

## CHAPTER 5

### DISCUSSION

#### 5.1 Introduction

This chapter discusses the findings of this study. Firstly, gut microbiota composition in different ethnic groups in nonDM were discussed. This is followed by a discussion of the diversity and gut microbiota composition between nonDM and T2DM groups by ethnicity as well as the associations of gut microbiota with the clinical characteristics of all study participants are discussed. Lastly, the systematic review of available literature on the gut microbiota composition in preDM and newDM is discussed.

#### 5.2 Discussion of Study Results

This is the first study to have analysed the gut microbiota composition of nonDM and T2DM individuals from the three main ethnic groups (Malay, Chinese and Indian) residing in the same geographical region in Malaysia. The T2DM participants in this study had significantly higher age, BMI and FPG in comparison to the nonDM participants. Similar studies have indicated altered clinical characteristics in participants with T2DM when compared with healthy controls, especially with increased age (Lê et al., 2012; Salamon et al., 2018; Wang et al., 2020), BMI and glycaemic profile (FPG and HbA1c) (Ahmad et al., 2020; Doumatey et al., 2020; Fassatoui et al., 2019; Li et al., 2020a; Navab-Moghadam et al., 2017; Salamon et al., 2018; Wang et al., 2020).

### 5.2.1 Gut Microbiota Composition in NonDM

The first aim of this study is to characterize and compare the gut microbiota composition of healthy adults of the three major Malaysian ethnic groups. In alpha diversity analysis, the measure of evenness or distribution of species abundance in a sample using Pielou's evenness index found that all three ethnic groups in nonDM exhibited evenness closer to 1 than to zero. This indicates that the bacterial species found in each ethnic group have similar abundance. Even so, the Chinese ethnic group had a subtly, but significantly lower abundance of bacteria in comparison to Malay and Indian ethnic groups ( $p=0.015$ ). Meanwhile, all measures of beta diversity showed no significant difference. This suggests that a similar gut microbiota structure is seen between ethnic groups in nonDM.

Overall, the dominant phyla found in nonDM participants were *Firmicutes* (73.75%), *Bacteroidetes* (21.17%), *Actinobacteria* (2.35%) and *Proteobacteria* (1.61%). These are the commonly reported four predominant phyla where a stable relative abundance of ~65% *Firmicutes*, ~25% *Bacteroidetes*, ~5% *Actinobacteria* and up to ~8% *Proteobacteria* are generally exhibited in a healthy human gut (Candela et al., 2012). However, past studies reported an inconsistent trend in the relative abundance of these dominant phyla.

In a study among healthy adults (N=193) aged 41-67.6 years from Nigeria (Doumatey et al., 2020), an increased abundance of *Firmicutes* (80.07%) was followed by *Actinobacteria* (14.80%) instead of *Bacteroidetes* (0.30%) while healthy adults (N=15), aged 42 to 69 years from the USA (Lambeth et al., 2015) exhibited an increased abundance of *Bacteroidetes* (55.0%) than *Firmicutes* (38.2%). Meanwhile, one study among healthy adults (N=20) aged 25-55 years, belonging to the Punjabi ethnic group

from Pakistan observed the increased abundance of *Firmicutes* (36.9%) and *Bacteroidetes* (32.1%) was followed by the markedly increased abundance of *Proteobacteria* (15.8%) (Ahmad et al., 2020). This varying gut microbiota abundance and composition found among healthy populations is probably due to host-specific factors, i.e. host ancestry, lifestyle, diet, geographical factors and ethnicity (Gupta et al., 2017). Each factor may shape the gut microbiota profile over time, thus contributing to the inter-individual dissimilarities of gut microbiota abundance and composition in a community (Candela et al., 2012).

In the present study, no significant difference in gut microbiota composition at the phylum and genus levels were found between the three ethnic groups among the nonDM participants. This could be a reflection of the insignificant diversity analyses among ethnic groups in nonDM which proves that healthy adults residing in a similar geographical region shared the same gut microbiota profile. In support of this study's findings, in one Malaysian study, Khine et al. (2019) analysed the gut microbiota composition of healthy children between the age of 7 to 12 years old found in three cities. The Malay children from Penang (N=21) and Kelantan (N=33) were compared with Southern Han Chinese children from Penang (N=21), Kelantan (N=45) and Guangzhou city in China (N=18). It was found that, regardless of ethnicity, children that were living in the same urban city had no significant differences in their gut microbiota composition. This suggests that they could be sharing a similar gut microbiota composition as communities living in a similar region tend to have an overlap in dietary practices (Khine et al., 2019). Hence, dietary patterns seen in a particular region may well be an important determinant factor in shaping the gut microbiota composition of different communities.

