CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter presents a comprehensive review of the existing literature relevant to our study. As a start, a thorough review of *Blastocystis* has been discussed focusing on *Blastocystis* infection in type-2 diabetic mellitus. Background information on T2DM, gut microbiota and specific markers that had been observed in this study has also been described briefly. The recent studies help to summarize the association of *Blastocystis* in gut microbiota.

2.2 *Blastocystis*

Blastocystis has arisen as a major discourse among researchers due to its unknown status for being pathogenic or commensal over decades. There has been an intensive review that requires a thorough historical description to appreciate events leading to its reclassification and consideration as a respected human pathogen. The history of this complex parasite is a panorama of early advance followed by decades of retreat into obscurity and then reintroduction into the modern era. Various observations and research have been made over the years to generate the present taxonomical status of *Blastocystis*. Initially, the unique mimicry of form *B. hominis* led to a state of confusion as the predominant hollow spherical form was and still is confused with unrelated cell

from many animals. It had falsely been reported as occurring *Blastocystis* in almost every insect, reptile, bird, fish and mammal examined (Zierdt and Charles, 1991).

However, the first observations of the organism were made independently by Brittan and Swayne while studying the infamous cholera epidemic in London in 1849. They had discovered unknown parasitic ova and cysts collectively and marked them as cholera bodies. They wrongly identified their observation to be the causative agent of cholera. It was the moment of research about *Blastocystis* had been specifically observed by Alexeief and Emile Brumpt in the early 1900s, who proposed that it has to be a harmless saprophytic yeast in the intestinal tract. Then, the genus of *Blastocystis* itself as a distinct organism was proposed by Alexeieff who derived a specific name *B. enterocola.* As an extensive morphological description was developed and a life cycle was discovered, Brumpt proposed the name *B. Hominis* for the organism isolated from faecal material and this name has been recognized until the current literature on that time (Stenzel & Boreham, 1996).

The taxonomy of *Blastocystis* spp. remains contentious, and the history of the organism reflects the difficulty in defining its taxonomy position. In the early 1920s, several groups confirmed *B. Hominis* had been classified as a yeast due to the several structures and had been assigned the organism to different yeast genera, for example, *Schizosacchararomyces* and *Saccharomyces* (Mehlhorn, 1988). The organism had a yeast-like glistening appearance in fresh wet faecal mounts, no pseudopia in the usual unheated wet preparations, and no evidence of locomotion. Plus, the size range was more extreme than protozoan as was the diversity of forms seen in a single specimen. The division by binary fission was easily mis-constructed as budding. Apart from an

occasional case report, the classification of *B. hominis* and its role as a harmless yeast was not questioned for another 50 years (M. Johnson et al., 1989). Then, Zierdt *et al*, 1991 first described the protozoan characteristic of this intestinal yeast after further observation which is the character of *B. hominis* is more similar to the protozoa while there is no single strongly allied with the yeast. Table 2.1 indicates the characteristics that have been used to reclassify *B. hominis* from fungi to protozoa morphologically and physically.

Table 2.1: Characteristics to reclassify *Blastocystis* hominis from fungi to protozoa

Characteristics	Fungi	*B. hominis*
Strict anaerobe	No	Yes
Growth on fungal or bacteriological media	No	Yes
Growth on media developed for intestinal protozoa	No	Yes
No growth on surface solid media	No	Yes
Requires presence of bacteria for growth; axenic growth achieved slowly under carefully controlled conditions.	No	Yes
Capable of ingesting bacteria and other particulate matter	No	Yes
Cultures die in 2-3 days at 22°C or overnight at 4°C	No	Yes
No growth below 33°C, but cell death at 30°C	No	Yes
Optimal growth at 37°C	No	Yes
Resistant to 400 ug of amphotericin per ml	No	Yes
Sensitive to drugs effective against intestinal protozoa	No	Yes
No growth at 37°C	No	Yes
No cell wall	No	Yes
Reproduction by endodyogony, schizogony, binary fission, and plasmotomy (cutting off) individual *B. hominis* from the amoeba form	No	Yes
No budding	No	Yes
Supports stable bacterial endosymbiont (an obligate mutualism)	No	Yes
Slow-feeding pseudopods	No	Yes
Rapid locomotion pseudopods	No	Yes
Limiting membrane with micropinocytocic vesicles	No	Yes
Membrane-bound CB, a reproductive organelle (vacuole)	No	Yes
Mitochondria in all cells	No	Yes

Since *B.hominis* is difficult to classify due to the unique diversity of its shape, size, physiology and reproductive modes, continued research has been conducted to strengthen the need for this change in classification (Zierdt, 1967). Therefore,

reclassification of *Blastocystis* as a protozoan in the subphylum Sporozoa was suggested and it should be considered as a potential pathogen (Zierdt and Tan, 1976). A new class *Blastocystea*, and a new order, *Blastocystida* were proposed (Zierdt, 1978). However, the findings of motility by pseudopodia as well as feeding pseudopodia, extracellular location in the host, lack of an apical complex and other minor characteristics indicate a better assignment is to Sarcodina, order Amoeiba, in a separate suborder, blastocystina (Zierdt, 1991). Nonetheless, there is no supported data has been provided for this reclassification. According the classification scheme by Levine et al, 1985, classified and recognized the description of *B. hominis* based as follows; kingdom Prostita, subkingdom Protozoa, phylum Sarcomastigophora, subphylum Sarcodina, superclass Rhizopoda, class Lobosea, subclass Gymnamoeba, order Amoebida, new suborder Blastocystina, genus *Blastocystis*, species *hominis*. Recurrently, the molecular sequencing studies on a single-cell axenic human isolate were performed by small-subunit rRNA sequencing techniques for further reclassification which showed that *B. hominis* is not monophyletic with the yeasts Saccharomyces spp., the fungi *Neurospora* spp., the ameaba Naeglaria, *Acanthamoeba* and *Dictyostelium* spp., or the flagellates T*rypanosoma* and *Euglena* spp. These data conclude that *B. hominis* is not closely linked to yeast, fungi, sarcodines, or sporozoans. Small-subunit rRNA sequence comparison is ideal for the determination of phylogenetic affinities due to its ubiquitous, and segments of it evolve at different rates. Some sections are conserved, unchanged over millions of years of evaluation, and the remainder shows wide variation amongst even closely related organisms (Johnson *et al*., 1989).

Then, a new subphylum was subsequently proposed Blastocysta, which included the class Blastocystea, the order Blastocystida, the family Blastocystidae, and the genus

Blastocystis, with *B. hominis* as the type species (Jiang and He, 1993). Yet *Blastocystis* does not fit well into existing protozoan classification as there is still a lack of evidence to construct a new classification for the organism (Boreham & Stenzel, 1993; hollebeke et al, 1994). Recent debates on the systematic of protists have not included the genus *Blastocystis* in any of the suggested classifications. Further morphological and molecular data are required for an explicit classification.

At the end of the 1900s, the extended phylogenetic study had classified the organism among eukaryotic phylum, Heterkontophyta type is based on the molecular analysis of small subunit (SSU) rRNA (SSU-RNA) and elongation factor 1 (EF-1α) (Silberman et al., 1996). Later study by Arisue *et. al*, (2002), the present SSUr-RNA and HSP70c phylogenic demonstrated that *B. hominis* HE87-1 strain is positioned within stramenophiles being comparable with the previous SSUrRNA phylogeny by Silberman et al., (1996). The two data sets have reached one conclusion that the close relationship of *Blastocystis* with stramenopiles is supported. Although the taxonomy such as this was debated when related to the other studies demonstrating a similarity of *Blastocystis spp.* parasites with protists, the subsequent studies confirmed the assertion presented above by using multiple molecular sequence data (Arisue *et al.*, 2002). There are over 100,000 members in the phylum Heterokontophyta commonly called heterokonths or stramenophiles comprising algae, diatoms, slime moulds and oomycetes, while *Blastocystis* forms the newer member of this complex group of 'botanical Protists" (Tan, 2008; Riisberg et al.,2009). The studies show all stramenophiles possess mitochondria with tubular cristae and, unlike all other eukaryotes have tripartite tubular hairs either on their cell surface or more commonly on their long anterior flagellum. However, *Blastocystis* lacks flagella and flagella hairs

despite its clear phylogenic placement within the stramenophiles (Zierdt, 1991). This probably results from a secondary loss of these features on the line as its closest relatives Proteromonadidae and Bicosoecida have flagella and tubular hairs (Silberman et al., 1996; Simpson et al., 2002; Hoevers et al., 2005). Therefore, *Blastocystis* is placed in a newly created class as below in Table 2.2 (Cavelier-Smith, 1998):

Table 2.2: Classification of *Blastocystis*

Class	*Blastocystea*
Subphylum	*Opalinata*
Infrakingdom	*Heterokana*
Subkingdom	*Chrompbiota*
Kingdom	*Chromista*

The more recent classification into subtypes (ST) is equivalent to the earlier species classification. According to recent studies, a total of 40 subtypes have been reported vary from different hosts; humans, animals of various orders and sources of water based on genetic diversity in the small subunit ribosomal RNA (SSU-rDNA) gene (Darwish *et al*., 2023; Maloney *et al*., 2021, 2023; Christen Rune Stensvold & Clark, 2020; Santin et al., 2024).

2.2.1 Biology

Blastocystis is a strict anaerobe which is lives in oxygen-poor environments and is characterized by the presence of some double membrane surrounded-organelles showing elongate, branched and hooked cristae called mitochondria-like organelles (MLOs) (Zierdt, 1991; Nasirudeen *et al*.,2004). Although numerous organelles resembling mitochondria are seen, they are completely devoid of these organelles have the property of both the mitochondria of aerobes and the hydrogenosome. Recently, *Blastocystis* MLOs were reported to contain a circular genome, including genes encoding 10 of 20 complex subunits, but they lack all genes encoding cytochromes, cytochrome oxidases and ATP synthase subunits, unlike mitochondrial DNA from other sequenced stramenopiles such as *Phytophytora* sp.. They are also responsible in various metabolic pathways including tricarboxylic acid cycle, amino acid metabolism and iron sulphur cluster biogenesis (Wawrzyniak et al., 2008; Denoeud et al., 2011).

Furthermore, the organism can synthesize certain essential cellular phospholipids and accumulate them commonly within the storage vacuoles (Jeremiah & Parija, 2013). The average time of generation is about 17-22 h but also different among *Blastocystis* subtypes reliant on the medium used for cultivation (Zierdt and Swan, 1981; Natasa *et al*, 2017). Besides, the organism also has been observed to undergo apoptosis when exposed to adverse conditions, like exposure to ambient air and an antiparasitic agent such as metronidazole. This phenomenon serves as a mechanism to increase the number of viable cells during adverse conditions. There are four known modes of division proposed which are binary fission, plastomotomy, schizogony and dodyogony. Usually, binary fission is a wide mode that is used in the host for reproduction (Zierdt, 1991).

2.2.2 Morphology

Blastocystis is a polymorphic protozoan with various morphological forms including vacuolar, granular, amoeboid, cyst, multi vacuolar and avacuolar forms. Among these forms, the vacuolar form is the most commonly identified in stool and in vitro culture while the cysts are recognized as the only transmissible form and the transmission of *Blastocystis* by consumption of food and water contaminated with the cyst form through the faecal oral route. Recently, all forms can be found in a stool sample by various microscopic techniques such as phase contrast microscopy of wet mounts, bright field microscopy of wet mount and stained smears and also electron microscopy (Zierdt, 1991). However, only an electron microscope is used to identify particular forms like avacuolar and multivacuolar.

2.2.3 Life cycle and transmission

In the early 1900s, a life cycle for *Blastocystis* was first proposed by Alexeieff who identified a complex cycle involving binary fission of a binucleate stage (plastomotomic division) and autogomy, a sexual process that forms primary cysts. These cysts were formed by numerous budding spores. The spores or secondary cysts were thought to be resistant forms and were smaller than the primary cysts, uninucleate and surrounded by a thick membrane. Merogony (schizogony), an asexual mode of reproduction and sporogony, a sexual phenomenon also were described (Boreham & Stenzel, 1993). However, more recent reports have indicated that binary fission and plasmotomy were two distinct modes of reproduction (Zierdt, 1988). As proposed by Zierdt (1973), based on his light microscopical observations, this life cycle expected the vacuole form to differentiate into either the granular form from the central body or the

amoeboid form which produced prospective vacuole daughter cells by budding. He suggested that there were four modes of division asexually; binary fission, plasmotomy, endodyogeny and schizogony (merogony) but these were not illustrated in the proposed life cycle and little evidence was presented for their existence.

