Gut Microbiota In Human Type 2 Diabetes: 
*In-vivo* and *In-vitro* Studies

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Abstract

The gut microbiota plays an important role in the development of type 2 diabetes (T2D), which is an alteration in the diversity and abundance of the gut microbiota, favouring the growth of Gram-negative bacteria. Although a lot of studies have shown this to be the case, most of this work has been done in animal models with few studies in humans. In animal models of T2D, it is known that a high-fat diet alters the gut microbiota in favour of the growth of Gram–negative bacteria. The outer membrane of Gram-negative bacteria contains lipopolysaccharide (LPS) which is an endotoxin that can trigger inflammation leading to metabolic disorders such as insulin resistance and T2D, hence T2D is considered a low grade inflammatory disorder.

In this thesis, the effect of Galactooligosaccharide (GOS), a prebiotic, on the composition of the gut microbiota was investigated. Next generation sequencing (NGS) of the gut microbiota of T2D and healthy control subjects showed no significant difference at the phylum level between the two groups. Furthermore, T2D patients in the prebiotic group had a significant increase in the level of Firmicutes compared to the placebo group. Also, although not significant, T2D patients on metformin had increased level of Bacteroidetes, Proteobacteria and Actinobacteria compared to those not on metformin. The ability of human faecal water (FW) to distinguish between healthy and T2D patients using an in vitro model of the intestinal mucosa was studied. FW from T2D patients decreased Caco-2 cell monolayer integrity when compared to the
healthy controls and in the T2D patients, FW activity *in vitro* correlated with biological markers of T2D severity measured *in vivo*. Additionally, cytokines were measured in T2D faecal samples using a human cytokine array. Finally, GOS anti-cytotoxic activity was also assessed *in vitro* using cell viability assays and the anti-cytotoxic effect of GOS was time and concentration dependent.

Together, the thesis explored potential new ways of using faecal samples as biomarker for T2D *in vitro* and relating it to *in vivo* parameters of the patients. Also future work in this area may reveal mechanistic insight to the use of FW as a non-invasive biomarker for T2D.
Acknowledgement

I am eternally grateful to God for the opportunity to embark on this PhD journey, his grace has been sufficient for me.

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Dedication

This thesis is dedicated with all my love and thanks

To my beloved mother, Deaconess Gladys U. Jaiyeola, who passed away months before I started my PhD at Surrey, her encouragement got me started on this journey and her inspiring life kept me pushing hard even when the going got tough. You are and will forever be greatly loved and missed.

My Wonderful Father- His love and sacrifice has propelled me to great heights I never imagine I could attain.

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<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<td>BP</td>
<td>Base Pair</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>FW</td>
<td>Faecal water</td>
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<td>FOS</td>
<td>Fructooligosaccharide</td>
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<td>GOS</td>
<td>Galactooligosaccharide</td>
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<td>IL-1</td>
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<td>IL-8</td>
<td>Interleukin-8</td>
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<tr>
<td>IFNg</td>
<td>Interferon gamma</td>
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<tr>
<td>L-group</td>
<td>Prebiotic group</td>
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<tr>
<td>SCFA</td>
<td>Short chain fatty acids</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>Wt/wt</td>
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<td>Wt/vol</td>
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</table>
Vol/vol  Volume/volume
B·GOS  Bimuno galacto-oligosaccharide
GOS  Galactooligosaccharide
NGS  Next generation sequencing
PCR  Polymerase chain reaction
rRNA  Ribosomal Ribonucleic acid
RCT  Random controlled trial
SEM  Standard error of mean
spp.  Species
T·group  Placebo group
T1D  Type 1 Diabetes
T2D  Type 2 Diabetes
TNFα  Tumor necrosis factor alpha
TER  Transepithelial electric resistance
CHAPTER 1

INTRODUCTION
1. Introduction

1.1. Diabetes Mellitus

1.1.1. Overview

Diabetes mellitus is a metabolic disease associated with by hyperglycaemia arising from defects in insulin secretion, insulin action, or both [1, 2]. All forms of diabetes are associated with substantial co-morbidity with the associated costs for both the individual patient and the NHS. In the UK, the total cost of direct patient care for diabetes in 2010/2011 is estimated at £9.8 billion, while the indirect costs associated with diabetes are estimated at £13.9 billion. The cost of screening, diagnosis, treatment, interventions and complications management accounts for over £2 billion of the direct cost. For complications experienced by those with Type 1 or Type 2 diabetes, the cost was estimated at £7.7 billion. It is projected that the cost will range between £13.8 billion and £20 billion by year 2035/2036 [3, 4].

The severe and repeated hyperglycaemia of diabetes is associated with damage, dysfunction, and failure of multiple organs, especially the kidneys, eyes, heart, nerves and blood vessels [1, 5]. Moreover, diabetes can result in long-term complications such as retinopathy with potential loss of vision; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms; and nephropathy leading to renal failure. Furthermore, diabetic
patients have an increased incidence of atherosclerotic cardiovascular, cerebrovascular and peripheral arterial disease [1, 5]. In addition, abnormalities of lipoprotein metabolism and hypertension are also associated with diabetes [1, 2].

The two most common forms of diabetes are type 1 Diabetes (T1D) and type 2 Diabetes (T2D). Other common types of diabetes include gestational and maturity onset of disease in the young (MODY) [1, 5]. T1D occurs when there is an absolute deficiency of insulin secretion and accounts for 5–10% of cases was historically referred to as insulin-dependent diabetes or juvenile-onset diabetes [1, 5]. This type of diabetes is an autoimmune condition and people at risk of development of T1D are usually identified by an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers [1, 5]. T2D is a combination of resistance to insulin action which finally leads to an inadequate insulin secretion [1, 2, 5]. It accounts for 90–95% of cases and was historically known as non–insulin dependent diabetes or adult-onset diabetes [1, 2, 6]. These terms are no longer in common usage due to the number of patients with T2D who will eventually move onto insulin treatment, and with the age of onset of T2D now considerably younger than in previous generations. Although, a degree of hyperglycaemia enough to cause pathologic and functional changes in various target tissues may be present, there is often no clinical symptoms other than those associated with changes in plasma osmolarity (thirst, frequent urination) and the condition may be undetected for a long period of time [1, 2]. However, it
can be detected by measurement of plasma glucose in the fasting state or after an oral glucose load challenge, which demonstrates an abnormality in carbohydrate metabolism [1, 2].

1.1.2. Epidemiology

Factors such as population growth, age, urbanization, and increased prevalence of obesity and physical inactivity are leading to an increase incidence of diabetes [7]. Since approximately 1 in 22 people worldwide have diabetes, it is necessary to understand the aetiology of diabetes, now and in the future, to allow rational planning and allocation of resources [7]. In 2010, it was estimated that 285 million adults had diabetes, which was 67% higher than the published estimate in 2004 [8]. In 2015, approximately 415 million adults have diabetes and it is estimated that the number of people with diabetes will further increase by 2030 and 2040 to approximately 439 million and 642 million, respectively. The World health organisations (WHO) also projected that by 2030, diabetes will be the seventh leading cause of death [9].

Although the overall prevalence of diabetes prevalence is similar in men and women, its prevalence is slightly higher in men under 60 years of age and in women at older ages (over 65 years). Also, it is estimates that 90 percent of people around the world who have diabetes have type 2. In the least developed countries, the majority of people with diabetes are between 45- to 64-years of age while in developed countries majority of the people are 64 years of age or older [7]. It is
estimated that by 2030, the number of people with diabetes over 64 years of age will be greater than 82 and 48 million in the least developed and developed countries respectively, [7].

This represents a mean annual increment of 20,000 cases [8]. In the UK, the Figures (Fig 1.1, 1.2, and 1.3) below represents the data for diabetes prevalence in both women and men for year 2014, projection and probability of halting the increase of diabetes by 2025 [11].
Figure 1.1: A sunburst plot showing the number of adults with diabetes in High-income English speaking countries.
In the UK, 1.78 million women and 2.06 million men have diabetes which accounts for 12.6% and 12.9% of the total population of women in the region, respectively [11].

Figure 1-2: World map showing projection of the prevalence of diabetes for adults 18 years and older, in different countries by 2025

It is projected by 2025, the prevalence of diabetes will be 5.4% for women and 7.8% for men in the UK [11].
Figure 1-3: World map showing probability of achieving the target of halting the rise in diabetes by 2025 compared to its 2010 level if post 2000 trends continues.

In the UK, the probability is estimated at 48.4% for women and 35.6% for men [11].
1.1.3. Pathogenesis

T2D is defined by an increasing decline in pancreatic beta cell function and chronic insulin resistance [1, 5, 12]. One major risk factor for the development of T2D is obesity although most people who are obese do not develop diabetes. T2D is a highly heterogeneous disorder but can be inherited, which accounts for about 50% of the disease susceptibility [5, 13]. Over 40 diabetes-associated loci have been identified, most of which are associated with impaired β-cell function. Also gene defects that affect islet β-cell function are most common in patients with the monogenic form of the disease [5, 14].

The islet β cells are sensitive to nutrient induced damage and play a vital role in the development of T2D [5, 13]. They maintain the synthesis of proinsulin with correct post-translational modification; affect the secretion of secretory granules; monitor nutrient concentrations in blood, usually by intracellular metabolism and production of nutrient-secretion coupling factors; and the release of insulin granule by activation of a complex exocytosis machinery [5, 15, 16]. However, in human T2D, islet β cells have the tendency to develop islet amyloid polypeptide and triglyceride deposits, and whilst this may not initiate the disease, it may contribute towards disease progression [5, 17]. Although it is difficult to identify the reasons for the failure of the beta cells, it is possible that a combination of β-cell susceptibility factors causes the initial mechanism of damage. Likewise, the development of severe hyperglycaemia leads to glucotoxic and gluco-lipotoxic activity which further affects the rate of failure [5, 18-20].
Insulin resistance may induce abnormalities in adipose tissue which could occur independently from β-cell impairment [5, 21]. Healthy white adipose tissue protects against metabolic disease and acts as a fatty acid “sink” preventing the uptake and storage in ectopic tissues. In T2D, adipose tissue function may be compromised in many ways such as reduced rates of adipocyte differentiation and adipogenesis, reduced adiponectin expression and secretion, increased expression and secretion of inflammatory cytokines (e.g. tumour necrosis factor α, interleukin-1β, and monocyte-chemo-attractant protein-1) and increased tissue inflammation (e.g. macrophage infiltrates). Impaired secretion of adiponectin and increased non-esterified fatty acids and concentration of inflammatory cytokines can aggravate insulin resistance in muscle and cause pathology in non-alcoholic steatohepatitis [5, 15]

In the liver, increased endogenous glucose production is a major determinant of fasting hyperglycaemia in T2D and is considered to be one of the earliest detectable defects in the condition [15, 17]. The production of glucose is not suppressed by insulin in the postprandial state and this contributes to a fed-state hyperglycaemia. The mechanism that explains this is varied and complex but includes increased supply of gluconeogenic substrate from peripheral tissues, the hepatic response to raised concentrations of glucagon, and activation of hepatic gluconeogenesis by raised concentrations of non-esterified fatty acids [5, 22, 23].
1.2. Treatment of Type 2 Diabetes

1.2.1. Metformin

Metformin belongs to the biguanide class of antidiabetes drugs and it is an orally administered drug. It is used for lowering blood glucose concentrations in patients with T2D, particularly in those overweight and obese as well as those with normal renal function [24-26]. The origin of biguanides can be traced from the use the plant Gallega officinalis, which was traditionally used in medieval Europe as a drug treatment for diabetes for centuries. The synthesis of galegine-like compound called Guanidine derived from the plant led to the discovery of metformin [10, 16]. The other two biguanides, phenformin and buformin, were withdrawn in the early 1970s due to the risk of lactic acidosis and increased cardiac mortality. The usefulness of metformin was discovered in 1950 when it was observed to reduce glucose levels in a dose-response manner [13, 27, 28].

The mode of action is primarily at the liver where glucose output is reduced and aiding the uptake of glucose in the peripheral tissues, especially muscles [10, 16]. Metformin indirectly regulates and inactivates the downstream kinase adenosine monophosphate co-activator and transducers of regulated CREB protein 2 (TORC2) by activation of an upstream kinase, liver kinase B1 (LKB-1). This results in downregulation of transcriptional events that promotes the synthesis of gluconeogenic enzyme [29-31]. Other than glycaemic control, metformin has an effect on inflammatory pathway [4, 16], endothelial function
[18], blood pressure [19], cancer [20] and most recently, the gut microbiota [21, 22].

Metformin is the most prescribed drug by doctors for patients with T2D due to its efficacy, safety profile and beneficial metabolic and cardiovascular effects. It is the first line treatment based on the National institute for Health and Care Excellence (NICE) guidelines [29, 32-35]. To support this, in the UK primary care, a total of 8,838,031 individuals with T2D aged 0-99 years were included in a retrospective cohort study between 2000 and 2013. In 2000, 45.1% of T2D patients were administered metformin and it increased gradually on an annual basis to 91.0% for newly diagnosed T2D patients and 79.9 % of add-on therapy for patients on sulfonylureas by 2013 [36]. Furthermore, in 2005, 8.6 million metformin hydrochloride drug were dispensed, and this increased to 18.1 million items dispensed in 2014. This accounts for a 101.5% increase in 9 years, hence the leading anti-diabetic medicine used in the UK [37].

Metformin can also be given in combination with sulfonylureas, glinides, insulin, thiazolidinediones (TZD), alpha-glucosidase inhibitors, glucagon-like peptide-1 receptor agonist (RA-GLP1), sodium-glucose co-transporter 2 inhibitors (iSGLT2) and dipeptidylpeptidase 4 inhibitors (iDPP4). However adverse side effects have been associated with the use of metformin [38, 39]. The most commonly reported side effect is gastrointestinal intolerance which occurs in the form of anorexia, abdominal pain, flatulence and diarrhoea [31, 34, 39]. Although these effects subside once the dose is reduced or when administered with meal,
about 5% of patients do not tolerate metformin even at the lowest dose [38·40] and many others simply learn to tolerate the side-effects.

Metformin (50-60%) is absorbed mainly from the small intestine and gets into the blood in about 1·2 hours after an oral dose of 500·1000mg [32]. The maximal plasma concentration is 1·2µg per millilitre and it remains in the blood for about 1.5 to 4.9 hours [32]. Metformin (approximately 90%) is eliminated in urine in 12 hours unchanged as no metabolites have been found in urine [32].

1.3. Normal microbiota – Diabetes/obesity

The human body is colonised on every surface that is exposed to the external environment by microorganisms. This includes the skin, respiratory, oral cavity, urogenital and gastrointestinal tract [41]. However, a large and varied population of microorganism inhabit the human large intestine including bacteria, fungi and archaea. Many of the microorganisms are beneficial to the host; however given the correct conditions some have been linked to clinical diseases [41, 42]. These microorganisms are collectively referred to as the gut microbiota [41].

The gut microbiota as a community consists of over 1500 species of microorganism with about 40 trillion bacterial cells in a human body [41, 43]. The Gram-positive bacteria are able to ferment undigested dietary polysaccharides such as dietary fibre and resistant starch to short chain fatty acids which are absorbed by the host and serves as energy [41]. Additional sources of substrates
to Gram-positive bacteria are provided by the host including desquamated epithelial cells, mucins and digestive enzymes [44]. Intestinal microbiota also aid in the conversion of secondary plant metabolites such as polyphenols in fruits, vegetables, cereals, chocolate, tea, coffee, or wine and glucosinolates in brassica vegetables. In addition, intestinal bacteria also play a role in the conversion of bile acids and metabolism of xenobiotics. Bile acids (cholic acid and chenodeoxycholic acid in humans) are synthesised in the liver, conjugated with either glycine or taurine and then secreted into the intestinal tract, where they undergo deconjugation and partial dehydroxylation by intestinal bacteria. While, xenobiotics are oxidised and subsequently sulphated or glucuronidated to render them water soluble and thereby facilitate their urinary excretion [45-49]. Also, the gut microbiota is able to synthesize vitamins such as vitamin B12, vitamin K, and folic acid and protects its host against the harmful effect of pathogenic bacteria. Furthermore, commensal gut microbiota also plays a role in maturation of immune cells and normal development of immune function [50, 51].
Figure 1-4: The independent and dependent effect of the gut microbiota on host metabolism

The independent effect of the gut microbiota results in the production of pro-inflammatory molecules, such as lipopolysaccharide and peptidoglycan, which may affect host metabolism through proteins produced by the host to mediate the immune response. While the dependent effect involves the metabolism of Choline, cholesterol and polysaccharides obtained from the diet by the gut microbiota and either directly or through further host–microbial co-metabolization generate bioactive compounds. In the case of choline, this can lead to cardiovascular disease; for cholesterol, activation of TGR5 can increase energy expenditure and GLP-1 secretion or protection against heart disease; and for polysaccharides, short-chain fatty acids can be used as an energy source or can bind to GPR41 or GPR43 to regulate hormones and modulate inflammation. FMO, flavin-containing monooxygenase; TLR4, Toll-like receptor 4; TMA, trimethylamine; TMAO, trimethylamine-N-oxide Reproduced from [49, 52].
The human gut consists of bacteria groups belonging to 5 major phyla; the Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia [43]. The Firmicutes contain the genera *lactobacillus* which includes several strains of probiotics, butyrate producers such as *Eubacterium, Feacalibacterium, Roseburia, Ruminococcus and Clostridium* [43, 44]. The Bacteroidetes contains the genera *Bacteroides, Prevotella* and *Xylanibacter*. The Actinobacteria phylum contains *Bifidobacteria* (probiotic strains) and *Collinsella*. Proteobacteria phylum contains *Desulfovibrio*, which constitutes sulphate reducing bacteria and *Escherichia* from the Enterobacteriaceae family. Verrucomicrobia phylum contains *Akkermansia* which is known for mucus degradation [43]. The phyla Firmicutes and Bacteroidetes constitute over 90% of the bacteria in the gut [53]. The beneficial intestinal microflora includes *bifidobacteria* and *Lactobacilli* [42]. Although most adults carry *bifidobacteria* in their colon, it is found mostly in the faeces of breast-fed infants [54-56]. This is a major pointer to the benefit of *bifidobacteria* to its host and the need to increase its population in the colon of adults. In adults, the Firmicutes and Bacteroidetes dominate the gut and it is stable over a long period of time [42, 54, 55].

Gut microbiota varies between individuals due to factors such as host genotype, age and geographical location [56-59]. Even though gut microbiota are shared among family members, there still exists, a variation in bacterial lineages [60]. This was shown in a study where characterization of the gut microbial communities in 31 monozygotic twin pairs, 23 dizygotic twin pairs and, where
available, their mothers, showed that the gut microbe was similar among family members but with a variation in the gut microbial community of specific bacterial lineages present in each person [60]. The gut microbial community of the adult monozygotic and dizygotic twin pairs showed variation with a comparable degree but there was a vast array of microbial genes shared among sampled individuals [60]. This may indicate that no two people have exact microbiota.

1.4. Development and function dysbiosis in obesity and diabetes

In both animal and human models, several studies have shown that alteration in the composition and activity of the gut microbiota may play a role in the development of T2D and obesity. This alteration is known as dysbiosis [51, 61-64]. In mice, high levels of Bifidobacteria in the gut have been shown to improve intestinal permeability and reduce intestinal endotoxin levels [67, 69]. However, the genus *Bifidobacterium* belongs to the phylum Actinobacteria, which is found mostly in infants than adults and may be outnumbered by other bacteria [39]. In the human gut, the two major phyla are the Firmicutes and Bacteroidetes and the former and latter are composed mostly of Gram-positive and Gram-negative bacteria, respectively [39]. The level of these phyla has been shown to differ in obesity and T2D. In obesity, the phylum Firmicutes is reported to be higher than the phylum Bacteroidetes. The level of Firmicutes are proposed to be the reason for weight gain in obesity because of the ability of the bacteria to reduce the fermentation of polysaccharides [82-84]. One of the principal end
products of colonic fermentation are SCFA, which are more in overweight/obese individuals’ faecal samples. This may be due to increased SCFA production, decreased SCFA absorption or fewer microbial species that utilize SCFA as an energy source in the obese microbiota. SCFA produced account for 5–10% of total dietary energy [85]. In lean and overweight/obese individuals, the level of Firmicutes and Firmicutes/Bacteroidetes ratio was higher in the overweight/obese than lean individuals. Also, faecal SCFA was higher in the latter than former. This difference was due to change in colonic microbiota and not dietary intake or SCFA absorption. This was evident in the study as Firmicutes and not fat intake positively correlated to faecal SCFA [86]. Contrary, in T2D, the phylum Bacteroidetes (contains mostly Gram-negative bacteria) is higher compared to the phylum Firmicutes [87, 88].

Factors such as diet, intake of prebiotics, probiotics and antibiotics are thought to affect the gut microbiota [65-67], as well as the host immune functions [68-70] in T2D and obesity. Although T2D and obesity are now considered as low grade inflammation conditions, the exact mechanism through which this occurs is unknown. However, from several animal model studies, it is hypothesised that high-fat diet feeding leads to altered gut microbiota, and then the translocation of LPS or whole bacteria into the blood is followed by metabolic endotoxaemia and onset of metabolic disorders [71-76]. However, why high fat foods change the gut microbiota and result in the onset of metabolic disorders is unknown. It could be
possible that some diets promote the growth of Gram-positive bacteria while other diets could promote the growth of Gram-negative bacteria [77].