Besides that, in another Malaysian study led by Dwiyanto and team analysed the gut microbiota composition of four major ethnic groups (Malay, N=46, Chinese, N=65, Indian, N=49 and Jakun, N=54) residing in sub-districts in Segamat (Dwiyanto et al., 2021). A total of 39 lifestyle factors, categorized into either demographic, dietary behaviour, hygiene practices or health conditions were recorded from the study participants in this study. In the PERMANOVA analysis, ethnicity was found to be significantly associated with the gut microbiota changes and exhibited the largest effect size in comparison to the other factors investigated. This significance remained even after adjusting for demographic covariates (age, BMI, sex, income, occupation) and the inclusion of additional factors, i.e., household earning, good water source and red meat consumption. Meanwhile, when associating demographic, dietary, health or hygiene index with gut microbiota composition, gut-ethnic variation associated with hygiene index was seen between Indian – Malay and Jakun - Indian where Malay, Jakun and Indian ethnic groups each had increased abundance of unclassified *Clostridiales*, *Klebsiella quasipneumoniae* and *Bifidobacterium longum*, respectively. This shows that the significance of ethnicity on gut microbiota composition was likely contributed by unique lifestyle patterns in each ethnic group (Dwiyanto et al., 2021).

In addition, in a study done among Chinese (N=5) and Indian (N=11) ethnic groups, Jain et al. (2018) analysed the gut microbiota composition of healthy university students from China and India, ages 22 to 35 and studying in Singapore for the past 1 to 3 years. While each ethnic group was found to be similarly diverse, the beta diversity indices reveal that both groups have significantly different gut microbiota composition. The dominant phyla found in both groups were *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* where a non-significant increase of *Firmicutes* (68.08%) and *Actinobacteria* (25.48%) was found in Chinese when compared to the

Indians (60.5% and 20.57%, respectively). Significant increases in the abundance of *Bacteroidetes* (16.39%) and *Cyanobacteria* (0.22%) were found among Indians in comparison to the Chinese ethnic group (4.27% and 0.12%, respectively) (Jain et al., 2018). At the genus level, the relative abundance of genera *Prevotella*, *Megasphaera*, *Catenibacterium*, *Lactobacillus*, *Mitsuokella*, *Carnobacterium* and *Lachnospira* were significantly increased while *Bifidobacterium* and *Blautia* were significantly decreased among the Indians when compared to the Chinese ethnic group (Jain et al., 2018). Even though residing in a similar region, the ethnic-specific diet intake among Chinese and Indians was related to the variation of gut bacteria found among both groups (Jain et al., 2018). Similar to the Malaysian and the Singaporean study, the present study also noted a trend towards higher abundances of *Bifidobacterium*, *Prevotella*, *Lactobacillus*, *Megasphaera* and *Catenibacterium* among Indian participants when compared to Chinese (as well as Malay) participants. However, these were not significant findings.

Hence, all three studies by Jain et al.(2018), Khine et al.(2019) and Dwiyanto et al. (2021) had reported varying gut microbiota composition among healthy adults from different ethnic groups residing in a similar geographical region. Moreover, these differences in gut microbiota composition were mostly associated with the diet intake and other lifestyle factors, i.e. age, sex, BMI, income, occupation, health condition, hygiene index of the respective ethnic groups residing in the respective area of study (Chong et al., 2015; Dwiyanto et al., 2021). This could probably be related to the gut microbiota composition of healthy Malay, Chinese and Indian participants in the present study. All study participants recruited were residing in the same urban region under Ampang Jaya municipality in Selangor and likely consumed similar food types with similar environmental exposure or lifestyle practices. Since the lifestyle or other environmental factors of the ethnic groups in this study were not enumerated (as these

were beyond the scope of the present study), no link between these factors with gut microbiota changes could be established. Furthermore, the sample size is a limitation of this study. This could be another possible reason for the differential abundance being insignificant between the ethnic groups, although a higher or lower trend was noted. Thus, further studies with larger sample sizes are needed to understand the role of ethnic-associated environmental factors, including diet and geography on gut microbiota composition among healthy individuals in different ethnic groups in Malaysia.

### **5.2.2 Gut Microbiota Composition in T2DM when compared to NonDM**

The gut microbiota composition of T2DM participants was found to be altered when compared to the nonDM groups. Firstly, alpha diversity was significantly decreased in the T2DM group when compared to the nonDM group. This was also noted specifically among the Malay and Indian T2DM groups in comparison to their respective nonDM healthy controls. A decrease in alpha diversity i.e. a decrease in the diversity of gut microbiota among the disease groups generally indicates an underlying disease state (Manor et al., 2020) while the increasing severity of gut microbiota dysbiosis has been associated with disease severity and development of complications (Iatcu et al., 2021). Studies on gut microbiota in nonalcoholic fatty liver disease (Mouzaki et al., 2013), cardiovascular diseases (Jie et al., 2017), inflammatory bowel disease (Joossens et al., 2011; Morgan et al., 2012) and colorectal cancer (Chen et al., 2012; Wang et al., 2012a) have all reported a less diverse gut microbial composition in the disease groups in comparison to healthy controls. Secondly, a significant association of beta diversity with diabetes status noted in this study suggests that the gut microbiota

composition between nonDM and T2DM groups is influenced by the diabetes status of the study participants. Moreover, the PCoA analysis revealed a clear separation of gut bacteria found between nonDM and T2DM groups indicating a difference in gut microbiota structure in both groups. These findings were reflected among nonDM and T2DM of Malay and Indian ethnic groups, suggesting that both ethnic groups with T2DM have a different gut microbiota structure compared to their respective healthy nonDM groups.

