CHAPTER 5

DISCUSSION

5.1 Introduction

This chapter explores the findings of the study. Initially, this study observed the prevalence of *Blastocystis* subtype infection among individuals with type 2 diabetes mellitus (T2DM) and non-diabetic individuals (NonDM). Following this, an association of *Blastocystis* infection with gastrointestinal symptoms was discussed in both groups. This chapter also highlighted the gut microbiota composition associated with *Blastocystis* subtypes in T2DM and NonDM groups. Lastly, the composition of the gut microbiota was associated with clinical characteristics focusing on two targeted markers (IL-6 and IL1- β) was discussed.

5.2 *Blastocystis* subtypes prevalence in Type 2 Diabetes Mellitus (T2DM) and non-diabetic (NonDM)

Blastocystis is one of the most prevalent parasites found in human faecal specimens, particularly among immunocompromised individuals, such as those with type 2 diabetes mellitus (T2DM). While its clinical importance and pathogenicity remain uncertain, recent research suggests that *Blastocystis* may have potential clinical

significance in these immunocompromised populations. Due to the growing research interest in *Blastocystis*, the prevalence observations, as well as subtype identification, have been performed in this study focusing on the Malaysian population. This present study evaluated the prevalence of *Blastocystis* in T2DM, a group that is known to be susceptible to infectious diseases, and non-diabetic (NonDM) individuals.

According to the results, a high prevalence rate of *Blastocystis* infection was observed in T2DM (25.5%) compared to non-diabetic (NonDM) individuals (17.8%) by polymerase chain reaction (PCR), which were similar to previous studies that were conducted in various regions. Recent studies have compared the prevalence observed in a comparison between diabetic group and healthy control groups that highlighted *Blastocystis* infection between diabetic patients and healthy control groups. These studies revealed varying rates of *Blastocystis* infection with a high prevalence rate in diabetic patients compared to healthy controls. For instance, Tangi et al., (2016) reported rates of 2.7% and 1.2% among diabetic patients and the control group. Also, the higher prevalence rate of *Blastocystis* (35.5%) was reported by Ali et al., (2018) among diabetic patients in Iraq. Aourarh et al., (2020) observed a significantly higher prevalence in diabetic patients (42%) compared to healthy controls (4%) ($p<0.05$), while Mohtashamipour et al., (2015) also noted a significant difference, with rates of 9.3% and 2.5% among diabetic and healthy groups, respectively ($p<0.05$). Additionally, Popruk et al., (2020) and de Melo et al., (2021) reported rates of 12.3% and 9%, and 12.1% and 7.9%, respectively. A study conducted in Egypt by Ibrahim et al., (2020) also reported that diabetic patients had higher *Blastocystis* infection (87%) compared with non-diabetic patients (42%) by Modified Jones's culture while in Turki, higher *Blastocystis* infection has also been observed in diabetic patients (25.3%, 14%) than

control (22.7%, 10%) in both PCR and microscopic method respectively (Şahin et al., 2023). Furthermore, most studies have reported patients with diabetes mellitus have a weakened immune system because the risk of infection is higher (Popruk et al., 2020). It was discovered by Ibrahim et al., (2020) that diabetic patients may have a higher risk of acquiring *Blastocystis* infection due to their chance of infection increased by more than nine times. Mohtashamipour et al., (2015) also revealed that the risk of intestinal parasites particularly *Blastocystis* was 3.6 times higher in diabetics than non-diabetics. In immunocompromised hosts, *Blastocystis* serves as a potential opportunistic pathogen that can impair intestinal mucosal integrity, thereby explaining the elevated risk observed in diabetic patients (Olusegun et al., 2013). These findings demonstrate that *Blastocystis* should be considered as a potential risk factor in patients with diabetes.

However, some researchers have drawn different conclusions where *Blastocystis* infection was higher in the control group (9.1%) than in diabetic patients (10.1%) in Iran (Poorkhosravani et al., 2019). This variation could be related to the health status of the study population, geographic distribution, and detection method (Khanna et al., 2022; Matovelle et al., 2022; Popruk et al., 2020). Specifically for the diagnosis of *Blastocystis*, most previous studies used parasitological techniques by microscopic including stained smears and culture methods while our present study used molecular analysis to identify *Blastocystis* in T2DM and NonDM. It has been found that molecular analysis has higher positivity rates than microscopic examination (de Melo et al., 2021; Matovelle et al., 2022; Şahin et al., 2023). This is because molecular techniques are significantly more sensitive than microscopic techniques and xenic and vitro culture for the detection of *Blastocystis* in faecal specimens in both humans and animals

(Mahmoud et al., 2023; Tamalee Roberts et al., 2011). Therefore, the rate of *Blastocystis* infection reported might be underestimated depending on the detection method.

Through molecular analysis of the barcode region, this present study had successfully isolated different *Blastocystis* subtypes including ST1, ST2, ST3, ST4, ST6 and ST7 among diabetic patients and non-diabetic individuals. A thorough evaluation of the studies in the literature reported that ST1, ST2, ST3 and ST4 constitute 90% of the *Blastocystis* infections in humans where the most common subtype detected worldwide was ST3 (P. A. Jiménez et al., 2019). However, *Blastocystis* ST4 was lowly detected among the common STs in our study and this was consistent with the previous studies reporting a low prevalence of ST4 in Asia, America and Africa (P. Jiménez et al., 2022; Popruk et al., 2020). Interestingly, there was a predominance of ST3 (34.62%) followed by ST1 (26.92%), ST2 (15.39%), ST4 (11.54%), ST6 (3.85%) and ST7 (7.69%) in T2DM patients. While, ST3 (33.33%) followed by ST1 (27.77%), ST2 (22.22%), ST4 (11.11%) and ST6 (5.56%) were observed in the non-diabetic group. It was similar to some previous studies on *Blastocystis* subtypes in diabetic patients as ST3 had been highly detected in both groups. In Thailand, ST3 (9.3%) followed by ST1 (3%) were detected in diabetic patients while ST3 (6%) followed by ST1 (2%) and ST4 (1%) in the control group (Popruk et al., 2020). In contrast, de Melo et al., (2021) reported ST1 (38.2%) had been prevalent in diabetic patients followed by ST3(35.3%), ST2 (11.8%), ST6 (2.9%) and ST7 (2.9%) while in control, ST3 (43.5%) was higher followed by ST1(21.8%), ST2 (21.8%) and ST6 (4.4%) in Brazil. Generally, the identification of ST6-ST7 subtypes in *Blastocystis* can indicate a potential area for zoonotic transmission, where these subtypes are frequently found in birds (P. A. Jiménez et al., 2019). Upon evaluating participant data in this study, we observed that