Later on, further analysis by extensive electron microscopical studies was made to reveal the division modes specifically. Nevertheless, the studies have failed to expose sporogony, endodyogeny or merogony (Boreham & Stenzel,1993). Those proposed modes are not likely due to the pleomorphic nature of the organism and not true modes of reproduction (Tan, 2008). Membrane-bound projections and cytoplasmic bridges which appear to divide the central vacuole may have given the appearance of these supposed means of division when examined by light microscopy. Thus, binary fission is the only proven method of reproduction for *B. hominis* at present. *B. hominis* cells dividing by binary fission are seen commonly by light microscopy and less frequently by electron microscopy (Sienzel et al., 1991; Stenzel & Boreham, 1996; Y Matsumoto, 1987).

Yet the life cycle and transmission of the parasite are still under intensive investigation (Lepczy et al., 2017). A life cycle comprising thick and thin-walled cysts has been hypothesized on its function, the thick one is important for external transmission while the thin-walled cysts were auto-infectious. There are two types of production have been described; asexual reproduction by binary fission and sexual reproduction by autogamy to form a primary cyst (Basak et al., 2014; Stenzel & Boreham, 1996) which is the only transmissible form of *Blastocystis.* After getting into the intestinal lumen, the cysts first developed into vacuolar form. In humans, vacuolar

form divide by binary fission and may be developed into amoeibod, multivacuolar, or granular forms before they become pre-cyst (Jeremiah & Parija, 2013). The primary route of transmission is faecal oral with an indefinite incubation period and infection duration but the cyst is postulated to be its infective stage.

2.2.4 *Blastocystis* is one of the Intestinal parasite infections (IPIs)

Intestinal parasitic infections (IPIs) are among the major public health issues of high morbidity and mortality worldwide which vary from intestinal protozoa and helminths. According to the World Health Organization (WHO), it has been classified as a Neglected Tropical Disease (NTDs) where about 340 parasites infect more than 3.5 billion persons and cause apparent clinical signs and symptoms in 450 million approximately with high prevalence in developing countries (WHO, 2015). It has since been extensively spread throughout the world and is more prevalent in tropical and sub-tropical regions. Several intestinal parasites are known to cause morbidity in humans, including several protozoans such as *Entamoeba* histolytica, *Giardia* lambia, *Blastocystis* hominis, and soil-transmitted helminths (STHs) such as hookworm, *Trichuris trichiura*, *Ascaris lumbricoides* and *Strongyloides stercoralis* (Bahrami et al., 2018; Htun et al., 2018).

Parasitosis influences the body's metabolism, nutrition absorption and gut ecosystem (Almugadam et al., 2021; Htun et al., 2018). Besides, individuals infected by *G. duodenalis*, *Cryptospodium* spp., *Blastocystis*, and *E. bieneusi* can also develop a wide range of symptoms ranging from asymptomatic to acute or chronic diarrheal disease including clinical signs of diarrhoea, nausea, weight loss, blood in the stool and fever are less in common (Certad et al., 2017; Darlan et al., 2020; W. Li et al., 2019).

While occasionally life-threatening, approximately 200 million people in low and middle countries have symptomatic giardiasis particularly in infants, young children and young adults being the groups most at risk of infection (Torgerson et al., 2015). It also has been proven that infectious agents like parasites can alter certain enzymes and metabolic factors in the infected host (Zibaei et al., 2023). For example, acute *Cryptosporidium* infections are estimated to cause 48,000 annual deaths worldwide in children under five years of age (Khalil et al., 2018).

Regarding *Blastocystis*, more than 1 billion people carry the parasites globally and are linked with intestinal (irritable bowel syndrome) and extra-intestinal (urticaria) disorders. Additionally, *E. bieneusi, Giardia Lambia, Cryptosporidium* and *Blastocystis* spp. are regarded as an emerging public health concern in both immunocompromised patients (HIV-infected patients, cancer patients, organ transplant recipients) patients and immunocompetent individuals (Esteghamati et al., 2019; Muadica et al., 2020). The pathogenicity of several intestinal protozoa is well established and their presence is usually associated with diarrhoea and other intestinal manifestations (Stark et al., 2010). However, the pathogenesis of *Blastocystis* is controversial but the colonisation with this organism also be linked to gastrointestinal symptoms (T. Roberts et al., 2014).

In Malaysia, previous epidemiology showed that *Blastocystis* infection was more prevalent in the rural (13.7%) than the urban population (3.4%) (Nithyamathi et al., 2016). Commonly, *Blastocystis* infection has been reported in numerous studies involving aborigines and school children in Malaysia with more asymptomatic symptoms. This suggests that *Blastocystis* could thrive as a harmless commensal organism in the gut of individuals, without causing overt disease in certain populations.

However, there were some studies on patients with comorbidities like dengue patients, cancer patients and patients with diarrhoea who had been reported with gastrointestinal symptoms (Kumarasamy et al., 2023). The distribution of *Blastocystis* among dengue patients was 23.6% with a high fever rate when compared to non-*Blastocystis* infection patients (Thergarajan et al., 2019). The host's immune status appears to be a risk factor for symptomatic *Blastocystis* infection. Moreover, migrant labourers, animal handlers and inmates living close by were also exposed to *Blastocystis* infection, which leads to increased symptomatic infections in this population. Overall, the *Blastocystis* infection pattern in Malaysia has been reported in various states with a high prevalence, especially in patients with comorbidities, which were up to 25% prevalent (Kumarasamy et al., 2023).

Concerning the *Blastocystis* infection, there are different means of exposure to intestinal parasitic infections including consumption of undercooked meat, drinking contaminated water and skin absorption and interaction with livestock (Mudassar *et al*., 2018). Even in some countries where education excels and adequate sanitation conditions, some intestinal parasites have played a significant role in causing diseases in specific groups such as immunocompromised individuals, organ diseases such as diabetes and also among renal failure patients with ambiguity mechanisms (Peña et al., 2020; Darlan et al., 2020; Li et al., 2018). As a consequence, ending epidemics of NTDs by managing the transmission of IPIs and the mitigation of potential risk factors is one of the sustainable development goals (SDG) of the United Nations (2030 Agenda; Goal 3.3) (Sitotaw & Shiferaw, 2020).

Nonetheless, even though there is an immense mobilisation on health progress following SDGs and substantial changes in the diagnosis of parasitic diseases and subsequent therapies (Momčilović *et al*., 2019), no reduction has been seen and IPIs in low-income countries such as Ethiopia or rural areas especially in aboriginal communities where it has continued to be a major public health issue mainly affecting school children and immunocompromised individuals (Noradilah et al., 2017; W. Li et al., 2019; Al-Jawabreh et al., 2019; Sitotaw & Shiferaw, 2020). It has been revealed that any related factors to poverty, lack of awareness and unavailability of sufficient healthcare as well as the prevailing bad climatic and environmental conditions are the most aggravating risk factors of IPIs. Notably, *Blastocystis* is one of the major concerns as it is part of intestinal parasitic that infect people, particularly in children and immunocompromised patients.

2.2.5 Genetic diversity and *Blastocystis* pathogenicity

Molecular research has revealed different subtypes of *Blastocystis*, based on genetic heterogeneity of the small-subunit ribosomal RNA gene (*SSU* rRNA) sequences which currently 40 subtypes (STs) have been identified (ST1- ST38). All of these subtypes have been found in mammalian and avian hosts while there is no nomenclature subtyping as yet for amphibian, insects and reptilian hosts although several genetically distinct *Blastocystis* lineages have been identified (Santin, 2024; Maloney et al., 2021, 2023; Jinatham et al., 2021; Maloney & Santin, 2021; Osorio-Pulgarin et al., 2021; Christen Rune Stensvold & Clark, 2020; Yoshikawa et al., 2016).

Currently, 14 subtypes (STs) (ST1-ST9, ST10, ST12, ST14, ST16 and ST23) have been isolated from human range with varying prevalence; about 90-95% of human

infections can be attributed to one of the ST1-ST4 with a predominance of ST3 (Guilavogui et al., 2022; Khaled et al., 2020, 2021; A. D. S. Zanetti et al., 2020). Meanwhile, all the subtypes found in humans, except for ST9 have also been identified in animals including non-human primates, mammals and birds (Valenca et al.,2019; Zanetti et al., 2020). Nine subtypes (ST1-ST9 and ST12) have been potentially zoonotic where ST5 and ST6 are frequently harboured by hoofed animals and birds respectively (Forsell et al., 2017; Hublin et al., 2021). More recently, 15 additional subtypes (ST18 to ST32) were identified from birds, Bovidae and Cervidae (Higuera et al., 2021; Khaled et al., 2020; Maloney, Lombard, et al., 2019; Maloney et al., 2020, 2021). However, ST18-ST20 and ST22 are not currently considered as valid as they represent potential chimaeras arising during PCR amplification (Christen Rune Stensvold & Clark, 2020). While, four novel subtypes (ST35-ST38) have been reported its validation using future full-length reference sequences (Maloney et al., 2023).

Blastocystis has been the subject of controversy for many years due to its role as both a commensal and potential pathogen. Some of the previous studies did not report any conclusive evidence for the pathogenic role of *Blastocystis* as it did not cause any clinical symptoms, especially in healthy individuals (Chabé et al., 2017; Deng et al., 2021; Kumarasamy et al., 2023). Conversely, depending on the study, the prevalence of *Blastocystis* infection has been sporadically shown to be higher in either immunocompromised or immunocompetent in other cohort studies (Barçın Öztürk et al., 2023; Saadah et al., 2022; Sarzhanov et al., 2021. A metagenomic survey by Beghini et al., (2017) highlighted that *Blastocystis* may be important for host health because it was discovered to be more prevalent in healthy control individuals than in patients with obesity, Crohn's disease and colorectal cancer. Interestingly, *Blastocystis*

colonisation has been proposed to have a beneficial role in the gut which is associated with increased microbial diversity and richness indicating a commensal relationship (Deng et al., 2021). A study conducted in Korea observed that *Blastocystis* was more frequent in the non-diarrhoea group compared to diarrhoea patients which ST3 was the most common subtype detected (M. J. Kim et al., 2020). This previous finding resulted in the commensal of *Blastocystis* which could potentially be beneficial to gut health.

Moreover, most of the Blastocystosis clinical characteristics such as abdominal pain, diarrhoea, flatulence, constipation, anoxeria, nausea, vomiting and fatigue, that have been ascribed to *Blastocystis* are non-specific. However, some studies reported to have a strong association with non-specific gastrointestinal symptoms likes diarrhoea, abdominal pain and vomiting among *Blastocystis* infected patients especially in irritable bowel syndrome (IBS), cancer patients and urticaria (A. Asghari et al., 2020; Kesuma et al., 2019; Lepczyńska et al., 2016). Looking at *Blastocystis* subtypes, it has been proposed that different subtypes are linked to *Blastocystis* pathogenicity (Ajjampur & Tan, 2016). Up till now, a correlation between pathogenicity and subtypes (STs) of *Blastocystis* is still lack of information and it has been demonstrated in recent findings that not all strains of given subtypes are pathogenic. Table 2. shows the possible subtype contribution to *Blastocystis* pathogenicity.

Table 2.3: Possible subtype contribution to *Blastocystis* pathogenicity

Subtype	Pathogenic Characteristics	References
ST1	• Associated with symptomatic infections; Diarrhoea, abdominal pain • Mostly seen in several diseases including patients with Irritable bowel disease (IBS), Cancer • Pathologic outcomes in experimentally-infected rats (Inflammation response)	(Zhang et al., 2017; Kesuma et al., 2019; E. M. Hussein et al., 2023)
ST2	• Associated with symptomatic (diarrhoea and abdominal pain) and asymptomatic infection • Mostly seen in several diseases (IBS, colitis ulcerous, cancer)	(Bednarska et al., 2018; Labania et al., 2023)
ST3	• Possibly variant: pathogenic and non-pathogenic strains within the same subtype	(Rojas-Velázquez et al., 2022)
ST4	• Associated with symptomatic infection • Pathologic outcomes experimentally-infected rats (Increased anti-inflammatory response) • Expression of protease activity in experimentally-infected rats	Christen Rune Stensvold et al., 2011; Deng et al., 2021)
ST6	• Expression of protease activity from symptomatic human sample	(Karamati et al., 2021)
ST7	• Pathologic outcomes from human samples and experimentally-infected rats (Increased oxidative stress, inflammation response) • Expression of protease activity in experimentally-infected rats	(M. X. Lim et al., 2014; Deng et al., 2021)

It had been reported that *Blastocystis* ST1 is the most prevalent where it was associated with IBS-diarrhoea with a risk factor of 2.9 times, indicating that ST1 is likely to be correlated with the clinical presentation of diarrhoea and probably is a pathogenic strain (Kesuma et al., 2019; Vargas-Sanchez et al., 2015). Interestingly, Vargas-Sanchez et al., (2015) suggested that the generation times of *Blastocystis* might be susceptible to alterations in the intestinal environment caused by IBS. This could be attributed to the potential selection of virulent strains with slower growth rates, leading to a reduction in their genetic variability. However, a recent study did not agree as they found an insignificant correlation between individuals infected with ST1 and gastrointestinal symptoms (Darwish et al., 2023). Furthermore, an experimental study by Hussein et al., (2023) demonstrated that certain *Blastocystis* subtypes, particularly ST1 and wild ST3 are associated with being polyp inducers as *Blastocystis* infection with ST1 and ST3 led to goblet cell depletion of 33% and 60% respectively. The conflict in results may be attributed to chronic exposure of goblet cells in the gastrointestinal tract to mucus pathogens, triggering increased mucin protein (MUC2).

