CHAPTER 1

INTRODUCTION

1.1 Introduction

This chapter introduces the current study, which aims to assess the differentiation of *Blastocystis* subtypes in type-2 diabetes mellitus patients at Universiti Kebangsaan Malaysia Medical Centre in comparison to non-diabetic individuals and explore its association with gut microbiota. This chapter consists of the background of the study, problem statement, justification of the study, research question, research objectives, hypotheses, significance of the study, scope of study, conceptual framework, operational definition, and conclusion.

1.2 Background of the Study

The epidemic of diabetes is one of the major concerns for public health globally that has reached alarming levels. Currently, it is expected that 700 million adults aged 20-79 (10.9% of the population) will have diabetes by 2045 (Saeedi et al., 2019). Furthermore, it has been estimated that about 90% of the 425 million people living with diabetes worldwide are from type 2 diabetes. The prevalence is growing rapidly, especially in Asian countries, with about 60% of diabetes patients worldwide currently living in Asia. Moreover, the estimated prevalence of diabetes has increased from

11.6% in 2006 to 17.5% in 2015 and it is estimated that over 3.5 million adults in Malaysia are living with diabetes (NIH, 2019). Among Asian countries, Malaysia has one of the highest comparative prevalences of type 2 diabetes with other neighbouring countries such as Singapore (12.8%), Indonesia (6.2%) and Thailand (8.0%) (International Diabetes Federation, 2015).

Type 2 diabetes mellitus (T2DM) is a complex and multifactorial metabolic syndrome that is characterised by hyperglycaemia, lipid profile dysfunction, insulin resistance and chronic inflammation state would exist throughout the progression of T2DM (Donath & Shoelson, 2011; Sircana et al., 2018). Chronic exposure to high levels of glucose and lipids activates a variety of pathways that are responsible for prompting the impaired insulin secretion from β-cells of pancreatic islets, insulin resistance in peripheral tissues, decreased glucose utilization of peripheral tissue and abnormal hepatic glucose production (Rehman & Akash, 2017). The release of lipopolysaccharide (LPS) from Gram-negative bacteria in the gut followed by its passage into the bloodstream, induces inflammations that lead to insulin resistance in T2DM (Galicia-Garcia et al., 2020; Raetz & Whitfield, 2008). Based on previous studies, infectious pressure by parasites also potentially be a modifier of insulin resistance that would facilitate the development of inflammatory diseases including T2DM (Molan et al., 2020; Saeed & Al-aubaidi, 2020; Tahapary et al., 2015). With these shreds of evidence, parasitic infections become one of the main concerns that might affect T2DM patients, particularly in gut health.

Moreover, insufficient sanitation, lack of access to safe water and other facilities lead to contamination of food and water sources which in turn sustains the life cycle of

parasites particularly in developing countries. There are two main types of gastro-intestinal parasites; protozoans and helminths (Siddiqui, 2017; Sinha et al., 2012). Interestingly, some parasites play an important role in triggering diseases in specific groups such as immunocompromised individuals and young children even in countries that have adequate sanitation conditions and education. Certainly, intestinal parasites are part of the important cause of morbidity and mortality (Gil et al., 2013). As has been observed in previous studies, diabetic patients were more likely to get parasitic infections as compared to non-diabetic people primarily due to immune dysregulation, age, metabolic control and long-term complications. In developing countries, infectious pressure might be one of the particular modifiers of insulin resistance (Tahapary et al., 2015). However, it is not clear whether parasitic infections can increase the risk of diabetes or diabetes can lead to an increased risk of parasitic infections (Moudgil et al., 2019). Thus, the role of intestinal parasites in the development of human disease in immunocompromised individuals has been established (Siddiqui, 2017).

