ST3; all were downregulated (Table 6.ii).

Both down regulated ST4 pathways were involved in lipid metabolism and within the beta-oxidation, acyl-CoA synthesis module (M00086), both possible pathway components (K01897 and K15013; EC:6.2.1.3) were downregulated.

The only complete downregulated ST3 pathway was identified as phosphoribosyl pyrophosphate (PRPP) biosynthesis (M00005) and included only one step with the enzyme ribose-phosphate pyrophosphokinase (K00948 EC:2.7.6.1), Figure 6.iv provides an illustration of the complete pathways identified here.

Table 6.ii Summary of complete pathways identified from the KEGG module mapper.

Subtype	Gene regulation	Module	Biochemical process	Pathway
ST4	Down	M00089	Lipid metabolism	Triacylglycerol biosynthesis
ST4	Down	M00086		Beta-oxidation, acyl-CoA synthesis
ST3	Down	M00005	Carbohydrate metabolism	Phosphoribosyl pyrophosphate biosynthesis

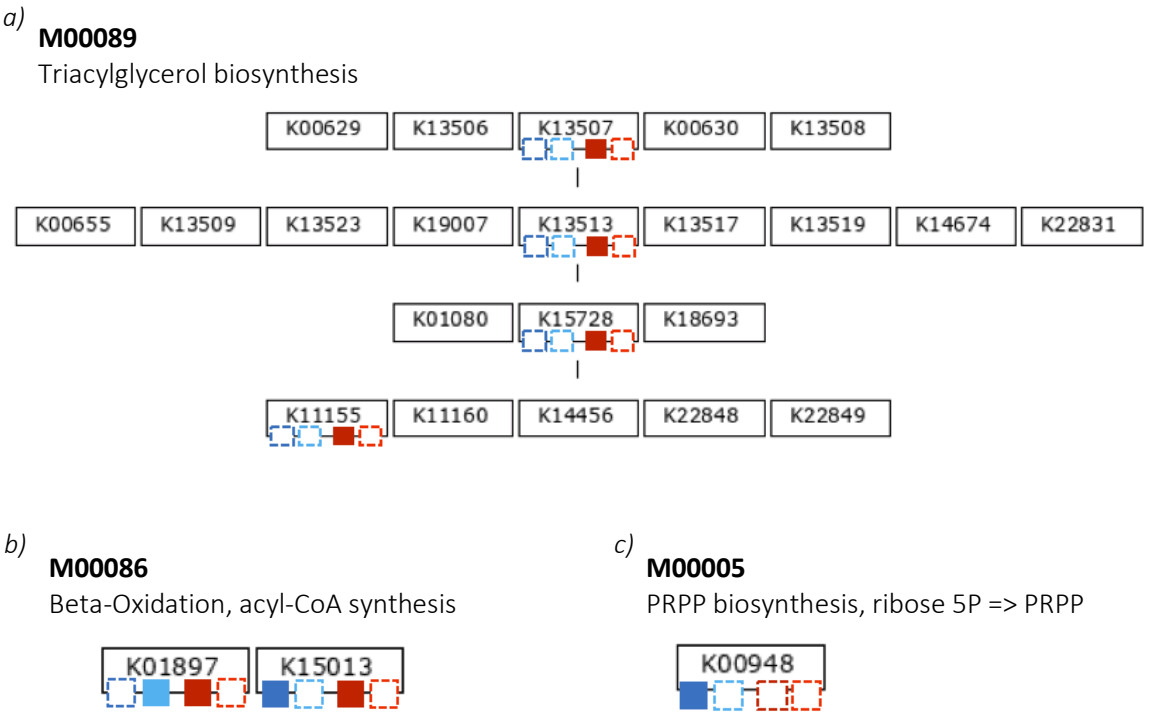

Figure 6.iv Complete module pathways for all three biochemical process identified as complete from transcriptomics data regarding *Blastocystis* ST3 and ST4.

a) Module M00089 representing the triacylglycerol biosynthesis which was completely downregulated in ST4. *b)* Module M00086 representing the beta-oxidation, acyl-CoA synthesis pathway which was completely downregulated in ST4, in with both long-chain fatty-acid-CoA ligases (K01897, K15013 EC:6.2.1.3) *c)* Module M0005 representing the single step pathway for phosphoribosyl pyrophosphate (PRPP) biosynthesis, which was downregulated in ST3

Dark blue solid boxes represent a negative log fold change (LFC) for ST3, Light blue solid boxes represent a positive LFC for ST3, Burgundy solid boxes represent negative log fold change for ST4 and red solid boxes represent positive LFC for ST4. Empty dashed boxes represent an absence of LFC across identified KO identifiers.

Abbreviations: Phosphoribosyl diphosphate (PRPP), ribose 5-phosphate (ribose 5P)

2.3 Incomplete pathways

Analysis revealed a total of 17 incomplete pathways missing no more than one component/protein. Nine of these were partially downregulated in ST4 under oxygen stress (Supplementary figure 6.i illustrates these) and included two carbohydrate metabolism pathways, two lipid metabolism pathways, one pathway involved in biosynthesis of terpenoids and polyketides, one glycan metabolism pathway, one amino acid metabolism pathway and two energy metabolism, both of which belonged to the Crassulacean acid metabolism contained the enzyme malate dehydrogenase (K00025, K00026 and K00029 EC:1.1.1.37 and EC:1.1.1.40).

Two pathways were incompletely upregulated for ST4 under oxygen stress, these belonged to the metabolism of cofactors and vitamins and carbohydrate metabolism. Table 6.iii provides an overview of the different gene expression results and corresponding pathways in ST4.

Table 6.iii Summary of incomplete pathways identified from the KEGG module mapper for ST4. The missing pathway component necessary for module completion is listed along with its KO identifier and the module it belongs to.

Gene expression	Module	Biochemical Process	Pathway	Missing component
Down	M00741	Carbohydrate metabolism	Propanoyl-CoA metabolism to succinyl-CoA	Methylmalonyl-CoA mutase (K01847/K01848 or K01849 EC:5.1.99.1)
Down	M00132		Inositol phosphate metabolism to phytate	1D-myo-Inositol-trisphosphate 6-kinase (K00913/K01765 EC:2.7.1.159)
Down	M00082	Lipid metabolism	Fatty acid biosynthesis	Acetoacetyl-[acyl-carrier protein] synthase (K00648/K18473 EC:2.3.1.180)
Down	M00092		Phosphatidylethanolamine biosynthesis	Ethanolamine-phosphate cytidylyltransferase (K00967 EC:2.7.7.14)
Down	M00367	Biosynthesis of terpenoids	C10-C20 isoprenoid biosynthesis	Isopentenylpyrophosphate Delta-isomerase (K01823 EC:5.3.3.2)

		and polyketides		
Down	M00073	Glycan metabolism	N-glycan precursor trimming	Alpha 1,3-glucosidase (K05546 EC:3.2.1.84)
Down	M00134	Amino acid metabolism	Polyamine biosynthesis	Arginase (K01476 EC:3.5.3.1)
Down	M00168	Energy metabolism	Crassulacean acid metabolism (CAM)	Phosphoenolpyruvate carboxylase (K01595 EC:4.1.1.31)
Down	M00169			Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) (K00029 EC:1.1.1.40)
Up	M00141	Metabolism of cofactors and vitamins	C1-unit interconversion	Methylenetetrahydrofolate dehydrogenase (NADP+) (K00288 EC:1.5.1.5 3.5.4.9 6.3.4.3) / Methylenetetrahydrofolate dehydrogenase (NAD+) (K13403 EC:1.5.1.5 3.5.4.9) and C1-tetrahydrofolate synthase (K13402 EC:6.3.4.3)
Up	M00549	Carbohydrate metabolism	UDP-glucose biosynthesis	Phosphoglucomutase (K15779, K01835 or K15778 EC:5.4.2.2)

Acylglycerol degradation (M00098) was the only module identified as partially downregulated across both subtypes, acylglycerol lipase (K01054 EC:3.1.1.23) was downregulated for both ST3 and ST4, and triacylglycerol lipase was absent (K01046, K12298, K16816, K13534, K14073, K14074, K14075, K14076, K22283, K14452, K22284, K14674, K14675, K17900 EC:3.1.1.3).

Analysis revealed a total of three pathways downregulated in ST3 as a result of oxygen stress and included biochemical processes involved in carbohydrate metabolism, glycan metabolism and cofactor and vitamin metabolism. Upregulated pathways included one carbohydrate metabolism and one energy metabolism pathway (Table 6.iv, Supplementary figure 6.ii).

Table 6.iv Summary of incomplete pathways identified from the KEGG module mapper for ST3. The missing pathway component necessary for module completion is listed along with its KO identifier and the module it belongs to.

Gene expression	Module	Biochemical Process	Pathway	Missing component
Down	M00549	Carbohydrate metabolism	UDP-glucose biosynthesis	Hexokinase (K00844 EC:2.7.1.1) / glucokinase (K00845, K12407 EC:2.7.1.2) / polyphosphate glucokinase (K00886 EC:2.7.1.63)
Down	M00070	Glycan metabolism	Glycosphingolipid biosynthesis	Hexosyltransferase (K03766 EC:2.4.1.206)
Down	M00120	Cofactor and vitamin metabolism	Coenzyme A biosynthesis via pantothenate	Pantetheine-phosphate adenylyltransferase (K02318,K00954,K02201 EC:2.7.7.3) / dephospho-CoA kinase (K02318,K00859 EC:2.7.1.24)
Up	M00554	Carbohydrate metabolism	UDP-galactose biosynthesis	UDPglucose--hexose-1-phosphate uridylyltransferase (K00965 EC:2.7.7.12)
Up	M00168	Energy metabolism	Crassulacean acid metabolism (CAM), dark	Phosphoenolpyruvate carboxylase (K01595 EC:4.1.1.31)

To further investigate incomplete pathways, missing module components were input into the BLAST nonredundant (nr) protein database (NCBI) against *Blastocystis* to firstly see if the missing pathway component had been previously characterised in *Blastocystis.* A total of 45 KO identifiers were investigated, of these 13/45 were confidently matched with corresponding proteins from published *Blastocystis* genome data. 28/45 gave no significant relevant BLAST hit and were thus assumed to be absent or currently uncharacterised proteins from *Blastocystis* (Eme *et al.* 2017). The remaining 4/45 results were positive BLAST hits from the *Blastocystis* genome which correlated to enzymes belonging to the same functional class as the missing component.

Positive KO identifiers were then analysed through BLAST against our transcript data to identify possible pathway components that were present but did not reach the LFC

threshold in analysis. This helped identify seven incomplete modules (Table 6.v). Of the identified pathways, four belonged to ST4 and included: Inositol phosphate metabolism (M00132) in which 1D-myo-Inositol-trisphosphate 6-kinase (K00913 EC:2.7.1.159) was identified; Phosphatidylethanolamine biosynthesis (M00092) with the missing cytidylyltransferase (K00967 EC:2.7.7.14); N-glycan precursor trimming (M00073) with Alpha 1,3-glucosidase (K05546 EC:3.2.1.84) and UDP-glucose biosynthesis (M00549) which was incomplete and upregulated in ST4, here phosphoglucomutase (K15779 EC:4.4.2.2) was identified. This module was also completed for ST3, yet here it was downregulated and the missing hexokinase (K00844 EC:2.7.1.1) was found. Two other ST3 modules were also considered complete when investigating below the LFC threshold; glycosphingolipid biosynthesis (M00070) was initially missing a hexosyltransferase (K03766 EC:2.4.1.206), and Coenzyme A biosynthesis (M00120) was missing pantetheine-phosphate-adenylyltransferase (K02318 EC:2.7.7.3).

Table 6.v Summary of incomplete pathways identified from the KEGG module mapper which were further assessed the expression of missing transcripts, identified components represent the missing KO identifiers which were initially put into the BLAST nonredundant (nr) protein database against *Blastocystis* in order to determine whether genes were presently characterised.

Gene expression	Module	Biochemical Process	Pathway	Identified component	Log2FC
ST4 Down	M00132	Carbohydrate metabolism	Inositol phosphate metabolism to phytate	1D-myo-Inositol-trisphosphate 6-kinase (K00913 EC:2.7.1.159)	-0.92
ST4 Down	M00092	Lipid metabolism	Phosphatidylethanolamine biosynthesis	Ethanolamine-phosphate cytidylyltransferase (K00967 EC:2.7.7.14)	-0.73
ST4 Down	M00073	Glycan metabolism	N-glycan precursor trimming	Alpha 1,3-glucosidase (K05546 EC:3.2.1.84)	-0.75
ST4 Up	M00549	Carbohydrate metabolism	UDP-glucose biosynthesis	Phosphoglucomutase (K15779 EC:4.4.2.2)	0.38
ST3 Down	M00549	Carbohydrate metabolism	UDP-glucose biosynthesis	Hexokinase (K00844 EC:2.7.1.1)	-0.52
ST3 Down	M00070	Glycan metabolism	Glycosphingolipid biosynthesis	Hexosyltransferase (K03766 EC:2.4.1.206)	-0.33

ST3 Down	M00120	Cofactor and vitamin metabolism	Coenzyme A biosynthesis via pantothenate	pantetheine-phosphate adenylyltransferase (K02318 EC:2.7.7.3)	**-0.91**

2.4 Pathways with mixed differential regulation

Analysis revealed three complete pathways that were differentially expressed, but had both up and down regulated KO identities within them. Additionally, four incomplete mixed pathways with also identified, with enzymes that were both up and down regulated, thus giving contradictory results.

These pathways included the pentose phosphate pathway (M00006) in which a complete upregulated pathway was identified for ST4, but also included downregulated 6-phosphogluconate dehydrogenase (K00033 EC:1.1.1.44 1.1.1.343). This enzyme was also both up and down regulated in ST3 as illustrated in Figure 6.v.

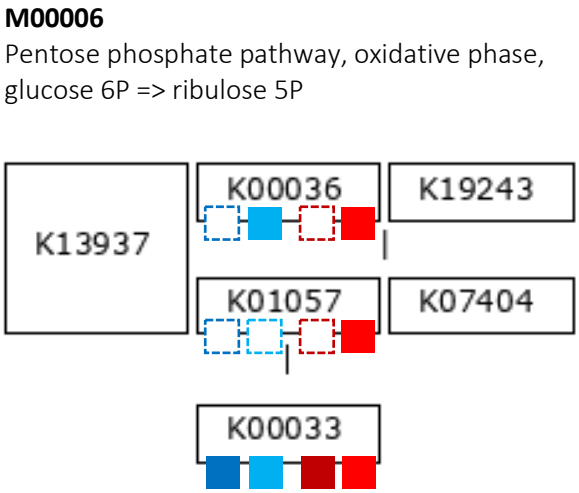

Figure 6.v Complete module pathway M00006 for ST4 identified by the KEGG module mapper with both upregulated and down-regulated differential gene expression. Here 6-phosphogluconate dehydrogenase (K00033 EC1.1.1.44 1.1.1.343) was identified as upregulated and down regulated in the complete ST4 pathway and incomplete ST3 pathway.

Dark blue solid boxes represent a negative log fold change (LFC) for ST3, Light blue solid boxes represent a positive LFC for ST3, Burgundy solid boxes represent negative log fold change for ST4 and red solid boxes represent positive LFC for ST4. Empty dashed boxes represent an absence of LFC across identified KO identifiers.

Abbreviations: glucose 6-phosphate (glucose 6P), ribulose 5-phosphate (ribulose 5P)

Glutathione biosynthesis from glutamate (M00118) was incompletely up and downregulated module in ST4, here the glutamate--cysteine ligase catalytic subunit (K11204 EC:6.3.2.2) was up- and down regulated in ST4 and upregulated in ST3. The other glutamate--cysteine ligase regulatory subunit (K11205 EC:6.3.2.2) was absent from both subtypes. Glutathione synthase (K21456 EC:6.3.2.3) was present and differentially up and down expressed in both subtypes.

Beta-Oxidation, acyl-CoA synthesis (M00086) was also identified as both upregulated and down regulated in ST3. This module consists of a single step with two possible KO identifiers of which long-chain fatty-acid-CoA ligase (K01897 EC:6.2.1.3) was upregulated in oxygen stress in ST3, yet long-chain-fatty-acid--CoA ligase ACSBG (K15013 EC:6.2.1.3) was downregulated in ST3.

Figure 6.vi offers an illustrated view of the differential expression results for these mixed pathways.