**Figure 1-5: The hallmarks of obesity, metabolic syndrome and diabetes**

High-fat/high–glycaemic load diet in hyperglycaemia (HG) and increased free fatty acids (FFA), could result in increased activation of the inflammasome complex as well as increase the activation of macrophages via increased TLR activation and NF-κB activation. Also the diet can cause changes to the gut microbiota by altering the content of histidine, glutamate, SCFAs, and other factors and promote gut barrier dysfunction and conditions prevalent in obesity, metabolic syndrome, and diabetes by altering the host response Adapted from [51].
1.5. Prebiotic Concepts: Animal models and Human Studies

Prebiotics are defined as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health.”[78] However, the effects of prebiotics in the colon are not permanent and disappear from faeces few days after oral dose is stopped [42]. In contrast, probiotics are live organisms that when ingested in sufficient amounts confers a health benefit to the host. Several supplements containing viable microorganisms with probiotic properties are available commercially [79]. The commonly used probiotics are bifidobacteria, lactobacilli, and non-pathogenic yeasts.

The human breast milk contains oligosaccharides, which is referred to as human milk oligosaccharides (HMOs) and are described as the prototypic prebiotic [80, 81]. In the colon of exclusively breast-fed neonates, they facilitate the preferential growth of bifidobacteria and lactobacilli, which may be a strong reason for some of the immunological and other benefits observed in breast-fed infants [80]. Prebiotics are not digested in the small intestine due to their chemical structure (they are considered under the Codex definition of dietary fibre), but are fermented in the colon [79, 80]. The endogenous bacteria ferment prebiotics to energy and metabolic substrates, with lactic and short-chain carboxylic acids as end products of the fermentation [79]. The current prebiotics in use are carbohydrates, which cannot be easily digested, present naturally in
food, but increasingly taken by the consumer as either a supplement or found fortified within a range of foodstuffs. These include galactooligosaccharides (GOS), mannoooligosaccharides (MOS), pectic-oligosaccharides (POS), transgalactosylatedoligosaccharides (TOS), chitooligosaccharides and xyloooligosaccharides (XOS) [79, 80]. The two commonly used prebiotics in most studies are inulin and oligofructose (FOS), however, the prebiotic property of GOS is becoming established with some efficacy demonstrated in healthy humans [42, 82, 83].

1.5.1. Galactooligosaccaride (GOS)

GOS is produced by the enzymatic transgalactosylation of lactose using β-galactosidase (lactase, EC 3.2.1; 2.4), forming several oligomers of different chain lengths, between DP2 to DP10 with a terminal glucose [81]. Whey-derived lactose is the primary raw material for the commercial production of GOS and it is formed in large amounts as a by-product of the dairy industry [80]. Furthermore, GOS can be produced using β-galactosidases of microbial sources in the forms of crude enzymes, purified enzymes, recombinant enzymes, immobilized enzymes, immobilized cells, toluene treated cells, and whole-cell biotransformation [80, 84]. Bifidobacteria and lactobaccili are termed ‘generally recognized as safe’ for use in food applications, hence they are of interest to many researchers as regards their enzymes for GOS production [84]. This is because it is mostly likely to selectively
promote the growth and metabolic activity of these bacterial genera in the gut, which will have health benefits to the host [80] [84, 85].

GOS has good moisture retention, highly soluble and has only about one third of the sweetness of sucrose. The molecules are highly stable in acidic conditions, room and high temperature conditions and this makes it easy to be incorporated into a wide variety of foods [80, 81]. In food, they have a pleasant taste, improve the texture and mouth-feel of foods, and act as bulking agents. GOS are used in a wide range of commercial products, including infant formulas, dairy products, soups, sauces, breakfast cereals, beverages, animal feeds, and as sugar replacements [80]. The fact that GOSs are synthesized from milk sugar and traditional dairy foods makes it ‘generally recognized as safe’ (GRAS). However, consumption of GOS at high levels has adverse effects of transient osmotic diarrhoea and flatulence [80].

1.5.2. Relevant studies on the efficacy of prebiotics

Since the gut microflora attached to the gut mucosa (e.g. *bifidobacteria*) acts as barriers preventing the invasion of pathogenic bacteria, it is important that nutrients that can promote their growth are provided. Oligosaccharides such as well studied FOS, GOS, inulin and the disaccharides lactulose have been used as prebiotics in human and mice studies to remodel the gut microbiota [80, 86, 87]. These carbohydrates are fermented by gut bacteria (e.g. *bifidobacteria*) for growth and these bacteria can competitively inhibit the growth of pathogenic
bacteria by reducing the luminal pH from an alkaline to acidic environment and the production of mucins and antibiotics is stimulated [88].
### 1.5.2.1. Animal Studies using prebiotics

Table 1-1: Example of Animal Studies (diabetes/obesity) designed to determine the prebiotic effect of inulin and oligofructose

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Component</th>
<th>Dose</th>
<th>Time</th>
<th>Main outcomes</th>
<th>Ref.</th>
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</table>
| Diabetic C57BL6/J Mice          | Oligofructose   | 90:10 wt/wt (HF-OFS)  | 14 weeks | - Improved blood glucose  
- Normalised endotoxaemia  
- Normalised plasma and adipose tissue proinflammatory cytokines  
- Restore bifidobacteria | [72] |
| Obese JCR:LA-cp rats            | Inulin and Oligofructose | 20% wt/wt           | 10 weeks | - Bacteroidetes decreased  
- Firmicutes, Bifidobacteria and lactobacilli increased | [89] |
| Obese fa/ffa Zucker rats         |                 | 10g                   | 10 weeks | - Slowed the increase in body weight  
- Decreased food intake  
- Lower glycemia  
- Phospholipemia, triglyceridemia and cholesterolemia remained unchanged  
- Lessen severe hepatic steatosis | [90] |
<table>
<thead>
<tr>
<th>Subjects</th>
<th>Component</th>
<th>Dose</th>
<th>Time</th>
<th>Main outcomes</th>
<th>Ref.</th>
</tr>
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</table>
| Diabetic C57BL6/J Mice           | Oligofructose     | 90:10 wt/wt (HF-OFS)  | 4 weeks | - Improved glucose tolerance and fasting blood glucose  
- Improved glucose-stimulated insulin secretion, and insulin-sensitive hepatic glucose production  
- Reduces hepatic phosphorylation of IKK-β and NFκ-B  
- Reduced body weight gain                                                                                                      | [91]  |
| Pubs                             | Inulin and oligofructose | 1:1 wt/wt             | 6 weeks | - Increased mass of the small intestine, colon and cecum  
- Decreased glucose and increased GLP-1                                                                                                           | [92]  |
| Naïve Mice                       | Native chicory Inulin | 3 weeks               |       | - Reduced sucrase activity in the small intestine mucosa  
- Higher caecal content and tissue weight  
- No changes in body weight gain, fat mass or liver weight  
- No shift between bacterial phyla  
- Higher abundance of Lachnospiraceae and decreased abundance of vadin BB60 and Ruminococcaceae                                                 | [93]  |
1.5.2.2. Relevant studies on the efficacy of prebiotics

Human Studies using prebiotics

The following studies illustrate the benefits of prebiotics such as oligofructose (FOS), galacto-oligosaccharides (GOS), and inulin. The benefits included changes in gut microbiota composition, improved immune function, metabolic biomarkers and anthropometric parameters. In a study where healthy men and women fed with FOS showed that satiety was increased after breakfast and dinner, hunger was reduced and food intake following dinner was reduced [94]. Another study of the effects of GOS on the composition of the human faecal microbiota in healthy adults showed that GOS increased the level of *bifidobacteria* species in half of the subjects at a magnitude of five- to ten-fold. Accordingly, increased Firmicutes was observed only in few subjects and decrease in Bacteroidetes as the *bifidobacteria* increased in the subjects [95]. The above findings are indicators that prebiotic feeding could be used to improve the composition of the gut microbiota, and reduce host susceptibility to diseases caused by pathogenic bacteria.

The efficacy of oligofructose enriched inulin on reducing inflammatory cytokines and increasing anti-inflammatory cytokines in T2D was determined. The study included 70 females with T2D in a double-blinded placebo–controlled trial. The participants were between 30-65 years old, had T2D for more than 6months and BMI between 25 and 34.99 kg/m². Participants were allocated randomly into prebiotic treated and placebo group. The prebiotic treated group
had a daily intake of 10g oligofructose enriched inulin for two months, which resulted in a significant increase in anti-inflammatory cytokine IL-4 and reduction in inflammatory cytokine IL-12 and IFNγ, thereby improving the immune system in patients with T2D. Also, BMI, waist circumference, hip circumference, serum lipid, fasting serum glucose, HbA1c and diastolic blood pressure were improved in the prebiotic treated group. However, no significant change was observed for cell count of CD3⁺, CD4⁺, CD8⁺ and CD 11b⁺T after prebiotic treatment. In conclusion, this study highlighted the benefits of oligofructose enriched inulin on the improvement of immune function, glycaemic status and serum lipid, although underlying mechanism still needs to be investigated [96].

Another study using females with T2D between ages 30-65 years, assessed the effect of resistant dextrin as a prebiotic to improve insulin resistance and inflammation. The randomised controlled clinical trial included 30 and 25 patients in the intervention and control group respectively and had 10g per day (5grams each during breakfast and dinner) of resistant dextrin in the intervention group or maltodextrin as placebo for 8weeks. The authors reported no significant baseline change for body weight and BMI between the two groups but significant decrease was observed after 8weeks supplementation in the resistant dextrin group when compared with those at baseline. Also, the resistant dextrin group had significant decrease in total fat and intakes of energy compared with those at baseline, but not significantly decreased in the
maltodextrin group. The glycaemic status did not differ significantly between the two groups at baseline but at the end of the trial, the resistant dextrin group had a significant decrease in fasting insulin concentration, quantitative insulin sensitivity check index, HOMA-IR, IL-6, TNFα, malondialdehyde, and endotoxin. However, the levels of fasting plasma glucose, HbA1c and hs-CRP, were not significantly reduced between the two groups. The findings reported in this study supports the conclusion that resistant dextrin supplementation can efficiently improve insulin resistance and inflammation in women with T2D [97].

1.6. Tight Junction and Intestinal Permeability

The body’s largest interface with the external environment is the gastrointestinal epithelium. The epithelium primarily forms the intestinal barrier, with the individual epithelial cell membranes forming majority of the barrier. Its function is to allow the absorption of nutrients and provide a physical barrier to the translocation of pathogens, toxins and antigens which are pro-inflammatory from the luminal environment into the mucosal tissues and circulatory system [71]. The barrier are impermeable where specific transporters exist. Also the barrier can be severely compromised when epithelial cells are lost, as seen in erosions and ulcerations[98].

Selective permeability by the epithelial cells is created by two pathways: the transcellular and paracellular pathway [71]. Transcellular transport is dependent on cell-specific profile of transporters and channels positioned on the
apical and basolateral cell membranes. Also, it is directional and energy
dependent. Paracellular transport is passive and results from diffusion,
electrodiffusion, or osmosis down the gradients created by transcellular
mechanisms. In contrast to transcellular transport, paracellular route is non-
directional, shows small differences in ionic selectivities and it varies extensively
among epithelia in terms of electrical resistance. Thus, by defining the degree
and selectivity of back leak for ions and solutes, the paracellular pathway
complements transcellular mechanisms [99]
Figure 1-6: Transepithelial transport pathways

The transcellular route is both active and passive. It is also based on the activity of transmembrane pumps, channels, and carriers expressed in a polarized fashion. However, paracellular transport is only passive and driven by the gradients secondary to transcellular transport mechanisms. The important barrier in the paracellular route is the tight junction, and it is regulated and varies among epithelia in tightness and ion selectivity. This regulated back diffusion can significantly modify the molecular composition of transcellular transport [99].

One of the apical junctional complexes that regulate the paracellular pathway is known as tight junctions [71]. The paracellular space between adjacent cells, would provide barrier function only where it is sealed by tight
junctions, which is most critical. An important factor for paracellular transit is the permeability of the tight junction that defines the overall barrier function of an intact intestinal epithelium. Tight junctions, also known as desmosomes, are composed of transmembrane proteins such as claudins and occludins [100]. Tight junctions determine the selective paracellular permeability to solutes and provides barrier to harmful molecules [98, 101]. Although pro-inflammatory cytokines, antigens and pathogens contribute to barrier impairment under pathophysiological conditions, other factors such as food and nutrients can modify tight junctions’ barrier functions and paracellular permeability which is associated with health and susceptibility diseases [102, 103]. Food consumed can either have beneficial or adverse effect on intestinal microbiota which also determines the “leakiness” of the gastrointestinal mucosa. However, the intestinal barrier function can also be improved by several protective measures such as antioxidants, epidermal growth factor, enteral feeding, fibre and oats, probiotics and trefoils [102].

1.7. High-fat diet, gut microbiota, impaired intestinal permeability and type 2 diabetes

A link between high-fat diet, gut microbiota, impaired tight junction and T2D has been established in several mice and human studies [71, 74, 104, 105] which have been shown to be LPS dependent [72-75]. A change in the gut microbiota may result in impaired intestinal permeability which is commonly
referred to as ‘leaky gut’ thereby increasing the concentrations of endotoxins such as LPS in the circulation in the blood [74]. The reduced expression of tight junction proteins such as ZO-1 and Occludin leads to increased intestinal permeability [74]. Several studies has also revealed that increased plasma concentration of bacterial LPS during a fat–enriched diet was responsible for the onset of metabolic endotoxaemia [71, 74, 75].

The importance of bacteria population in the onset of metabolic disorders has been studied in both animal and human models. A previous study using animal models of obesity, showed that compared to the control mice, obese mice fed prebiotic carbohydrates had increased *Bifidobacteria* spp., *Lactobacillus* spp. and *Clostridium coccoides-Eubacterium rectale* in the caecum. These species were linked to reduced gut permeability and lower level of LPS measured *in vivo* [106]. This was due to increased Z0-1 and occluding mRNA in the jejunum section which are major markers of tight junction integrity [106]. Furthermore, systemic and hepatic inflammation was shown to be improved given that the plasma cytokine and chemokine concentration decreased in obese mice that was fed prebiotic [106]. The reduced oxidative stress markers such as PAI-1, CD68, NADPHoX and iNOS mRNA concentrations led to decreased TLR4 and TNFα mRNA concentration [106]. Also, these markers were greatly reduced as the number in *bifidobacteria* spp. increased. Furthermore, as the gut microbiota changed in the prebiotic fed mice, the gastrointestinal peptide, GLP-1, significantly increased and led to reduced appetite [106].
Furthermore, the study by Cani et al., (2008) also showed that high-fat diet greatly changed the gut microbiota content in obese mice [74]. There was a reduction of *Bifidobacterium* spp., *Lactobacillus* spp., and *Bacteroides-Prevotella* spp. High-fat diet also reduced intestinal permeability by reducing the expression of epithelial tight junctions such as ZO-1 and occludin [74]. The reduced intestinal permeability was the mechanism through which high-fat diet induced metabolic endotoxaemia by increased intestinal LPS permeability followed by increased mRNA concentration of PAI-1, IL1 and TNFα, which are oxidative stress marker and pro-inflammatory cytokines [74]. Furthermore, during the glucose challenge, it showed that high-fat feeding induced glucose intolerance. This is evident in the study as high-fat fed obese mice had higher blood glucose concentration compared to the control obese mice [74]. Also glucose-induced insulin secretion, insulin resistance index, body weight gain, total energy intake, and visceral and subcutaneous adipose weight were all higher in high-fat diet fed obese mice [74]. Interestingly, high-fat fed obese mice that received antibiotic treatment had a complete reversal of the above mentioned bacterial composition and/or metabolic activity [74]. The reason is that antibiotic treatment reduced the number and effect of pathogenic bacteria and the number of Gram-positive bacteria increased and improving all adverse conditions noticed [74]. These results led to a conclusion that gut microbiota, intestinal permeability and endotoxemia by increased LPS levels during high-fat diet leads to the onset of metabolic disorders such as obesity and diabetes [74]. Another aim of the study was to show that gut microbiota does contribute to metabolic endotoxaemia in the
absence of high-fat diet. The obese mice were not fed with high-fat diet yet they had higher inflammatory tone and plasma LPS concentrations. This could be as a result of the ability of the gut microbiota in obese phenotype to harvest energy from the diet and general energy storage [74].

This was further illustrated by Bäckhed et al. (2004) in their study of a mice model of obesity and showed that in 14 days adult germ-free C57BL/6 mice conventionalized with a normal microbiota harvested from the cecum of conventionally raised animals, produced a 60% increase in body fat and insulin resistance. This happened despite reduced food intake [107].

In humans with obesity and diabetes, Creely et al., (2006) showed that in T2D there is a decrease in the phylum Firmicutes, class Clostridia, and Bifidobacterium genus. The ratio Bacteroidetes to Firmicutes and Betaproteobacteria positively correlated with plasma glucose concentration (but not BMI). T2D patients also had higher circulating serum LPS compared to the lean healthy subjects. This was expected given that the T2D patient had higher levels of phylum Bacteroidetes and Proteobacteria compared to the healthy controls. Furthermore, in obese and T2D patients, in isolated abdominal subcutaneous adipocytes, LPS activated the innate immune pathway and stimulated the secretion of pro-inflammatory cytokines (16). Likewise, in adipocytes increased expression of innate immune system pathway factors was observed. This clearly suggests that LPS causes inflammation in T2D and diabetic patients appear to be at greater risk of developing inflammation through
endotoxins (16). Similarly, an association between insulin and endotoxin was observed as hyperinsulinemic / insulin-resistant in obese phenotype. However, this association is not clearly understood. These results presented in this study suggested a strong link for gut microbiota in the pathogenesis of obesity-related T2D and the innate immune response.

Similarly, in humans with T2D, Larsen et al., (2010) showed that there was a significant reduction in the level of the phylum Firmicutes and Clostridia and increase in the level of phylum Bacteroidetes and Proteobacteria. The level of the latter negatively correlated with plasma glucose concentration. Also, there was a positive correlation between the ratio of Bacteroidetes to Firmicutes with glucose intolerance but not Body mass index (BMI). This is an indication that the dysbiosis in T2D and obesity differs [108].

1.8. Metformin – inflammation and gut microbiota

Metformin is known for its ability to lower glucose production in the liver, but much more it has been shown to have anti-inflammatory effect as well as altering the gut microbiota [6, 109, 110]. In mice models of T2D and obesity, it has been shown that increased intestinal LPS permeability led to increased mRNA concentration of IL1, IL6 and TNFα which are pro-inflammatory cytokines [72, 74]. The nuclear transcription factor-kappa B (NF-kB) plays a central role in mediating cytokines, which when activated transcriptionally activates multiple
pro-inflammatory cytokines including IL1, IL-6, IL-8, and TNFα [6]. In a cell culture model of atherosclerosis, the anti-inflammatory effect of metformin was investigated in human vascular smooth muscle cells (SMC), macrophages (Møs), and endothelial cells (ECs) [6]. Since, cytokines such as IL-8 and IL-6 may likely contribute to monocyte recruitment and adhesion to ECs in atherosclerosis, as well as drives the acute phase response respectively, the study aimed at demonstrating that metformin inhibits IL-1 induced IL-6 and IL-8 expression by impaired NF-κB nuclear activation [6]. Metformin inhibited IL-1 induced cytokine production. This was evident in study as IL-6 and IL-8 expression from SMCs, ECs, and Møs was reduced in a concentration dependent manner. IL-6 and IL-8 production were lower at low concentrations. Furthermore, IL-1 stimulated SMCs showed activation of all three mitogen-activated protein (MAP) kinases (p38, JNK, and Erk) and Akt which are all part the inflammatory signalling pathway. The p38 kinase regulates various transcription factors, including NF-κB. Metformin treatment inhibited IL-1–induced p38, JNK, and Erk phosphorylation but not Akt in SMCs. These results were obtained using metformin at a concentration that exceeded its therapeutic plasma concentration (max 20µmol/L). In ECs, high glucose leads to activation of PI3K and Akt, as well as NF-κB activation. ECs stimulated with high glucose induced phosphorylation of Akt. To show that metformin inhibitory effect occurs at therapeutic plasma concentrations (20µmol/L), ECs pre-treated with metformin and then exposed to high glucose, showed decreased Akt phosphorylation. The findings of this study support the author’s conclusion that metformin attenuate inflammation in
atherosclerosis, which may explain why it reduces macrovascular complications of diabetes aside its glycaemic effect [6].

Secondly, Napolitano et al. (2014) believed metformin could alter bile acid circulation and gut microbiota which could enhance entero-endocrine hormone secretion. To validate this, 14 T2D subjects were studied at for time points: i) patients on metformin sampled at baseline (visit 1), ii) 7 days after metformin was stopped (visit 2), iii) when fasting blood glucose raised by 25% after metformin was stopped (visit 3) and iv) when fasting blood glucose reached baseline after metformin intake was resumed (visit 4). This study established that total bile acids in serum and secondary bile acid (lithocholic and deoxycholic acids) in faeces greatly increase upon metformin withdrawal. This indicates that metformin indirectly inhibits the reabsorption of bile acid in the gut through the sodium–dependent intestinal bile acid transporter. Also, the activity and amount of GLP-1 was reduced which suggests that metformin may act on the metabolizing enzyme, DPP-IV which is a GLP-1 and PYY inhibitor. Furthermore, this study established a change in the gut microbiota in T2D patients when on or off metformin. The microbiota analysis showed that the T2D Patient’s on-metformin had significantly high levels of the phylum-genus; Firmicutes - SMB53, Actinobacteria-Adlercreutzia and low level Firmicutes-Eubacterium. However, Firmicutes-Eubacterium was significantly higher in patients’ off-metformin. This significant difference was not observed for the interaction of metformin treatment and bacterial species after multiple factor correction. Also taxa level varied
between subjects as evident on the PCoA plots which had no distinct sample clustering. Although this study had interesting findings, the limitation was the small number of subjects studied which made it difficult to determine subgroup differences and if these groups increased or reduced the efficacy or side effect of metformin [109].