Blastocystis, an anaerobic protozoan is among the most frequently observed intestinal parasites in humans. It has been estimated that the number of infections from this parasite has risen to more than 1 billion worldwide (Andersen, 2016; Özkan-Ahmetoğlu et al., 2023). Currently, the *Blastocystis* route of transmission is linked to environmental contamination with human or animal faecal directly via hand or mouth or indirectly through the ingestion of contaminated food or water (Castañeda et al., 2020; Perea et al., 2020). It had been remarkable to have up to 40 subtypes of genetically distinct strains by analysis of the ribosomal RNA gene (SSU rDNA) to colonize a range of hosts including humans and animals (Darwish et al., 2023; Maloney et al., 2023; Maloney & Santin, 2021; Matovelle et al., 2022; Santin, 2024). Since there are 14

subtypes found in humans including those with and without intestinal disorders, ST1-ST4 account for 90-95% of human carriage with ST3 and ST1 have been observed to be the most common subtypes (Cristanziano et al., 2019; Khaled et al., 2020; Maloney et al., 2023; C. R. Stensvold & Clark, 2016; Yoshikawa et al., 2004).

Despite its description approximately 100 years ago, still, not much information is available regarding their commensal or pathogenesis, genetic diversity, host range and available treatment options, especially in diabetes clinical epidemiology (Perea et al., 2020; Roberts et al., 2014). Until now, there is still heavy debate on the pathogenesis of *Blastocystis* as most infections are asymptomatic even if it seems to be the only explanation for other symptoms in other cases (Renelies-Hamilton et al., 2019). *Blastocystis* is considered a normal part of the intestinal microbiome in healthy humans, as it can reside in the human gut for extended periods without causing any symptoms (Aykur, 2024). Some studies also showed that *Blastocystis* may enhance the diversity of gut microbiota in healthy individuals, potentially preventing intestinal disorders (Billy et al., 2021; Kodio et al., 2019). In recent decades, several epidemiology studies have shown *in vitro* evidence in animal models and humans strongly suggesting the pathogenic of this parasite due to a quite prevalence of *Blastocystis* in the human population. However, not all strains of a specific subtype are clinically relevant, and there may be a correlation biologically between the different subtypes such as drug resistance, immune response, pathogenicity and effects on gut microbiota (Ajjampur & Tan, 2016; Karamati et al., 2021; Yason et al., 2019).

Undeniably, the presence of this parasite has been linked to various gastrointestinal symptoms and signs such as diarrhoea, abdominal pain, vomiting,

constipation and flatulence (Gabrielli et al., 2020; Perea et al., 2020; Tan, 2008). Accumulating from recent studies, it was indicated that it may play a significant role in several chronic diseases with unknown causes including irritable bowel syndrome (IBS), cancer and diabetes mellitus (Beiromvand et al., 2017; de Melo et al., 2021; Mülayim et al., 2021). Most of the studies have revealed ST3 to be the most frequent subtype detected in symptomatic patients followed by ST1 and ST2 (Cristanziano et al., 2019; Jeremiah & Parija, 2013).

Several studies reported the prevalence of intestinal parasites in the diabetic group was higher than in the non-diabetic group with a p-value <0.05 with *Blastocystis* being one of the highest detected parasites (Ali et al., 2018; de Melo et al., 2021; Fattahi Bafghi et al., 2015; Mohtashamipour et al., 2015; Tangi et al., 2016). The studies also showed that the risks of infection with intestinal parasites were higher in patients with diabetes mellitus than in healthy patients (Aourarh et al., 2020; Fattahi Bafghi et al., 2015; Mohtashamipour et al., 2015). Phylogenetic analyses and BLAST searches also revealed six subtypes among *Blastocystis* isolates in the diabetic group which were represented by ST1, ST2, ST3, ST6, ST7 and ST8. *Blastocystis* ST3 and ST1 were the most prevalent subtypes detected in the diabetic group compared to other subtypes (de Melo et al., 2021; Popruk et al., 2020). According to de Melo et al. (2021), *Blastocystis* 18S alleles retrieved for each ST showed particular alleles in patients with diabetes mellitus such as alleles 9 and 12 (ST2), allele 37 (ST3) and allele 122 (ST6). It is noted that the presence of particular alleles in diabetic patients should be linked to the clinical implication where it is still unclear.