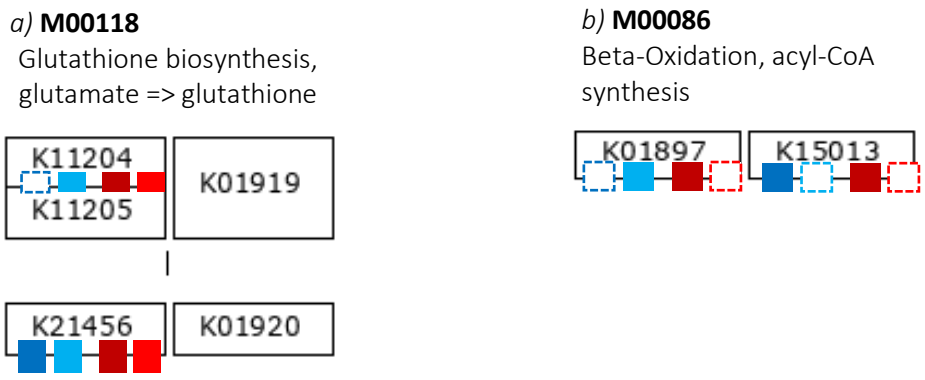

Figure 6.vi Complete module pathway for ST4 and ST3 identified by the KEGG module mapper with both upregulated and down-regulated differential gene expression results for both. *a)* Glutathione biosynthesis from glutamate, here one glutamate--cysteine ligase regulatory subunit (K11204 EC: EC:6.3.2.2) was identified across both subtypes and upregulated in both, in addition to downregulation in ST4. Glutathione synthase (K21456 EC: EC:6.3.2.3) was identified in all samples with both regulation results. *b)* Beta-Oxidation, acyl-CoA synthesis, both possible long-chain fatty-acid-CoA ligases (K01897, K15013 EC:6.2.1.3) are downregulated in ST4 and K01897 EC:6.2.1.3 was upregulated in ST3.

Black rectangles represent the KO identified obtained from KEGG module mapper. Dark blue solid boxes represent a negative log fold change (LFC) for ST3, Light blue solid boxes represent a positive LFC for ST3, Burgundy solid boxes represent negative log fold change for ST4 and red solid boxes represent positive LFC for ST4. Empty dashed boxes represent an absence of LFC across identified KO identifiers.

The glycosphingolipid biosynthesis (M00070) module was incompletely differentially regulated in both subtypes with hexosyltransferase (K03766 EC:2.4.1.206) absent. Although the data for ST3 revealed the enzyme beta-1,3-galactosyltransferase 1 (K07819 EC:2.4.1.86) as downregulated, analysis revealed that this was both up and down regulated for ST4.

Lastly, module UDP-galatcose biosynthesis (M00554) is a two-step pathway in which UDPglucose--hexose-1-phosphate uridylyltransferase (K00965 EC:2.7.7.12) is absent from both subtypes but galactokinase (K00849 EC:2.7.1.6) was differentially up and down regulated in ST4. A visual representation of these incomplete modules are represented in figure 6.vii.

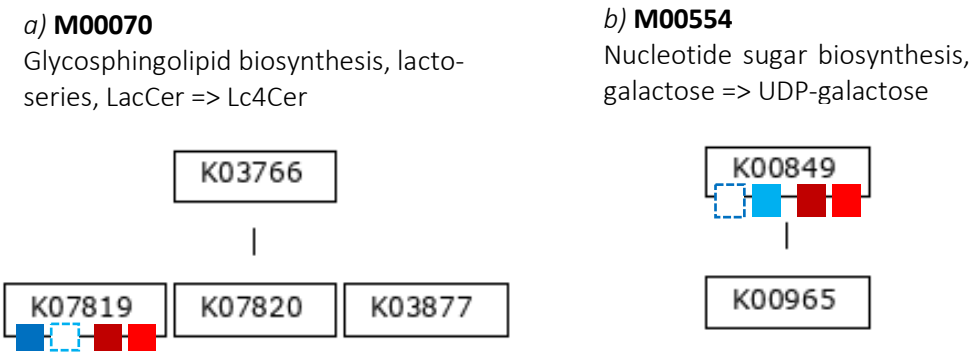

Figure 6.vii Incomplete module pathways for ST3 and ST4 identified by the KEGG module mapper with upregulated and down-regulated differential gene expression results for both. *a)* Glycosphingolipid biosynthesis, here beta-1,3-galactosyltransferase 1 (K07819 EC:2.4.1.86) was the only enzyme identified for both subtypes and was up and downregulated for ST4. *b)* pathway module representing UDP-galatcose biosynthesis (M00554) galactokinase (K00849 EC:2.7.1.6) is upregulated for both subtypes but also downregulated in ST4.

Black rectangles represent the KO identified obtained from KEGG module mapper. Dark blue solid boxes represent a negative log fold change (LFC) for ST3, Light blue solid boxes represent a positive LFC for ST3, Burgundy solid boxes represent negative log fold change for ST4 and red solid boxes represent positive LFC for ST4. Empty dashed boxes represent an absence of LFC across identified KO identifiers.

Abbreviations: Lactosylceramide (LacCer), Lc4Cer (Le4Cer metabolite)

2.5 ^{1}H NMR metabolomics analysis of *Blastocystis* cultures under different conditions

The different subtype and condition culture supernatants were analysed via ^{1}H NMR spectroscopy in order to assess metabolite profiles in response to oxygen stress compared with the anaerobic controls. Compound concentration data from each subtype was analysed separately using MetaboAnalyst 4.0 after being processed in Chenomx to assign metabolite profiles and concentrations. Analysis revealed 174 features from ST3 samples and 221 features from ST4 with a constant or single value that were subsequently deleted.

Statistical analysis using univariate analysis on normalized metabolite concentrations revealed nine significant metabolites that were relevant from anaerobic against oxygen treated samples. Of these, four were elevated in anaerobic samples and five metabolites were elevated in samples under oxygen stress; this is visually represented in Figure 6.viii by way of a scatter-plot and summarised in Table 6.vi. Pathway analysis was attempted to further support univariate analysis and identify metabolites associated with differentially regulated pathways using Metaboanalyst 4.0, however, no statistically relevant pathways were identified from this dataset from either subtype (p-adj<0.1) (Supplementary table 6.i and 6.ii).

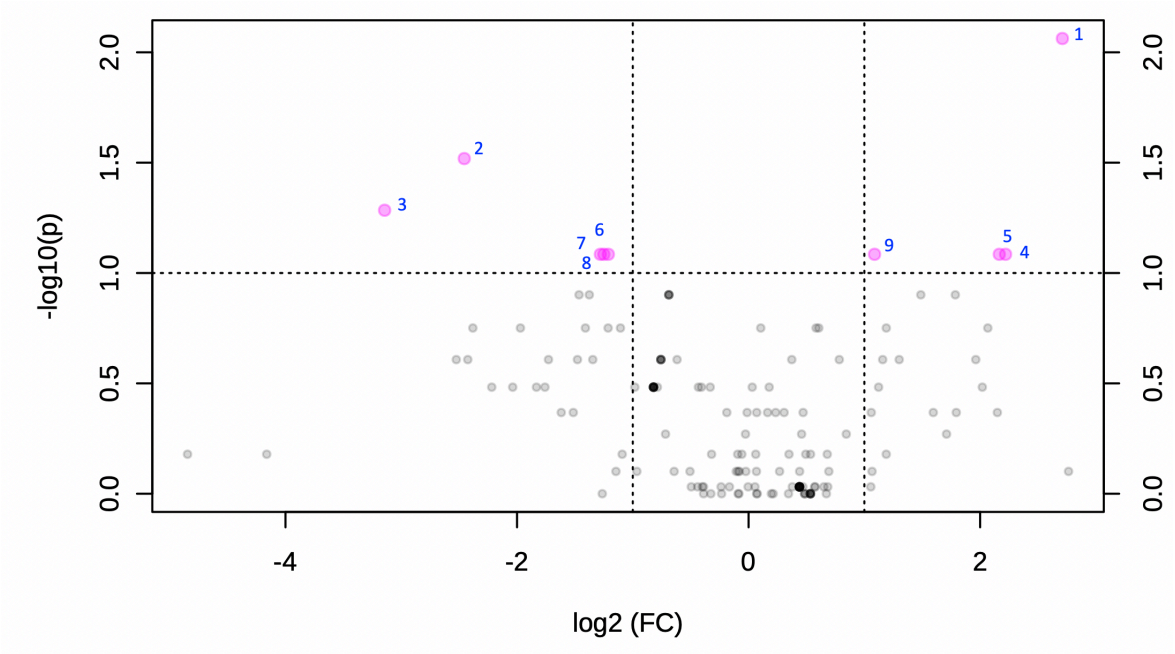

Figure 6.viii Volcano scatter-plot for metabolites identified in form ST3 cultures. Metabolites identified with a fold change threshold of 2 (x axis) and t-tests threshold of 0.1 (y-axis). Anaerobic samples were compared with oxygen treated samples and significant features are represented by pink circles which exceed the fold change threshold, with points further away from 0,0 representing more statistically significant observations. Plots were drawn using Metaboanalyst 4.0.

Table 6.vi Identified metabolites which were statistically increased in anaerobic samples when anaerobic and oxygen samples were compared in ST3. Plot ID represents the metabolites position in the corresponding volcano plot

Plot ID	Metabolite	FC	log2(FC)	raw.pval	-log10(p)
1	trans-Aconitate	6.5449	2.7104	0.008658	2.0626
2	3-Hydroxy-3-methylglutarate	0.1823	-2.4556	0.030303	1.5185
3	2-Furoylglycine	0.11316	-3.1436	0.051948	1.2844
4	Trimethylamine	4.6581	2.2197	0.082251	1.0849
5	N-Acetylserotonin	4.4862	2.1655	0.082251	1.0849
6	Xanthine	0.41174	-1.2802	0.082251	1.0849
7	Salicylurate	0.42046	-1.25	0.082251	1.0849
8	3-Chlorotyrosine	0.43185	-1.2114	0.082251	1.0849
9	5-Hydroxyindole-3-acetate	2.1259	1.0881	0.082251	1.0849

Univariate analysis of the ST4 sample supernatants revealed a total of five metabolites that were significantly altered between anaerobic and oxygen stress. Four of these were elevated in the anaerobic samples and one was increased from oxygen stress. Figure 6.ix and Table 6.vii summarises these findings

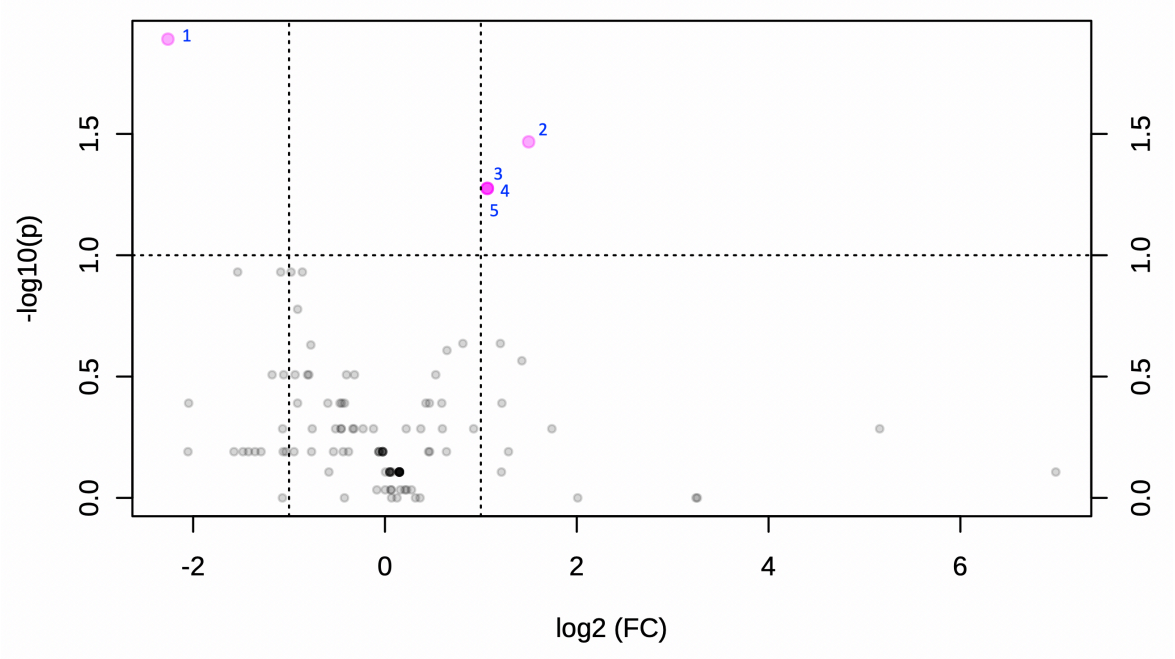

Figure 6.ix Volcano scatter-plot for metabolites identified from ST4 cultures. Metabolites identified with a fold change threshold of 2 (x axis) and t-tests threshold of 0.1 (y-axis). Anaerobic samples were compared with oxygen treated samples and significant features are represented by pink circles which exceed the fold change threshold, with points further away from 0,0 representing more statistically significant observations. Plots were drawn using Metaboanalyst 4.0.

Table 6.vii Identified metabolites which were statistically increased in oxygen treated samples when anaerobic and oxygen samples were compared in ST4. The Plot ID relates to the position of the metabolite in the corresponding volcano plot

Plot ID	Metabolite	FC	log2(FC)	raw.pval	-log10(p)
1	N-Methylhydantoin	0.20834	-2.263	0.012866	1.8906
2	3-Chlorotyrosine	2.8253	1.4984	0.034094	1.4673
3	Cellobiose	2.0957	1.0675	0.053027	1.2755
4	Glucose-1-phosphate	2.0957	1.0675	0.053027	1.2755
5	Sucrose	2.0957	1.0675	0.053027	1.2755

Subtypes were also compared regardless of treatment to see metabolite profile difference between *Blastocystis* subtype cultures and surrounding bacterial environment. Herein, 159 features with a constant or single value across samples were found and deleted before statistical analysis. Univariate analysis revealed no significant metabolites between ST3 and ST4 (p<0.05).

3 Discussion

An increased level of oxygen in the gut has been linked to dysbiosis in a number of circumstances, and may result in detrimental shifts in intestinal communities towards facultative anaerobes and certain diseases including inflammatory bowel disease (Rigottier-Gois 2013; Henson and Phalak 2017; Zhu and Li 2012). Existing knowledge of the impact of oxygen on anaerobic protozoa in the gut is limited; previous reports reveal oxygen-stress investigations of few protozoa including *Giardia, Entamoeba* and *Spironucleus* (Stairs *et al.* 2019; Ma'ayeh, Knörr and Svärd 2015; Vicente *et al.* 2009). Among these parasites, a number of antioxidant proteins and protection mechanisms have been characterised which function to combat periods of increased oxygen exposure and reactive oxygen species (ROS) (Vonlaufen *et al.* 2008; Sen *et al.* 2007). In 2018, Tsaousis et al. showed that *in vitro Blastocystis* NandII (ST1) responds to fluctuations in oxygen levels via Complex II and Alternative Oxidase (Tsaousis *et al.* 2018). A number of other reports also identify enzymes predicted to protect *Blastocystis* against increasing oxygen levels, some of which likely arose from lateral gene transfer events and include dimeric iron-containing superoxide dismutase, Thioredoxin reductase and 2-Cys peroxiredoxin, alcohol dehydrogenase (ADH3) and SufCB respectively (Denoeud *et al.* 2011; Eme *et al.* 2017; Tsaousis *et al.* 2012). All of these likely act in an attempt to support survival under oxygen stress and oxidative bursts, but may not confer long-term protection to fluctuating oxygen levels.

Our results offer an initial look into differentially expressed genes caused by oxygen stress in *Blastocystis* subtypes 3 and 4, both of which are known to colonize humans. We identified a total of 20 complete and incomplete biochemical pathways in the two subtypes that had differential expression under increased oxygen. Identified pathways included amino acid metabolism (2), biosynthesis of terpenoids and polyketides (1), carbohydrate metabolism (6), energy metabolism (2), glycan metabolism (2), lipid metabolism (5) and metabolism of cofactors and vitamins (2). Among the identified pathways, exposure to oxygen caused a general decrease in the vast majority, most of which had no identifiable roles in oxygen stress responses.

From comparing the pathways to previous reports regarding oxygen stress and oxidative stress in a range of organisms (Siauciunaite *et al.* 2019; Eme *et al.* 2017; Gentekaki *et al.* 2017; Wu *et al.* 2017; Perl *et al.* 2011; Vonlaufen *et al.* 2008). Some of those described here were able to be affiliated with possible stress responses exerted by *Blastocystis* in attempt to combat rising oxygen levels. It should be noted however, that none of these were

complete and most involved mixed enzyme expression, we will nevertheless discuss their possible roles below.