Thirdly, the role of metformin in changing the composition of the gut microbiota in diet-induced obese and diabetic C57BL/6 mice was investigated [111]. The significant reductions in the proportions of Akkermansia and Alistipes and the increases in the proportions of Anaerotruncus, Lactococcus, Parabacteroides, Odoribacter, Lawsonia, Blautia and Lactonifactor, seen in high-fat diet fed mice, were reversed by metformin to a similar genera proportion in the normal chow diet mice. Akkermansia are mucin–degrading bacteria that have a protective effect on the intestinal tract by increasing the number of goblet cells [111]. The level of Akkermansia in high-fat diet fed mice after treatment with metformin greatly increased the number of mucin-producing goblet cells. Also, the role of the gut microbiota in aiding the effectiveness of metformin was shown in high-fat diet mice treated with Akkermansia. The mice had improved glucose intolerance and increased goblet cell number. Hence it can be that metformin improves glucose intolerance by increasing the level of Akkermansia, which increases the number of goblet cells [111].
1.9. **Methods used to study human gut microbiota**

Most studies used culture independent methods to characterise the gut microbiota. This is because most bacteria in the gut are not easily or cannot be cultured in the laboratory[53]. Culture independent methods are high throughput methods and do not require the growth of bacteria which makes it less susceptible to contamination [53, 112]. The methods include polymerase chain reaction (PCR) based DNA profiling, quantitative PCR (QPCR), fluorescent in situ hybridization (FISH), flow cytometry, DNA microarray and DNA sequencing. Of these methods, DNA sequencing is the most commonly used method. The molecular marker used for genetic diversity of bacteria is the 16s ribosomal RNA (16s rRNA) [113].

Recent technologies with high throughput systematic sequencing are referred to as Next generation sequencing (NGS). NGS systems include Sanger sequencing, Illumina Genome Analyzer II system, Roche 454, GS FLX Genome Analyzer (Pyrosequencing), Applied Biosystems's solid system, and Helicos HeliScope [112]. These high throughput sequencing techniques have different features and principles but their common advantages include higher throughput efficiency, increased sensitivity, and sequencing of multiple samples at once [112, 114]. However, a huge amount of sequence data is generated by the high throughput sequencing techniques and this requires extensive bioinformatics analysis. Also, the high throughput sequencing techniques has higher error rate due to the shorter sequence reads (<500 bp) produced [112].
The NGS technologies have been used mainly for metatranscriptomics and metagenomics studies. Metatranscriptomics and metagenomics rely on RNA and DNA sequencing method respectively [112]. Although both methods can be used to determine the function of the gut microbiota, metatranscriptomics provides information on alterations in gene expression in relations to changes in the host and diet impact. Metagenomics gives insight about the composition by giving sequence information from the collective genomes of the microbiota in a single experiment [112, 115, 116]. The metagenomic technique has advantages such as its high throughput and ability to identify new functional genes. However, given that collective genome of the microbiota is sequenced, the DNA from dead cells cannot be distinguished from the DNA of live cells [117]. This challenge does not apply to metatranscriptomics because it’s an RNA based techniques which only expressed genes have been transcribed to RNA and then translated to protein [112]. In both methods, faecal samples are used because of the easy collection [112].

1.10. **Aims of the research project**

The gut microbiota can be a potential therapeutic target for metabolic diseases such as T2D. Since most studies have been conducted in animal models, the need for more human studies becomes imperative to further understand the role disease play in shaping the gut microbiota. Many studies have successfully differentiated the gut microbiota in both healthy individuals and T2D. However,
the role of the participant’s genetics, diet, and geographical location and research methods used in the various studies can lead to varying outcome. The need for more research on this topic to contribute to the growing knowledge of the study of the gut microbiota in human T2D. Furthermore, there is a need to use faeces experimentally as faecal water, which may be a potential research approach to investigate the activity of bacteria in human in-vitro. Thus the overall aim of this study was to investigate the impact of T2D on the gut microbiota, an important ‘organ’ that had the potential to influence disease progression and the activity of faecal water from T2D patients on colon cells as a measure of disease state by correlating the results with markers of T2D.

This thesis aimed to:

I. Elucidate the differences in the gut microbiota of type 2 diabetic patients and age, sex, and BMI matched healthy controls using next generation sequencing.

II. Investigate the benefit of GOS on gut microbiota composition after prebiotic intervention in a double-blinded human studies involving T2D patients and matched healthy controls.

III. Investigate the impact of T2D and healthy controls FW samples prepared in-vitro, to mimic a complex mixture of both host and bacteria compounds using a Caco-2 monolayer cell culture model of intestinal epithelium and linked it to T2D patient’s metabolic markers measured in the blood.
IV. Investigate the protective effect of GOS against the cytotoxic effect of FW extracted from the faeces of T2D patients and healthy controls on Caco-2 cells using cell viability assays as a measure of cytotoxicity.
CHAPTER 2

MATERIALS AND METHODS
2. Materials and Methods

2.1 Faecal bacteria DNA extraction

The DNA was extracted, according to the manufacturer’s protocol, using the Cambio PowerFeacal\textsuperscript{TM} DNA isolation Kit (Cambio, UK), with slight modifications. Briefly, 0.25g of the faecal sample was added to a 2ml Dry Garnet Bead Tube. Samples were lysed by both chemical and mechanical disruption. The sample was chemically disrupted by addition of the provided bead solution and subsequent incubation at 65\textdegree\text{C} for 10 minutes. Modification to the protocol (in place of vortex for 10 minutes) included mechanical disruption of the sample using beads grinding at 5.0 speed for 30 seconds using a FastPrep FP120 (Thermo Savant). PCR inhibitors were removed by binding the DNA on a silica spin filter membrane; samples were washed to remove contaminants and pure genomic DNA eluted in TE buffer. The DNA concentration and quality was determined using a Nanodrop 2000 (Thermo Scientific) and Qubit 2.0 fluorometer (Invitrogen).

2.1.1 Determination of DNA concentration using Qubit\textsuperscript{®} 2.0 Molecular probe fluorometer Invitrogen (Life Technologies)

DNA sample concentrations were measured using a Qubit and samples of 50ng/\mu l were sent to Animal and Plant Health Agency (APHA) for next generation sequencing as described in Section 3.4. For the standards, two assay
tubes were set up and one for each of the samples. The Qubit working solution was prepared by diluting the Qubit reagent 1:200 with the Qubit buffer. 200μL of working solution was prepared for each standard and sample (190μL of working solution was added to 10μL of the standard while 198μL of working solution was added to 2μL of each sample). The tubes were vortexed for 3 seconds and incubated for 20 minutes at ambient temperature. Each tube was then inserted into the Qubit fluorometer and the DNA concentration was measured.

2.2 DNA polymerase chain reaction (PCR)

PCR inhibitors generally exert their effects through direct interaction with DNA or interference with thermosTable DNA polymerases. Direct binding of agents to single stranded or double-stranded DNA can prevent amplification and facilitate co-purification of inhibitor and DNA (Promega). The absence of PCR inhibitors in the extracted DNA was confirmed by amplification of bacterial and archaea 16S rRNA V4-V5 region as follows: A 50 µl reaction contained 5x Go Taq G2 buffer (Promega), 10 mM of each Deoxynucleotide Triphospahe (dNTPs) (Promega), 10 pmol each of the forward (U515F- 5′-GTGYCAGCMGCCGCGGTAG and reverse primer (U927R- 5′-CCCGYCAATTTCMTTTRAGT) [57], 5 u of GoTaq G2 DNA polymerase (Promega), and 2 µl of the template DNA. The volume was then adjusted with nuclease free water to a total volume of 50µl. Amplification was performed using a thermal cycler (Applied Biosystems, UK), under the following cycling conditions: 94°C for 3 minutes; 30 cycles of 94°C for 30 seconds,
55°C for 45 seconds, 72°C for 1 minute; followed by a final extension at 72°C for 8 minutes.

2.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis uses an electric field to separate DNA fragments according to their sizes. Agarose (Fisher Scientific, UK) was weighed and dissolved in a solution of 1 X Tris/Boric EDTA (TBE) by heating, resulting in a final concentration of 1% (w/v) and then 5 µl of Redsafe™ (Ecogen), a nucleic acid stain, was added to the 1% agarose gel.

For detection of the PCR amplicon, 5 µl of the reaction products were mixed with loading buffer (at a ratio of 5:1) and loaded on the gel. A corresponding 1 Kb DNA ladder (Promega), were also loaded each time to estimate the size of the desired bands. The gel was run at 100V for 45 minutes-1 hours to allow separation of bands in 1 X TBE. DNA bands were visualised with a Gel Documentation system (GeneFlash Syngene, Bio Imaging).

2.3 Tissue Culture Methods

The human adenocarcinoma cell line (Caco-2) was used. The cells were kindly provided by Dr. Jenny Ritchie at passage number 12. Cells were used for experiments between passages 14-25. Cells were cultured in DMEM (with L-glutamate) supplemented with 10% Foetal Bovine Serum, 1% Non-essential
amino acids, 1% Penicillin-Streptomycin and maintained at 37°C in a humidified environment supplied with 5% CO₂. Depending on the type of experiment being conducted, cells were grown in T75 flasks, T25 flasks, 12-well plates (transwells) and 96-well plates. The culture media used is also described in Table 2.2. Passage of confluent adherent cells was done by removing the existing medium, washed with phosphate buffered saline (PBS) and covered in 0.25% Trypsin-EDTA for five minutes to allow cells to detach. Trypsin action was stopped by adding culture medium and the cells were pelleted by centrifugation (4 minutes, x 2500 rpm) in a swinging bucket rotor for the Hereaus Megafuge 16R (Thermofisher Scientific). Pellets were re-suspended in fresh media, counted and plated at the required number and stored in the incubator at 37°C. For cryopreservation, aliquots of the culture cells in 10% DMSO were stored in liquid nitrogen (−196°C) for long-term storage. Cells were counted prior to seeding the culture plates as described below.

2.3.1 Cell Enumeration

Cell suspensions were diluted in 0.4% Trypan blue solution and cell medium at a ratio of 1:1:5 and 10μL of this mixture was transferred to each chamber of a haemocytometer (Neubauer chamber). The cells were counted under an inverted microscope. Non-viable cells take up Trypan blue dye, hence, it is possible to distinguish between non-viable and viable cells. Each square of the haemocytometer with 13mm cover slip in place represents a total volume of 0.1
mm3. 1 cm3 is equivalent to 1 mL, so that cell concentration per mL was determined by multiplying the average number of cells per square by the dilution factor and also by the area covered by the grid lines (104) using the formula below:

\[
\text{Cells per mL} = \text{the average count per square} \times \text{dilution factor} \times 10^4 \text{ (a constant)}.
\]

For example: if the average count per square is 50 cells \( \times 5 \times 10^4 = 2.5 \times 10^6 \text{ cells/mL} \).

2.4 Transwell tissue culture assay

Polyester transwell (PET) Clear inserts have microscopically transparent polyester membranes that are tissue culture treated for optimal cell attachment and growth. Transwell-Clear inserts provide better cell visibility under phase contrast microscopy. It also allows for assessment of cell viability and monolayer formation (Corning Incorporated, Life Sciences). Caco-2 cells were grown on transwell filter inserts (0.4 μm pore size, polyester, 12-mm inside diameter; Corning Incorporated, Corning, NY) at a density of 1 x 10^5 cells per well. The culture medium was changed every 2 days for 15 days until a differentiated Caco-2 monolayer was achieved. The monolayer integrity was evaluated by measuring the transepithelial resistance (TER) using an EVOM2 Epithelial Voltohmmeter and wells with TER reading above 900 ohms were used in the experiment. Cell
medium was gently aspirated from the apical and basolateral compartments. 500µl of FW (2.5% w/v) for sample well or cell medium for control well were added to the apical side of the inserts of the transwells and the basolateral side was bathed in 1.5ml cell medium. The resistance of a blank (culture insert without cells) was measured to correct for background resistance. The plates were incubated at 37°C with 5% CO².

2.5 Transepithelial electric resistance (TER) measurements

TER is used as a measure of cell monolayer integrity for confluent cells grown on transwells. The higher electric resistance indicates the formation of good cell monolayer. TER measurements for all samples were performed within 5 min after taking the culture plates out of the incubator to eliminate the influence of temperature. Prior to taking the measurements, the electrodes were sterilized using 70% ethanol and equilibrated in pre-warmed cell medium. The electrode was then inserted into the EVOM2 Epithelial Voltohmmeter (World Precision Instruments). The change in TER was measured in ohms before the experiment (Initial), at the start of experiment, at 24 and 48 hours. The TER of the sample well was obtained by subtracting the blank value from the total resistance of the sample. Uninfected well TER readings were adjusted to 100% and the values of sample wells expressed as a percentage of the uninfected well.
2.6 Human faecal water sample preparation

Faecal water was extracted by adding 40ml PBS to 1g of faeces (2.5% w/v) and homogenised using a Masticator® Homogenizer Blender (MG Scientific) for 5mins. The homogenate was transferred to a tube and centrifuged at 39,800 g in an Avanti J-26S XP centrifuge (Beckman Coulter) for 2 hours at 4°C. 35ml of the supernatant was transferred to a clean tube and centrifuged at 3725g in an Allegra X-15R centrifuge (Beckman Coulter) for an additional 20 minutes at 4°C to remove remaining debris present in the supernatant. The clear supernatant was then filtered using a 0.22µm filter and aliquots of the supernatants (faecal water) were placed into 2ml Eppendorf tubes and stored at −80°C prior to analysis.

2.7 Determination of faecal cytokines using Human Cytokine array

2.7.1 Faecal Protein Extraction

For each sample, 0.1 g of faeces was weighed into a sterile eppendorf tube, and 0.4 mL RIPA lysis buffer was added. The samples were then vortexed for 1 min, followed by a 5-min incubation in an ice bath until no visible faeces granules remained. The samples were then centrifuged at 13,000 rpm for 5 minutes at 4°C to remove cell debris. The supernatant fraction was collected into a fresh tube and placed on ice for 1 hour, centrifuged for 30 min at 13,000 rpm at 4°C. The
supernatant fraction was collected again and stored at -80 °C until further analysis.

2.7.2 Determination of Protein Concentration

Thermo Scientific Pierce BCA Protein Assay Kit (Catalogue No. 23225) was used to determine protein concentration according to the manufacturer’s instruction. Briefly, bovine serum albumin (BSA) standards of known concentrations were made up in distilled water. The working reagent (WR) was prepared by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B (50:1, Reagent A: B). For standards and unknown samples, 10μL of each was added to a sterile 96-well microplate in triplicate and 200μL volume of the WR was added to each well and mixed thoroughly on a plate shaker for 30 seconds at ambient temperature. The plates were then incubated at 37°C for 30 minutes and the absorbance was measured at 540nm using a spectrophotometer (Omega Fluorostar, Switzerland).

2.7.3 Cytokine Array

RayBio® C-Series Human Cytokine Antibody Array 1 Kit (RayBiotech·AAH·CYT·1·8) detects 23 Human Cytokines and suitable for all liquid sample types (Figure 2.1). It is a membrane, sandwich based, semi-quantitative method for detection of protein expression of cytokines in biological fluids. This assay was
used to identify the cytokines present in the stool samples of T2D patients and was performed according to the manufacturer's protocol. Briefly, membranes are blocked with blocking buffer for 30 minutes, incubated with faecal protein (50 µg) overnight at 4°C, membranes washed and then incubated with Biotinylated Detection Antibody Cocktail. Also, membranes were washed, incubated with HRP-Conjugated Streptavidin, washed again and then incubated with detection Buffers. All incubations were performed for 2 hours at ambient temperature, unless otherwise stated. Images of the membranes were taken with chemiluminescent imaging system, and Image was used to quantify each blot on the membrane and analysed using the Software tool for RayBio Human Cytokine Antibody Array 1 (S02-AAH-CYT-1).

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<th>Each antibody is spotted in duplicate vertically</th>
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<td>IL-3</td>
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<td>IL-5</td>
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<td>5</td>
<td>IL-13</td>
<td>IL-15</td>
<td>IFN-gamma</td>
<td>MCP-1 (CCL2)</td>
<td>MCP-2 (CCL8)</td>
<td>MCP-3 (CCL7)</td>
<td>MIG (OXCL9)</td>
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**Figure 2-1: RayBio® C-Series Human Cytokine Antibody Array 1 Kit**

List of 23 human cytokines detected by the array using the human faecal protein from type 2 diabetes patients.
2.8 Cell viability assay to measure faecal water cytotoxicity

To determine the cytotoxicity of FW, cells were seeded at $2 \times 10^4$ cells/100µl in a 96well plate for 48 hours and the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), a colorimetric method for determining the number of viable cells was used according to the manufacturer’s instructions. The CellTiter 96® AQueous One Solution is MTS and an electron coupling reagent (phenazine ethosulfate; PES. The $3\cdot(4,5\cdot$dimethyl-2-yl)$\cdot5\cdot(3\cdot$carboxymethoxyphenyl)$\cdot2\cdot$(4-sulfophenyl)$\cdot2H\cdot$tetrazolium, inner salt; MTS assay is based on the cellular reduction of MTS by the mitochondrial dehydrogenase of viable cells, to a formazan product that is soluble in tissue culture medium, because combined with PES, MTS forms a stable solution.

After 2 hours incubation with FW in triplicate, the FW was removed from the cells, replaced with 100µl of sterile medium, then 20µL of MTS solution was added into each of the 96-well plates, and incubated for 4 hours at 37°C. Following incubation, optical density was measured using a spectrophotometer (Omega Fluorostar, Switzerland) at 540nm. Cells with media were used as positive controls, while cells lysed with 1% Triton X-100 were used as negative control. The percentage viability of each sample was derived by dividing the sample OD by the OD of the positive control, hence the positive control is used as 100% cell viability.
2.9  Cell viability assay to investigate the effect of GOS against FW cytotoxicity

2.9.1  Galactooligosaccharide (Bimuno) Preparation

Bimuno is a daily food supplement containing unique patented GOS developed by scientist working at the University of Reading [78]. Bimuno ingredients as detailed on product includes GOS, lactose (from milk), glucose syrup, thickener (gum Arabic), galactose, and acidity regulator (trisodium citrate). Galactooligosaccharide stock solutions of 20% w/v were prepared by weighing 5.5g of commercially available GOS powder (Bimuno) and 25ml of cell culture media. From the stock, a 5%, 10% and 15% v/v solution was then prepared by diluting in cell culture media. The solutions were thoroughly mixed by inverting the tube several times until solutions had no visible particulate matter.

2.9.2 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) Assay

Caco-2 cells were seeded at 2x10^4 cells/100µl in a 96well plate and incubated for 48 hours. At 48 hours, cells were pre-treated in duplicate with either 5%, 10% or 15% v/v (100µl) GOS solution and incubated for 2 or 12hours. At the end of the pre-treatment, in order to assess GOS effect on cell viability, MTS assays were performed on the cells. For investigating GOS effect against FW cytotoxicity, GOS was either removed or left on cells and 100µl of each FW
was added to the wells and incubated for 2 hours. After incubation, FW was removed from the cells and MTS assay performed. MTS assay was performed as described in section above. Cells with culture medium were used as positive controls, while cells killed with 1% Triton X-100 were used as negative control. OD of the MTS assay was measured and analysed as described above in section 2.8.

2.10 Statistical analysis

Experimental data were analysed as stated in the method section of the individual chapters of this thesis. Dr Huihai Wu (Department of Bioinformatics, University of Surrey) and Peter Williams (Department of Mathematics, University of Surrey) were consulted to analyse the data in Chapter 3 and 4 respectively. Otherwise, data were analysed using the One-way ANOVA, T-test and linear regression analysis using the statistical package of GraphPad7 Prism software (Graphpad, California, USA)
CHAPTER 3

UNDERSTANDING THE GUT
MICROBIOTA IN HUMAN TYPE 2 DIABETES
3. Understanding the Gut Microbiota in Human Type 2 Diabetes

3.1. Introduction

T2D is considered a low grade inflammatory disease, but the exact mechanism through which this occurs is unknown. A number of animal models have been used to investigate the gut microbiota in T2D, and results showed an increased bacteria population belonging to the phyla Bacteroides. LPS is an endotoxin that on entering the circulation of the host, can lead to endotoxaemia and inflammation [73, 108].

It has been proposed that high-fat feeding is a major trigger leading to bacterial population shifts in the human gut, in addition to chylomicron formation as a source of blood LPS per se [72]. More recently human studies have confirmed these early bacterial composition changes in T2D patients, however, as the aetiology of human and animal diabetes is not always comparable, many of the features of this microbial alteration remain to be understood. When analysing the literature, bacterial populations have been shown to be affected by the host genetics, age, gender, ethnicity and geographical location. Additionally, sample storage, DNA extraction and analysis methods such as qPCR, FISH, and next generation sequencing (NGS) can contribute to the considerable variation found between individual studies [114, 115]. These diverse factors make it necessary for more human clinical studies to be performed, possibly to broaden the scope and further understand the role these microbial changes may have for host metabolism.
3.2. **Hypothesis**

This chapter aimed to test the hypothesis that the gut microbiota in patients with T2D would be significantly altered in age, gender and BMI matched healthy controls and that the dietary supplementation with a pre-biotic carbohydrate with documented effects in healthy participants (galacto-oligosaccharide) would normalise the gut microbial population back to the non-diabetic “state” when used in a group of T2D patients.

The aims and objectives of this chapter were to:

1. Extract faecal DNA from both healthy controls and T2D subjects and assess the integrity of DNA for application in NGS using nanodrop and Qubit
2. Investigate the difference in gut microbiota composition between healthy controls and T2D patients using the healthy control and T2D extracted DNA sequenced data and differential statistical analysis.
3. Investigate the effect of GOS treatment on the gut microbiota in T2D using the T2D faecal extracted DNA sequenced data and differential statistical analysis.
4. Investigate the potential effect of the oral diabetic agent metformin on the gut microbiota using the T2D faecal extracted DNA sequenced data and differential statistical analysis.
5. Investigate the gut microbiota composition in healthy controls split by BMI using the healthy controls faecal extracted DNA sequenced data and differential statistical analysis.