The dominant phyla found among nonDM and T2DM participants were *Firmicutes* (73.75%, 67.97%), *Bacteroidetes* (21.17%, 22.02%), *Proteobacteria* (1.61%, 7.32%) and *Actinobacteria* (2.35%, 1.83%). This study found that the significant increase in phylum *Proteobacteria* among participants with T2DM was primarily represented by an increase in genus *Escherichia-Shigella*. Also, although present at a low detection level, the phylum *Synergistetes* was found to be significantly increased in terms of both prevalence as well as abundance in T2DM participants.

*Proteobacteria* is a bacterial phylum consisting of mainly pathogenic members including *Escherichia* and *Shigella* which are often found to be increased in disease (Rizzatti et al., 2017). Past studies have also observed an increased abundance of Gram-negative *Proteobacteria* among T2DM participants. In a study conducted in China, Wang et al. (2020) found that T2DM participants (N=134) had an increased abundance of *Proteobacteria* along with increased *Actinobacteria* and F/B ratio as well as a decreased abundance of *Bacteroidetes* than the control group (N=37). They also reported that at the genus level, genera *Escherichia-Shigella* (phylum *Proteobacteria*), *Bifidobacteria* (phylum *Actinobacteria*), *Faecalibacterium* and *Lactobacillus* (both of phylum *Firmicutes*) were increased while genera *Prevotella* (phylum *Bacteroidetes*)

and *Roseburia* (phylum *Firmicutes*) were decreased among T2DM participants in comparison to the control groups (Wang et al., 2020). In another study, Pushpanathan and their team from India found that when T2DM participants (N=17) were compared with nonDMs (N=13), an increase in genera *Escherichia* and *Lactobacillus* was found (Pushpanathan et al., 2016). The same study also reported that the abundances of genera belonging to phylum *Firmicutes*, namely *Faecalibacterium*, *Eubacterium* and *Clostridium* as well as *Bifidobacterium* of phylum *Actinobacteria* in T2DM participants were decreased than the nonDM group (Pushpanathan et al., 2016). These findings are comparable to the present study whereby T2DM participants had a higher trend of F/B ratio and genus *Lactobacillus* as well as a lower trend of genera *Faecalibacterium*, *Eubacterium*, *Roseburia* and *Bifidobacterium*, although these were all statistically insignificant.

In studies on germ-free mice and human subjects, an increased abundance of Gram-negative bacteria, especially those belonging to the phylum *Proteobacteria* resulted in the occurrence of metabolic endotoxemia that precedes the development of obesity and insulin resistance (Magne et al., 2020). Metabolic endotoxemia is characterised by increased levels of lipopolysaccharide (LPS), an endotoxin found in the outer membranes of Gram-negative bacteria (Vallianou et al., 2018). LPS influences the signalling pathway via Toll-like receptor 4 (TLR4) on macrophages and triggers the innate immune response to increase secretion of pro-inflammatory molecules, i.e. TNF- $\alpha$ , IL-1 and IL-6 in the body (Mohammad et al., 2021). The pro-inflammatory cytokine, i.e. TNF- $\alpha$  was found to mediate insulin resistance by deactivating the insulin receptors in muscle and fat tissues and impairing  $\beta$ -cell function (Cani et al., 2007). Hence, measuring the levels of pro-inflammatory markers such LPS, LBP, CRP and interleukins could determine the occurrence of metabolic endotoxemia with diabetes in

body. Both CRP and LBP are acute-phase proteins synthesized in the liver and are used as inflammatory markers (Gomes et al., 2017; Wang et al., 2012b). This is an indication that metabolic endotoxemia-induced inflammation directly causes insulin resistance.

Furthermore, the increase in the genus *Escherichia-Shigella* is considered an important cause of mortality and morbidity in T2DM as it directly increases invasive infections among diabetics (Graff et al., 2002). This is probably due to lower cellular immune function seen with increased blood glucose levels in T2DM patients which exacerbate microbial pathogenicity that contributes to systemic infections (Madacki-Todorović et al., 2018). Moreover, the increased risk of complications due to *Escherichia* and other *Enterobacteriaceae* have been observed among participants with T2DM (Thomsen et al., 2005).

