A Study to Explore the Effects of Probiotics on Endotoxin Levels and Cardiometabolic Indices in Patients with Type 2 Diabetes Mellitus

By

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DECLARATION

I declare that this thesis is an accurate record of my results obtained by myself within the labs at University of Warwick, Clinical Science Research Laboratories and the data that has arisen is detailed in this thesis. All sources of support and technical assistance have been stated in the text of the acknowledgments. None of the work has been previously submitted for a higher degree.

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Special thanks to Professor Philip McTernan, my mentor and my big brother. Not a day passed where I felt I am the luckiest PhD candidate alive to be under your supervision. My sincerest gratitude also goes to my fellow PhD candidates and scientists at the Division of Biomedical Sciences at the Clinical Sciences Research Institute (CSRI), Warwick Medical School, your passion to your studies is an inspiration to me.

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DEDICATION

Dedicated to the bravest warrior and survivor I know,

2nd Lieutenant Pedro F. Sabico, my father.

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SYNOPSIS

Low-grade chronic inflammation in patients with type 2 diabetes mellitus (T2DM) may be influenced by circulating endotoxin levels, acting as an inflammatory stimulus. Health- promoting live microorganisms, such as probiotics, may influence circulating endotoxin levels and reduce inflammation. Limited information is available whether or not probiotics do so in patients with T2DM. The aim of this study was to characterise the beneficial effects of a multi-strain probiotics on circulating endotoxin levels and other biomarkers related to systemic low-grade inflammation and cardiometabolic status in patients with T2DM.

A total of 150 adult Saudi T2DM patients (naïve and without co-morbidities, aged 40-60 years) were initially recruited, 96 of whom were randomized, 78 completed 3 months, and 61 completed the entire clinical trial. They were randomized to receive twice daily placebo or probiotics [(2.5×10^9cfu/gram) containing the following bacterial strains: *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58 (Ecologic®Barrier)] in a double-blind manner over a 6 month period. Anthropometrics, glycaemic and lipid profiles, as well as inflammatory and other markers, including adipocytokines, were measured. Measurements/samples were obtained at baseline and after 3 and 6 months of treatment.

After 12/13 weeks of intervention and using intention-to-treat analysis, no difference was noted in endotoxin levels between groups [Placebo -9.5% vs Probiotics -52.2%; (CI: -0.05-0.36; p=0.15)]. Compared with the placebo group however, participants in the probiotics groups had a significant but modest

improvement in WHR [Placebo 0.0% vs Probiotics 1.11%; (CI: -0.12- -0.01; p=0.02)] as well as a clinically significant improvement in HOMA-IR [Placebo -12.2% vs Probiotics -60.4%; (CI: -0.34- -0.01; p=0.04)].

After 6 months of intervention, significant improvements were observed in endotoxin levels, glycaemic, lipid, inflammatory and adipocytokine profiles in the probiotics group, which were not seen in the placebo group. Between group analyses, however, revealed that only HOMA-IR demonstrated a clinically significant reduction in favour of the probiotics group after adjusting for baseline covariates [Placebo % change: 0.80 vs. Probiotics % change: -3.40 (CI: -0.59 - -0.17); p=0.001].

The current thesis expanded our knowledge on the beneficial effects of a multi-strain probiotics intake in improving insulin resistance among Saudi patients with T2DM and is therefore recommended as a promising adjuvant anti-diabetes therapy. Larger trials may causally confirm whether the beneficial effects of probiotics in reducing endotoxin levels may extend in preventing complications of T2DM.