3.3. **Study Design**

3.3.1. **Study Plan**

This study was undertaken (funded by the European Funds for the Study of Diabetes) in the format shown below (Fig. 2.1). This was performed using a mixed study design:

- Cross-Sectional Study
- Randomized Controlled Parallel (Parallel RCT) Study
A total of 60 human subjects were recruited comprising of T2D patients (30) and healthy controls (30). The T2D group was randomised into two groups, prebiotic or placebo treatment groups. Baseline measurements were taken for all subjects, and treatment with prebiotic or placebo (n=15 per group) lasted for a period of 12 weeks. The control group consisted of both obese and lean individuals (n=15 per group).

### 3.3.2. Inclusion and exclusion criteria

Eligible subjects were between the ages of 40 and 65 years and with/without T2D. Reasons for exclusion from study were:

- Current or previous use of antibiotics in the previous three months
- Concurrent use of Non-steroidal Anti-inflammatory drugs (NSAID’s)
- A positive history of inflammatory bowel disease (IBD)
- Impaired renal function (Estimated Glomerular Filtration Rate, eGFR < 60).
3.3.3. Treatment plan- 12 weeks prebiotic/placebo

In the parallel RCT study consisting of 30 T2D subjects, prebiotic (Galactooligosaccharide (GOS)) or placebo (Maltodextrin) was provided in individual sachets (5.5g powder) enough to last for a period of 12 weeks. GOS used in this study is the commercially available Bi2muno ® (B-GOS, 52% GOS content) supplied by Clasado Ltd, (Milton Keynes, United Kingdom). The major importance of B-GOS is the use of a specific probiotic (Bifidobacterium bifidum NCIMB 41171). It is produced from the activity of galactosidase enzymes isolated from this strain, hence it has increased bifidogenic property [78, 83].

Patients were instructed to take one sachet daily (GOS or Maltodextrin) and to stop the consumption of yoghurt for the period of treatment. The group taking GOS or Maltodextrin were known as L and T, respectively. However, the content of the sachet taken in L and T groups was unknown during the study, making it a double-blind study.

3.3.4. Faecal Sample Collection

Subjects were provided with sterile collection bottles and bed pans to ensure collection of samples were done in sterile holders. Subjects were instructed to keep samples cold after collection and for < 2 hours before their study visits. The healthy controls had no treatment and provided one sample while the T2D patients in the L and T groups provided two samples, before and
after treatment for 12 weeks. The samples provided by the T2D patients were labelled as pre and post samples. Samples consisted of 90 samples from T2D (n=30) and matched (n=30) adult males (age 45-60 years old). Faecal samples were collected and stored initially at -20°C and at -80°C for long term storage, until further processing.

3.4. **Next generation sequencing (NGS)**

Genomic DNA samples were extracted and sent to the Animal and Plant Health Agency (APHA) for next generation sequencing. Only DNA samples with concentration ≥ 50ng/µl were eligible, hence 27/30 control subjects and 52/60 T2D patients DNA samples were included in the NGS. The sequences were processed in Qiime using the AmpliconNoise pipeline that utilises flowgram information of the sequences to correct for errors. The samples were demultiplexed by exact matching of both barcode and primer and the sequences filtered and trimmed based on identification of low quality signals. The filtered flowgrams were clustered to remove platform-specific errors and converted into sequences using the PyroNoise algorithm. The sequences had barcodes and degenerate primers removed prior to trimming at 500 base pairs (bp). They were then further clustered by SeqNoise to remove PCR single base errors. In the final step, the Perseus algorithm was used to identify chimeras. The de-noised sequences were classified using the standalone RDP classifier. From this, taxa frequencies at five different levels: Phylum, Class, Order, Family
and Genus were calculated. Additionally, a non-supervised approach was used, operational taxonomic units (OTUs) were generated at 3% divergence following pair-wise global sequence alignment and hierarchical clustering with an average linkage algorithm. To improve resolution at the OTU level, sequences were also compared with databases at the NCBI website (http://blast.ncbi.nlm.nih.gov/blast.cgi). Further statistical analyses were performed in R using the Tables and data generated as above, in addition to the meta-data associated with the study. For community analyses (including alpha and beta diversity analyses) we used the vegan (http://cran.r-project.org/web/packages/vegan/) package. To calculate Unifrac distances (that account for phylogenetic closeness), we used the phyloseq, ape, and phangorn packages. To determine significant differences in bacterial abundances between the groups, we used DESeqDataSetFromMatrix function from DESeq package with a significance value cut-off of 0.05. This function allows negative binomial GLM fitting (as abundance data from metagenomic sequencing is overdispersed) and Wald statistics for abundance data and identifies species with log-fold changes between different conditions [118].

3.5. Statistical analysis

The statistical analysis was kindly performed by Dr Huihai Wu (Experimental officer in Bioinformatics, University of Surrey). Baseline values were compared between the healthy controls and T2D group using an unpaired t
test or Mann-Whitney test, while treatment effects were assessed by comparing the differences in changes from baseline within the T2D group using a paired t test or Wilcoxon’s matched pairs signed–rank test.

The cross-sectional study data analysis was performed for T2D patients (pre samples) versus the healthy controls. Furthermore, the parallel RCT study data analysis was done for treatment (L) versus placebo (T) groups of both the pre and post samples of the T2D group. Although 27/30 control subjects DNA samples were sent for sequencing, only 26 were included in the statistical analysis as one subject was taking antibiotic during the study. In the T2D group, for it to be included in the analysis it had to be a complete pair, pre and post samples of an individual subject. Only 23 subjects had complete pairs with 11 and 12 in the treatment and placebo groups respectively.
3.6. **Results.**

3.6.1. **Amplification of extracted faecal DNA**

In this study, although both the Nanodrop and Qubit fluorometer were used to determine the quality and quantity of the DNA, the results of the Qubit fluorometer was relied on, which was used to determine those samples to be included in the sequencing. PCR was used to amplify the DNA and PCR products with bands on a gel electrophoresis of expected product size were considered suitable for sequencing. PCR amplification of the V4-V5 region of 16s ribosomal RNA gene for both bacteria and archaea was performed for 79 DNA samples (≥50ng/µl). As expected, bands for the amplification of the V4-V5 region of 16s rRNA genes were successful for the 79 PCR amplified products and negative control, containing no DNA had no band (see Figure 3.1). The DNA samples (not PCR product) were sent to APHA for 16s rRNA next generation sequencing.
Figure 3-2: 1% agarose gel PCR amplification fragments of the 16S V4-V5 region amplicon

1kb ladder (Promega): Lane 1· 1kb DNA ladder: lanes 2-9 and 12-14 are PCR products for the DNA extracted from the subjects both T2D and healthy controls while lane 10 is a negative control which has no band. This shows that the extracted DNA were free of PCR inhibitors and suitable to be sent for next generation sequencing. The amplicon size was approximately 400bp. This is a representative gel image of the PCR amplicons from all healthy controls and T2D subjects, which were sent to APHA for next generation sequencing.
3.6.2. 16s rRNA Sequences constituted of mostly bacteria products

The primers used were optimised to identify the V4-V5 of 16s rRNA of both bacteria and archaea and the result showed that approximately 99.99% of sequence from the sequencer are bacteria products. This implies that the genomic DNA extracted from faecal samples was mostly that of bacteria (see Figure 3.2) compared to archaea. Given that the interest of this study is to classify the groups of bacteria present in the gut of healthy controls and T2D subjects, this result is a positive starting point for further analysis.

Figure 3.3: Direct sequencer data showing the kingdom classification of the microbial community
The bars represent both healthy control and T2D subjects in the study. Bacteria sequences (blue bar) constitutes 99.99% of the sequences.

3.6.3. Beta Diversity Comparisons between healthy controls and type 2 diabetes group (pre- and post-samples).

The analysis of the microbial community by 16S rRNA gene sequencing using weighted UniFrac analysis of the communities showed no difference in the overall community diversity between the healthy controls (Red dots), T2D pre samples (Orange dots) and T2D post samples (blue dots). The communities had no separation by group (healthy controls vs T2D) or treatment (pre- vs post-samples) as can be seen on the PCoA plots.
Figure 3-4: The PCoA plot of the weighted UniFrac distances for the 16S rRNA analysis of DNA samples derived from healthy controls and T2D (pre and post samples) groups.

No distinct difference was observed as clustering did not occur by either group (T2D patients and healthy controls) or treatment (Placebo and treatment).
3.6.4. **Gut microbiota in healthy controls**

The healthy control subjects had a higher level of Bacteroidetes than Firmicutes (mean 54.35% versus mean 40.92%). The lowest bacteria phyla were cyanobacteria, Euryarchaeota and Fusobacteria (mean 0.01%, 0.01%, and 1.58e-003% respectively) (Fig 3.4).

![Gut Composition in Healthy Controls](image)

**Figure 3-5: Composition of the gut microbiota in healthy controls (n=27).**

The above chart shows the composition of the gut microbiota at phyla level in healthy controls. The values are mean values taken from the statistical data and used to plot charts for visual interpretation. From the graph, the prominent four divisions are the phyla Firmicutes, Bacteroidetes, Proteobacteria and other. The abundance of Bacteroidetes is higher than that of the Firmicutes.
3.6.5. Gut microbiota in healthy controls split by BMI - Normal weight ($\leq 25$kg/m$^2$) versus overweight ($> 25$kg/m$^2$)

In this study, there was a lower level of Firmicutes in overweight controls compared to normal weight controls (mean 37.8$\%$ versus mean 43.1$\%$; $P=0.16$ and $Q=1.0$). Also, the Bacteroidetes level was higher in overweight controls compared to normal controls (mean 56.4$\%$ versus mean 53.1$\%$; $P=0.86$ and $Q=1$). Furthermore, the level of Actinobacteria was the same for both overweight and normal weight controls (mean 0.16$\%$ versus mean 0.16$\%$; $P=0.90$ and $Q=1$), but the level of Proteobacteria was higher in the overweight controls than normal weight controls (mean 2.70$\%$ versus mean 0.76$\%$; $P=0.96$ and $Q=1$). However, the above differences were not significant for the $P$ and $Q$ values.
Figure 3-6: Difference at the phyla level between normal weight (n=14) and overweight (n=13) healthy controls.

The four phyla compared between the T2D patients and healthy controls are the Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. There was no significant difference between the normal weight and overweight group for the phylum Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria,
3.6.6. Gut microbiota in T2D subjects

The pre samples of T2D subjects had a higher level of Bacteroidetes (P=0.007; Q=0.04) and decreased level of Firmicutes (P=0.003; Q=0.03) (mean 52.54% versus mean 39.70%) (Fig 3.3). The lowest bacteria phyla were the Cyanobacteria, Euryarchaeota and Deferribacteres (mean 0.00%, 0.01%, and 0.01%, respectively) (Fig 3.6).

![GUT MICROBIOTA COMPOSITION IN T2D](image)

**Figure 3-7: Composition of the gut microbiota in T2D (n=27).**

The chart shows the composition of the gut microbiota at phyla level in T2D. The values are mean values taken from the statistical data and used to plot graphs for visual interpretation. From the graph, the prominent five divisions are the phyla Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and other. The abundance of Bacteroidetes is higher than that of the Firmicutes in T2D subjects.
3.6.7. Difference in gut microbiota between healthy controls and T2D (Pre-samples) patients

The Wilcoxon Signed–Rank test (Mann–Whitney test) was used to test for significant differences in the gut microbiota between the two groups. There was no significant difference between the healthy control and T2D subjects (pre) in the phyla Firmicutes (P=0.85; Q=0.88), Bacteroidetes (P= 0.54; Q=0.88), and Actinobacteria (P=0.90; Q=0.88). Reporting the p value, the phylum Proteobacteria was significantly different (P=0.02; Q=0.19) between the two groups which was higher in the T2D subjects compared to healthy controls.
Figure 3-8: Difference at phylum level in the gut microbiota of healthy controls (n=27) and T2D subjects (n=27) (pre samples).

The bars represent the mean values taken from the statistical data and used to plot graphs for visual interpretation. The four phyla compared between the T2D patients (pre samples) and healthy controls were the phyla Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. From the graph, Firmicutes and Bacteroidetes were slightly lower in T2D subjects compared to healthy controls. Actinobacteria level was low in both groups but a little higher in T2D subjects. Proteobacteria is higher in T2D patients (pre samples) than in healthy controls.
3.6.8. Difference in gut microbiota between healthy controls and T2D (post-samples) patients

The Wilcoxon Signed –Rank test (Mann –Whitney test) was used to test for significant differences in the gut microbiota between the two groups. There was a significant difference between the healthy control and T2D patients (post intervention) in the phyla Firmicutes (P=0.01; Q=0.08), Bacteroidetes (P=0.006; Q=0.06), and Proteobacteria (P=0.03; Q=0.11) but no significant difference for Actinobacteria (P=0.22; Q=0.44). GOS supplementation in the T2D patients resulted in higher levels of the phyla Firmicutes compared to the healthy controls. However, the level of Proteobacteria was higher in the T2D patients compared to the healthy controls.
Figure 3·9: Difference at phylum level in the gut microbiota of healthy controls (n=17) and T2D subjects (n=26) (post samples).

The bars represent the mean values taken from the statistical data and used to plot graphs for visual interpretation. The four phyla compared between the T2D patients (post samples) and healthy controls were the Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. From the graph, Firmicutes and Bacteroidetes were higher and lower (respectively) in the T2D patients after GOS supplementation. Actinobacteria level was low in both groups but Proteobacteria was higher in T2D patients compared to the healthy controls.
3.6.9. **Gut microbiota in T2D - Placebo and prebiotic treatment group**

The difference between the pre and post samples of both placebo and treatment groups is shown below (Table 3.1). Four major phyla had either decreased or increased in post samples of placebo and treatment groups. For the placebo group, the level of Firmicutes increased in the post samples and Bacteroidetes level decreased. Also the level of Actinobacteria and Proteobacteria were increased in the post samples. For the prebiotic group, the level of Firmicutes and Bacteroidetes increased and decreased respectively post treatment, while the levels of Actinobacteria and Proteobacteria were both decreased. However, only the post treatment increase in the level of Firmicutes for the prebiotic group was significant reporting for the P and Q value, while the post treatment decrease of Bacteroidetes was significant reporting the P but not Q value (Table 3.1, Figure 3.9). Therefore, the prebiotic treatment had a significant effect on the gut microbiota in T2D by increasing the level of phylum Firmicutes.
Table 3-1: Mean values for the placebo and treatment group, showing the phyla mean difference between pre and post samples. Statistical significance were reported as P and Q values.

<table>
<thead>
<tr>
<th>Placebo Group</th>
<th>Pre</th>
<th>Post</th>
<th>Mean diff.</th>
<th>P value</th>
<th>Q value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes</td>
<td>39.80%</td>
<td>50.30%</td>
<td>10.50%</td>
<td>0.02</td>
<td>0.23</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>52.20%</td>
<td>42.60%</td>
<td>-9.60%</td>
<td>0.07</td>
<td>0.28</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.10%</td>
<td>0.20%</td>
<td>0.10%</td>
<td>0.06</td>
<td>0.28</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>3.70%</td>
<td>3.90%</td>
<td>0.20%</td>
<td>0.56</td>
<td>0.89</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Pre</th>
<th>Post</th>
<th>Mean diff.</th>
<th>P value</th>
<th>Q value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes</td>
<td>40.10%</td>
<td>48.90%</td>
<td>8.60%</td>
<td>0.001</td>
<td>0.02</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>51.50%</td>
<td>43.70%</td>
<td>-7.80%</td>
<td>0.05</td>
<td>0.19</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.49%</td>
<td>0.35%</td>
<td>-0.14%</td>
<td>0.44</td>
<td>0.68</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>5.10%</td>
<td>2.50%</td>
<td>-2.60%</td>
<td>0.08</td>
<td>0.22</td>
</tr>
</tbody>
</table>


The gut microbiota in T2D subjects’ taking metformin (n=18) and metformin naïve (n=8) showed no significant difference at the phylum level for Firmicutes (P= 0.51, Q=0.99), Bacteroidetes (P= 0.81, Q= 0.99), Actinobacteria (P= 0.58, Q=0.99) and Proteobacteria (P= 0.48, Q=0.99). However, the subjects on metformin had slightly higher level of Bacteroidetes (mean 54.0% versus 51.4%), Actinobacteria (mean 0.33% versus 0.12%), and Proteobacteria (mean 4.09% versus 3.20%) than those not taking metformin. Firmicutes level was higher in subjects’ off-metformin than those on metformin (mean 38.5% versus 40.6%).
3.7. Discussion

The primary aim of the overall study was to investigate the effects of prebiotic supplementation on intestinal bacteria, intestinal permeability, endotoxaemia, and glucose tolerance concurrently in T2D patients. To achieve that, intestinal permeability, anthropometric parameters, blood pressure, glucose tolerance, inflammatory markers and lipids were assessed at baseline and post-interventions. The study by Pedersen et al., (2016) (see appendix A for publication) detailed the findings of the primary aim, where it was reported that prebiotic supplementation had no significant effect on clinical outcomes. However, for this chapter, only the effect of prebiotic supplementation on intestinal microbial community structure were investigated and reported [118].

DNA samples were sent for 16s rRNA sequencing and sequenced data files were statistically analysed. The sequencing yielded mainly bacteria products (approximately 99.99%) which make it possible to identify the bacteria groups residing in the gut of T2D patients and healthy controls. Looking at the sequenced data, over 90% of the bacteria products sequenced belonged to the phyla Firmicutes and Bacteroidetes, which has been reported in previous studies [70, 77, 79]. Furthermore, beta diversity comparisons between healthy controls and T2D group (pre and post samples) showed no significant difference as distinct clustering was not observed for groups or treatment.

The cross sectional study analysis was to highlight the composition of the gut microbiota in T2D and healthy controls and differentiate between the two
groups. The healthy controls in this study had a higher level of Bacteroidetes than Firmicutes, which is contrary to the findings in the study by Larsen et al., 2010, although the difference was not significant. Also, the healthy controls were split by BMI, normal weight (below 25kg/m²) versus overweight (over 25kg/m²) and there was no significant difference between the two groups. T2D patients have been reported to have higher level of Bacteroidetes than Firmicutes and healthy controls have higher level of Firmicutes than Bacteroidetes [75, 108]. This is in agreement with the present study, the T2D subjects had significantly higher level of Bacteroidetes (P=0.007; Q=0.04) than Firmicutes in the pre samples. The pre samples, which were collected before prebiotic or placebo treatment, serves as a true presentation of the gut microbiota in the T2D subjects. The level of Firmicutes were significantly lower in T2D subjects (P=0.003; Q=0.03). This finding is in agreement with the findings of Larsen et al. (2010), which showed that the diabetic group had a significantly low levels of Firmicutes. Also, Proteobacteria level was significantly higher in the T2D patients than the controls (mean 3.9% versus mean 1.7%; P= 0.01 and Q=0.19). This is in agreement with the findings of Mrozinska et al., (2016) and Creely et al., (2007) showed that at the phylum level, the amount of Proteobacteria was higher in the T2D group than in healthy controls [75, 119]. Therefore, this suggest that the gut microbiota in T2D and controls are not different, which is contrary to the findings of the study of Larsen et al., (2010) that reported higher level of Firmicutes than Bacteroidetes in healthy controls.
Furthermore, the Firmicutes sequence primarily belonged to the class Clostridia, which was higher in the T2D group than controls but not significant (mean 37.0 % versus mean 36.3%; P=0.47 and Q= 0.85) (see appendix). However, reporting the P value but not Q, the class Erysipelotrichi was significantly higher in the diabetic group than controls (mean 1.6% versus 1.2%; P=0.04 and Q= 0.48) (see appendix). The phylum Bacteroidetes was mainly presented by the class Bacteroidetes, which was relatively higher but not significant in the control group than in T2D group (mean 51.9% versus 49.1%; P=0.27 and Q=0.85) (see appendix). Also, the class Gammaproteobacteria, was significantly (reporting P but not Q values) higher in the T2D group than controls (mean 3.2% versus mean 1.3%; P=0.02 and Q=0.48). Therefore, our findings shows that the gut microbiota in T2D and control subjects does not differ at the class level.

Prebiotics have been reported as a dietary tool to remodel the gut microbiota, which may have secondary effects on improving glucose tolerance, normalising endotoxaemia, and restoring Bifidobacteria spp. levels [54]. In this study, the T2D group was split into prebiotic and placebo treatment groups. Each group had 12 weeks of supplementation with Maltodextrin or GOS. The GOS treatment had a significant effect on the gut microbiota in T2D by significantly increasing the level of the phylum Firmicutes and decreasing the phylum Bacteroidetes in the post samples. Although not significant, the prebiotic group also had higher level of Actinobacteria in the pre samples but it decreased in the
post samples. Also, the placebo group had low level of Actinobacteria in the pre samples, which increased in the post samples.

The main importance of using GOS was due to its bifidogenic properties, however there was no increase in the number of bifidobacteria belonging to the phylum Actinobacteria, which might be attributed to dosage and/or duration of study, but studies in humans have shown bifidogenic effects in individuals after taking above 5g of GOS per day for over 10 weeks. Vulevic et al., (2008) showed in 44 elderly subjects randomly assigned to receive either a placebo or the B·GOS treatment 5.5 g/day for 10 weeks that B·GOS significantly increased the numbers of important bacteria, especially bifidobacteria, at the expense of less beneficial groups compared with both the baseline and placebo [66]. Furthermore, a similar study by Vulevic et al., (2013), showed that in 45 overweight adults randomly assigned to receive either a placebo or the B·GOS treatment 5.5 g/day for 12 weeks, that B·GOS significantly increases the number of bifidobacteria in faeces, whereas decreased the number of Bacteroides spp., Desulfovibrio spp., C. histolyticum group and beta-Proteobacteria compared with placebo at 12 weeks [120]. With even much lower dosage and treatment time, thirty-seven volunteers, men and women of 50 years and above, included in a randomised, double-blind, placebo-controlled crossover trial were given juice containing 4 g GOS or placebo, consumed twice daily for 3 weeks found bifidobacteria that were significantly more abundant compared to post-placebo [121]. In addition, the method of analysis used in these studies may have play a role in the positive finding.
Methods such as FISH [66, 120] and qPCR [121] were used in the studies discussed above compared to 16s rRNA sequencing method used in this study. FISH and qPCR methods are specific for the bacteria of interest and may explain why these studies have positive outcomes, while 16s rRNA makes it possible to identify broad bacteria groups and for taxonomic purpose [122, 123].