Although the LPS from Gram-negative bacteria has been related to the occurrence of metabolic endotoxemia, recent evidence has found that a distinct type of LPS is produced by different Gram-negative bacteria (Anhê et al., 2021). The pro-inflammatory subtype of LPS induced by *Proteobacteria* has higher endotoxic activity than that of the LPS induced by the most abundant group of Gram-negative in the gut, *Bacteroidetes* that has immunoinhibitory functions and prevents inflammation (d'Hennezel et al., 2017). Being a subdominant group of bacteria in the gut, the increase in the abundance of *Proteobacteria* could be a result of the decrease in either of the two most dominant gut bacterial phyla, *Firmicutes* or *Bacteroidetes*, thus increasing the production of pro-inflammatory subtype of LPS (Magne et al., 2020). Moreover, gut dysbiosis seen with the alterations to the abundance of the *Firmicutes* and *Bacteroidetes*, as well as the increase or decrease of the F/B ratio are often linked with several diseases. Few studies have related the increased abundance of *Firmicutes* or an

increased F/B ratio with the development of obesity (Magne et al., 2020). This is because, in comparison to *Bacteroidetes*, *Firmicutes* are more efficient in harvesting energy from food that will be absorbed in the gut circulation into the body, eventually increasing the body calories intake resulting in obesity (Magne et al., 2020). Obesity, a significant risk factor for T2DM, is also characterised by the appearance of low-grade inflammation that is seen present before the onset of insulin resistance (Magne et al., 2020). Therefore, it could be said that an increase in the abundance of *Proteobacteria* could serve as an important indicator of gut dysbiosis that precedes the occurrence of low-grade inflammation, a characteristic feature found in obesity and T2DM.

The significant increase in prevalence and relative abundance of phylum *Synergistetes* found among T2DM participants in this study agrees with past studies. In a study in the USA, Lambeth et al. (2016) found a significant increase of phylum *Synergistetes* among T2DM participants (N=14) in comparison to the healthy controls (N=15) that was lost after adjustment with FDR. However, in another USA study, Maskarinec et al. (2021) found that the increased abundance of *Synergistetes* among T2DM participants (N=307) in comparison to the controls (N=735) was inversely related with T2DM status. One study by Nuli et al. (2019) in China found that out of 15 phyla prevalent among the participants with T2DM (N=20), impaired glucose tolerance (N=20) and normal glucose tolerance (N=20), phylum *Synergistetes* was one of the minor phyla that positively correlated with FPG and the intake of cholesterol, vitamin (nicotinic acid) and mineral (selenium). In another study by Das et al. (2021) in India, the abundance of phylum *Synergistetes* was slightly increased in both T2DM participants with (N=28) and without (N=25) diabetic retinopathy in comparison to the healthy controls (N=30). Although consistently found among T2DM participants and

had been related to glycaemic parameters as well as T2DM status, the relevance of this low-abundant bacteria to T2DM development is still unclear.

On the other hand, T2DM participants in this study had a significantly decreased relative abundance of the genus *Fusicatenibacter*. Also, the significantly decreased prevalence and relative abundance of genus *Anaerostipes* as well as the decreased prevalence of genus *Clostridium* were noted in T2DM participants. The genera *Fusicatenibacter*, *Anaerostipes* and *Clostridium* belong to phylum *Firmicutes* and are known SCFA producers in the colon (Rivière et al., 2016; Takada et al., 2013). Short-chain fatty acids (SCFA) such as butyrate, acetate and propionate are metabolic products of microbial fibre fermentation in the gut and are found to exert beneficial effects on human metabolism and the immune system (Lin et al., 2012). Traditionally, SCFAs from biological samples could be quantified by gas chromatography (Kim et al., 2022; Zhao et al., 2006; Zheng et al., 2013) and recently, an isotope-based strategy for absolute quantification of SCFA with reverse-phase liquid chromatography mass spectrometry was developed (Bihan et al., 2022).

Most butyrate-producers belong to phylum *Firmicutes* and protect against inflammation by reducing pro-inflammatory cytokines production, maintaining the gut epithelium integrity as well as serving an important role in blood glucose and lipid regulation (Lin et al., 2012). Furthermore, the decreased abundance of butyrate-producing bacteria are observed in various studies on T2DM (Doumatey et al., 2020; Larsen et al., 2010; Navab-Moghadam et al., 2017; Qin et al., 2012). There is evidence that SCFAs butyrate, propionate and acetate protect against diet-induced obesity and improve metabolic functions in T2DM (Lin et al., 2012). Remely et al. (2014) suggested that gut microbiota dysbiosis alters SCFA production with different signalling

pathways. Consequently, this affects the epigenetic regulation of genes modulating insulin resistance and inflammatory reactions seen in T2DM .