individuals infected with ST6 and ST7 reported engaging in animal rearing, specifically owning cats as pets. Moreover, it has been suggested that *Blastocystis* ST1 is also associated with zoonotic transmission to humans particularly by exposure to animal stools. This suggests that animal exposure may represent a potential mode of transmission for *Blastocystis* infection (Popruk et al., 2020). In the current study, ST7 (2/1.7%) was only detected in T2DM. The previous study also reported similar findings where ST7 was found exclusively in diabetic patients (de Melo et al., 2021). This consistency across the studies underlines the potential association between ST7 and diabetes disease warranting a further evaluation into the specific mechanism. However, ST7 was not strongly associated with T2DM, as it was observed in only a small percentage of individuals in this study.

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5.3 Association of *Blastocystis* subtypes with gastrointestinal symptoms

Blastocystis is commonly found in asymptomatic individuals but it can occasionally cause several gastrointestinal symptoms, particularly in immunocompromised individuals. Clinical manifestations of *Blastocystis* infection may include bloating, constipation, abdominal pain, flatulence, vomiting, diarrhoea and fatigue (A. Asghari et al., 2020; Noradilah et al., 2017; Safadi et al., 2016). Despite ongoing debates about whether *Blastocystis* is a commensal or pathogenic protist, some studies have reported no association between gastrointestinal symptoms and *Blastocystis* infection or its subtypes (Delshad et al., 2020; Mardani Kataki et al., 2019). A recent study in Iran found that *Blastocystis* infection was more prevalent in healthy individuals compared to those with gastrointestinal disorders, suggesting that most infections are asymptomatic (Mardani Kataki et al., 2019). Additionally, a review study by Aykur et al., (2024) highlighted that, *Blastocystis* could reside in the human colon for a long period without developing any symptoms, supporting the controversy of whether it should be classified as a pathogen or a commensal microorganism.

In this present study, flatulence (93.2%) was the most frequent symptom detected in the presence of *Blastocystis* among T2DM and NonDM followed by bloating (81.8%), watery and loose stool (79.5%) abdominal pain (75%) and diarrhoea (65.9%). These symptoms were often reported in association with symptomatic *Blastocystis* infection by several studies including patients with T2DM (Khorshidvand et al., 2021; Mohtashamipour et al., 2015; Noradilah et al., 2017; Waly et al., 2021). Furthermore, in this present study, *Blastocystis* was significantly associated with bloating (p=0.037) with a risk factor of three times (OR = 2.937, 95% CI: 1.104-7.811) higher in *Blastocystis*-positive while there were no association of *Blastocystis* infection and other

symptoms. This finding was supported by a study from Peru Hospital which observed the likelihood of presenting dyspepsia, bloating and abdominal pain increased with the presence of *Blastocystis* (Robles-Cabrera et al., 2021). However, in previous studies on diabetic patients, *Blastocystis* was observed to be associated with diarrhoea (p<0.001) (Mohtashamipour et al., 2015; Waly et al., 2021). In the study by Noradilah et al., 2017 in the Malaysian aboriginal community, flatulence was among the prominent symptoms detected and significantly observed more common in individuals positive with *Blastocystis*.

Pathogenicity of *Blastocystis* is believed to be subtype-dependant. In this present study, no significant difference was observed in the distribution of STs among the study groups. However, all participants in this study who were infected by *Blastocystis* experienced some gastrointestinal symptoms within 6 months. ST3, ST1 and ST2 were the most common subtypes observed among the symptomatic T2DM and NonDM. It was similar in recent studies that reported ST1 (22.2%), ST2 (2.8%), ST3 (72.2%) and ST4 (2.8%) were most dominant in the symptomatic group of children with diarrhoea (Zulfa et al., 2017). Interestingly, this study observed that ST3 was the most common subtype detected in T2DM patients with abdominal pain (34.6%), flatulence (34.1%), mucus in stool (35.7%) and cramping (36.4%). Meanwhile, ST2 was predominant in symptomatic individuals with blood in stool (40%).

In addition to gastrointestinal symptoms, previous studies associated the *Blastocystis* infection with cutaneous disorders and chronic or acute urticaria belonging to the ST2 or ST3 in the patient's faecal samples (Kumarasamy et al., 2018). ST1 has also been observed with all gastrointestinal symptoms in a slightly lower detection compared to ST3. These results were in agreement with other studies that denied any

association between the presence of symptoms and *Blastocystis* subtypes (Abaza et al., 2014; Dogan et al., 2017; Kumarasamy et al., 2018). Individuals that were infected with ST3 and ST6 may result in a range of clinical outcomes with some individuals experiencing symptomatic infection while others remain asymptomatic or experience mild symptoms (E. M. Hussein et al., 2008; Karamati et al., 2021; Rojas-Velázquez et al., 2022). ST3 infection has also been significantly associated with flatulence while ST1 infection has been observed in patients with gastrointestinal symptoms particularly nausea in the infected individuals (Noradilah et al., 2017; Tan et al.,2008). Meanwhile, other studies stated that ST1 may be associated with more severe clinical outcomes compared to other subtypes (E. M. Hussein et al., 2023; Kesuma et al., 2019). Regarding ST4 and ST6, recent studies reported that ST4 and ST6 have been common subtypes in patients with diarrhoeal (Deng et al., 2022; Stensvold et al., 2011). In this study, all the participants that infected with ST4 and ST7 experienced watery and loose stool. A previous review highlighted that watery loose stool is a part of chronic diarrhoea which frequently occurs due to a parasite infection (D. Friday et al., 2023). However, this study showed, that there were no significant association between *Blastocystis* subtypes and gastrointestinal symptoms.

Recently, several studies have even proposed that this protozoan may be promoted as an indicator of a healthy gut (Deng et al., 2021). In addition to that, accumulating evidence over recent years has shown that *Blastocystis* colonisation is associated with a higher bacterial diversity of gut communities particularly in individuals without any comorbidities (M. J. Kim et al., 2022; C. R Stensvold et al., 2022). Therefore, the variation in clinical findings among individuals with *Blastocystis* infection is acknowledged to be influenced by many factors including genotype of

Blastocystis, microbiota composition, host immune response, *Blastocystis* density, and other concurrent infections (C. Rune Stensvold & Clark, 2016).