Furthermore, it may be that our cohort were non-responders to GOS. To support this, the study by Davis et al., (2011), involving 18 healthy human volunteers during a 12 week period using GOS dosages administered at increasing levels of 0 g, 2.5 g, 5 g, and 10 g GOS per day, showed a dose dependent bifidogenic effect of GOS. The 2.5 g dose of GOS was not sufficient to induce a response, while 5 and 10 g doses induced a response. Although the difference between 5 and 10 g was not statistically significant, there was a further increase in bifidobacteria in several subjects when the dose of GOS was increased to 10 g. This findings points to the fact that there is a dose response element to GOS. In addition, it was observed that not all eighteen subjects showed a response of the taxa that were significantly affected by GOS. Some subjects responded highly to GOS, which may be due to the presence of specific GOS-metabolizing strains at baseline that confer responder status on that individual. While others who are non-responders might simply not harbour strains of Bifidobacteria that are able to utilize GOS [95].

The effect of metformin on the gut microbiota was also determined by comparing the composition of the gut microbiota in T2D subjects taking
metformin or not. T2D subjects on metformin, although not significant had higher level of Bacteroidetes (mean 54.0% versus 51.4%), Actinobacteria (mean 0.33% versus 0.12%), and Proteobacteria (mean 4.09% versus 3.20%) compared to those not taking metformin. Firmicutes level was lower in subjects’ taking metformin compared to those not taking metformin (mean 38.5% versus 40.6%), suggesting metformin affects this taxon. Also, the T2D patients taking metformin had increased levels of Enterobacteriaceae compared to those not taking metformin (mean 3.30% versus 2.3%; P=0.02; Q=0.28). This finding is in line with a previous study that reported that fifty three T2D women (70 years) using metformin had increased levels of Enterobacteriaceae (Escherichia, Shigella, Klebsiella and Salmonella) and decreased levels of Clostridium and Eubacterium [81]. However, no significant difference was observed in our study for this findings as the effects of metformin were only beginning to be found after we had our study funded, hence we didn’t recruit based on metformin status and so looking at this variable would be underpowered.

Overall, the results in this chapter suggest that the gut microbiota in healthy controls and T2D patients differ in the phyla Proteobacteria. Since the bacteria belonging to this phyla contains mostly Gram-negative species, which will have LPS as its outer membrane, the chances of this leading to endotoxaemia is high, especially if they suffer a ‘leaky gut’ [75]. Although, the placebo was also seen to have similar effect, GOS did improve the numbers of the phyla Firmicutes, which contains probiotic species that are beneficial to the host.
CHAPTER 4

ASSESSING THE RELATIONSHIP BETWEEN
FAECAL WATER ACTIVITY, METABOLIC MARKERS OF TYPE 2 DIABETES AND
ANTHROPOMETRIC PARAMETERS
4. Assessing the Relationship between Faecal Water Activity, Metabolic Markers of Type 2 Diabetes and Anthropometric Parameters

4.1. Introduction

The intestinal epithelium is the largest mucosal surface that provides an interface with the external environment, thereby providing protection against foreign antigens, toxins and molecules entering the body by the oral/enteric route. This defensive barrier in the healthy state allows only a minimal fraction of antigens across, but during prematurity, or following exposure to toxins, the integrity of the TJ becomes compromised. This leads to the induction of an immune responses towards environmental antigens [100-102]. Experimentally, measurement of transepithelial electric resistance (TER), which is the electrical resistance across cellular monolayer and the ability of TJs to restrict the passage of small molecules such as inulin, mannitol, or dextran through the paracellular space are used to assess TJ barrier function [124]. In this chapter, TER was used to measure FW disruption of in vitro epithelial barrier function, which is a potentially useful non-invasive biomarker of diseases such as colorectal cancer and inflammatory bowel diseases. However, this has yet to be investigated in T2D, as a potential tool to investigate the exposure of the colonic mucosa to compounds which would be found in vivo and in patients, as a novel mechanistic discriminator between those with well managed glycaemia and those with uncontrolled T2D. The rationale behind this approach is that components of the FW fraction of faeces are more likely to have deleterious effect on the colonic
mucosa than substance bound to insoluble food particles or colonic bacteria [125, 126]. Additionally, FW contains the bioactive compounds such as bile salts, polyphenols, SCFA and fatty acids, which may interact with stem cells at the base of colonic crypts. Factors such as intake of prebiotics and probiotics, drugs and diet have been shown to alter the activity of FW on cultured epithelial cells [127, 128]. This may not be the best model because the FW is not sterile. Synthetic FW sample that contains compounds similar to the human FW sample may be suitable since it can be made sterile. However, other unknown compounds that may be important, which are present in human FW samples will be omitted in the synthetic FW samples, hence using human FW samples was preferred.

This study focuses on understanding the interaction between the gut microbiota and the host in T2D. To enable this understanding, this chapter describes the in-vitro activity of faecal water prepared ex-vivo from T2D patients then added to a Caco-2 cell monolayer as a model of the colonic mucosa. The aim was to test the hypothesis that faecal bioactive compounds will differ between healthy controls and T2D patients, which may have a direct impact on the gut, hence FW will reflect this in-vitro. Additionally, a specific aim was to determine whether there was any the relationship between metabolic markers of T2D, anthropometric parameters and the in vitro measurement of TER in the T2D group.

The objectives of this chapter are to:
1. Investigate the effect of FW from healthy controls and T2D patients on Caco-2 cell monolayer integrity using a transwell and TER assay over a two day period.

2. Determine the relationship between metabolic markers, anthropometric parameters and TER in the T2D group using linear regression analysis.

4.2. Methods

Tissue culture methods: cell enumeration, human faecal water sample preparation, transwells tissue culture assay, and transepithelial electric resistance (TER) measurements were used in this chapter as described in chapter 2.

4.2.1. Anthropometric measurements

Anthropometric parameters were measured non-invasively using the Tanita TBF-300A body composition analyser (Tanita Europe BV, Netherlands). 1.0kg was subtracted to allow for the weight of clothes. The Tanita TBF-300A assesses body composition indirectly by bioimpedence analysis (BIA) through the lower half of the body. A safe electrical signal is sent through the body via the pressure contact foot pad electrodes, which participant are asked to step onto in bare feet after the analyser has been switched on and calibrated. The analyser directly measured body weight and the bioelectric impedance using the conductive
properties of the legs, percentage body fat, body fat %, fat mass (kg), total body water (kg), free-fat mass (kg) and muscle mass (kg) are then calculated on the basis of the measured body weight, bioelectric impedance and other variables including age, height, gender and body type.

Height was measured using a Harpenden stadiometer without shoes, socks and hats. Participants stood erect, facing ahead in a horizontal plane with their back to the stadiometer with their heels, buttocks, shoulder blades and back of the head touching the wall, arms by their side, knees straight and ankles together. The measuring bar was lowered to touch on top of the participant’s head and height was recorded to the nearest 0.1cm

4.3 Data analysis

For the TER data, difference between healthy control and T2D was performed using two-tailed unpaired t-test. For time differences were analysed by one-way ANOVA, with post-hoc Tukey’s multiple comparisons test. The relationship between monolayer integrity (TER) and metabolic markers of T2D were assessed using non-linear regression analysis (one phase decay). Non-linear regression analysis was performed using an X and Y Table format where X values were the independent variable (variable causing the effect) such as patient’s measured fasting insulin, total cholesterol, TNF alpha and LPS values, while Y values were the dependent variable (the variable on which the effect is observed) such as mean TER data for each time point for an individual patient’s FW
sample. The use of this approach was discussed with a statistician in the Mathematics Department at the University of Surrey (Peter Williams) and he noted that the sampling number was small, but the $r^2$ and $p^2$ values should be used as an indicator of how good the model fits is. The statistical significance is represented as $R^2$ (denotes goodness of the slope fit) and $P$ value ($\leq 0.05$). $P \leq 0.05$ was considered statistically significant. All statistical analysis were performed using Prism 7 GraphPad software.
4.4 Results

4.4.1 Faecal water samples from type 2 diabetes patients had significant effect on cell monolayer integrity

When compared with the FW samples of healthy controls, the T2D patients FW samples had significant impact on cell monolayer integrity as TER were decreased at 24 hours and 48 hours post treatment (Figure 4.1) \( (p<0.001 \) and \( p=0.003 \), respectively), but not at 0 hour (baseline)\( (p=0.8745) \).

![Figure 4.1: FW from T2D patients (n=9) at 24 and 48 hours (b and c respectively) had significant impact on TER compared to FW samples from non-diabetic healthy controls (n=3).]

At baseline, TER for healthy controls and T2D group were not significantly different. At 24 and 48 hours, FW samples from T2D patients significantly decreased TER \( (p<0.0001 \) and \( p= 0.03 \) respectively). The values are expressed as percentage of negative control (without FW set as 100% TER integrity). The data represents the mean ± SEM of at least three independent experiments. Two tailed unpaired t test. \( P \leq 0.05 \)
4.4.2 Type 2 diabetes patients’ faecal water samples reduced cell monolayer integrity in a time-dependent manner

Caco-2 cell monolayer grown on transwell inserts on the apical compartment were challenged with FW from 9 T2D patients. The experiment was performed over a 24 hours periods with TER readings taken at 0, 12 and 24 hours. The results showed that FW samples had a significant impact on cell monolayer integrity as TER decreased in a time dependent manner. At the start of the experiment, TER remained unchanged for 8 out of 9 samples (Figure 4.2 b-I), while 1 sample significantly decreased TER (-27.76%, p=0.0072) (Figure 4.2a) compared to the TER before treatment (initial TER) with FW sample. However, compared to the TER at 0 hour, 7 out of 9 samples significantly reduced TER at 24 hours (-41.95%, p= 0.0005; -45.91%, p=0.0041; 43.48%, p=0.0124; -44.02%, p=0.0134; -46.87%, p=<0.001; -59.97%, p=0.01; and -54.21, p=0.03) and 48 hours (-38.61%, p= 0.0009; -13.9%, p= 0.4559; -31.33%, p= 0.0626; -18.76%, p=0.3501; -32.55%, p= 0.0012; -78.87, p= 0.003; and -59.32, p= 0.0183) (Figure 4.2 a-f, I ) but the extent varied between different patient samples. The extent of TER increase or decrease is shown in Table 4.1.
Figure 4.2: Effect of faecal water on transepithelial resistance.

FW samples from T2D patients (n=7, a-i) had an effect on TER causing decrease in a time dependent manner. The values are expressed as percentage of control (without FW). The data represents the mean ± SEM of three independent experiments. ^,* and #, denotes, statistical significance to initial, 0 hour and, 24 hours respectively. P ≤ 0.05 ANOVA followed by Tukey's multiple comparisons test.
Table 4.1: TER % increase/decrease for T2D faecal water sample.

These values are for FW sample in samples represented in Figure 4.2 with the label shown in the serial number A-I (s/n) below. All values are comparison with 0 hour unless otherwise stated.

<table>
<thead>
<tr>
<th>S/N</th>
<th>0 Houra</th>
<th>P value</th>
<th>24 Hours</th>
<th>P value</th>
<th>48 Hours</th>
<th>P value</th>
<th>48 Hoursb</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-27.76</td>
<td>0.0072</td>
<td>-41.98</td>
<td>0.0005</td>
<td>-38.61</td>
<td>0.0009</td>
<td>3.34</td>
<td>0.9417</td>
</tr>
<tr>
<td>B</td>
<td>-17.8</td>
<td>0.2708</td>
<td>-45.91</td>
<td>0.0041</td>
<td>-13.9</td>
<td>0.4559</td>
<td>32.01</td>
<td>0.0303</td>
</tr>
<tr>
<td>C</td>
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<td>0.9642</td>
<td>-43.48</td>
<td>0.0124</td>
<td>-31.33</td>
<td>0.0626</td>
<td>12.12</td>
<td>0.6544</td>
</tr>
<tr>
<td>D</td>
<td>-13.82</td>
<td>0.5834</td>
<td>-44.02</td>
<td>0.0134</td>
<td>-18.76</td>
<td>0.3501</td>
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<td>-46.87</td>
<td>&lt;0.0001</td>
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<td>F</td>
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<td>-59.97</td>
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<td>-78.87</td>
<td>0.003</td>
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<td>-28.74</td>
<td>0.2505</td>
<td>-29.48</td>
<td>0.2319</td>
<td>0.76</td>
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<td>-50.32</td>
<td>0.0183</td>
<td>-5.11</td>
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</tr>
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</table>

a Comparison with Initial TER
b Comparison with 24 hours

4.4.3 Association of in-vivo metabolic markers of type 2 diabetes with faecal water activity in-vitro

Non-linear regression was used to assess the relationship between in-vivo markers of T2D and the FW samples (n=5) impact on cell monolayer integrity. The range of means for the markers of T2D for all patients is shown in Table 4.3. The TER means for 0, 24 and 48 hours for all samples were used in a linear regression model with patient values for insulin, total cholesterol, LPS and TNF
alpha, with the r² and p values shown below (Table 4.3). At baseline, only one sample (Figure 4.2.a.) had an effect on TER, which significantly decreased TER compared to the initial (pre-faecal water addition) TER. The sample was from a T2D patient who had significant hyperinsulinaemia (213.5 pmol/L fasting insulin) compared to other patients (Figure 4.2 b-e). At 24 hours of FW incubation, all samples had significantly decreased TER to approximately 27-47 % of control TER, which was significantly associated with elevated plasma total cholesterol and TNF alpha (Figure 4.2 a-e). However, compared to the TER measured after 24 hours of incubation, the TER actually increased for all samples by 48 hours of incubation (Table 4.1) but the extent varied among patient samples and was only significant for one T2D patient (Figure 4.4b.). The TER increase was inversely associated with the level of plasma LPS (Figure 4.6), with one patient’s FW sample having the lowest TER increase and highest LPS level (+3.34% and 4.45 EU/ml) compared to other T2D patient samples (Figure 4.2a, Table 4.3).
Table 4.2: Mean Values of metabolic markers for T2D patients

<table>
<thead>
<tr>
<th></th>
<th>T2D patients mean values</th>
<th>Range</th>
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<tr>
<td>N</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>98.79</td>
<td>44.214</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>4.53</td>
<td>3.6 – 5.4</td>
</tr>
<tr>
<td>LPS (EU/ml)</td>
<td>1.598</td>
<td>0.3 – 4.5</td>
</tr>
<tr>
<td>TNF-a (pg/ml)</td>
<td>25.57</td>
<td>13.48</td>
</tr>
</tbody>
</table>

Table 4.3: Non-Linear regression analysis assessed the relationship between TER (mean of 3 values per patient) and metabolic markers in T2D patients. The R² value for 0, 24 and 48 hours are presented (n=5)

<table>
<thead>
<tr>
<th>TIME (hours)</th>
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<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R² Value</td>
<td>R² Value</td>
<td>R² value</td>
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<tr>
<td>Insulin (pmol/L)</td>
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<td>-</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
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<td>-</td>
</tr>
<tr>
<td>LPS (EU/ml)</td>
<td>-</td>
<td>-</td>
<td>0.8521</td>
</tr>
<tr>
<td>TNF-a (pg/ml)</td>
<td>-</td>
<td>0.9427</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4-3: Regression analysis assessed the relationship between the TER (48 hours) and Clinical parameters.

The TER mean values of the T2D patients (n=5) for three independent transwell experiments were used in a non-linear regression model with patients’ blood measurements of Insulin, total cholesterol, TNF alpha and LPS at A) 0 hour B) 24 hour and C) 48 hour. The decrease in TER associated with increasing levels of A) insulin in the T2D patients. While a saturation effect was observed between TER and B) total cholesterol C) TNFα and D) LPS, where the decrease in TER occurred with low concentrations and remained stable as the concentration increased.
4.4.4 Anthropometric parameters of type 2 diabetes patients associated with faecal water decrease of cell monolayer integrity.

Linear regression was used to assess the relationship between anthropometric measurements of T2D patients and the FW (n=4, Figure 4.2 F-I) impact on cell monolayer integrity. Anthropometric parameters were measured for four T2D patients (Figure 4.2 F-I), which was used in a linear regression with means of TER values (3 independent experiment). The TER means for 0, 24 and 48 hours for all samples were used in a linear regression model with age, body weight, BMI, body fat %, fat mass (kg), total body water (kg), free-fat mass (kg) and muscle mass (kg). The r² and p values are shown in Figure 4.7. At 24 hours, two FW samples (fig 4.2 a & d) significantly decreased TER which was significantly associated with elevated level of body fat % and decreased level of total body water (Figure 4.4 a & b). Additionally at 48 hours, TER decreased for 2 samples which was associated with elevated level of body fat % and decreased level of total body water (Figure 4.7 c& d; 4.4 c & d).
Figure 4-4: Linear regression analysis assess the relationship between TER (24hours, a-b; 48 hours, c-d) and anthropometric parameters of T2D patients.

The TER mean values of the T2D patients (n=4) for three independent transwell experiments were used in a linear regression model with age, weight, body mass index, body fat %, fat mass (kg), total body water (kg), free fat mass (kg) and muscle (kg) measured using the bioimpedence. The decrease in TER associated significantly with high body fat % and low total body water in the T2D patients at 24 and 48 hours. R² and p values are displayed on the Figures.
4.5 Discussion

Tight junctions protect the body from passage of harmful substances across the intestinal barrier. The concept of ‘leaky gut’ in human T2D had previously been investigated but the exact mechanism is not known. However, high-fat diet and gut microbiota has been thought to play a role by altering its composition favouring growth of bad bacteria over good bacteria. The overgrowth of Gram-negative bacteria in turn disrupts the tight junctions by producing harmful metabolites which negatively impact the integrity of the epithelial cell monolayer [129-131].

It was hypothesised that the FW samples from healthy controls will not decrease cell monolayer integrity, which maybe a related to non-diseased state. The healthy controls FW samples did not significantly decrease cell monolayer integrity, although very few samples were used, which may not be representative of healthy states. These findings was also observed in another study [125], where two FW samples from healthy controls did not change Caco-2 TER over a 24 hours period. This may suggest that in contrast to T2D, the observed effect on cell monolayer integrity by the FW from healthy controls likely indicates it is dependent of disease state and associated with elevated levels of insulin in T2D. Also, the impact of individual T2D patients FW on TER over a 48 hours period was investigated and it was observed that 77% (7 out of 9 samples) of FW samples from T2D patients had significant impact on TER at 24 hours, with TER increase noticed at 48 hours for most samples (7 out of 9 samples). To
demonstrate that the *in vitro* FW activity (n=5) of the T2D patient’s may likely be associated with the physiological aspects of the condition, we used non-linear regression (one phase decay) to assess the relationship between TER (assessed at different time points) and markers of T2D. This suggests a possible saturation effect as the decrease in TER appears to occur rapidly at low concentration of total cholesterol ($r^2= 0.7715$), TNF alpha ($r^2=0.8670$), and LPS ($r^2= 0.8346$), with a steady state as the concentration increased. However the decreased TER seems to be decreasing as the insulin concentration increased. All markers had over 70% relationship with decreased TER, which may suggest that metabolic state of T2D patients may possibly play a role in the activity of FW in-vitro. Furthermore, linear regression assessed the relationship between TER of FW of T2D patients (n=4) and anthropometric parameters, which showed that TER decrease at 24 and 48 hours was associated with higher body fat % and lower total body water (kg).

This study identified FW activity might help explain physiological features of the condition, although further investigation as to the underlying mechanism is needed. One possibility is that T2D impacts upon the gut microbiota and its metabolites such as SCFA, LPS, bile acids and TNF alpha, which may determine the FW activity *in vitro*, assuming that the plasma level may reflect the faecal level of these bacterial metabolites. Prior to faecal sample collection, the T2D patients had no prebiotic and probiotic intake, therefore the two likely patient activities that can affect the FW components used in this study are diet and use.
of drugs such as metformin and NSAIDs. These factors had been previously shown to affect the FW activity on cultured cells such as Caco-2 causing genotoxic, cytotoxic and apoptotic effects [128, 132, 133] and preventing bacterial cell adhesion to cells [134].

Whilst the present study didn’t investigate the components of the FW from T2D patients, it has been previously shown that FW activity can be altered by diet high in fat [137], high calcium [138], high protein, red meat and carbohydrate [139, 140], dietary meat and fish [141], dietary copper [142], high concentration of bile acids [143], vegetables (carrot and tomatoes) [144], and prebiotics [135, 136]. Bacterial metabolites such as SCFA, bile acids, LPS, TNF alpha, phenols and ammonia play a role in determining barrier function using epithelial cell culture models such as Caco-2 and T84. A study of diet on the cellular toxicity of FW, identified bile acids and fatty acids as the major compounds responsible for the activity of FW on cultured colonic cells. This study included 18 healthy male and females, who were involved in a cross over study where they changed from a dairy–rich diet to a dairy–free diet [126].

