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THE ROLE OF NUTRITIONAL STATUS AS AN EPIGENETIC MODULATOR IN TYPE 1 DIABETES IN PEDIATRIC POPULATION OF QATAR

BY
AMIRA KOHIL

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COMMITTEE PAGE

The members of the Committee approve the Thesis of

Amira Kohil defended on 15/04/2021.

Dr. Mashael Al-Shafai
Thesis/Dissertation Supervisor

Dr. Annalisa Terranegra
Committee Member/Co-supervisor

Dr. Maha Al-Asmakh
Committee Member

Dr. Nasser Rizk
Committee Member

Approved:

Hanan Abdul Rahim, Dean, College of Health Science

ABSTRACT

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Type 1 Diabetes Mellites (T1DM), is an autoimmune disorder caused by the destruction of pancreatic β-cells and is considered to be among the most prevalent chronic conditions in Qatar. This study aimed to identify the differential methylation status in pediatric T1DM subjects from the population of Qatar. Also, to explore the correlation between nutritional factors and gut microbial composition and its metabolites with DNA methylation. In this study, we recruited a total of 72 subjects that were divided into four groups (T1DM = 35, T1DM-OB = 9, obese = 16, and healthy = 12). Different measurements were collected from the study subjects, which are 24-hour dietary recall, physical and biochemical data along with blood samples. Nutritionist Pro software (Axxya Inc) was used for the determination of the micro-and macro-nutrients intake for each study subject. CpG DNA methylation level was measured by Illumina Infinium EPIC Array and analysis of the generated data was conducted through the use of GenomeStudio. Differential methylated genes were identified using ParteK Genomic Suite software and then analyzed using Ingenuity Pathway Analysis (IPA) for functional pathway analysis. Network analysis was performed to identify the potential correlation of DNA methylation with dietary factors, gut microbiome, and SCFAs was explored in pediatric T1DM subjects. Based on the dietary analysis, the T1DM group was found to have a lower intake of SFA and vitamin K compared to healthy and obese groups. DNA methylation analysis showed the up-regulation of the *SAPCD1* gene in T1DM patients and the down-regulation of the *DNAJC7* gene in T1DM-OB subjects, in comparison to healthy and obese subjects.

The significant canonical pathways identified to be downregulated in T1DM-OB are aldosterone signaling in epithelial cells, xenobiotic metabolism CAR/PXR pathways and NRF2 mediated oxidative stress response. Furthermore, T1DM patients were found to have low gut microbial abundance compared to healthy controls . Our network analysis, showed a positive correlation of DNA methylation level with folate and thiamin intake in healthy controls. We have also identified a positive correlation between the microbial genus *Lachnospira* with DNA methylation in obese subjects. In this study, we were able to shed the light on the possible interaction between dietary components, DNA methylation and gut microbiome in T1DM development in children. However, more studies are needed for further exploration of such association.

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Chapter 1: Introduction

Type 1 diabetes mellitus (T1DM) is an auto-immune inflammatory disorder that destroys pancreatic β-cells and leads to insulin deficiency. T1DM patients are at a high risk of developing chronic and acute complications due to impaired glucose metabolism (hyperglycemia or hypoglycemia) [1]. The pathophysiology behind T1DM development is complex and involves the interaction between genetics, environment, and the immune system [2, 3]. Various genetic loci are associated with the disease susceptibility, mainly the human leucocyte antigens-DR/DQ (*HLA-DR/DQ*) locus [4]. Moreover, different environmental factors, such as infection, diet, early nutrition, mode of delivery, antibiotic use, and psychological stress are implicated in the onset and progression of T1DM [5]. Obesity has been long known as a concurrent phenotype observed with T2DM, but its association and development in T1DM cases are still under investigation. A study conducted between 2010 - 2012 found that overweight and obesity account for 22.9% and 13.1%, respectively in T1DM adolescents [6]. Several epigenetic modifications, mainly DNA methylation, histone modification, and micro-RNA (miRNA) are found to have an essential role in the development of several autoimmune disorders including T1DM [7]. DNA methylation is found to be involved in the pathophysiology behind T1DM development as it alters the expression of certain genes involved in insulin secretion, β-cell survival and autoimmunity [7]. A genome-wide DNA methylation study generated from T1DM discordant monozygotic twins identified 132 T1DM–associated methylation variable positions mostly in genes involved in apoptosis, inflammation, and the immune system [8]. Although T1DM patients are found to have unique DNA methylation patterns compared to healthy controls [9], the mechanism behind the influence of such patterns on T1DM is not

clearly understood. Several studies conducted in other metabolic disorders, such as obesity and Type 2 Diabetes mullites (T2DM) have found that the interaction between gut microbiome and immunity acts through epigenetic mechanisms [10]. However, it is not clear yet whether such mechanism is relevant for T1DM development as well. The development of obesity seen in T1DM patients may be attributed to different factors, including epigenetics, nutrition and gut microbiome [11]. However, such association is yet to be confirmed.

Hypothesis

We hypothesize that T1DM patients have a unique methylation status, and it plays an important role in the onset and progression of the disease. External factors, such as different dietary habits, BMI, and gut microbiome composition, can affect the DNA methylation contributing to explain the pathogenesis of T1DM and its high incidence seen in the young Qatari population.

Aim and Objectives

The study aimed to identify any differential methylation status in T1DM patients compared to T1DM obese, not diabetic obese, and lean healthy subjects. The study objectives were the following:

- To measure the levels of DNA methylation on blood samples from T1DM patients, T1DM obese, obese, and lean healthy controls, using the Illumina Infinium EPIC Array
- To identify differentially methylated signatures in T1DM patients compared to T1DM obese, obese, and lean healthy controls.
- To correlate DNA-methylation patterns with clinical and dietary parameters.

- To correlate the DNA methylation levels with the gut microbiome (data previously measured).

Study Design

This study aimed to determine the influence of DNA methylation on T1DM development and to link its effect with nutrient intake and gut microbiome composition. The target subjects were T1DM children compared to T1DM obese (T1DM-OB), obese and healthy lean participants. Different physical and biochemical tests were performed along with the 24-hours dietary recall. Blood samples were obtained from each subject for the DNA methylation analysis using the Illumina Epic Array. Differentially methylated genes were identified using Partek software and canonical and disease pathway analysis was conducted using Ingenuity pathway analysis (IPA) to define the affected pathways. Gut microbiome and microbial metabolite analysis were available from previous analyses and correlated with the methylation data. Finally, an integration analysis was applied to show the effect of diet and gut microbiome on DNA methylation in the development of T1DM disease.

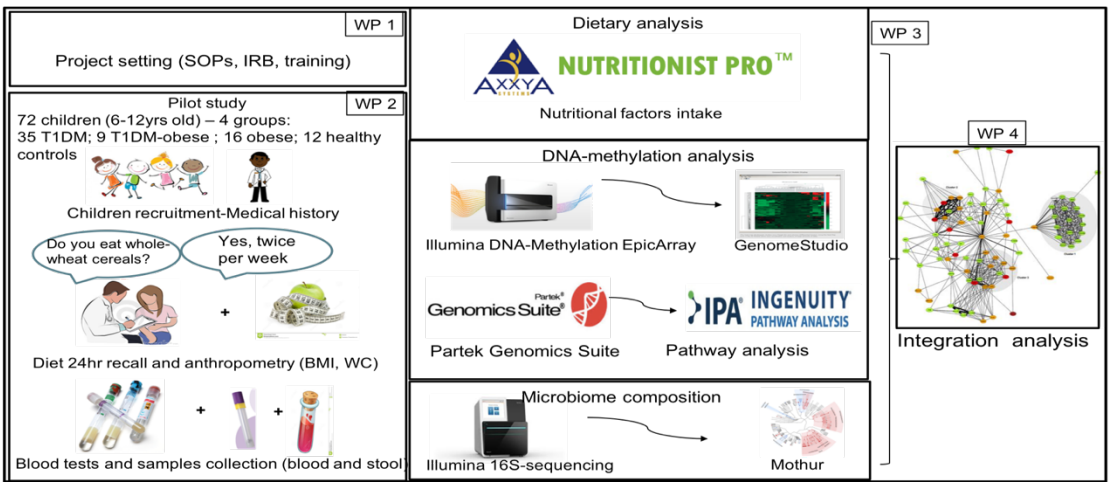


Figure 1. Summary of the study design.

Chapter 2: Literature Review

2.1 Background

Diabetes mellitus (DM) is a group of metabolic disorders characterized by impaired glucose control and the development of hyperglycemia. The classic symptoms associated with DM are polyuria, polydipsia, susceptibility to infection, and fatigue [12]. Based on the American Diabetes Association (ADA), diabetes is classified into four main types, which are T1DM, T2DM, gestational diabetes mellitus (GDM), and diabetes due to other causes [13]. T1DM is a chronic disease caused by the autoimmune destruction of pancreatic β-cells and insulin deficiency. The presence of T1DM associated auto-antibodies and HLA susceptibility variants are considered important risk factors in T1DM development and progression [4]. T1DM patients have a higher risk of developing co-morbidities, such as cardiovascular disorders (myocardial infarction and stroke), retinopathy, neuropathy, and nephropathy [14].

According to the International Diabetes Federation (IDF), T1DM incidence is increasing, with approximately one million cases present annually [17]. In the US, the prevalence of T1DM disease among adolescence (< 20 years old) increased by 21% from 2001 to 2009 [18]. Also, in Korea, the annual incidence rate of T1DM from 2007 and 2013 was reported to increase from 2.73 to 5.02 per 100,000 [19]. However, in Japan, a lower incidence rate is reported between 1.5 – 2.5 per 100,000 among children (< 15 years old) [20]. The prevalence rate of T1DM in India is 31.9 per 100,000, where higher rates are seen in urban areas [21]. Moreover, in the Middle East Region, a high prevalence of T1DM is detected in young adults (< 19 years old), in which 12.2% of T1DM cases are detected in Qatar, 33.5% in Saudi Arabia and 44.5 % in Kuwait. The reason behind the increased incidence over the years could be attributed to the recent

improvement seen in regards to early diagnosis, disease monitoring, and treatment [14]. Another factor that could contribute to the increased rate of T1DM worldwide is the predominance of a sedentary lifestyle (mainly in westernized countries), which has been found to have a strong influence on autoimmune disease development, including T1DM [23]. A western lifestyle characterized by high fat/low fiber dietary intake and absence of physical activity, is considered to be a strong promoter of T1DM development by modulating the function of the immune system, mainly T-cells [24, 25]. T1DM prevalence and incidence continue to increase worldwide and particularly in countries that have started to be exposed to a western-like lifestyle, such as Qatar and other countries in the Middle East.

Diagnosis of diabetes is usually based on the concentrations of random blood glucose (> 11.1 mmol/L), fasting blood glucose (> 7.0 mmol/L), and abnormal oral glucose tolerance test. Measurement of HbA1c is considered a less sensitive tool for T1DM diagnosis and is usually used to monitor glycemic control [13]. Clinical features by themselves cannot differentiate between T1DM and other types of diabetes; therefore other factors, such as the age of diagnosis, genetic susceptible variants, and pancreatic autoantibodies should be considered [26]. Patients with T1DM, usually have autoantibodies against insulin, islet antigen 2, glutamate decarboxylase, and zinc transporter 8 [26]. A systematic review and meta-analysis study showed that the presence of islet autoantibodies increases the risk of developing T1DM both in the presence or absence of susceptible genetic variants [27].

Regarding the health care management of T1DM patients, different types of treatments were developed, to preserve insulin secretion [28]. One of the most recommended therapeutic approaches for T1DM patients is insulin therapy (basal-bolus

regimen), which is known as multiple daily injection therapy. In this treatment, patients are given a basal insulin dose (long-acting) and prandial insulin dose (rapid-acting) in the form of multiple injections at various time intervals [28]. In addition, the doses are calculated according to the patient's body weight, insulin sensitivity, and carbohydrate intake [29, 30]. A study showed that patients with T1DM treated with basal-bolus therapy have improved glycemic control and an HbA1c level of < 7 % [31]. Another common treatment used by T1DM patients is the insulin pump (known as subcutaneous insulin infusion, SCII), which provides a steady level of insulin through the use of an insulin pump device [28]. It has been shown that T1DM patients treated with insulin pump have a lower risk of having severe hypoglycemia or hyperglycemia episodes [32]. Also, the use of insulin pump therapy in children and adolescence with T1DM showed safety and effectiveness, where significant decrease in HbA1c level was detected and improved glycemic control was achieved [33].

2.2 Pathogenesis of T1DM

T1DM disease is a complex disorder as it involves the interaction of both genetic and environmental factors. T1DM pathophysiology involves also environmental factors, such as diet and microbiota. We hypothesize that the triple interaction between epigenetics, dietary factors, and gut microbiota can explain the disease development and the response to the treatment. Several dietary factors are found to potentially influence T1DM by affecting gut microbiota and their metabolites [34, 35]. Also, different epigenetic modifications are found to have a significant role in T1DM development [7, 36].

2.2.1 Genetic Factors

Based on the genome-wide association studies (GWAS), different genes have been detected to be associated with increased risk for the development of T1DM, such as insulin gene *(INS)*, non-receptor type 22 gene *(PTPN22)*, and cytotoxic T-lymphocyte associated protein gene *(CTLA-4*) [37, 38]. However, the strongest association was detected with specific haplotypes of human leukocyte antigens DR/DQ (*HLA-DR/DQ*) [4].

In addition, based on a twin study, identical twins have a higher risk of developing T1DM (69%) in comparison to siblings (47%) in the presence of autoantibodies [39] highlighting that genetics have an important role in T1DM pathogenesis.

2.2.2 Environmental Factors

Despite the inheritance of T1DM susceptible variants, genetic predisposition alone cannot explain the pathophysiology and sub-phenotypes of T1DM [5, 40-42].

Environmental factors, such as infections, nutrition, mode of delivery, maternal age, and medication play a major role in the T1DM development [5]. This is supported by the phenomena of high T1DM incidence in genetically stable populations with a low genetic risk of developing T1DM [40-42]. Environmental factors trigger the development of T1DM either by its effect on intestinal function and the gut microbiota [43, 44] or on epigenetic mechanisms [45]. Since T1DM is a multifactorial metabolic disorder, its pathogenesis involves both genetic and environmental factors; however, recently the environmental determinants are getting more relevant in the development of T1DM.

2.2.2.1 The role of nutrition in T1DM development and treatment

An important environmental trigger associated with T1DM development and other metabolic diseases is diet [46-48]. Although different nutritional factors, such as

fiber, fat and protein are known to be involved in the pathophysiology and management of T1DM, the exact molecular mechanism is not clearly understood (Table 1). Since T1DM and its pre-clinical autoimmunity appear at an early age (3-9 months), the implication of early nutrition has been suggested to have a role in T1DM pathogenesis [49-52]. Breastfeeding was found to be an essential determinant of T1DM risk, where breastfed children showed a lower risk of developing the disease [49, 53]. This could be due to the presence of certain components in breast milk, such as lactoferrin, secretory immunoglobin A, lysosomes, and macrophages [50]. These components are known to have antimicrobial properties and are involved in the protection against different microbial antigens along with regulating the function of immune T-cells and B-cells [50]. On the other hand, cow's milk or formula milk intake at an early age (6-9 months) increases the risk of T1DM development [50, 51], which could be as a result of specific fatty acids present in the cow's/formula milk (myristic, monounsaturated palmitoleic acid and penta-decanoic) that induce pancreatic islet autoimmunity [54]. A similar finding was observed in an animal-based study, in which the A1 beta-casein present in cow’s milk altered glucose handling capacity by activating islet inflammation [55]. Furthermore, early introduction of cereals (age < 3 months) with or without gluten was found to promote T1DM development, while late introduction has no effect [52]. The effect of early cereal intake could be attributed to the immature immunity and undeveloped gut microbiota in children less than 6 months [52]. However, another study found that late introduction of gluten (age > 9 months) containing cereals is also associated with a high risk of autoimmunity and T1DM development [56]. Moreover, early probiotic supplementation within the first year of life is found to be associated with a reduced risk of developing T1DM in high-risk patients [57]. The intake of

linoleic acid (vegetable oil) is also found to be associated with reduced risk of β-cell autoimmunity in children (1-6 years old), while increased intake of myristic acid, monounsaturated palmitoleic acid, conjugated linoleic acid and pentadecanoic present in milk and ruminant meat is associated with a higher risk [46].

Diet in adult age can contribute as well to the pathogenesis and the progression of T1D. Vitamin D has an important role in the regulation of immune and metabolic related pathways, along with its association with a lower risk of T1DM development [58, 59]. Vitamin D is known to down-regulate the response of T helper-1 lymphocytes [60], which could explain the possible association between vitamin D intake and the reduced risk of T1DM development. On the other hand, several other studies did not observe this effect of vitamin D supplementation [61, 62]. These discrepancies between studies could be attributed to a number of variables, which are type of supplement (i.e., calcitriol, alpha-calcidiol, or cholecalciferol), vitamin dosage, study participants age group, and diabetes duration. Furthermore, it is suggested that glycemic control and HbA1c could be modulated in T1DM patients based on their nutritional intake. An improvement in glycemic control was observed with the intake of a low carbohydrate/high-fat diet in T1DM patients [63]. However, increased risk of hypoglycemia and dyslipidemia development was associated with this type of diet [63]. Furthermore, a poor glycemic control characterized by elevated levels of HbA1c was found to be associated with the intake of low dietary fibers in T1DM patients [64].

Understanding the role and effect of different dietary components on T1DM development may aid in improving T1DM management and the development of a personalized therapeutic nutritional approach. One example is medical nutrition therapy (MNT), which is a therapeutic approach developed to be used in the

management of T1DM patients. The goal of MNT is to improve glycemic control, address individuals' needs, provide the needed calories, and create a plan for follow-up care for patients [28]. It is recommended that patients with T1DM use a carbohydrate diabetes meal planning system, which provides consistency in the carbohydrate content, thus reducing the fluctuation of blood glucose [65].

Table 1. The effect of different dietary components on the risk of developing T1DM.

Dietary component	Study subjects	Age	Major finding	Ref.
Breastfeeding	Children with increased risk of T1DM based on HLA typing	< 5 years old	Breastfeeding during the introduction of new solid food, such as wheat/barley protected against development of T1DM in genetically susceptible children.	[49]
	Children with risk of developing T1DM	7 months – 16 years old	Breastfeeding reduces the risk of developing T1DM. Children who were not breastfed have a twofold increase risk of developing T1DM.	[53]
Cow's milk	Children with increased genetic risk of developing islet autoimmunity or T1DM	-	High intake of cow's milk protein is associated with an increased risk of developing islet autoantibodies in patients with a low and moderate risk of developing T1DM. No association was found between children with high risk and cow's milk intake.	[54]
	NOD mice fed with milk containing A1 or A2 beta-casein component	-	Subclinical insulitis and signs of T1DM were seen in mice fed with A1 beta-casein diet. A1 beta-casein diet altered glucose handling capacity by activating islet inflammation.	[55]
Gluten-containing food	Children with increased risk of T1DM	9 months – 17 years	Early introduction of gluten-containing food (age < 3 months) increases the risk of developing autoimmunity and T1DM. However, the introduction of gluten after 6 months of age does not affect the incidence of autoimmunity or T1DM development.	[52]
Probiotics	Children with increased risk of T1DM based on HLA typing	4- 10 years old	Early supplementation of probiotics is associated with a reduced risk of developing T1DM in high-risk patients.	[57]

Milk and ruminant meat fat	Children positive for HLA-DQβ1	1 -6 years old	increased levels of myristic acid, conjugated linoleic acid, monounsaturated palmitoleic acid, and pentadecanoic are positively associated with the risk of advanced β-cells autoimmunity.	[46]
Vegetable oil	Children positive for HLA-DQβ1	1 -6 years old	Increased level of linoleic acid reduces the risk of advanced β-cell autoimmunity.	[46]
Vitamin D	T1DM patients	> 5 years old	Sufficient levels of vitamin D preserves β-cells function and insulin secretion indicating that it provides a protective effect against T1DM development.	(59)
low carbohydrate/high fat diet	T1DM patients	-	Improvement in glycemic control observed by low levels of HbA1c and less glycemic variability Increased risk of hypoglycemia and dyslipidemia	[63]
Low fiber intake	T1DM patients	15-60 years old	Elevated levels of HbA1c were observed in T1DM patients, thus leading to a poor glycemic control	[64]

2.2.2.2 Immune dysregulation and microbiota dysbiosis

Under normal conditions, gut microbiota influences the immune system through activating adaptive immunity, compartmentalizing pathogens, and enhancing lymphoid tissue development [66]. As seen in many other pathological conditions, gut microbial dysbiosis associated with T1DM enhances gut permeability [67], resulting in the leakage of antigens and microbes into the systematic circulation and leading to β-cell damage and autoimmunity [68-70]. Different human and animal studies have been conducted to determine the microbial composition associated with T1DM. It has been found that T1DM patients have low gut microbiota diversity, low ratio of Firmicutes to Bacteroidetes, increased abundance of *Bacteroides,* and decreased *Faecalibacterium prausnitzii and Lactobacillus* [71]. A study done by Leiva and colleagues found that children with T1DM have an increased level of *Bacteroides, Veillonella, Streptococcus, Ruminococcus,* and *Blautia* compared to healthy controls. However, *Bifidobacterium,*

Faecalibacterium, Roseburia, and *Lachnospira* were reduced [72]. The immunopathogenesis of T1DM due to microbial dysbiosis is not well understood, where both the innate and adaptive immune systems have been found to contribute to the development of T1DM [73, 74]. A study conducted using non-obese diabetic mice (NOD) found that TIR-domain containing adaptor inducing interferon-ß (TRIF) (a key adaptor molecule in the innate immune system) deficiency protected against T1DM development possibly through altering the gut microbiota composition, as a significant reduction and increase in *Proteobacteria* and *Firmicutes,* respectively, was detected in TRIF deficient mice [74]. In MyD88-deficient NOD mice under normal conditions, no signs of diabetes were detected. However, under germ-free conditions, an advanced stage of diabetes was developed [73, 75]. This effect was also seen in other autoimmunity disorders, such as Celiac Disease, T2DM, obesity, and autoimmune uveitis [75]. In a study using the RIP-B7.1 C57BL/6 mouse model, diabetes was induced mainly due to microbial modulation through TLR3 and MyD88 pathways [76]. In general, there are two possible proposals on how microbiota could contribute to T1DM development: first, the enhanced intestinal permeability results in microbial leakage, thereby activating antigen-presenting cells and T-cells [67]. Secondly, the molecular homology of certain microbial products to Langerhans islets autoantigens could lead to the destruction of pancreatic β-cells and T1DM development [77]. Various research studies have proven the significant role of gut microbiota in the pathophysiology of T1DM [78, 79].