3.1 Pentose phosphate pathway

Firstly, the pentose phosphate pathway (oxidative phase) (M00006) was identified in both subtypes and is a metabolic pathway in which $NADP^+$ is reduced to NADPH and glucose-6-phosphate is converted to ribulose 5-phosphate. The generated carbohydrate intermediates are used in various biosynthetic pathways and the pool of NADPH has been seen to offer protection against oxidative stress in protozoa (Barrett 1997; Stincone *et al.* 2015; Siauciunaite *et al.* 2019). It would therefore be expected that this pathway would be over expressed during exposure to oxygen as a combative mechanism, the pathway was identified in both subtypes yet mixed expression levels of 6-phosphogluconate dehydrogenase (K00033 EC1.1.1.44 1.1.1.343) was observed. ST4 had a complete overexpressed pathway with 6-phosphogluconate dehydrogenase showing mixed regulation and ST3 had an incomplete overexpressed pathway with mixed expression of 6-phosphogluconate dehydrogenase as well. The 6-phosphogluconate dehydrogenase is a highly conserved oxidative carboxylase that catalyses the reduction of 6-phosphogluconate to ribulose 5-phosphate with NADP (Kruger and von Schaewen 2003). The query regarding its mixed expression is contradictory and an involvement in alternative pathways associated with oxygen stress response was explored, however none were identified.

3.2 Polyamine biosynthesis (putrescine)

Secondly, the putrescine synthesis pathway (M00134) has also been linked to oxidative stress responses. This pathway was incomplete and downregulated in ST4. Putrescine is a polyamine which has been linked to a number of environmental stress responses in a range of organisms (Wu *et al.* 2017; El-Halfawy and Valvano 2014; Smirnova *et al.* 2012) and includes experimental proof of it reducing reactive oxygen species, therefore it could be speculated that its synthesis would be upregulated in *Blastocystis* in an attempt to combat oxygen levels and ROS. However, the incomplete pathway identified was in fact decreased in ST4 with the one characterised enzyme: ornithine decarboxylase (K01581 EC:4.1.1.17) which is a key enzyme involved in putrescine biosynthesis. No published reports revealed other roles linking this enzyme to oxygen stress and this pathway has not been explored in *Blastocystis* making further assumptions arbitrary.

3.3 Glutathione biosynthesis

Lastly, glutathione biosynthesis (M00118) was incompletely up and down regulated in both subtypes. In both subtypes a glutamate—cysteine ligase regulatory subunit was missing (K11205 EC:6.3.2.2). Again, like the pentose phosphate pathway, the mixed expression meant no conclusions could be drawn about its role here. However, glutathione is known to offer protection against oxidative stress in mammals and protozoa (Lu 2013; Vonlaufen *et al.* 2008) and has been experimentally shown to be critical for the survival of anaerobic bacteria in aerobic conditions (Million *et al.* 2020) indicating that overexpression of this pathway could prove beneficial for survival.

3.4 Mixed expression pathways

Our analysis revealed that a number of biochemical pathways had mixed expression. These results were generally uninformative, contradictive and offered no viable results explaining the responses of *Blastocystis* to oxygen. A possible hypothesis for mixed regulation may include different components being oxygen sensitive and involved in other oxygen response pathways. For example, in pathways with upregulated and downregulated components, upregulated genes may have roles in uncharacterised pathways which are involved in oxygen-stress responses (Eme *et al.* 2017). Furthermore, downregulated components within these mixed expression pathways and in other downregulated pathways could be involved in encoding proteins that are non-essential and the downregulation may function as an energy saving mechanism under periods of stress. The study of oxygen stress response in *Blastocystis* is a relatively recent area of research and transcriptomics studies are currently limited in number. This means that most of the results presented here were investigated using reports that focus on other eukaryotes and inhabitants of the microbiota, and this greatly limited the conclusions we could draw. Nonetheless each pathway was investigated in detail and proteins from these pathways that may possibly be linked to oxygen stress included. UDP-glucose phosphorylase and malate dehydrogenase were identified and are discussed in detail below.

The enzyme UDP-glucose phosphorylase (K00963 EC:2.7.7.9) has been shown to be involved in oxidative stress responses and survival in yeast via trehalose and possible trehalose synthesis biosynthetic pathways have been proposed in *Blastocystis* (Yi and Huh 2015; Gentekaki *et al.* 2017). Although this enzyme was not identified in UDP-glucose synthesis

(M00549) in our study, UDP-sugar phosphorylase (K12447 EC:2.7.7.64) was found in both subtypes; this carries out an analogous role in UDP-glucose synthesis to UDP-glucose phosphorylase, possibly inferring a similar protection mechanism may exist.

Malate dehydrogenase (MDH) (K00025 and K00026 EC:1.1.1.37) has two main isoforms in eukaryotes including MDH1 and MDH2 which have been characterised from *Blastocystis* (Wawrzyniak *et al.* 2015; Denoeud *et al.* 2011; Lantsman *et al.* 2008). The results indicate that *Blastocystis* ST3 and ST4 encode different differentially expressed malate dehydrogenase enzymes: ST3 harbouring an upregulated MDH1 (cytoplasmic) isoform, whereas ST4 was encoding (mitochondrial) MDH2 which was downregulated.
In humans with Alzheimer's disease oxidative stress is a known feature, and there has been a link to increased malate dehydrogenase in response to oxidative stress, however its functional significance is unknown (Shi and Gibson 2011). The identification of the two differentially expressed transcripts here indicates that perhaps both MDH genes have possibly different functions in oxygen stress but the reasons and roles requires further investigation. One possible reason for the upregulation of cytosolic MDH in ST3 may involve the malate-aspartate shuttle and could be upregulated in an attempt to maintain levels of glutathione which is a known antioxidant and where the synthesis pathway was upregulated in ST3 (C. Wang *et al.* 2014).

3.5 Downregulated pathways

Additionally, results revealed that oxygen stress caused a gene repression of the remainder of complete and incomplete pathways identified here. Such attenuated pathways were mainly involved in lipid, carbohydrate and glycan metabolism and cofactor and vitamin metabolism and have been shown to be repressed by oxygen stress in human and yeast studies (Morel and Barouki 1999). This may be the result of an unfavourable redox status of the cell as well as a direct result of ROS causing changes in gene expression, yet many of the mechanisms underpinning this are currently unresolved, and the majority of studies investigating gene repression in response to oxygen are limited to humans (Allen and Tresini 2000; Chang *et al.* 2012; Siauciunaite *et al.* 2019).

3.6 Metabolomics

In addition to investigating gene expression responses to increased oxygen levels in *Blastocystis*, ^{1}H NMR metabolomics was employed to accompany the transcriptomics data. This was done to establish possible antioxidant roles of distinguished metabolites in order to assess associated roles in oxygen stress. An attempt was also made to map each identified metabolite to the annotated pathways to evaluate associations between transcriptome end products and metabolome. Results revealed a number of possible significant metabolites that were differentially altered between conditions.

In ST3 a total of nine metabolites were identified, four of these were increased in anaerobic supernatants and the remaining five were increased in samples exposed to oxygen.

For ST4 a total of five significant metabolites were found, four of these were increased in anaerobic samples and one: N-Methylhydantoin was increased in the oxygen treated samples. Published reports investigating the possible significance of these metabolites was assessed in order to identify roles in oxygen stress and antioxidant properties. A total of four metabolites were revealed to have possible roles relating to oxygen stress and these included: N-Acetylserotonin, N-Methylhydantion, N-acetylcysteine and N-Acetylglucosamine. These are further discussed in more detail below.

Firstly, N-Acetylserotonin has known antioxidant properties in humans where it has been implicated in interfering with free radical formation and promoting antioxidant enzymes (Oxenkrug 2005). It's presence here may suggest a possible role as an antioxidant, yet analysis revealed a significant increase in the ST3 anaerobic samples. Furthermore, its roles in the microbiota are currently unstudied and thus no conclusions can be currently drawn about its increase in the anaerobic supernatants.

Secondly, published data revealed that N-Methylhydantion has been linked to antioxidant and anti-inflammatory effects in humans (Liu *et al.* 2019), leading to the possibility that it is increased here in an attempt to combat rising oxygen. This metabolite was significantly increased in the oxygen treated samples form ST4, however synthesis of this has also not been characterised in *Blastocystis*.

Lastly, N-acetylcysteine and N-Acetylglucosamine were identified in the comparison between subtypes. These metabolites described antioxidant activities *in vitro* and *in vivo* by direct and indirect mechanisms (Aldini *et al.* 2018) and N-Acetylglucosamine is produced by some bacteria (Azam *et al.* 2014). This metabolite has been identified on the surface of *Blastocystis* where it is likely involved in mediating adhesion, invasion and immune responses (Lanuza, Carbajal and Borrás 1996; Yason and Tan 2018), no antioxidant roles

have been described for this metabolite in *Blastocystis* thus far. Its identification may be due to a build-up of the metabolite as a result of downregulation of glycan and carbohydrate metabolism pathways.

The remainder of the identified metabolites showed no direct correlation to the differentially expressed biochemical pathways observed. This was further supported by pathway analysis carried out in Metaboanalyst 4.0, where no statistically significant pathways were identified matching those detailed above (*p*-adj<0.1) (Supplementary table 6.i and 6.ii). This was likely influenced by the lack of available related reference protozoan libraries making a meaningful metabolome view hard to decipher. The lack of useful data here is also largely influenced by the use of xenic cultures. Although we were able to filter bacterial contamination from the transcriptomics data, here it was not possible to quantify the difference between bacteria and *Blastocystis* meaning that the metabolite profiles of these cultures represent the entire xenic environment. Ideally, if this were to be repeated axenic cultures of *Blastocystis* would be used as this negates metabolites resulting from bacterial responses to oxygen. Additionally, it would not be unreasonable to assume that some differentially regulated pathways within *Blastocystis* could be the result of changes in environmental metabolite concentrations caused by the response of the bacteria to increased oxygen levels. This may provide data on indirect responses of *Blastocystis* to increased oxygen and would be interesting to investigate further with gut associated bacterial communities; especially ones that are associated with IBD in order to explore *Blastocystis*-bacteria interactions.

Herein, we described some gene expression pathways affected by increasing oxygen levels in xenic *Blastocystis* cultures. Although biochemical pathways were identified with potential oxygen stress response roles, the majority of mixed expression pathways provided contradictory and uninformative data. The +/- 1 LFC threshold may account for this ambiguity and a higher threshold was also employed yet yielded no pathway results. Expected upregulated pathways, such as those utilising superoxide dismutase and other characterised antioxidant enzymes were not identified here.

Possibly repeating the experiment with the addition of ROS such as hydrogen peroxide as done with *Entamoeba* (Husain *et al.* 2012) or by inducing oxidative stress and monitoring responses over a prolonged time period could allow us to investigate response and recovery to oxygen and may provide more meaningful results detailing how *Blastocystis* may cope

with continual oxygen stress and if this affects its capability of long-term colonisation in the gut.

Pathways that included mixed expression of genes can further be investigated to assess the possibility of proteins being involved in different pathways by carrying out gene knock-out experiments, which have recently been facilitated in *Blastocystis* by the development of a transfection system (F.-J. Li *et al.* 2019); through making selected genes inoperative the responses of *Blastocystis* can be further investigated to determine if these genes have roles in oxygen stress responses.

Another option is to try an *in vitro* type of organoid gut system which has previously been used to study other protozoa (Heo *et al.* 2018) as this may provide a means of studying *Blastocystis* responses in an environment similar to the gut. It is also important to note that as *Blastocystis*-host interactions are not very well studied, many of the resulting assumptions made here are based largely on *in silico* predictions, meaning conclusions cannot confidently be drawn and further experimental validation would help develop our understanding.

The transcriptomics data was also accompanied by ^{1}H metabolomics that was likely heavily influenced by the xenic nature of the cultures and would yield more meaningful data if carried out on axenic cultures. This was initially trialled with *Blastocystis* NandII, but unfortunately RNA extraction had not provided a sufficient yield (data not shown).

Lastly, a large number of differentially regulated transcripts were unable to be characterised to known proteins and pathways, suggesting the need for further investigation into the roles of *Blastocystis* under oxygen stress.

Altogether, our results further build upon what is known about *Blastocystis* oxidative stress responses and contributes to our understanding of the what mechanisms it may employ to cope with rising oxygen levels as likely seen in the gut of IBD patients.

Chapter 7. Discussion

The gut microbiota is often referred to as the 'forgotten organ' (O'Hara and Shanahan 2006). In recent years, the increase in research investigating its roles towards health and disease have led to fascinating discoveries regarding its influence on host homeostasis, or lack of, in humans (Shreiner, Kao and Young 2015) and animals (Bahrndorff *et al.* 2016). Herein, we investigated the ubiquitous protozoon *Blastocystis,* which is a common gut coloniser of a range of vertebrate and invertebrate taxa and has a questionable pathogenic status. Firstly, the prevalence and genetic diversity of *Blastocystis* was explored across a range of captive animals (Betts *et al.* 2018; Betts, Gentekaki and Tsaousis 2020). Next, the impact of this protozoon on bacterial community abundance and diversity in the gut was explored. Here associations between *Blastocystis* and the bacterial microbiota was investigated in order to explore the relationship between microbial abundance and *Blastocystis* carriage. As the bacterial microbiota has been thoroughly investigated previously, certain communities can provide an indication to the overall gut health (Clemente *et al.* 2012; de Alencar Junior *et al.* 2020; Hermann-Bank *et al.* 2013; Kasper 2009; Ott *et al.* 2004). The role of *Blastocystis* in the gut was further expanded and an investigation of gut metabolite profiles from infected and non-infected human hosts was explored. Lastly, the roles of oxygen on two human associated subtypes were assessed. This was carried out to understand what responses *Blastocystis* may employ in response to the rising oxygen levels thought to occur in IBD with the hope of understanding if *Blastocystis* can persist in a dysbiotic gut with fluctuating oxygen.

1 Molecular epidemiology of *Blastocystis* in animals (Betts *et al.* 2018; Betts, Gentekaki and Tsaousis 2020)

1.1 *Blastocystis* subtype prevalence and diversity

The first objective of this thesis was to address the molecular epidemiology of *Blastocystis* across a range of host taxa in order to build upon current findings regarding host specificity and expand the range of identified hosts. A total of 230 samples from 38 different vertebrate species were investigated. The majority of positive identifications came from members of the artiodactyla, primates and rodentia taxa. However, in the case of rodentia and primates, the high prevalence was likely exacerbated by the large number of samples obtained from these animals. Overall, in excess of 40% of the species sampled from were positive for at

least one *Blastocystis* subtype. This observation is comparable, if not slightly higher, than that of similar animal-based prevalence studies of *Blastocystis* in animals which ranges from 6.0% - 37.9% in captive animals (Cian *et al.* 2017; Li *et al.* 2020; J. Li *et al.* 2019; Zhao *et al.* 2017).

The proposed subtype distribution and host specificity of *Blastocystis* has been detailed in a number of reports; the results presented here are largely in concordance with this existing data, thus supporting the notion of a degree of host range and specificity among the characterised subtypes (Ramírez *et al.* 2014; Valença-Barbosa *et al.* 2019; Wang *et al.* 2018; Wawrzyniak *et al.* 2013). Figure 7.i (Tsaousis *et al.* 2020) represents a illustrative overview of the subtype distribution observed form the results presented in Chapters 2 to 4 (Betts *et al.* 2018; Betts, Gentekaki and Tsaousis 2020). Subtypes displayed in red indicate findings that were unusual or unique to these results, the remainder in black represent the general *Blastocystis* host specificity based on published literature.