In various studies, ST3 has consistently emerged as the predominant subtype in numerous diseases, such as IBS, among transplant recipients, HIV patients, individuals with dengue, and others (R. Das et al., 2016; Fontanelli Sulekova et al., 2019; Silva et al., 2020; Thergarajan et al., 2019). Many researchers suggested that ST3 correlates with gastrointestinal symptoms. However, it is important to note that ST3 is extensively documented in both symptomatic and asymptomatic individuals. Furthermore, a study conducted by Bednarska et al., (2018) observed that *Blastocystis* ST3 and ST2 were detected in individuals who suffered from intestinal disorder and colitis ulcerous respectively. ST3 is associated with weight loss while ST2 with diarrhoea and

abdominal pain. *Blastocystis* ST2 infections are most often asymptomatic, but gastrointestinal problems and chronic urticaria sometimes occur (Vogelberg et al., 2010). In Italy, ST2 accounted for 14% of isolates in mildly symptomatic individuals, who had irritable bowel syndrome (IBS), experienced chronic diarrhoea, or had various forms of immunosuppression (Mattiucci et al., 2016).

While, *Blastocystis* ST4 is significantly associated with infectious diarrhoea in European patients, particularly in Denmark, where it constitutes the majority (76%) of *Blastocystis*-positive cases in individuals with acute diarrhoea lasting more than two weeks (Christen Rune Stensvold et al., 2011). A recent review study found out that, ST4 and ST7 are associated with pathogenic potential as they activate IL-8 gene expression in human colonic epithelial cells and derived cysteine protease. Cysteine protease can degrade immunoglobulin A (IgA), which allows the parasite to colonise the intestinal environment while protecting itself from the host's immune defences (Puthia et al., 2005). In addition, ST7 induces a strong expression of pro-inflammatory cytokines IL-6, IL-1β and TNFα (Deng et al., 2021; Puthia et al., 2008). Furthermore, this particular subtype was observed in several immunocompromised patients including transplant patients (4.2%), cancer patients (8.3%) and ulcerative colitis (14.93%) (Andersen et al., 2015; A. Asghari et al., 2020; Silva et al., 2020). In most cases, *Blastocystis* subtypes are thought to have the ability to modify the tight junctions between intestinal cells and intestinal content, impairing barrier function and contributing to the pathogenesis of autoimmune disease (E. M. Hussein et al., 2008; Mahmoud et al., 2023).

The results suggested that not all strains of a given subtype are pathogenic. Despite conflicting results between *Blastocystis* subtypes and clinical findings, ST1,

ST4 and ST7 were generally associated with symptoms, while ST2 and ST3 were considered pathogenic (Darwish et al., 2023; Kosik-Bogacka et al., 2021; Jeremiah & Parija, 2013; Mehlhorn et al., 2012). The pathogenicity of *Blastocystis* depends on several factors, including the pan-genome of infected subtypes which may code for virulent factors and toxins as well as the host's immune response and interactions with the intestinal microbiota (Rojas-Velázquez et al., 2022). This indicates that subtype alone is not the only factor linked to the pathogenicity of these parasites (Tito et al., 2019; Roberts et al., 2014). Furthermore, a recent study observed that asymptomatic *Blastocystis* in a human gut can be triggered to develop pathogenic characteristics when influenced by the intestinal microbiota (Rajamanikam et al., 2023). Genomic data in combination with *in-vitro* and *in-vivo* studies allowed the identification of recognised virulence factors and revealed the harmful influences of this parasite on the intestinal barrier, leading to reliable models of pathogenesis. The pathogenicity and commensal of *Blastocystis* has been summarised in the concept map in Figure. 2.1.

Although the presence of *Blastocystis* in both symptomatic and asymptomatic patients raised concerns about whether it is a pathogen or commensal, this parasite is now recognised as a both pathogen and parasite and is listed in "Water Sanitation and Health Program" of the World Health Organization (WHO, 2011; Mülayim *et al*., 2021). *Blastocystis* also matched all of the pathogenicity criteria that *Giardia* and *Entamoeba histolytica* do, including fulfilment of Koch's postulates, demonstration of a treatment that eliminates both the organism and the symptoms, and enhanced immune response in symptomatic patients (Sekar & Shanthi, 2015; T. Roberts *et al*., 2014).

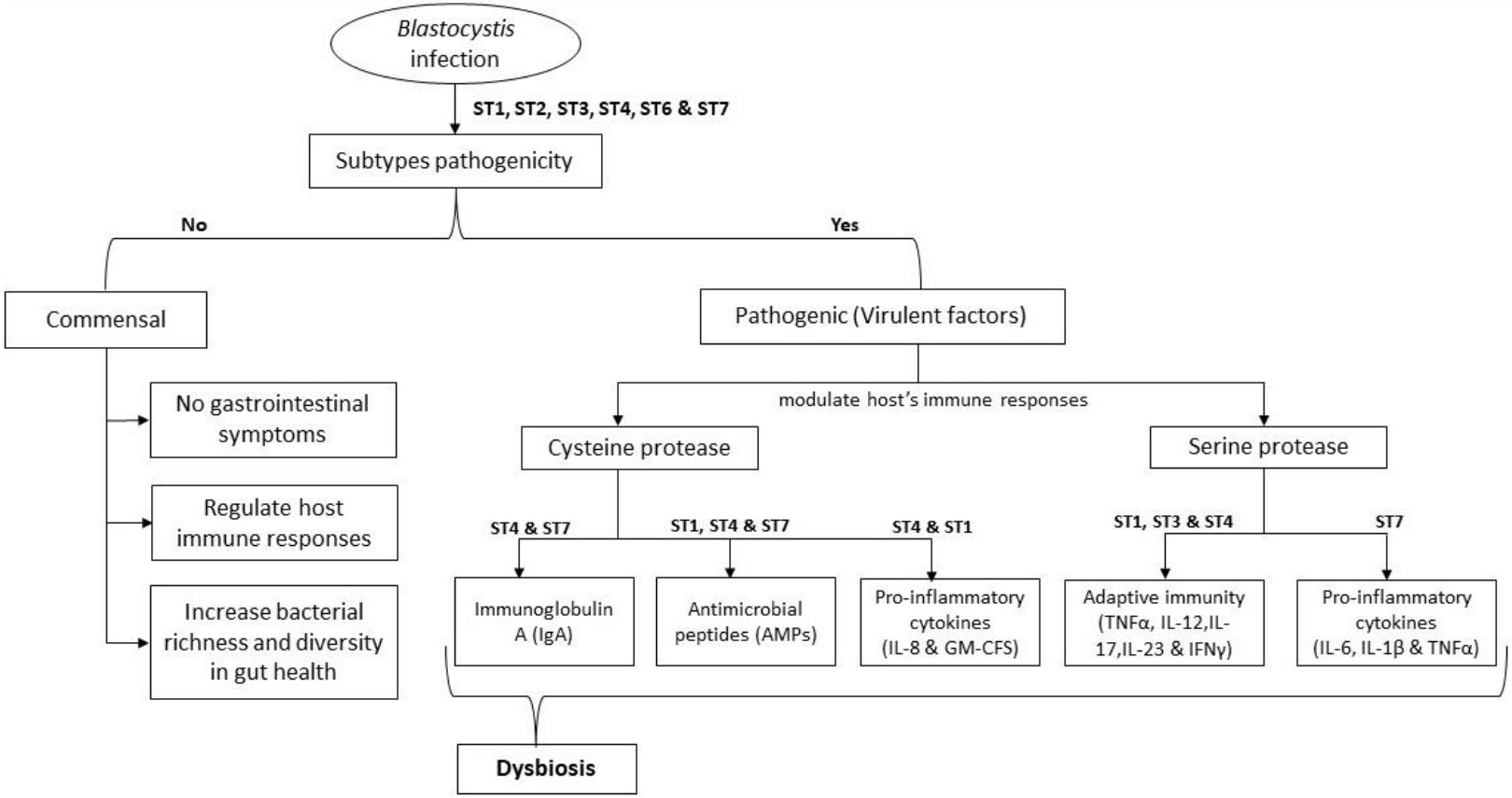

Figure 2.1: Concept map summarising the pathogenicity and commensal nature of *Blastocystis* infection based on the literature review

2.2.6 Prevalence of *Blastocystis* infection in diabetes diseases

Type 2 Diabetes Mellitus (T2DM) is a prevalent disease across the world. T2DM is characterised by the inability of the body to produce sufficient insulin to maintain a proper glucose level. It will lead to chronic hyperglycaemia resulting in impairment in innate and acquired immunity, ending with the immunocompromised condition that makes it susceptible to parasite infection (Berbudi et al., 2019; Ibrahim, 2020). Most recent findings demonstrated that *Blastocystis* infection may be prone to certain populations with immunocompromised conditions including diabetes disease. Due to the general immunocompromised conditions during diabetes mellitus, a high prevalence of various infections is expected in diabetics, but surprisingly, epidemiology data in this regard are scarce and there are few studies on the prevalence of intestinal parasites in diabetic patients especially *Blastocystis* infection (Aourarh et al., 2020). Table 2. shows a list of studies that observed on prevalence of *Blastocystis* in T2DM participants when compared to healthy controls.

Blastocystis hominis parasite has been revealed in many cases with uncontrolled diabetes as diabetic patients are known to be immunocompromised which puts them at higher risk of infectious by intestinal parasites (Marcos & Gotuzzo, 2013; Popruk et al., 2020). There was a study that demonstrated that the risk of infection with intestinal parasites was 3.6 times greater for patients with diabetes mellitus (DM) rather than healthy people. It was found that the most infection detected in this study was *Blastocystis hominis* (Mohtashamipour et al., 2015). This result was supported by the study that was conducted in Morrocco which parasite infection was significantly higher in patients with DM (48%) than in controls (10%) which the most detected parasite in

Blastocystis, 42% in diabetic patients and 10% in controls patients respectively (Aourarh et al., 2020). In another study conducted in India, the rate of parasitic infection was observed in equal numbers of diabetic and non-diabetic patients. Among diabetics, the most common parasite infection is *Blastocystis* (23.2%) (Khanna et al., 2022). However, it was not similar to a study that was observed in Cameroon, Africa as it has been demonstrated that the risk of intestinal parasites among diabetics was 10% compared to healthy control was 23.5%. Nevertheless, the rate of *Blastocystis* infection was still higher in the diabetic patients rather than control group about 2.7% and 1.2% respectively. This can be explained by the greater number of physician visits incurred by diabetic patients than by non-diabetic patients where diabetic patients consult frequently and are treated for possible intestinal parasitic infections (Tangi et al., 2016). Overall, the microscopic examination from recent studies only revealed the present of *Blastocystis* which may contributed to the low positivity but was mainly used to improve the visualization and distinction of *Blastocystis* forms (C. Rune Stensvold & Clark, 2016).

However, there are limited studies have been reported on *Blastocystis* subtypes of infection in diabetic patients. Amplification of *Blastocystis* DNA from recent studies revealed that *Blastocystis* in diabetic patients is also higher than in controls (de Melo et al., 2021; Popruk et al., 2020). A study conducted in Brazil observed that there were several *Blastocystis* subtypes were identified including ST1, ST2, ST3, ST6, ST7 and ST8. Interestingly, there was a predominance of ST1, followed by ST3 and ST8 in diabetic groups while ST3 was observed followed by ST1 and ST2 in the control group (de Melo et al., 2021). While a recent study from Turkey reported higher rates of *Blastocystis* infection in the control group compared to the diabetic group, molecular

techniques were employed to identify *Blastocystis* subtypes, revealing the presence of ST3, ST2, ST1 and ST6. However, there was no significant difference between the groups in terms of *Blastocystis* positivity (Barçın Öztürk *et al*., 2023).