The relationship between dietary influence and gut microbiome composition in the development of T1DM in infants and children is not clearly understood and few studies have been published addressing this concept. A study conducted by Endesfeler and

colleagues to study the association between diet and microbiome in high-risk children found that *Bacteroides* abundance was found in children characterized by early introduction of non-milk diet and complex diet intake [80]. They also found that the level of *Bacteroides* positively correlated with a high risk of early autoantibody development and low abundances of genes involved in butyrate production [80]. Another study found that early introduction of probiotics dietary supplement (0-27 days) in high-risk children is associated with decreased risk of autoimmunity [81].

2.3 The Role of Epigenetics in T1DM Development

Epigenetics is the mechanism of activating and inhibiting the expression of genes by external factors, without affecting the DNA sequence [82]. Therefore, epigenetic changes are seen as non-genetic factors that interact with genes and are affected by genetic variations. In addition, epigenetic modifications are considered as consequences of the interaction between genetics and environment that results in making the DNA accessible for transcription factors and thus regulating gene expression [9, 83]. Several features could support the influence of epigenetics in T1DM development, which are high rates of discordance in monozygotic twins, increased risk of T1DM development in offspring of the affected father rather than the affected mother, and the increased T1DM incidence in a genetically stable population [7, 36]. It has been shown that epigenetic modifications, mainly DNA methylation, play an important role in the development of different autoimmune disorders, including T1DM [7, 36].

2.3.1 The Role of DNA Methylation

DNA methylation is the process of methyl group binding to the fifth carbon of cytosine preferable in the CpG dinucleotides, leading to the formation of 5-

methylcytosine [84]. Epigenetic mechanisms are found to be implicated in the early development and maturation of β-cells [85]. In non-endocrine tissues, promoters of Glucagon and Insulin 2 [INS2] genes were found to be hypermethylated as compared to pancreatic β-cells [86]. Moreover, the different endocrine cell subtypes (α-cells, β-cells, and δ-cells) were found to have different CpG methylation levels, indicating possible gene expression regulation in these cells [86]. DNA methylation is also found to be implicated in T1DM development as it alters the expression of genes responsible for insulin secretion, β-cell survival and autoimmunity [7]. The genome-wide DNA methylation profile generated from T1DM discordant monozygotic twins identified 132 T1DM–associated methylation variable positions mostly in genes involved in apoptosis, inflammation, and the immune system [8]. Another study involving T1DM-discordant monozygotic twins detected 88 CpG sites that are significantly methylated [87]. Therefore, genetics is not the only determinant of T1DM development, and DNA methylation plays an essential role in it, which is supported by twin studies (Table 2).

Insulin (*INS)* gene and Interleukin 2 receptor α-chain gene (*IL2RA*) are important loci associated with the development of T1DM, and various methylation studies found that methylation of these genes are implicated in T1DM development. T1DM patients have an elevated level of methylation in CpG -180 and a decreased level of methylation in CpG -19, -135, and -234 in the *INS* gene, in comparison to healthy subjects [88]. Moreover, it has been found that the expression of the *INS* gene is regulated by the methylation of *Ins1* exon 2 and *Ins2* exon 1, which are induced by pro-inflammatory cytokines through activation of methyltransferases [89]. Regarding *IL2RA*, which encodes IL-2 receptors, T1DM patients are found to have elevated CpGs −456 and −373 methylation level in comparison to healthy controls. In addition to that, the methylation

at CpGs −373 was found to be associated with sixteen SNPs known to be implicated in T1DM [90].

There is evidence that shows the further implication of DNA methylation in T1DM associated complications, such as diabetic nephropathy [91]. One study showed that 19 CpG sites were found differentially methylated in diabetic nephropathy and one of them located close to the transcription start site of *UNC13B* (rs13293564), which is implicated in diabetes nephropathy development [91].

Table 2. Changes in DNA methylation associated with T1DM development in twins' studies.

Study subjects	DNA methylation profile	Cell type	Affected genes/pathways	Ref.
Monozygotic twins discordant for T1DM	132 T1DM associated methylation variable positions were identified and found to be significant before and during clinical diagnosis.	CD 14 $^{+}$ Monocytes	Apoptosis, inflammation, and the immune system	[8]
Two groups of monozygotic twins discordant and concordant for T1DM	88 CpG sites have been found to be significantly methylated in monozygotic T1DM discordant twins.	B-cells	Immune system (*APOA4, C4BPA, CLEC7A*) cell signaling (*APITDI, CALCA, GATA4, SLC12A5*)	[87]
Monozygotic twins discordant for T1DM	DNA methylation did not differ between T1DM patients and their healthy co-twins, except for cg01674036 in CD 4 $^{+}$ T-cells.	CD 4 $^{+}$ T-cells CD 19 $^{+}$ B-cells CD 14 $^{+}$CD16^{-} Monocytes	Immune system Cell cycle (mTOR pathway)	[92]
Monozygotic quadruplet discordant for T1DM (case report)	The prediabetic twin shows a differential methylation status between healthy and T1DM.	CD 14 $^{+}$ Monocytes CD 4 $^{+}$ T-cells	-	[93]

2.3.2 The role of other post-transcriptional and epigenetic regulatory events in T1DM development

A micro-RNA (miRNA) ranges from 18-22 nucleotides and is a non-coding RNA molecule that acts as a post-transcriptional silencer [94]. MiRNAs are involved in

different processes, such as proliferation, mitotic cell division, and programed cell death [95]. The miRNAs' regulation of gene expression is implicated in various autoimmune disorders [96, 97]. Regarding T1DM, it has been found that T1DM patients have up-regulation of miR-155-5p, miR-103a-3p, miR-210-3p and down-regulation of miR-146a-5p [98]. The alterations in miRNAs could contribute to the pathophysiology of the disease through its influence on insulin secretion, the immune system, apoptosis, and the mitogen-activated protein kinase (MAPK) signaling pathway [98]. In children with T1DM, an up-regulation of a set of miRNA (miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, miR-200a) was observed and found to be involved in ß-cell function and apoptosis [99]. The effect of miRNA in T1DM development through modulating the immune system is observed by the upregulation of miR-155-5p, which targets the mRNA of the transcriptional and immune response regulator gene (*TCIM*) [100]. The upregulation of miR-155-5p is also associated with inflammation through targeting the mRNA of toll-like receptors leading to the NF-κB pathway activation [101]. The downregulation of miR-146a-5p usually detected in patients with T1DM is found to be involved in the overproduction of IL-6 (a pro-inflammatory cytokine), thereby, suggesting the possible involvement of miR-146a-5p as a negative regulator of the NF-κB pathway in T1DM [101, 102].

Different modifications, such as acetylation, methylation and other mechanisms (ubiquitination and sumoylation), occur in the N-terminal region of histones [103]. Histone methylation defined as the attachment of methyl groups to arginine or lysine residues, it results in transcription regulation (activation /inhibition) based on the affected region and modification level [104]. In regards to histone acetylation, it

involves the function of histone-acetyltransferases (HATs) and histone-deacetylases (HDACs) enzymes responsible of the addition and removal of the acetyl group on lysine residues respectively [83]. Histone ubiquitination involves the function of ubiquitin ligases responsible for the addition of ubiquitin molecules to the conserved lysine residues [105]. For sumoylation, it involves the covalent binding of ubiquitin-like modifier proteins to histones through ubiquitin analog enzymes [106].

Different studies have shown the association between histone modification and T1DM development, in which H3 lysine 9 di-methylation (H3K9me2) was significantly increased in lymphocytes of T1DM patients [107]. There is also a strong association between increased H3K9me2 promoter activity with autoimmune genes, inflammatory pathway genes (TLR,p38-MAPK, and NF- κB), and T1DM susceptibility gene (*CLTA4*) [107]. Significant variations in H3K9 acetylation (H3K9Ac) levels at the upstream regions of *HLA-DRB1* and *HLA*-DQB1 were also detected in T1DM patients [108]. Furthermore, THP-1 monocytes under the treatment of TNF-α and interferon-γ showed an enhanced expression of *HLA-DQB1* and *HLA-DRB1* with changes in H3K9Ac as seen in T1DM patients [108].

Table 3. a summary of research studies showing the association between other post-transcriptional and epigenetic modifications (miRNA and histone modification) with T1DM

Type of modification	Study subjects	The epigenetic marker	Major findings	Ref.
miRNA	Children newly diagnosed with T1DM	Up-regulation of miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, miR-200a	The up-regulated miRNA detected is involved in the regulation of β-cell functions and apoptosis. miR-25 has been found to be associated with glycemic control and β-cell function improvement.	[99]
	Two groups of T1DM patients:	Up-regulation of miR-103a-3p, miR-155-5p, miR-200a-3p, and	These miRNAs could be considered as TIDM biomarkers since they target genes from the immune	[98]

	Diagnosed < 5 years Diagnosed >5 years	miR-210-3p and down-regulation of miR-146a-5p in newly diagnosed T1DM patients (< 5 years)	system, apoptosis, and insulin-related pathways.	
Histone Modification	Patients with the diagnosis of T1DM > 10 years	H3K9me2	Elevation of H3K9me2 in lymphocytes of T1DM patients a strong positive association between H3K9me2 promoter activity and genes involved in autoimmunity and inflammatory pathways	[107]
	Patients with the diagnosis of T1DM > 10 years THP-1 cells	H3K9Ac	Variations in H3K9Ac levels at the upstream regions of *HLA*-DQB1 and *HLA-DRB1* was observed in T1DM patients Enhanced expression of *HLA-DQB1* and *HLA-DRB1* with changes in H3K9Ac after treatment with TNF-α and interferon-γ in THP-1 monocytes (in vitro) as observed in T1DM patients	[108]

2.4 The Interplay between Diet, Epigenetics and Gut Microbiome in T1DM

The link between diet and the epigenome and its contribution to T1DM development is not clearly understood. However, several studies suggested the possibility of specific nutrients to act as epigenetic effectors in T1DM development. Different studies showed the association between high fat intake and the risk of poor glycemic control, islet autoimmunity, and development of T1DM-related complications during both childhood and adulthood in T1DM patients [109, 110]. It was also found to act as an epigenetic modulator, where it impacts a transcription factor essential for β-cell survival known as TCF7L2 [111]. Aberrant TCF7L2 promoter methylation in β-cells was observed in mice treated with a high-fat diet (HFD - 45kcal% fat) in comparison to mice given a normal diet [111]. Even though the implication of TCF7L2 was commonly detected in T2DM patients, it was also observed in a subset of non-obese T1DM patients [112]. Furthermore, the maternal high fat diet was found to negatively impact pancreatic β-

cells of male mice offspring leading to proliferation defects and insulin degranulation [113]. However, early transition from a high-fat diet to a normal diet protected pups from developing insulin resistance [113]. It has been found that maternal high fat diet causes insulin receptor substrate-2 gene (*IRS-2*) hypermethylation and mitogen-activated protein kinase kinase-4 gene (*MAP2K4*) hypomethylation in mice offspring. This results in a decreased expression of *IRS-2* and increased expression of *MAP2K4*, thus elevating the pup's risk of developing diabetes [114].

The interplay between diet and epigenetics was also found to be related to insulin signaling pathways and glucose metabolism. Through the use of the grass carp, *Ctenopharyngodon idellus,* whole-genome DNA methylation analysis revealed no significant difference in methylation levels under different dietary conditions (high carbohydrate diet/ normal diet) [115]. In the case of high carbohydrate intake, the differentially methylated genes in *Ctenopharyngodon idellus* were enriched in pathways related to insulin signaling, glucose and lipid metabolism. Moreover, the differentially methylated genes involved in obesity and T2DM detected in *Ctenopharyngodon idellus*, were similar to those found in mammals [115].

Although, the effects of diet on the microbial composition of the gut have been demonstrated in many animal models of T1DM, little is known regarding such effects in humans, due to the lack of long-term and placebo-controlled trials [68-70]. Specific dietary factors identified as potentially influencing T1DM, such as breastfeeding, and low-fat/high-fiber diet, are known to influence the gut microbiota composition and its products [34, 35]. As dietary fibers are digested only by gut microbiota present primarily in the lower gastrointestinal tract, fermentation of these fibers produces short-chain fatty acids (SCFAs) that contribute to the gut microbial diversity [117, 118]. The

proposal that SCFAs play an important protective role against T1DM is supported by the greater capacity for synthesis of SCFAs in healthy subjects compared to T1DM patients [79]. These SCFAs are found to activate specific free fatty acid receptors 2/3 (FFAR2 and FFAR3) leading to the inhibition of the histone deacetylase, and thus resulting in the activation of T-regulatory cells and inhibition of the inflammatory cascade [119].

The link between the three factors, which are diet, epigenetics, and microbiome, and their mechanistic contribution to T1DM development is yet to be identified (Figure 2). However, as observed in T2DM, it can be proposed that SCFAs (butyrate, acetate, and propionate) cause-specific activation of FFAR2 and FFAR3, which inhibit histone deacetylase. This results in the inhibition of NF-κB [120] and the activation of ERK and MAPK pathways [121] in intestinal regulatory T-cells, therefore down-regulating the inflammatory cascade [122]. Moreover, binding of SCFA to the promoter region of FFAR3 was found to reduce methylation of the CpG islands in T2DM patients [123]. Since T1DM and T2DM share similar genetic and environmental factors, further studies are needed to identify potential common underlying epigenetic mechanism

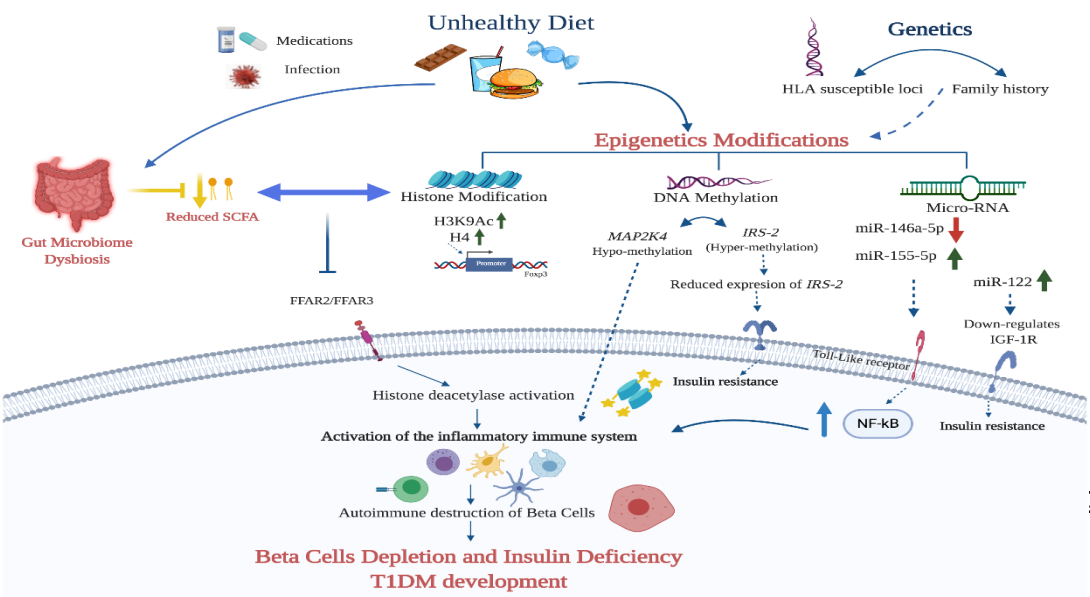


Figure 2. Schematic representation of the interplay between diet-epigenetic-microbiome in T1DM development [124].

Chapter 3: Materials and Methods

3.1 Study subjects' recruitment and sample collection

This study was conducted at Sidra Medicine. The study has been approved by the institutional review board at Sidra Medicine (IRB number: #1708012734) and Qatar University (IRB number: QU-IRB1411-E/20).

T1DM patients were approached and introduced to the study after their medical appointment in Sidra's endocrinology clinic. T1DM patients were selected according to the following criteria: age ranges from 6 – 12 years, had no chronic condition other than T1DM, no history of cancer or any familiar genetic disease, no history of antibiotic treatment in the last three months and the onset of T1DM diagnosis is more than one year. For the T1DM-obese subjects, the same criteria were followed along with a BMI percentile value higher than 95th percentile. In regards to the obese group, the same inclusion criteria were applied, along with a BMI value higher than 95th percentile and no history of diabetes diagnosis or glucose intolerance. Healthy lean controls were recruited from children of Sidra's staff who volunteered to participate in the study. Healthy lean controls should have no history of any chronic condition including diabetes diagnosis or glucose intolerance. Consent/assents forms were provided by the subjects and their parents who agreed to participate in this study.

The recruited subjects were divided into four groups: 35 T1DM patients, 16 obese subjects, 9 T1DM-obese patients, and 12 as lean healthy subjects. The subject's clinical information, such as family history of diabetes, medication, diabetes duration, and insulin treatment were provided by the physician during the visit. Also, 24-hrs dietary recall was collected during the interview to determine the dietary intake of the study subjects. Physical measurements were also collected, including body weight, height,

and waist circumference. Several biomedical tests were performed, including a lipid profile, liver function tests, thyroid tests (TSH, T4), and HbA1c.

From each study subject, blood samples were collected by the phlebotomist (3 ml of blood in a 6 ml Tempus Thermo Fisher Scientific tube containing RNA preservative solution) for DNA and RNA extraction. Samples were transported to the lab within 24 hours upon collection, in which aliquots of each sample were transferred into 1.5 micro-centrifuge tubes and stored at -80ºC immediately. A total of 72 participants were recruited in the study and their blood samples were processed and analyzed.

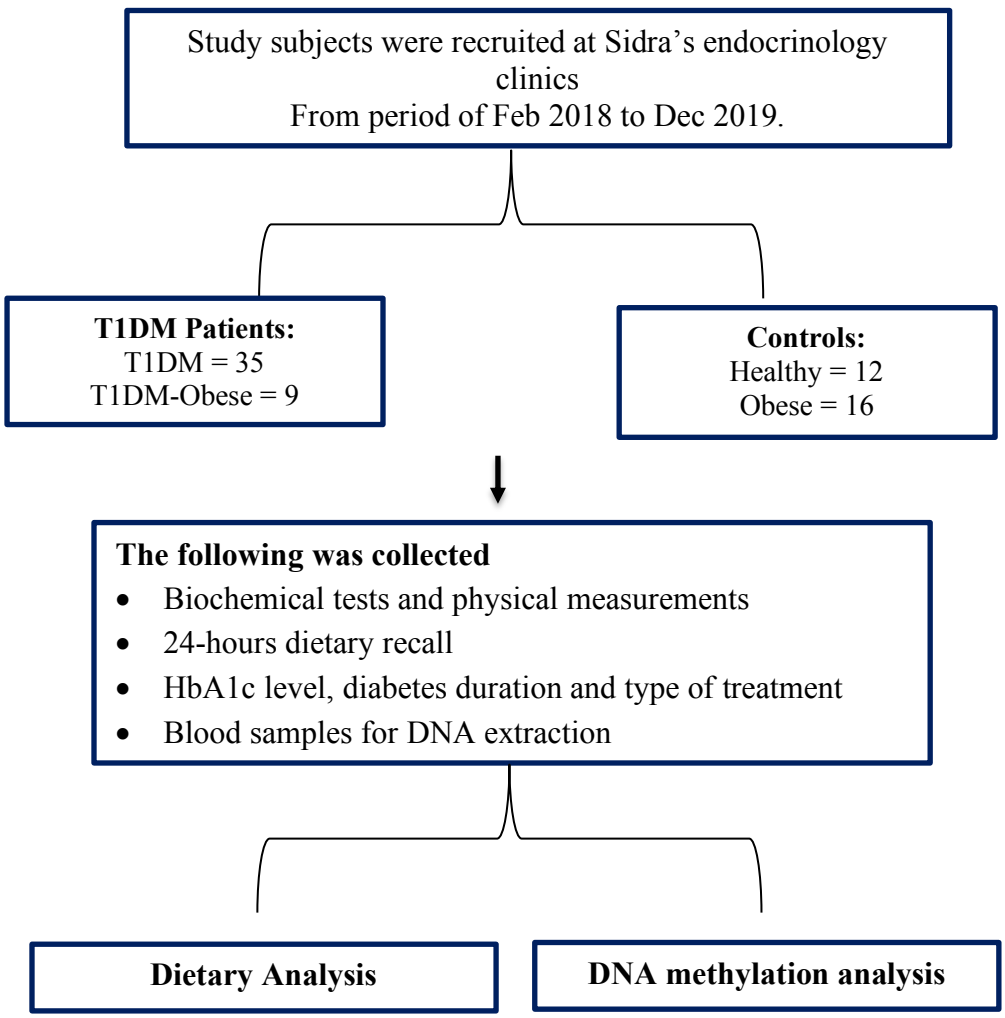


Figure 3. Schematic flow chart of the research plan.