T2DM has recently shifted from a metabolic disorder to an inflammatory disorder, as the effects of pro and anti-inflammatory cytokines such as tumour necrosis factor alpha (TNF), interleukin-6 (IL6), and C-reactive protein (CRP) have been reported in insulin signalling pathways, cross-linking and ultimately developing insulin resistance in pancreatic B-cells, which increases the risk of T2DM (Bashir et al., 2020; Hameed et al., 2015). Stability among these pro and anti-inflammatory cytokines is necessary to make B-cells immune to any infection that may lead to T2DM (Belfki et al., 2012). Interestingly, in vitro and animal studies have shown that intestinal protozoa such as *Blastocystis* trigger TNF- α promoting an inflammatory response but the mechanism is not known where favouring changes in the bacterial composition of the gut microbiota (Nourrisson et al., 2021; Zavala et al., 2018). A study conducted by (Yousif, 2021) demonstrated that *Blastocystis* affects stimulating the immune response by increasing the level of IL-6, IL1-β, IgG and IgM antibodies in patients with gastrointestinal disorders compared to control. In this regard, it is relevant to know the association between T2DM and *Blastocystis* infection due to the possibility of immune dysfunction in diabetes as certain parasitic infections occur more frequently in patients with diabetes mellitus than non-diabetics.

In addition, *Blastocystis* has been suggested to modulate the gut microbiome. Recently, the gut microbiota (GM) has been intensely studied over the past few years for its involvement in developmental aspects of the immune system and the disturbance can lead to autoinflammatory disorders (Round & Mazmanian, 2009). It is known that gut microbial communities include protozoa, fungi, bacteria and viruses, which share the same environment and live in close relationships. Numerous studies in healthy humans found that specific bacteria are favourable for immune system development and

function and that the immune system can impact the composition or function of gut microbiota, all of which are related to inflammation, which could lead to the occurrence of several chronic diseases such as inflammatory bowel disease, type-2 diabetes and atherosclerotic cardiovascular disease (Deng et al., 2021). In type-2 diabetes (T2DM), insulin resistance does not solely result from being overweight but also involves a complex interplay of multiple factors such as gut ecosystem and immune response which might be potentially influenced by the pathogenesis of T2DM (Almugadam et al., 2020). The recent studies demonstrated that the diversity of gut microbiota had significantly declined in T2DM compared to healthy control (Düzgün et al., 2022; Navab-Moghadam et al., 2017).

However, the infection of *Blastocystis* in diabetic patients associated with gut microbiota is not completely discovered. Several pieces of evidence suggest that *Blastocystis* modulates gut microbiota composition. A study from analysis of microbial communities in healthy individuals or those affected by intestinal disorders evidenced three main microbiota patterns, named enterotypes, characterized by three dominant bacteria clusters: *Bacteroides* (enterotype 1), *Prevotella* (enterotype II) or *Ruminococcus* (enterotype III) (Arumugam et al., 2011; Gabrielli et al., 2020). The recent NGS study associated *Blastocystis* with a higher diversity of gut microbiota, low Body Mass Index (BMI) and enterotypes II and III. Furthermore, *Blastocystis* was also linked to a eubiotic state where it has been characterized by a preponderance of potentially beneficial species, belonging mainly to the phyla Firmicutes and Bacteroides instead (Iebba et al., 2016).

Nevertheless, their interactions may also result in altered microbiota composition referred to as dysbiosis, however, it remains poorly understood (Kodio et al., 2019). Remarkably, *Blastocystis* is commonly detected in the gut of asymptomatic humans and it has been shown that colonisation is stable over 6-10 years (Scanlan & Stensvold, 2013). A study showed a direct association between *Blastocystis* and gut dysbiosis in the absence of gastrointestinal diseases or inflammation (Nieves-Ramirez et al., 2018). However, the majority of bacterial dysbiosis has been reported in many health problems associated with *Blastocystis* infection. A study proposed a decrease of protective faecal microbiota in *Blastocystis* colonised individuals which were frequently found in immunocompromised individuals (Piranshahi et al., 2018). Still, the *Blastocystis* colonisation may be dependent on the organism's ST and certain isolates of *Blastocystis* like ST7 potentially lead to the imbalance of the gut microbiota and induce the expression of IL-1β and IL-6 proinflammatory cytokines (Lim et al., 2014; Tito et al., 2019; Yason et al., 2019).