Primary bile acids (cholic acid and chenodeoxycholic acid) are metabolised in the liver from cholesterol and the gut flora in the colon then hydroxylate them to secondary bile acids (mainly deoxycholic acid and lithocholic acid). These secondary bile acids cause DNA damage and apoptosis to cultured cells, which links the gut microbiota to colon pathogenesis [48, 127]. Assuming that the level of total cholesterol observed in T2D patients (n=5) , could possibly lead to the
metabolism of primary bile acids, which the gut microbiota may convert to secondary bile acids and may potentially be present in high levels in the patients faecal samples. This may explain why cholesterol was associated with a decrease in TER after 24 hours of incubation with FW samples from T2D patients. Another study also observed bile acid reduction in TER in a time-dose dependent manner when added directly to the cell model. Bile acids were used at concentration that were physiologically relevant to the faecal aqueous concentrations (200µM). All bile acids reduced TER but cholic acid and hyodeoxycholic acids strongly decreased TER, which were due to alteration in metabolic energy, protein synthesis or transcription in Caco-2 cells [137].

Also, at 24 hours after incubation with faecal water samples from T2D patients, the drop in TER was associated with high TNF alpha and it has been previously shown to affect epithelial cell monolayer integrity. The addition of TNF-α (5 ng/ml), when Caco-2 monolayers were preconditioned for 24 h with IFN-γ (10 ng/ml) induced the expression of TNF-α receptors and caused a significant drop in TER within 6 h [138]. Additionally, Caco-2 cells displayed decreased TER of the cell monolayer when treated with TNF alpha for 6 h and the TER of Caco-2 cells dropped to the lowest level after stimulation by TNF-α for 48 h when compared to the baseline. Also, further incubation for 96 h, showed that TNF alpha induced changes on the TER of Caco-2 cells could not be reversed. This was due to TNF alpha in Caco-2 cells initiating NF-κB activity (lasted for 24 hours), cells exhibiting paracellular gaps between epithelial cells after 6 h of TNF-α.
treatment, induced stress fibre formation and sizeable gaps after Caco-2 cells stimulated by TNF-α for 24 h and were worsened after 48 h of TNF-α treatment. Finally, decreased cellular protein levels of ZO-1 was observed after treated by TNF-α for 6 h, while the down-regulation of occludin occurred much more lately and inhibition of NF-κB reversed the loss of tight junction proteins [139]. Another study investigated the effect of TNF alpha induced intestinal epithelial barrier dysfunction in Caco-2 cell monolayer and the underlying mechanism. Caco-2 monolayers treated with 100ng/ml TNF alpha for 72hours caused reduced TER and increased phenol red flux. This was due to the distribution of occluding and ZO-3, which were broken and fluorescence intensity was weak. Also, the NFkB signalling pathway was activated when Caco-2 cell monolayers were exposed to 100ng/ml TNFalpha for 12hours [140]. The above studies indicates that decreased TER caused by TNF alpha occur in a time dependent manner, hence the incubation time used in the present study (0-48 hours) made it possible to observe the effect, which was linked with the level of TNF alpha in FW at 24 hours. Also, inferring from the above studies mentioned, the possible mechanism of action of TNF alpha in FW of T2D patients used in this study might be disruption of TJ and activation of the NFkb pathways.

The drop in TER at 48 hours after incubation was associated with LPS. There was an increase in TER at this time point but only one sample had a lesser increase compared to other T2D patients and this patient had higher level of LPS compared to other patients. Assuming that high level of serum LPS could imply
high faecal LPS, it can potentially be due to LPS impact on cell monolayer integrity. In agreement with our findings, TER of Caco-2 cell monolayer pre-treated with LPS for 48 hours was significantly decreased [141]. Similarly, LPS significantly decreased TER when Caco-2 cells were treated with LPS for 24 hours [142]. LPS added to the basolateral but not apical side of Caco-2 cell monolayer caused a significant decrease in TER and increased penetration of FD-4 across the monolayers after a 2 hours treatment [143]. Likewise, TER was consistently decreased in LPS induced cells at 1, 3, 6 and 12 hours [144]. Also LPS at clinically relevant concentrations (1, 5, 10 ng/ml) significantly reduced TER after day 4 [145]. The above findings highlights the relevance of incubation time in the action of LPS as the time ranged from 1 hour to 7 days, which may suggest that the LPS in FW of T2D patients used in the present study took effect at 48 hours post incubation possibly due to the LPS concentration present in the FW. In addition, the possible mode of action of the LPS in FW in disrupting barrier function by decreasing TER, could be by inducing cell inflammation by increased production of pro-inflammatory cytokines (such as IL-1beta, TNF alpha, IL-6, IL-8) and decreased anti-inflammatory cytokines (such as IL-10) [141, 142, 144, 146-148]. Also LPS in FW could have decreased cell viability by acting as an anti-proliferating agent and increase oxidative stress, which can induce cell apoptosis by directly damage cell membrane or important genetic material [142]. Lastly, LPS in FW could induce reduced expression and redistribution of tight junction proteins (such as occludin, ZO-1 and claudins), and NFkB activation in Caco-2 cells [142, 144, 145, 147, 149-151].
Furthermore, although the cohort in this study was divided by disease state, healthy controls and T2D, SCFA may potentially differ between the two groups, hence the FW from healthy controls had no significant impact on TER. The role of SCFA contained in FW in maintaining barrier function was investigated in a study to assess the factor of age as a major risk factor for colorectal cancer [152]. The findings from the study by Gill et al., (2007) showed that treatment of Caco-2 cells with FW samples had significant impact on TER over a 48 hours period, as FW from adults increased TER (+ 4%), whereas FW from elderly subjects decreased TER (−5%). Also, their findings showed several components of FW potentially associated with modulation of TER, namely, SCFA and ammonia were investigated and SCFAs (propionic, acetic, and n-butyric) were significantly lower in the elderly population (−30%, −35%, and −21%, respectively). Likewise the effect of SCFA on barrier function was determined in-vitro, and the result showed that SCFA mixture improved TER at 24 hours but abated at 48 hours. However, SCFA used individually did not improve TER at any time point [152].

Although this study presents a pilot character, it is a novel idea as to the use of FW in-vitro to differentiate between disease state, how well managed it is in a person, and it needs to be further studied in a large cohort. This approach could serve as a non-invasive quick screen that can be done with faecal samples in-vitro to speculate on how well the disease was managed long term. Although permeability assay was not performed to determine whether the extent of the FW
impact can lead to cell monolayer permeation of solutes, but it is likely that continuous decreased cell monolayer integrity will be harmful to barrier function.

Most studies extract a compound of interest and test it using this model of barrier function but this study has used FW as a possible representation of the colonic content consisting of compounds from both human and bacteria. Therefore, this study represents a novel in-vitro model and human in-vivo markers are needed to better explain this concept. The correlation between the Caco-2 cells decrease in TER and in-vivo markers of T2D could be because the gut is the common source of components such as inflammatory mediators and endotoxins. Also, ‘live gut bacteria’ has been found in the blood of T2D patients [153], hence, it is likely that plasma and faeces may be similar in content and concentration.

In future studies, to best suit the human situation, it may be ideal to perform the experiments within a 24-hour duration as the time point to determine the optimal effect of FW on Caco-2 cell monolayer integrity. This is because an increase in TER was observed at 48 hours post incubation with most FW samples. It has been shown that inflammatory mediators such as TNF alpha, induced the release of acute phase proteins such as LPS binding protein (LBP) and Serum amyloid A (SAA) by intestinal epithelial cells such as Caco-2, which indicates that tissues other than the liver are involved in the acute phase response and play an important role in the local inflammatory process in the gut [154]. Therefore, incubation with FW for over 24 hours may not be relevant as the
cells may have released LBP and SAA into circulation in local defence against endotoxins such as LPS. Although this depends on the local concentration of LBP, for most FW samples, the toxicity of endotoxins will be greatly reduced, hence variable outcome may be observed at time points over 24 hours. In addition, the faeces contains compounds from the colon, and the normal colon transit of 24 hours is reflected by activity in the right colon [155, 156], hence the colon mucosa will be in contact with the content of the colon for approximately 24 hours and this time point may best explain the difference between healthy controls and diseased state as well as the management of diseased state.

The limitation of this study was the few FW samples used especially from the healthy control group (n=3), but in the future where more FW samples are used in similar studies, the proposed ideal incubation time will be 24 hours.
CHAPTER 5

NON-PREBIOTIC EFFECT OF GOS AGAINST FAECAL WATER CYTOTOXICITY
5 NON-PREBIOTIC EFFECT OF GOS AGAINST FAECAL WATER CYTOTOXICITY

5.1 Introduction

The effect of prebiotic via the action of the gut microbiota has been previously studied both \textit{in vivo} and \textit{in vitro} [71, 89, 157], however the non-bacteria effect of prebiotic has also been investigated [158]. The work described in this chapter focused on investigating the faecal sample cytokine profile, cytotoxicity of FW samples, and the effect of the prebiotic GOS on FW Caco-2 cell cytotoxicity. Although the source of the cytokines present in the faecal samples used in this study was not established, it is known that the adipose tissue, liver, muscle and the pancreas are sites of inflammation in the presence of T2D and the production of pro-inflammatory cytokines including IL-6, IL-1Beta and TNF alpha are produced at these sites [159].

Pro-inflammatory cytokines promote insulin resistance in an autocrine and paracrine manner by interfering with insulin signalling in peripheral tissues through the activation of c-JUN N-terminal kinase (JNK and nuclear factor-Kappa B (NFkB) pathways [160]. In T2D, these pathways are activated in multiple tissues and play a central role in promoting tissue inflammation [159, 161-163].

FW cytotoxicity has been previously studied, by a number of researchers and they focused on the impact of diet consumed in healthy volunteers [126, 132,
However, FW cytotoxicity has not yet been explored as a potential biomarker for diseases such as T2D, where low-grade inflammation is known to play a major role in disease progression.

The aims of this study were as follows:

i) Investigate the cytokine profile of T2D patients using a non-invasive sample and

ii) To elucidate the impact of FW samples as a complex inflammation mixture on cytotoxicity in a caco-2 cell model.

The specific objectives of this chapter were to:

1. Investigate the faecal cytokine profile using faecal protein extracted from T2D patient’s faecal samples in a human cytokine array.

2. Investigate the effect of GOS on Caco-2 cell viability using MTS assay

3. Investigate the FW cytotoxic effect on Caco-2 using MTS assay

4. Investigate the potential protective or preventive effect of GOS on Caco-2 cells in a presence/absence assay using MTS assay.
5.2 Methods

The methods used in this chapter includes faecal protein extraction, determination of protein concentration, cytokine array and cell viability assay to measure faecal water cytotoxicity, GOS preparation and MTS assay as described in chapter 2.

5.3 Results

5.3.1 Pro and anti-inflammatory cytokines measured in type 2 diabetes patients faecal samples

The detection of a broad spectrum of cytokines in human faecal samples (n=4) was assessed in faecal protein medium using the RayBio Human Cytokine Antibody Array 1 analysis. Figures 5.1 and 5.2 demonstrates cytokine levels expressed as a percentage of the positive control of the array (set as 100%). The positive control was a controlled amount of biotinylated antibody printed onto the array. All of the 23 selected cytokines (Figure 2.1) were detected in all FW samples (Figure 5.1). TNF-alpha and TNF-beta were lower in all samples. In addition, anti-inflammatory cytokines such as IL-13, TGF-beta 1 and IL-10 were detected in all patients’ samples.
Faecal protein were used to determine the cytokine present in the T2D patients’ faecal samples and all 23 cytokines were detected. Data are expressed in % versus the positive control of the array (100%) used for normalization and to orientate the arrays. The data represents the mean ± SEM of duplicates of two independent experiments.

Figure 5-1: Measurement of 23 cytokines in faeces of human T2D patients (n=4) using the RayBio Human Cytokine Antibody Array 1.
5.3.2 Cytokines that differed between individual type 2 diabetes patients

The cytokines IL-6, G-CSF, IL-1alpha, IL-2, IL-3, IL-15, IFN-gamma, RANTES, TGF-beta 1, IL-7, IL-8 and IL-13 were either significantly higher or lower between patients (Figure 5.2 and 5.3). Furthermore, typical triggers of inflammation in T2D such as IL-6 and RANTES varied between individuals.

Patient 1 had significantly higher levels of IL-6 (p=0.0124, Fig 5.2a), IL-3 (p=0.0328, Fig 5.2c), IL-8 (p=0.0466, Fig 5.3e) and IL-13 (p=0.0264, Fig 5.3f) when compared to patient 3. Also, patient 2 had significantly higher levels of IL-2 (p=0.0406, Fig 5.2d) and RANTES (p=0.0212, Fig 5.3b), when compared to patient 3. Likewise, patient 3 had significantly higher levels of G-CSF (p=<0.0001; Fig 5.2b), TGF-beta1 (p=0.0001, <0.0001 and <0.0001 respectively; fig 5.3c) and IL-1 alpha (p= <0.001, 0.0284 and 0.001 respectively; fig 5.2c) when compared to patient 1, 2 and 4, IL-15 (p=0.0030 and 0.0003 respectively; fig 5.2f) when compared to patient 1 and 4. Finally, patient 4 had significantly higher levels of IL-6 (p=0.0169), IFNg (p=0.0134), IL-7(p=0.0212), and IL-13 (p=0.0284) when compared to patient 3.
Figure 5.2: Measurement of inflammatory cytokines IL-6 (A) G-CSF (B), IL-alpha (C) IL-2 (D), IL-3 (E) and IL-15 (F) in faecal sample from T2D patients (patients 1-4) (n=4) (RayBio Human cytokine Antibody Array 1 analysis).

The above cytokines were significantly different between T2D patients. Data are expressed in % versus the positive control of the array (100%). The data represents the mean ± SEM of duplicates of two independent experiments. ****p= < 0.0001, ***p= < 0.001, **p=<0.01 *p=< 0.05 ANOVA followed by Tukey's multiple comparisons test.
Figure 5-3: Measurement of inflammatory cytokines IFN-gamma (A) RANTES (B), TGF-beta (C), IL-7 (D), IL-8 (E) and IL-13 (F) in faecal samples from T2D patients (patient 1-4) (n=4) (rayBio Human Cytokines Antibody Array 1 analysis).

The above cytokines were significantly different between individual T2D patients. Data are expressed in % versus the positive control of the array (100%). The data represents the mean ± SEM of two independent experiments. ****p= < 0.0001, *p=< 0.05 ANOVA followed by Tukey’s multiple comparisons test.
5.3.3 Bimuno galactooligosaccharide and Caco-2 cell viability

To determine whether B-GOS altered cell viability, Caco-2 cells were incubated in the presence and absence of B-GOS for 2 and 12 hours. The different volume/volume (v/v) concentrations of GOS was prepared in cell culture media from the weight/volume B-GOS preparation. At 2 hours post-incubation, 10% and 15% cell viability was not significantly decreased when compared to the untreated control. Treatment with 5% v/v significantly increased cell viability compared to control, 10% and 15% v/v B-GOS treatment (Fig 5.5 A). Also, at 12 hours post-incubation, 5% and 10% v/v B-GOS treatment significantly increased cell viability compared to control and 15% v/v B-GOS treatment (Fig 5.4 B).
Figure 5.4: Galacto-oligosaccharide did not decrease cell viability.

Cells were pre-treated with 5%, 10% and 15% (v/v) GOS for A) 2 hours and B) 12 hours. Cell viability was measured using MTS assay after the incubation with GOS. GOS did not significantly reduce cell viability after 2 and 12 hours, but increased cell viability compared to control. The values are expressed as percentage of control, without GOS which was used as 100% cell viability. The data represents the mean ± SEM of triplicates of six independent experiments. **p<=0.01, ***p= < 0.001 ANOVA followed by Tukey's multiple comparisons test.

5.3.4 Cytotoxic effect of type 2 diabetes FW samples on Caco-2 cells

To determine the cytotoxicity of FW samples, Caco-2 cells were incubated with FW for 2 hours and cell viability was assessed using MTS assay. Two samples were cytotoxic to cells post-incubation, which significantly decreased cell viability to 42% (Figure 5.5 a) and 43% (Figure 5.5 d) of the non-infected control. However, 2 samples were not cytotoxic to cells as no significant cell viability was observed and were 83% and 85% of the negative/non-infected control (Figure 5.5 b&
Figure 5-5: Effect of FW from Type 2 diabetes patient on cell viability.

Cell viability was measured using MTS assay after Caco-2 cells were challenged with FW for 2 hours. FW of patient 1 A) and patient 4 D) significantly reduced cell viability of Caco-2 cells. The value expressed as percentage of control (without FW) which was used as 100% cell viability. The data represents the mean ± SEM of triplicates of nine independent experiments. **** p< 0.0001ANOVA followed by Tukey's multiple comparisons test.

5.3.5 Bimuno galactooligosaccharide attenuated the cytotoxic effect of type 2 diabetes FW samples on Caco-2 cell

The Figures 5.6-5.9 showed the cytotoxic effect of 2 out of 4 T2D patents FW samples, hence the potential protective effect of GOS on Caco-2 cells against the cytotoxicity of the FW samples was assessed in a dose and time dependent
manner. Pre-treatment of cells with B·GOS at 5%, 10%, and 15% was performed for a duration of 2 and 12 hours either in the presence or absence of B·GOS when incubated with FW for 2 hours.

5.3.5.1 Two-hour pre-treatment with galactooligosaccharide before FW cytotoxicity assay:

5.3.5.1.1 Type 2 diabetes patient 1

For sample 1, the result showed that cells pre-treated with B·GOS for 2 hours, followed by incubation for 2 hours with FW in the presence of B·GOS increased cell viability from 42% of the non-infected control (Figure 5.5a) to 99%, 93%, and 73% of the control for 5%, 10% and 15% v/v concentration respectively (Figure 5.6a). Incubation of cells with FW in the absence of B·GOS caused significant decrease in cell viability (p= < 0.05) to 53%, 54% and 46% of the control for 5%, 10% and 15% v/v concentration, respectively compared to the control (Figure 5.6b).
Cells were pre-treated with 5%, 10% and 15% (v/v) GOS for 2 hours and cells were then incubated with FW for additional 2 hours a) in the presence of GOS and b) in the absence of GOS. Cell viability was measured using MTS assay after incubation with FW. FW did not significantly reduce cell viability (p = < 0.05) in the presence of GOS, but did decrease cell viability in the absence of GOS. The values are expressed as percentage of control, without FW which was used as 100% cell viability. The data represents the mean ± SEM of three independent experiments. ****p= < 0.0001 ANOVA followed by Tukey's multiple comparisons test.

5.3.5.1.2 Type 2 diabetes patient 2

The result showed that cells pre-treated with B-GOS for 2 hours, followed by further incubation for 2 hours with FW in the presence of B-GOS, resulted in increased cell viability from 83% of the non-infected control (Figure 5.5 b) to 87%, 119%, and 72% of the negative control for 5%, 10% and 15% v/v concentrations respectively (Figure 5.7 a). Also, in the presence of B-GOS, the 15% v/v
concentration significantly decreased cell viability (p < 0.05) compared to 10% v/v. However, the incubation of cells with FW in the absence of B·GOS had no significant effect on cell viability as it was 94%, 100% and 92% of the control for 5%, 10% and 15% v/v concentration respectively (Figure 5.7 b). Also, in the absence of B·GOS, the cell viability was not reduced as it was 94%, 100% and 92% of control.

**Figure 5-7: The effect of FW on cell viability in the presence or absence of GOS (patient 2).**

Cells were pre-treated with 5%, 10% and 15% (v/v) GOS for 2 hours and cells were incubated with FW for additional 2 hours in a) in the presence of GOS and b) in the absence of GOS. Cell viability was measured using MTS assay after the incubation with FW. FW did not significantly reduce cell viability (p < 0.05) in the presence and absence of GOS. The values are expressed as percentage of control (without FW) which was used as 100% cell viability. The data represents the mean ± SEM of three independent experiments. *p < 0.05, not significant p > 0.05 ANOVA followed by Tukey’s multiple comparisons test.
5.3.5.1.3 Type 2 diabetes patient 3

For patient 3, the result showed that cells pre-treated with B-GOS for 2 hours, followed by incubation for 2 hours with FW in the presence of B-GOS resulted in increased cell viability from 85% of the non-infected control (Figure 5.5 c) to 135%, 116%, and 81% of the control for 5%, 10% and 15% v/v B-GOS respectively (Figure 5.8 a). Also, in the presence of B-GOS, 15% v/v concentration significantly decreased cell viability (p= < 0.05) compared to 10% v/v. However, the incubation of cells with FW in the absence of B-GOS had no significant effect on cell viability as it was 94%, 100% and 92% of the control for 5%, 10% and 15% v/v concentration respectively (Figure 5.8 b). Also, in the absence of B-GOS, the cell viability that was significantly decreased for 15% v/v B-GOS compared to 10% v/v B-GOS was reversed as B-GOS absence during incubation with FW was beneficial to cells.
Figure 5·8: The effect of FW on cell viability in the presence or absence of GOS (patient 3).

Cells were pre-treated with 5%, 10% and 15% (v/v) GOS for 2 hours and cells were incubated with FW for additional 2 hours in a) in the presence of GOS and b) in the absence of GOS. Cell viability was measured using MTS assay after the incubation with FW. FW did not significantly reduce cell viability (p > 0.05) in the presence and absence of GOS. The values are expressed as percentage of control (without FW) which was used as 100% cell viability. The data represents the mean ± SEM of three independent. *p = < 0.05, not significant = p > 0.05 ANOVA followed by Tukey’s multiple comparisons test.

5.3.5.1.4 Type 2 diabetes patient 4

For sample 4, the result showed that cells pre-treated with B·GOS for 2 hours, followed by incubation for 2 hours with FW in the presence of B·GOS increased cell viability from 43% of the non-infected control (Figure 5·5 D) to 85%, 80%, and 61% of the control for 5%, 10% and 15% v/v concentrations respectively (Figure 5·9 A). Otherwise, the incubation of cells with FW in the
absence of B·GOS caused significant decrease in cell viability (p= < 0.05) to 53%, 42% and 42% of the control for 5%, 10% and 15% v/v concentration, respectively when compared to the non-infected control (Figure 5.9B).