ABBREVIATIONS

µg	Microgram
ADA	American Diabetes Association
AHA	American Heart Association
AMPK	AMP-activated Protein Kinase
ANCOVA	Analysis of Covariance
BMI	Body Mass Index
BP	Blood Pressure
BRP	Biomarkers Research Program
CHOD/POD	Cholesterol Oxidase/Peroxidase
CI	Confidence Interval
cm	Centimetre
CRF	Case Report Form
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DM	Diabetes Mellitus
ECL	Electrochemiluminiscence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FFA	Free Fatty Acids
FPG	Fasting Plasma Glucose
GDM	Gestational Diabetes Mellitus
HBA	Hydroxybenzoic Acid
HbA1c	Glycated Haemoglobin
HDL	High Density Lipoprotein
HMW	High Molecular Weight
HOMA-IR	Homeostasis Model Assessment for Insulin Resistance
IBS	Irritable Bowel Syndrome
IDF	International Diabetes Federation

IFG	Impaired Fasting Glucose
IL-6	Interleukin 6
iNOS	Inducible Nitric Oxide Synthase
ITT	Intention-to-Treat
IU	International Units
JNK	c-Jun NH2-terminal kinase
kg	Kilogram
Kinetic-QCL	Kinetic Quantitative Chromogenic LAL
KSU	King Saud University
l	Litre
LAL	Limulus Amoebocyte Lysate
LDL	Low Density Lipoprotein
LOCF	Last Observation Carried Forward
LPS	Lipopolysaccharides
m	Metre
M/F	Males/Females
MAP	Mean Arterial Pressure
MD	Maryland
MDC	Minimum Detectable Concentration
MENA	Middle East and North Africa
MetS	Metabolic Syndrome
mmHg	Millimetres Mercury
mmol	millimole
MN	Minnesota
MS	Microsoft
NAFLD	non-Alcoholic Fatty Liver Disease
NEC	Necrotizing Enterocolitis
NFκB	Nuclear Factor-kappaB
ng	Nanogram
NIH	National Institute of Health
NOD	Non Obese Diabetic
Ob	Obese
PEG	Polyethylene Glycol-Modified

pg	Picogram
pNA	p-Nitroaniline
PPA	Per Protocol Analysis
PPARα	Peroxisome Proliferator Activator Receptor Alpha
R	Coefficient
RCT	Randomised Control Trial
RETN	Resistin Gene
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SBP	Systolic Blood Pressure
SD	Standard Deviation
SFDA	Saudi Food and Drug Administration
SPSS	Statistical Package for the Social Sciences
SST	Serum Separator Tube
STZ	Streptozotocin
T2DM	Type 2 Diabetes Mellitus
TC	Total Cholesterol
TEER	Transepithelial Electrical Resistance
TER	Transepithelial Resistance
TG	Triglycerides
TJ	Tight Junction
TLR	Toll-like Receptor
TNF-α	Tumour Necrosis Factor Alpha
TPA	Tripropylamine
TX	Texas
USA	United States of America
USNLM	US National Library of Medicine
VA	Virginia
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization
WHR	Waist-Hip Ratio

Chapter 1

Introduction

1.1 Obesity

The definition of overweight obesity according to the Centre for Disease Control (CDC) and Prevention was based on weight that is higher than what is average or normal for a given height in both sexes as measured by the body mass index (BMI) (CDC, https://www.cdc.gov/obesity/adult/defining.html). BMI was also previously called the Quetelet's index or formula as a reliable indicator of fatness based on the study of Garrow and Webster (1985). As of 2016, an estimated 1.9 billion people above 18 years old were considered overweight, 650 million of whom were considered under the category of obese (≥30kg/m^2) (WHO Fact Sheet, http://www.who.int/mediacentre/factsheets/fs311/en/). Currently, obesity is considered by the most respectable international medical associations as a disease that needs treatment (Kilov and Kilov, 2017).