In conclusion, the relationship between *Blastocystis* and the gut microbiota is complex and remains the subject of ongoing research. While some studies have shown a possible link between *Blastocystis* and healthy and unhealthy gut composition, it is essential to acknowledge that the nature of this association may vary significantly among individuals. Further investigation into the interaction of *Blastocystis* with the gut microbiota in diabetic patients may provide valuable insights for future studies. These findings may contribute to a more nuanced understanding of the effects of *Blastocystis* on the mammalian immune system potentially leading to conflicting perspectives (Billy et al., 2021).

1.3 Problem Statement

Blastocystis infection has been frequently observed in both healthy individuals and immunocompromised patients. However, the pathogenesis of *Blastocystis* remains unclear as the infection can be observed in both immunocompromised patients and healthy individuals and is thought to be related to *Blastocystis* subtypes infection (Beiromvand et al., 2017; Ibrahim, 2020; Zhang et al., 2019). From recent studies, immunocompromised patients are under constant scrutiny mainly due to the possibility of immune dysfunction which allows the development of more severe parasitic infections (Alemu et al., 2018).

Diabetes is known as a metabolic disease that has become more common throughout time, accounting for 90% of all the occurrences of type 2 diabetes mellitus (T2DM). This disease has been considered a serious health with more people suffering in Malaysia and worldwide. It can cause systemic immunosuppression; therefore, a high prevalence of various infections is expected in diabetic patients. However, epidemiological data on this topic is sparse, and there is only a little research on the incidence of *Blastocystis* infections in diabetic patients with a lack of focus on the subtype variations of *Blastocystis* (Aourarh et al., 2020; de Melo et al., 2021; Mohtashamipour et al., 2015).

Furthermore, the complex interplay between parasites and the gut microbiota is poorly understood as the gut microbiota may modulate parasite virulence and host response to infection, particularly in *Blastocystis* infection. Recent research has suggested that different subtypes of *Blastocystis* may colonise distinct niches, implying a wide range of potential interactions with the human gut microbial community. It has

been widely reported that *Blastocystis* diversifies the gut microbiota of healthy humans. It has also been suggested that *Blastocystis* can induce intestinal dysbiosis in immunocompromised patients (Tito et al., 2019). However, it is not yet clear whether *Blastocystis* subtypes exploit the state of immunosuppression in diabetic patients, triggering inflammation and potentially altering the composition of the gut microbiota, leading to an unhealthy environment. There is also increasing evidence from previous studies suggesting that gut flora has a significant impact on the development of metabolic diseases, which is attributed to the dysbiosis of microbial communities and metabolites (Yang et al., 2018). On the other hand, the prevalence of *Blastocystis* in patients with diabetes in Malaysia is unknown. Since data from previous studies on the evaluation of *Blastocystis* subtypes infection and gut microbiota composition in diabetic patients remain controversial and insufficient, a study was conducted to determine the association between *Blastocystis* subtypes infection and gut microbiota composition in patients with type 2 diabetes mellitus (T2DM) and non-diabetic group (NonDM).