Past studies had also reported a decrease in genera *Anaerostipes* and *Clostridium* in T2DM. In studies by Doumatey et al. (2020) in Nigeria, Salamon et al. (2018) in Poland and Diener et al. (2021) in Mexico, genus *Anaerostipes* was noted to be significantly decreased among T2DM participants (N=98, N=23 and N=65) in comparison to their respective healthy controls (N=193, N=23 and N=214, respectively). The study by Diener and team also found that the gradual decrease in abundance of genus *Anaerostipes* from healthy controls to individuals with varying glucose intolerance and fully developed T2DM shows that this genus decreases with T2DM progression (Diener et al., 2021). The same study also associated the higher abundance of genus *Anaerostipes* with improved beta cell function and insulin efficiency (Diener et al., 2021). In another study, the *Anaerostipes sp.*, i.e. *Anaerostipes hadrus* which inversely correlated with BMI and blood glucose levels was found to also have a region that encodes a butyrate biosynthesis pathway-the composite inositol catabolism which is linked to the reduction of metabolic disease risk in a host (Zeevi et al., 2019).

Meanwhile, *Clostridium* is one of the dominant bacteria under phylum *Firmicutes* and represents a substantial number of butyrate-producing bacteria from *Clostridial clusters IV* (also known as *Clostridium leptum* group) and *XIVa* (also known as *Clostridium coccoides* group) (Eckburg et al., 2005). Recent findings showed that the area between the mucosal fold in the colon is majorly occupied by strict anaerobes from *Clostridium Cluster IV* and *XIVa* (Lopetuso et al., 2013). This specific position in the colon helps this *Clostridial* subphylum to interact closely with gut cells, influence

intestine structure and carry out vital metabolic functions such as the release of butyrate to provide energy for colonocytes (Lopetuso et al., 2013). *Clostridium sp.* are also necessary to maintain gut barrier integrity by fighting against pathogenic organisms to resist colonization. The bacteria from *Clostridium Cluster IV* and *XIVa* are mostly flagellated and protect the host guts against invading pathogens (Kelly et al., 2005). In a study by Afolayan et al. (2020) in Nigeria, the decreased prevalence of genus *Clostridium* was found in the gut microbiota of T2DM participants (N=20) than in the healthy urban elderly participants (N=22). This is supported by Pushpanathan et al. (2016) from India that also noted a decrease in the prevalence of genus *Clostridium* among T2DM participants (N=17) in comparison to the healthy controls (N=13). Hence, it is evident that the butyrate-producing *Clostridium* are commonly decreased in the diabetic gut.

The genus *Fusicatenibacter* found decreased in this study is a relatively new genus classified under the family *Lachnospiraceae* in 2013 (Takada et al., 2013). The abundance of the only species found under this genus, *Fusicatenibacter saccharivorans* was decreased with ulcerative colitis in both humans (Gryaznova et al., 2021) and mice (Maslowski et al., 2009; Takeshita et al., 2016). The increased abundance of this bacterial species exerts an anti-inflammatory effect which reduces inflammatory responses (Takeshita et al., 2016). However, its biological significance in T2DM is yet to be known.

In addition, gut-ethnic variation was found among ethnic groups with T2DM in this study. The significant increase in *Proteobacteria* was reflected in Chinese and Indian ethnic groups with T2DM. Past studies that investigated the gut microbiota composition of different ethnic groups with T2DM had also reported varying gut microbiota alterations in comparison to their respective healthy controls.

In a study conducted in China, the gut microbiota composition of T2DM and healthy nonDM participants from Chinese minority ethnic groups, Uygurs and Kazaks were analysed (Wang et al., 2017). An increase in *Proteobacteria* and a decrease in *Actinobacteria* were found among Kazak T2DM in comparison to the healthy Kazak group. Meanwhile, only an increased abundance of phylum *Firmicutes* represented by the family *Erysipelotrichaceae* was found among Uygur T2DM when compared to the healthy Uygurs (Wang et al., 2017). In another study among the Punjabi population with T2DM in Pakistan, a decreased abundance of genera *Escherichia-Shigella*, *Bacteroides*, *Prevotella*, *Bacillus* and *Ruminococcus* were found in comparison to the nonDM groups (Ahmad et al., 2020). The study also noted an increased abundance of genera *Lactobacillus*, *Subdoligranulum* and *Eubacterium* along with increased phylum *Actinobacteria* represented by class *Coriobacteriia* in T2DM (Ahmad et al., 2020). Meanwhile, in a study conducted in the Netherlands, the gut microbiota composition of healthy and T2DM participants from African-Surinamese and South-Asian Surinamese ethnicity residing in a similar geographical region was determined (Balvers et al., 2021). Likewise other studies of T2DM, both the ethnic groups with T2DM had a significant increase in *Escherichia-Shigella* as well as decreased abundance of *Peptostreptococcaceae* and *Clostridium* in comparison to their respective control groups. Meanwhile, *Anderostipes hadrus* was only prevalent in African Surinamese, while the prevalence of bacteria belonging to families *Lachnospiraceae*, *Lactobacillaceae*, *Christensenellaceae*, *Erysipelotrichaceae* and *Ruminococcaceae* were unique to South-Asian Surinamese (Balvers et al., 2021). The varying gut microbiota composition seen across these studies could be due to ethnic-specific differences in genetic diversity, culture, diet, socioeconomic variation and geography (Brooks et al., 2018; Gupta et al., 2017). Thus, ethnicity could be potentially considered

a major factor when analysing the gut microbiota composition of different ethnic groups in health and disease.