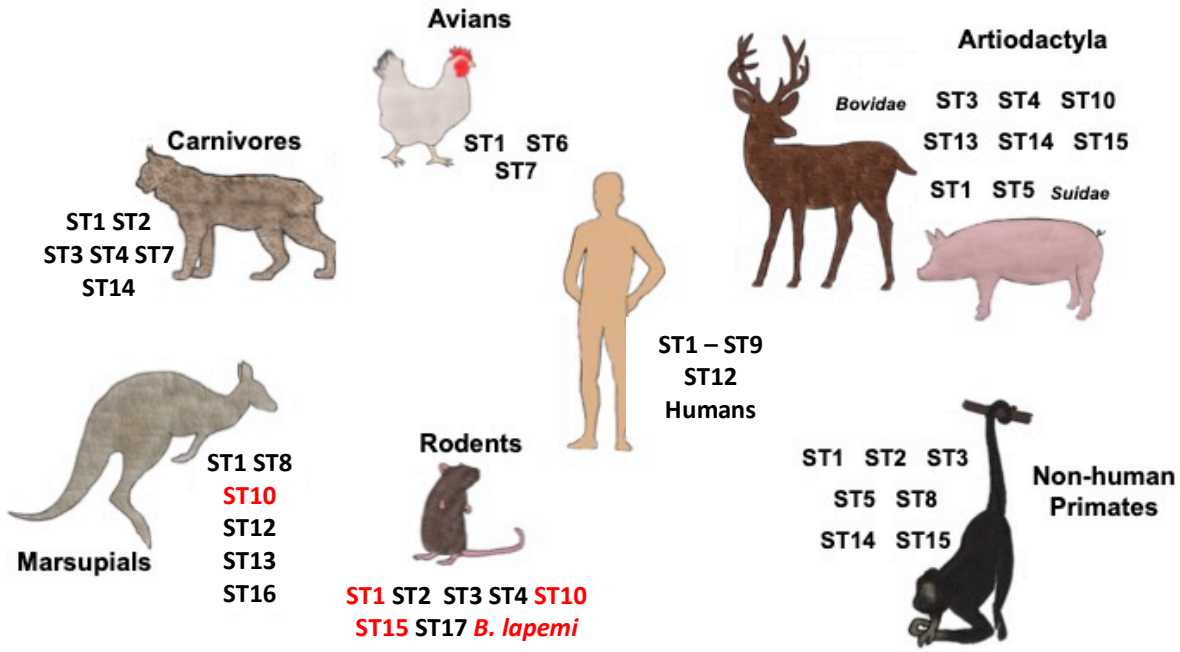

Figure 7.i *Blastocystis* subtype with various host specificities. Those in red represent unique or 'unusual' findings from the results of this thesis. Unusual results are based upon those that do not match prevalence findings of similar hosts in published literature. Adapted from Eukaryome impact on human intestine homeostasis and mucosal immunology (61-74), by A. Tsaousis, 2020, Springer, Copyright 2020 Springer Nature Switzerland AG 2020, adapted with permission.

Results from this investigation support the notion of *Blastocystis* having a relatively low prevalence or relative absence in carnivorous animals, with captive inhabitants having lower positive reports than wild/stray animals (Abe *et al.* 2002; Betts *et al.* 2018; Calero-Bernal *et al.* 2020; Cian *et al.* 2017; Paulos *et al.* 2018; Ramírez *et al.* 2014; Wang *et al.* 2013). Sporadic

reports have identified ST1 in the California sea lion (*Zalophus californianus)*, ST2 in the Cheetah (*Acinonyx jubatus*) and ST3 in Grey Wolf (*Canis lupus)* and Polar bear (*Ursus maritimus*) (Cian *et al.* 2017). ST1, ST3, ST4 and ST7 were identified in a range of carnivorous hosts including the Artic fox (*Vulpes lagopus)* (ST1, ST4 and ST7). The Grey wolf and Artic fox were included in our studies, yet both were negative for *Blastocystis* throughout the duration of study, but we were able to report *Blastocystis* from the Lynx (*Lynx* lynx) (ST2, ST14) and Scottish wild cat (*Felis silvestris*) (ST4, ST14) for the first time. Interestingly, ST14 was identified in both of these hosts and has been recently described in other carnivores (Calero-Bernal *et al.* 2020). It is speculated that the overall lack of positive identification in this group of animals may be a result of diet (Forsell *et al.* 2017). In captivity, the meat is often refrigerated and is unlikely to provide a source of infection. Furthermore, *Blastocystis* colonisation may in fact favour the gut microbiota of individuals with high fibre and/or vegetarian diets, and perhaps the differentially driven community enterotypes of meat orientated diets provide an unsuitable environment for *Blastocystis* colonisation. This theory would also support increased colonisation rates in other animal groups such as artiodactyls. However, this is largely based on speculation and loose associations between *Blastocystis* and certain bacterial communities and is a possible an avenue for future investigations (Forsell *et al.* 2017).

The subtype distribution in marsupials detailed here does not generally reflect that of other published reports which includes ST1, ST8, ST12, ST13 and ST16 (Parkar *et al.* 2010; Ramírez *et al.* 2014; Wawrzyniak *et al.* 2013). Additional sporadic accounts of different subtypes have been documented in marsupials, but this taxa remains poorly studied in comparison to others and thus further prevalence studies are needed before a host range can be confidently inferred (Roberts *et al.* 2013).

The lack of marsupial-related subtypes identified in our cohort may possibly be attributed to the small sample size of marsupials; only three samples were obtained from the wallabies (*Macropus rufogriseus*) of which there were a number free-roaming in a large paddock shared with pygmy goats (*Capra aegagrus hircus*). It is possible that the ST10 isolate identified from the wallaby and goat could be from the same source and a sequence alignment revealed over 99% similarity between the two; possibly indicating a transmission event between the two species at some point. ST10 has been identified in goats previously (Alfellani, Stensvold, *et al.* 2013) and is a common coloniser of artiodactyls, possibly indicating the goat was the original host of this subtype. This may present evidence of a

wider host range for ST10, which although is usually associated with artiodactyls, persisted asymptomatically in the wallaby population across multiple time points.

The artiodactyla represent a large reservoir for multiple subtypes. Numerous publications identify a broad subtype range with some limited host specificity, for example: ST5 is usually found is *Suidae* (W. Wang *et al.* 2014). These results largely support the current proposed subtype distribution with a high prevalence of ST10 and ST14 (Alfellani, Taner-Mulla, *et al.* 2013; Betts *et al.* 2018; Fayer, Santin and Macarisin 2012; Lee *et al.* 2018; Masuda *et al.* 2018; Ren *et al.* 2019). The high incidence of *Blastocystis* in the artiodactyla could be influenced by favoured diets or ecological conditions in the gut that support *Blastocystis*. Herbivorous diets have been linked to increased microbial diversity in the gut in animals and humans (Nishida and Ochman 2018; Tomova *et al.* 2019; Youngblut *et al.* 2019). In humans, it has been casually observed that *Blastocystis* has some positive association with high fibre and vegetable diets opposed with those constituting mainly of protein and fats (Forsell *et al.* 2017). Perhaps this association is further reflected in the lower prevalence of *Blastocystis* in high income countries which typically favour 'westernised diets' that are high in saturated fats, meat-based proteins and are low in fibre. Although other factors must also be considered including antimicrobial treatment, genetics and a reduced exposure to water-borne protozoan infection, it is likely that diet and the accompanying associated bacterial communities further influence the favourability of *Blastocystis* colonisation in the gut (Hills *et al.* 2019; Statovci *et al.* 2017). One could thus speculate that the increased microbial diversity, richness and dominating bacterial communities in herbivorous animals may then provide favourable conditions for *Blastocystis* colonisation, possibly explaining the high prevalence in the artiodactyla and NHPs and low prevalence in carnivores.

Within NHP centric studies of *Blastocystis*, a number of studies encompass captive and wild populations. This offers a unique opportunity to compare the impact captivity may have on *Blastocystis* colonisation. The prevalence recorded from these results: Gorilla (*Gorilla gorilla gorilla)* at 64% and Javan Gibbon (*Hylobates moloch*) at 85% are in accord with other published data of captive and wild primates; previous reports demonstrate that *Blastocystis* prevalence in NHP ranges from over 50% in a zoo in Australia (Parkar *et al.* 2010) and 59%-60.3% across two French zoos (Cian *et al.* 2017) to 85% in Japan (Abe *et al.* 2002) and 87.6% in Italy (Zanzani *et al.* 2016). Wild primate studies ranged from 38.6% (Villanueva-Garcia *et al.* 2017), 29%-64% in Guinea-Bissau (Sá *et al.* 2013), 60.4% in Ecuador (Helenbrook, Shields and Whipps 2015) and 79.9% in NHPs from a national park (Petrášová *et al.* 2011).

Our results also support the host specificity of *Blastocystis* within the NHPs, for example ST1-ST3 are commonly isolated from most NHPs including New World and Old World Monkeys and Apes. ST5 is often identified in African apes and ST8 from arboreal primates both in captivity and in the wild (Alfellani, Jacob, *et al.* 2013; Cian *et al.* 2017; Parkar *et al.* 2007; Parkar *et al.* 2010; Valença-Barbosa *et al.* 2019). Overall, the subtype diversity in published reports and the results here support the notion of a degree of host specificity that is not greatly impacted by captivity and may reflect the environment resembling that of their natural habitat (Oliveira *et al.* 2020). It should be noted however that ST1-ST3 are also human associated subtypes and thus it is possible that identified isolates may differ between captive and wild NHP populations due to the close contact with humans. Further work could expand the sampling from captive animals to encompass keepers working with the NHPs, as well as trying to expand sampling from wild counterparts. By including wild hosts and keepers we can better establish subtype similarity between captive vs wild animals in addition to identifying subtypes that may be the result of abnormal exposure due to captivity. Subtype isolates can be further investigated by including haplotype data where possible, which will aid in the assessment of subpopulations that are otherwise indistinguishable at the subtype level (particularly between humans and NHPs) and this may aid in determining the acquisition/transmission of particular isolates between humans and NHPs (Villanueva-Garcia *et al.* 2017).

Our results also report ST5 from the Javan Gibbon for the first time. This primate is an ape endemic to South-east Asia. The occurrence of ST5 in this population may indicate a wider scope of colonisation of this subtype than previously thought, which currently includes apes found solely in Africa (Alfellani, Jacob, *et al.* 2013; Cian *et al.* 2017; Santín *et al.* 2011; Wang *et al.* 2018). However, the impact of captivity and possibility of transient infection cannot be ruled out. Although these NHPs were sampled from multiple times, ST5 was found on one occasion in two different family groups, meaning further sampling is necessary before conclusions can be drawn about the long-term colonisation of this subtype in this host.

Rodents, namely water voles (*Arvicola amphibious*), which had not been previously characterised in *Blastocystis* prevalence studies represented a large portion of this study cohort. Due to involvement in breeding programs and re-wilding projects during the duration of the project, the voles represented a unique opportunity to study both the

transition of wild voles brought into captivity, and to monitor individuals housed separately which allowed for repeat sampling on an individual basis.

The sample population comprised of two main groups including those sampled from between 2016 and 2017 (Betts *et al.* 2018) which were involved in a capture, breeding and release program over the course of one year, and a group of 28 voles sampled from twice in 2019 (Betts, Gentekaki and Tsaousis 2020). The results revealed a high prevalence of *Blastocystis* ST4 in both the captive and wild caught voles. This subtype is commonly identified in rodents (Yoshikawa, Tokoro, *et al.* 2016) and these results support its defined host range and specificity. Interestingly, ST1 was only identified in captive voles and may represent an example of a captivity-acquired subtype as it was never identified from the wild voles which were captured from two separate locations over the entire sampling duration. ST10 was also found in one captive vole but was not isolated from subsequent collections and may be a transient infection as this subtype is not usually associated with rodents (Chai *et al.* 2020). ST15 and an isolate clustering in the *B. lapemi* clade were also identified. ST15 was isolated in a wild vole across a prolonged period of time revealing long-term colonisation, whereas *B. lapemi* was isolated from two voles at one collection point only, to our knowledge this is the first instance of a *B.* lapemi-like isolate being identified form a non-reptilian host (Teow *et al.* 1991). The source of infection is unknown as food, bedding and water for the voles all originates from the same source. Repeat sampling from this individual, sequencing of the entire 18S rRNA gene and establishing a culture for this isolate would help determine if it is *B. lapemi,* or possibly a new subtype as well assessing whether the isolate represented a transient infection.

Other rodents sampled included the Red squirrel (*Sciurus vulgaris),* which had not been previously identified as a host of *Blastocystis,* and Black and Brown rats (*Rattus rattus* and *Rattus norvegicus)*. Both rat species gave no positive results for any protozoa studied, they were housed in large group enclosures with an unknown number of inhabitants that likely exceeded 30. To our knowledge, the rats were not undergoing any anti-microbial treatment and their diet was largely herbivorous, thus the lack or protozoal identification was somewhat surprising, especially since rats are often identified as hosts in prevalence studies (Chai *et al.* 2020; Cian *et al.* 2017; Katsumata *et al.* 2018; Ramírez *et al.* 2014). A possibility for the negative identification may be due to their enclosures being exclusively inside. All other animals were housed in enclosures that included both indoor and outdoor areas which

were likely exposed to wild animals and birds and had natural vegetation which may provide sources of infection.

The majority of positive *Blastocystis* sequences grouped within existing clades of previously published subtypes, and most of these mirrored published host-range data; with the results presented here supporting existing evidence of host specificity and host ranges (Cian *et al.* 2017; Parkar *et al.* 2010; Wawrzyniak *et al.* 2013). The Elk (*Alces alces*) initially gave rise to an unresolved *Blastocystis* isolate (Betts *et al.* 2018), however when included in an updated phylogenetic tree which contained additional samples and more reference sequences, including more basal sequences including those representing reptiles, insects, ST15, ST16 and ST17, its position was resolved and placed in ST14 (Accession: MF186665.1). A positive isolate from the muntjac (*Muntiacus reevesi*) also underwent a rearrangement from ST14 to ST13 (Accession: MF186700.1). These rearrangements highlight the importance of monitoring advances in the field as well as the general ambiguity and unclear distinction between the boundaries of subtypes. This is further amplified by phylogenetic trees being based on partial (18S rRNA) genes, thus limiting our appreciation and understanding of the distinctions between subtypes. Furthermore, under-sampled clades are the most poorly resolved, making accurate phylogenetic analysis of these problematic; this has been highlighted in a number of reports, for example, Alfellani et al. speculated that ST14 may need to be split into two subtypes (Alfellani, Taner-Mulla, *et al.* 2013). Data obtained in Chapters 2 and 3 further demonstrate this obscurity and support continued sampling from underrepresented populations and depositing findings into sequence databases will benefit subtype resolution in the future.

1.2 Co-colonisation of *Blastocystis* subtypes

Mixed subtype infections are documented intermittently across human populations and are often identified in animals (Cian *et al.* 2017; Tito *et al.* 2019). Our results revealed 24% (9/38) species were positive for co-colonisation with two or more *Blastocystis* subtypes. The distribution of these subtypes generally fit host specificity patterns as detailed in the introduction (Ramírez *et al.* 2014; Valença-Barbosa *et al.* 2019; Wang *et al.* 2018; Wawrzyniak *et al.* 2013).

ST4, ST10 and ST14 were isolated form the elk, of which there was only one possible host, subsequent sampling throughout the project duration revealed long-term colonisation with

ST14, the other two subtypes were only identified once and thus cannot be ruled out as transient infections.

The co-colonisation from the pygmy goat samples came from two samples across one collection. Although three subtypes were isolated, there were multiple goats per enclosure meaning that the subtypes may be spread across multiple hosts. It was found that one goat was positive for ST1 and ST10 and the other ST14, these goats share the same pen, food and water source as the wallabies, possibly providing interspecies transmission events, and may explain the unusual occurrence of ST10 in the wallabies, as detailed above.

The red deer (*Cervus elaphus)* was sampled across two time points but was only positive for ST4 and ST10 in the first sampling despite there being multiple faecal samples collected. Therefore, it cannot be determined if infection was long-term and if the identified subtypes were consistent throughout the herd as sampling from an individual was not possible. It would be interesting to examine if the herd shared a particular subtype pattern which may circulate via vertical and horizontal transmission as, to our knowledge, this has not been investigated previously within herd communities.

The water voles represented an interesting study of co-colonisation. The PP0- and TB- cohorts were sampled bi-monthly after being brought into captivity and this allowed the unique opportunity to monitor *Blastocystis* prevalence during the transition into captivity. Upon introduction into the conservation park, voles from both wild collection areas were only infected with ST4; a common rodent associated subtype (Wawrzyniak *et al.* 2013). The voles were kept in a quarantined area during their time in captivity, yet ST15 was also identified in two of the wild caught voles on three occasions. The quarantined area consisted of individual, separated enclosures positioned outdoors and away from the rest of the park and the general public. Keepers used sterile, disposable gloves and aprons when cleaning and feeding the voles, yet there were opportunities for other wild animals and birds to access the enclosures and possibly provided a source of transmission; this was documented on numerous occasions where other small rodents had invaded the enclosures. Conversely, the captive voles had a larger subtype diversity range which included ST1, ST4 and ST10 from the first sample cohort, with one vole positive for all three subtypes. ST1 and ST10 are not generally associated with rodents and may be the result of captivity-acquired infections, even though all of the voles are in a quarantined area (Betts *et al.* 2018; Mohammadpour *et al.* 2020; Wawrzyniak *et al.* 2013; Yoshikawa, Tokoro, *et al.* 2016). Later collections from a

different captive cohort revealed a wider subtype range of *Blastocystis* with ST4 and an isolate clustering with *B. lapemi* being identified from one host.