5.4 Gut Microbiota Composition in T2DM Compared to NonDM

In this present study, a comprehensive analysis by next gene sequencing (NGS) showed a notable difference in alpha diversity in type-2 diabetic mellitus (T2DM) when compared to non-diabetic (NonDM). The gut microbiota was seen to be altered in T2DM, as alpha diversity was significantly decreased in the T2DM group compared to the NonDM group. Regarding alpha diversity, a significant reduction in species richness, diversity and abundance of gut microbiota in this study suggested a less diverse microbial composition within the gut ecosystem of T2DM. A decrease in alpha diversity of the gut microbiota will lead to an underlying disease state, as in T2DM, previous studies reported that lower alpha diversity levels related to the development of glucose intolerance (T. Das et al., 2021; Maskarinec et al., 2021). This finding was supported by other studies that demonstrated the gut composition of patients with T2DM showed statistically significant differences concerning alpha diversity compared to healthy individuals (Larsen et al., 2010; Petakh et al., 2023). Moreover, a recent study by Almugadam et al., (2020) observed that the abundance of gut microbiota was higher in T2DM compared to healthy controls, as noticed in alpha diversity although there were no significant differences between both groups. However, one study showed that alpha diversity was higher in cases than in control, in contradiction to another previous finding. This observation may be due to treatment or lifestyle changes that are prescribed following a diabetes diagnosis, which may contribute to increasing alpha diversity (Doumatey et al., 2020). From the relative bacterial abundance, the dominant

phyla observed among T2DM and NonDM groups in this study were Firmicutes (55.37%, 55.73%), followed by Actinobacteria (21.19%, 11.71%), Bacteroidetes (14.75%, 25.87%) and Proteobacteria (6.9%, 5.18%). Interestingly, the current study revealed numerous changes in the gut composition as there was a significant difference in bacteria composition between the T2DM and NonDM groups. This difference appeared at the level of certain phyla and genera.

The current study highlighted this finding which NonDM was enriched significantly with the Bacteroidetes phylum (p<0.001, FDR=0.0001) consisting of *Bacteroides* (p<0.014, FDR=0.016). As the *Bacteroides* genus was significantly lower in T2DM, it can be explained by a previous study reported that a reduction in the genus *Bacteroides* has been linked to insulin resistance due to a decrease in branched amino acids and short-chain fatty acids (SFCAs) (Pedersen et al., 2016). Generally, gut Bacteroidetes produce butyrate, a byproduct of colonic fermentation. It is believed to have antineoplastic properties and contribute to gut health (Y. S. Kim & Milner, 2007). Like *Prevotella*, it was recognised as positively associated with the production of health-promoting compounds such as SFCAs, where it improved glucose metabolism or had an overall anti-inflammatory effect (De Vadder et al., 2016). In this study, *Prevotella 9* appeared to have a decreasing trend in T2DM patients. Thus, this explained the abundance of Bacteroides phylum in NonDM compared to T2DM. However, these findings were contradictory to the animal model study mainly in *Prevotella 9,* where genetic T2DM Wistar rats were observed in their enrichment of the genera *Prevotella 9*, *Blautia*, *Roseburia*, *Allobaculum* and *Prevotella 1* compared with normal Wistar rats (Peng, 2020). Another study also revealed a genus of *Prevotella 9* sequences consisting of *Prevotella* copri whose presence of this species could increase the risk of diarrhoea

and irritable bowel syndrome (Su et al., 2018; Wutthi-in et al., 2020). It is noteworthy that *Prevotella 9*, a particular strain within the *Prevotella* genus, may be linked to specific disease endotypes. Additionally, *Prevotella* is classically thought of as a commensal bacteria because of its wide genetic diversity, ecology and ability to interact with other commensal bacteria from the gut to regulate its levels (Ley, 2016).

Meanwhile, the relative abundance of the Actinobacteria phylum increased significantly in T2DM compared to NonDM (p=0.001, FDR=0.005). Actinobacteria is a bacterial phylum comprising of genera *Bifidobacterium* and *Collinsella*. The increased Actinobacteria phylum in this study was reflected in the significantly higher abundance of *Bifidobacterium* (p=0.008, FDR<0.001) and increased trend of *Collinsella* among T2DM. Interestingly, this finding was consistent with previous results stated that the levels of the Actinobacteria phylum consisting of *Bifidobacterium* as well as *Collinsella* genus were significantly increased in patients with hypertension (HT), hyperlipidemia (HL) and type-2 diabetes mellitus (T2DM) whereas the level of Bacteroidetes phylum was significantly decreased in all disease groups (Adachi et al., 2019; Cunningham et al., 2021; Hashimoto et al., 2020; Takagi et al., 2020) compared to control individuals. In contrast, Zhang et al., (2021) found that the proportion of *Bifidobacterium* was lower in T2DM. While *Collinsella is* often associated with individuals suffering from T2DM. Recent studies reported that *Collinsella* was associated with serum cholesterol and triglyceride levels as this genus had a high abundance in patients with obesity and T2DM, but the precise molecular mechanisms are unknown (Frost et al., 2019; Lahti et al., 2013; Lambert et al., 2015; Nirmalkar et al., 2018). This suggests that the changes in both Actinobacteria and Bacteroidetes phyla may be associated with the development of T2DM.

Furthermore, Firmicutes was the phylum with the greatest number of the genera in the gut including *Lactobacillus*, *Megamonas*, *Dorea*, *Faecalibacterium* and *Blautia*. In this study, the relative abundance of the Firmicutes phylum was almost similar in the T2DM and NonDM groups. While, some previous studies from different regions showed a lower abundance of Firmicutes observed in T2DM patients (Chávez-Carbajal et al., 2020; Larsen et al., 2010; Letchumanan et al., 2022; Maskarinec et al., 2021). Generally, Firmicutes is a pivotal bacteria present in the gut microbiota, which could metabolise indigestible polysaccharides and increase the production of short-chain fatty acids (SCFAs). Short-chain fatty acids (SCFAs), including acetate, butyrate and propionate along with the bacterial taxa responsible for their production, have been of particular interest in the diabetic gut due to the beneficial effects of these molecules on the host function (Blaak et al., 2020; Sanna et al., 2019). Production of SCFAs will influence different metabolic pathways and therefore will contribute to preventing and modulating T2DM (Abdul Rahim et al., 2019). Thus, a decrease in SCFAs-producing taxa could be comorbid with T2DM and may cause or worsen the disease (Nayman et al., 2024).