Despite there is potential significance of the association between *Blastocystis* infection and diabetes disease, still has a lot of unknowns, especially when it comes to pathogenicity (Dubik et al., 2022). *Blastocystis* may be an opportunistic infection in immunocompromised hosts, potentially contributing to reduced intestinal mucosal integrity and increased risk of diabetes (Fattahi Bafghi et al., 2015). The prevailing consensus suggests that the clinical manifestations induced by *Blastocystis* might be related to factors such as *Blastocystis* subtypes, the host immune system, parasite density, intestinal microbiota, and the presence of other concomitant pathogens (Deng et al., 2021; Tan & Mirza, 2012). Nonetheless, a review of studies indicated that T2DM in humans can be associated with compositional changes in the intestinal microbiota, favouring the occurrence of *Blastocystis* in this group of patients depending on its subtype (Crudele et al., 2023; Deng et al., 2021).

Table 2.4: Studies on the prevalence of *Blastocystis* in Diabetic patients and non-diabetic group

No	Location	Diabetes Disease	Study Design	Number of Samples (N)	Prevalence (%)		Diagnostic Approach	Subtypes	References
					DM	NonDM			
1.	Iraq	DM	Cross sectional	419	35.48%	N/A	Microscopy	N/A	(Ali et al., 2018)
2.	Iran	T2DM	Case control	236	9.3%	6.8%	Microscopy	N/A	(Mohtashamipour et al., 2015)
3.	Cameroon	DM	Cross sectional	235	2.7%	1.2 %	Microscopy	N/A	(Tangi et al., 2016)
4.	Iran	DM	Cross sectional	500	2.4%	2.4%	Microscopy	N/A	(Fattahi Bafghi et al., 2015)
5	Iran	DM	Case control	100	42%	4%	Microscopy	N/A	(Aourarh et al., 2020)
6	Brazil	T2DM	Case control	175	12.1%3	7.9%	Microscopy	N/A	(de Melo et al., 2021)
					4.3%	30.3%	PCR	ST1, ST2, ST3, ST6, ST7 & ST8	
7	India	DM	A Retrospective study	625	23.2%	N/A	Microscopy	N/A	(Khanna et al., 2022)
8	Iran	DM	Case control	501	9.1%	10.3%	Microscopic	N/A	(Poorkhosravani et al., 2019)
9	Thailand	DM	Case control	230	12.3%	9%	PCR	ST3, ST1 & ST4	(Popruk et al., 2020)
10	Turkey	T2DM	Case control	200	9%	9%	Microscopy	N/A	(Barçın Öztürk et al., 2023)
					9%	11%	PCR	ST3, ST2, ST1 & ST6	
11	Egypt	DM	Case control	80	27.5%	12.5%	Microscopy	N/A	(Fadl et al., 2021)
12	Egypt	DM	Case control	200	29%	N/A	Microscopy	N/A	(Waly et al., 2021)

N/A; Not available, PCR; Polymerase chain reaction

2.2.7 Laboratory Diagnosis for *Blastocystis*

2.2.7.1 Microscopy

The microscopic method is usually made through the observation of *Blastocystis* morphological in faecal samples with stained specimens (Tan, 2008). Diagnosis by direct examination in fresh form with physiological saline solution and diluted lugol solution is widely used in developing countries for *Blastocystis* cultivation due to the insufficient infrastructure and cost (Roberts et al., 2011; Devera et al.,2014). Stains usually used in *Blastocystis*'s detection include Lugol's iodine for wet mount and permanent-stained smears with acid-fast, Giemsa, Field's and trichome stains. However, previous studied discovered that, it would be a low sensitivity for cysts detection (Tamalee Roberts et al., 2011). In contrast, trichome stain is routinely addressed in many clinical microbiology laboratories and some studies have shown it is more sensitive for the detection of intestinal protozoa rather than iodine- stained wet mounts (Tan, 2008; Gardner et al., 1980).

The documented forms of *Blastocystis* consist of vacuolar, multivacuolar, amoeboid, granular and cysts that may be detected by using this technique. By some means, microscopic detection by fresh faecal smears is difficult mainly due to the morphological diversity of the parasite which leads to false analysis. The classic vacuolar form may be intermittent and the rarer cystic, amoeboid, avacuolar and multivacuolar forms may be predominant. The cysts different in size and appearance concerning the vacuolar form may be undetected (C. Rune Stensvold & Clark, 2016). As been observed by a previous study conducted by Roberts et al., (2011), the finding showed about 50% of the infections with *Blastocystis* are missed by using permanent

stains although the method is the gold standard for diagnosis of enteric protozoa. The low sensitivity of this method will lead to misleading observation if the inexperienced individual is unable to identify the boundless morphological diversity of this protozoan (vacuolar, granular, amoeboid, and cyst) and to differentiate it with other protozoa, yeasts, human cells and artefacts. Also, the low sensitivity may result to low numbers of parasites in the samples. Thus, they are not detected by the staining procedure. It likely to underestimate the prevalence of the stramenophiles. So, this technique is not adequate to differentiate *Blastocystis* subtype but it can be improved by in-vitro cultivation of faecal specimen in culture medium (Jose R.V., 2019; Noradilah et al., 2018; Roberts et al., 2011).

2.2.7.2 In vitro cultivation

Xenic or monoxenic laboratory cultures of *Blastocystis* isolates which are cultures of *Blastocystis* cells grown in association with non-standardized or single known species of microorganisms, respectively can be maintained in Jones' or Boeck and Drbohlav's inspissated egg medium. However, a previous study that isolated from Australian marsupials reported Jones' medium may not support faecal cultures from certain animal hosts (Tan, 2008). Some modifications were made to utilize a diphasic agar slant medium which was useful for the culture of *Blastocystis* isolates from cattle, pigs, and chickens (Abe, 2004).

Axenic cultures are cultures of *Blastocystis* cells that do not associate with any other living organism which are demonstrate luxuriant growth in a variety of media such as Iscove's modified Dulbecco's medium, minimal essential medium or biphasic inspissated egg slant overlaid with Locke's solution (Ho et al., 1993). The doubling

time of axenic isolates can be variable ranging from 6 to 23 hours depending on the isolate, the study and the type of medium used (Boreham & Stenzel, 1993; Zierdt et al.,1981). Some techniques can be used including by the pour plate method which was the colony growth of *Blastocystis* cells can be established in soft agar and clonal growth was achieved by the addition of sodium thioglycolate as a reducing agent. It was useful for isolating the parasite colonies from the bacterial ones and for screening surface reactive antibodies for cytotoxic activity (Moe et al., 1996; Tan, 2008). In addition, it also can be grown in a solid medium but it has been indicated that the growth characteristics of the same *Blastocystis* isolate in a solid medium are markedly different from those of the isolate in a liquid medium.(Tan et al.,2000; Tan, 2008). Axenization also can be accomplished by the addition of antibiotic cocktails to eliminate contaminating bacteria and yeasts a variety of antibiotic mixtures have been described with various levels of success. However, the process is generally laborious and may take weeks to months and the successful elimination is not guaranteed (Tan, 2008; Zierdt, 1991).

Most studies tended to apply the Jones' medium with 10% horse serum because of its convenience and sensitivity to detect more numbers of *Blastocystis* infections in large samples in field studies. It had been observed in the previous study, XIVC was more sensitive than any traditional parasitological techniques which were able to detect the different morphological stages including the cysts stage in culture since it might be expected that cysts would excyst when incubated in culture under anaerobic conditions at 37℃ (C. Stensvold, 2013). Plus, IVC in Jones' medium followed by Wheatley Trichome staining method showed a significantly higher detection rate (86.3%) of *Blastocystis* infection in comparison to fixed-PVA stool specimen (PVA) followed by

Wheat Trichome staining (68.6%). It has been discovered that the negative predictive values of IVC suggested the detection of true *Blastocystis*-free was 93.2% in comparison to 91.8% with PVA. Meanwhile, the positive predictive value to determine the probability of having Blastocystis was 78.2% for IVC and 37.4% for PVA which displayed that IVC followed by Wheat Trichome staining was superior in *Blastocystis* detection (Noradilah et al., 2018). It was supported by other studies that demonstrated when both microscopic techniques (Wheatley's trichome stain and in-vitro culture) were used together, the overall prevalence and sensitivity of the *Blastocystis* detection were markedly improved and increased, which were 27.9% and 60.6% respectively. Being solely depends on one technique could lead to misinterpretation and under reported results if the molecular technique is not adopted in the study (Mohammad et al., 2018).

2.2.7.3 Conventional Polymerase Chain Reaction (PCR) and Real-time Polymerase Chain Reaction (Rt-PCR)

Concerning the extensive genetic variations among *Blastocystis* subtypes, the PCR-based method has become the most favourable diagnostic method. PCR was widely used as a reference technique due to its high sensitivity and specificity over other microscopy methods (Stensvold, 2013; Parkar et al., 2007; Stensvold et al., 2006). However, most developing countries cannot afford to use PCR as a part of diagnostic tools as it requires expensive and complex tools which also require specialized and highly trained technicians to promote intended results.

The first diagnostic by PCR for detection of *Blastocystis* was introduced in 2006 before the introduction of the consensus terminology (Stensvold et al., 2006). This

conventional PCR was based on sequences present in GenBank at that time and targets~300 bp toward the 3'-end of the SSU rRNA gene but later observations suggest that it may exhibit preferential amplification of ST3 over others due to the ST- specific polymorphisms on both primer annealing regions. But now, the subtype-specific diagnostic primers have been determined and these are useful to identify them distinctly which provides information on the distribution of various genotypes among human and animal populations and the zoonotic nature of certain genotypes (Sekar & Shanthi, 2015; Tan, 2008; Li et al.,2007b; Yoshikawa et al.,2004b).

Numerous studies have used STS primers in subtyping methodology that appeared to be insensitive especially those targeting ST4 for which the common 18S allele (allele 42) resulting to be undetected (Stensvold, 2013; Stensvold, 2012). Moreover, STS primer pairs are only available for ST1-ST7. Hence, the other STs remained undetected by using this method as STS PCR products are rather long (~300-700 bp) which is a clear disadvantage since the primers are sometimes used diagnostically (Stensvold, 2013). It has been reported in a previous study that ST4 accounts for 1% of *Blastocystis* carriage by using STS primer and is mainly absent in those countries typically Asia and the Middle East. Furthermore, a survey that has been conducted across the globe reported that those using STS primers indicate a lower of infection level of mixed ST infections compared to the prevalence of the parasite, probably <10% of all cases (Stensvold, 2013).

Another approach of *Blastocystis* isolates by PCR of SSU rRNA genes followed by reaction-restriction fragment length polymorphism (RFLP) analysis, dideoxy sequencing, or nested amplification of intragenic regions (Abe, 2004; Tan, 2008). In the

last decade, *Blastocystis* strains have been subcategorized using PCR-RFLP analysis which is usually used in prevalence studies and a variety of primers for its amplification. It has been called 'riboprinting', each 'riboprint' pattern identifies the 'ribosomes' to which the investigated parasite belongs (Clark, 1997). However, there are several limitations in a lack of standardization of the condition and choice of primers, mutation at restriction sites and the difficulty in interpreting RFLP profiles from mixed infection.