The diversity presented in the vole cohort supports the concept of repeat sampling and culturing to aid in *Blastocystis* identification. Unfortunately, culturing was not possible for all samples here due to the volume of samples collected. It has been previously proposed that an initial *Blastocystis* screen and then subsequent culturing of those that are PCR positive is a beneficial screening method and may be something to consider in future studies (Leelayoova *et al.* 2002; Stensvold, Arendrup, *et al.* 2007; Termmathurapoj *et al.* 2004).

The Scottish wild cats' (*Felis silvestris silvestris*) samples comprised of a family with parents and kittens living in the same enclosure and were sampled from across multiple time points. Of the 13 samples, only one sample was sequence positive with ST4 and ST14. *Blastocystis* is relatively uncommon or absent in carnivores, and the subtype range is largely unknown (Alfellani, Taner-Mulla, *et al.* 2013; Calero-Bernal *et al.* 2020; Cian *et al.* 2017). The lynx housed in the same park was found to be co-colonised with ST2 and ST14. Mixed subtype colonisation in carnivores is recorded sparsely (Calero-Bernal *et al.* 2020) and an alignment of the ST14 isolates revealed a 98% sequence similarity, likely indicating separate isolates. As mentioned above, the meat fed to these animals is an unlikely source of infection, however the enclosures for both animals are housed within a wooded area where small animals, birds and rodents could possibly enter and be eaten. This offers a reasonable explanation for an infection source of ST2 and ST4, yet ST14 is generally restricted to larger hosts. Repeat screening possibly over consecutive days may provide answers as to whether colonisation was established and would also account for intermittent shedding.

Focusing on a different family group, *Blastocystis* was sampled from a Bongo (*Tragelaphus eurycerus)* mother and calf living together and father who was housed separately. All three were positive for ST14 and the mother and calf were also co-colonised with ST10 and ST14. Sequence similarity between the Mother and Calf for ST10 and ST14 revealed similarities of over 99% for both subtypes. The ST14 similarity between the mother and father was 100% and was therefore likely transmitted before the mother and father were separated. This subtype likely established a long-term infection and could have been transmitted vertically to the calf.

These results also showed that both the gorilla and gibbons accounted for a high percentage of the mixed subtype colonisation data and presented an interesting case as separate family groups were included in this study. In total, four gorillas were positive for mixed subtypes with either ST2 and ST3 or ST2 and ST5. The ST2 and ST3 colonisation was identified in one family across both collection times and the other in one family in one collection. Although mixed colonisation was observed, it cannot be determined for sure that the faecal sample represented one individual. However, multiple samples from each enclosure were obtained in order to create an accurate representation of the subtype diversity within the family. Between families (which are housed in separate enclosures), the genetic difference between isolates was minimal or absent indicating the same isolates persist among the different families irrespective of their separate accommodation and are possibly transmitted by the keepers or from a food source.

The gibbons were one of the most often identified hosts of *Blastocystis,* mixed subtypes were identified across three family groups which ranged from two individuals (groups C, E and F) to four individuals (groups B and D) and five individuals (group A). Family C was the only one to yield consistent colonisation of ST1 and ST2 between collections, family A identified ST1, ST5 and ST8 on the first collection but ST1 and ST3 on the second and family B had ST5 and ST15 on the first and ST1 and ST2 on the second. The disparity may be due to sampling from different individuals within a family as correlating faecal sample to a given host was not possible here, meaning the extent of co-colonisation is unknown. However, the subtypes identified within the NHP primate populations coincide with that of other published works (Alfellani, Jacob, *et al.* 2013).

Investigations of co-subtype colonisation can likely be improved by a number of methods. Firstly, subsequent continual collections over multiple days can minimise impact of possible intermittent shedding, however this may not always be feasible. The use of culturing prior to screening samples could also improve sensitivity, as it may help identify subtypes that were not identified by cloning methods and could be useful to consider in future studies, but does introduce the possibility of preferential growth of certain subtypes and loss of presently uncultured subtypes in mixed infection samples. Additional procedures could include qPCR and metagenomics-based identification. qPCR is often employed for *Blastocystis* identification technique and provides a quantitate method of detection but is currently limited to certain subtype (Forsell *et al.* 2017; Stensvold *et al.* 2012). A

metagenomics-based approach could prove beneficial for providing an unbiased, broad identification technique for a range of protozoa. This method has recently been used to identify intestinal protozoa, including *Blastocystis* from swine, in which subtyping was also achieved (Wylezich *et al.* 2019).

1.3 Co-parasitism of *Blastocystis* and other protozoa

Co-parasitism of *Blastocystis* with other intestinal protozoa has been documented throughout the literature in humans and animals (Forsell *et al.* 2016; Osman *et al.* 2016; Osman *et al.* 2015; Sánchez *et al.* 2017). The animals investigated here were screened for a range of intestinal protozoa including *Cryptosporidium* sp.*, Entamoeba* sp. and *Giardia* sp.. Results demonstrate a relatively high incidence of co-infection with at least one additional protozoon. The prevalence of these protozoa among the study cohort was not unusual, for example *Entamoeba* sp. is commonly found in members of the artiodactyla, and these isolates are likely *E. dispar* and *E. bovis* related species (Barbosa *et al.* 2020; Stensvold, Lebbad and Clark 2010). The identification of *Cryptosporidium* sp. and *Giardia* sp. in this cohort had all been documented in the same or similar species previously (Helmy *et al.* 2018; Jiménez Martínez *et al.* 2018; Sak *et al.* 2013; Tangtrongsup *et al.* 2019).

All animals were asymptomatic at the time of sampling indicating that the presence of these microbial eukaryotes were not causing symptomatic disease, however symptoms prior and after this investigation are unknown meaning that disease before or after sampling cannot be ruled out. These animals also present possible sources of zoonotic infection; especially to keepers who come into close frequent contact with them. Determining the source of these infections in the animals was not possible, however all animals (except the wild caught water voles) had all been in captivity for a prolonged period of time, possibly indicating that these infections were either contracted in captivity, for example through interaction with wild animals or birds, via contaminated food and drink or from the keepers and may have persisted for a prolonged period in these hosts. Future monitoring of these protozoa and further 'omics-based studies investigating the impacts of these mixed infections on the gut, via monitoring bacterial diversity and assessing metabolite profiles in infected and uninfected hosts may shed some light on the roles co-colonisation can have on the microbiota and host.

To further investigate *Blastocystis* within the microbiota, a metagenomics-based approach was used on a cohort of water voles monitored over two collection points (Chapter 4) and discussed below.

2 Influence of *Blastocystis* on gut microbial community abundance

In the introduction, the associations between *Blastocystis* and the bacterial gut microbiota were summarised. It was hypothesised that there were some casual relationships between *Blastocystis* colonisation and the abundance (or lack thereof) of certain bacterial communities in humans (Andersen *et al.* 2015; Andersen *et al.* 2016; Audebert *et al.* 2016; Beghini *et al.* 2017; Forsell *et al.* 2017; Kodio *et al.* 2019; Nash *et al.* 2017; Nourrisson *et al.* 2014; Tito *et al.* 2019; Yason *et al.* 2019). Some studies documented positive correlations between *Blastocystis* and a number of bacteria ranging from the phylum to species level (Andersen *et al.* 2015; Andersen *et al.* 2016; Kodio *et al.* 2019). Currently, limited studies investigate bacterial community abundance and *Blastocystis* in animals (Renelies-Hamilton *et al.* 2019), thus direct comparisons between the work presented here has, in many instances, been compared to human and murine studies under the assumption that there are intrinsic similarities and differences between different host species (Ley *et al.* 2008; Nguyen *et al.* 2015; Wang *et al.* 2019). The results documented a decrease in the overall abundance of a number of bacteria, none of which were associated with *Blastocystis* as summarised in Chapter 1 (Andersen *et al.* 2015; Andersen *et al.* 2016; Audebert *et al.* 2016; Beghini *et al.* 2017; Forsell *et al.* 2017; Kodio *et al.* 2019; Nash *et al.* 2017; Nourrisson *et al.* 2014; Tito *et al.* 2019; Yason *et al.* 2019). The decline in certain bacteria included a number belonging to the Firmicutes phylum and also Spirochetes (in particular *Treponema*) which has been reported to have roles in plant material degradation in the rumen and has been found in voles (Bekele, Koike and Kobayashi 2011; Bo *et al.* 2019) and is enriched in the gut of rural humans and wild apes (Angelakis *et al.* 2018; Bittar *et al.* 2014). The relative decline in these taxa across *Blastocystis* infected voles may indicate an impaired ability for these voles to degrade fibrous foods, yet no gastro-intestinal symptoms were observed, possibly indicating that this was not impairing the voles to a noticeable extent.

The cause of such changes within the microbiota cannot be known for sure. Both direct and indirect associations with *Blastocystis* may account for the observed results. For example, the amoeboid form of *Blastocystis* has been shown to consume bacteria *in vitro* (Singh *et al.* 1995) and one could reasonably assume that this may be the case in the gut too. A decline in bacteria community abundance may also be an indirect consequence of inflammatory

responses caused by the host in response to infection which can promote a dysbiotic state in the gut (Wang, Chen and Wang 2020). Additionally, the infecting subtype or isolate may play a role in influencing the microbiota (Yason *et al.* 2019). Although the role of *Blastocystis* in the gut is largely unknown, evidence exists for both modulation of inflammatory cytokine production as well as induction of low-level inflammation in the gut (Iguchi *et al.* 2009; Long *et al.* 2001; Stark *et al.* 2007). Therefore, it would not be unreasonable to speculate that these bacteria are affected by disturbances in homeostasis caused by host immune responses or from direct interactions with *Blastocystis* (and other protozoa) (Barash *et al.* 2017; Kirkpatrick *et al.* 2002; Long *et al.* 2001). Another possible explanation is that a decline in certain bacterial species could facilitate *Blastocystis* infection/colonisation via competition for ecological niches.

Unfortunately, the majority of bacteria identified in this research have currently uncharacterised roles in the gut, thus the implication of their altered abundance is unknown. Although direct consequences of changes in these community profiles cannot be determined in the vole cohort, generally a reduced microbial diversity is associated with dysbiosis (Manichanh *et al.* 2006; Ott *et al.* 2004; Wohlgemuth *et al.* 2009). By taking this approach, the results may indicate that the presence of *Blastocystis* is associated with a decrease in bacterial diversity. Whether this is because *Blastocystis* is causing this change or just preferentially inhabits the gut of those voles is unknown. By applying a 'top-down' ecological approach it could be presumed that *Blastocystis* does influence bacterial communities, but this is merely speculation. As stated above, the lack of knowledge regarding the roles of the bacteria and microbe-host interactions limits what inferences can be drawn, as although these bacteria are reduced their absence may not negatively impact the host.

3 Limitations of molecular epidemiological and co-abundance studies

The work conducted as part of this thesis provides an in-depth record of the prevalence, diversity and co-parasitism of *Blastocystis* in the gut across a range of taxa. However, perhaps the most confounding limitation of this work, from a conservation point of view, is the fact that these hosts represent a relatively small sample size of captive populations. This is of importance for a number of reasons, for example, the usefulness of subtype

identification is limited as the potential for abnormal subtype acquisition (i.e. from keepers, or close proximity to other animals) likely influences subtype diversity in captive populations. Thus, the results of this work may not represent the diversity and breadth of *Blastocystis* in wild (free living) animals. Many epidemiological studies of this nature are limited to captive cohorts, and although there is an increase in free-living animal studies (Calero-Bernal *et al.* 2020; Renelies-Hamilton *et al.* 2019; Wang *et al.* 2018), it is often difficult to obtain fresh, non-contaminated faecal samples from wild animals. Additionally, sampled animals from captive cohorts generally represent species found across the world; obtaining the same taxon diversity in wild animals would likely be unfeasible and therefore limited the scope to a few taxa. Furthermore, if wild samples were to be obtained the ability to sample repeatedly from them would be near impossible, meaning captive populations an attractive model for monitoring known individuals in controlled environments.

Herein, the identification of *Blastocystis* and other intestinal protozoa was largely carried out by molecular approaches (Betts *et al.* 2018; Betts, Gentekaki and Tsaousis 2020). The use of short-term culturing is considered to be useful in *Blastocystis* screening studies to help increase sensitivity when accompanied by molecular characterisation (Leelayoova *et al.* 2002; Stensvold, Arendrup, *et al.* 2007; Termmathurapoj *et al.* 2004). Initially, we attempted to sub-culture faecal samples using TYM and LYSGM at either room temperature or 37^{o} C, however the large number of samples proved problematic and unfeasible to maintain long-term and quickly became overgrown as antibiotics were not used. Culturing was again attempted using the water vole cohort (at room temperature) and proved useful in the identification of mixed subtype infections that were not identified from those characterised straight from the faecal sample, signalling the possible usefulness of employing this approach. Perhaps, if repeating such work with a large sample cohort, an initial PCR screen and subsequent culturing of positive samples or short-term culturing of all samples at a temperature that reflects the host before PCR would help resolve the issue of large culture collections.

In order to screen for multiple subtypes, we employed the use of cloning PCR products and up to 10 positive clones sent for sequencing for each positive PCR result. This method helped identify multiple-subtype infections, but is likely influenced by subtype abundances within the faecal sample meaning there is possibility that subtypes were missed. Nevertheless, this is still a beneficial method to use as it filters out chimeric sequences which are often mistaken for novel subtypes (Stensvold and Clark 2020).

For unresolved subtypes, we followed the strategy reported in Stensvold and Clark in 2020 and sampled from the hosts again, in addition to sequencing entire SSU rRNA gene (Stensvold and Clark 2020). Unfortunately, additional sampling was only capable from a few hosts and some lived in large family groups meaning individual identification was not possible since some of the voles had been released into the wild.

4 Roles of *Blastocystis* in the gut

To expand the work carried out involving the prevalence of *Blastocystis* and associations with the bacterial microbiota, further 'omics-based approaches were employed in Chapters 5 and 6 in order to investigate the impact of *Blastocystis* infection on the metabolome and investigate *Blastocystis'* responses to oxygen. Firstly, *Blastocystis* in the gut of infected and uninfected humans was explored using metabolomics; this is an approach that has not currently been applied to *Blastocystis* research. Secondly, the impact of oxygen treatment on this protozoan was assessed to aid in the understanding of its responses to oxygen, which in turn could be linked to its ambiguous roles towards IBD.

4.1 Metabolic profiles of *Blastocystis* infected cohorts

The metabolome refers to the collection of small molecules found in a biological sample which can display the phenotypic changes in an environemnt and provides an attractive method for drug-discovery in addition to investigating disease biomarkers (Wishart 2007; Zhang, Sun and Wang 2017). To date parasitological based metabolomic studies encompass *Cryptosporidium* (Hublin *et al.* 2012; Miller *et al.* 2019), *Giardia* (Allain *et al.* 2018) and *Entamoeba* (Jeelani *et al.* 2012), however, currently there are no published reports using this method to investigate *Blastocystis.*

In Chapter 5, faecal metabolite profiles from *Blastocystis* infected and uninfected humans were investigated in order to determine the impact of infection on the metabolome and the possible consequences that may arise from this.

Overall, the data from both countries highlights a distinct metabolome profile associated with infection, which is characterised by a shift in observed metabolites and a general 'clustering' effect of the infected profiles. This observation was further verified using supervised methods of statistical analysis. The distinct metabolome profile provides an interesting case as it highlights a divergent pattern observed only in infected samples. This

shift could reflect a change in metabolites caused by a number of factors, for instance certain metabolites may be associated with the body's response to infection/colonisation. Another possibility is shifts in microbial communities associated with *Blastocystis* in the gut which produce different metabolite phenotypes. Furthermore, a decline in certain metabolites may be the result of scavenging by *Blastocystis* or may be increased as a direct result of production by *Blastocystis*.

An investigation into these distinguished metabolites revealed a number of similarities between the infected groups between the two countries, in addition to a number of dissimilarities. Unfortunately, an inherent issue with metabolomics-based studies, especially in faecal samples, is the lack of homogeneity within samples. Published reports highlight a number of influences that impact metabolome of faecal samples (Smith *et al.* 2020). Thus, the data is unlikely to provide a direct response of infection but rather the impact of *Blastocystis* in relation to the gut environment as a whole; yet the variance between infection status implies a difference that possibly impacts the entire gut environment.

Of the identified metabolites, key interest was paid into assessing their roles are biomarkers for eubiosis and dysbiosis, as such data can greatly help contribute to the debated questionable pathogenicity of this protozoan. Interestingly there was a general lack of pro-inflammatory metabolites, which includes amino acids and inflammatory related metabolites.