3.2 Clinical data analysis

The clinical data obtained from study subjects include anthropometric measurements (height, weight, waist circumference), liver function tests, lipid profile, thyroid and pituitary gland hormones, micro-albumin/creatinine ratio, and blood pressure. Tests related to diabetes include glucose level, HbA1c, insulin/carbohydrates ratio, insulin dose (Kg/unit), and treatment type.

3.3 Dietary analysis

The dietary data obtained from the subjects' 24-hrs recalls were uploaded on Nutritionist Pro software (Axxya Inc). Dietary meals that were not found in Nutritionist Pro software, such as Arabian meals, were added as a new recipe by adding the ingredients and the serving amounts in the software. After uploading the dietary data, the average daily intake of macronutrients and micronutrients was calculated per subject and per group.

3.4 DNA extraction from a blood sample

DNA extraction was conducted using QIAamp® DNA kit (catalog no #51106, Qiagen, Germany) according to the manufacturer's instructions. However, a different concentration was used for proteinase K (20mg/ml) and the sample (40µl and 400µl respectively) in order to obtain a higher DNA concentration. Measurements of the other reagents were adjusted accordingly, except for the washing buffer reagent. In the elution step, 100 to 200 µl of the buffer AE was added in the QIAamp Spin Column and centrifuged for 1 minute. NanoDrop One (catalogue no #ND-ONE-W, Thermo

Scientific, USA) was used to measure DNA quantity and quality. The eluted DNA was then transferred into a new Eppendorf tube and stored at -80ºC for further analysis.

3.5 DNA methylation analysis

The extracted DNA samples (200-500 ng) were distributed in a 96-well plate and processed using the Zymo EZ DNA Methylation Kit for bisulfite conversion as per the manufacturer's instructions. This kit converts unmethylated cytosines (C) in genomic DNA to uracil (U), though leaving methylated cytosines unchanged for methylation analysis. First, the bisulfite converted DNA was denatured and neutralized before amplification. The samples were denatured for 10 minutes at room temperature using 0.1 N NaOH. The denatured DNA was then amplified in an overnight step at 37°C for 20-24 hours. Afterwards, a controlled enzymatic fragmentation was performed, which uses an endpoint fragmentation process to prevent over fragmentation. DNA fragmentation was accomplished by centrifuging the plate at 280 xg, and addition of 50µl FMS followed by incubating the plate into a preheated heat block for 1 hour at 37°C. The fragmented DNA then underwent isopropanol precipitation with centrifugation at 4°C before being resuspended in a hybridization buffer. The resuspended DNA was loaded onto one of 8 positions on a barcoded BeadChip slide and incubated overnight in the hybridization oven at 48°C. A washing step was performed to remove unhybridized DNA from the BeadChip slide using PB1 at room temperature. Finally, BeadChips was coated with XStain BeadChip Solution 4 (XC4) and dried prior to scanning using the iScan system.

Then, genome-wide DNA methylation profiling from the study subjects was performed in the Genomic Core at Sidra Medicine using the Illumina Infinium EPIC Array according to the manufacturer's protocol. The array profiles methylation levels

of over 850,000 methylation sites across the genome at single-nucleotide resolution, imaged with the Illumina iScan platform. A number of quality control steps were performed on the raw methylation data. First, probes with a mean detection level P-value <0.05 were used for subsequent analysis. Second, the data were normalized and the background was corrected using the methylation module (1.9.0) available on GenomeStudio (v2011.1) software. The DNA methylation level at each CpG site was given for each sample by the following formula: $\beta = \frac{M}{M+U} . 100\%$, where M represents signal strength of methylated CpG and U is the signal strength of unmethylated CpG.

The generated β-value was exported to Partek Genomics Suite version 7.0 in the form of *.idat* files for differential methylation analysis and data quality/quality control analysis (Appendix B, figure S2). The data was normalized within and between the groups using Illumina normalization. Probes located on X and Y Chromosomes were excluded from the analysis. A two-way ANOVA test was conducted to detect differential methylation analysis, in which M-values were generated from the β-values using the following formula $M = \text{Log2}\,(\frac{\beta}{1-\beta})$. The gene marker list, with the significant methylated genes, was generated using ANOVA with a P-value <0.05 and FDR >2 and by measuring the fold change.

3.6 Functional classification and gene network analysis

To cluster differentially regulated genes based on their common functionality, a set of genes was analyzed for gene ontology and pathway analysis using Ingenuity Pathway Analysis (IPA). The differentially methylated genes that are FDR adjusted were added to the software to investigate canonical pathways, disease and bio-functions, and network analysis.

3.7 Gut microbiome and Short Chain Fatty Acid (SCFA) analysis

The metagenomic analysis data (16S rDNA sequencing) of the gut microbiome for the study subjects were already available from previous data analysis**.** Alpha diversity and beta diversity analyses were performed using the R package. To identify microbial biomarkers among the different groups, Linear discriminant analysis effect size (LEfSe) analysis was performed with the cut-off value of LDA >2.0.

SCFA analysis was performed in Deep Phenotyping Core. Internal standard solutions containing deuterated fatty acids were mixed with the aliquots of the study subjects' stool samples to undergo derivatization with ethyl chloroformate producing ethyl esters. Analysis of ethyl esters was performed by Agilent 7890B Gas Chromatograph with 5977 Single Quadrupole Mass Spectrometer. Data analysis was conducted using a MassHunter WorkStation and SCFAs concentration was expressed in μmol/g of stool.

3.8 Integration analysis

The back-end of the network analysis was primarily based on Python and C++. Through the use of PHP, server connections were established, and front-end design was based on HTML, CSS, and Javascript. In this analysis, both prevalence and occurrence-based filtration criteria were used. Prevalence indicates the minimum abundance at which a given feature must be present in a sample. Whereas occurrence indicates the minimum number of samples in which the given feature must prevail at the prevalence threshold. To normalize and filter the data, total sum scaling method was performed. Microbial OTU abundance with the metadata was correlated using NAMAP correlation matric [125]. Finally, data were visualized based on Cytoscape.js, jVenn, D3.js and in-house customizations for a better user experience.

3.9 Statistical analysis

Generated results were evaluated using logistic regression with covariates such as age, and sex to derive adjusted odds ratios and 95% confidence intervals. For the differential CpG data analysis, a statistical one way ANOVA was performed to compare the average of DNA methylation level between the four groups: T1DM, T1DM-OB, obese and healthy controls. Clinical and dietary data were further analyzed by ANOVA- Turkey multi-comparison analysis for normally distributed data and Kruskal-Wallis test for not-normally distributed data. A two-sided P-value of < 0.05 was considered statistically significant. Categorical data were reported as the number or proportion of subjects within a category, while continuous data were reported as mean (standard deviation). All statistical analyses were performed using GraphPad Prism.

Chapter 4: Results and Discussion

4.1 Results

4.1.1 General characteristics of the study subjects

We recruited a total of 72 subjects divided into four groups: 35 T1DM patients, 9 T1DM-obese patients, 16 obese subjects, and 12 as lean healthy subjects. In the T1DM group, the mean age was 11.5 years old with a mean BMI value of 18.8±3.7 kg/m^2 in the average of normal weight and half of the study subjects being males (50%). Qatari nationals accounted for 54.3% of the study subjects and expatriates living in Qatar accounted for 45.7%. The HbA1c average and diabetes duration of the T1DM study subjects were 7.811% , 9 years, respectively. Out of the 36 participants, only 9 subjects are given the Continuous Subcutaneous Insulin Infusion (CSII) treatment. On the other hand, nine participants were in the T1DM-obese group, with a mean age of 10.5 years and a mean BMI value of 28.95±2.0 kg/m^2. The HbA1c average and diabetes duration of the T1DM-obese study subjects were 8.42% and5 years, respectively. Also, only 5 subjects (55.5%) are using CSII. For the obese group (n= 16), the mean age was 11 years with a mean of 33.9±7.4 kg/m^2 for the BMI. Most of the study subjects in this group were males (62.5%) and Qatari nationals (75%). Regarding the healthy group, the mean age was 11 years with an HbA1c mean value of 4.86%. In this group, males account for 41.6% and most of the participants were expatriates living in Qatar (83.3%). The baseline characteristics of the study subjects are shown in table 4.

Table 4. General characteristics of the study subjects.

Parameters	T1DM (n=35)	TIDM-OB (n=9)	Obese (n=16)	Healthy (n=12)
Age in years (mean±std dev)	11.5±2.12	10.5±3.53	11±4.24	11±1.4
Male gender; n (%)	18 (50%)	3 (33.3%)	10 (62.5%)	5 (41.6%)
Nationality; n (%)	Qatari n= 19 (54.3%) Expats n= 16 (45.7%)	Qatari n= 6 (66.6%) Expats n= 3 (33.4%)	Qatari n= 12 (75%) Expats n= 4 (25%)	Qatari n= 2 (16.6%) Expats n= 10 (83.3%)
BMI kg/m^2 (mean±std dev)	18.8±3.7	28.95±2.0	33.9±7.4	17.1±2.7
HbA1c (mean±std dev)	7.811±1.27	8.42±2.44	5.49±0.27	4.86±0.37
CSII n (%)	9 (25.7%)	5 (55.5%)	NA	NA
Diabetes Duration (years; mean±std dev)	9±4.24	5±2.82	NA	NA
Dietary data; n (%)	33 (94.2%)	9 (100%)	16 (100%)	12 (100%)
Clinical data; n (%)	35 (100%)	9 (100%)	14 (87.5%)	9 (75%)
DNA methylation ; n (%)	30 (85.7%)	9 (100%)	16 (100%)	12 (100%)
Gut microbiome data; n (%)	24 (68.5%)	4 (44.4%)	10 (62.5%)	11 (91.6%)
SCFAs data; n (%)	21 (60%)	5 (55.5%)	10 (62.5%)	11 (91.6%)

Note: Values are reported as mean±std dev and in percentage, wherever applicable. CSII: Continuous Subcutaneous Insulin Infusion; HbA1c: Hemoglobin A1c; SCFAs: Short Chain Fatty Acids; NA: not applicable.

4.1.2 Clinical Characteristics analysis of the study subjects

Different physical and chemical measurements were obtained from the study subjects, which are blood pressure measurements, triglycerides, HDL, LDL, liver function tests, vitamin D, and thyroid-related hormones (Table 5). Significance was observed in the measurements of systolic and diastolic blood pressure (SBP/DBP) (One Way ANOVA SBP p-value = 0.0048 / DBP p-value = 0.0060). T1DM-OB and obese subjects had a significantly higher SBP (p-value= 0.0386) and DBP (p-value= 0.0032) respectively in comparison to the T1DM group based on multi-comparison Tukey's test

(Figure 4). For the biochemical analysis, significance was observed in the measurements of LDL, AST, ALT, and TSH (Table 5). T1DM-OB showed a significantly higher level of LDL in comparison to T1DM and obese subjects with a multi-comparison Tukey's p-value of 0.0227 and 0.0116, respectively (Figure 5A). Measurements of TSH level was also observed to be significantly different between the four groups (One way ANOVA p-value = 0.023), but non-parametric Kruskal-Wallis test showed non-significance (Figure 5B). For the liver function tests, significance was only observed in the measurements of AST and ALT (One way ANOVA p-value = 0.012 and 0.0092, respectively). T1DM subjects have significantly lower AST levels in comparison to obese (Kruskal-Wallis test p-value = 0.0287) and higher ALT levels compared to healthy subjects (Tukey's p-value = 0.017), respectively (Figure 5C and D).

Table 5. Physical and clinical characteristics of study subjects

Parameter (mean±std dev)	T1DM	T1DM-OB	Obese	Healthy	One Way ANOVA P-value
Systolic blood pressure (mmHg)	104.0±7.31	112.5±5.31	111.3±9.0	103.3±10.02	0.0048
Diastolic blood pressure(mmHg)	66.39±5.18	69.75±4.92	72.25±5.66	68.67±5.41	0.0060
Triglycerides (mmol/l)	1.04±0.49	1.68±1.3	1.09±0.47	1.31±0.97	ns
HDL (mmol/l)	1.71±0.43	1.35±0.21	1.52±0.62	1.40±0.23	ns
LDL (mmol/l)	2.31±0.68	3.11±0.81	2.12±0.84	2.33±0.60	0.0152
AST (IU/L)	17.91±5.70	22.4±5.83	26.79±10.97	17.22±4.79	0.012
ALT (IU/L)	21.49±5.40	22.2±8.09	26.79±10.97	29.67±4.38	0.0092
TSH (mIU/L)	2.28±1.13	4.58±4.64	3.47±1.87	1.77±1.01	0.023
T4 (pmol/L)	12.12±1.52	11.38±2.60	11.47±1.85	12.44±1.48	0.04

Vitamin D (nmol/L)	51.06±16.81	37.56±8.56	45.11±19.81	39.89±12.63	ns

Note: Values are reported as mean±std dev. P-value was calculated using One Way ANOVA. HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; AST: Aspartate Transaminase; ALT: Alanine Transaminase; TSH: Thyroid Stimulating Hormone; T4: Thyroxine; ns: non-significant.

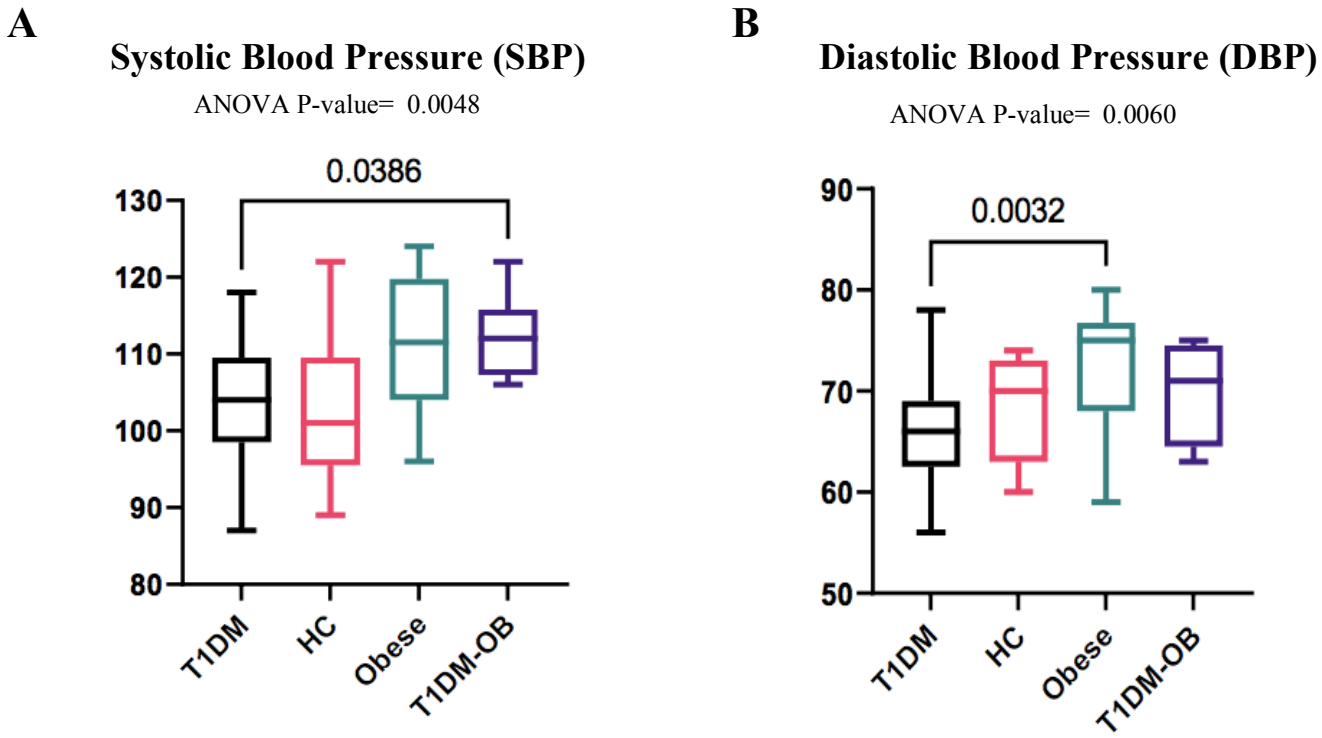


Figure 4. Significant systolic and diastolic blood pressure measurements SBP/DBP between the different study groups.: T1DM, T1DM-OB, obese and healthy controls. T1DM subjects have significantly lower systolic and diastolic blood pressure. P-value was calculated using one-way ANOVA and multi-comparison Tukey's test.

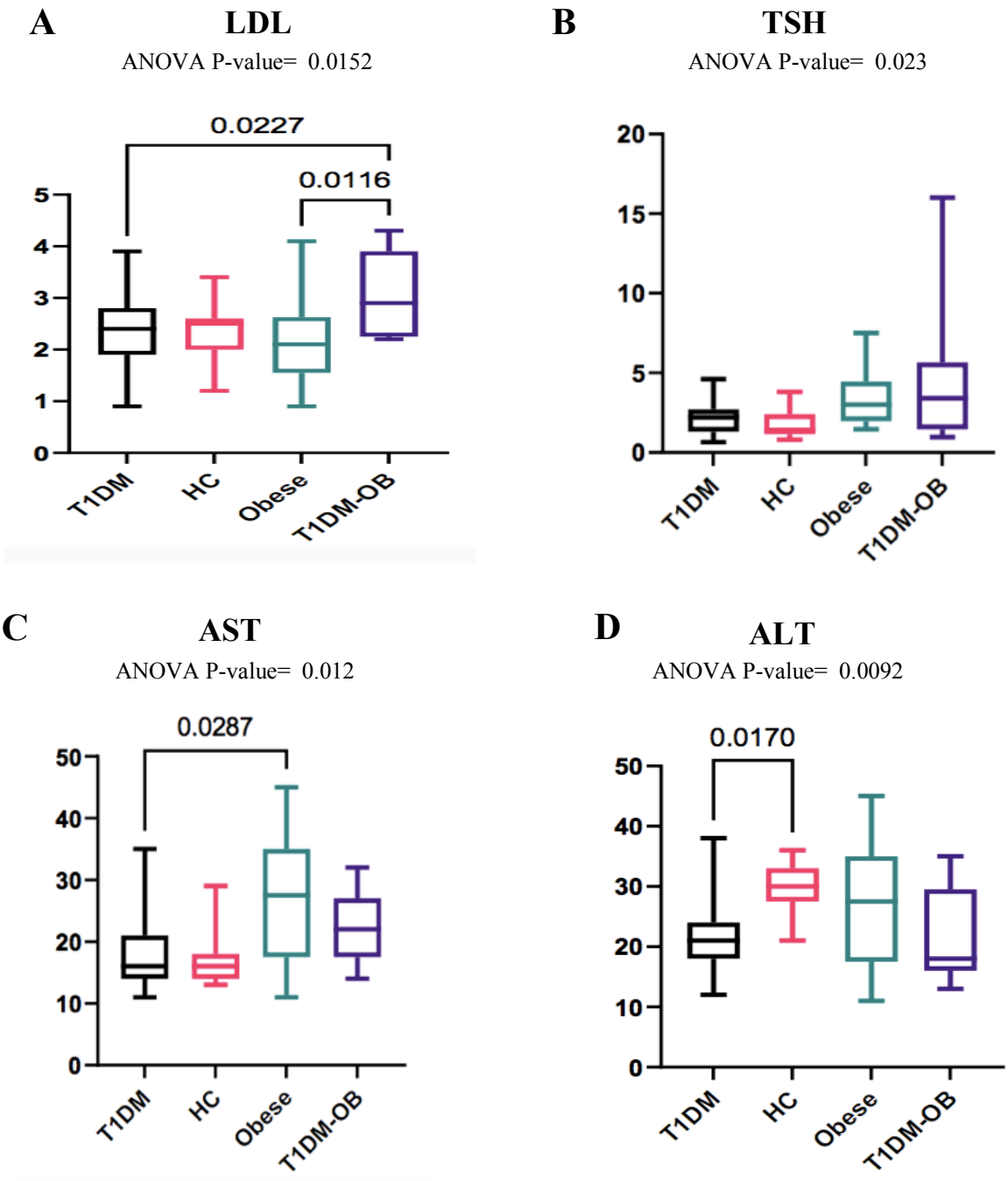


Figure 5. Significant clinical data analysis between the different study groups: T1DM, T1DM-OB, obese and healthy controls. A: LDL levels significantly increased in T1DM-OB compared to T1DM and obese groups. B: difference in the TSH level between the different four groups. C: decreased levels of AST in T1DM subjects compared to obese subjects. D: decreased levels of ALT in T1DM subjects compared to healthy controls. P-value was calculated using Tukey's p-value and the Kruskal-Wallis test was used for non-parametric data (AST and TSH). LDL: Low-Density Lipoprotein; AST: Aspartate Transaminase; ALT: Alanine Transaminase; TSH: Thyroid Stimulating Hormone.