Due to the limitation of several approaches, the extensive genetic diversity of *Blastocystis* was clearly and independently noticeable by (Clark, 1997). C. Stensvold, (2013) indicated that pairwise genetic distances among some STs amount to at least 14.8% and this situation has made the design of genus-specific primers that apply to faecal DNA template amplification much more challenging than for most other parasites encountered in the clinical microbiology laboratory. Barcoding appears vigorous for genetic characterization combining the use of a forward primer of broad eukaryotic specificity (RD5) and a genus-specific reverse primer (BhRDr) (Scicluna et al., 2006; C Rune Stensvold & Clark, 2016). Sequence traces from PCR products obtained by barcoding may be difficult to interpret in cases of mixed ST carriage. However, the barcode fasta files can be interrogated individually or by bulk submission at the "*Blastocystis* ST (18S) and Multi Locus Sequence Typing (MLST) Multi Locus Sequence Typing Databases at www.pubmlst.org/*blastocystis* (Jolley & Maiden, 2010; C. Rune Stensvold et al., 2012). Apart from identifying on ST level, this technique also covers 18S allele analysis which offers higher resolution than subtyping alone (C. Stensvold, 2013) By using this barcoding method, a previous study by Noradilah et al., (2017) discovered that the isolated *Blastocystis* ST1, ST2, ST3 and ST4 were successfully detected from Aboriginal communities in Malaysia with an overall

prevalence of 40.2% where 42.6% and 27.8% of the subjects were infected with *Blastocystis* ST1-ST4 during the wet and dry seasons respectively. However, barcoding PCR relies on the protocol originally described by Scicluna et al., (2006) and involves amplification of partial SSU rRNA genes. When used on genomic DNA from stool, amplification of non-*Blastocystis* DNA may occur, especially in the absence of a *Blastocystis*-specific DNA sample. Hence, the primer used in the PCR reaction; forward primer of broad eukaryotic specificity (RD5) and a genus specific reverse primer (BhRDr) are not fully specific to *Blastocystis* and may amplify fungal DNA, especially in the absence of *Blastocystis* DNA in the human samples. Due to that reason, this PCR should not be used diagnostically unless for molecular characterization of positive samples has been detected from previous screening. It has been highlighted that barcoding primers are usually very specific when used on DNA extracted from *Blastocystis*-positive cultures (Stensvold & Clark, 2016).

More recent methods have been used which as real-time PCR for the detection of *Blastocystis* on faeces. Initially, "only two pan-*Blastocystis"* real-time PCR assays were published and the first PCR assay had low sensitivity and a specificity of 95% (Stensvold, 2013; Poirier et al.,2011). The other assay published was based on Taqman technology including a specific probe and internal process control to identify cases of PCR inhibition. It has been shown to have a sensitivity of 2.5 parasites per reaction and can detect all subtypes up until ST9 in humans (Stensvold & Clark, 2016b). Primers and probes targeting the SSU rRNA gene with a specificity of 100% can help to delineate carriers for non-carriers. (Stensvold, 2013; Stensvold et al.,2012; Stensvold & Clark, 2016).

2.2.7.4 Next gene sequencing (NGS) – *Blastocystis*

Recent molecular approach tools have identified that the mixed infections of *Blastocystis* may not be detected because of the preferential PCR amplification of the predominant subtypes. Before this, sanger sequencing coupled with cloning had been previously used to detect mixed infections when mixtures were suspected to chromatograms. However, this approach risks overlooking subtypes present in the low abundance causing genetic heterogeneity within a specimen to be underreported even when cloning is used. Therefore, the application of next-generation sequencing (NGS) to genomic DNA offers a unique and cost-effective opportunity to study the genetic diversity of intestinal parasites and can detect mixed infections.

Next-generation sequencing (NGS) technologies provide powerful tools for the characterization and quantification of microbial communities. For *Blastocystis*, the SSU rRNA gene is currently the best phylogenetic marker available as it is the most commonly sequenced *Blastocystis* gene with widely available references that include all subtypes previously reported. Also, next-generation amplicon sequencing generates a vast number of individual sequences from a single simple allowing for a highly sensitive population of level analysis (Maloney, Molokin, et al., 2019).

It has been reported by Maloney and colleagues (2019) that, the NGS approach by using specific primers has a greater sensitivity compared to Sanger sequencing coupled with cloning. The study showed among 75 *Blastocystis* positive that obtained from cattle faeces, a total of 14 subtypes were detected by using NGS while Sanger sequencing was not able to detect another three subtypes. It also showed the agreement between the two sequencing technologies and demonstrated that NGS was as accurate

as Sanger sequencing for subtype detection while being a far more sensitive tool for the identification of mixed infections and low abundance subtypes. Similarly, other studies also revealed that the NGS method efficiently identified the mixed infection of *Blastocystis* in human samples. A mix of ST2 and ST3 was observed in 29.4% (n=5) of mixed infections and a mix of ST1, ST2 and ST3 was observed in 17.6% (n=3) of the mixed infection samples (Rojas-Velázquez et al., 2019).

2.3 Diabetes disease

Diabetes has been seen as a severe disease, a long-term disorder with a significant effect on the lives and well-being of people, families and communities worldwide. It is among the top 10 causes of death in adults and was estimated to have caused millions of deaths globally in 2017 with health expenditure of around USD 727 billion (International Diabetes Federation, 2017; Saeedi et al., 2019). Thus, both the United Nations and the World Health Organization (WHO) are focused on diabetes as a significant public health issue, considering the immense global epidemic of the most critical non-communicable global disease (Standl et al., 2019). Diabetes mellitus (DM) has been referred to as a clinical syndrome manifested by chronic hyperglycaemia in the absence of treatment. The heterogeneous aetio-pathology includes defects in insulin secretion, insulin action or both and disturbance of carbohydrate, fat and protein metabolism (WHO, 2019). This high blood sugar produces the classical symptoms of polyuria, polydipsia and polyphagia.

There are three main types of diabetes based on the World Health Organization which are Type 1 diabetes, insulin-Dependent Diabetes Mellitus (IDDM), Type 2 diabetes mellitus (T2D) or Non -Insulin-Dependent Diabetes Mellitus (NIDDM) and gestational diabetes mellitus (GDM). Type 2 diabetes mellitus (T2DM) is a complex and multifactorial metabolic syndrome characterised by an irregular metabolism of carbohydrates, fats, and proteins that leads to increased levels of glucose and lipids in the blood. Chronic exposure to elevated levels of glucose and lipids activates some pathways that cause impaired insulin secretion from pancreatic β-cells, insulin tolerance in peripheral tissues, reduced peripheral tissue glucose utilisation and abnormal hepatic glucose output (Rehman & Akash, 2017).

In terms of global diabetes prevalence, type 2 diabetes (T2DM) has increased significantly in recent decades. Global diabetes prevalence is estimated to be 9.3 percent of the global adult population (20-79 years) in 2019, rising to 10.2 percent (578 million) by 2030 and 10.9 percent (700 million) by 2045. Prevalence of diabetes differed by World Bank income group with a higher prevalence among high-income countries (10.4%) and middle-income countries (9.5%) compared to low-income countries (4.0%). Nevertheless in 2045, an increasing movement it projected to reach 11.9%, 11.8% and 4.7% in high, middle and low-income countries respectively (Saeedi et al., 2019). Diabetes prevalence numbers are largely determined by people with type 2 diabetes mellitus (T2DM) which comprised about 90% of the total population ranging from youth and adults (Lascar et al., 2018). In Malaysia, the National Health and Morbidity Survey (NHMS) 2019 reported there were almost 2 million (1,999,450) adult individuals with known diabetes ranging from 7.3% to 23.8% resulting from a variety

of causes including population expansion, population aging, rising rates of obesity and physical inactivity (Akhtar et al., 2022; Harris et al., 2019; Samsudin et al., 2016).

2.3.1 Development and Pathogenesis of T2DM

Historically, DeFrenzo (2009) presented the idea of the Triumvirate that resulted from deficits where it involves three factors; the pancreatic β cell, liver and the muscle that lead to the pathogenesis of diabetic and hyperglycaemia. DeFronzo noted that in a normal-weight patient with T2DM, the relationship between fasting glucose and the insulin response to an oral glucose tolerance test follows an inverted u-shape. This pattern summarises the natural progression of T2DM in an individual as the increase in fasting glucose initially stimulates a compensatory increase in insulin production from β cells but as fasting glucose increases further the β cell cannot maintain this increased output. As a result, insulin output is excessively low relative to the patient's high fasting plasma glucose. In addition to disproportionate insulin response in patients with T2DM, the effect of insulin has blunted throughout the body. In other words, patients with T2DM are insulin resistant which further increases the demands on the β cells.

In the last decade, in light of the growing complexity of the understanding of T2DM, DeFronzo expanded his model of T2DM from the triumvirate into the ominous octet. The ominous octet model recognized the role of fat cells, the gastrointestinal tract, pancreatic α cells, kidneys and the brain together with muscle, liver and β cells in the pathogenesis of T2DM (DeFronzo, 2009). In fat cells of healthy individuals, insulin inhibits lipolysis thereby suppressing the release of free fatty acids. However, in patients with T2DM, insulin resistance prevents insulin from acting on fat cells, leading to increased lipolysis and elevated free fatty acid concentration. The excess free fatty acids

cause lipotoxicity which further promotes insulin resistance, inducing inflammatory and atherosclerotic provoking adipocytokines. It also decreased the insulin-sensitizing adipocytokines (adiponectin). In T2DM, it is also found to have enlarged fat cells which ironically will decrease the capacity to store fat adequately. As they cannot store adequately, lipids overflowed into the liver or muscle will produce insulin resistance. Furthermore, the lipid overfilled β cells and arterial smooth cells which results to an impaired insulin secretion and also atherosclerosis (Sears & Perry, 2015).

After several years of research, scientists discovered that gut microbiota plays a role in the pathogenesis of hyperglycaemia in diabetes. Obesity, non-alcoholic fatty liver (NAFL), insulin resistance, and chronic inflammation are all associated with the development of T2DM (Mouries et al., 2019; Saad et al., 2016; Z. Zhou et al., 2022). The gut microbiota can influence glucose homeostasis through multiple mechanisms (Gérard & Vidal, 2019). Furthermore, there may also be additional unidentified novel risk factors, such as subclinical inflammation caused by infectious agents, that contribute to this rising prevalence of T2DM (Molan et al., 2016). In developing countries, infectious pressure might be one particular modifier of insulin resistance. For example, intestinal parasitic infections like Helminth may affect the whole body and tissue-specific insulin sensitivity owing to their immunomodulatory properties (Tahapary et al., 2015). Other recently reviewed studies also suggested the intestinal parasites like *Toxoplasma gondii* could be the inflammatory mediated destruction of pancreatic β cell mass that ultimately contributes to the failure of the β cell to produce enough insulin. This in turn would increase the risk of developing acute and chronic pancreatitis as well as diabetes (Molan et al., 2020). In this regard, an emerging field of research is beginning to investigate the potential of infectious and environmental

pathogens to cause low- grade inflammation that may facilitate the risk and development of various metabolic conditions, including diabetes and obesity. In fact, the development of Type 2 Diabetes Mellitus (T2DM) is influenced by underlying abnormal conditions such as nutritional factors, physical activity, gut dysbiosis, metabolic memory, and mitochondrial dysfunction (Galicia-Garcia et al., 2020).

2.3.2 Pro-Inflammatory Cytokines: IL-1 and IL-6 in T2DM

Type 2 diabetes mellitus (T2DM) is thought to be caused in part by subclinical chronic low-grade inflammation. The pathogenesis of diabetes is characterized by hyperglycaemia regulated by various mechanisms including immune cells and impaired insulin secretion. Inflammasomes are important sensors of T2DM metabolic dysfunction and β-cell dysregulation because they can activate an inflammatory response in response to a variety of signals (Yang *et al*., 2021). Accumulating evidence points out that the inflammation status of T2DM islets has two characteristics; elevated cytokines levels and increased activation of immune cells, mostly macrophages (Herder *et al*., 2015). It is stated that the risk of T2DM can increase due to the level of inflammatory biomarkers that are chronically elevated which directly enhance insulin resistance (IR) and lead to disruption of insulin sensitivity in turn impairing glucose homeostasis (Calle & Fernandez, 2012). T2DM is associated with increased oxidative stress and low-grade inflammation mediated by mast cells (MCs) which can worsen insulin sensitivity, β-cell activity and cardiovascular events (Todingan et al., 2023). Proinflammatory mediators or cytokines, particularly interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-), and various other IL-1-dependent pro-inflammatory cytokines and chemokines, are major contributors to

insulin resistance by inducing various inflammatory responses (Rehman & Akash, 2016). However, the relationship between inflammation and diabetes mellitus and insulin resistance is still being studied (Fadaei et al., 2020).

Interleukin-6 (IL-6) is one of the main pro-inflammatory cytokines that involved in various inflammatory processes by controlling differentiation, migration, proliferation and cell apoptosis (Rehman & Akash, 2016). It has complex roles and is expressed in many cells including immune cells, skeletal muscle cells and pancreatic cells. It is also considered as a predictor of T2DM that contributes to insulin resistance and deteriorating glucose homeostasis (Sari et al., 2019; Qu et al., 2014; Bastard et al.,2000). One of the causative factors is the progressive failure of pancreatic islet cells and decreased cell mass, which eventually leads to increased apoptosis, which is the primary cause of impaired insulin secretion (Rehman & Akash, 2016). It is well-established that chronic inflammation promotes insulin resistance in T2DM. Accumulating from research studies, T2DM patients have higher levels of inflammatory markers such as IL-6 and C-reactive protein (CRP) and likely related to excess adipose tissue. Macrophages in such tissue express IL-6, which promotes fatty acid oxidation and thus lipolysis (Kreiner et al., 2022; Rodrigues et al., 2017). Whilst, TNF-and IL-1β, two major upstream pro-inflammatory cytokines, are major inducers of IL-6 expression (Kreiner et al., 2022).