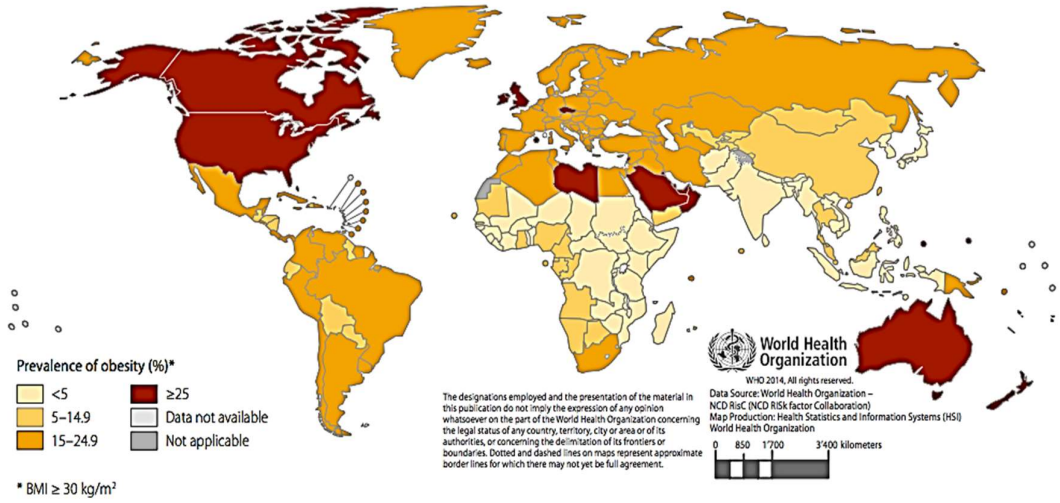


Figure 1.1.1 Age-standardized prevalence of obesity in men aged 18 and over (BMI≥18kg/m^2) in 2014.

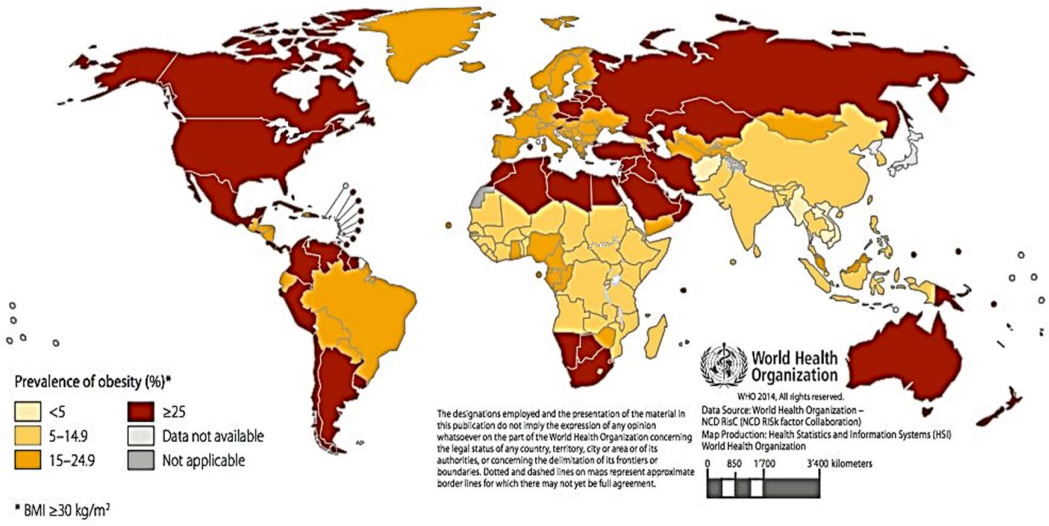


Figure 1.1.2 Age-standardized prevalence of obesity in women aged 18 and over (BMI≥18kg/m^2) in 2014.

Whilst the prevalence of obesity is highest among highly industrialized nations, it was observed that it has started to plateau. This is in opposition to emerging economies such as the Middle East, including Saudi Arabia, with obesity trends continuing to grow especially amongst children and adolescents (NCD Risk Factor Collaboration 2017).