In the present study, the specific bacterial genera found to be significantly altered in T2DM generally in terms of prevalence or abundance and further noted to be significantly altered in T2DM by ethnicity were *Escherichia-Shigella* (increased in Malay and Indian T2DM), *Fusicatenibacter* (decreased in Indian T2DM), *Anaerostipes* (decreased in Chinese and Indian T2DM) and *Clostridium* (increased in Malay T2DM). Besides these, the abundance of genus *Dorea* was also found to be decreased in Malay T2DM and genus *Bifidobacterium* in Chinese T2DM. Studies by Almagadam et al. (2020) in Sudan and Salamon et al. (2018) in Poland had reported increased abundance of genus *Dorea* among T2DM participants. However, Que et al. (2021) in China found that this Gram-positive bacterium of phylum *Firmicutes* was decreased in T2DM and they suggested it to be an important gut microbial signature to predict T2DM. On the other hand, as reviewed by Gurung et al. (2020), genus *Bifidobacterium*, a Gram-positive bacterium under phylum *Actinobacteria* appears to be consistently reduced among T2DM patients. This bacterial genus produces SCFA, namely acetate and lactate during carbohydrate fermentation which suggests it has a protective role in T2DM (Parada Venegas et al., 2019).

Meanwhile, although increasing or decreasing trends of other specific bacterial genera were noted by ethnicity, these alterations were not significant, most likely due to the small sample size comparing the three ethnic groups (each of Malay, Chinese and Indian ethnicity, N=15). For instance, in comparison to the nonDMs, all ethnic groups with T2DM exhibited a higher trend of *Lactobacillus* abundance. *Lactobacillus* are common gut inhabitants and the abundance of this genus has been associated with

several inflammatory diseases including T2DM (Candela et al., 2016; Qin et al., 2012). Furthermore, the increased abundance of this genus is often found among T2DM participants (Gurung et al., 2020). Besides *Lactobacillus*, in comparison to their nonDM groups, a higher trend of *Bacteroides* and *Clostridium* were observed among Malay and Indian T2DM whereas Chinese T2DM had a higher trend of *Subdoligranulum* and *Eubacterium* while Indian T2DM had a higher trend of *Ruminococcus*. In both Malay and Chinese groups, the higher trend of *Prevotella* was found in comparison to the Malay and Chinese nonDM groups. The small sample size could have resulted in a lower statistical power in analysing the differences in gut microbiota composition between the ethnic groups with T2DM and the healthy nonDMs. Hence, a larger cohort of adults is needed to identify if there are reproducible differences concordant with this study's findings across major ethnic groups in Malaysia.

### 5.2.3 Correlation Analysis

The gut bacteria found to be significant in the present study were also tested for correlation with the clinical characteristics (anthropometric, demographic, diabetic profile and biochemical parameters) of all study participants.

The increased abundance of *Proteobacteria* and *Escherichia-Shigella* seen among T2DM participants in this study correlated positively with age. Most of the T2DM patients attending the diabetes clinic in Ampang Health Clinic were over the age of 50, which is common as T2DM is usually diagnosed in middle-aged patients (Diabetes Atlas, 2019). Moreover, the prevalence of diabetes is higher among Malaysian adults of 60 years or older than among adults in the 20 to 29-years age group (Akhtar et al., 2022). Past studies observed that along with age-related modifications in

diet and lifestyle, the increased immunosenescence, frailty, metabolic syndrome, diabetes and sarcopenia seen with ageing could be related to the changes in gut microbiota composition and the occurrence of inflammatory processes (Biagi et al., 2012). Moreover, in a few studies, the increased abundance of *Proteobacteria* found among older T2DM participants in comparison to the control groups was not associated with age (Salamon et al., 2018; Wang et al., 2020). Thus, no conclusive evidence showed age as an independent factor affecting the gut microbiota composition (Biagi et al., 2012).

On the other hand, BMI was correlated positively with *Escherichia-Shigella* as well as negative correlations with the genera found to be in lower abundance among T2DM participants in this study, *Fusicatenibacter* and *Anaerostipes*. In this study, the T2DM Indian and Malay had a BMI greater than 27.5kg/m<sup>2</sup> which is the obese category in the Asian-Pacific classification of BMI (WHO Expert Consultation, 2004). However, when comparing the three ethnic groups with T2DM, the BMI among Indians was found to be significantly increased. Similarly, past studies have reported ethnic-specific differences in BMI whereby Indians with T2DM tend to have the highest BMI and increased prevalence of obesity, followed by Malay and Chinese ethnic groups (Abdullah et al., 2014). Obesity is a known risk factor for T2DM. Obesity leads to a state of insulin resistance seen in T2DM (Abdullah et al., 2014). The rising prevalence of obesity as indicated by increased BMI in parallel to the rising incidence of T2DM is seen in Asian countries, including Malaysia. This could be due to differences in diet, physical inactivity and sedentary lifestyle found among different populations (Abdullah et al., 2014; Institute for Public Health, 2015). Hence, the abundance of gut bacteria

seen to correlate with BMI could be important indicators of obesity and T2DM progression.