Looking at the genus level belonging to the Firmicute phylum, this study observed a significant increase in *Blautia* (p=0.001, FDR<0.001) and *Dorea* (p=0.029, FDR=0.001) genera whereas there was a decrease in *Megamonas* (p=0.001, FDR=0.005) among T2DM patients compared to NonDM. This finding appeared to be well substantiated by previous studies stating that *Blautia* and Dorea had a positive association with T2DM. The increase in *Blautia* and *Dorea* with a decrease in *Megamonas* suggested that these particular bacterial taxa may play a role in the pathogenesis and progression of T2DM as they may influence host metabolism and

inflammation through the production of metabolites such as SCFAs and modulate gut barrier function (Almugadam et al., 2020; Egshatyan et al., 2016; Gurung et al., 2020; Li et al., 2020; Vallianou et al.,2019). Additionally, this study also observed a decreasing trend in *Faecalibacterium* and an increasing trend in *Lactobacillus* among T2DM compared to NonDM, although no significant differences were found. Most recent studies observed a significantly higher *Lactobacillus* level in patients with diabetes compared to healthy controls (Larsen et al., 2010; Marlene, 2013; Sedighi et al., 2017). It was also supported by previous substantial body and experimental research that the *Faecalibacterium* genus was inversely correlated with T2DM, while the genera *Blautia* and *Lactobacillus* were positively associated with T2DM. This indicates that as the abundance of genera increases, the likelihood of T2DM occurring also increases (M. Gurung et al., 2020; Sedighi et al., 2017; D. Wang et al., 2022; Jun Wang et al., 2012; Zewen Zhang et al., 2021).

On the other hand, T2DM participants are commonly associated with an increase in pathogenic bacteria, mainly from the Proteobacteria phylum. Proteobacterial species are frequently recognised as a typical indicator of microbial dysbiosis (Shin et al., 2015). In contrast, a healthy gut is dominated by Bacteroidetes and Firmicutes but also includes smaller proportions of Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia (Hollister et al.,2014). Interestingly, this study observed an increasing trend in the *Escherichia-Shigella* genus belonging to the Proteobacteria phylum among T2DM compared to NonDM, although not statistically significant. This is consistent with past studies, as the proportion of Proteobacteria was higher followed by *Escherichia-Shigella* was common in patients with T2DM but not significantly (Larsen et al., 2010; Zewen Zhang et al., 2021). A recent study by Maskarinec et al., (2021)

found that *Escherichia-Shigella* was elevated in T2DM patients and also associated with the use of Metformin in T2DM. Taking metformin was associated with a change in the composition of the faecal microbiome in subjects with T2DM including enrichment in Escherichia (Maskarinec et al., 2021; Wu et al., 2017). It is thought a high abundance of *Escherichia-Shigella* may enhance chronic systemic inflammation in T2DM disease as it is considered an endotoxin-producing bacteria (Maskarinec et al., 2021). The explanation for this could be that in T2DM, glucose metabolic irregularities and elevated glucose metabolites increase the number of pathogenic bacteria in the gut, resulting in inflammation and insulin resistance (Larsen et al., 2010).

According to Sun et al., (2010), the outer member of these bacteria contains lipopolysaccharide (LPS) which is a cellular membrane component of gram-negative bacteria that is elevated in both obesity and T2DM patients. LPS can cause metabolic endotoxemia, which is linked to oxidative stress, macrophage-secreted components and inflammatory markers that induce insulin resistance (Momin et al., 2016). In this state of endotoxemia, the mechanisms are likely to involve both increased LPS uptake and decreased LPS clearance. The chylomicrons secreted by enterocytes exhibit heightened LPS uptake, while cooperated tight junctions between enterocytes lead to increased intestinal permeability, commonly referred to as leaky gut. Thus, this ultimately causes more LPS to translocate into the blood circulation (Gomes et al., 2017; Sohail et al., 2017). Rodent investigation revealed that LPS-TL 4 signalling promotes an innate immune response that mediates systemic inflammation and insulin resistance (Cani et al., 2008). However, a growing body of research on humans suggested that LPS may cause a systemic inflammatory response in T2DM participants, characterised by elevated neutrophil counts, elevated body temperature and heart rate, increased plasma

concentrations of cytokines, and increased adhesion molecule production (Andreasen et al. 2010; Dong et al., 2022). According to a recent study by L. Zhang et al., (2022), there was a significant increase in LPS in T2DM participants compared to the controls (p<0.05).

Indeed, a review of previous findings observed an increase in pathogenic bacteria such as *Klebsiella*, *Salmonella*, *Enterobacteria*, *Escherichia coli*, *Bacteroides caccae*, *Prevotella copri* and *Bacteroides vulgates* that have been found in the microbiota of diabetic patients, as well as Proteobacteria phylum which are considered highly pro-inflammatory (Kumar et al., 2022; Pedersen et al., 2016). Additionally, it is widely recognised that the development of insulin resistance and type 2 diabetes is triggered by this subclinical pro-inflammatory state, which is caused by the LPS-dependent synthesis of inflammatory cytokines like interleukin-1 (IL-1), IL-6, and tumour necrosis factor-α (TNF-α) (Crudele et al., 2023; M. Gurung et al., 2020; Kumar et al., 2022; Jingjing Wang et al., 2015). Interestingly, a correlation analysis in the present study also demonstrated that pro-inflammatory cytokines (IL6 & IL1-β) were strongly correlated with T2DM compared to NonDM. Thus, an increase in the abundance of Proteobacteria could serve as an essential signal of gut dysbiosis that precedes the formation of low-grade inflammation, which is an indicator feature identified in obesity and T2DM. As a consequence, high levels of opportunistic pathogens such as *Escherichia-Shigella* in the T2DM group could support the observation that monitoring the gut microbiome may be a valuable technique in monitoring and managing bacterial infections in diabetics (Kumar et al., 2022).