4.1.3 Macronutrient and micronutrient dietary analysis of the study subjects

A 24-hour dietary recall was obtained from each subject and then uploaded on Nutritionist Pro software (Axxya Inc) to determine the significant dietary factors and dietary patterns present in the four groups. Macro- and micro-nutrients were measured, including Kcal, carbohydrates, proteins, fats (SFA, MUFA), sugars (glucose, lactose, etc.), vitamins (vitamin A, C, D, etc.), and minerals (iron, calcium, etc.). The ANOVA analysis showed a significant difference in the intake of saturated fatty acids (SFA), vitamin-K, and pantothenic acid with a p-value of 0.018, 0.0312, and 0.0273, respectively (Table 6). Multigroup comparison test confirmed that T1DM subjects were found to have a significant decrease in the intake of SFA compared to healthy controls (Tukey's p-value= 0.0170) (Figure 6). Whereas vitamin K was significantly lower in the T1DM group compared to the obese (Kruskal-Wallis's p-value = 0.0214) (Figure 6). The intake of SFA in the T1DM group is 9.7% (14.89 g), which is considered close to the recommended intake of SFA (10% of the total Kcal intake) [126]. However, T1DM subjects have a mean value of 12.3 g fiber of the total dietary intake, which is lower than the recommended value (25 g per day) [126].

Table 6. The difference in nutrients intake between study groups.

	T1DM	T1DM-OB	Obese	Healthy	p-value
Kcal	1369±431.4	1331±556.6	1506±686.2	1509±515.7	ns
Protein (g)	60.6±25.23	70.24±39.0	59.1±16.46	49.7±16.9	ns
Carbohydrate (g)	171.2±71.6	147.4±68.3	167.9±102.2	176.8±62.6	ns
Total Fats (g)	47.24±19.5	52.17±23.6	62.17±27.5	63.3±28.65	ns
Cholesterol (g)	229.8±161.8	263.1±195.4	194.8±84.2	130.9±105.3	ns
MUFA (g)	16.8±8.27	18.55±10.83	22.1±11.69	17.9±9.7	ns
PUFA (g)	10.59±6.22	10.1±5.53	10.68±7.3	11.01±7.1	ns
TSFA (g)	0.30±0.33	0.46±0.53	0.76±0.85	0.17±0.2	ns
SFA (g)	14.89±6.298	17.90±9.734	21.01±9.427	24.43±15.67	0.018
Dietary Fiber (g)	12.38±8.8	12.36±6.3	12.64±7.75	13.04±6.03	ns

Total Sugars (g)	53.5±37.2	54.9±45.6	55.6±42.5	71.7±32.5	ns
Calcium (mg)	558±345.2	719.6±496	716.7±385.2	461.2±226.6	ns
Iron (mg)	11.75±8.7	12.9±10.9	16.48±13.9	10.79±5.14	ns
Vitamin A (IU)	3047±4363	5057±7212	2447±1436	2008±3392	ns
Vitamin A (RAE)	400.3±357.9	468.7±423.3	318.2±209.8	312.9±256.9	ns
Beta-Carotene (mcg)	1076±2052	2342±3791	993.1±736.8	1626±2625	ns
Vitamin C (mg)	41.2±32.9	57.2±51.4	69.3±60.8	90.8±107.9	ns
Vitamin D (mcg)	3.76±3.25	3.67±3.4	3.5±3.9	2.87±2.4	ns
Vitamin-K (mcg)	56.37±121.5	91.65±159.6	138.5±177.2	57.20±42.46	0.0312
Vitamin E (mg)	0.81±1.5	0.25±0.28	0.55±0.90	0.74±1.4	ns
Vitamin E (Alpha – Tocopherol) (mg)	4.37±2.3	3.95±2.2	6.3±4.3	4.56±2.9	ns
Thiamin (mg)	1.14±0.7	1.05±0.74	1.09±0.47	1.03±0.39	ns
Folate (mcg)	98.08±50.9	121.6±47.8	125.1±57.5	141.6±88.7	ns
Pantothenic acid (mg)	3.649±1.931	3.669±1.899	2.498±1.319	2.166±0.7914	0.0273

Note: Values are reported as mean±std dev. P-value was calculated using One Way ANOVA. SFA: Saturated Fatty Acid; ns: non significance >0.05

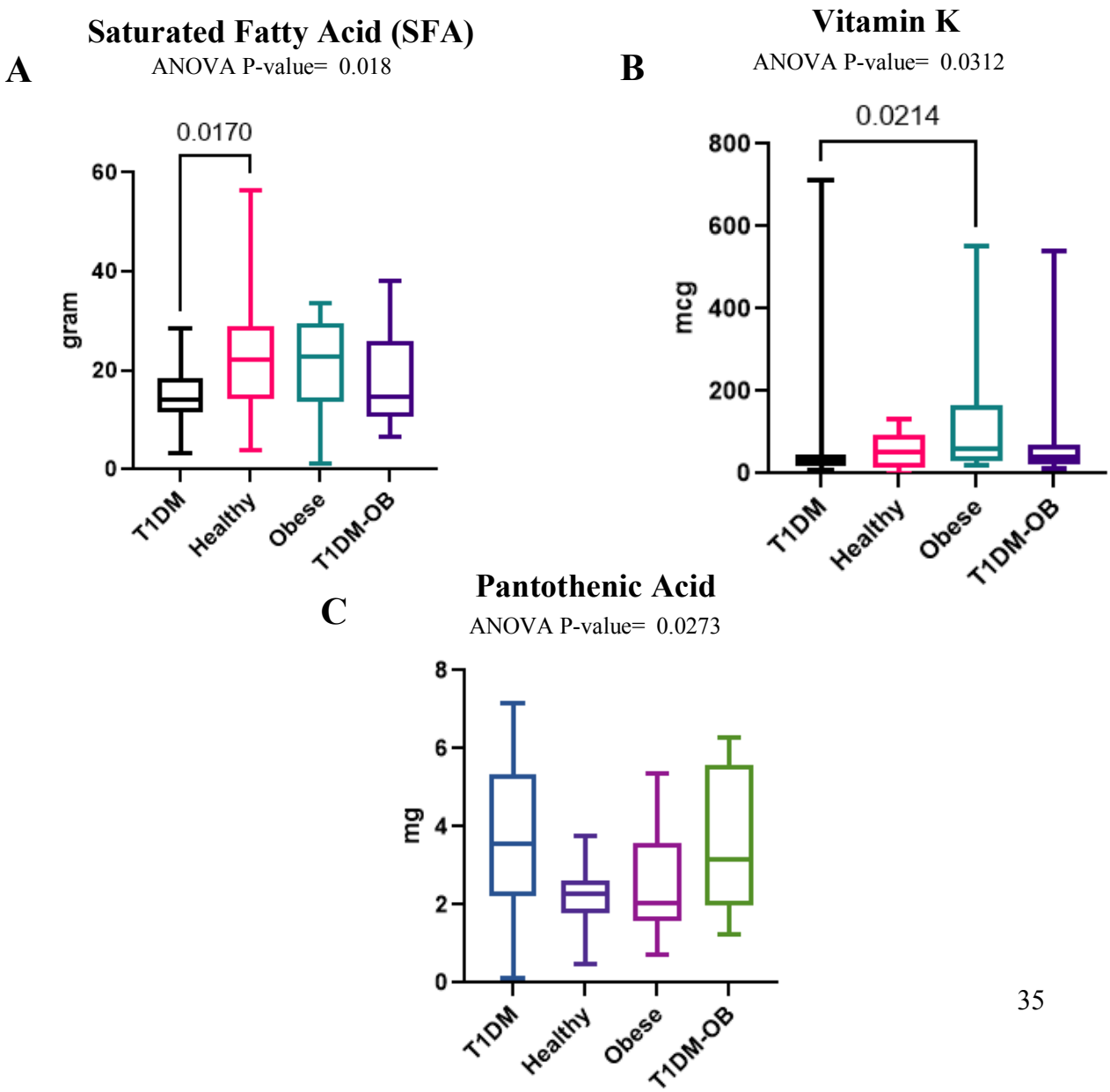


Figure 6. Significant nutrients intake between the different study groups: T1DM, T1DM-OB, obese and healthy controls. A: SFA intake was significantly lower in T1DM subjects compared to healthy. B: vitamin K intake was significantly lower in T1DM subjects compared to obese. C: the difference in the pantothenic acid between the different four groups: T1DM T1DM-OB, obese and healthy. P-value was calculated using Tukey's p-value for SFA and Kruskal-Wallis test for non-parametric data (vitamin K and pantothenic acid). SFA: Saturated Fatty Acid.

4.1.4 Differentially methylated genes detected between the different groups

In order to detect the DNA methylation level at each CpG site from each study subject, raw methylation data generated from the Illumina Infinium array were imported to the Genome Studio software, in which global methylation level was measured from the Beta values. We did not observe any significant variation in the DNA methylation level detected in the four groups. Most of the patients have a methylation level of over 800,000 probes (Appendix B, figure S2). In addition, the comparison between the four groups (T1DM, T1DM-OB, healthy and obese) showed no significance in the CpG methylation levels (Figure 7).

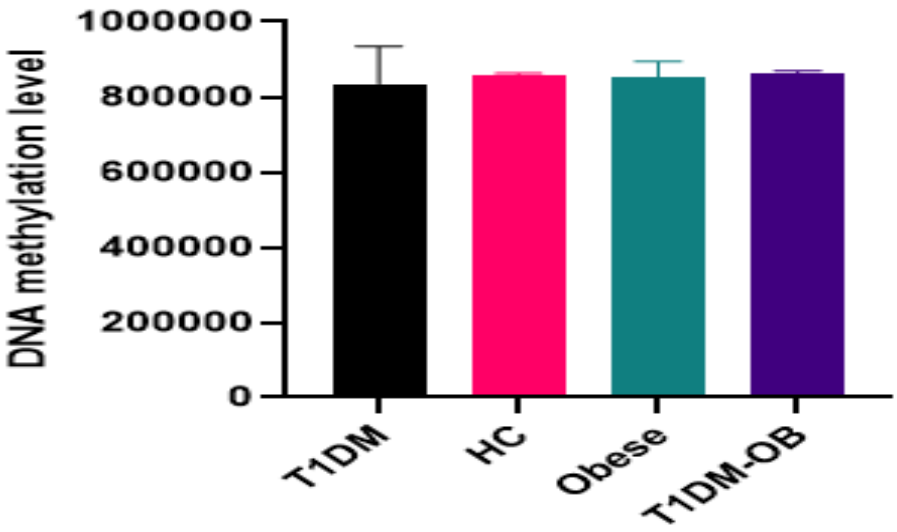


Figure 7. CpG DNA methylation levels between study groups: T1DM, T1DM-OB, Healthy, and Obese with probes with a mean detection level P-value <0.01. One way ANOVA was used to compare between groups. ns: non-significant.

Our next analysis was to determine the differentially methylated genes between the different groups focusing on three main comparisons, which are T1DM vs Healthy, T1DM-OB vs T1DM, and T1DM-OB vs Obese. A total of 1153, 955, and 946 genes with a p-value<0.05 were differentially methylated between T1DM vs Healthy, T1DM-OB vs T1DM, and T1DM-OB vs Obese respectively (Appendix B, Table S1). However, using a more statistically stringent conditions of a P-value <0.05 and a FDR >2 only two differentially methylated genes were detected (Table 7). In our analysis, we have identified the differential methylation of the suppressor APC domain containing 1 (*SAPCD1*) in T1DM patients compared to healthy controls a fold change of 4.9 (P-value= 0.00349). Moreover, we have also observed the differential methylation of DnaJ heat shock protein family (HSP40) member C7 (*DNAJC7*) in T1DM-OB subjects compared to obese with a fold change of -9.1 (P-value = 0.0000000262). In addition, the mean difference (mean±SD) was calculated from Beta values of each gene. For the *SAPCD1* gene a mean difference of 2.29±71.9 was observed between T1DM and healthy control. Whereas, the *DNAJC7* showed a mean difference of 0.22±111.9 between T1DM-OB and obese subjects. No FDR-adjusted genes were identified between T1DM-OB and T1DM.

Table 7. Differentially methylated genes detected between T1DM vs Healthy and T1DM-OB vs Obese.

Gene Symbol	Gene name	Fold change value	Fold change description	Adjusted P-value

SAPCD1	suppressor APC domain containing 1	4.9	T1DM compared to healthy controls	0.00349
DNAJC7	DnaJ heat shock protein family (HSP40) member C7	-9.1	T1DM-OB compared to obese	0.0000000262

Our functional pathway analysis of the FDR-adjusted gene between T1DM and healthy controls conducted by IPA showed no significant canonical pathways. However, three disease pathways were found significant between the two groups, which are cardiovascular diseases, organismal injury and abnormalities, and reproductive system diseases (Figure 8). In the case of T1DM-OB, we have identified some significant canonical pathways in comparison to the obese group, which are aldosterone signaling in epithelial cells, xenobiotic metabolism CAR/PXR pathways, NRF2 mediated oxidative stress response, and protein ubiquitination pathway (Figure 9). Moreover, a number of significant diseases were identified between the T1DM-OB and the obese group, which are gastrointestinal disease, hepatic system, metabolic disease, organismal injury and abnormalities, cancer, and reproductive system diseases (Figure 10A). Molecular and cellular function analysis showed several functions to be significantly different between the two groups, which are post-translational modifications, protein folding, lipid metabolism, molecular transport, and small molecule biosynthesis (Figure 10B).

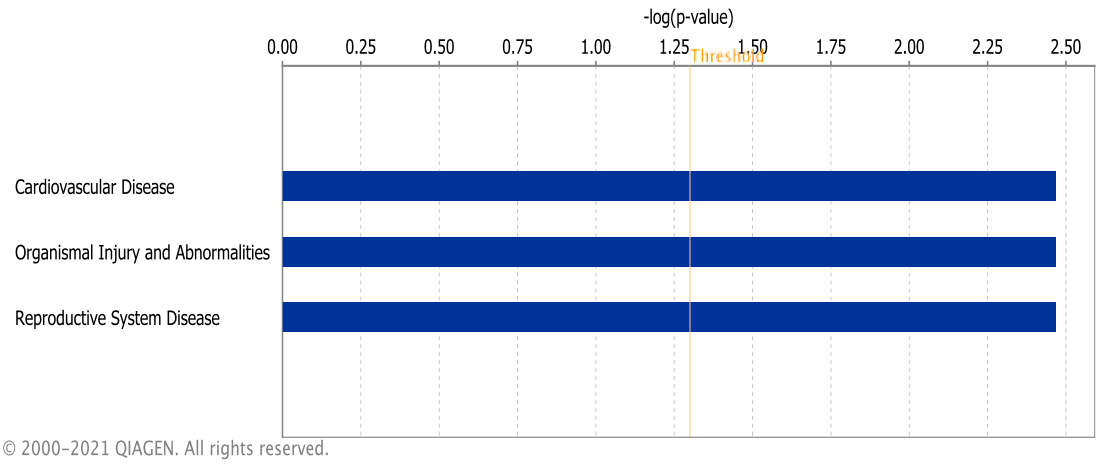


Figure 8. Significant disease pathways detected between T1DM and healthy controls generated by IPA

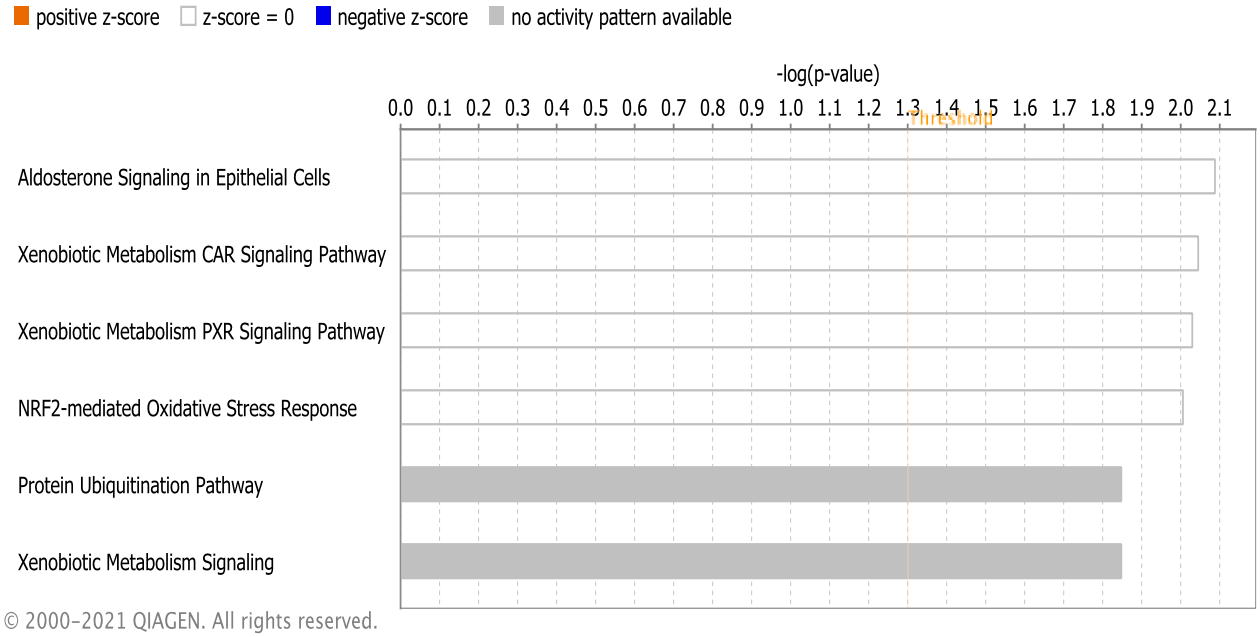
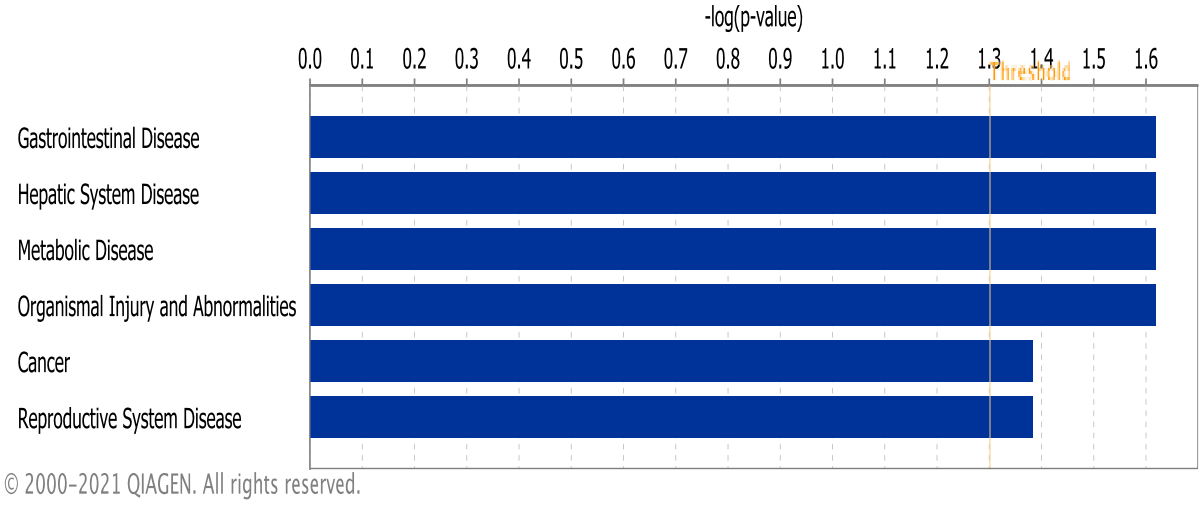


Figure 9. Significant canonical pathways detected between T1DM-OB and obese subjects generated by IPA.

A



B

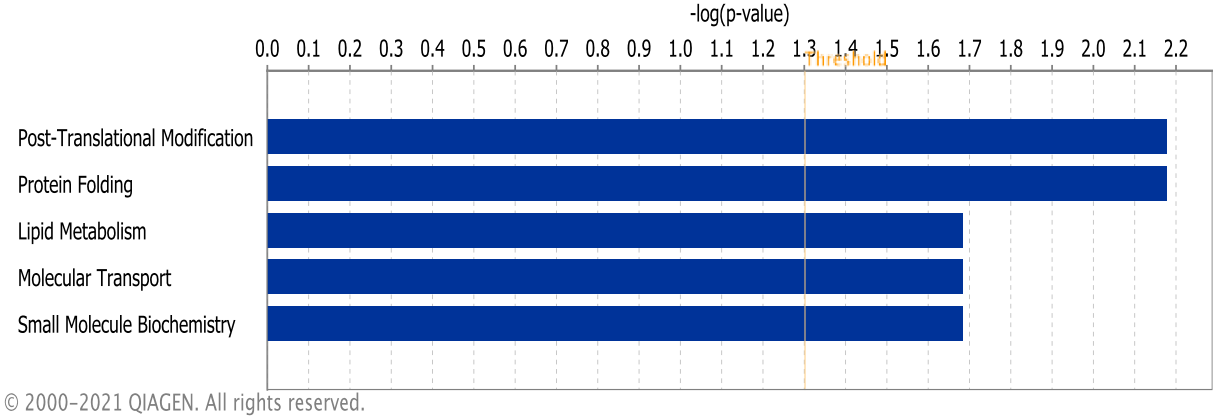


Figure 10. Significant disease pathways (A) and molecular and cellular functions (B) detected between T1DM-OB and obese subjects generated by IPA.