Among cytokines, interleukin-1β (IL-1β) emerges as an important player in β-cell mass and secretion of insulin. It belongs to the IL-1 family and has agonist activity. Hematopoietic cells, such as monocytes, tissue macrophages, skin dendritic cells, and brain microglia, produce IL-1β in response to Toll-like receptors (Dinarello, 2011). It

has been stated that the presence of IL-1β in genetically predisposed individuals worsens insulin sensitivity via insulin secretion, thus contributing to the development of T2DM and macrovascular complications (GELEN et al., 2017). Chronically elevated IL-1β levels in T2DM individuals cause β- cell dysfunction (Tong et al., 2017). It also induces an acute phase response, low blood pressure, dilation of the blood vessel and fever which can ultimately result in large inflammatory events (Yongjie Wang et al., 2020). From the recent studies, it has been indicated that proinflammatory cytokine IL-1β levels are higher in T2DM compared to non-diabetic individuals (Tong et al., 2017; Q. Zhou et al., 2018). These show that inflammatory processes play a decisive role in the pathogenesis of T2DM.

Unfortunately, in T2DM, the host's immune response in interrupted due to insulin deficiency and hyperglycaemia. Thus, it suppressed cytokines production resulting from an immune response against invading pathogens like intestinal parasites. Therefore, T2DM is known to be more susceptible to infections (Berbudi et al., 2019). As suggested from a recent study, protozoa infection especially *Blastocystis* ST7 may exhibit immunocompromising functions and several potential virulence factors have been identified that could support the view of pathogenicity. It had been reported that *Blastocystis* ST7 induced the mitogen-activated protein kinase-dependent expression of pro-inflammatory cytokines such as IL-6 and IL-1β and tumor necrosis factor in macrophages, mouse intestinal explants and colonic tissue (M. X. Lim et al., 2014). In summary, it has been suggested that the observed expression of pro-inflammatory cytokines appears to be associated with a generalised immunity weakness and alteration of gut microbiota. The changes may have consequences on the short and long-term of diabetes-related complications (Almugadam et al., 2021).

2.3.3 Diagnosis and Management of T2DM

Type 2 diabetes mellitus (T2DM) commonly is described as a group of metabolic disorders characterized and identified by the presence of hyperglycaemia in the absence of treatment. The heterogenous aetio-pathology includes defects in insulin secretion, insulin action or both and also from disturbances of carbohydrate, fat and protein metabolism. Diabetes can also be more severe, with long-term specific effects such as retinopathy, nephropathy and neuropathy among other complications (World Health Organization, 2019). Diabetes patients are also at higher risk of developing other diseases such as heart, obesity, erectile dysfunction, cataracts and non-alcoholic fatty liver disease (Z. Hussein et al., 2015). Furthermore, they are also at increased risk of some infectious diseases and are prone to parasitic infections (Zibaei et al., 2023).

Diabetes can manifest with noticeable symptoms like increased thirst, frequent urination, blurred vision, and weight loss. However, in T2DM, symptoms may be mild or even absent due to the gradual progression of hyperglycaemia. Without biochemical testing, pathological and functional changes from elevated blood sugar levels may persist unnoticed for an extended period, leading to complications at the time of diagnosis. The prevalence of undiagnosed diabetes varies across countries, contributing to a substantial percentage of cases with delayed identification (World Health Organization, 2019).

Diabetes may be diagnosed based on plasma glucose criteria, either the fasting plasma glucose (FPG) value, 2-hour (2-h) post-load plasma glucose after a 75g oral

glucose tolerance test (OGTT) or criteria in the presence of signs and symptoms of diabetes. People with fasting plasma glucose values ≥ 7.0 mmol/L (126mg/dl), 2-h post-load plasma glucose ≥ 11.1 mmol/L (200 mg/dl) or HbA1c ≥ 6.5% (48mmol/mol) in the presence of sign and symptoms are considered to have diabetes. If elevated values are detected in asymptomatic people, the testing will be repeated with the same test to confirm the diabetes (WHO, 2019). It should be noted that the different screening tests could produce different detection rates that vary in populations and individuals.

Diabetes care is generally available at major public hospitals at a state level. Patients will be referred for consultation with dietitians, diabetes nurse educators and pharmacists, mostly on an individual basis. The management of T2DM includes lifestyle management and pharmacologic management. It has been stated that T2DM patients in Malaysia have poor adherence to lifestyle. Thus, successful diabetes management results from proper integration of healthy eating, physical activity and when needed, pharmacotherapy (Z. Hussein et al., 2015). Engaging in physical activity, such as regular exercise, among adults with T2DM has been linked to improvements in blood glucose levels, metabolic profile, adiposity, body mass index, inflammatory markers, cardiovascular health, and overall well-being (Nadeau, 2014). In addition, weight loss is an important therapeutic objective for patients with diabetes to reduce insulin resistance (Z. Hussein et al., 2015). Recently, cardiovascular outcome trials (CVOTs) have confirmed several drug therapies in T2DM management including oral glucose-lowering drugs (OGLDs) and injectable agents. Metformin, Sulphonylureas, Meglitinides, Alpha-glucosidase inhibitors, Thiazolidinediones, Incretins, sodium-glucose Cotransporter 2 inhibitors (SGLT2-i) are part of OGLDs. While the injectable agents include insulin and *Glucagon like peptide-1 receptor agonist* (*GLP1-RA*). In

brief, treatment of T2DM is begun with metformin as it is the first-line oral therapy for T2DM according to guidelines from several medical professional groups. However, if monotherapy fails, the combination of other agents is recommended referring to established guideline (Malaysia Endocrine & Metabolic Society, 2020; Nadeau, 2014). Maintaining a long-term integration of lifestyle modification and pharmacological management is important for achieving good metabolic control in diabetes (Wake, 2020).

2.4 Human Gut Microbiota

Ecosystems comprise masses of interacting species living in several body niches such as skin, vagina, nose and mouth. The great gut microbiome (GM) which is home to trillions of prokaryote and eukaryote cells is no exception which is at least 100 trillion (10^{14}) microbial cells and quadrillion viruses in and on us (Whitman et al.,1998; Haynes and Rohwer, 2011). Our microbiota is communally constituted by the microbial associates that live in and reside in the human body and the genes they encode are known as our microbiome. This diverse culture contains taxa from across the tree life, bacteria, eukaryotes, viruses and at least one archaeon that interact with one another and with the host. Research into microbiomes has been explicitly observed, where the complex relationship between various GM members and their association with hosts is beginning to be revealed; intestinal eukaryotes are associated with particular GM communities (Leung et al., 2018).

In the past years, new technology has allowed researchers to phylogenetically identify or quantify the components of the gut microbiota by analysing nucleic acids (DNA and RNA) directly extracted from stools. The majority of these approaches are based on DNA extraction and 16S ribosomal RNA gene (rRNA) amplification (Poretsky *et al*., 2014; Rosselli *et al*., 2016). 16 The sequencing of rRNA has been the most effective tool for illustrating microbiome complexity and abundance. Polymerase chain reaction (PCR) and metagenomics sequencing can be used to classify the microbial strains in the 16S rRNA gene sequences (Nelson et al., 2010).

In fact, due to its ability to either poorly or well serve the host, microbiota plays an important role in human health and disease and is often referred to as a "forgotten organ" (O'Hara & Shanahan, 2006). Intestinal microbiota characteristics such as great variety, stability and durability, and symbiotic relationship with the host can be described as a "superorganism" that performs immune and metabolic functions (Laterza et al., 2016).

2.4.1 Development of Gut Microbiota

Gut microbiota is made up of a variety of microorganism organisms, including bacteria, yeast and viruses. Taxonomically, bacteria are categorised by phyla, class, order, family, genus and species. As the bombarding research is being interested in the human gut microbiota on a large scale, the association of traditional cultural techniques with new molecular techniques based on the analysis of the small subunit ribosomal RNA (SSU rRNA) gene sequence as phylogenetic markers made bacteria the most known components of gut microbiota identifying more than 1000 species. Bacteria together with Archaea and Eukaryota constitute the three kingdoms in which life is

classified. Bacteria are subclassified into many phyla (plural of phylum, a major taxonomic division that contains one or more classes Just a few phyla are described, accounting for more than 160 species. The main intestinal microbial phylae are Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia, with the two phylae Firmicutes (consisting mainly of Gram-positive clostridia) and Bacteroidetes (consisting mainly of Gram-negative bacteria) comprising 90% of the gut microbiota (Laterza et al., 2016; Marchesi et al., 2016). The Firmicutes phylum is composed of more than 200 different genera such as *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus* and *Ruminicoccus* with *Clostridium* genera representing 95% of the Firmicutes phyla. Bacteroidetes consist of predominant genera such as *Bacteroides* and *Prevotella*. The Actinobacteria phylum is proportionally less abundant and mainly represented by the Bifidobacterium genus (Arumugam et al., 2011; Rinninella et al., 2019).

There are three stages of composition of the gut microbiota in human life: from birth to weaning, from weaning to obtaining a normal diet; and finally, during old age. Generally, the microbiota diversity increases in the time between childhood and adulthood and decreases at older age (over 70) (Rinninella et al., 2019). At birth, the intestine is sterile and devoid of bacteria. After birth, a rich and dynamic ecosystem develops from microbiota colonisation varies according to the type of delivery. Recent literature had observed those are vaginally delivered infants are characterized by predominant groups such as *Bifidobacterium longum* and *Bifidobacterium catenulatum* compared to Caeseran born babies. Moreover, babies who were solely breastfed until weaning were observed to have a more stable and less diverse bacterial community with higher proportions of bifidobacteria than babies fed formula milk (Aggarwal et al.,

2023; Rinninella et al., 2019). Specifically, the genus *Bifidobacterium* is typically a dominant microbial taxon during the first six months after birth (Mancabelli et al., 2020).

Moreover, the diversity of children's gut microbiota is dominated by *Akkermansia muciniphila*, *Bacteroides*, *Veillonella*, *Clostridium coccoides* spp., and *Clostridium botulinum* spp. before the formation of a relatively stable gut microbiota composition. Then, the gut microbiota in children at approximately three years becomes similar to those of adults which has a stable adult microbiota composition dominated by three bacterial phyla; Firmicutes, Bacteroidetes and Actinobacteria (Amabebe et al., 2020; Derrien et al., 2019). These results are influenced by genetics, environment, diet, lifestyle and gut physiology (Tidjani Alou et al., 2016). Concerning older people over the age of 70, the gut microbiota composition potentially changes due to the dietary profile and immune system. The shift in diet may also exacerbate the immune system. The decrease of prominent commensal taxa like *Prevotella*, *Faecalibacterium*, *Eubacterium rectale*, *Lachnospira*, *Coprococcus*, and the health-associated genus *Bifidobacterium* characterizes these broad aging-related alterations (Ghosh et al., 2022). Particularly, a lower level of *Bifidobacterium* and higher levels of Clostridium and Proteobacteria have been observed in elder people which has been associated with inflammatory reactions (Ghosh et al., 2022; Odamaki et al., 2016). Overall, the composition of the human gut microbiota varies with age since it plays an important role in human health and is also actively involved in several biological processes as well as the onset of disease.

2.4.2 Gut Microbiota and T2DM

Diabetes is a complex metabolic disease comprehensively influenced by genetic and environmental risks. It has been characterized by excessive hyperglycaemia, which can result in insulin resistance or damage to the β-cells that produce insulin in the pancreas (Slouha et al., 2023). According to recent research, dysbiosis of the gut microbiota is a significant environmental component that may cause diabetes (Que et al., 2021). The gut microbiota of diabetic individuals is usually associated with pathogenic and opportunistic gram-negative species that can weaken the immune system to cause infections. The shift occurs at the expense of commensal bacteria, which are normally present in the gut and contribute to overall health (Crudele et al., 2023). Moreover, metabolites and components of gut microbiota affect the progress of various diseases specifically in T2DM patients which involves the important regulators in T2DM including SCFAs, bile acid, branched-chain amino acid (BCA As), imidazole propionate and lipopolysaccharide (LPS) (Lili Zhang et al., 2021). Therefore, an imbalance in the gut microbiota such as decreased or increased levels of certain bacteria can influence immune responses (LPS, IL-6, IL-1β, TNF-α) which contributes to the development of T2DM (Craciun et al., 2022; Z. Zhou et al., 2022).