**Figure 5.9:** The effect of FW on cell viability in the presence or absence of GOS (patient 4).

Cells were pre-treated with 5%, 10% and 15% (v/v) GOS for 2 hours and cells were incubated with FW for additional 2 hours in a) in the presences of GOS and b) in the absence of GOS. Cell viability was measured using MTS assay after the incubation with FW. FW did not significantly reduce cell viability (p= > 0.05) in the presence of GOS but did decrease cell viability in the absence of GOS. The values are expressed as percentage of control, without FW which was used as 100% cell viability. The data represents the mean ± SEM of three independent experiments. ****p= < 0.0001, not significant p=>0.05 ANOVA followed by Tukey's multiple comparisons test.
5.3.5.2 12 hours pre-treatment with galactooligosaccharide before FW cytotoxicity assay:

5.3.5.2.1 Type 2 diabetes patient 1

For sample 1, the result showed that cells pre-treated with B·GOS for 12 hours, followed by incubation for 2 hours with FW in the presence of B·GOS, increased cell viability from 42% of the non-infected control (Figure 5.5 A) to 85%, 94%, and 157% of the control for 5%, 10% and 15% v/v concentration respectively (Figure 5.10 A). Also, incubation in the presences of 15% v/v B·GOS, significantly increased cell viability compared to control (p=0.0198), 5% (p=0.0032) and 10% (p=0.0092). However, the incubation of cells with FW in the absence of B·GOS caused significant decrease in cell viability for B·GOS 5% v/v concentration which decreased to 41% of the control (p=0.001) and also compared to 10 (p=0.0001) and 15% (p=0.007) v/v concentrations significantly decreased cell viability (Figure 5.10B).
Figure 5-10: The effect of FW on cell viability in the presence or absence of GOS (patient 1).

Cells were pre-treated with 5%, 10%, and 15% (v/v) GOS for 12 hours and cells were incubated with FW for additional 2 hours in a) in the presence of GOS and b) in the absence of GOS. Cell viability was measured using MTS assay after the incubation with FW. FW did not significantly reduce cell viability ($p > 0.05$) in the presence of GOS but did decrease cell viability in the absence of GOS only at 5% v/v. The values are expressed as percentage of control (without FW) which was used as 100% cell viability. The data represents the mean ± SEM of triplicates of three independent experiments.

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ ANOVA followed by Tukey’s multiple comparisons test.

5.3.5.2.2 Type 2 diabetes patient 2

For sample 2, the result showed that cells pre-treated with B·GOS for 12 hours, followed by incubation for 2 hours with FW in the presence of B·GOS decreased cell viability from 83% of the non-infected control (Figure 5.5B) to 62%
and 69%, for 5%, 10%, respectively, while 15% v/v increased cell viability compared to the negative control (Figure 5.11A). Also, in the presence of B-GOS, 15% v/v concentration significantly increased cell viability compared to 5% v/v (p=0.0331). However, the incubation of cells with FW in the absence of B-GOS, 5% (p=0.0367) and 15% (p=0.007) v/v concentrations significantly decreased cell viability as it was 69%, and 65% of the positive control, respectively (Figure 5.11 B).

**Figure 5.11:** The effect of FW on cell viability in the presence or absence of GOS (patient 2).

Cells were pre-treated with 5%, 10% and 15% (v/v) GOS for 12 hours and cells were incubated with FW for additional 2 hours in a) in the presence of GOS and b) in the absence of GOS. Cell viability was measured using MTS assay after the incubation with FW. FW did not significantly reduce cell viability (p=>0.05) in the presence of GOS but did decrease cell viability in the absence of GOS at 5% and 15% v/v. The values are expressed as percentage of control, (without FW) which was used as 100% cell viability. The data represents the mean ± SEM of triplicates of three independent experiments. *p=< 0.05 ANOVA followed by Tukey's multiple comparisons test.
5.3.5.2.3 Type 2 diabetes patient 3

For sample 3, the result showed that cells pre-treated with B·GOS for 12 hours, followed by incubation for 2 hours with FW and in the presence of B·GOS, resulted in increased cell viability from 85% of the non-infected control (Figure 5.5C) to 114%, 135%, and 87% of the negative control for 5%, 10% and 15% v/v B·GOS, respectively (Figure 5.12A). However, the incubation of cells with FW in the absence of B·GOS had no significant effect on cell viability (p= >0.05) as it was 85%, 106% and 106% of the negative control for 5%, 10% and 15% v/v concentration, respectively (Figure 5.12B).
Figure 5-12: The effect of fecal water on cell viability in the presence or absence of GOS (patient 3).

Cells were pre-treated with 5%, 10% and 15% (v/v) GOS for 12 hours and cells were incubated with FW for additional 2 hours in a) in the presence of GOS and b) in the absence of GOS. Cell viability was measured using MTS assay after the incubation with FW. FW did not significantly reduce cell viability in the presence and absence of GOS. The values are expressed as percentage of control, (without FW) which was used as 100% cell viability. The data represents the mean ± SEM of triplicates of three independent experiments. Not significant p=> 0.05 ANOVA followed by Tukey’s multiple comparisons test.

5.3.5.2.4 Type 2 diabetes patient 4

For patient 4, the result showed that for cells pre-treated with B-GOS for 12 hours, followed by incubation for 2 hours with FW, and in the presence of B-GOS, resulted in increased cell viability from 43% of the non-infected control (Figure 5. 5D) to 92%, 117%, and 150% of the negative control for 5%, 10% and
15% v/v concentration, respectively (Figure 5.13 A). Also, in the presence of B-GOS, 15% v/v concentration significantly increased cell viability compared to control (p=0.0468) and 5% (p=0.0176) v/v concentration. However, the incubation of cells with FW in the absence of B-GOS caused significant decrease in cell viability to 51% of the control for 5% (p=0.0010) v/v concentration and 15% v/v increased cell viability when compared to the control (p= 0.0248), 5% (p= < 0.001) and 10% (p= 0.0004) (Figure 5.13 B).

Figure 5.13: The effect of FW on cell viability in the presence or absence of GOS (patient 4).

Cells were pre-treated with 5%, 10% and 15% (v/v) GOS for 12 hours and cells were incubated with FW for additional 2 hours in a) in the presences of GOS and b) in the absence of GOS. Cell viability was measured using MTS assay after the incubation with FW. FW did not significantly reduce cell viability in the presence of GOS but did decrease cell viability in the absence of GOS only at 5% v/v. The values are expressed as percentage of control, (without FW) which was used as 100% cell viability. The data
5.4 Discussion

The present investigation found that both pro-inflammatory and anti-inflammatory cytokines can be measured in vitro in T2D patients using protein extracted from faecal samples. Although all 23 selected cytokines were detected in all T2D patients faecal samples (n=4), 12 cytokines were significantly different (p= < 0.05) between individual patients. This suggests that faecal samples could be used as a non-invasive tool to evaluate cytokines/inflammation profiles in T2D patients. However, the cytokines may not have originated from a single site rather multiple sites of inflammation, which may or may not have degraded before reaching the faeces. The differences observed between patients faecal samples in terms of the levels of cytokines present could potentially have contributed directly to the cytotoxic effect of the FW samples used in the cell viability assay in vitro.

Cytokines have been previously measured in faecal samples collected from children with inflammatory bowel disease or infective diarrhoea [166] and Shigella dysenteriae 1 infection [167], International travellers with diarrhoea due to Noroviruses [168], burn patients [169], and patients with Clostridium difficile infections [170], but to the best of my knowledge no study has been performed on human T2D faecal samples. However, to support the results observed in this study, several in vivo inflammatory cytokines measurements in T2D have shown that pro-inflammatory cytokines including TNF alpha, IL-6, IL-15, MCP-1 and

represents the mean ± SEM of triplicates of three independent experiments. **p = < 0.0001, ***p= < 0.001, *p=< 0.05 ANOVA followed by Tukey's multiple comparisons test.
IL-1beta play a key role in initiating inflammatory responses and insulin resistance [159, 171-176]. Chronically elevated levels of TNF alpha and IL6 can affect insulin sensitivity by altering different key steps in the insulin signaling pathway such as stimulate phosphorylation of serine residue instead of tyrosine in insulin receptor substrate-1 (IRS1), leading to inactivation of insulin signaling, thereby causing IR [177-180]. Furthermore, hyperglycemia has been shown to acutely increase the levels of circulating TNF alpha and IL-6, while IL-6 was reported to be increased in women who later developed T2D [181, 182]. Also, TNF alpha and IL6 were showed to be increased in T2D women with and without cardiovascular diseases [171]. Likewise, TNF alpha and IL6 were significantly increased in T2D patients compared to normal subjects [183]. Also a study reported that TNF alpha and IL6 concentrations were increased in obese non-diabetic and diabetic patients with high insulin resistance. Serum concentration of TNF alpha and IL6 significantly correlated with BMI. IL6 was proportional to insulin resistance and blood glucose. Although TNF alpha remain unchanged in obese patients placed on a very low-calorie diet (VLCD) for a 3-week period, IL6 decreased significantly in both serum and adipose tissue, thereby concluding that increased IL6 is associated with severe diabetes [178]. Similar to the above study, it was reported that serum pro-inflammatory cytokine IL-6 concentrations but not TNF alpha were higher in subjects with impaired glucose tolerance and T2D than in the control subjects. The change in IL-6 level seem to also have a relevant immune changes because the acute phase proteins C-reactive protein, serum amyloid A protein and fibrinogen were also increased. Again the authors
concluded that IL-6 associated rather than TNF alpha associated responses were
unregulated in patients with T2D [184]. All the above studies showed that IL-6
play a key role in inflammation in T2D, which was also one of the cytokine that
differed between individual T2D patients in this study.

The cytokine profiling results follow on to the next series of experiments
presented in this chapter which determined the cytotoxicity level of all T2D
patients FW samples and demonstrated how the prebiotic GOS , can protect
epithelial cells in a Caco-2 model . Initially, the cytotoxicity of the various
concentration of B-GOS and incubation time used in the experiment were
investigated. Caco-2 cells were pretreated with B-GOS at 5%, 10% and 15% v/v
concentration for 2 hours and 12 hours using MTS assay. At these concentrations
and incubation times, B-GOS had no cytotoxic effect as cell viability was not
decreased, when compared to the control, rather 5% v/v (p=0.006) for 2 hours and
5% and 10% v/v for 12 hours post incubation, increased cell viability compared to
the control (Fig5.4). The next step was to investigate the cytotoxicity of the T2D
patients FW samples on Caco-2 cells. It was observed that 2 of 4 patients FW
samples (1 and 4) were cytotoxic to the cell while the other 2 patients FW
samples (2 and 3) had no cytotoxic effect as cell viability decreased and increased
respectively. It is worth noting that the 2 T2D patients (1 and 4) FW samples had
similar cytokine profile with a significantly higher level of IL-6 and IL-13, which
are known pro-inflammatory cytokines. Also, patient 1 and 4 combined had
higher pro-inflammatory cytokines such as IL-3, IL-8, IFNg8, and IL-7. These
cytokine profile is similar to the inflammatory response that has been previously reported in T2D patients measured in-vivo [178, 184]. In addition, all T2D patients faecal sample had low level of TNF alpha. Patient 1 had higher level of 9.5% compared to 7%, 3.5% and 2% for patient 2, 3, and 4 respectively. The higher level of TNF alpha in patient 1 maybe the reason for its higher level of IL6 as TNF alpha has been found to increase the expression of the gene encoding IL6 and MCP-1 [173, 185]. Also, relating it to the patients measured anthropometric parameters a trend was observed. Although no correlation analysis was performed, it was observed that the anthropometric characteristic of the 2 T2D patients (1 and 4) whose FW samples were cytotoxic, consisted of high body fat (39.40% and 31.10%, respectively), fat mass (36.80kg and 17.90kg respectively), and low level of total body water (43.10kg and 48.20kg, respectively). While, the 2 T2D patients (2 and 4) whose FW samples were not cytotoxic had low body fat (21.60% and 16.90% respectively), fat mass (17.45kg and 15.25kg) and high level of total body water (54.30kg and 58.20kg respectively). Furthermore, the 2 T2D patients FW samples were among the FW samples that caused decreased TER over a 48 hours period, which significantly associated with elevated level of body fat % and decreased level of total body water (chapter 4, fig 4.2g and h; 4.7 a-d).

Postulating from these observed trends, there may be a possible link between pro-inflammatory cytokines leading to cell death and decreased TER.

Lastly, the ability of prebiotic, B-GOS to offer either protection or preventive effect against the cytotoxicity of T2D FW samples on Caco-2 cells was
investigated. Again, cells were pretreated with B·GOS at 5%, 10% and 15% v/v concentration for 2 hours and 12 hours before infection with FW samples for 2 hours. To determine protective or preventive effect, cells were either incubated in the presence or absence of B·GOS during a 2 hours incubation with FW samples. For 2 hour pretreatment with B·GOS, the cytotoxic FW samples (T2D patients 1 and 4) (Fig 5.10 and 5.13), B·GOS effect on cells was preventive than protective, as the absence of B·GOS resulted in a significant decrease in cell viability compared to the control. While the 2 non-cytotoxic FW sample (T2D patient 2 and 3) (fig 5.11 and 5.12), B·GOS had a protective effect as the absence of B·GOS during incubation with FW had no impact on cell viability. For 12 hours pretreatment with B·GOS, the absence of B·GOS during infection with FW for cells pretreated with 10% and 15% v/v concentration had a protective effect as cell viability did not decrease compared to control but at 5% v/v concentration, cell viability significantly decreased compared to control, 10% and 15% v/v concentration.

To the best of our knowledge, this is the first study to explore the concept of B·GOS protecting epithelial cells in a T2D model of infection in vitro, using FW as a complex mixture containing both pro and anti-inflammatory cytokines. These data suggest that B·GOS effect on cells against the cytotoxicity of FW may be advantageous in a dose and time dependent manner. Higher dose and longer pre-incubation time offers protective effect even in the absence of B·GOS during infection. Although this study did not measure the level of either the cytokines
measured in this study or new pro-inflammatory cytokine produced by the Caco-2 cells after exposure with GOS and FW, B-GOS protective effect appear quite potent to act against a possible increase in pro-inflammatory cytokines released by the cells. The study by Vendrig et al., 2013, investigated the effects of three different products containing either GOS alone, a combination of GOS with FOS, and a triple combination of GOS and FOS with acidic-oligosaccharides (AOS), at different concentrations on the LPS induced inflammatory response in equine peripheral blood mononuclear cells (PBMCs). The exposed cultured PBMCs to either GOS or GOS/FOS fractions resulted in a substantial dose-dependent increase of TNF-α production in LPS challenged PBMCs, while incubation with GOS/FOS/AOS resulted in a dose-dependent reduction of both TNF-α and interleukin-10 production following LPS challenge. Also, incubation with GOS/FOS/AOS significantly increased the apparent PBMC viability, indicating a protective or mitogenic effect [186]. Similar to the above study, human milk-derived oligosaccharides and plant-derived oligosaccharides were reported to stimulate cytokine production of cord blood T-Cells *In vitro* [187]. However the method of action remains unknown in both studies.

It may be postulated that the cytotoxic effect of 50% of the FW samples used in this study arose from the pro-inflammatory cytokines present in the patient’s samples as well as induced production of cytokines by the Caco-2 cells. T2D patient 1 and 4 FW samples which were cytotoxic to cells had a similar cytokine profile with significantly higher pro-inflammatory cytokines including
IL6 which has been implicated in low grade inflammation in T2D [173, 184]. The 2 non-cytotoxic FW samples (T2D patients 2 and 3) had lower level of IL6 measured: patient 2 had only 2 pro-inflammatory cytokines that was significantly higher compared to other patients and patient 3 had higher level of TGF-beta 1, which is an anti-inflammatory cytokine. These profiles may have attributed to the non-cytotoxic effect observed in-vitro on cell viability by these FW samples. The possible mechanism could be the inactivation of NFKb which is a transcription factor that modulates the expression of many inflammatory cytokines. The activation of NFKb occurs when pattern-recognition receptors activates the NFKb signaling pathways, thus leading to an inflammatory cytokines [184, 188]. Also, it can be postulated that the activity of B·GOS on the Caco-2 cells may be by acting as pro-proliferation agent by inactivation of NFKb thereby preventing cell death, hence increasing the number of viable cells even in the presence of FW as this was observed during the experiment when viewed using a light microscope (data not shown). This observation is also in agreement with the study by Miriam et al. (2015), which observed an increased cell proliferation of intestinal epithelial cells as a direct impact of dietary fibers in vitro [158].

In conclusion, this chapter presents an important aspect to the use of FW samples of T2D patients as a model of inflammation in T2D, which is quite complex but may be a potential way to investigate patients inflammatory state in
a non-invasive manner and the mechanism of action is worth investigating in future studies.

Chapter 6

GENERAL DISCUSSION
6. General Discussion

The experimental work described in this thesis were primarily designed to investigate the role of the gut microbiota in human type 2 diabetes. This thesis does not follow a sequence, where the result of a study prompted further investigation, rather it was a way of exploring the gut microbiota produced compounds in an in vitro context to finding possible new ways of understanding the host-microbiota interaction.

However, the results presented in chapter 3, indicated that there were no significant difference in the gut microbiota between T2D and healthy controls, although the T2D group had higher levels of phylum Proteobacteria, which contain more bacteria that are Gram-negatives. Gram-negative bacteria are known to have an outer cell membrane containing LPS, which can cause inflammation [72, 154, 189]. This lead to the idea that the faecal samples could potentially contain compounds from bacteria and host, that maybe different between T2D patients and healthy controls, hence can distinguish between healthy and diseased state.

The method of preparation of FW was an important factor to carefully consider as it was the experimental medium used in Chapter 4 and 5. It is important to note that the method of FW preparation (section 2.6) resulted in a very dilute solution (1:40 wt/vol), hence it was used for incubations without dilution. This is contrary to the study of Gill et al. (2007), where faecal sample
was weighed and mixed with ice cold PBS 1:1 wt/vol, but in agreement with the finding of the study, which was used to determine the effect of FW on colonic mucosal barrier function at 0 and 48 hours, TER was affected by samples from elderly when compared with young adults. Also a study used the extraction of faeces in PBS method of preparation in a 1:1 wt/vol dilution. But contrary to the finding of the study, TER remained unchanged after 24 hours post-incubation. Other methods of preparation as observed in several studies was to weigh faecal samples, homogenised, centrifuged and either diluted before incubations [132, 134] or used directly [126, 133]. However, it has been reported that the method of preparation does not affect experimental outcomes. A study by Klinder et al. (2007), looked at 3 method of FW preparation which were direct centrifugation, extraction of faeces in PBS before centrifugation and centrifugation of lyophilised and reconstituted faeces. All preparation methods were used to assess genotoxicity and 4 out of 7 samples were non-genotoxic irrespective of the FW preparation methods, while 2 out of 7 samples were similarly genotoxic when using direct centrifugation and extraction of faeces in PBS before centrifugation. Also TER was measured at 24 hours for all 3 preparation method and no difference was observed for all methods, which had no effect on TER [125]. Since extraction of faeces in PBS before centrifugation allows for more FW preparation that can be filtered sterilized, stored and used in further experiment, this method was considered suitable for use in this thesis.
Focused mainly on diet, several studies has explored the activity of FW *in vitro* using colonocyte-based models of cytotoxicity, genotoxicity, and barrier function. Also, these studies are mostly in terms of the role in the development and progression of colon cancer risk [127, 132, 190, 191]. However, in chapter 4 of this thesis, FW from T2D was assessed on Caco-2 cell monolayers to evaluate the impact on monolayer integrity as an index of paracellular permeability in the context of T2D disease. The purpose was to investigate if there was a discrimination between healthy and diseased individuals based on FW. TER was chosen to be used alone because to continuously monitor the barrier integrity, non-invasive techniques are best suited. Also, it is a very sensitive and reliable method to confirm the integrity and permeability of the monolayer. Although the best indicators of the integrity of the tight junctions and of the cell monolayer are measurements of TER and of transepithelial passage of marker molecules, they determine different experimental parameters [192-194].

The result observed in chapter 4 was able to discriminate between healthy and diseased state as FW from T2D patients (n=9) had a significant impact on TER causing decrease at 24 and 48 hours post incubation when compared to healthy controls (n=3). This is in agreement with a study where 2 FW from healthy volunteers had no effect on TER after 24 post incubation [125]. Although literature search did not produce results for effect of FW from T2D on TER, it certainly appears possible given the significant difference observed in this thesis,
that the activity of FW from the T2D patients can be a good tool to identify diseased state when compared with healthy state.

In addition, a novel aspect of this thesis (chapter 4) was to identify patients, whose T2D have been ‘well managed’ over time and those with possibly ‘not so well managed state’. Again, literature search produced no result to support this aspect of this investigation directly but other *in-vitro* studies on the effect of LPS, TNF alpha and bile acids on barrier function was used to explain the findings in this thesis. Patients measurements of *in-vivo* markers of T2D including insulin, total cholesterol, LPS and TNFalpha were correlated with TER measurements for 0, 24 and 48 hours post incubation with FW samples (n=5). It was observed that there was a relationship between all markers of T2D with TER at 0, 24 and 48 hours. It is worth noting that patients with high levels of markers of T2D, had FW samples that reduced TER over a 48 hours period. At 48 hours post-incubation with FW, 4 out of 5 samples had an increase in TER, except 1 sample for the patient who had higher level of LPS. LPS is contained in the outer membrane of bacteria belonging to the phylum Proteobacteria, which was higher in T2D patients when compared to healthy control (chapter 3). These observations can be used to postulate that the blood level of these markers of T2D can directly or indirectly impact the gut-microbiota, hence the composition of faecal samples. This also suggests that T2D management maybe an important way of achieving a stable and healthy gut microbiota. Furthermore, the relationship between anthropometric parameters of the T2D patients and TER
measurements for 0, 24 and 48 hours was assessed. It was observed that high body fat (%) and low total body water (kg) were significantly associated with decreased TER at 24 and 48 hours. Since the consumption of high fat diet is known to favour the growth of pathogenic bacteria in the gut, hence the level of endotoxins such as LPS might be on the increase in the gut [71, 72, 74], this may possibly address the result observed in this thesis.