1.4 Justification of the Study

Despite being the most common parasite in humans, there is still controversy regarding the pathogenicity of *Blastocystis* (Roberts, 2014). It has come to a consensus that the pathogenic potential of *Blastocystis* correlates with subtype variation of the SSU-rRNA gene (Skotarczak, 2018). Investigating the effects of colonisation by *Blastocystis* on the composition of the gut microbiota may clarify the question of *Blastocystis* pathogenicity. Diversification of the gut microbiota following *Blastocystis* colonisation has been reported in healthy individuals (Audebert et al.,2016; Kodio et

al., 2019). However, little has been reported on the diversification of the microbiota following *Blastocystis* subtype infection causing gut dysbiosis in immunosuppressed. Since diabetic patients are immunosuppressed, they are at risk of acquiring communicable diseases, including parasitic infections. Previous studies have shown that *Blastocystis* infection was high in diabetic patients compared to other intestinal parasites with prevalence ranging from 2.7% to 42% (Tangi et al., 2016).

The high rate of *Blastocystis* infection in diabetic patients may suggest its pathogenic role in humans as it is believed to disrupt the immune status of the host. Abnormal metabolic activities and immune dysregulation in diabetic patients may be induced by colonisation of certain *Blastocystis* subtypes which may further aggravate the suffering of diabetic patients. Late diagnosis and mismanagement of such infections may result in serious outcomes (Mudassar et al., 2018). Furthermore, accumulated data from previous studies provide inadequate specific *Blastocystis* subtypes evaluation of diabetic patients.

The intestinal microbiota is highly variable among individual humans and its diversity is affected by factors like diet, socio-geographic setting, antibiotic use, disease, age and to a lesser degree genetics (Nieves-Ramirez et al., 2018). In conjunction with alteration in the immunocompromised host, *Blastocystis* infection has been reported to diversify the gut microbiota in healthy individuals and those interactions may have downstream effects on host immunity and gut homeostasis (Fredensborg et al., 2020; Rapin et al.,2018; Zais et al.,2015). A previous study from the Flemish Gut Flora Project (FGFP), a Western population cohort found a strong association between *Blastocystis* subtypes and microbiota community composition with higher explanatory power than

host characteristics. The results suggest that *Blastocystis* subtypes play the main role associated with human health (Tito et al., 2019). There was also a report of asymptomatic individuals without gastrointestinal disease and inflammation which had a direct association between the presence of *Blastocystis* and changes in the gut bacterial and eukaryotic microbiome, as faecal calprotectin was significantly decreased in association with *Blastocystis* colonisation. This result proposed that the ecological shift induces subclinical immune consequences in the asymptomatic host (Nieves-Ramirez et al., 2018).

However, microbiota differentiation concerning *Blastocystis* subtypes remains unclear, especially in immunocompromised individuals including diabetic patients, whether *Blastocystis* subtypes may take advantage of the immunosuppression state of the diabetic patient, induce inflammation and potentially alter the composition of the gut microbiota, leading to an unhealthy gut environment. If *Blastocystis* can cause an unhealthy gut environment in diabetics, a baseline mechanism on how it can do so should be explored and determined. Interestingly, intestinal parasites have been associated with the same molecules involved in promoting an inflammatory response by regulating the concentration of inflammatory cytokines (Rook, 2009; Zavala et al., 2018; Zavala et al.,2016). Instead, pathogenic protozoa have been shown to cause intestinal inflammation and tissue damage where they impair the systematic inflammatory process (Naveed & Abdullah, 2021). On the other hand, there is still a lack of information regarding the association between *Blastocystis* and systematic inflammation, particularly in T2DM disease. Recent studies revealed that *Blastocystis* ST7 induces the expression of IL-6 and IL-1β pro-inflammatory cytokines through the activation of mitogen-activated protein kinases in mouse intestinal explants (Lim et al.,

2014). *Blastocystis* ST1, ST2, and ST3 were found to have higher levels of the cytokine IL-6 in IBS patients (Azizian et al., 2016).