Changes in the composition of the gut microbiota in patients with T2DM have been studied in recent years. Table 2.5 shows a list of studies of gut microbiota composition using 16S rDNA sequencing technology among T2DM participants and non-diabetic (NonDM) groups. An early metagenomic study conducted by Larsen et al., (2010) observed that T2DM was associated with dysbiosis, with enrichment in the Proteobacteria phylum and the alteration occurred in the proportion of Firmicutes and

Bacteroidetes phyla. Moreover, several studies reported, patients with T2DM had a disorder level of short-chain fatty acid, bile acids and lipids as well as a higher level of Proteobacteria and a higher proportion of Firmicutes and Bacteroidetes (Jiajia Wang et al., 2020; L. Zhao et al., 2019). These specific changes of butyrate-producing bacteria have been related to insulin resistance in T2DM patients (Doumatey et al., 2020).

In the healthy individuals, the beneficial bacteria like *Bifidobacterium* were significantly more abundant compared to the patients with T2DM. However, there was no significant difference between the groups in the level of *Fusobacterium* and *Prevotella* (Sedighi et al., 2017). Interestingly, Navab-Moghadam and colleagues (2017) found out *Faecalibacterium prausnitzii* was significantly decreased in patients with T2DM while *Bacteroides fragilis* also decreased but not significantly compared to the healthy individuals. They stated that the reduction of both bacteria proportions could potentially serve as a therapeutic target in T2DM.

Overall, dysbiosis commonly occurs in T2DM patients with an increase in the pathogenic bacteria and a decrease in the beneficial bacteria. However, the changes in gut composition are not fully understood as they could influenced by several factors like modern lifestyle, medications, diet, parasite colonisation and diseases. Therefore, it is critical to characterise the intestinal environment as an ecosystem in which biological and biochemical interactions occur at several organisational levels between parasites, microbial communities, and host immune responses (Beyhan & Yıldız, 2023; Sirisinha, 2016). By understanding the specific gut changes in T2DM, a better treatment could be addressed involving a complex interaction of parasites in the host.

Table 2.5: Gut Composition in T2DM and NonDM in clinical studies using 16S rDNA sequencing

No	Study design	Study location	Sample size (N)		Change in abundance of gut composition		References
			T2DM	HC	T2DM	Healthy control/NonDM	
1.	Case control	Denmark	18	18	• INC in p. Proteobacteria (c. *Betaproteobacteria*), p. Bacteroidetes (g. *Bacteroides*) and p. Firmicutes (c. Bacilli & g. Lactobacillus) • DEC in p. Firmicutes (o. *Clostridiales*)	• INC in p. Firmicutes (c. *Clostridia and Erysipelotrichi*)	(Larsen et al., 2010)
2.	Case control	Iran	18	18	• INC in c. *Lactobacillus*	• INC in c. *Bifidobacterium*	(Sedighi et al., 2017)
3	Case control	Iran	18	18	• DEC in *Faecalibacterium prausnitzii*	• INC in *Bacteroides fragilis*	(Navab-Moghadam et al., 2017)
4	Case control	Sudan	24	24	• INC in p. Firmicutes (g. *Blautia*, g. *Catenibacterium*, g. *Holdemanella* & g. *Parvimonas*) • INC in Actinobacteria (g. *Bifidobacterium*) • INC p. Fusobacteria (g.*Fusobacterium*)	• INC in Firmicutes (g. *Faecalibacterium* & g.*Dialister*) • INC in p. Proteobacteria, p. Verrucomicrobia (g. *Succinivibrio*) & p. Elusimicobia	(Almugadam et al., 2020)
5.	Case control	Poland	23	23	• INC in p. Firmicutes (g. *Ruminococcus*) • INC in p. Proteobacteria (f. *Enterobacteriaceae*)	• INC in p. Firmicutes (f. *Clostridiaceae*, f. Lachnospiraceae, f. *Peptostreptococcaceae* g.*Anaerostipes*, & g. *Roseburia*)	(Salamon et al., 2018)

						• INC in p. Bacteroidetes (f. *Flavobacteriaceae* & g. *Bacteroides*)	
6.	Case control	China	134	37	• INC in p. Firmicutes (g. *Faecalibacterium* & g. *Lactobacillus*) • INC in p. Proteobacteria (g. *Escherichia-Shigella*) • INC in Actinobacteria (g. *Bifidobacterium*)	• INC in p. Firmicutes (g. *Roseburia)* • INC in p. Bacteroidetes (g. *Prevotella)* •	(Jiajia Wang et al., 2020)
7.	Case control	China	137	139	• INC in Proteobacteria (g. *Escherichia-Shigella*, g. *Verrucomicrobia* & g. *Klebsiella*) • INC in p. Firmicutes (*Lachnospiraceae_incertae_sedis, Subdoligranulum, Enterococcus)*	• INC in g. *Bacteroidetes (g. bacteroides & g. Prevotella* •	(X. Zhao et al., 2020)
8.	Case control	Nigeria	98	193	• DEC in p. Firmicutes (f. *Clostridiaceae* and g. *Peptostreptococcus*) • INC in p.Proteobacteria	• INC Firmicutes, Actinobacteria, and Bacteroidetes • INC *in Collinsella, Ruminococcus lactaris, Anaerostipes* & *Clostridium*	(Doumatey et al., 2020)

p, Phylum; c, Class; f, Family; o, Order; g, Genus; sp., Species

2.4.3 Association of *Blastocystis* in the gut microbiota composition

Blastocystis is an intestinal parasite that can be commensal or pathogenic, depending on the subtypes. It is postulated that different subtypes of *Blastocystis* may colonise in different niches, indicating a diverse potential association with specific human gut microbiome (Tito et al., 2019). Recent studies demonstrated that *Blastocystis* infection may be associated with changes in the abundance of both beneficial and pathogenic intestinal bacteria. *Blastocystis* can contribute to improving gut microbiota balance (eubiosis) and also modulating the gut composition quantitatively and qualitatively resulting in microbial imbalance (dysbiosis). The association of *Blastocystis* subtypes and gut microbiota composition studies is listed in Table 2.6.

Generally, *Blastocystis* is a common eukaryote that has been associated with beneficial bacteria that promote a healthy gut environment. The diversity and bacterial species composition of the microbiota of healthy individuals differs from those of patients with metabolic or infectious diseases. An early metagenomic study showed that the enrichment of Firmicutes, *Prevotella* or *Ruminococcus*-driven enterotype and higher bacterial richness were associated with the presence of *Blastocystis* (Andersen et al., 2015). There was a higher abundance of Clostridia class, *Ruminococcacea* and *Provotellacea* families in *Blastocystis*-infected individuals while *Enterobacteriacaea* was enriched in *Blastocystis*-free patients (Audebert et al., 2016). A study on healthy Malian children reported that *Blastocystis* colonisation is significantly associated with higher bacterial diversity, characterised by the abundance of beneficial bacteria such as Firmicutes, Elusimicrobia, Lentisphaerae, and Euryarchaeota at the phylum level. Indeed, this study discovered that *Roseburia* sp. (family *Lachnospiraceae*) and

Faecalibacterium prausnitzii (family *Ruminococcaceae*) were relatively more abundant in children colonized by *Blastocystis.* Other study was also observed the presence of *Blastocystis* increased the proportion of *Faecalibacterium* spp., *Ruminococcaceae* and *Prevotella* in Korean population (M.J. Kim et al., 2022).

Furthermore, *Blastocystis* subtypes were strongly linked to gut composition with higher explanatory power in a non-clinical cohort and were associated with both *Blastocystis* prevalence and subtype variation (Tito et al., 2019). Despite this association, ST3 and ST4 differed significantly with ST4 being more prevalent in the most diverse *Ruminococcaceae* entrotyped among *Blastocystis* carriers. ST2 was found to be more frequent than ST3 in *Ruminococcaceae* compared to *Prevotella* enterotype, whilst ST4 was shown to be positively associated with Methanobrevibacter relative abundances. The high richness and enterotype distribution were observed in *Blastocystis* carriers which indicates that *Blastocystis* is frequent in stable, resilient and health-associated microbiota depending on the subtype level (M. J. Kim et al., 2022; Tito et al., 2019). Moreover, Gabrielli *et al*. (2020) also discovered that *Blastocystis* ST3 carriers were associated with higher bacterial diversity which enriched in *Prevotella*, *Methanobrevibacter* and *Ruminococcus* while a high proportion of *Bacteroides* was found in *Blastocystis*-free subjects. Interestingly, the enrichment of *Akkermansia* genus belonging to Verrucomicrobia phylum was observed to be inversely associated with *Blastocystis* ST3 and positively correlated with ST4 suggesting differential associations between subtypes and host health. This study suggested that ST4 could be linked to markers of healthy gut microbiota such as *Akkermansia* (Tito et al., 2019). In a previous Korean study, a higher prevalence of *Blastocystis* ST3 was reported. The study found that the relative abundance of *Akkermansia* (>2%) was

greater in the *Blastocystis*-negative group compared to the *Blastocystis*-positive group although no significant difference was observed in ST3 (M. J. Kim et al., 2022). In an experimental study, the proportion of *Akkermansia* increased in mice colonised by *Blastocystis* ST1. This colonisation exerted beneficial effects on host health by modulating the gut microbiota (Deng et al., 2023). Thus, these recent findings convey that *Akkermansia* is a part of a beneficial member of gastrointestinal bacteria and may interact with *Blastocystis* subtypes to promote a healthy gut phenotype.

However, dysbiosis of the gut microbiota may be associated with the presence of *Blastocystis* which can cause a variety of diseases including gastrointestinal disorders with an unknown mechanism. Dysbiosis, also known as an imbalanced microbiome, is marked by pathobiont development, commensal loss, and reduced diversity, which may be associated with the aetiology of numerous diseases (Levy et al., 2017). The composition of gut microbiota can alter under some circumstances such as acute diarrhoea, antibiotic treatments, psychological stress, modern diet and hygiene. These changes are typically characterised by a decrease in bacterial diversity, a reduction in *Clostridia* abundance and an increase of facultative anaerobic *Gammaproteobacteria* such as *Enterobacteriaceae* (Hawrelak & Myers, 2004; Bernstein & Shanahan, 2008; Winter & Bäumler, 2014; Defaye et al., 2020).

According to a study by Nagel et al., (2016), it was reported that there was a change in the faecal microbiota in the dominant phyla with an increase in *Firmicutes* and a reduced significantly in *Bacteroidetes* in diarrhoea-predominant IBS patients than healthy subjects. The Firmicutes/Bacteroidetes (F/B) ratio is commonly considered to have an essential influence in sustaining normal intestinal homeostasis and the change

of the ratio can lead to dysbiosis. However, there is no significant difference in *Blastocystis* carriage seen in the phyla or genus level of the gut microbiota among IBS patients. Moreover, another study found out *Blastocystis* colonisation was correlated with significant changes in bacterial alpha and beta diversity, which significantly showed a difference in the abundances of predominant bacterial taxa, including *Prevotella stercorea*, *Prevotella copri*, *Alistipes putredinis*, *Ruminoccoccus bromii*, *Bacteroides* species, *Oscillospira* species and *Bifidobacterium longum*. Surprisingly, *Blastocystis* ST3 was shown to be the most significantly different in bacterial community abundance between *Blastocystis* positive and negative individuals (Nieves-Ramirez *et al*., 2018).

Several *in vivo* rodent experiments revealed the pathogenicity that involved a rare subtype ST7 and the common ST4 displays minor inflammation and pathology to the host's tissue. A study using Wistar rats reported that there was a significantly greater change in bacterial richness in chronically infected rats by 10^5 ST4 cysts, which was observed in increasing relative abundance of *Tenericutes* and *Proteobacteria* in infected animals by *Blastocystis*. The decrease of *Firmicutes*, *Bacteroidetes*, *Clostridium* with increasing *Oscillospira*were significantly correlated with colonic hypersensitivity. A decrease in the overall content of SFCAs in the faeces composition of infected rats was observed (Defaye *et al*., 2020). SCFAs are important metabolites in the maintenance of intestinal homeostasis that enhance epithelial barrier function and immune tolerance and have been produced dominantly by *Bacteoidetes* (gram-negative) and *Firmicutes* (gram-positive) in the human gut (Rooks & Garrett, 2016; Venegas *et al*., 2019).