4.1.5 Gut microbial composition and SCFA analysis of the study subjects

The gut microbial composition and abundance were previously analyzed in our study groups. Gut microbial relative abundance in the phylum and genus level is presented in Appendix C, figure S3. Gut microbial diversity analysis, measured by the alpha-diversity, showed significance in the genus richness (Observed and Chao1 indexes) among the four different groups (Observed p-value = 0.0004; Chao1 p-value = 0.0003) (Figure 11). We have also observed a significant lower microbial abundance and genus richness in T1DM subjects in comparison to healthy controls (Observed p-value = 0.002; Chao1 p-value = 0.002; Shannon p-value = 0.045; Simpson p-value= 0.020) (Figure 10). No difference was observed in the beta-diversity analysis among the four different groups (Figure 12).

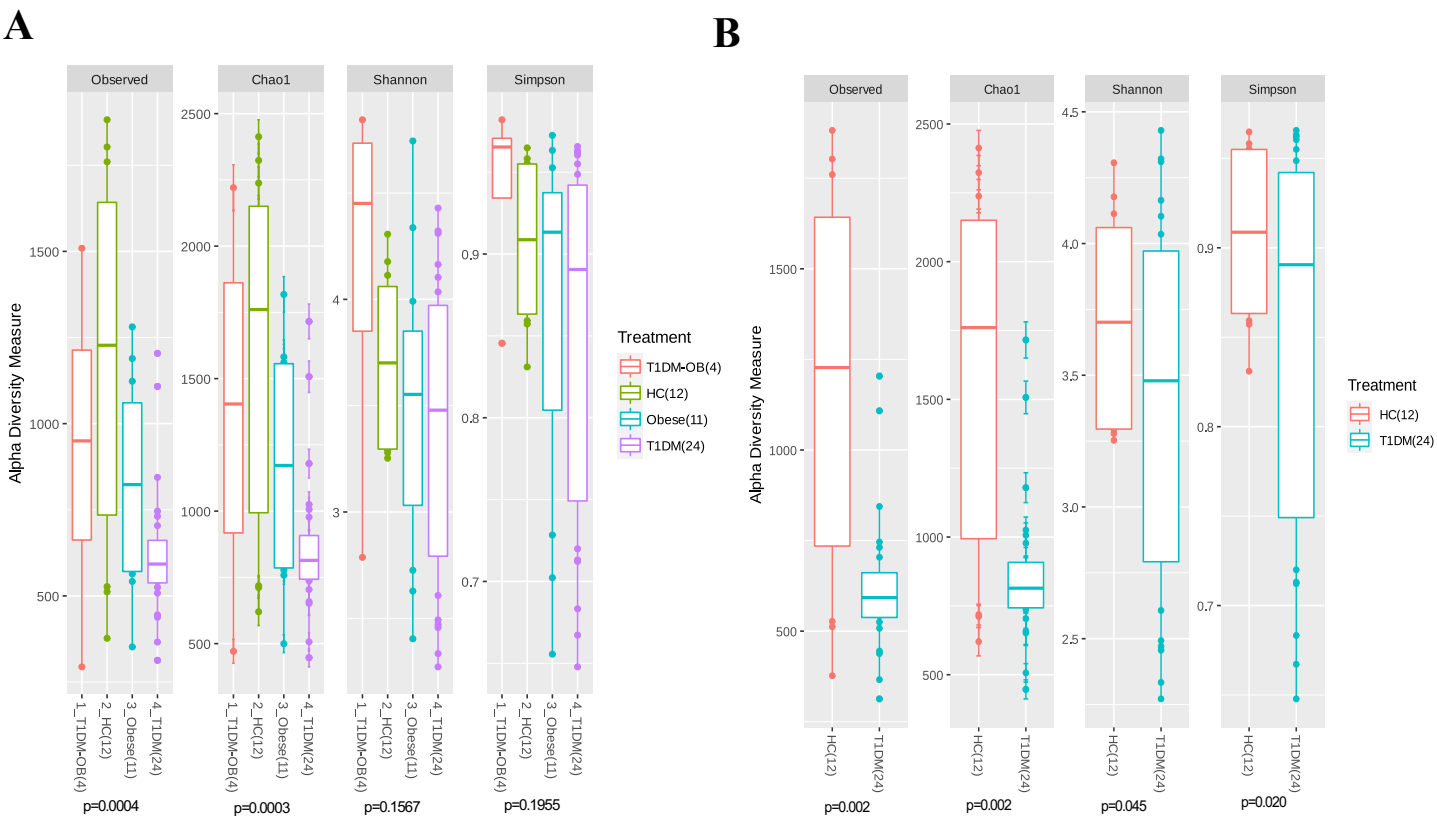


Figure 11. Alpha-diversity (gut microbiome) analysis of the study subjects. A: significance in the genus richness (Observed and Chao1 indexes) among the four different groups. B: T1DM subjects showed lower microbial abundance and genus richness compared to the healthy group.

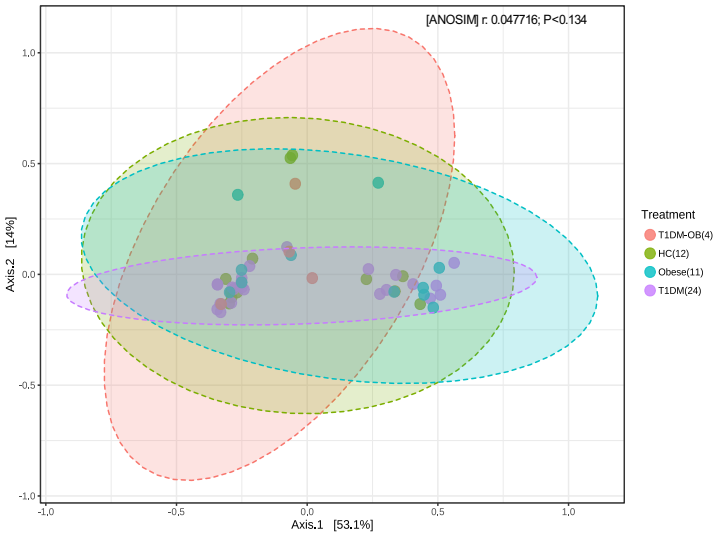


Figure 12. Beta-diversity (gut microbiome) analysis of the study subjects. No difference was observed in the beta diversity among the four groups. Beta-diversity was visualized using Principle co-ordinations generated with the Bray–Curtis distance metric by QIIME. Analysis of group similarity (ANOSIM) was measured between categories included in this study using 1000 permutations.

Furthermore, in this study, LEfSe analysis was used for the identification of the significant gut microbial markers between the different study groups with the cutoff value of LDA >2.0. *Rikenellaceae*, *Christensenellaceae*, *Weissella*, *Lactobacillales*, *Anaerofustis*, *Leuconostocaceae,* and *Brachyspira* were found to be significantly enriched in the healthy controls compared to the T1DM patients (Figure 13). No specific gut microbial marker was identified for the T1DM group. In regard to the T1DM-OB group, *Roseburia*, *Bifidobacterium*, *Neisseria*, *Blautia*, *Lachnospiraceae*, among others, were found to be significantly enriched in comparison to obese subjects that is characterized only by *Odoribacter*. (Figure 14A). Whether *Roseburia*,

Bifidobacterium, *Blautia*, *Lachnospiraceae, among others,* were found to be significantly enriched in T1DM-OB compared to T1DM patients (Figure 14B).

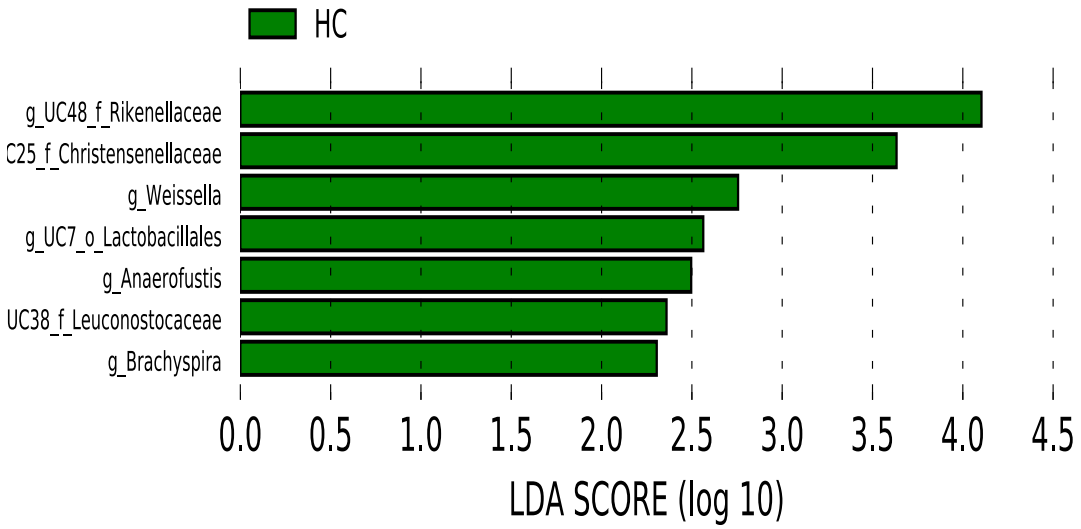
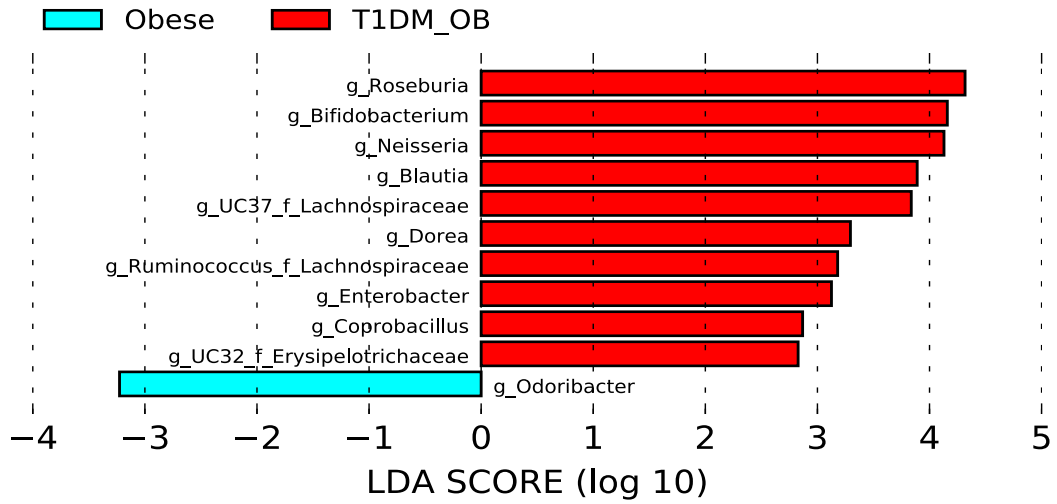


Figure 13. Significant gut microbial markers between T1DM and healthy controls. Significant enrichment of *Rikenellaceae*, *Christensenellaceae*, *Weissella*, *Lactobacillales*, *Anaerofustis*, *Leuconostocaceae,* and *Brachyspira* in the healthy controls. A cutoff value of LDA >2.0.

A



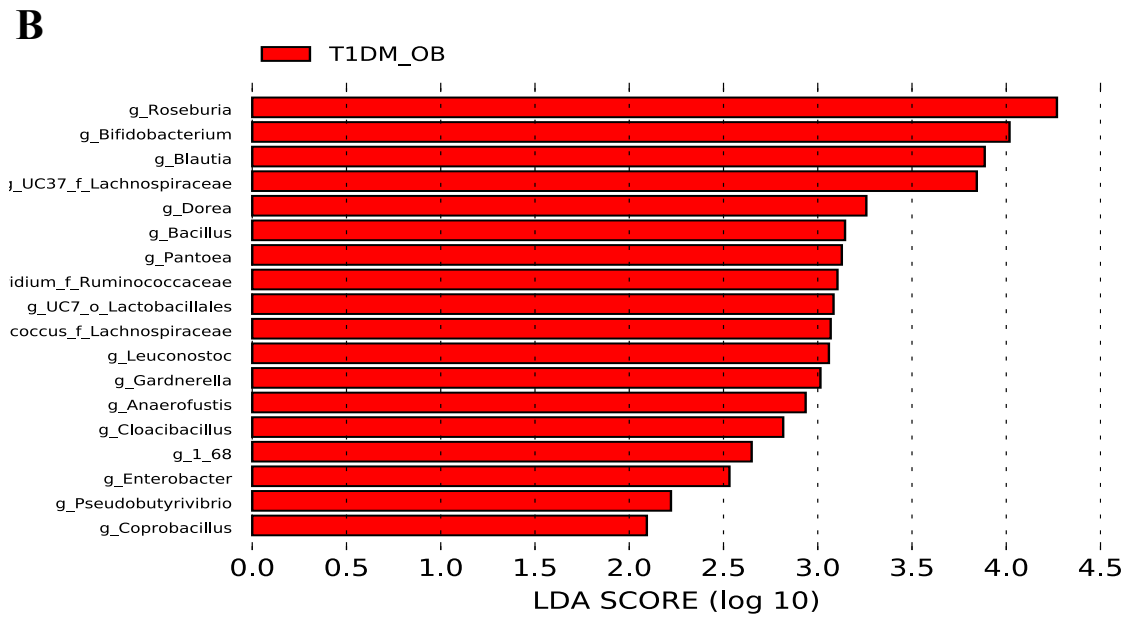


Figure 14. Significant gut microbial markers between T1DM-OB with obese and T1DM subjects. A: significant enrichment of *Roseburia*, *Bifidobacterium*, *Neisseria*, *Blautia*, *Lachnospiraceae*, *etc.* in T1DM-OB compared to obese subjects. B: significant enrichment of *Roseburia*, *Bifidobacterium*, *Blautia*, *Lachnospiraceae*, *etc*. in T1DM-OB compared to T1DM subjects. A cutoff value of LDA >2.0.

The metabolic analysis was also conducted to measure the concentration of SCFAs in the study subjects and determine the significant microbial metabolites among the four groups. The SCFAs measured in this study were ethanoic acid, propionic acid, isobutanoic acid, butanoic acid, 2-methylbutanoic acid, isopentanoic acid, pentanoic acid, 3-methylpentanoic acid, 4-methylpentanoic acid, and hexanoic acid. Based on ANOVA analysis only butanoic acid showed significance that was significantly increased in T1DM-OB compared to the healthy control (One Way ANOVA p-value = 0.0253; Kruskal-Wallis p-value = 0.016) (Figure 15).

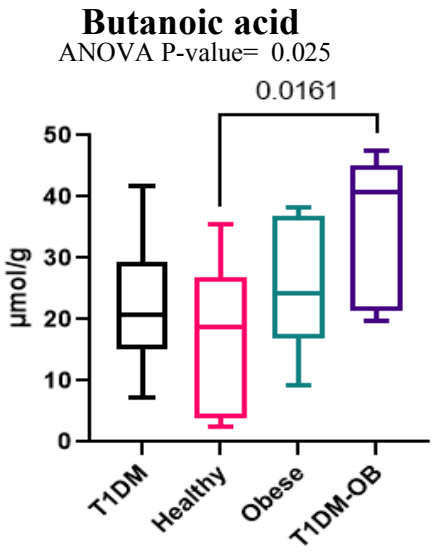


Figure 15. The difference in the butanoic acid between the different four groups: T1DM T1DM-OB, obese and healthy controls. Butanoic acid was significantly increased in the T1DM-OB group compared to healthy controls. P-value was calculated using the Kruskal-Wallis test.

4.1.6 Correlation between DNA methylation with dietary factors, gut microbiome, and SCFAs using network analysis

To identify the potential link of DNA methylation with diet and gut microbial composition in the development of T1DM disease, we have conducted network analysis between CpG methylation levels from the study subjects of the four groups with dietary factors and the gut microbiome. No network association was found between diet and DNA methylation in T1DM, T1DM-OB, and obese groups. However, in the healthy controls, a positive correlation was detected between CpG methylation level and folate and thiamin intake (Figure 16).

Regarding the association between DNA methylation level and gut microbiome, no network association was found in the T1DM-OB group. In contrast, T1DM patients showed a negative correlation between detected CpG methylation level and *Faecalibacterium* (Figure 17 A). In the healthy controls, a negative correlation was detected between methylation level with *Butyricimonas*, *Lachnospira*, and *Clostridium*

(Figure 17 B). However, *Lachnospira* was found to be positively correlated with methylation level in the obese group. Network analysis also showed a negative correlation between methylation and *Odoribacter* in Obese subjects (Figure 17 C). Furthermore, network analysis showed no association between CpG methylation levels and SCFAs.

We have also conducted network analysis of CpG methylation levels with HbA1c and diabetes duration, however no significant correlation was observed. CpG methylation level was found to be positively correlated with HbA1c and negatively correlated with diabetes duration in T1DM-OB subjects (P-value = < 0.1) (Appendix B, figure S3).

Figure 16. Network analysis between CpG methylation level and dietary factors in healthy controls. Note: blue line indicates a positive correlation, the red line indicates negative correlation.

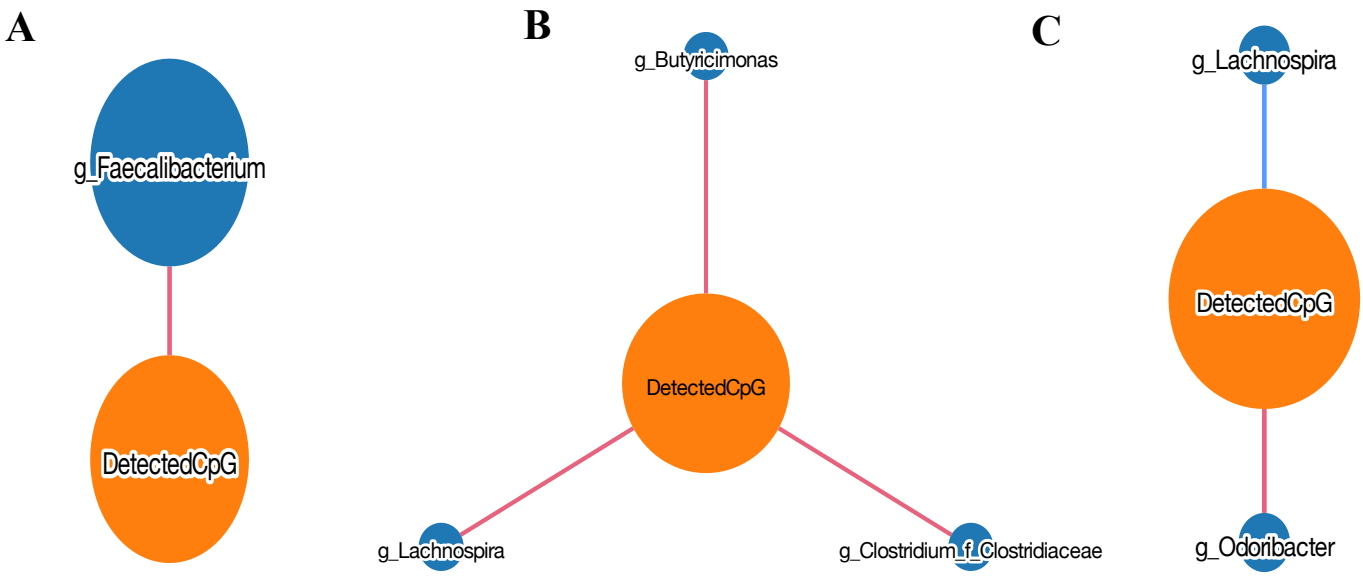


Figure 17. Network analysis between CpG methylation level and the gut microbiome. A: correlation observed between methylation level and microbial genus in T1DM subjects. B: correlation observed between methylation level and microbial genus in healthy controls. C: correlation observed between methylation level and microbial genus in obese subjects. Note: blue line indicates a positive correlation, the red line indicates a negative correlation.