In Saudi Arabia, it was previously observed that as of 2010, the prevalence of obesity plateaued, with a reported over-all prevalence of 40% in Saudi adults similar to the year 2000. This was despite the increasing incidence of T2DM (from 28.6% in 2000 to 31.6 in 2010), hypertension (from 30% in 2000 to 32.6% in 2010) and coronary artery disease (from 6.2% to 6.9%) (Al-Daghri et al., 2011) (Figure 1.2.1). More recent epidemiologic studies now suggest that the incidence of T2DM continues to rise as the prevalence of being overweight or obesity among Saudi adults increases from 52.6-55.1% (Azzeh et al., 2017; Ahmed et al., 2017) and there is a significant increase in the prevalence of childhood obesity from 12.6% in 2008 to 15.3% in 2013 as well, affecting population data (Al-Daghri et al., 2016).

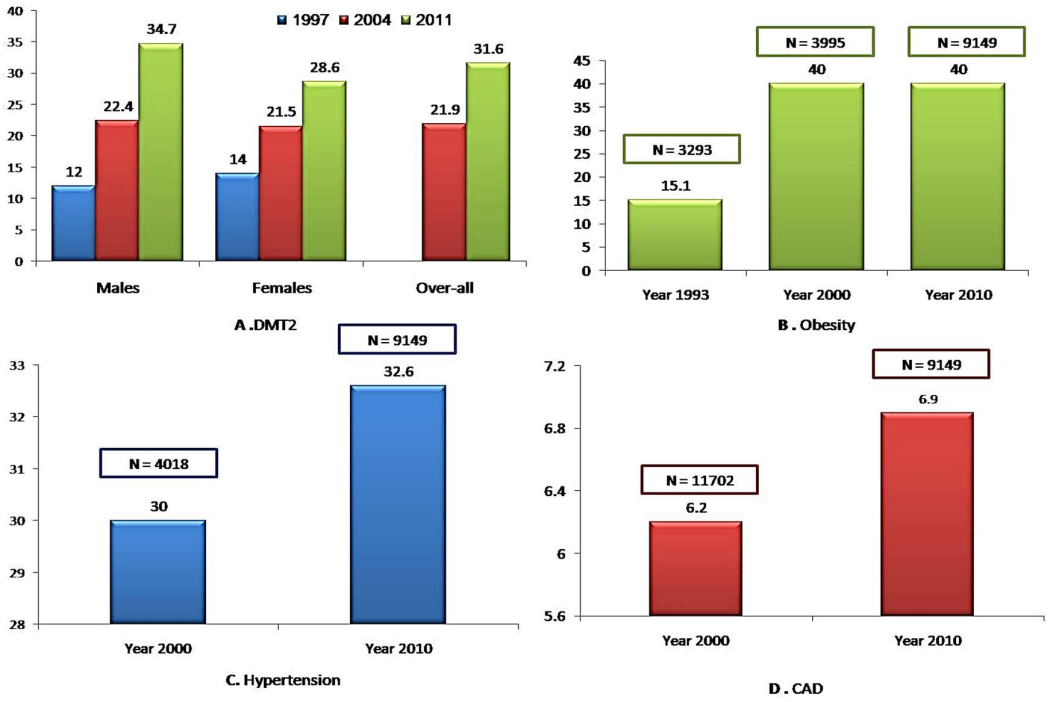


Figure 1.1.3 Trends in the prevalence of non-communicable diseases in Saudi Arabia (Adopted from Al-Daghri et al., 2011).

Obesity has been consistently considered as the single biggest risk factor for type 2 diabetes mellitus (T2DM), as obesity was hypothesized to induce insulin resistance and β-cell failure (Eckel et al., 2011). On the other hand, diabetes is defined as *"a group of metabolic diseases characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels"* (ADA, 2010). The two most common types of diabetes mellitus include type 1 (T1DM), caused by cellular-mediated auto-immune destruction of the β cells of the pancreas and accounts for 5-10% of all people with diabetes (ADA, 2010). The most common type is type 2 (T2DM), and most patients with this type are obese, having chronic insulin resistance with comparative insulin deficiency to mostly an insulin secretory defect with insulin

resistance (ADA, 2010). T2DM accounts for 90-95% of patients considered to have diabetes.