In addition, the association between the presence of *Blastocystis* and *Clostridium difficile* infection in diarrhoeic patients has been reported by Vega and colleagues (2020), indicating a reduction in beneficial bacteria while increasing the abundance of bacteria belonging to the Enterobacteriacea family or other groups of bacteria pathogens. It has been suggested that *Blastocystis* may be able to adapt to dysbiosis and oxidative stress (Vega *et al*., 2020). Nourrisson and colleagues (2014) suggested that *Blastocystis* might be correlated to the pathophysiology of IBS-C and intestinal flora imbalance since the result showed a reduction of beneficial bacteria in *Blastocystis*-positive patients with IBS. Overall, the changes in the feasibility of these bacterial species may be caused by *Blastocystis* subtypes could lead to potential disturbances in the intestinal composition which would have a major impact on several inflammatory cases in clinical diseases.

Overall, gut microbiota studies confirm that colonisation of *Blastocystis* subtypes would influence gut microbiota composition which is linked with a eubiotic gut characterised by beneficial bacteria and also can lead to dysbiosis state.

Table 2.6: Studies on Association of *Blastocystis* Colonisation in Gut Composition infected in Humans and Mices

No	Subtype	Gut microbiota composition shift	Status	References
1	*Blastocystis* (ST1-4,7)	• INC bacterial richness in immunocompetent compared to immunocompromised patients. • INC in Firmicutes, Bacteroidetes and Proteobacteria (Phylum). • INC Bacteroida(Class) in *Blastocystis* free subjects. • INC in *Prevotellaceae*, *Methanobacteriaceae*, *Clostridiaceae Lachnospiraceae*, *Erysipelotrichaceae* and *Pasteurellaceae* (Family). • DEC in *Bacteroidaceae* and *Veillonecel* (Family). • INC in *Prevotella*, *Methanobrevibacter*, *Ruminococcus*(Genus) in *Blastocystis* carrier; INC in *Bacteroides* (Genus) in free *Blastocystis*	Eubiosis	(Gabrielli et al., 2020)
2	*Blastocystis*	• INC bacterial diversity in *Blastocystis* carrier of healthy children • INC in Firmicutes, Elusimicrobia, Lentisphaerae, Euryarchaeota, *and* IHU_PP_Bacteria (Phylum) in *Blastocystis* carrier; INC Actinobacteria, Proteobacteria, unassigned bacteria, and Deinococcus–Thermus (Phylum) in free *Blastocystis.* • INC of *Clostridia*, IHU_PC_PC_Bacteria, *Elusimicrobia, Lentisphaeria, Metanobacteria, and Deltaproteobacteria* (Class) in *Blastocystis* carrier; INC *Planctomycetacia, Rubrobacteria, Deinococci, Gammaproteobacteria, Actinobacteria*, unassigned bacteria and *Bacilli* (Class) *in* free *Blastocystis* • INC Clostridiales, IHU_PO_Bacteria, Victivallales, Methanobacteriales, Elusimicrobiales, Aeromonadales, Acidaminococcales, and Desulfovibrionales in *Blastocystis* carrier (Order); INC in Planctomycetales, Rhodobacterales, Sphingomonadales, Rubrobacterales, Veillonellales, Pasteurellales, Micrococcales, Pseudonocardiales, Enterobacteriales, Myxococcales, Bifidobacteriales, unassigned bacteria, and Lactobacillales (Order) in free *Blastocystis*. • INC Clostridiaceae, Ruminococcaceae and Lachnospiraceae in *Blastocystis* carrier (Family); INC in Streptococcaceae, Bifidobacteriaceae, Enterobacteriaceaeand Leuconostocaceae in free *Blastocystis.* • INC in *Ruminococcus* and *Clostridium* (Genus) in *Blastocystis* carrier; INC *Streptococcus, Bifidobacterium* and *Shigella* (Genus) in free *Blastocystis.*	Eubiosis	(Kodio et al., 2019)

		• INC in *Clostridium saudii*, *Methanobrevibacter smithii*, *Faecalibacterium prausnitzii*, *Roseburia* sp. (Species) in *Blastocystis* carrier; INC *Streptococcus* sp., *Bifidobacterium* sp., *Shigella* sp. (Species) in free *Blastocystis.*		
3	*Blastocystis* (ST1-8)	• There were no significant differences between the IBS-*Blastocystis* postive, IBS-*Blastocystis* negative, healthy control-*Blastocystis* positive and healthy control-*Blastocyctis* negative. • INC in Firmicutes (Phylum) in the IBS group compared to healthy control.	Dybiosis	(Nagel *et al*., 2016)
4	*Blastocystis* (ST2-ST3)	• INC bacterial diversity in *Blastocystis* positive of asymptomatic individuals. • INC *Prevotella copri, Prevotella stercorea*, *Ruminoccoccus bromii, Alistipes putredinis, Bacteroides species, Bifidobacterium longum*, and *Oscillospira* sp. (Species); INC *Debaryomyces hansenii*, *Mucor mucedo*, *Aspergillus flavus*, *Mucor racemosus*, and *Issatchenkia terricola* (Species) • DEC in *Hymenolepis nana* (Species).	Dybiosis	(Nieves-Ramirez *et al*., 2018)
6	*Blastocystis* (ST1-4,7,8)	• INC bacterial diversity and richness • DEC prevalent in *Bacteroides* enterotyped samples (Genus); INC in Methanobrevibacter relative abundances (Genus) • ST4 is more prevalent in *Ruminococcaceae entrotyped* samples and associated with *Akkermansia* (Genus) • ST2 was more prevalent in *Ruminococcaceae entrotyped* samples (Genus) • ST3 inverse with *Akkermansia* (Genus).	Eubiosis	(Tito, et al., 2019)
7	*Blastocystis*	• INC in Firmicutes, Bacteroidetes and Proteobacteria all patients' faecal samples (Phylum) • INC in Clostridia and Mollicutes in colonized patients (Class); DEC in Bacilli (Class) • INC in Clostridiales, Erysipelotrichales Burkholderiales (Order). DEC in Lactobacillales, Bacteroidales (Order) • INC in Ruminococcaceae and Prevotellaceae (Family); INC Enterococcaceae**,** Streptococcaceae, Lactobacillaceae and Enterobacteriaceae (Family) in free-*Blastocystis* • INC in *Prevotella*, *Acetanaerobacterium, Acetivibrio, Coprococcus, Hespellia, Oscillibacter, Papillibacter, Sporobacter, Ruminococcus, Roseburia* and *Faecalibacterium* (Genus).	Eubiosis	(Audebert et al., 2016)

8	*Blastocystis* ST 7	• DEC in *Lactobacillus* and *Bifidobacterium* in infected mice.	Dybiosis	(Yason et al., 2019)
9	*Blastocystis* ST4	• INC bacterial richness in chronically infected rats. • INC in Proteobacteria and Tenericutes in infected animals (Phylum). • DEC in *Firmicutes, Bacteroideted, Clostridium, Pseudomonas and Rhodoplane (Genus); INC Anaerovorax, Oscillospira* and *Parabacteroides*(Genus)	Dybiosis	(Defaye *et al*., 2020)
10	*Blastocystis*	• There is a significant association between the presence of *Blastocystis* and *Clostridiodes difficile* infection.	Dybiosis	(Vega *et al*., 2020)
11	*Blastocystis* (ST1-4, ST6)	• INC bacterial richness, *Blastocystis* mainly presence in individuals with *Prevotella* and *Ruminococcus* enterotypes (Genus)	Eubiosis	(Andersen et al., 2015)
12	*Blastocystis*	• INC in *Prevotella*; DEC of *Bacteroides* and *Clostridial* cluster XIVa (Genus)	Eubiosis	(O'Brien Andersen *et al*., 2016)
13	*Blastocystis* (ST1-ST5)	• INC in *Bacteroides* sp. in IBS-C; DEC in *Bifidobacterium* sp., *Desulfovibrio* sp., *C. leptum* and *F. prausnitzii* (Species)	Dybiosis	(Nourrisson et al., 2014)
14	*Blastocystis*	• Firmicutes and Bacteriodetes were enriched in carriers while Proteobacteria were enriched in non-carriers (Phylum). • *Prevotella*, *Faecalibacterium*, *Flavonifracter*, *Clostridium*, *Succinivibrio*, and *Oscillibacter* among others were enriched in carriers, while *Escherichia*, *Bacteroides*, *Klebsiella*, and *Pseudomonas* were enriched in non-carriers among others (genus).	Eubiosis	(Christen Rune Stensvold *et al*., 2022)
15	*Blastocystis*	• There was no significant difference between *Blastocystis* free and *blastocystis*-colonised children in the composition of the microbial community. • INC in Firmicutes (Phylum). • INC in Bacteroidetes (Phylum) in *Blastocystis*-free individuals. • INC in Proteobacteria (Phylum) in *Blastocystis*- colonized group.	Eubiosis	(Castañeda *et al*., 2020)
16	*Blastocystis* (ST1–ST5, ST7)	• Higher prevalence of *Blastocyctis* sp. in UNIME compared to FASCA cohort. • Firmicutes/ Bacteroideted ratio (F/B ratio) is high in UNEME cohort subjects.	Eubiosis	(Yañez et al., 2021)

17	*Blastocystis*	• INC in c. *Clostridia*, f. *Ruminococcaceae*, g. *Faecalibacterium*, *Faecalibacterium sp. Bifidobacterium sp.* g. *Prevotella 9*, *Ruminococcaceae UCG-002*, *Ruminococcaceae UCG-005*, *Muribaculaceae*, *Rikenellaceae*, *Acidaminococcaceae*, *Phascolarctobacterium* in *Blastocystis* positive group • INC c. *Bacill*i, *Bacteroides*, *Lactobacillales*, *Enterococcus* species in *Blastocystis* negative group	Eubiosis	(M. J. Kim et al., 2022)

p, Phylum; c, Class; f, Family; o, Order; g, Genus; sp, Species; INC; increase; DEC: decrease

2.4.4 Next-Generation Sequencing (NGS), 16S rDNA Sequencing

Next-Gene Sequencing (NGS) is a powerful and high-throughput technology that has revolutionized genomic research. Due to its high throughput capability and affordability, next-generation sequencing (NGS) has emerged as a vital tool for researchers in a wide range of fields, from clinical diagnostics to basic biology (Goodwin et al., 2016). As gut microbiome has been correlated with many diseases such as diabetes and others (Salamon et al., 2021), more findings and characterization of microbes have increased dramatically in recent years due to advancements in NGS technology. This is because NGS approaches do not depend on conventional culture techniques, making them capable of detecting uncultured microbes and unique species (Wensel et al., 2022).

Amplicon-based NGS of the 16S ribosomal RNA (rRNA) gene is the most widely used method of taxonomic identification. The prokaryotic 16S rRNA gene is approximately 1500 bp long and has conserved and hypervariable nine regions (V1-V9), which are used for the phylogenetic classification of genera and in diverse populations (Bei Gao et al., 2021). Due to the limitation of short-read NGS technologies, various universal primers targeting the partial sequences in hypervariable regions such as V1-V2, V1-V3, V3-V4, V4 and others have been developed for microbiome analysis (Fricker et al., 2019). However, the choice of optimal variable regions may differ based on the analysis target, the primer's specificity, the GC contents in the chosen region and the bacterial compositions of different samples (Kameoka et al., 2021). Recently, NGS sequencing of the entire 16S rRNA gene has emerged, and advanced analytical methods are being used to propose a species and strain resolution

in microbiota communities.(J. S. Johnson et al., 2019). Since 2011, most laboratories have employed Illumina NGS technology, particularly the MiSeq system (San Diego, USA), which allows for the accurate evaluation of relatively short paired-end sequencing reads, providing for a thorough examination of changes in microbiota composition. (Salamon et al., 2022).

Besides, several bioinformatics tools have been developed in the last decade to analyse the 16S rRNA sequencing data, with a majority of them consisting of three main steps, including data preprocessing and quality control, taxonomic assignment and community characterisation (Y. X. Liu et al., 2020). Taken together, the advancements in bioinformatics and data analysis will be essential for deriving valuable insights from the massive amounts of NGS data collected. As a result, the future of NGS promises to reveal new knowledge that will have a significant influence on a wide range of fields, including human health, agriculture, environmental conservation and others (Satam et al., 2023).

2.5 Conclusion

This chapter reviewed the existing literature related to *Blastocystis* prevalence in individuals with Type 2 Diabetes Mellitus (T2DM) associated with gut microbiota. This review summarized the available findings that could help in identifying any gaps in the current understanding, especially in the Malaysian population. Thus, the expected results in this study could be evaluated based on the recent findings.