4.1.7 Correlation between dietary factors and gut microbiome using network analysis

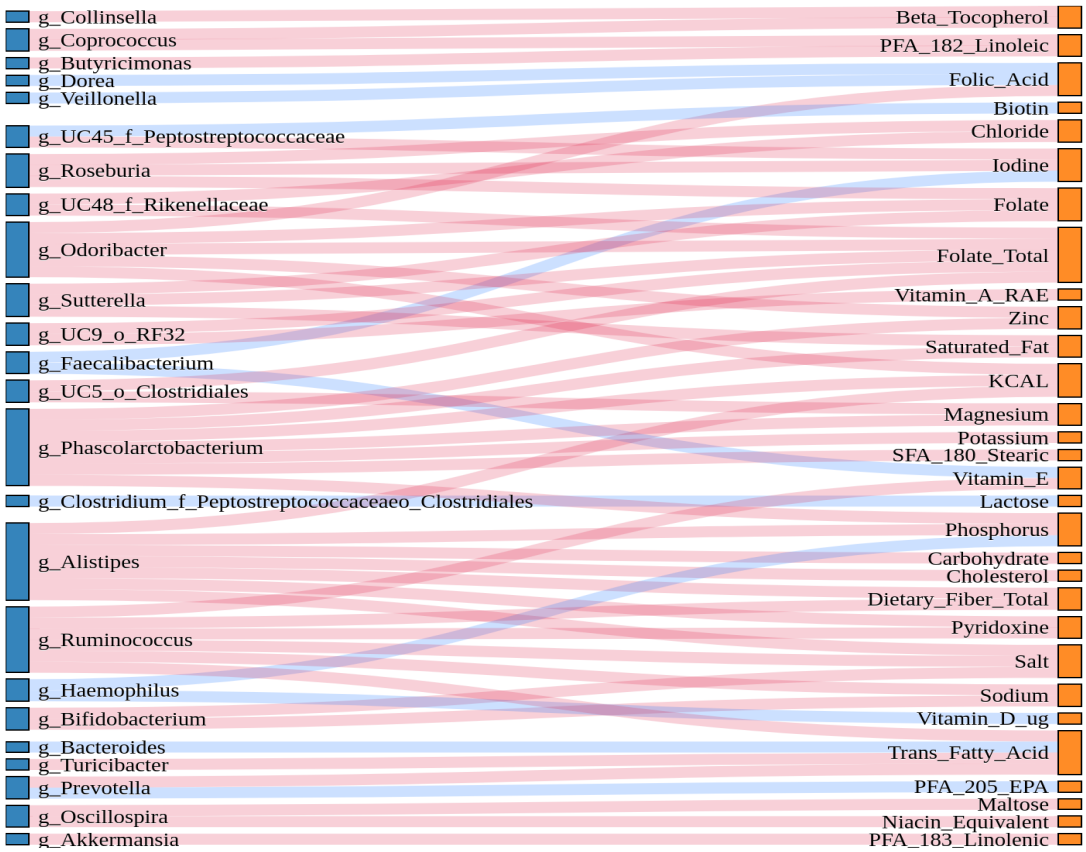
Network analysis was conducted to identify the potential correlation of gut microbiome with different nutritional factors in all the study groups. In the T1DM group, a positive correlation was found with few dietary factors, which are folic acid, biotin, vitamin E, iodine, lactose, vitamin D, phosphorus, and trans-fatty acids (Figure 18A). Folic acid was found to be positively correlated with *Dorea*, and *Veillonella* in the T1DM subjects. However, a negative correlation was observed between total folate intake with *Suttterella, Odoribacter, Rikenellaceae,* and *Clostridiales*. Also, both iodine and vitamin E were positively correlated with *Faecalibacterium*. In regard to vitamin D and phosphorus, both were positively correlated with *Haemophilus*. A

positive correlation was also observed between biotin and *Peptostreptococcaceae*; lactose and *Peptostreptococcaceaeo*; and trans fatty acids with *Bacteroides*. On the other hand, SFA was found to be negatively correlated with *Sutterella* and *Phascolarctobacterium* in the T1DM subjects. Dietary fiber was found to be negatively correlated with *Alistipes* and *Ruminococcus*.

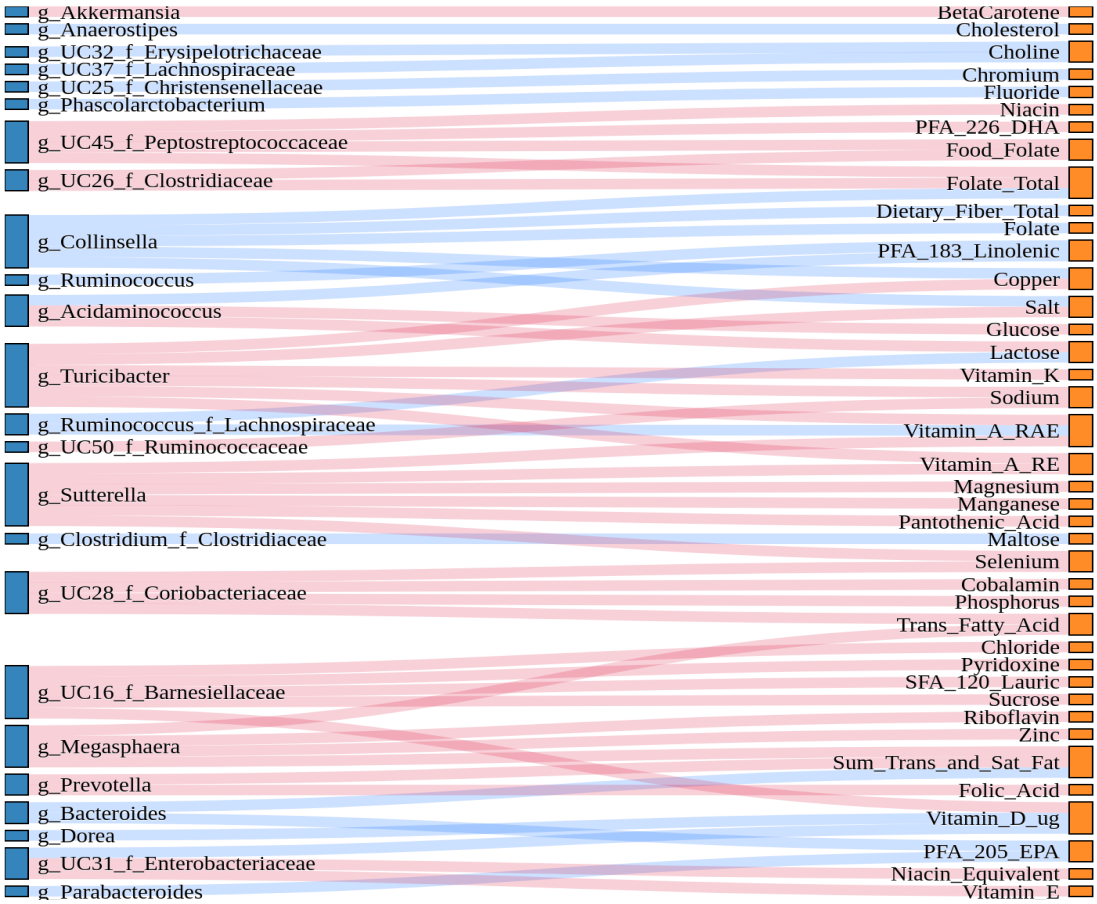
Similar to the T1DM group, healthy controls showed a positive correlation with vitamin D, but with a different microbial genus, which is *Dorea.* Unlike the T1DM group, healthy controls showed a negative correlation between folic acid and *Prevotella*. Total folate was observed to be negatively correlated with *Peptostreptococcaceaeo* and *Clostridiaceae*. In addition, in healthy controls, both vitamin K and pantothenic acid were found to be negatively correlated with *Turicibacter* and *Suttterella*, respectively (Figure 18B).

In regard to the obese group, no network association was observed between dietary intake and microbial composition. In the case of T1DM-OB subjects, SFA was found to be positively correlated with *Coprococcus* and *Dialister* and negatively correlated with *Bacteroides*. Unlike T1DM patients, a positive correlation was observed between total folate level and *Lachnospira.* Also, pantothenic acid showed a positive correlation with *Lanchnospiraceae* and a negative correlation with *Parabacteroids*. In T1DM-OB, *Suttterella* was only observed to be negatively correlated with riboflavin (Figure 18C).

A


g_Collinsella
g_Coprococcus
g_Butyricimonas
g_Dorea
g_Veillonella
g_UC45_f_Peptostreptococcaceae
g_Roseburia
g_UC48_f_Rikenellaceae
g_Odoribacter
g_Sutterella
g_UC9_o_RF32
g_Faecalibacterium
g_UC5_o_Clostridiales
g_Phascolarctobacterium
g_Clostridium_f_Peptostreptococcaceaeo_Clostridiales
g_Alistipes
g_Ruminococcus
g_Haemophilus
g_Bifidobacterium
g_Bacteroides
g_Turicibacter
g_Prevotella
g_Oscillospira
g_Akkermansia
Beta_Tocopherol
PFA_182_Linoleic
Folic_Acid
Biotin
Chloride
Iodine
Folate
Folate_Total
Vitamin_A_RAE
Zinc
Saturated_Fat
KCAL
Magnesium
Potassium
SFA_180_Stearic
Vitamin_E
Lactose
Phosphorus
Carbohydrate
Cholesterol
Dietary_Fiber_Total
Pyridoxine
Salt
Sodium
Vitamin_D_ug
Trans_Fatty_Acid
PFA_205_EPA
Maltose
Niacin_Equivalent
PFA_183_Linolenic

B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g_Anaerostipes
g_UC32_f_Erysipelotrichaceae
g_UC37_f_Lachnospiraceae
g_UC25_f_Christensenellaceae
g_Phascolarctobacterium
g_UC45_f_Peptostreptococcaceae
g_UC26_f_Clostridiaceae
g_Collinsella
g_Ruminococcus
g_Acidaminococcus
g_Turicibacter
g_Ruminococcus_f_Lachnospiraceae
g_UC50_f_Ruminococcaceae
g_Sutterella
g_Clostridium_f_Clostridiaceae
g_UC28_f_Coriobacteriaceae
g_UC16_f_Barnesiellaceae
g_Megasphaera
g_Prevotella
g_Bacteroides
g_Dorea
g_UC31_f_Enterobacteriaceae
g_Parabacteroides
BetaCarotene
Cholesterol
Choline
Chromium
Fluoride
Niacin
PFA_226_DHA
Food_Folate
Folate_Total
Dietary_Fiber_Total
Folate
PFA_183_Linolenic
Copper
Salt
Glucose
Lactose
Vitamin_K
Sodium
Vitamin_A_RAE
Vitamin_A_RE
Magnesium
Manganese
Pantothenic_Acid
Maltose
Selenium
Cobalamin
Phosphorus
Trans_Fatty_Acid
Chloride
Pyridoxine
SFA_120_Lauric
Sucrose
Riboflavin
Zinc
Sum_Trans_and_Sat_Fat
Folic_Acid
Vitamin_D_ug
PFA_205_EPA
Niacin_Equivalent
Vitamin_E

C

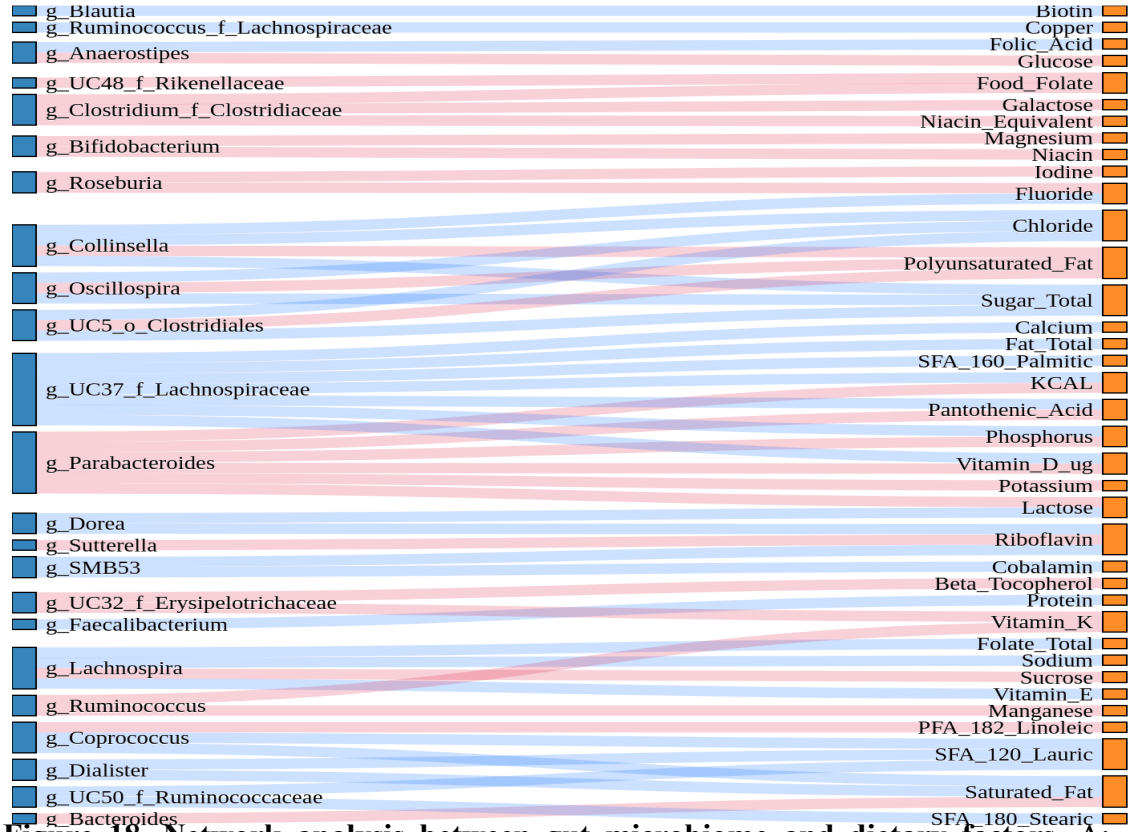


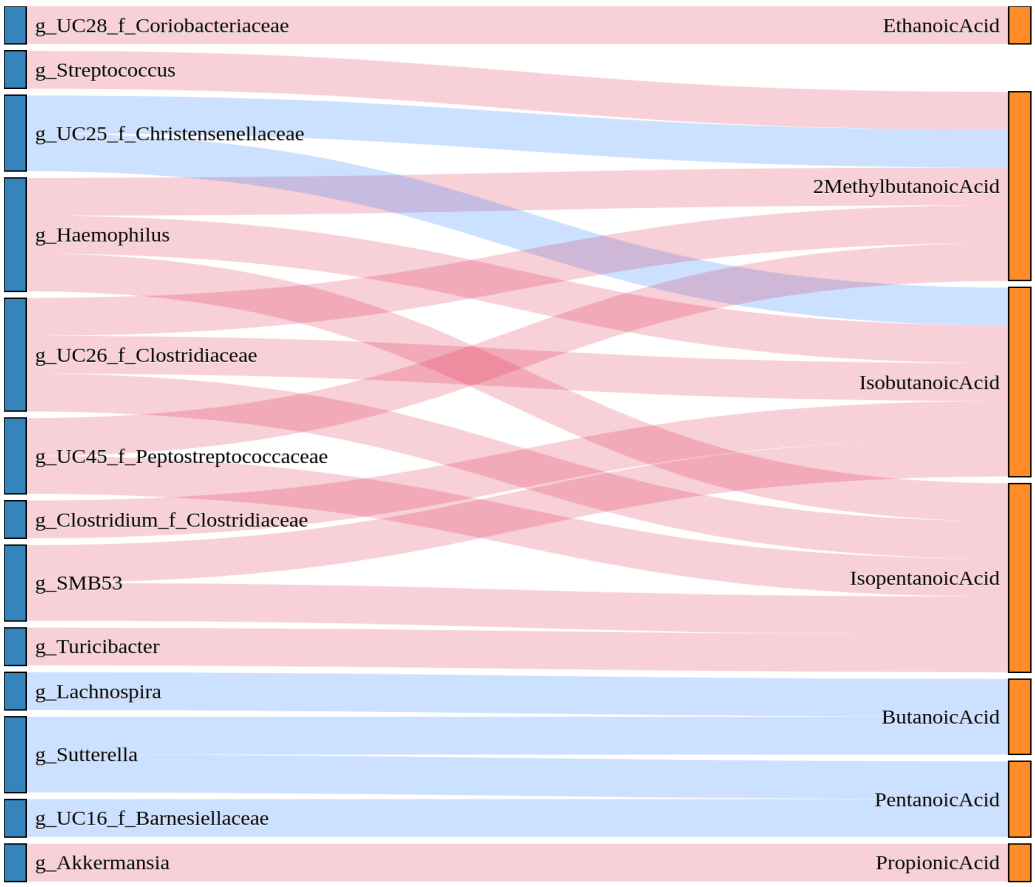
Figure 18. Network analysis between gut microbiome and dietary factors. A: correlation observed between microbial genus and diet in T1DM subjects. B: correlation observed between microbial genus and diet in healthy controls. C: correlation observed between microbial genus and diet in T1DM-OB subjects. Note: blue line indicates positive correlation, red line indicates negative correlation.

4.1.8 Correlation between SCFAs and gut microbiome using network analysis

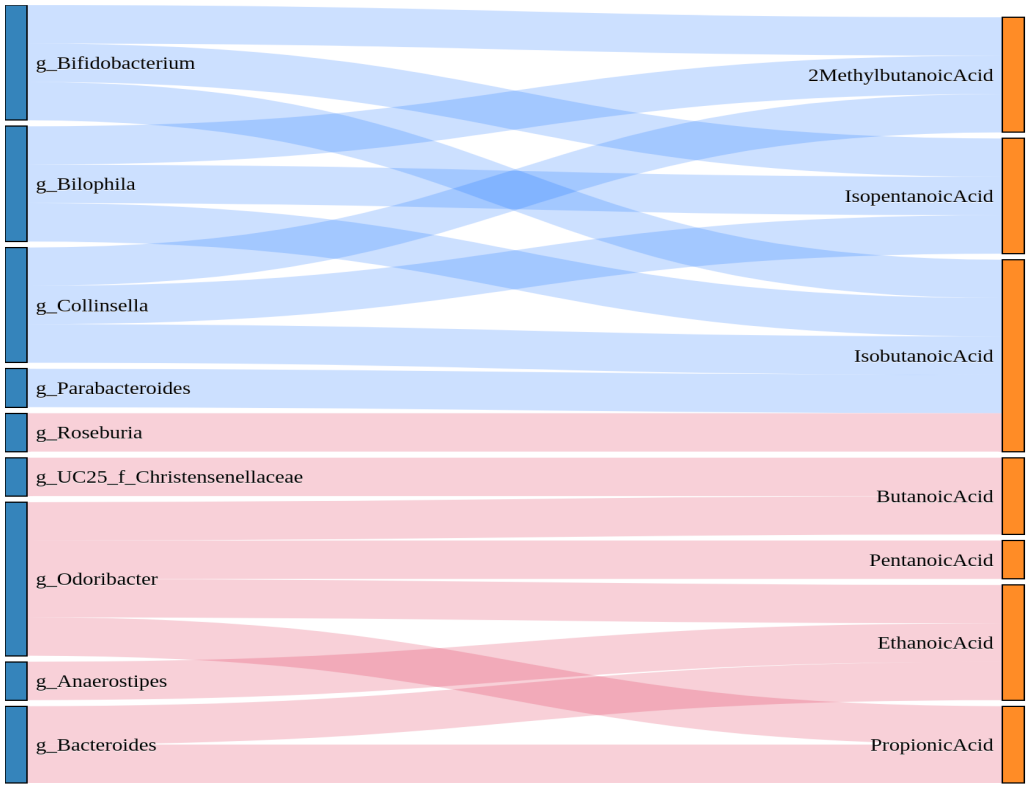
Network analysis was also conducted to determine the possible correlation of gut microbiome with SCFAs for the four study groups. The butanoic acid was found to be positively correlated with the genus *Lachnospira* and *Sutterella* in the T1DM subjects (Figure 19A). In addition, positive correlation was found between pentanoic acid with *Sutterella* and *Bamesiellaceae*. However, in the healthy controls, butanoic acid and pentanoic acid were found to be negatively correlated with *Christensenellaceae* and

Odoribacter (Figure 19B). Similar to the T1DM group, T1DM-OB subjects showed a positive correlation with pentanoic acid, but with different microbial genus, which are *Blautia* and *Erysipelotrichaceae* (Figure 19C). Unlike T1DM, *Sutterella* showed a negative correlation with propionic acid in the T1DM-OB group. In addition, 2-methylbutanoic acid was found to be positively and negatively correlated with Ruminococcaceae and Bacteroides respectively. However, in the obese group, 2-methylbutanoic acid was found to be negatively correlated with *SMB53* (Figure 19D).