An increase in amino acids can be associated with impaired ability of absorption in the gut, likely due to inflammation as described in humans with IBD (Bosch *et al.* 2018), however the results documented a significant decrease in a number of amino acids. The decline cannot be ruled out as a result of nutrient scavenging, which has been reported in *Blastocystis.* This is especially relevant as a number of the biosynthesis pathways for these amino acids and other nutrients are absent or currently uncharacterised (Gentekaki *et al.* 2017; Zhang and Rubin 2013). The possibility of *Blastocystis* scavenging nutrients may account for the decline in a number of metabolites, and this in turn may promote a competitive environment, not just between the microbiota and *Blastocystis* but also between *Blastocystis* and the host. By taking this into account, although the decrease in amino acids may indicate a lack of inflammation, the competition for nutrients may mean that significant proliferation of *Blastocystis* in the gut could cause harm by depriving the host and gut flora of essential nutrients. This would be interesting to examine in future studies, where metabolomics coupled with qPCR to quantify *Blastocystis* infection could be used to determine if the

parasite load contributes to pathogenicity which has been documented previously (Seguí *et al.* 2017).

That being said, there was also a lack of other pro-inflammatory biomarkers in infected individuals such are primary bile acids and sphingolipids (Ahmed *et al.* 2013; Franzosa *et al.* 2019; Lavelle and Sokol 2020; Marchesi *et al.* 2007; Zhgun and Ilina 2020). Furthermore, a lack of pro-inflammatory metabolites was supported by pathway analysis data which identified a decrease in the histidine metabolism pathway in infected individuals. This was accompanied by a decrease in histamine, which can act as a pro-inflammatory metabolite in the intestine (Smolinska *et al.* 2014).

In fact, a number of the identified metabolites from the pathway and univariate analysis may elicit positive effects in the gut and are associated with gut health. This included l-arginine, l-glutamine and propanoate. Thus, the data here may indicate that *Blastocystis* infection has a positive association with gut health. Although the mechanisms for this warrant further investigation, such positive associations could be a direct response to the presence of *Blastocystis* – which may synthesise these metabolites directly or may aid in the establishment of bacterial communities that produce the aforementioned metabolites. However, the association of *Blastocystis* and eubiotic biomarkers may also be the result of *Blastocystis* preferentially colonising the gut which contains such bacterial communities anyway. For example, *Blastocystis* is not thought to be associated *Bacteroides*-dominated enterotypes in the gut due to them not being main butyrate producers, which helps maintain anaerobic conditions, thus leading to less-than-optimal conditions for *Blastocystis* to persist (Stensvold and van der Giezen, 2018).

Repeating this study with additional metatranscriptomics-based work investigating gastrointestinal microbiome gene expression patterns between infected and uninfected humans could further clarify intestinal physiology and gene regulation of the host and *Blastocystis.* This could accompany published reports in aiding the identification of phenotypic metabolites associated with *Blastocystis* directly (Eme *et al.* 2017; Gentekaki *et al.* 2017; Sharma *et al.* 2019).

Additionally, including subtype information and comparing metabolomes of different subtype infections could aid in identifying links between pathogenic and non-pathogenic subtypes, which is a likely contributing factor to the debated pathogenicity (Nagel *et al.* 2012; Roberts *et al.* 2013). Furthermore, an investigation of asymptomatic and symptomatic

infected individuals would offer an attractive model to study roles of *Blastocystis* in symptomatic disease, however here it was not possible to draw comparisons as the symptomology differences were between country only and not within.

Overall, although the results have shown a metabolome that is significantly altered in infected individuals, yet this may not be a cause for alarm. There was an overall absence of pro-inflammatory and dysbiotic biomarkers and instead, infection was associated with some possible eubiotic metabolites possibly indicating a link between *Blastocystis* and positive gut health.

4.2 *Blastocystis* and oxygen stress

Blastocystis has a questionable role in the gut and current 'omics studies have highlighted possible virulence factors and adaptations to the gut, some of which were obtained through lateral gene transfer (Eme *et al.* 2017; Gentekaki *et al.* 2017). In Chapter 6, the aim was to investigate genes that had differential expression as a result of increased oxygen. Prior reports document a number of such proteins including alternative oxidase, dimeric iron-containing superoxide dismutase, thioredoxin reductase and 2-Cys peroxiredoxin, alcohol dehydrogenase and SufCB (Denoeud *et al.* 2011; Eme *et al.* 2017; Tsaousis *et al.* 2012). Such an investigation may help elucidate mechanisms used that allow it to persist in the dysbiotic gut which may be experiencing fluctuations in levels of oxygen, as thought to occur in IBD.

The results from xenic cultures of ST3 and ST4 reveal a number of pathways possibly differentially regulated as a response to oxygen stress, including some that have been identified in other intestinal protozoa (Vonlaufen *et al.* 2008). Unfortunately, the majority of these demonstrated incomplete or mixed gene expression highlighting the need for further work in order to determine the extent of their role in oxygen stress. Identified pathways that may have been upregulated in *Blastocystis* included; i) the pentose phosphate pathway that can control oxidative stress by NADPH (Perl *et al.* 2011); ii) the putrescine synthesis pathway which may act to reduce oxygen species in bacteria (El-Halfawy and Valvano 2014) and iii) glutathione biosynthesis that could offer protection against oxidative stress in both protozoa and bacteria (Million *et al.* 2020; Vonlaufen *et al.* 2008). A number of additional pathways were downregulated as a result of oxygen treatment and mainly involved lipid, carbohydrate, glycan, cofactor and vitamin metabolism. A possible reason for

this may be due to unfavourable redox states of the cells and reactive oxygen species interference, however an assessment of this needs further investigation in *Blastocystis* (Allen and Tresini 2000; Chang *et al.* 2012; Siauciunaite *et al.* 2019).

The transcriptomics work was accompanied by NMR metabolomics in the hopes of identifying phenotypic outcomes of alterations in the transcriptome in metabolites from the culture environment. Unfortunately, an investigation into each metabolite revealed none had a direct association with the differentially regulated pathways, including a possible build-up of metabolite intermediates. The metabolites identified herein were most likely a product of the bacteria in the culture medium which unfortunately could not be filtered out.

A total of four of the identified metabolites had possible antioxidant properties (Aldini *et al.* 2018; Azam *et al.* 2014; Liu *et al.* 2019; Oxenkrug 2005) yet, characterization of these in *Blastocystis* was largely unknown. Initially this experiment was trialed on axenic cultures of the *Blastocystis* isolate NandII which would exclude the issue of bacterial contamination. This was in addition to it being an attractive isolate to work with due to the prior published transcriptomic data (Gentekaki *et al.* 2017). Unfortunately, a successful RNA extraction was not achieved.

Although, oxygen treatment on *Blastocystis* revealed a number of differentially expressed pathways, the usefulness of this data was largely limited due to incomplete and inconsistent data. It has been experimentally proven, however, that *Blastocystis* does employ certain strategies to protect itself against oxygen (Tsaousis *et al.* 2018) possibly in an attempt to survive oxygen fluctuations that may occur in the dysbiotic gut. Further work on this possibly employing gene knock-out approaches to individually investigate genes of interest or using an organoid gut system may provide more meaningful data.

5 Overall conclusions and future directions

Blastocystis is a globally distributed protozoan which has been of increasing interest in recent years largely due to its debated role as a pathogen. This thesis aimed to investigate two main areas of *Blastocystis* research (Figure 7.ii), firstly the prevalence was investigated to develop our current understanding of subtype diversity and host range. Results generally fit that of published work and also expanded the host range to include novel hosts.

Blastocystis co-abundance with bacterial communities in water voles was then investigated in order to identify possible bacterial communities that are associated with *Blastocystis*. Here, it was found that the overall species richness was not impacted by infection, yet *Blastocystis* did exert some modulation over certain taxa. Some of these were associated with essential roles in the gut of these rodents including dietary-fibre metabolism, possibly indicating a negative outcome of *Blastocystis* infection and the opportunity for a shift towards dysbiosis.

The *Blastocystis* 'omics-based work was subsequently built upon further by the use of metabolomics and transcriptomics. These approaches provided novel and useful insights into the altered metabolome of *Blastocystis* infected/colonised humans and responses of *Blastocystis* to oxygen stress. Interestingly, the metabolome of infected humans demonstrated an apparent lack of dysbiotic biomarkers. This could possibly indicate that although infection caused a shift in the metabolite phenotype, infection may be associated with eubiosis due to the increase in a number of potentially beneficial metabolites linked to gut positive gut health.

Lastly, *Blastocystis* was investigated under oxygen stress in order to determine mechanisms employed by this protozoan to combat fluctuating oxygen. A number of possible oxygen-stress related pathways were identified herein, however these results are preliminary and provide a foundation for further work in this area.

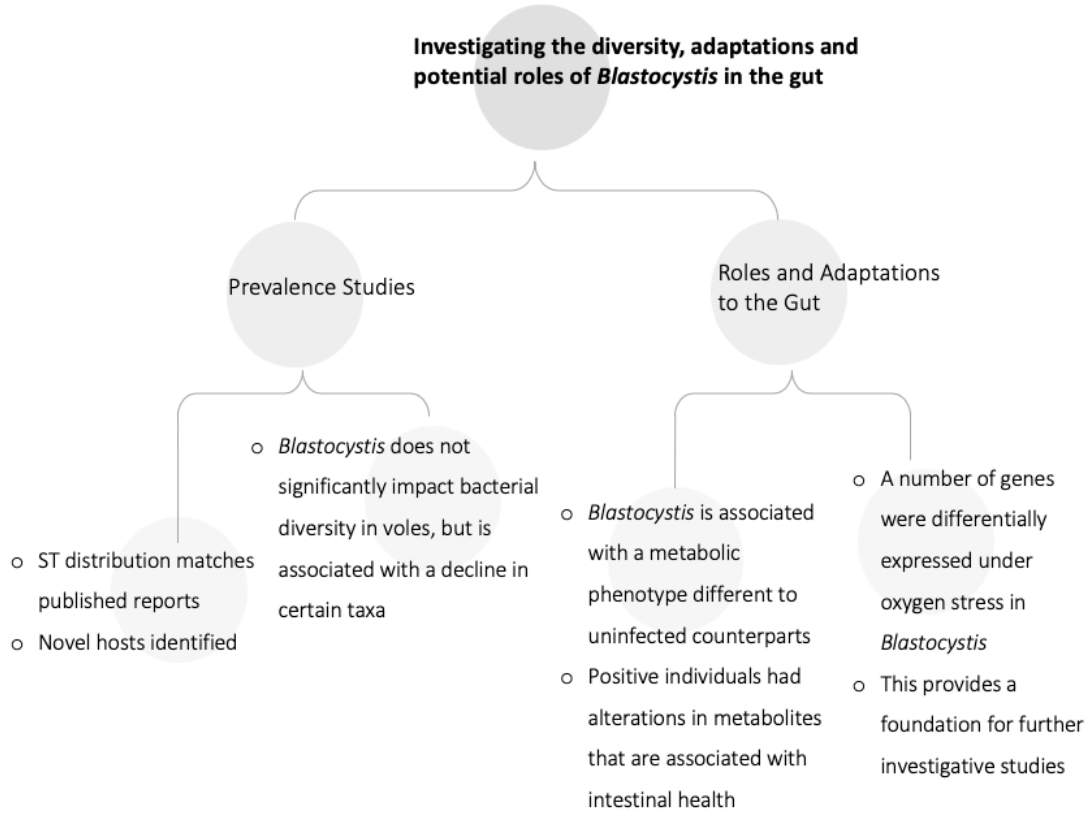

Figure 7.ii Diagram summarising overall findings of this Ph.D.

There are a number of questions about the roles of *Blastocystis* in host health and disease that still remain unsolved. Through epidemiological studies of this protozoan, a general pattern has emerged regarding its global prevalence in which animals offer a reservoir for infection. Although the subtype diversity has been relatively well established, there is no doubt that other subtypes remain to be discovered, meaning that continued sampling from a range of taxa - especially those representing understudied population (reptiles, amphibian and insects) is imperative. Furthermore, continued sampling will aid in the resolution of under sampled clades and provide better resolution on basal groups.

One of the most widely debated topics regarding *Blastocystis* is its pathogenicity. Although numerous studies document symptomatic disease, there is an equal number that propose infection/colonisation causes an increase in bacterial diversity and has a positive impact on gut health. Thus, further work needs to be carried out to better elucidate its roles in gastrointestinal health. A possible way to do this is by further metabolomics-based studies which are accompanied by an assessment of the accompanying bacterial microbiota. Considering the complex relationships and feedback loops between microbiota and host. It is sensible to investigate *Blastocystis* infection/colonisation in relation to the sorrounding microbiota and host collectively as this likely influences infection status and disease outcome. Moreover, by including subtype information in such work could also provide an insight into the possibility of pathogenic subtypes which may help to explain the disparity in its current pathogenic status.

Finally, *Blastocystis* is often associated with inflammatory bowel disease, yet the mechanisms contributing to infection are largely uncharacterised. In addition, the proliferation of facultative anaerobes provide indication that oxygen levels in the gastrointestinal tract are elevated, meaning that the persistence of *Blastocystis* challenges its strict anaerobic status. Further work needs to be done to clarify the ability of *Blastocystis* to withstand oxygen, including further transcriptomic-based studies to clarify its currently unknown roles towards IBD.

Chapter 8. Methods and Materials

Herein, the methods used in this thesis including those presented in publications are described thoroughly.

1 Microbiology techniques

1.1 Media

All media was prepared with ultra-pure deionised water (dH_20; Thermo scientific Barnstead NanoPure Diamond System). All media and other liquid sterilisations were carried out using a bench top Prestige Medical autoclave at 121°C unless stated otherwise.
In many cases media was supplemented with horse serum (Gibco), which was heat inactivated before use by placing it in a water bath at 56 °C for 30 minutes.

1.1.1 LB media (Bertani 1951)

Tryptone (Oxoid)	1% w/v
Yeast extract (Oxoid)	0.5% w/v
NaCl (Fisher)	1% w/v
Agar (SLS) for solid media	1.5% w/v

1.1.2 Iscove's Modified Dulbecco's Medium (IMDM) (Ho *et al.* 1993)

IMDM stocks were prepared directly by adding powdered Gibco Iscove's Modified Dulbecco's Media (Thermo scientific) to 900 ml autoclaved dH_2O and filter sterilizing via a 0.2 µM vacuum driven bottle filter. 100 ml of heat inactived horse serum was added and media was stored at 4 °C for a maximum length of one month.

1.1.3 Stone's modification of Locke's solution (Zierdt *et al.* 1988)

NaCl (Fisher)	0.8% w/v
$CaCl_2$ (Melford)	0.02% w/v
KCl (Melford)	0.02% w/v
$MgCl_2$ x $6H_2O$ (Melford)	0.001% w/v
Na_2HPO_4 (Melford)	0.2% w/v
$NaHCO_3$ (Melford)	0.04% w/v
KH_2PO_4 (Melford)	0.03% w/v
Heat inactivated horse serum	25% v/v

1.1.4 Egg Medium

Per fresh egg, 12.5 ml of modified Locke's solution was added and homogenised for a minimum of 1 hour with a stirrer under sterile conditions. The homogenised mixture was filtered through a gauze in a funnel into a vacuum flask. This was then placed under vacuum for a minimum of three hours. The media was aliquoted into sterile 125 mm screw capped glass test tubes with a total volume of 4 ml per tube and inspissated at 80 °C for 30 minutes at a 30 ° slant. Solidified slants were removed from the oven and cooled before a 4.5 ml modified Locke's solution overlay was added. Sealed tubes were autoclaved, and when cooled, 25% heat inactivated horse serum was added before storage at 4 °C (Zierdt *et al.* 1988).