1.2 Diabetes Mellitus in the Middle East and Saudi Arabia

Diabetes mellitus (DM) is a chronic, non-communicable disease that debilitates not only the overall well-being of the individual affected but also impacts the general health of the society involved in terms of productivity and economy. According to the International Diabetes Federation (IDF) and as of 2015, one out of every 11 human adults has diabetes. By the year 2040 this incidence will increase to one out of 10, or 640 million (IDF, 2015) (Figure 1.1.1).

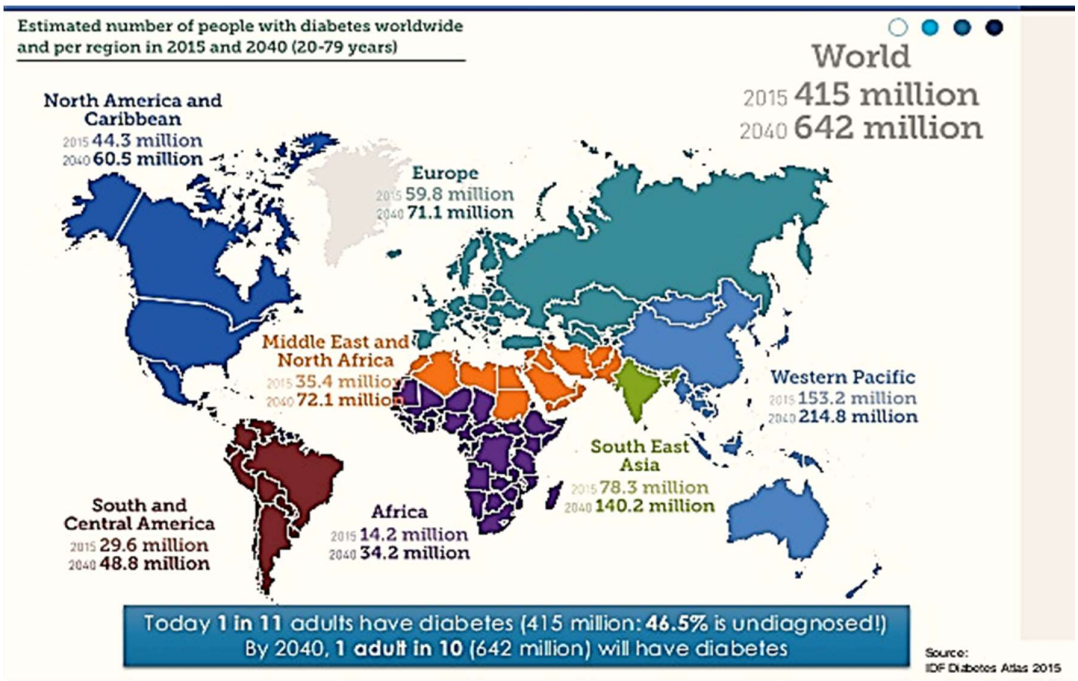


Figure 1.2.1. The Growing Diabetes Pandemic

Whilst globally the trend for diabetes incidence is clearly rising, the rate of escalation is fastest in developing nations, particularly in the Middle East and North Africa (MENA) region (NCD-RisC, 2016). Furthermore in the MENA region, the

highest prevalence of DM is observed in the Gulf nations, with Saudi Arabia topping the list at 23.87% followed by Kuwait at 23.09%, Qatar at 22.87% and Bahrain at 21.84% (Majeed et al., 2014; Meo et al., 2017) (Table 1.1.1).