Moreover, this study also found an increasing trend in the abundance of Lentisphaerae phylum among T2DM and NonDM. Commonly, the Lentisphaerae

phylum is among the less prominent phyla in the gut including Tenericutes, Fusobacteria and others. This finding was similar to a study conducted by Maskarinec and colleagues (2021) observed on Lentisphaerae that was positively associated with T2DM status (p<0.05). In contrast, women with hyperglycaemia in pregnancy (HIP) had a lower abundance of Lentisphaerae compared to healthy subjects. Lentisphaerae and Tenericutes phyla were enriched in the healthy control (p<0.05) (Beibei Gao et al., 2020). In other studies, Tenericutes was higher in controls than in individuals with polycystic ovary syndrome (PCOS) and metabolic syndrome (M. Y. Lim et al., 2017). Consistent with the previous studies, the current study found that the Tenericutes phylum was more abundant in non-diabetic individuals compared to T2DM.

Overall, this present study demonstrated that bacterial composition may be altered in T2DM, influenced by several predominant bacteria in the gut. The variations in gut microbiota composition between T2DM and NonDM were inconsistent across the included studies. However, it should be noted that some conflicting evidence regarding the dysbiosis profile associated with diabetes, as well as the relative abundance or reduction of specific genera, suggests that these alterations are more species-specific than genus-specific. Moreover, the effect of individual bacterial strains is often improved in the presence of other species, developing a complex gut microbiota environment (Crudele et al., 2023).

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5.5 Association between *Blastocystis* subtypes and the gut microbiota and its correlation with IL-6 and IL1-β

Blastocystis has been reported to have a complex interaction with the gut microbiota. Most studies demonstrated that colonisation of *Blastocystis* could affect the gut microbiota in both composition and richness. It has also been stated that the diversity and bacterial species composition of the microbiota of healthy individuals and those of patients with metabolic or infectious diseases differ. Regarding *Blastocystis* infection, recent studies have indicated that it could be associated with alterations in the abundance of both beneficial and harmful gut microbiota (Aykur, 2024; M. J. Kim et al., 2022; Olyaiee et al., 2022). Thus, this study provides insight into the association between *Blastocystis* subtypes and gut microbiota richness and diversity, indicating that *Blastocystis* could potentially contribute to gut health and could lead to dysbiosis depending on the subtypes among type 2 diabetes mellitus (T2DM) and non-diabetic (NonDM) groups.

Interestingly, this study observed that there was a significant difference in alpha diversity with or without the presence of *Blastocystis* among T2DM and NonDM participants, confirming the enrichment of bacterial diversity in *Blastocystis* carriers. This finding was similar to a previous study that stated a higher bacterial diversity in the faecal microbiota of patients colonised with *Blastocystis* was detected compared to *Blastocystis*-free subjects (Audebert et al., 2016). Several studies had observed a significant difference in beta diversity of the microbial community composition across the groups (Kim et al., 2022; C. R. Stensvold et al., 2022), which was consistent with the findings of this study. The higher gut composition diversity appears to be favourable to gut health, which may indicate that *Blastocystis* is a component of a stable, resilient

and health-associated microbiota. This will also be influenced by subtype-level analysis of *Blastocystis* which revealed significant differences within the genus (M. J. Kim et al., 2022).

The current study observed several significant abundances of phyla and genera including Bacteroidetes (p<0.001, FDR<0.0001) among groups with *Blastocystis* and without *Blastocystis*. Plus, the enrichment of *Bacteroides* (p=0.010, FDR<0.020) and *Prevotella 9* (p=0.006, FDR<0.015) genera belonging to Bacteroidetes phylum were significantly higher in the presence of *Blastocystis* especially in T2DM. A significant shift in the *prevotella*-driven enterotype in children and adults with *Blastocystis* has been reported in previous studies (Alzate et al., 2020; Nieves-Ramirez et al., 2018; Tito et al., 2019). In a study of the Korean population, *Prevotella 9* was also highly enriched in the *Blastocystis*-positive group (M. J. Kim et al., 2022). *Prevotella* species are typically the dominant colonisers of the gut in non-westernised populations that consume plant-rich diet (De Filippo et al., 2010). *Prevotella* strains are linked to plant-rich diets including fibres, simple sugars and carbohydrates indicating their role as beneficial bacteria (Ley, 2016). Certain *Prevotella* strains could produce SCFAs, which protect the gut barrier and reduce the possibility of inflammation diseases such as T2DM (Abdelsalam et al., 2023; Bedarf et al., 2017). This suggests that *Blastocystis* could promote a healthy gut composition and may reflect the homeostasis of the gut. In the healthy gut, metabolites such as short-chain fatty acids (SCFAs) are developed by fermentation by anaerobic bacteria and possibly enhanced SCFA production by *Blastocystis* (Aykur, 2024). In contrast, a recent experimental study conducted by Defaye et al., 2020 reported that the faecal composition of infected rats was characterised by a decrease in overall SCFA production, including acetate, propionate

and butyrate. This gut alteration included an increase in the relative abundance of *Oscillospira* and a decrease in *Clostridium,* which appear to be associated with lower levels of SCFAs in the infected rats' faeces (Defaye et al., 2020).

Furthermore, this study found a significantly higher abundance of *Bifidobacterium* (p=0.019, FDR=0.032) belonging to Actinobacteria phylum (p=0.002, FDR=0.005) in *Blastocystis*-negative individuals which T2DM group exhibiting even greater Actinobacteria abundance compared to the NonDM group. Previous studies also observed that *Bifidobacterium* was inversely associated with *Blastocystis* in healthy children and males with intestinal bowel syndrome (IBS) (Beghini et al., 2017; Cinek et al., 2021; Nourrisson et al., 2014). A study conducted by (Kodio et al., 2019) also observed that Actinobacteria was overrepresented in *Blastocystis*-non colonised children in healthy Malian children. Generally, *Bifidobacterium* is an essential genus since its key roles include stabilising the early bacteriome, preserving the intestinal barrier, immunological education, and the switch between the newborn and childhood microbiomes (Kato et al., 2017). Despite *Bifidobacterium* being widely utilised as a probiotic, the current study discovered a negative association between *Bifidobacterium* and *Blastocystis*.