1.1.5 Modified Jones' Medium (Clark and Stensvold 2016; Jones 1946)

Separate stock solutions of each Na_2HPO_4 (66 mM), KH_2PO_4 (67 mM) and NaCl (154 mM) were made with dH_20 and autoclaved. Working stock was as follows and uses these stock concentrations:

Na_2HPO_4 stock (Melford)	13.6% w/v
KH_2PO_4 stock (Melford)	4.6% w/v
NaCl stock (Fisher)	81.8% w/v
Yeast extract (Oxoid)	0.1% w/v
Heat inactivated horse serum	10% v/v

1.1.6 LYSGM (Clark and Stensvold 2016; Diamond 1982)

K_2HPO_4 (Sigma)	0.28% w/v
KH_2PO_4 (Melford)	0.04% w/v
NaCl (Fisher)	0.75% w/v
Yeast extract (Oxoid)	0.25% w/v
Neutralized liver digest (Oxoid)	0.05% w/v
Heat inactivated horse serum	5% v/v

1.1.7 TYM (Diamond 1957; 1983)

Trypticase peptone (Oxoid)	2.22% w/v
Yeast extract (Oxoid)	1.11% w/v
$C_{12}H_{22}O_{11}$ (Melford)	0.56% w/v
$C_3H_7NO_2S$ (Sigma)	0.11% w/v
$C_6H_8O_6$ (Sigma)	0.02% w/v
K_2HPO_4 (Sigma)	0.09% w/v
KH_2PO_4 (Melford)	0.09% w/v
Heat inactivated fetal bovine serum	5% v/v

1.1.8 Liver Digest medium

Liver extract (Oxoid)	0.05% v/v

1.1.9 Ringer's solution

NaCl (Fisher)	0.86% w/v
$CaCl_2$ (Melford)	0.03% w/v
KCl (Melford)	0.03% w/v
Heat inactivated horse serum	10% v/v

1.1.10 Agar slant (Clark and Diamond 2002; Feng *et al.* 2012; Yoshikawa, Kuwayama and Enose 1995)

Each agar slant was composed of 5 ml Ringer's solution with Asparagine (0.1% w/v) (Sigma) and agar (SLS) (1.5% w/v) which was autoclaved and aliquoted into sterile 15 ml conical tubes (Sarstedt) at a 30 ° slant until set. Afterwards 5 ml of Ringer's solution was added. Tubes were kept at 4 °C until use.

1.2 Maintenance of *Blastocystis* cultures

Blastocystis strain	Subtype	Reference	Culture media	Culture Type
HJ96-1	2	(Yoshikawa *et al.* 2003)	Agar slant	Xenic
HJ96A-26	3	(Yoshikawa *et al.* 2000)	Agar slant	Xenic
SY94-3	5	(Yoshikawa *et al.* 1998)	Agar slant	Xenic
HJ96AS-1	6	(Yoshikawa *et al.* 2000)	Agar slant	Xenic

MJ99-132	8	(Abe 2004)	Agar slant	Xenic
HJ00-4	9	(Yoshikawa *et al.* 2004a)	Agar slant	Xenic
ST3	3	C.R. Stensvold	Modified Jones' Medium	Xenic
ST4	4	C.R. Stensvold	Modified Jones' Medium	Xenic
NUH9	1	(Wong *et al.* 2008)	IMDM	Axenic
WR1	4	(Noël *et al.* 2005)	IMDM	Axenic
ST7B	7	(Ho *et al.* 1993; Moe *et al.* 1997)	IMDM	Axenic
NandII	1	ATCC 50177	Egg Medium	Axenic

1.2.1 Axenic culturing

Axenic cultures (ST1-NUH9, ST4-WR1 and ST7-ST7B) were maintained in IMDM with 10% heat inactivated horse serum. Cultures were maintained at 37 °C in sterile round-bottom polystyrene test tubes (VWR) in a Gas-Pak anaerobic jar with Gas-pak sachets. Fresh media was de-gassed a minimum of five hours before cultures were passaged and warmed to 37 °C. Culture passaging was carried out in a biosafety cabinet under aseptic conditions. Disposable, sterile Pasteur pipettes were used to transfer 1 ml of gently homogenised culture to 9 ml of fresh media. Exposure to air and introduction of air bubbles was minimised to ensure maintenance of anoxic conditions. Cultures were left five days between passages. ST1-NandII was maintained in egg medium slants with 25% heat inactivated horse serum. Samples were stored in a hypoxic chamber at 37 °C with oxygen levels set to minimum. Fresh media was de-gassed and warmed prior to passaging. One ml of the old culture was removed with a disposable, sterile Pasteur pipette along the media and slope interface and gently resuspended in the fresh media without introducing air. Cultures were passaged at a maximum of every 3 – 5 days to avoid over-growth (Clark and Diamond 2002; Ho *et al.* 1993). Routine light microscopy examination was carried out on 10 µl aliquot of the culture at a minimum of X400 magnification to monitor *Blastocystis* morphology check for contaminants.

1.2.2 Xenic culturing

Xenic cultures: ST2-HJ96-1, ST3-HJ96A-26, ST5-SY94-3, ST6-HJ96AS-1, ST8-MJ99-132 and ST9-HJ004 were maintained in agar slants with Ringer's solution with 10% heat inactivated horse serum. Cultures were kept at 37 °C with the bacteria providing an anaerobic environment and passaged in a biosafety cabinet under aseptic conditions. Before passaging, the fresh media was kept at 37 °C for a minimum of two hours before passaging. Upon passaging, the culture media was gently resuspended with a disposable, sterile Pasteur pipette ensuring an equal homogenisation throughout the liquid media, 1 ml of this was aliquoted into 5 ml fresh media maintaining anaerobic conditions, cultures were passaged every five days and growth was monitored via light microscopy (Clark and Diamond 2002; Feng *et al.* 2012).

Xenic cultures ST3 and ST4 (kindly provided by C.R. Stensvold) were maintained in Jones' media with 10% heat inactivated horse serum. Cultures were kept at 37 °C with the bacteria providing an anaerobic environment and passaged every 3 – 5 days. Prior to passaging fresh media was warmed to 37 °C, passaging was carried out in a biosafety cabinet under aseptic conditions. cultures were gently resuspended using a disposable, sterile Pasteur pipette and 1 ml was inoculated into 9 ml of fresh media, cultures were routinely monitored via light microscopy.

For some animals, when fresh faecal samples were collected a small quantity was inoculated into 15 ml conical tubes containing 13 ml LYSGM, 0.5% Liver digest or TYM. These were stored at the following temperatures: Room temperature (~21 °C), 35 °C and 37 °C (Clark and Stensvold 2016; Diamond 1982; Jones 1946).

1.2.3 *Blastocystis* counting and staining

Both axenic and xenic cultures were routinely monitored via microscopic observation in order to monitor growth, morphology and for axenic cultures to screen for contaminants. Cell counts were gathered using a haemocytometer with neutral red stain which was prepared as follows:

0.33% Neutral Red Dye (Sigma) was warmed to room temperature before use. *Blastocystis* was resuspended anaerobically using a sterile, disposable Pasteur pipette and 94 µl of the culture sample from the suspension and add 6 µl neutral red solution. The sample was incubated at room temperature for 10 minutes and centrifuged at 6500 rpm for 30 seconds,

the supernatant was removed and the pellet was gently resuspended in 20 µl Phosphate buffered saline (PBS) (Oxoid).

2 Molecular techniques

2.1 Genomic DNA Extraction from faecal samples

A minimum of 250 mg of faecal sample was used for DNA extraction. Faecal samples were collected and stored in sterile 15 ml conical tubes (Sarstedt) at 4 °C before extraction. Genomic DNA was extracted using the Microbiome DNA Purification Kit Purelink (Fisher, United Kingdom) to the manufacturer's instructions. DNA was eluted in 100 µl elution buffer. The working stock was stored at −20◦ C, while the rest was placed at −80 °C for long-term storage. DNA concentration ranged between 10-50 ng/µl and was diluted accordingly with nuclease free water (Qiagen).

2.2 Genomic DNA Extraction from culture

Genomic DNA from xenic and axenic cultures was extracted using the Microbiome DNA Purification Kit Purelink (Fisher, United Kingdom) to the manufacturer's instructions. The final elution volume was 100 µl and a working stock was stored at -20 °C, the rest was stored at -80◦ C. DNA concentration ranged between 10-50 ng/µl and was diluted accordingly with nuclease free water (Qiagen).

2.3 DNA Amplification, visualisation and gel extraction of *Blastocystis*

The extracted DNA was subjugated to PCR with target primers. For *Blastocystis* a nested reaction was carried out targeting the SSU rRNA, two different PCR reaction kits were used: GoTaq G2 Flexi DNA Polymerase (Promega: M7801); reagents per 50 µl PCR reaction were as follows: 10 µL 5× buffer, 1 mM MgCl2, 0.4 µM forward primer, 0.4 µM reverse primer, 0.2 mM dNTPs (Promega), 0.25 µL Taq polymerase, 30.75 µl HPLC grade water 2 µL DNA.

2X PCRBIO Taq Mix Red (PCRBIOSYSTEMS: PB10.13); reagents per 50 µl reaction were as follows: 25 µl PCRBIO Taq mix, 0.4 µM forward primer, 0.4 µM reverse primer, 19 µl nuclease free water (Qiagen) and 2 µl DNA.

The broad specificity primers RD3 5′ -GGGATCCTGATCCTTCCGCAGGTTCACCT AC-3′ and RD5 5′ -GGAAGCTTATCTGGTTGATCCTGCCA GTA-3′ (Clark 1997) were used for the first PCR with

the following conditions: 95 °C 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing 55 °C for 30 s, extension at 72 °C for 1 min 40 s and final extension at 72 °C for 5 min. The second PCR used the forward primer: RD5F 5′ -ATCTGGTTGATCCTGCCAGT-3′ and reverse BhRDr 5′ -GA GCTTTTTAACTGCAACAACG-3′ (Scicluna, Tawari and Clark 2006). One µl of the first reaction was used as the template for the second, reactions gave a fragment of approximately 650 bp. 10 µL of the PCR product was run on a 1.5% agarose gel (Melford) which was stained with ethidium bromide (Sigma) (0.5 µg/ml), gels were visualised and recorded using a Syngene G-box transilluminator.

30 µl of positive reactions were run again on a 2% agarose gel (Melford) stained with ethidium bromide (Sigma) (0.5 µg/ml) and were excised, DNA fragments were extracted using the Thermo Scientific GeneJET Gel Extraction Kit (following manufacturer's instructions), the final elution was between 30 – 50 µl.

2.4 PCR product Transformation and Cloning

If purified DNA was left for seven days prior to ligation, a polyadenylation reaction was carried out with the following per 10 µl reaction: 7 µl Gel extraction, 0.25 µl GoTaq DNA Polymerase (Promega), 2 µl 5X GoTaq Buffer (Promega), 0.5 mM $MgCl_2$, 2.5 mM dATP (Promega) and 0.3 µl nuclease water (Qiagen) at 72 °C for 30 min.

Either 1.5 µl of the gel extract or polyadenylation reaction were used cloned using the pGEM-T Easy Vector System I (Promega: A1360) (following manufacturer's protocol), reactions were incubated overnight at 4 °C. Ligation products were used to transform *DH5-α Escherichia coli* with the following conditions: 2.5 µl ligation product and 50 µl *DH5-α* competent cells were incubated for 10 minutes on ice then heat-shocked for 1 minute at 42 °C, followed by 2 minutes on ice. Cells were recovered in 250 µl LB medium at 37 °C for 1 hour under agitation.

Cells were plated on LB plates containing Ampicillin at 100 µg/ml (Melford), IPTG at 0.1mM (Melford) and X-Gal at 40 µg/ml (Melford) and were grown overnight at 37 °C. Positive transformants were represented by white colonies, between 5 – 10 positive colonies per transformation were grown in 5 ml overnight LB and Ampicillin 100 µg/ml cultures. Plasmids were purified using the Thermo Scientific Genejet Plasmid Miniprep Kit (to manufacturer's instructions) with a final elution of 50 µl. Fragment insertion was confirmed by a restriction digest using *Eco*RI (Promega), per 10 µl reaction: 0.25 µl *Eco*RI, 5 µl miniprep, 1 µl 10X buffer H and 3.75 µl dH20 was incubated at 37°C for 2 h and visualized on a 1.5% agarose gel

(Melford) stained with ethidium bromide (Sigma) (0.5 µg/ml), and visualised on the Syngene G-box transilluminator.

2.5 Genomic DNA Sequencing

Positive samples were sent for sanger sequencing via Eurofins Genomics, United Kingdom with 2 µl 10 µM of T7 or SP6 universal sequencing primers (Eurofins) with 15 µl of the purified DNA.

2.6 Phylogenetic Analysis

Nucleotide sequences obtained from sanger sequencing (Eurofins) were visualized and trimmed using Snapgene Viewer to remove vector fragments and when applicable forward and reverse sequences were joined using Sequencher. Trimmed sequences were input into BLAST (NCBI) against the non-redundant (nr) database and sequences which were not positively identified as *Blastocystis* were discarded. All positive sequences and reference sequencing representing the diversity of *Blastocystis* subtypes were compiled into a dataset and were aligned via MAFFT v.7 (Katoh and Toh 2010). Genetic distance of the barcoding region was calculated using the Kiruma2 parameter criteria and was carried out using MEGA X (Stecher, Tamura and Kumar 2020), where gaps were defined as complete deletions. Ambiguous positions were removed using trimAI (Capella-Gutiérrez, Silla-Martínez and Gabaldón 2009) (http://trimal.cgenomics.org). A maximum likelihood tree was constructed with RAxML version 8 (Stamatakis 2014) via the CIPRES online resource (Miller, Pfeiffer and Schwartz 2010) (http://www.phylo.org). Bootstrap supports was calculated from 1000 replicates.

2.7 16S Microbiome Profiling

For extracted genomic DNA samples submitted for microbiome profiling, analysis was carried out via INVIEW Microbiome Profiling 3.0 (Eurofins). Genomic DNA purity and concentration was measured on using a nanodrop (BioDrop) and DNA was diluted with nuclease free water (Qiagen) to give a resulting concentration between 10 – 45 ng/µl where necessary. A total volume of 20 µl was sent for analysis. The bacterial taxonomic profiling targeted the 16S rRNA gene V1 – V3 hypervariable region forward sequence: fD2

AGAGTTTGATCATGGCTCAG (Weisburg *et al.* 1991), reverse sequence S-D-Bact-0008-a-S-20, S-*-Univ-0519-a-A-18: GTATTACCGCGGCTGCTG (Leser *et al.* 2002). Initial bioinformatics analysis was carried out as part of the INVIEW microbiome profiling pipeline, this is summarised as follows: Demultiplexing was done according to index sequences and was carried out on all reads that passed the illumina chastity filter. Merging was carried out on any suitable overlapping reads using the FLASH (V2.2.00 http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg 2011). Quality Filtering of merged reads was implemented by length filtering according to expected sequence length and known length variations according to the specifications determined by the INVIEW microbiome profiling pipeline. Any merged reads containing ambiguous bases were discarded. Chimera filtering was done to remove any sequences with portions of multiple sequence; removal was carried out based on the algorithm of UCHIME via the VSEARCH package (Edgar *et al.* 2011; Rognes *et al.* 2016). Primer and adaptor sequences were removed using Cutadapt (Martin 2011).

High quality reads were processed using Minimum Entropy Decomposition (MED) which provides an unsupervised methods for sensitive clustering of OTUs and does not rely on arbitrary sequence similarity thresholds to petition sequences into OTUs (Eren *et al.* 2015; Eren *et al.* 2013). Taxonomy assignment to each OTU was carried out by using discontiguous Mega BLAST (DC-MEGABLAST) to match alignments of cluster representative sequences to the sequence database. Specific taxonomic assignment for each OTU was then transferred from the best-matching reference sequence set, a sequence identity of 70% across a minimum of 80% of representative sequences was the threshold for consideration of a reference sequence (representing a 97% threshold). Further processing of OTUs was performed using QIIME (version 1.9.1 http://qiime.org/) and OTU abundance normalisation was employed using CopyRighter (Angly *et al.* 2014).

Further bioinformatics analysis, filtering, normalization, diversity measures and visual representation of data were conducted via the MicrobiomeAnalyst 4.0 pipeline (Dhariwal *et al.* 2017) (https://www.microbiomeanalyst.ca), this included low count filtering to remove low prevalence counts which are likely due to sequencing errors or low-level contamination. The threshold was set to a minimum count of 4 with a prevalence of 20% in samples, meaning that for a feature to be retained a minimum of 20% if its values require at least 4 counts. Data filtering also included low variance filtering, used to remove feature that were constant across the different variables; this filtering was carried out based on the inter-quartile range (IQR). Data normalization was applied to the samples to address sampling

depth variability and sparsity. Data rarefication was considered but not carried out as this is usually beneficial for datasets with significant variability between library sizes (<10 X). Data scaling was applied via total sum scaling (TSS) to address uneven sequencing depth.

Microbial diversity was analysed at the different taxonomic levels, here groups of data were compared based on *Blastocystis* infection status.

Diversity analysis included alpha (within sample) and beta (between samples) diversity measures, before analysis the Shapiro-Wilk test for normality was carried out across each taxa to measure the normality of abundance. Alpha diversity was measured using the Observed species (determines amount of unique OTUs within a sample) and Chao1 indices (determines observed OTUs and accounts for unobserved species based on low-abundance OTUs) for measuring diversity richness and Shannon indices which accounts for OTU richness and evenness, the corresponding statistical significance was determined using a Mann-Whitney U test. Beta diversity indices were measured using Bray-Curtis Index distances, the corresponding statistical significance was assessed via Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA). Results were presented as principal coordinates analysis plots (PCoA) to illustrate relationships between the vole microbiomes based on infection category.