Prebiotic has been shown to be beneficial as it improve various metabolic markers in-vivo [93, 157]. However in this thesis, non-prebiotic B-GOS effect on cell resistance to FW cytotoxicity was assessed in a time and dose dependent manner (chapter 5). Before using the FW from the T2D patients (n=4), the cytokine profile of the faecal samples were measured using a cytokine array. This was performed because it was thought that the combinations of cytokine present in the faecal sample will be different between individual T2D patients, which will determine the FW cytotoxicity on Caco-2 cells in-vitro. The results observed showed that the cytokine profile of T2D patients with cytotoxic FW were different from those patients with non-cytotoxic FW. Again, literature search produced no result for studies that could directly be related to the findings here but in-vivo studies have shown IL-6, TNF alpha , IFNg, IL-13 as pro-inflammatory cytokines present in T2D patients that play a key role in driving inflammation. It is also possible that using faecal samples as a non-invasive way to determine the inflammatory tone in T2D maybe a good way to determine those with well managed T2D. The protective effect of B-GOS against FW cytotoxicity was also
observed when B·GOS was present during the incubation with FW samples. Contrary to this study, the prebiotic Elixor, a GOS, was found to be less effective in increasing the cellular resistance to FW genotoxicity [128] . However, this was not the same type of GOS used in this thesis. Also, despite this study was performed on cells in-vitro, prebiotic supplementation has been shown to effect faecal water genotoxicity in a human study involving 38 healthy male volunteers (smokers and non-smokers), after a dietary intervention study with bread supplemented with prebiotics (6% inulin) for 5 weeks. Faecal samples were collected from volunteers and FW genotoxicity was assayed in human colon HT29 clone 19A cells. Faecal water genotoxicity was reduced only in non-smokers but not in smokers [195]. In addition, prebiotic ameliorated the effect of FW on cells in an animal study where rats were administered Azoxymethane (AOM) to initiate tumours, treated with inulin/oligofructoses (100g/kg wt/wt) and faeces were collected at 0 and 10 days and 2, 4, and 8 months. FW were prepared and tested for genotoxicity in HT29 colon cells using the comet assay. Treatment with inulin/oligofructoses significantly reduced faecal genotoxicity on cells [191]. These studies showed that prebiotic treatment both in-vivo and in-vitro affect the activity of FW. However, these studies were not related to T2D or GOS, but shows that GOS might also offer non-prebiotic effect independent of the gut-microbiota. Overall, this thesis work presents possible novel ways to study gut microbiota in human T2D in the laboratory in-vitro models and as well linked with in-vivo works in T2D.
6.1 Study Limitations

A number of limitations were identified in the studies performed in this thesis. Despite the novelty of the studies presented in chapter 4 and 5, the small sample size used exhibits pilot characteristics and therefore it will be necessary to conduct this in a larger cohort. However, the small sample size enabled the required number of biological replicates for the experiments in chapter 4 to be performed as the transwells are expensive and cannot be reused. Although the idea of using the FW was to explore a way to use it as a complex mixture and relate it to *in vivo* markers, it would have been an added advantage if the composition of the FW was measured. Identifying what compounds were implicated in the activity of FW from those with supposed ‘well managed T2D’ that had not effect on paracellular permeability would be beneficial to know if they are diet related or not. Finally, using B-GOS (chapter 5) may suggests that other components (such as glucose) may have influenced the observed outcome of the experiment. However, commercially available B-GOS are consumed by individuals and not pure GOS, hence the method of preparation (vol/vol concentrations) was as used in this thesis. Also, GOS was chosen to be used in the study (chapter 5) as it was the prebiotic consumed by the participants in the intervention studies in chapter 3, but it might be useful to include other prebiotics such as inulin and FOS.
6.2 Future studies

Recommendation for future works might be to conduct the studies (chapter 3, 4, and 5) in a larger cohort to address study and experimental sample power. Also addressing the limitations of the studies (Section 6.1) such as measurement of FW composition and use more prebiotics can improve the study outcomes. Finally, an important aspect will be to investigate the mechanism behind the FW effect on cell monolayer and cytotoxicity, which was not done in this thesis as follows:

1. The role of FW in the expression and redistribution of tight junction proteins (such as occludin, ZO-1 and claudins), which may explain the reason for decrease cell monolayer integrity in Caco-2 cells can be performed using fluorescence microscopy (confocal microscopy).

2. Determine the activation of immune response in Caco-2 cells, which may lead to production of inflammatory mediators that can influence the cell response to FW. This can be achieved by measuring cytokines in the cell culture media and using western blotting experiment to investigate the upregulation / downregulation of inflammatory pathways.
BIBLIOGRAPHY


17. van Raalte, D.H. and C.B. Verchere, **Improving glycemic control in type 2 diabetes: stimulate insulin secretion or provide beta-cell rest?** Diabetes Obes Metab, 2017.


36. Sharma, M., I. Nazareth, and I. Petersen, Trends in incidence, prevalence and prescribing in type 2 diabetes mellitus between 2000 and 2013 in


APPENDIX
Appendix A – Publications, presentations and awards

Publications


- Camilla Pedersen, Edith Gallagher, Felicity Horton, Richard Ellis, Umer Ijaz, Etana Jaiyeola, Thibaut Duparc, David Russell-Jones, Paul Hinton, Patrice Cani, Roberto LaRagione, and Denise Robertson. "Enterobacteriales enrichment in type 2 diabetes is associated with impaired intestinal permeability". (Submitted to Clinical Science-Under peer review)

Presentations


• 06/2015 University of Surrey, Faculty of Health and Medical Sciences
  Research Festival. Guildford, Surrey. Poster.

Awards


3. Travel grant to attend Schlumberger Foundation Faculty for the Future Fellows Forum. Cambridge (UK) from October 30th to November 2nd, 2016.

4. Travel Grant to attend the Annual meeting of the Society for General Microbiology (SCG/15/037). Birmingham, UK (March, 2015).

5. Awarded Funds for Women Graduates (FfWG) - Emergency Grants. March 2015
Appendix B: Statistically analyzed sequencing data from which mean, p and q-values were reported in chapter 3.

Table A-1: Statistic data at the phylum level - T2D versus healthy controls

The phylum Proteobacteria was significant for the P but not Q values.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Mean T2D-PRE</th>
<th>Mean CON</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root:Bacteria:Proteobacteria</td>
<td>0.039778662</td>
<td>1.75E-02</td>
<td>0.017780931</td>
<td>0.195590236</td>
</tr>
<tr>
<td>Root:Bacteria:Deferribacteres</td>
<td>0.000100422</td>
<td>0.00E+00</td>
<td>0.317310508</td>
<td>0.887143714</td>
</tr>
<tr>
<td>Root:Bacteria:Fusobacteria</td>
<td>0</td>
<td>1.58E-05</td>
<td>0.317310508</td>
<td>0.887143714</td>
</tr>
<tr>
<td>Root:Bacteria:Bacteroidetes</td>
<td>0.525482539</td>
<td>5.43E-01</td>
<td>0.418616316</td>
<td>0.887143714</td>
</tr>
<tr>
<td>Root:Bacteria:Cyanobacteria</td>
<td>3.77E-05</td>
<td>5.35E-05</td>
<td>0.556057769</td>
<td>0.887143714</td>
</tr>
<tr>
<td>Root:Archaea:Euryarchaeota</td>
<td>0.000140257</td>
<td>1.22E-04</td>
<td>0.59465903</td>
<td>0.887143714</td>
</tr>
<tr>
<td>Root:Bacteria:Verrucomicrobia</td>
<td>0.000663681</td>
<td>1.96E-04</td>
<td>0.596475901</td>
<td>0.887143714</td>
</tr>
<tr>
<td>Root:Bacteria:Other</td>
<td>0.034010269</td>
<td>2.78E-02</td>
<td>0.709857667</td>
<td>0.887143714</td>
</tr>
<tr>
<td>Root:Bacteria:Actinobacteria</td>
<td>0.002829393</td>
<td>1.64E-03</td>
<td>0.775894859</td>
<td>0.887143714</td>
</tr>
<tr>
<td>Root:Bacteria:Firmicutes</td>
<td>0.39695707</td>
<td>0.409425941</td>
<td>0.806494285</td>
<td>0.887143714</td>
</tr>
<tr>
<td>Root:Other:Other</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
**Table A-2: Statistic data at the phylum level - T2D subjects in the prebiotic group**

The phylum Firmicutes was significant for the P and Q values, which was higher in the post samples of the prebiotic group. The phylum other bacteria was increased in the prebiotic group but not significant.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Mean PREBIOTIC</th>
<th>Mean POST-PREBIOTIC</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root;Bacteria:Firmicutes</td>
<td>0.401312683</td>
<td>0.4872494</td>
<td>0.001953125</td>
<td>0.021484375</td>
</tr>
<tr>
<td>Root;Bacteria:Other</td>
<td>0.025627351</td>
<td>0.0449443</td>
<td>0.018554688</td>
<td>0.102050781</td>
</tr>
<tr>
<td>Root;Bacteria:Bacteroidetes</td>
<td>0.515273542</td>
<td>0.4372489</td>
<td>0.053710938</td>
<td>0.196940104</td>
</tr>
<tr>
<td>Root;Bacteria:Proteobacteria</td>
<td>0.051326987</td>
<td>0.0258083</td>
<td>0.083007813</td>
<td>0.228271484</td>
</tr>
<tr>
<td>Root;Archaea:Euryarchaeota</td>
<td>0.000207648</td>
<td>0.0009009</td>
<td>0.285049407</td>
<td>0.581735931</td>
</tr>
<tr>
<td>Root;Other:Other</td>
<td>0</td>
<td>0.0000235</td>
<td>0.317310508</td>
<td>0.581735931</td>
</tr>
<tr>
<td>Root;Bacteria:Actinobacteria</td>
<td>0.00495209</td>
<td>0.0035289</td>
<td>0.444586739</td>
<td>0.687753353</td>
</tr>
<tr>
<td>Root;Bacteria:Verrucomicrobia</td>
<td>0.001062338</td>
<td>0.0001876</td>
<td>0.500184257</td>
<td>0.687753353</td>
</tr>
<tr>
<td>Root;Bacteria:Deferribacteres</td>
<td>2.37E-04</td>
<td>0.0001084</td>
<td>0.654720846</td>
<td>0.800214367</td>
</tr>
<tr>
<td>Root;Bacteria:Cyanobacteria</td>
<td>0</td>
<td>0.0000000</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Root;Bacteria:Fusobacteria</td>
<td>0</td>
<td>0.0000000</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table A-3: Statistic data at the phylum level - T2D subjects in the placebo group**
The Phylum Firmicutes was significant for the P but not Q values, and which was higher in the post samples of the prebiotic group.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Mean</th>
<th>PRE·PLACEBO</th>
<th>POST·PLACEBO</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root:Bacteria:Firmicutes</td>
<td>0.398943399</td>
<td>5.03E-01</td>
<td>0.020996094</td>
<td>0.230957031</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Actinobacteria</td>
<td>0.001084987</td>
<td>2.08E-03</td>
<td>0.063964844</td>
<td>0.282877604</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Bacteroidetes</td>
<td>0.522093638</td>
<td>4.26E-01</td>
<td>0.077148438</td>
<td>0.282877604</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Cyanobacteria</td>
<td>0</td>
<td>1.13E-04</td>
<td>0.179712495</td>
<td>0.494209361</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Other</td>
<td>0.039926615</td>
<td>2.95E-02</td>
<td>0.266113281</td>
<td>0.585449219</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Verrucomicrobia</td>
<td>0.000292697</td>
<td>0.000211794</td>
<td>0.500184257</td>
<td>0.894670759</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Proteobacteria</td>
<td>0.037545116</td>
<td>3.92E-02</td>
<td>0.569335938</td>
<td>0.894670759</td>
<td></td>
</tr>
<tr>
<td>Root:Archaea:Euryarchaeota</td>
<td>0.000113547</td>
<td>7.57E-05</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Deferribacteres</td>
<td>0.00E+00</td>
<td>0.00E+00</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Fusobacteria</td>
<td>0</td>
<td>0.00E+00</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Root:Other:Other</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Table A-4: Statistic data at the phylum level - T2D subjects pre and post samples

The phylum Bacteroidetes was significantly higher in the pre samples and this is a true representation of the gut microbiota in T2D subjects. Firmicutes were decreased in the pre samples.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Mean PRE-T2D</th>
<th>Mean POST-T2D</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root:Bacteria:Firmicutes</td>
<td>3.97E-01</td>
<td>0.497212854</td>
<td>0.003248257</td>
<td>0.035730827</td>
</tr>
<tr>
<td>Root:Bacteria:Bacteroidetes</td>
<td>0.525482539</td>
<td>0.427021742</td>
<td>0.007777278</td>
<td>0.042775031</td>
</tr>
<tr>
<td>Root:Other:Other</td>
<td>0</td>
<td>1.03E-05</td>
<td>0.307821472</td>
<td>0.653808328</td>
</tr>
<tr>
<td>Root:Bacteria:Cyanobacteria</td>
<td>3.77E-05</td>
<td>6.04E-05</td>
<td>0.312333123</td>
<td>0.653808328</td>
</tr>
<tr>
<td>Root:Bacteria:Other</td>
<td>0.034010269</td>
<td>3.75E-02</td>
<td>0.394487168</td>
<td>0.653808328</td>
</tr>
<tr>
<td>Root:Archaea:Euryarchaeota</td>
<td>0.000140257</td>
<td>5.45E-04</td>
<td>0.399445255</td>
<td>0.653808328</td>
</tr>
<tr>
<td>Root:Bacteria:Actinobacteria</td>
<td>0.002829393</td>
<td>2.76E-03</td>
<td>0.416059845</td>
<td>0.653808328</td>
</tr>
<tr>
<td>Root:Bacteria:Proteobacteria</td>
<td>3.98E-02</td>
<td>3.45E-02</td>
<td>0.772180313</td>
<td>1</td>
</tr>
<tr>
<td>Root:Bacteria:Verrucomicrobia</td>
<td>0.000663681</td>
<td>2.91E-04</td>
<td>0.942690469</td>
<td>1</td>
</tr>
<tr>
<td>Root:Bacteria:Deferribacteres</td>
<td>0.000100422</td>
<td>4.77E-05</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Root:Bacteria:Fusobacteria</td>
<td>0.00E+00</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Table A-5: Statistic data at the phylum level – healthy control subjects’ normal weight (NW) and overweight (OW)

No statistical significant difference between the two groups.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Mean</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL-NW</td>
<td>CONTROL-OW</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Cyanobacteria</td>
<td>9.94E-05</td>
<td>0</td>
<td>0.164914823</td>
</tr>
<tr>
<td>Root:Bacteria:Fusobacteria</td>
<td>2.93E-05</td>
<td>0</td>
<td>0.335234414</td>
</tr>
<tr>
<td>Root:Bacteria:Firmicutes</td>
<td>0.431212352</td>
<td>0.378258186</td>
<td>0.550177931</td>
</tr>
<tr>
<td>Root:Bacteria:Verrucomicrobi a</td>
<td>0.00017351</td>
<td>0.000205695</td>
<td>0.841493453</td>
</tr>
<tr>
<td>Root:Bacteria:Bacteroidetes</td>
<td>0.53129547</td>
<td>5.64E-01</td>
<td>0.867376498</td>
</tr>
<tr>
<td>Root:Bacteria:Other</td>
<td>0.027797025</td>
<td>2.85E-02</td>
<td>0.905062742</td>
</tr>
<tr>
<td>Root:Bacteria:Proteobacteria</td>
<td>0.007640601</td>
<td>2.70E-02</td>
<td>0.905062742</td>
</tr>
<tr>
<td>Root:Archaea:Euryarchaeota</td>
<td>7.91E-05</td>
<td>0.00159434</td>
<td>0.946851439</td>
</tr>
<tr>
<td>Root:Bacteria:Actinobacteria</td>
<td>0.001673165</td>
<td>1.68E-03</td>
<td>0.961178326</td>
</tr>
<tr>
<td>Root:Bacteria:Deferribacteres</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Root:Other:Other</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Table A-6: Statistic data at the phylum level - T2D subjects on metformin and off-metformin.

No statistical difference between the two groups.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Mean (PRE)</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metformin</td>
<td>non-metformin</td>
<td></td>
</tr>
<tr>
<td>Root:Bacteria:Cyanobacteria</td>
<td>0</td>
<td>1.23E-04</td>
<td>0.123291598</td>
</tr>
<tr>
<td>Root:Bacteria:Other</td>
<td>0.028418338</td>
<td>4.48E-02</td>
<td>0.237670349</td>
</tr>
<tr>
<td>Root:Bacteria:Actinobacteria</td>
<td>0.003381354</td>
<td>1.23E-03</td>
<td>0.488680305</td>
</tr>
<tr>
<td>Root:Bacteria:Firmicutes</td>
<td>0.385202038</td>
<td>4.07E-01</td>
<td>0.514726755</td>
</tr>
<tr>
<td>Root:Bacteria:Deferribacteres</td>
<td>1.37E-04</td>
<td>0.00E+00</td>
<td>0.516412268</td>
</tr>
<tr>
<td>Root:Bacteria:Proteobacteria</td>
<td>0.040955929</td>
<td>3.21E-02</td>
<td>0.584535207</td>
</tr>
<tr>
<td>Root:Bacteria:Verrucomicrobia</td>
<td>0.000831295</td>
<td>0.00182635</td>
<td>0.730487521</td>
</tr>
<tr>
<td>Root:Archaea:Euryarchaeota</td>
<td>0.000174198</td>
<td>4.21E-05</td>
<td>0.7311671</td>
</tr>
<tr>
<td>Root:Bacteria:Bacteroidetes</td>
<td>0.540899428</td>
<td>5.15E-01</td>
<td>0.815036429</td>
</tr>
<tr>
<td>Root:Bacteria:Fusobacteria</td>
<td>0</td>
<td>0.00E+00</td>
<td>1</td>
</tr>
<tr>
<td>Root:Other:Other</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Table A-7: Statistic data at the class level - T2D subjects (pre) and healthy controls.

The class Gammaproteobacteria and Erysipelotrichi are higher in the T2D subjects but not significant reporting the Q values.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Mean T2D-PRE</th>
<th>Mean CON</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria</td>
<td>0.032999021</td>
<td>0.01338151</td>
<td>0.028433062</td>
<td>0.480901705</td>
</tr>
<tr>
<td>Bacteria;Firmicutes;Erysipelotrichi</td>
<td>0.016944412</td>
<td>0.012210368</td>
<td>0.048090171</td>
<td>0.480901705</td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Alphaproteobacteria</td>
<td>0.000193758</td>
<td>2.55E-04</td>
<td>0.266793889</td>
<td>0.858106655</td>
</tr>
<tr>
<td>Bacteria:Bacteroidetes:Bacteroidetes</td>
<td>0.491408427</td>
<td>5.19E-01</td>
<td>0.270710129</td>
<td>0.858106655</td>
</tr>
<tr>
<td>Root:Bacteria:Deferribacteres:Deferribacteres</td>
<td>0.000100422</td>
<td>0</td>
<td>0.317310508</td>
<td>0.858106655</td>
</tr>
<tr>
<td>Root:Bacteria:Fusobacteria:Fusobacteria</td>
<td>0</td>
<td>1.58E-05</td>
<td>0.317310508</td>
<td>0.858106655</td>
</tr>
<tr>
<td>Root:Bacteria:Proteobacteria:Epsilonproteobacteria</td>
<td>1.52E-05</td>
<td>0</td>
<td>0.317310508</td>
<td>0.858106655</td>
</tr>
<tr>
<td>Root:Bacteria:Firmicutes:Other</td>
<td>0.007105284</td>
<td>0.005589701</td>
<td>0.399768642</td>
<td>0.858106655</td>
</tr>
<tr>
<td>Root:Bacteria:Proteobacteria:Other</td>
<td>0.00032744</td>
<td>0.000416123</td>
<td>0.44235268</td>
<td>0.858106655</td>
</tr>
<tr>
<td>Root:Bacteria:Firmicutes:Clostridia</td>
<td>0.370714017</td>
<td>0.363028668</td>
<td>0.472970147</td>
<td>0.858106655</td>
</tr>
</tbody>
</table>
Table A-8: Statistic data at the family level - T2D subjects (pre) and healthy controls.

The family Enterobacteriaceae decreased in the post samples of T2D subjects’ on metformin but not significant reporting the Q values.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>mean_POST-met</th>
<th>mean_POST-no met</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root;Bacteria:Firmicutes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptostreptococcaceae</td>
<td>0.000527568</td>
<td>0.003684141</td>
<td>7.16E-03</td>
<td>0.386899191</td>
</tr>
<tr>
<td>Root;Bacteria:Proteobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>: Oxalobacteraceae</td>
<td>7.28429E-05</td>
<td>0.000462838</td>
<td>0.019754768</td>
<td>0.431930127</td>
</tr>
<tr>
<td>Root;Bacteria:Proteobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>: Enterobacteriaceae</td>
<td>0.020119165</td>
<td>0.037855348</td>
<td>0.029987081</td>
<td>0.431930127</td>
</tr>
<tr>
<td>Root;Bacteria:Proteobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>: Other</td>
<td>0.000320684</td>
<td>0.000580707</td>
<td>0.031994824</td>
<td>0.431930127</td>
</tr>
<tr>
<td>Root;Bacteria:Firmicutes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veillonellaceae</td>
<td>0.017969968</td>
<td>0.049495103</td>
<td>0.047622378</td>
<td>0.514321678</td>
</tr>
</tbody>
</table>