In individuals with type 2 Diabetic Mellitus (T2DM), monoclonal cells and monocytes will secrete less IL-1 and IL-6 in response to lipopolysaccharide stimulation, which leads to an intrinsic defect of cells (Casqueiro et al., 2012; Frydrych et al., 2017; Geerlings et al.,2000; Pelag et al.,2007). Recent evidence elucidates that systematic inflammation is an important causal factor that precedes the development of T2DM, and inflammatory processes play a crucial role in the pathogenesis of T2DM. Somehow, through direct or indirect effects, it affects glucose metabolism and homeostasis in various organs of the body (Akash et al.,2013; Rehman et al., 2017; Kristiansen and Mandrup.,2005). According to Das and colleagues, they found that the number of anti-inflammatory bacteria (*Roseburia, Lacnospira, Caprococcus, Phascolarbacterium, Blautia* and *Anaerostipes*) decreased, while the number of pro-inflammatory bacteria (*Escherichia, Enterobacter, Methanobrevibacter* and *Treponema*) were increased in T2DM compared to healthy controls (Das et al., 2021). This suggests that T2DM is considered an inflammatory disease.

In light of this, a comparative cross-sectional study was conducted among T2DM patients at the Endocrine Clinic and Primary Medical Universiti Kebangsaan Malaysia, and the non-diabetic group to provide comprehensive information on the *Blastocystis* subtypes distribution with gut microbiota differentiation among both groups of study. This present study was performed by using specific "barcoded" primers, to identify *Blastocystis* subtypes in T2DM patients compared to NonDM group by molecular technique, polymerase chain reaction. Next gene sequencing (NGS) was conducted to

determine the gut microbiota composition among the groups. With this background in mind, the present study is the first to provide information on the relationship between *Blastocystis* infection with gut microbiota differentiation among T2DM patients and NonDM group in Malaysia.

1.5 Research Questions

This cross-sectional comparative study was based on three research questions:

1. What is the prevalence and subtype of *Blastocystis* in type-2 diabetes patients (T2DM)?
2. Are there any subtype variations of *Blastocystis* with gut microbiota?
3. What is the association between *Blastocystis* and gut microbiota in T2DM patients?

1.6 Objectives

1.6.1 General Objective

This study aims to evaluate the *Blastocystis* subtypes differentiation in type2 diabetic mellitus (T2DM) patients and non-diabetic (NonDM) individuals associated with gut microbiota.

1.6.2 Specific Objectives

1. To determine the prevalence and subtypes of *Blastocystis* infection in type-2 diabetic mellitus (T2DM) patients.

2. To identify the subtype variations of *Blastocystis* with gut microbiota.
3. To evaluate the association between *Blastocystis* and gut microbiota in T2DM patients.

1.7 Hypotheses

1. *Blastocystis* ST3 is prevalent in type 2 diabetes mellitus (T2DM) patients.
2. There are subtype variations of *Blastocystis* with gut microbiota.
3. *Blastocystis* induce gut dysbiosis in T2DM patients particularly *Blastocystis* subtype 7 (ST7).

1.8 The Significance of the Study

The findings of this study provide vital information on the relationship between colonisation of *Blastocystis* subtypes in T2DM patients and gut microbiota. Subtype classification provides further information on the pathogenicity of different *Blastocystis* subtypes. Despite that, the mechanism of how *Blastocystis* may disrupt host cells in immunocompromised individuals' infection remains ambiguous. Therefore, this study would contribute information on the potential inflammation triggered by *Blastocystis* infection which may associated with T2DM patients. Furthermore, this present study also revealed the possible association between *Blastocystis* subtype distribution and gut microbiota especially in T2DM patients. This added significant information on the impact and pathogenesis of *Blastocystis* infection in T2DM patients within subtypes variation associated with gut microbiota. Understanding the prevalence of infection and its influence on the prevalence will help to design effective solutions and integrate control measures. The outcome of this study is expected to shed more light on the pathogenic potential of this organism particularly in the T2DM population. The new evidence is hoped to pave the way for new and improved patient care for T2DM disease.