Furthermore, other clinical characteristics, i.e., FPG, ALP, ALT, urea, and TG seen to be altered among T2DM participants in this study were seen to be positively correlated with *Proteobacteria* while genus *Escherichia-Shigella* specifically with urea and TG. Also, genus *Fusicatenibacter* correlated negatively with TG while both FPG and TG correlated negatively with genera *Anaerostipes* and *Clostridium*. Additionally, genus *Clostridium* correlated positively with HDL.

Other similar studies also found increased FPG and TG (Ahmad et al., 2020; Wang et al., 2020) as well as decreased HDL (Doumatey et al., 2020; Li et al., 2020a; Wang et al., 2020) among T2DM participants. In the present study, when comparing the T2DM group by ethnicity, significantly increased levels of ALT were found in T2DM Malays. There were higher levels of HDL/TC ratio in T2DM Chinese in comparison to T2DM Malay and Indian. On the other hand, when comparing the nonDM group by ethnicity, higher levels of both HDL and HDL/TC ratio were found in nonDM Chinese as well as higher albumin and lower ALP in nonDM Malay.

Alterations in lipid profile, especially a change in TG, LDL and HDL levels seen in T2DM are a sign of abnormal lipid metabolism and a major risk factor for cardiovascular diseases (CVD) (Vergès, 2009). On the other hand, the level of HDL is a strong predictor of CVD than the TC, LDL or TG levels (Kostapanos et al., 2014). Low levels of HDL are found in insulin-resistant states and T2DM is associated with high levels of TG and obesity (Razak et al., 2005). The TG-rich HDL increases the activity of hepatic lipase, a lipolytic enzyme responsible for HDL catabolism, thus reducing HDL levels in T2DM (Vergès, 2009). Conversely, a high level of HDL is

believed to be beneficial and protects against the occurrence of CVD due to its anti-oxidative, anti-inflammatory, anti-thrombotic, anti-apoptotic and endothelium-dependent vasorelaxant properties (Vergès, 2009). Meanwhile, higher levels of hepatic enzymes, i.e. ALT found in T2DM participants are associated with increasing levels of BMI, and poor diabetic control contributed by higher FPG (Harris, 2005). This is likely due to a condition called fatty liver affecting the liver, resulting in increased ALT levels (Mouzaki et al., 2013).

Thus, the association of altered clinical characteristics found among participants with T2DM, in particular the ethnic-specific differences with the abundance of genera *Proteobacteria*, *Escherichia-Shigella*, *Fusicatenibacter* and *Anaerostipes* as well as the prevalence of genus *Clostridium* may serve as important indicators of gut dysbiosis seen in T2DM. Further interventional studies are needed to understand the specific role of each of these genera to serve as biomarkers in monitoring T2DM progression.

### 5.3 Systematic Review

This study aimed to systematically review the available literature reporting findings on the gut microbiota composition in preDM and newDM participants when compared to nonDM participants. This is hoped to highlight the changes occurring at earlier stages of T2DM development before beginning anti-diabetic treatment.

Overall, the dominant phyla found among preDM and newDM in comparison to the nonDM group were *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. Four studies found that the newDM participants had an increased abundance of *Firmicutes* along with a decreased abundance of *Bacteroidetes* (Bhute et al., 2017; Gaïke et al., 2020; Nuli et al., 2019; Zhao et al., 2019). Meanwhile, only three (Gaïke et al., 2020; Li et al., 2020a; Zhao et al., 2019) out of 18 studies reported an increased F/B ratio among participants in preDM and newDM groups. In diversity analyses, most studies found a lower alpha diversity as well as significantly different beta diversity between the disease groups and healthy controls.