In addition, a significant increase in relative abundance of less the prominent phylum Lentisphaerae (p< 0.001, FDR<0.001) was found among *Blastocystis* negative in T2DM participants and *Blastocystis* positive in NonDM participants. While Euyarchaeota (p<0.001, FDR<0.001) and Tenericutes (p=0.008, FDR=0.016) phyla were significantly higher in the *Blastocystis* positive NonDM group compared to other groups. It is consistent with a recent study that reported *Blastocystis* colonisation has a significant association with a higher bacterial diversity in the phyla Firmicutes,

Elusimicrobia, Lentispaerae and Euryacheota (Kodio et al., 2019). In the present study, the findings on Lentispaerae phylum were dominant in the T2DM group compared to NonDM which suggests that the presence of *Blastocystis* could help in enriching Lentispaerae phylum. However, there is currently not enough evidence to assign the influence of *Blastocystis* on these phyla. The bacterial classifications Lentisphaerae (phylum), Lentisphaeria (class), and Victivallales (order) are understudied (Zhi Zhang et al., 2023).

A recent study also revealed that these microbial communities were intimately related to immune modulation and could potentially improve symptom severity scores (Fan et al., 2020; Hemmings et al., 2017). In addition, this study correlates favourably with the finding of Yong Wang et al. (2020), further supporting the association between *Blastocystis* carriage and the high relative abundance of Tenericutes phylum, which consisted of both commensals and pathogens. This finding was also observed in an experimental infection study involving rats; those infected with *Blastocystis* had a higher abundance of the Tenericutes phylum. This indicates that parasites like *Blastocystis* may certainly modify this phylum, regardless of the underlying disease (Defaye et al., 2020). According to recent studies, Lentisphaerae and Tenericutes were abundant in healthy controls compared to individuals with polycystic ovary syndrome (PCOS), hyperglycaemia or metabolic syndrome when comparing less prominent phyla (Beibei Gao et al., 2020; Lindheim et al., 2017). Thus, it would suggest that bacteria like Tenericutes seem to play a protective role against several kinds of diseases (Beibei Gao et al., 2020). It is like the current study which observed that the NonDM group has a higher abundance of these phylum; Lentisphaerae, Euryarchaeota and Tenericutes when compared to T2DM. However, these phyla were lower in the presence of

Blastocystis in T2DM, which suggested that T2DM itself could alter the gut composition.

In this study, all groups with the presence of *Blastocystis* and without *Blastocystis* were dominated by phylum Firmicutes which was consistent with recent studies among the Malaysian population (Neoh et al., 2018; Rajamanikam et al., 2023). Generally, Firmicutes has been related to butyrate producers' bacteria as it is one of the most dominant phyla in the gut, consisting of a member of the *Faecalibacterium*, *Blautia*, *Dorea* and *Megamonas* genera. The increase in Firmicutes would lead to increased butyrate production. The metabolism of butyrate may errand a hypoxic intestinal lumen, which again favours colonisation by *Blastocystis* while disfavouring facultative anaerobic bacteria, providing a balance of gut environment (Christen Rune Stensvold & van der Giezen, 2018). Looking at the genus, *Faecalibacterium* was significantly higher in *Blastocystis* individuals ($p<0.001$, FDR=0.0001) while *Blautia* ($p=0.001$, FDR=0.003) and *Megamonas* ($p<0.001$, FDR=0.0001) were significantly higher in *Blastocystis*-negative in both T2DM and NonDM. Some butyrate-producing bacteria decreased, which suggested that in some unhealthy individuals, the types of Firmicutes bacteria present in the gut may change, leading to variations in Firmicutes abundance (Magne et al., 2020; Oliva et al., 2022). Regardless of the changes in T2DM, *Blastocystis* could potentially contribute to the enhancement of butyrate-producing bacteria like *Faecalibacterium*, leading to gut health.

In contrast, a positive association was suggested between the presence of *Blastocystis* and T2DM patients, which was also correlated with an increase in pathogenic bacteria. An increasing trend of the phylum Proteobacteria in *Blastocystis*-positive T2DM compared to NonDM has been seen to have probable pathogenic

bacteria consisting of *Escherichia-shigella* with significant differences (p=0.004, FDR=0.040). Specifically in this study, a pro-inflammatory strain like *Escherichia-shigella* genus was shown to be elevated in patients with T2DM, with or without *Blastocystis*. The *Escherichia-shigella* genus was once thought to be an opportunistic pathogen that could cause inflammation in the intestines to varying degrees and eventually trigger the host immune response, increasing the risk of type 2 diabetes (T2DM). This pro-inflammatory component production included the production of lipopolysaccharides (LPS) and peptidoglycans (PGN) (X. Zhao et al., 2020). Therefore, the *Escherichia-shigella* genus was increased in T2DM participants compared to NonDM participants, regardless of *Blastocystis* presence. In the NonDM group, *Escherichia-shigella* was more abundant in the presence of *Blastocystis*. However, there was no significant correlation found in this study between Proteobacteria as well as *Escherichia-shigella* genus with IL1-β and IL-6.

Commonly, colonisation with *Blastocystis* has been observed to be associated with both healthy gut composition and dysbiosis in this present study. This is due to the alteration of gut microbiota being associated with *Blastocystis* subtypes, which explains the conflicting reports on clinical significance (Alzate et al., 2020). In this study, ST3 was the most prevalent in the T2DM compared to other STs. Regarding *Blastocystis* subtypes, this study revealed that *Blastocystis* ST1 and ST3 were significantly associated with a less predominant phyla gut, including Lentisphaerae, Euryarchaeota and Tenericutes compared with non-colonised (NC) group. While *Collinsella* and *Dorea* genera were observed to have an increased trend in ST2 and ST3 infection, respectively, but not significant. In contrast, other studies showed *Blastocystis* ST1, ST2 and ST3 were strongly negatively associated with bacterial species belonging to the

Actinobacteria phylum, particularly from the *Actinomyces*, *Bifidobacterium* and *Collinsella* genera (Mayneris-Perxachs et al., 2022). Actinobacteria and Proteobacteria phyla had also been observed to have a decreased trend in the presence of *Blastocystis* ST3 but not significant. It was evident that the most abundant relative phyla were Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria for all ST infections, which agrees with previously reported findings (Audebert et al., 2016; Castañeda et al., 2020; Gabrielli et al., 2020). However, this current study did not find any significant differences in these dominant phyla including Firmicutes and Bacteroidetes, across individuals colonised by *Blastocystis* subtypes.