1.9 Scope of Study

This current study has evaluated the infection of *Blastocystis* would be influenced on gut microbiota composition in the type-2 diabetic patients (T2DM) and non-diabetic (NonDM) group, as the targeted inflammatory markers would be the proxy to determine the interaction by both. This study was conducted at Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur and a research laboratory at the Faculty of Medicine and Health Science, Universiti Sains Islam Malaysia (FPSK USIM, Nilai). A total of 203 samples were obtained from March 2021 to November 2022 following the inclusion criteria, those who were diagnosed with T2DM with non-complication with other underlying diseases and healthy individuals. All the volunteers were approached for their consent to participate in our study. The stool samples were analysed by a molecular technique by PCR to detect the infection of *Blastocystis* in both groups. Meanwhile, gut microbiota composition for targeted 100 samples was determined by Next Gene Sequencing (NGS) and was analysed in terms of its abundance and diversity. Blood samples were analysed for pro-inflammatory markers (IL-6 and IL-1β) in both T2DM and NonDM. The gut microbiota analysis also revealed a correlation between *Blastocystis* infection and elevated levels of IL-6 and IL-1β, indicating the presence of inflammation in the gut.

1.10 Conceptual Framework

In line with the objectives, the proposed conceptual framework in Figure 1.1 was developed to evaluate the *Blastocystis* subtypes colonisation in the type 2 diabetes mellitus (T2DM) and non-diabetic (NonDM) group associated with gut microbiota based on available works of recent literature.

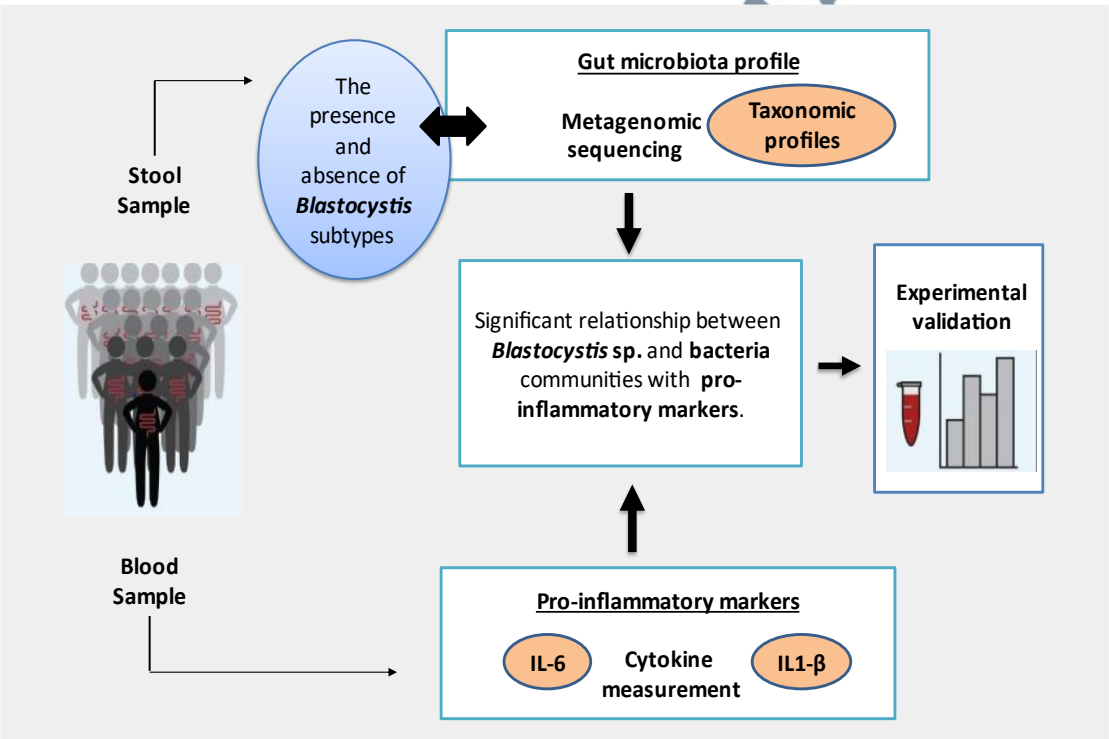

Figure 1.1: Conceptual framework showing the infection of *Blastocystis* in the (T2DM) and (NonDM) group that associated with gut microbiota by (PCR), (NGS) and ELISA