A



B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g_Parabacteroides
g_Roseburia
g_UC25_f_Christensenellaceae
g_Odoribacter
g_Anaerostipes
g_Bacteroides
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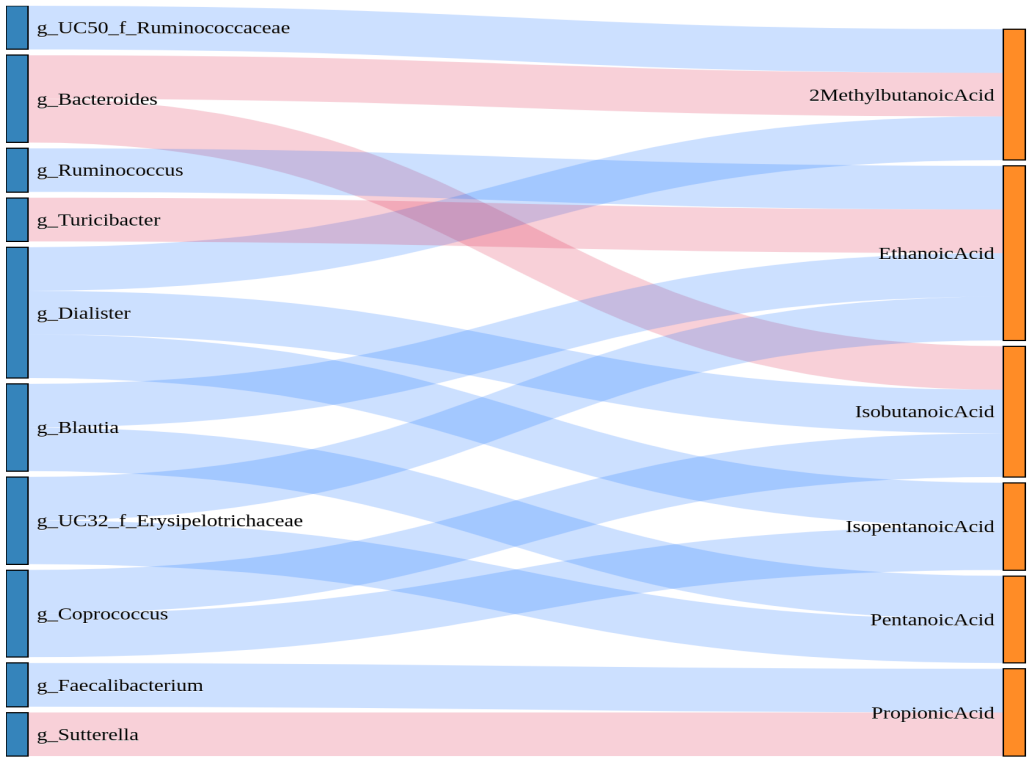
IsopentanoicAcid
IsobutanoicAcid
ButanoicAcid
PentanoicAcid
EthanoicAcid
PropionicAcid

C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g_Ruminococcus
g_Turicibacter
g_Dialister
g_Blautia
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g_Coprococcus
g_Faecalibacterium
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EthanoicAcid
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IsopentanoicAcid
PentanoicAcid
PropionicAcid

D

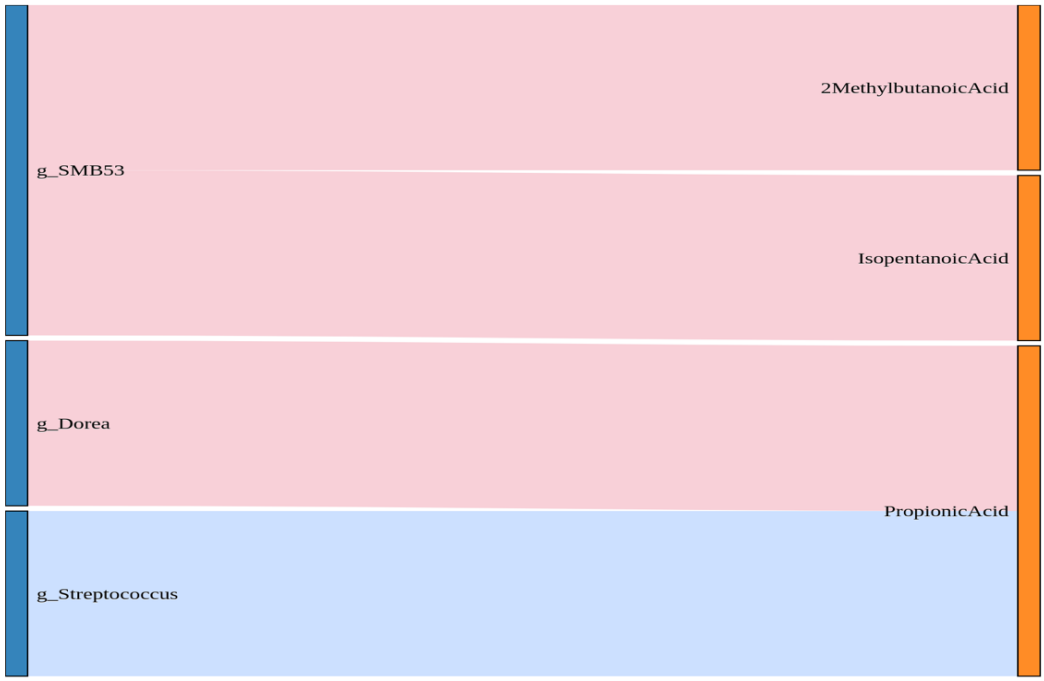

Figure 19. Network analysis between gut microbiome and SCFAs. A: correlation observed between microbial genus and SCFAs in T1DM subjects. B: correlation observed between microbial genus and SCFAs in healthy controls. C: correlation observed between microbial genus and SCFAs in T1DM-OB subjects. D: correlation observed between microbial genus and SCFAs in obese. Note: blue line indicates positive correlation, red line indicates negative correlation

4.2 Discussion

T1DM is a disease of childhood, affecting mainly children and adolescence [14] with an increasing incident rate worldwide [17]. The exact pathophysiology of the disease is complex and has been associated with the interaction of multiple factors, mainly genetics. However environmental factors, such as diet and infections can contribute to affecting the epigenetic modifications and the gut microbiome composition [5]. In this study, we aimed to identify the different diet intake and differentially methylated genes present in T1DM patients compared to healthy children. Secondly, we aimed to investigate the possible interaction between diet, DNA

methylation, and gut microbiome in the development of pediatric T1DM. Up to our knowledge, this study is considered the first study to identify differentially methylated genes in T1DM children in Qatar and to show a possible correlation with dietary habits and gut microbial profiles in the development of the disease.

Based on the clinical data analysis, a significant increase in blood pressure was observed in obese and T1DM-OB subjects compared to the T1DM group (Figure 3). Similarly, a study conducted by Parker and colleagues found that obese children and adolescents have high blood pressure and an increased risk of developing hypertension later in life compared with children with a low BMI [127]. Moreover, a systematic review analysis showed that the presence of overweight or obese phenotype during childhood is positively correlated with an increased risk of developing hypertension in adulthood [128]. Adipose tissue dysfunction and the imbalance between pro/anti-inflammatory activities of adipocytes observed in obesity are what contribute to high blood pressure and elevated risk of hypertension [129]. In addition, from the lipid panel tests, only LDL levels were significant between the four study groups, in which it was significantly elevated in T1DM-OB compared to obese and T1DM subjects (Figure 4). Various studies have shown that obese T1DM children have elevated levels of LDL along with high blood pressure, increasing their risk of developing hypertension and cardiovascular diseases [130, 131]. Therefore, indicating the possible involvement of obesity in modulating the risk of obese children with or without T1DM to develop hypertension and other cardiovascular morbidities.

In this study, we have also observed a significant elevation in the levels of TSH in T1DM-OB and obese subjects when using the Kruskal-Wallis test (Figure 4). However, the multi-comparison analysis showed no significance, which could be due

to the differences in the variability and sample size among the groups. Based on the literature, elevated levels of TSH is common among obese children and could result as a consequence of obesity since those patients have a low incidence of developing thyroid autoantibodies [132]. Regarding the liver function test panel, both AST and ALT levels were found to be significantly decreased in our T1DM children in comparison to obese and healthy subjects (Figure 4). A possible explanation for the increased ALT levels in our healthy controls could be due to their high dietary intake of SFA as 75% of them consume a western-like diet with a total SFA % intake being 14.6 % (>10% of the recommended). Various published data have shown that high intake of SFA induces liver fat content and liver enzymes [133, 134]. In regard to the elevated AST levels observed in our obese children, it aligns with the data from the literature. A recent study conducted by Pirimoglu and colleagues showed that obese and overweight children have elevated levels of both ALT and AST and it is positively correlated with liver fat fraction [135]. Another study showed that measurements of ALT/AST ratio along with family history could be characteristic biomarkers for the development of diabetes in obese Japanese children [136].

Furthermore, another possible explanation for the significant elevation observed in the clinical findings of our patients is due to the fact that they are in the growth and developmental stage of becoming young adolescence, which is associated with elevated levels of thyroid related hormones and liver related parameters. For example, several studies have showed that puberty could induce changes in thyroid hormone levels, and be markedly different between children and young adults [137, 138]. Similar to thyroid levels, liver function tests (AST and ALT) were also found to be affected by growth and development of children [139].

In the dietary analysis, we have observed a significant decrease in the intake of SFA and vitamin-K in the T1DM subjects compared to the control groups (Table 6; Figure 5). Based on different studies, the high intake of SFA is found to be positively correlated with advanced ß-cell autoimmunity, insulin resistance, and obesity in children [46, 140]. In this study, the % intake of SFA in the T1DM group is 9.7%, which is considered close to the recommended intake of SFA (10% of the total Kcal intake) [126]. Unlike our T1DM subjects, different studies have shown that T1DM patients don't follow the recommended SFA dietary guidelines. For example, studies conducted in Australia, Norway, and US showed that the majority of T1DM children have significant overconsumption of SFA [141-143]. However, the significant decrease seen in the intake of SFA in our T1DM group could be possibly due to the fact that the T1DM patients follow medical recommendations and the healthy controls don't have a healthy lifestyle as 75% of them consume a western-like diet with a high SFA intake being 14.6 % , thus explaining their elevated ALT levels (Table 7).

Various studies have shown the important role of vitamin-K in glycemic regulation and insulin sensitivity. It is suggested that vitamin-K intake is associated with improved glucose levels as higher intake was correlated with higher insulin sensitivity and better glycemic control [144]. In a T1DM rat model, administration of vitamin-K prevented the development of hyperglycemia and cancellous osteopenia [145]. Another study showed the same conclusion, where T1DM rats treated with vitamin-K have improved insulin secretion and normal levels of glucose and HbA1c [146]. Therefore, it is recommended that T1DM patients have a diet rich in vitamin-K.

Moreover, a significant higher intake of pantothenic acid was observed in T1DM and T1DM-obese patients when using the Kruskal-Wallis test. However, the

multi-comparison analysis showed no significance among the single groups, which could be due to the differences in sample size and the variability between the groups. Pantothenic acid (vitamin B5) is known to be involved in fatty acid metabolism, especially in the citric acid cycle [147]. In a diabetic rat model, pantothenic acid (Dexpanthenol) was found to restore endothelial function and reduce glucose levels [148]. The role and involvement of pantothenic acid in T1DM is not clearly understood, thus further studies are needed to understand its beneficial effect.

Various studies have been published that show the involvement and association of DNA methylation in the development of T1DM [7, 8, 87, 149]. In regard to our DNA methylation analysis, we have identified Suppressor APC domain containing 1 *SAPCD1* to be hypermethylated inT1DM children when compared to healthy controls (Table 7). We have also identified DnaJ heat shock protein family (HSP40) member C7 (*DNAJC7)* to be differentially methylated and hypomethylated in T1DM-OB patients compared to the obese group (Table 7). *SAPCD1* gene is known to be involved in the establishment of mitotic spindle orientation and the negative regulation of protein localization to the cell cortex. Based on the literature, there is no published data that correlate this gene with T1DM disease. However, there are studies that showed its correlation with different types of cancer, such as breast, lung, and familial papillary thyroid cancer [150-152]. The link between cancer and diabetes has long been investigated as diabetic patients have an increased risk of developing specific types of cancer, such as liver, kidney, and pancreatic cancer [153]. The link between T1DM and cancer is not well understood as most studies presented in the literature discuss the link between T2DM with cancer due to its increased prevalence compared to T1DM [154]. Hyperglycemia detected in diabetic patients could be the linking factor between T1DM

and cancer as it contributes to oncogenesis, tumor cell resistance, and cell death inhibition [155, 156]. A recent genome-wide association study done by Hebbar *et al.* (2020) found a significant association between high fasting plasma glucose levels and the Valyl-TRNA Synthetase gene (*VRAS*)[157]. In addition, when they performed genotype tissue expression analysis, they found that this gene was involved in the regulation of *SAPCD1* among other genes [157]. Further studies are needed to investigate and understand the involvement and role of the *SAPCD1* gene in T1DM pathogenesis.

Although the role of the *DNAJC7* gene is not well identified in T1DM pathogeneses, various studies investigated its involvement in T2DM and insulin resistance. *DNAJC7*, also known as cytoplasmic constitutive active/androstane receptor retention protein (CCRP), belongs to the heat shock protein 40 (HSP40) family [158]. Various studies have shown the effect of impaired heat shock response on insulin production and ß-cell function, due to dysregulated *DNAJC* that results in the progression of T2DM [159-161]. A study conducted using CCRO knockout mice found that the absence of *DNAJC7* expression resulted in liver steatosis and abnormal serum lipid values, including elevated LDL levels [158]. Based on our analysis, T1DM-OB patients were found to have an elevated LDL level, correlating with the data presented in the literature. In addition, pathway analysis revealed a number of pathways and molecular functions associated with liver function and lipid metabolism in T1DM-OB subjects. Furthermore, IPA analysis showed that gastrointestinal, hepatic and metabolic disorders were affected in T1DM-OB patients (Figure 10A), which were all seen in diabetes and obese related conditions based on the literature [162-165].

Furthermore, disease pathway analysis generated by IPA showed significance in reproductive system diseases in both T1DM and T1DM-OB subjects compared to controls (Figure 8, 10A). Based on the literature, different studies have shown that women with T1DM have menstrual disturbances and lower fertility rates compared to healthy controls [166, 167]. Also, hyperglycemia and hyperinsulinemia due to insulin deficiency and exogenous insulin injection are found to induce the risk of developing hypogonadism, hyperandrogenism, and polycystic ovarian morphology in T1DM patients [168].

Disease pathway analysis also revealed cardiovascular diseases in the comparison between T1DM patients and healthy controls (Figure 8). Various studies have shown that young T1DM patients have an increased risk of developing cardiovascular diseases later in life as the increased risk is associated with the diabetes duration [169-171]. Therefore, the diseases identified in this study are considered long-term morbidities commonly observed at the adult stage of T1DM patients.

In this study, we were able to identify canonical pathways between T1DM-OB and obese subjects, which are aldosterone signaling in epithelial cells, xenobiotic metabolism CAR/PXR pathways, and NRF2 mediated oxidative stress respond (Figure 9). Aldosterone overproduction is usually observed in T1DM patients with diabetic nephropathy due to impaired regulation of the renin-angiotensin-aldosterone system (RAAS) [172]. In a T1DM rat model, blockage of Aldosterone signaling in epithelial cells was observed to increase the expression of tight junction proteins, thus regulating the permeability of solutes across the epithelial membrane [173]. In regard to xenobiotic metabolism CAR/PXR pathways, which mainly take place in the liver, it was suggested by the literature to be involved in drug metabolism in T1DM patients

[174, 175]. A study conducted by Dong and colleagues found that T1DM mice showed an increase in the expression of drug-metabolizing cytochrome P450, whereas this induction was absent in CAR knockout T1DM-induced mice [174]. Therefore, xenobiotic metabolism through CAR receptors has a potential impact on the efficiency and toxicity level of different drugs. Moreover, the NRF2 signaling pathway was reported to be impaired in T1DM conditions, leading to insufficient protection against hypoglycemia-induced oxidative damage in the hippocampus associated with long-term cognitive abnormalities [176].

Regarding the gut microbiome composition, previous data from our group showed that T1DM patients have a low microbial abundance and richness compared to healthy controls (unpublished; Figure 11, supplementary figure S2). In the literature, there are conflicting results regarding the diversity and richness of the microbial flora in the case of T1DM development. Various human and animal-based studies have reported decreased alpha diversity in association with T1DM development [177, 178]. However, it was also reported that gut microbial diversity and richness are increased in T1DM patients compared to healthy controls [78].

In addition to its influence on the immune system, the gut microbial composition may have also an influence on different epigenetic modifications, including DNA methylation in the development of T1DM. The gut microbiome can affect DNA methylation pattern through the production of the epigenetically active metabolite, such as certain vitamins, butyrate, folate, and acetate [179]. In metabolic conditions characterized by gut microbial dysbiosis, such as T1DM, altered concentrations of these vitamins and cofactors are observed leading to aberrant DNA methylation patterns and thus affecting the development of the disease [179-182].

Besides, a study conducted by Ghadimi *et al.* found that probiotic bacteria, such as *Bifidobacterium breve* and *Lactobacillus rhamnosus* downregulate the expression of IL-17, IL-23, and CD40 enhancing the DNA methylation through its metabolites and inhibiting the NF-kB pathway [183]. Probiotic supplementation (containing *Bifidobacterium breve* Bb12 and *L. rhamnosus* GG) was also found to affect the DNA methylation pattern of obesity-related genes in pregnant women and their children. The authors of this study found that in response to probiotic intake a total of 38 and 68 genes were differentially methylated in women and their children, respectively [184]. The women supplemented with the probiotics showed a decreased methylation level in the promoter region of fat mass and obesity-associated (*FTO*) gene and methionine sulfoxide reductase A (*MSRA*) gene, which are important genes involved in obesity-related pathways [184].

Regarding the SCFA analysis in this study, butanoic acid concentration, also known as butyric acid, was found to be significantly elevated in T1DM-OB patients (Figure 15). However, based on the literature, different studies have shown that young children with T1DM have reduced levels of SCFAs, including butyrate [185, 186], which contrasts the results obtained in our analysis. A study conducted by Traisaeng and colleagues found that upon supplementation of *Leuconostoc mesenteroids*, increased levels of butyric acid were observed [187]. They also found that butyric acid was associated with elevated insulin levels and reduced glucose levels in vitro (Min 6 cell culture) and in vivo (T1DM mice model) [187].

The gut microbial composition is not the only factor affecting butyric acid levels, different dietary factors are found to influence its levels, such as fat and fiber intake [188]. A study conducted by Choi and colleagues found that specific free

pathogen (SFP) rats given a high-fat diet have significantly decreased levels of butyric acid when compared to rats given a normal chow diet [188]. A high-fat diet is known to be an important risk factor in various inflammatory and metabolic disorders, including obesity and diabetes [189]. In contrast, fiber intake was found to be associated with an increased level of butyric acid in obese children indicating its potential beneficial effect [190]. Besides, It has been found that dietary fiber-mediated modulation of the gut microbiome and SCFAs, including butyric acids, protect against the development of diabetic nephropathy through the action of GPR109A and GPR43 [191]. In our study, the T1DM-OB group has 11.6 % intake of SFA, which is slightly higher than the recommended intake (10%) [126]. Therefore, the increased level of butyric acid observed in our T1DM-OB group could be explained by their nutritional intake as they consumed normal to slightly elevated fat intake. Understanding the link between different dietary components and butyric acid could provide a better understanding of the pathophysiology of T1DM.

There is also evidence on the epigenetic effect of butyric acid and its possible influence on the development of T1DM [192, 193]. In a colitis model, butyrate was found to induce the differentiation of T-regulatory cells in colonic epithelium through enhancing Histone 3 acetylation in the promoter region of FOX-3 locus [194]. In a T1DM mice model, the salt form of butyrate (sodium butyrate) was found to reduce glucose levels and increase insulin levels through histone acetylation and inhibition of histone deacetylase (HADC) [195]. Further studies are needed to explore the effect of butyric acid on other epigenetic modifications, such as DNA methylation and its influence on T1DM development.

We have also performed network analysis to determine the potential link between DNA methylation, nutrition, and gut microbiome in the development of T1DM disease in children. In our analysis, an association between diet and detected CpG methylation level was only observed with folate and thiamin intake in the healthy controls (Figure 16). Folate is known to be involved in the process of DNA methylation as it acts as a methyl donor for the synthesis of S-adenosylmethionine (co-substrate required in DNA methylation) [196]. Based on the literature, folate intake was associated with beneficial effects on glucose homeostasis by acting as an epigenetic modulator. Studies have shown that folate supplementation is associated with methylation levels of *CAMKK2* and adipose-related genes, thus regulating pancreatic functions and insulin secretion [116, 197]. In addition, specific gut microbiome, such as *Bifidobacterium*, and *Enterococcus,* are known as a source of folate biosynthesis in the distal intestine [198]. In our analysis, healthy subjects had the highest intake of folate (an average of 141.6 mcg) compared to the other study groups, which could explain why an association with DNA methylation level was only observed in the healthy controls.

In this study, we have also observed a negative correlation between DNA methylation level and *Faecalibacterium* in T1DM subjects (Figure 17 A).). T1DM patients have the highest relative abundance of *Faecalibacterium* accounting for an average of 6.25% compared to the other study group. *Faecalibacterium* is an anti-inflammatory bacteria from the phylum Firmicutes involved in improving intestinal membrane integrity through the production of butyrate [199]. Different studies have reported the decrease in *Faecalibacterium* abundance in T1DM children associated with increased gut permeability [185, 199-201]. Based on the literature, no study

discuss the link between *Faecalibacterium* and DNA methylation in T1DM. However, since *Faecalibacterium* is a butyrate producer, it can act as an epigenetic modulator through the production of butyrate [194, 195]. In the obese group, a positive correlation was observed between DNA methylation and *Lachnospira* (Figure 17 C). According to the literature, *Lachnospira* is a gut microbial marker observed in obesity-related conditions [202]. Although no network association was identified between diet and gut microbiome in obese groups, in the T1DM-OB, *Lachnospira* showed a positive association with folate level, which is an epigenetic modulator (Figure 18C). This discrepancy in the network analysis could be attributed to the small sample size. More studies are needed to further understand the link between DNA methylation, folate intake, and *Lachnospira* as it could explain possible association between DNA methylation, nutrition and microbiome in the development of disease.

In the network analysis between diet and microbiota, we identified a negative correlation of the SFA with *Sutterella* and *Phascolarctobacterium* in T1DM subjects (Figure 16). According to the literature, *Phascolarctobacterium* was previously reported to be positively correlated with SFA intake in obesity [203]. In the case of *Sutterella*, no study reported its possible correlation with SFA intake. However, high levels of *Sutterella* were found to be associated with extra virgin olive oil (EVOO), which is characterized by high levels of Monounsaturated Fatty Acids (MUFA) and low levels of SFA [203, 204]. In this study, T1DM patients report a low intake of SFA, and the *Sutterella* relative abundance was 1.03%, which is lower in comparison to healthy subjects (2.04%) even if it didn't reach statistical significance (Appendix C, figure S4).