Blastocystis ST1 and ST3 are very common subtypes that have been suggested to promote beneficial bacteria such as *Bacillota* (Firmicutes), *Bacteroidata* (Bacteroidetes), *Bifidobacterium* (Actinobacteria) and *Lactobacillus* (Firmicutes) which indirectly influence to the host immune response (Behboud et al. 2022; Deng et al., 2023). ST3 was linked to a eubiotic state characterised by higher bacterial diversity and beneficial bacteria from the phyla Firmicutes and Bacteroidetes, such as those of the genera *Ruminococcus* and *Prevotella*, respectively while a high proportion of *Bacteroides* found in *Blastocystis*-free subjects (Simona Gabrielli et al., 2020; Yañez et al., 2021). An increased trend of the *Dorea* genus belonging to the Firmicutes phylum was observed in ST3 compared to other STs in this present study. Dorea has been shown to play a crucial part in protective action in the gut, boosting SCFAs and consequently elevating fatty acid oxidation in germ-free mice (Juárez-Fernández et al., 2023).

In an experimental study, Deng and colleagues (2023) reported that *Blastocystis* ST1 colonisation increased the abundance of beneficial bacteria *Alloprevotella* and *Akkermansia* and induced protective immune responses in normal, healthy mice. ST1-

colonised mice also showed decreases in the severity of Dextran sulphate sodium (DSS)-induces colitis when compared to non-colonised mice. The production of SCFA also increased with the abundance of *Alloprevotella* and *Akkermansia* in the ST1-colonised mice. While a study in Taiwan reported that *Akkermansia Muciniphilia* was higher in all *Blastocystis* groups (ST1, ST3 & ST4) compared to non-colonised group (Huang et al., 2023). Interestingly, this present finding also found that ST1 has an increasing trend in Verrucomicrobia phylum which comprises of genus *Akkermansia*. However, this study did not find any significance after FDR adjustment. However, this finding in agreement with previous studies indicated that *Akkermansia* belonging to Verrucomicrobia phylum potentially can be elevated with *Blastocystis* colonisation.

Regarding *Blastocystis* ST4, the present study did not observe any significant findings with gut phylum and genus. Numerous studies reported that ST4 had been associated with a healthy gut composition such as *Akkermansia* and *Methanobrevibacter* in healthy individuals (Tito et al., 2019; Yañez et al., 2021). These genera may potentially degrade intestinal mucin which is associated with weight loss and may play an important role in carbohydrate digestion that can protect from the weight gain (Le Chatelier et al., 2013). In the experimental study conducted by Deng and co-workers (2022), they observed that *Blastocystis* ST4 colonisation can maintain the stability of bacterial community composition and induce an immune response that facilitates faster recovery from experimentally induced colitis mice. Plus, it could increase the SCFA production and anti-inflammatory cytokines IL-10 while increasing the gut *Clostridiales* taxa and depleting *Bacteroidales*. Thus, ST4 has been identified as having a commensal strain of *Blastocystis* that is increasing in bacterial richness in individuals (Feranmi, 2022).

However, ST7 was only observed in T2DM to be significantly associated with an increase in pathogenic bacteria like *Escherichia-Shigella* belonging to the Proteobacteria phylum in the present study. *Bacteroides* genus belonging to Bacteroidetes phylum and *Bifidobacterium* belonging to Actinobacteria phylum were also decreased in the presence of *Blastocystis* ST7. This finding was concordant with a previous study reported that diarrheal patients infected with *Blastocystis* ST7 exhibited a higher proportion of *Escherichia-Shigella* and a lower abundance of *Bacteroides* and Parabacteroides in patients (Deng, Lee, et al., 2022). In obese individuals, the proportion of Firmicutes and Proteobacteria increased compared to Bacteroidetes which indicated that an inflammatory environment was present (Hand et al. 2016). In an experimental murine model, Yason et al., (2019) found that a decrease in the beneficial bacteria of the genera *Lactobacillus* and *Bifidobacterium* was associated with the presence of *Blastocystis* ST7. Some roles of *Bifidobacterium* are to maintain the epithelial barrier and to employ anti-inflammatory properties that can reduce the production of pro-inflammatory cytokines such as IL-6 and tumour necrosis alpha (TNF-α) (Ling et al., 2016). *Blastocystis* ST7 had a selective influence on specific bacterial populations. The growth of *Escherichia coli* (facultative anaerobe) was enhanced while inhibiting *Bifidobacterium longum* (an obligate anaerobe) and Lactobacillus sp. which can lead to dysbiosis (Deng et al., 2021). In addition, the *Escherichia-Shigella* belonging to phylum Proteobacteria and the family *Entrerobacteriaceae* have been correlated positively with gastrointestinal and systemic inflammation, commonly in IBD patients (J. Liu et al., 2021; Zhai et al., 2019).

The pathogenicity of *Blastocystis* ST7 has been determined both in *vivo* and in *vitro* experiments, which reported its impact on immune inflammatory response in the

gut microbiome. Parasites like *Blastocystis* produce cysteine proteases that reduce the zonulin protein and cause the reformation of F-actin, a structural protein cell. As a result, *Blastocystis* subtypes particularly ST7 caused a significant increase in intestinal permeability (Mirza et al., 2012). The proteases can also cleave secretory immunoglobulin A (sIg A), allowing the parasite to evade the host immune system, survive and colonise the intestinal tract. ST7 infection was found to induce the degradation of immunoglobulin A (IgA) and impair the epithelial barrier in colonic epithelial cell lines (Puthia et al., 2005; Yason et al., 2019). Thus, this pathogenic feature of *Blastocystis* appears to stimulate the secretion of pro-inflammatory cytokines (Karamati et al., 2021). Recent studies have observed that *Blastocystis* ST7 activates protein kinases (MAPKs) to increase the expression of proinflammatory cytokines such as IL-6, IL1-β and TNF-α. Then, it boosted the effect of lipopolysaccharide (LPS) on the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Lim et al., 2014; Teo et al., 2014).