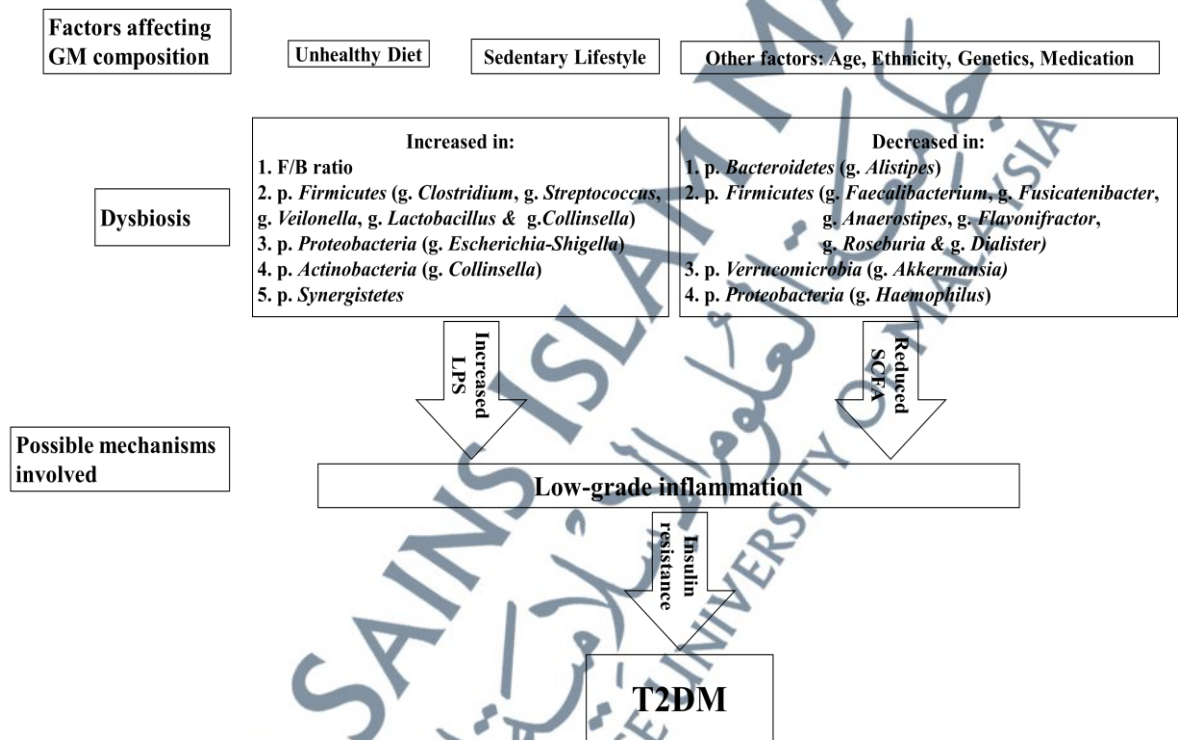
The gut microbiota composition among participants with preDM and newDM was also significantly altered, however the findings were not consistent across the 18 studies. Hence, this review focused on observations similar in two or more studies. In this way, genera *Faecalibacterium prausnitzii*, *Roseburia*, *Dialister* and *Flavonifractor* of phylum *Firmicutes* that has established role as biomarkers of health by producing SCFAs, especially butyrate were found to be depleted in the disease groups (Martín et al., 2018; Mukherjee et al., 2020; Tamanai-Shacoori et al., 2017). Meanwhile, the disease groups also observed a decrease in genus *Alistipes* of phylum *Bacteroidetes* which was found to have protective effects against certain diseases, including CVD, while also being pathogenic in others due to its inflammatory potential (Parker et al., 2020). Also, the genus *Akkermansia muciniphila* of phylum *Verrucomicrobia*, a mucin-

degrading bacteria that protects the gut barrier (Cani et al., 2008) was seen to decrease among preDM and newDM participants. On the other hand, the decreased abundance of *Haemophilus* of phylum *Proteobacteria* noted among the disease group was unexpected given that this bacterial genus is a known mucosal pathogen and its abundance has been associated with varying pathogenicity in infections (Nørskov-Lauritsen, 2014), multiple sclerosis (Chen et al., 2016) and colorectal carcinoma (Liu et al., 2020).

Conversely, an increased abundance of genera under phylum *Firmicutes*, namely *Streptococcus*, *Veillonella* and *Lactobacillus*, specifically *L.ruminis*, *L.gasseri* and *L.salivarius* were noted among the disease groups. In addition, the increased abundance of genera *Escherichia-Shigella* of phylum *Proteobacteria* and *Collinsella* of phylum *Actinobacteria* were also observed among the disease groups. The genera *Streptococcus*, *Escherichia coli* and *Collinsella* are all common gut inhabitants which are often linked with several inflammatory diseases including T2DM (Candela et al., 2016; Qin et al., 2012). Meanwhile, there is limited evidence on the role of genus *Veillonella*, a Gram-negative bacterium in health and disease to date. This review also found a noteworthy positive association between the genus *Lactobacillus* with glycaemic markers, namely FPG, HbA1c and HOMA-IR index.

Overall, this review observed increased levels of glycaemic and pro-inflammatory markers as well as low diversity of gut bacteria among preDM and newDM participants. The alterations of gut microbiota composition found among the disease groups in this review suggest that changes in the abundance of specific gut bacteria that occur at the early stages of T2DM development could probably induce the inflammation-based environment seen with T2DM progression.

The changes observed among T2DM patients in this study as well as in the systematic review were summarized in Figure 5.1. This figure also included the confounding factors that could affect gut microbiota composition as well as the possible mechanisms involved in gut microbiota dysbiosis that could lead to insulin resistance state and T2DM.



**Figure 5.1** The Changes in Gut Microbiota Composition in this Study with the Postulated Effects in T2DM.