In regard to the SCFAs and microbiome network analysis, *Sutterella* was also observed to be positively correlated with butanoic acid and pentanoic acid in T1DM patients (Figure 16). Based on the literature, *Sutterella* was found to be significantly increased in T1DM patients compared to controls [72]. Another study reported the same finding with high levels of *Sutterella* being observed in T1DM along with the impaired activity of IL-17 secretion [205]. *Sutterella* from the phylum *Proteobacteria* is considered a pro-inflammatory agent and has been frequently involved in different inflammatory and metabolic disorders, such as inflammatory bowel disease (IBD), and autism [206]. No study reported the possible correlation between *Sutterella* and SCFA production in T1DM. However, it has been found that subjects with overconsumption of alcohol have elevated abundance of *Sutterella* compared to controls, but no significant correlation was observed with SCFAs level [207].

There are several limitations associated with this study, mainly the small size of the study subjects, particularly for the healthy controls, the T1DM obese and the obese groups. Another limitation is in the use of 24-hour dietary recall, which records one day intake and it may not be a good representative of the patients' dietary habits. To overcome the uncertainties of the dietary records, our recruiting criteria involved a narrow range of age (6-12 years) to exclude variability in social and dietary habits exhibited by teenagers. Also, correction of blood cell composition wasn't performed, which affects the analysis conducted in this study. Another limitation of this study is the absence of gene expression validation to confirm the effect of DNA methylation.

Larger studies are needed to confirm the findings of this study, involving different bioinformatic and epigenetic software to provide a better understanding of the

correlation between DNA methylation, diet, and gut microbiome in the pathogenesis of T1DM.

4.3 Conclusion:

The risk of developing T1DM is increasing worldwide, especially in westernized countries. The exact pathophysiology of the disease is complex and involves the interaction of different genetic, epigenetic, and environmental factors. The interplay between DNA-methylation, diet, and gut microbiome and how this link contributes to the pathogenesis of T1DM is yet to be identified. In this study, we were able to determine the differentially methylated genes seen in T1DM patients in comparison to the T1DM obese, pure obese, and lean controls along with the significant functional pathways involved. Both *SAPCD1* and *DNAJC7* genes were found to be possibly implicated in T1DM disease. A possible link was identified between folate intake and DNA methylation as well as with microbial genus *Lachnospira.* In addition to the negative association between *Faecalibacterium* and DNA methylation in T1DM patients. Therefore, the conducted network analysis showed a possible interplay between diet, DNA-methylation and gut microbiome and its influence on the development of T1DM. More studies are needed to provide further information and better understanding of such association.

Chapter 6: Appendix

6.1 Appendix A: Study approvals

Qatar University Institutional Review Board **QU-IRB**
QU-IRB Registration: IRB-QU-2020-006, QU-IRB, Assurance: IRB-A-QU-2019-0009

October 28th, 2020

Dr. Mashael Alshafai
College of Health Sciences
Qatar University
Phone: +974 4403 5589
Email: malshafai@qu.edu.qa

Dear Dr. Mashael Alshafai,

Sub.: Research Ethics Review Exemption
Ref.: Student, Amira Kohil/ e-mail: ak1404654@student.qu.edu.qa
Project Title: "Role of epigenetics in type 1 diabetes in pediatric population of Qatar"

We would like to inform you that your application along with the supporting documents provided for the above project, has been reviewed by the QU-IRB, and having met all the requirements, has been granted research ethics **Exemption** based on the following category(ies) listed in the Policies, Regulations and Guidelines provided by MoPH for Research Involving Human Subjects:

Exemption Category 3: Research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified.

Documents Reviewed: QU-IRB Application Human Subject- Ver. 2_Bilingual_AT edits_18102020, QU-IRB Application Material Check List_AT rev, Thesis Proposal, Sidra Approval Letter, Copy of Data Collection Form_AT, Review Forms, responses to IRB queries and updated documents.

Please note that exempted projects do not require renewal; however, any changes/modifications to the original submitted protocol should be reported to the committee to seek approval prior to continuation.

Your Research Ethics Approval Number is: **QU-IRB 1411-E/20**. Kindly refer to this number in all your future correspondence pertaining to this project. In addition, please submit a closure report to QU-IRB upon completion of the project.

Best wishes,
Dr. Mohamed Emara

Vice Chair, QU-IRB

Institutional Review Board
(IRB)
Office Of Academic Research

Tel: +974-4003-7747
Email: irb@sidra.org

Sidra IRB MOPH Assurance: MOPH-A-Sidra-00100
Sidra IRB MOPH Registration: MOPH -Sidra-IRB-099
Sidra IRB DHHS Assurance: FWA00022378
Sidra IRB DHHS Registration: IRB00009930

March 18, 2020

Approval

Dear Annalisa,

On March 18, 2020 the IRB approved the following through March 17, 2021 inclusive.

Type of review:	Continuing Review
Protocol Title:	The triple interaction Diet -Microbiome-Epigenome in obese type 1 diabetic children
Principal Investigator:	Annalisa Terranegra, PhD
IRB Number:	1500755
Sponsor/ Funding Agency:	IRF 2017& IRF 2019
Grant title and ID, if any:	SDR200010/SDR200060
Documents reviewed:	• IRB-408 Modification Application Form-B_V2.0_7 October 2019 (UPDATED: 12/15/2019) • IRB-408 Modification Application Form-A_V2.0_7 October 2019 (UPDATED: 12/15/2019) • IRB-407 Continuing Review Application_V2_7 October 2019 (UPDATED: 12/15/2019) • IRB-413 Research Proposal (UPDATED: 12/15/2019) • IRB-402 Parental Permision Form_V1.5/Nov 2019_English (UPDATED: 03/16/2020) • IRB-402 Parental Permision Form_Arabic (UPDATED: 03/15/2020) • IRB-404 Assent Form_V1.2/Nov 2019_English (UPDATED: 03/16/2020) • IRB-404 Assent Form_Arabic (UPDATED: 03/15/2020) • Questionnaire/Survey - PWIND-SDQ-C_English (UPDATED: 12/15/2019) • Questionnaire/Survey - PWIND-SDQ-C_Arabic (UPDATED: 12/15/2019)
	• Questionnaire/Survey - SCOFF_English (UPDATED: 12/15/2019) • Questionnaire/Survey - SCOFF_Arabic (UPDATED: 03/15/2020) • Questionnaire/Survey - CHEAT_English (UPDATED: 12/15/2019) • Questionnaire/Survey - ChEAT_Arabic (UPDATED: 03/15/2020) • Sidra - IRB Application Form (UPDATED: 12/15/2019)
Level of review:	Expedited Review
Expedited Categories:	2, 3, 4 & 7
Pediatric Category:	Research does not involve greater than minimal risk

Before *February 17,2021*, you are to submit a continuing review to request continuing approval or closure. If the IRB does not grant continuing review, approval of this protocol ends after March 17,2021.

Copies of approved parental permission documents and assent documents are attached.

In conducting this study, you are required to follow Sidra's Policies and Procedures pertaining to Human Research Protection.

If you have questions or concerns, please call the IRB office at 4003-7747 or send an email to irb@sidra.org.

Sincerely yours,

Catherine H Cole

Catherine Cole, MD

Chair

Institutional review Board
Chief of Division,
Pediatric Hematology, Oncology and Hemopoietic Stem Cell Transplantation
Palliative Care Liaison Service
t. +974 40036567
PO BOX 26999 Doha, Qatar
ccole@sidra.org

6.2 Appendix B: DNA methylation in the four different study groups

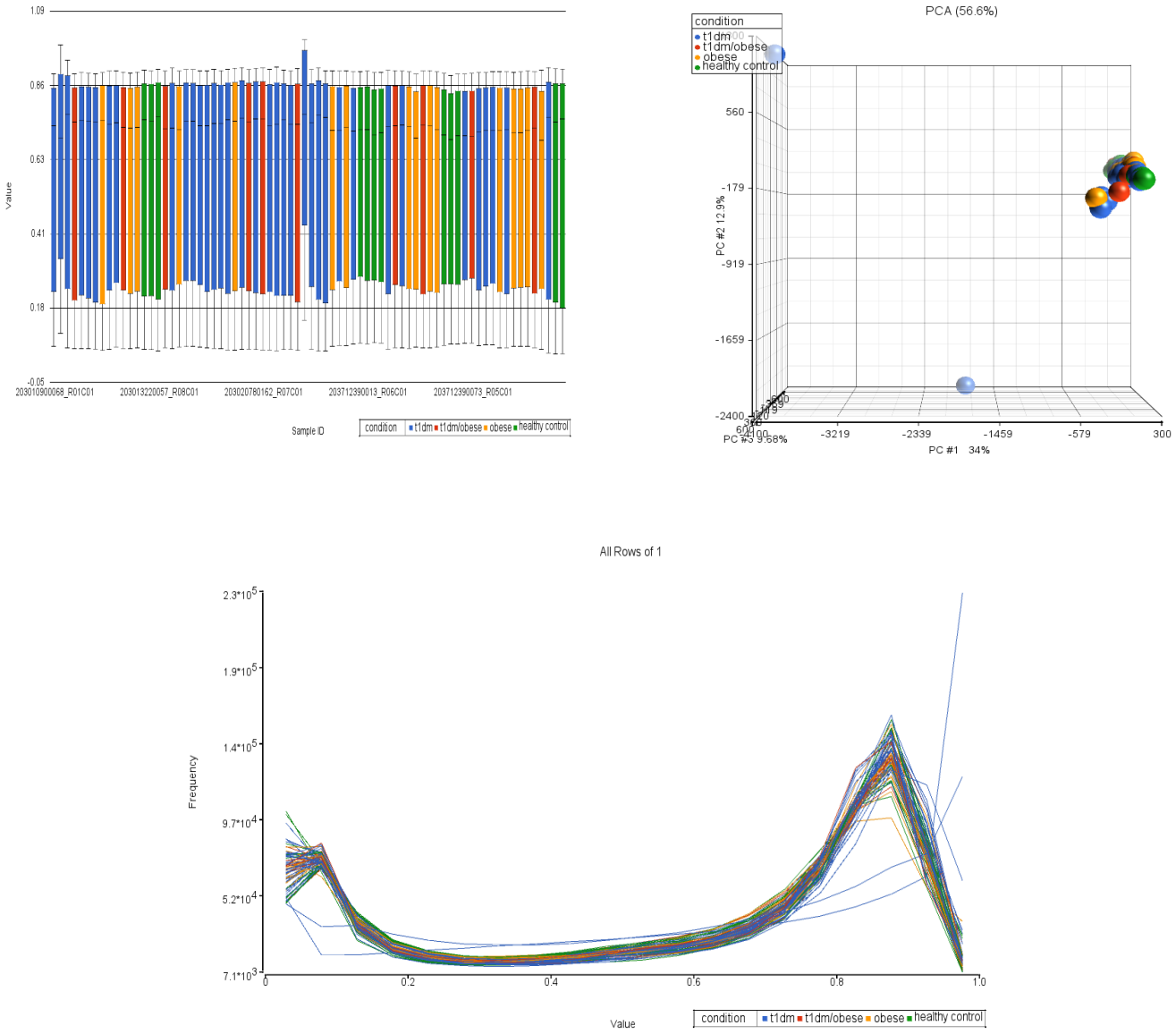

Figure S1. Data quality control analysis using Partek Genomics Suite version 7.0

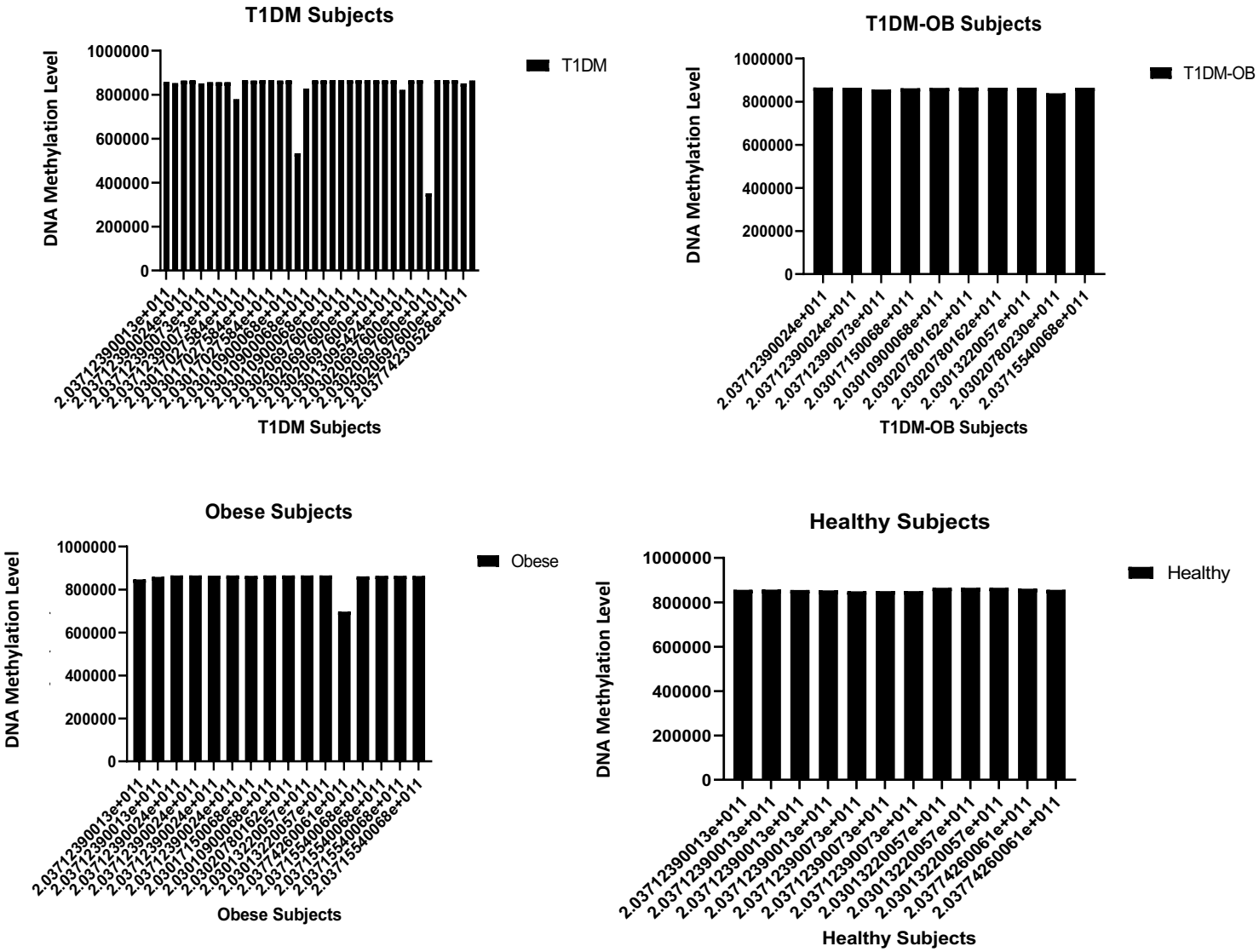

Figure S2. **CpG DNA methylation levels within study groups.** Raw methylation data generated from the Illumina Infinium array were imported to the genome studio where probes with a mean detection level P-value <0.01.

Table S1. Differentially methylated unadjusted genes

Group	Mapped genes	Up-regulated genes	Down-regulated genes	Comparison group
T1DM	854	589	265	Healthy
T1DM-OB	735	234	501	T1DM
	735	256	479	Obese

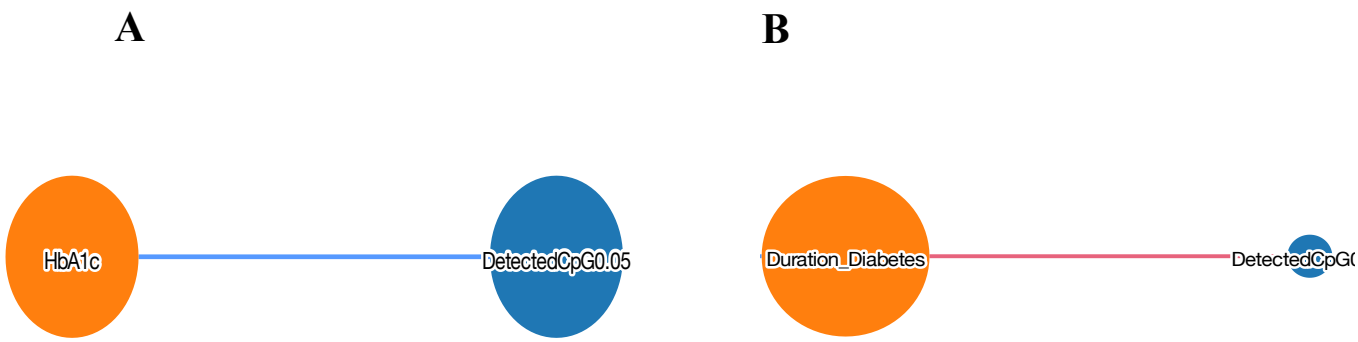

Figure S3. **Network analysis between CpG methylation level and HbA1c (A) and diabetes duration in T1DM-OB subjects.** Note: blue line indicates a positive correlation, the red line indicates negative correlation.

6.3 Appendix C: Gut Microbial composition

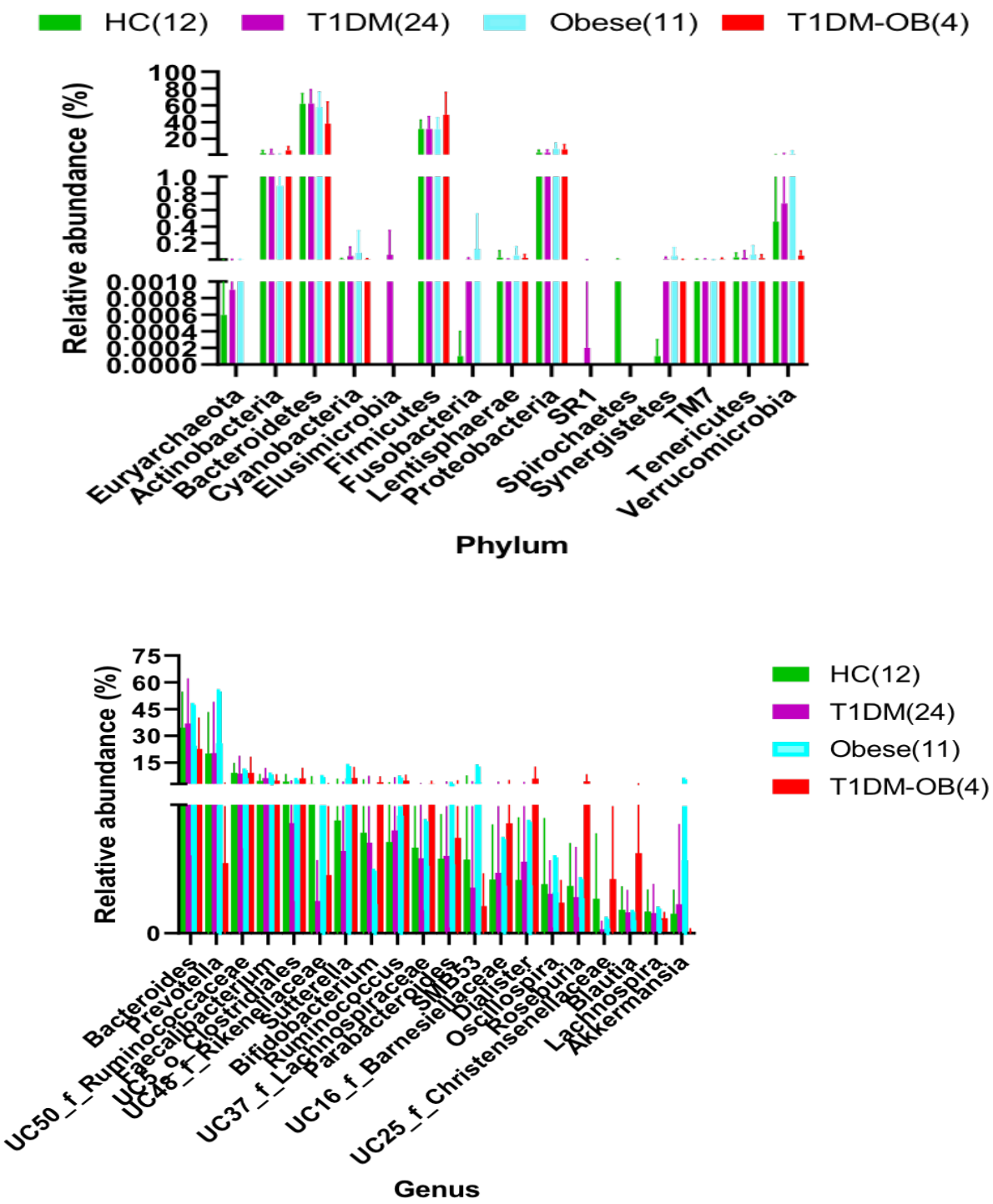

Figure S4. Relative abundance of gut microbial composition at both the phylum and genus level in the four groups.

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