Meanwhile, *Blastocystis* ST1 and ST2 were also associated with IL-6 and IL1-β in the present study. These findings are consistent with past study highlighted an increasing concentration of the cytokines IL- 6 and TNF-α in patients with IBS that infected with *Blastocystis* subtypes ST1, ST2, and ST3 as evaluated by serum enzyme-linked immunosorbent assays (Azizian et al., 2016). In IBS patients, *Blastocystis* ST1 and ST3 were associated with IL-12 and IL-8, resulting in a low goblet cell depletion and pro-inflammation response (Yakoob et al., 2014). A low goblet cell depletion in both case and control groups in this study may be explained by the replacement of Th2 reactions with proinflammatory response. The low IL-12 levels in coinfection with *Blastocystis* may be due to the immune modifying effect. This revealed that the

Blastocystis antigen-activated the humoral immune response in peripheral blood mononuclear cells (PBMCs), which could result in an inflammatory reaction and cell proliferation to confront the infection (Chandramathi et al., 2014). The expression of ST2 in IL-6 was observed to be significantly increased among symptomatic subjects after 24 hours of incubation. However, the protease activity of symptomatic isolates of ST1, ST3 and ST6 was significantly higher than ST2 (Karamati et al., 2021). Proteases released by *Blastocystis* are reported to provoke the immune response by activation of T cells, monocytes or macrophages and natural killer (NK) cells. These activations may produce pro-inflammatory cytokines such as IL-6, IL1-β and TNF-α (P. Gurung & Kanneganti, 2016). A recent *in vitro* study showed that *Blastocystis* infection may be associated with modifications of the resulting microbial pathway since significant changes in metabolic activities were detected. *Blastocystis* also has gut immunomodulatory properties and can produce proteases that affect integrity (Rajamanikam et al., 2023).

These contrasting findings suggest that different *Blastocystis* subtypes may have a distinct relationship with specific bacterial groups in the gut microbiota. The association of altered gut composition like *Escherichia-Shigella* belonging to Proteobacteria was observed specifically in colonisation of *Blastocystis* ST7 in T2DM, which potentially leads to dysbiosis. While certain *Blastocystis* subtypes like ST3, could contribute to the development of a healthy gut. The pro-inflammation (IL-6 and IL1-β) may serve as indicators in dysbiosis of T2DM as well as the infection from *Blastocystis* subtypes. Thus, further interventional studies are encouraged to specify the role of *Blastocystis* subtypes in the gut microbiota particularly in T2DM disease

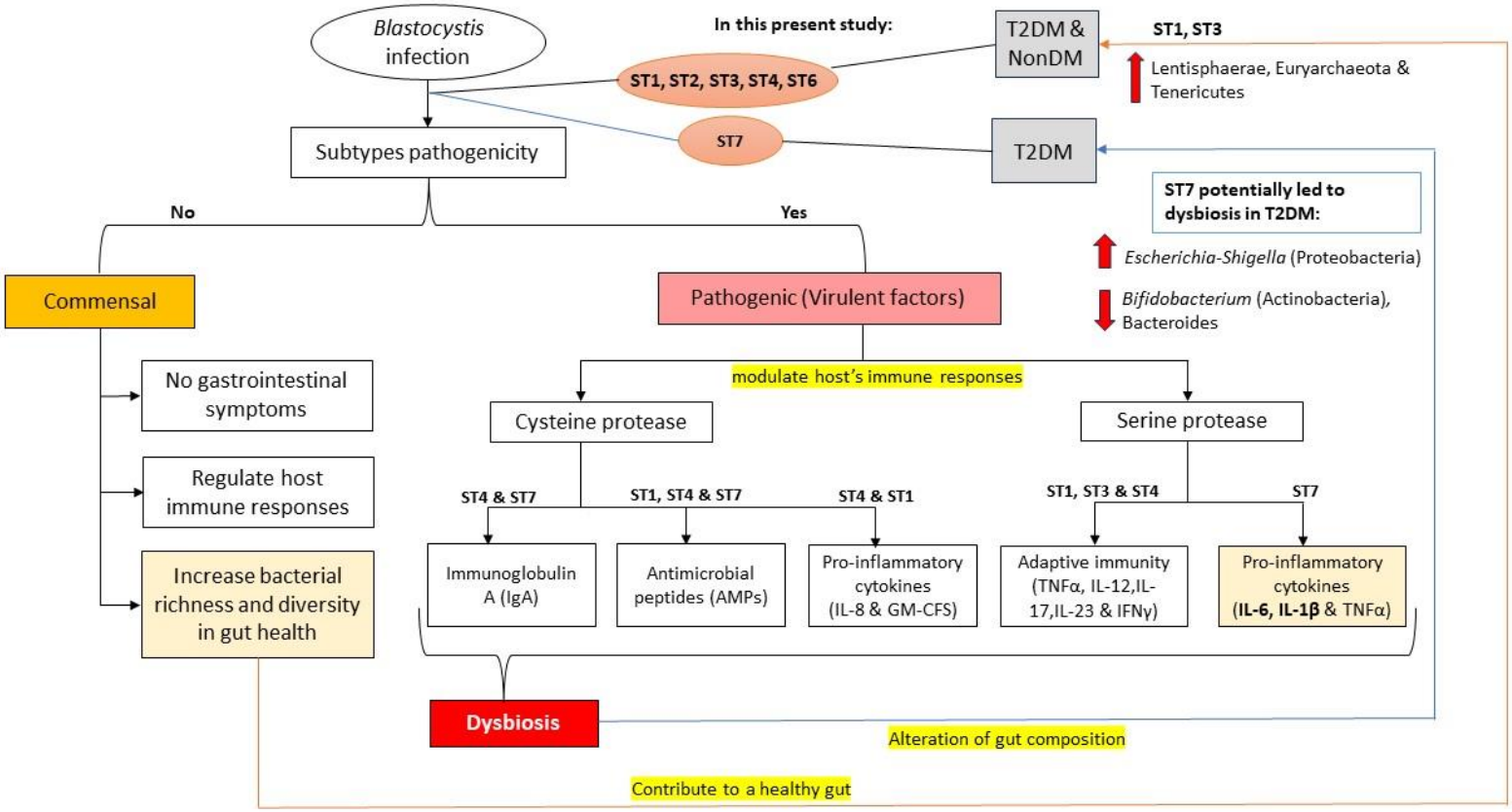

Figure 5.1: Concept Map Integrating the Commensal and Pathogenicity aspects of *Blastocystis* from Various Studies and Current Research Findings