Source: Developed from literature review

From Figure 1.1, it is assumed that the presence of *Blastocystis* gives rise to symptoms related to intestinal damage which vary in subtypes with unknown mechanisms. As a result, *Blastocystis* infection was previously considered an opportunistic disease associated with human immunodeficiency or occasionally found

in patients suffering from irritable bowel syndrome (IBS) or chronic inflammatory bowel disease (IBD) (Reviewed by Partida-Rodríguez et al., 2017). The increased prevalence of *Blastocystis* has been linked to changes in the composition of the microbiota in the human host (Audebert et al., 2016; Partida-Rodríguez et al., 2017). According to the study by Nagel et al., (2016), there was a change in the gut microbiota in the main phyla with the rise of Firmicutes and a statistically significant reduction in Bacteroides relative abundance in diarrhoea-predominant Inflammatory Bowel Disease (IBS) patients than healthy subjects. Besides, it has been hypothesised that the changes in gut bacteria would be associated with certain inflammatory markers responses when the participants were *Blastocystis*-positive and *Blastocystis*-negative. Certain cytokines were expressed during the innate and adaptive immune response with several functions. As discovered by Lim et al., 2014, *Blastocystis* antigens induced abundant expression of proinflammatory cytokines, including 1β (1l-1β), IL-6 and tumour necrosis factor-alpha (TNF-α) via the activation of MAP kinases and that infection with *Blastocystis* may contribute to the pathogenesis of inflammatory intestinal diseases through the activation of inflammatory pathways in host immune cells, such as macrophages. Thus, the immune changes induced by *Blastocystis* colonisation may directly influence the host gut microbiota composition (Deng et al., 2021). From Figure 1.1, there may be a significant relationship between *Blastocystis* and gut microbiota with pro-inflammatory markers (1l-1β & IL-6). The analysis of the relationship between gut microbiota and cytokine responses would be viewed as a proxy to understand the role of *Blastocystis* itself in T2DM.

1.11 Operational Definition

Variables	Operational Definition
Blastocystis	*Blastocystis* were identified through sequencing by polymerase chain sequences (PCR) amplification of SSU rDNA by using barcode primers RD5 and BhRDr that amplify an ~600 bp region to detect the presence of *Blastocystis* DNA. The frequency and prevalence of *Blastocystis* was expressed in proportion.
Subtypes	The PCR product containing sufficient information for phylogenetic analysis and subtype identification of *Blastocystis* is sequenced by using Sanger sequencing to detect its subtypes and alleles.
T2DM	Type 2 diabetes was diagnosed according to the Malaysian Diabetes Association criteria; patients with FBS ≥ 7.0 mmol/L or HbA1c ≥ 6.3% (≥ 45 mmol/mol)
Gut Microbiota Composition	The bacterial phylum and genus were characterized by 16S rRNA gene and were performed on the Miseq system with paired-end strategy constructed according to the 16S metagenomic sequencing library preparation (Illumina)
Alpha-diversity	The richness of single microbial taxa within a sample. Alpha diversity was measured using observed OTU, Chao 1, Fisher's index and Shannon's and Simpson's indexes and had been presented in mean or median.
Beta-diversity	The variation in microbiota composition between individual samples based on Bray-Curtis dissimilarity matrix (analyses dissimilar microbial species.

1.12 Conclusion

This chapter briefed a comparative cross-sectional study that was designed to address some of the shortcomings in the literature on *Blastocystis*. The overall aim of the study was to evaluate the *Blastocystis s*ubtypes colonisation in the type 2 diabetes mellitus patients (T2DM) and non-diabetic (NonDM) group associated with gut microbiota in the Malaysia population. By highlighting the problem statement, significance of the study, discussing relevant prior studies and outlining the objectives and methodologies, in-depth exploration was discussed in the following chapters revolving around *Blastocystis*, diabetes and the gut microbiota.