In order to assess the microbial community abundances between different experimental variables we employed two analyses to identify differentially abundant communities. Classical univariate analysis was used to identify differentially abundant community profiles using *t*-test based on a single grouping experimental variable. MetagenomeSeq analysis was implemented to apply a more robust method of abundance measure. This is designed to designed to address normalization and biases in measurements across taxonomic features by way of a zero-inflated Gaussian distribution model to account for the variety in sequencing depth. This method is beneficial as it aids in the detection of differentially abundant rare taxa.

LEfSE (Linear discriminant analysis Effect Size) was used to perform a non-parametric factorial Kruskal-Wallis sum-rank test to identify which community abundance features were significantly different with regard to the experimental factor and most likely to explain differences between experimental variables.

2.8 RNA Transcriptomics

All samples for use in RNA extraction were passaged between one and three days before use in experiments depending on average *Blastocystis* growth time. Samples extracted under anaerobic conditions were kept in 15 ml conical tubes (Sarstedt) with a total volume of 14 ml media to ensure anaerobic conditions. Samples extracted under 5% oxygen were flushed with 5% oxygen in a hypoxic chamber before being gently agitated for 30 minutes at room temperature prior to extraction.

Before RNA extraction, cell counts were performed on a 10 µl aliquot of the sample using a haemocytometer and *Blastocystis* cell counts over $x10^4$ cells/ml were considered sufficient. Extraction was carried out using the RNAqueous total RNA isolation kit (Invitgoren) (following manufacturer's instructions) unless stated otherwise. The RNA concentration and purity was measured on a nanodrop (BioDrop). The five replicates with the highest yield and lowest contamination levels were sent for illumina transcriptome sequencing (Macrogen Europe, Netherlands).

2.8.1 RNA Extraction from *Blastocystis* culture

Axenic *Blastocystis* cultures: ST1-NUH9 and ST1-NandII and xenic cultures ST3 and ST4 were passaged 48 hours before RNA extraction, 12 cultures per isolate were made and used in the extraction process. Extraction was carried out using the RNAqueous total RNA isolation kit (Invitrogen) (following manufacturer's instructions). A number of different sample preparation steps were implemented before extraction to aid cell lysis and maximise RNA yield, these are documented as methods 1 – 5 as follows:

1. Axenic culture was resuspended under anaerobic conditions and centrifuged at 800 g for 8 minutes at room temperature (RT), it was then gently resuspended in fresh culture medium and centrifuged for a second time under the same conditions before the RNAqueous total RNA isolation kit RNA extraction was used. The final elution volume was 50 µl of elution buffer followed by a subsequent 20 µl.
2. Axenic culture was resuspended under anaerobic conditions and centrifuged once at 800 g for 5 minutes at RT before extraction before the RNAqueous total RNA isolation kit RNA extraction was used. Final elution volume was 40 µl followed by 10 µl.
3. Axenic culture was subjugated to one centrifugation step at 800 g for 8 minutes at RT, the pellet was gently washed with Ringer's solution under aseptic conditions. A

centrifugation step with the aforementioned conditions followed by vigorous vortexing for 1 minute on a bench top vortex was carried out before sample was used in the extraction protocol.

4. Axenic culture was centrifuged at 800 g for 8 minutes at RT, the pellet was gently washed with Ringer's solution and resuspended under aseptic conditions. A second centrifugation with the aforementioned conditions was carried out followed by bead beating for 5 minutes.
5. Axenic culture was centrifuged at 800 g for 8 minutes, supernatant was removed and 100 µl lysis buffer (Invitrogen) was added, the sample was briefly vortexed to resuspend pellet, then the tube was snap-frozen by adding to liquid nitrogen. The sample was subsequently thawed gently in warm water and this freeze-thaw process was repeated between three and six times.

In addition to the aforementioned methods that were employed prior to use of the RNAqueous total RNA isolation kit (Invitgoren), RNA isolation using TRIzol reagent (Ambion) was trialed as an alternative method, following the procedure below:

Axenic culture was initially centrifuged at 800 g for 5 minutes, between 1 – 5 ml of TRIzol reagent was added until the pellet completely dissolved in a fume hood. 200 µl 1-Bromo-3-chloropropane (BCP) for every 1 ml TRIzol was added to the sample, the sample was shaken by hand for 2 minutes and then incubated at RT for 5 minutes. The sample was then centrifuged at 11,000 g for 25 minutes at 4^{o} C and the transparent layer was carefully moved to a new sterile 1.5 ml microcentrifuge tube (Eppendorf). The procedure from adding BCP until transferring the transparent layer to a new tube was repeated a total of two times. 100% isopropanol (Fisher) (0.5 ml per 1 ml TRIzol) was then added and gently mixed. The sample was incubated for 10 minutes at room temperature and then centrifuged at 11,000 g for 5 minutes at 4^{o} C, following this the supernatant was discarded and 1 ml of 70% ethanol (Fisher) was added and subsequently centrifuged at 11,000 g for 5 minutes 4^{o} C. The addition of 70 % ethanol and centrifugation was repeated three times. The supernatant was discarded and the sample was dried by centrifuging briefly to remove excess ethanol and then leaving the tube on its side to dry. The pellet was resuspended in 50 µl nuclease free water (Qiagen) and stored at -80^{o} C.

2.8.2 Xenic culture RNA extraction

Xenic cultures of *Blastocystis* subtype 3 (ST3) and subtype 4 (ST4) were cultured in Jones' media and 10% horse serum at 37° C, upon initiation of experiment 1 ml the confluent subtype culture was split into twelve separate sterile 15 ml conical tubes (Sarstedt) and topped with Jones' media plus 10% horse serum to maintain anaerobic conditions and were left for 24 hours to grow at 37° C. Before extraction each culture was first centrifuged at 8000 g for 8 minutes at room temperature, the supernatant was poured off with 500 µl aliquoted into a sterile microcentrifuge tube, and the remaining volume was kept in a 15 ml conical tube; both samples were snap-frozen in liquid nitrogen and stored at -80° C. The pellet was used in the RNA extraction protocol following the manufacturers guidelines as stated above. Samples were eluted in 50 µl elution buffer and stored at -80° C.

2.8.3 High-throughput RNA sequencing and analysis

All eligible samples were sequenced and TruSeq mRNA standard sequencing libraries were prepared and sequenced on the NovaSeq Illumina platform (Outsourced to Macrogen, Korea). Raw sequence data was quality checked using the online platform: FastQC (Babraham Bioinformatics) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and any samples not meeting the quality checks were discarded. The first 15 bases and last 5 bases of each forward and reverse sequence were removed using the trimmomatic software and any sequences that fell below 100 bp were subsequently removed. The trimmed paired-end contigs were assembled using the Trinity pipeline using RSEM for *de novo* transcriptome assemblies (Grabherr *et al.* 2011; Haas *et al.* 2013; Li and Dewey 2011) (https://github.com/trinityrnaseq/trinityrnaseq/wiki). Differential gene expression analysis was performed using DESeq2 (Love, Huber and Anders 2014) and visual plots were generated using RStudio v1.3.1093 (https://rstudio.com) with R v4.0.3 (https://www.r-project.org/). FASTA files for isoforms with +/- log fold change (LFC) of 1 and 5 were extracted, nucleotide sequences were translated into protein sequences using TransDecoder and the longest open reading frame for each sequence with a minimum length of 100 amino acids was extracted (Haas *et al.* 2013) (https://github.com/TransDecoder/TransDecoder/wiki). The amino acid sequence was fed into the KEGG: BlastKOALA v2.2 database (https://www.kegg.jp/blastkoala/) for functional characterisation of the genome, the resulting K number output files was input into KEGG Module Mapper for reconstruction of KEGG pathway maps

(https://www.genome.jp/kegg/tool/map_pathway.html). Complete modules and modules with one block missing were included in the final pathway annotation. Pathways were manually cross-validated by inputting the amino acid sequences into NCBI BLAST against non-redundant protein sequences (BLASTp) to ensure hits were not a result of bacterial contamination.

In the case of incomplete modules, missing KO values were assigned protein sequences from Swiss-Prot (UniProt) via KEGG and input into BLAST non-redundant protein sequences against *Blastocystis* (taxid:12967) to establish if similar proteins have been identified in *Blastocystis* subtypes. The missing KO values were also aligned against the transcript database of each condition to identify likely missing pathway components which had not met the LFC criteria.

2.9 ^{1}H NMR Metabolomics

2.9.1 Human sample collection

All faecal samples used in this study were obtained with an ethics agreement from the collaborating groups. Samples from South Korea were kindly provided by Dr. Eun Jeong Won (Department of Parasitology and Tropical Medicine, Chonnam National University Medical School, South Korea) and those from Turkey were kindly provided by Prof. Funda Dogruman AL (Department of Medical Microbiology, Gazi University Faculty of Medicine, Turkey).
Samples were submitted from medical examination as part of a diagnosis or 'general check-up' depending on the patient. *Blastocystis* was diagnosed via microscopical inspection and/or molecular characterisation via PCR (Subtype data not provided). Collected samples were stored in sterile collection tubes provided by the hospital and stored at 4^{o}C until metabolite extraction took place.

2.9.2 Faecal sample preparation

^{1}H NMR metabolomics was performed on human faecal samples and on *Blastocystis* subtype culture supernatants. The metabolite extraction protocol used was as follows:
Into a sterile 15 ml tube 200 mg of the faecal sample was added along with 6 ml of 75 % ethanol at 80^{o} C (Fisher) and 200 mg 2 mm diameter glass beads. The sample was then agitated via vortexing for 30 seconds. The mixture was incubated at 80^{o} C for 3 minutes and agitated further until the sample was completely homogenised. The homogenised sample

was aliquoted into 3 x 2 ml sterile microcentrifuge tubes (Eppendorf), and the glass beads were retained in the 15 ml conical tubes (Sarstedt) and washed with a further 2 ml of 75 % ethanol at 80 ^{o}C, this was aliquoted into another 2 ml sterile microcentrifuge tube and the glass beads were retained in the conical tube. The 2 ml samples were centrifuged at 16000 g for 10 minutes at room temperature and the supernatant was transferred to new sterile 2 ml microcentrifuge tubes. The samples were dried overnight in a rotorvac at 40 ^{o}C. The remaining desiccate was suspended in 330 µl double distilled water and vortexed at maximum speed for 30 seconds until the desiccate was fully dissolved before being centrifuged at 2500 g for 10 minutes at RT. The supernatant of each sample was recombined into a sterile 1.5 ml microcentrifuge tube (Eppendorf) and stored at -80 ^{o}C until analysis (Newton *et al.* Under Review).

2.9.3 *Blastocystis* culture preparation

650 µl of the snap-frozen supernatant from the xenic cultures, as described in section 2.8.2 above were used for ^{1}H NMR metabolomics analysis.

2.9.4 ^{1}H NMR spectroscopy

2.9.4.1 Preparation

650 µl of sample for analysis was mixed with 35 µl of premixed stock of 10 mM deuterium oxide (D_2O) (CortecNet) and Sodium trimethylsilylpropanesulfonate (DSS) (Sigma) to a final concentration of 0.5 mM. The pH of each sample was recorded (LAQUA twin pH meter, Qiagen) before being added to 535-PP7 NMR tubes (Wilmad).

2.9.4.2 Procedure

Samples were analysed using one-dimensional (1D) ^{1}H NMR spectroscopy on a 600 MHz AVANCE III spectrometer (Bruker) at 298 K with a transmitter frequency of 600.05 MHz locked to D_2O. Tuning and shimming were carried out automatically as was the 90 ^{o} pulse calibration. Soft pulses were set and receiver gain was limited to a maximum value of 128. A noesy of 512 scans and 8 dummy scans with a spectral width of 15.98 ppm (9590.75 Hz) was performed, with an acquisition time of 1.71 s and relaxation delay of 3 s and a data size of 32768 points. An excitation sculpting experiment was carried out with 256 scans and 8 dummy scans; the spectral width was 15.98 ppm (9590.79 Hz), acquisition time 1.7 s and

relaxation delay 3 s; with a data range of 32768 data points. For all experiments, water resonance was at 4.699 ppm.

2.9.4.3 Analysis of Spectra

The resulting spectra were phased, baseline corrected and line-broadened with a 1 Hz exponential window function in TOPSPIN 4.0.9 (Bruker). Processed spectra were imported into Chenomx 8.4 where the Chenomx Processor was used to perform standard profiling and calibration. A shim correction of 1.2 Hz was applied and the region between 4.56 ppm to 4.97 ppm was deleted to eliminate the water resonance peak. pH adjustments were made based on the recorded pH of the samples. Peak assignment, identification and quantification was achieved using the Chenomx profiler tool, spectra were automatically fitted to the 338 reference compounds in the Chenomx library, each spectrum was manually checked and adjusted to account for the non-homeostatic nature (varying pH and metal ion content) of faecal samples before the resulting compound concentrations were exported.

2.9.5 Statistical analysis of compound concentrations

Exported compound concentrations were input into the MetaboAnalyst 4.0 pipeline as one-factor, unpaired data (Chong, Wishart and Xia 2019) (https://www.metaboanalyst.ca) for statistical analysis and visual plots production.

2.9.5.1 *Blastocystis* culture analysis

For the *Blastocystis* cultures, supernatants subjected to NMR metabolomics analysis comparisons were made between anaerobic and 5% oxygen samples within each subtype, where each the subtype was analysed separately. Data normalization and scaling was carried out in MetaboAnalyst 4.0. Data normalization was implemented to reduce systematic bias and improve biological comparisons, normalization by the median was used. Data scaling was also applied to make metabolite concentrations more comparable, here, Auto-scaling of the data (mean-centred and divided by the standard deviation of each variable) was used. The resulting metabolite concentrations were visually inspected by assessing the Gaussian distribution and the Shapiro-Wilk test for normality was applied.

2.9.5.2 Human Sample analysis

The human faecal metabolite profiles were analysed based on *Blastocystis* infected vs uninfected (control group) per country. The results for each country were analysed independently from each other to reduce variability. The statistical analysis was performed using MetaboAnalyst 4.0. Data filtering by the mean of intensity values was implemented to remove non-informative results, including compound values which were close to the baseline. Sample normalization to remove undesired systematic biases to retain biologically relevant differences in the data and was implemented by normalizing by the sum. Data scaling was carried out by the Auto-scaling method. This was used to help balance signal intensity variances which originate from differences in the average abundance of metabolites (Giskeødegård, Bathen and Euceda 2015). Before statistical analysis, the overall data quality of metabolite concentration values was checked for any obvious outliers.

2.9.5.3 Statistical Analysis

Univariate analysis of metabolite profiles obtained from *Blastocystis* infected and uninfected patients was used to determine which metabolites are different between the two groups based on the fold-change (FC) values that exceeded the threshold of significance of 2. Data distribution was checked using the Shapiro-Wilk test for normality. Analysis was carried out on false discovery rate (FDR) adjusted *p*-values (based on Benjamini-Hochberg procedure) for a multiple comparisons approach with a *p* value of <0.1.

Multivariate analysis was carried out to investigate relationship patterns between *Blastocystis* infection and resulting metabolite profiles in comparison to uninfected controls. It facilitated the assessment of data homogeneity and was used to identify potential outliers. This was implemented using unsupervised Principal Component Analysis (PCA) which evaluates the sample variance and helped identify outliers, which, given suitable reasoning could be removed. Supervised analysis based on Partial Least-Squares Discriminant Analysis (PLS-DA) was also applied to the dataset in order to investigate the prediction of difference metabolite profile in infected and uninfected individuals. PLS-DA analysis was cross-validated using the leave-one-out (LOOCV) cross validation method to address the issue of possible overfitting, this is used to determine the ideal number of components for the PLS-DA model and uses three different performance measures for validation.

2.9.5.4 Pathway analysis

Pathway enrichment analysis was carried out on identified metabolites to determine if differences exist between metabolic pathways in *Blastocystis* infected and uninfected individuals. This was implemented by assessing normalized metabolite concentrations via MetPA's (Metabolomics Pathway Analysis) pathway topology analysis (Xia and Wishart 2010) available on the MetaboAnalyst 4.0 pipeline. This method is conducted via: pathway enrichments analysis; pathway topology analysis and pathway impact analysis.

This analysis is based on the centrality measures of a metabolite from a specific network against the '*Homo sapien*' pathway library (KEGG) using the Globaltest enrichment method (Goeman *et al.* 2004) and relative betweenness centrality for pathway topology analysis. Pathway impact is also calculated as the sum of the importance measures of matched metabolites which have been normalized by the sum of the importance measures of all metabolites in the pathway.

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