Table 1.2.1.1 Top 10 countries with the highest prevalence of T2DM in the MENA region

Rank	MENA Country	Prevalence (%)2013
1	Saudi Arabia	23.9
2	Kuwait	23.1
3	Qatar	22.9
4	Bahrain	21.8
5	United Arab Emirates	19.0
6	Egypt	16.8
7	Lebanon	15.0
8	Oman	14.2
9	Jordan	11.4
10	Iran	9.9

Note: Table adopted from Majeed et al., 2014

With regards to Saudi Arabia, there is no lack of updated epidemiologic data pointing to increased prevalence of DM, particularly T2DM in all populations including children and adolescents at 10.84% (Al-Rubeaan, 2015) and higher than 20% in adults (Al-Rubeaan et al., 2015; Al-Daghri et al., 2011). Even more alarming in the case of Saudi adults is the higher prevalence of those unaware they already have T2DM (>40%) as well as those with impaired fasting glucose (IFG) at >25% (Al-Rubeaan et al., 2015). The most recent observations from Meo (2016) indicates that the based on the current trends of DM in Saudi Arabia (Figure 1.1.2), the prevalence will continue to ascend by as much >45% by the year 2030, with higher rates among females, adolescents and those living in urban areas (Alotaibi et al., 2017). It was reported that in 2014 alone, direct expenses related to DM in Saudi Arabia was ~14%

of the entire health expenditure budget, or 25 billion Saudi riyals out of 180 billion (Robert et al., 2017). Among the major conventional risk factors for T2DM identified particularly in the Saudi population include obesity, sedentary lifestyle, unhealthy nutrition, smoking and aging (Alneami and Coleman, 2016).

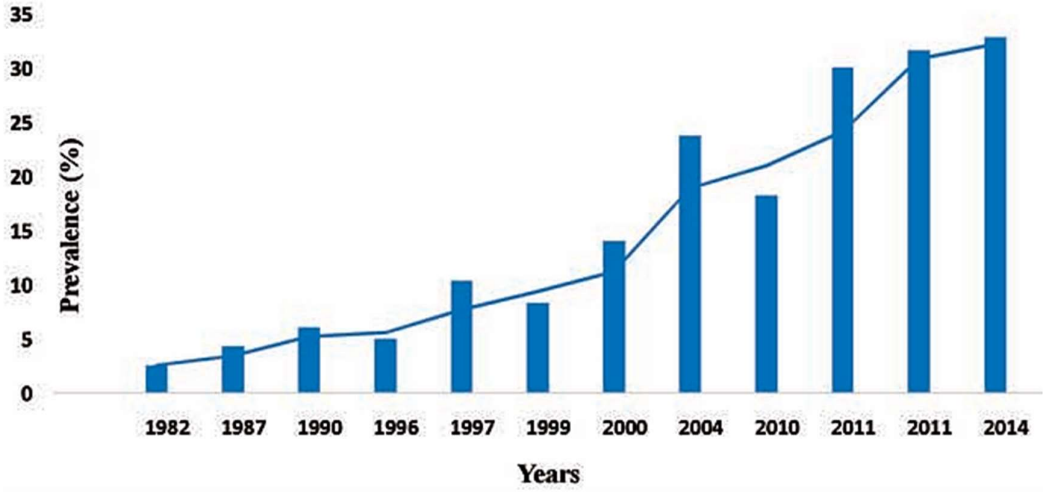


Figure 1.2.2. Ascending Prevalence of T2DM in Saudi Arabia (1982-2014) [Adopted from Meo, 2016].

Given the increasing incidences of DM, it is unfortunate that Saudi Arabia up to the present time still has limited interventional studies or clinical trials that would address the expanding T2DM epidemic in the populations. Amongst the limited prospective studies undertaken in Saudi Arabia a primary care study gave a 12 month dietary lifestyle program to improve management of DM patients in a primary care facility (Alfadda et al., 2011). This study showed no differences in glycaemic and HbA1c control and whilst management remained substandard, the intervention given was more efficacious in improving adherence (Alfadda et al., 2011). In another more recent study which was a non-randomised, single-blind trial, Badar and colleagues observed the lipid-lowering effects of one year *Nigella sativa* supplementation among Saudi T2DM subjects (Badar et al., 2017). Other local studies have reported modest improvements in cardiometabolic profiles with the use of moderate exercise (Abd El-

Kader et al., 2013), self-monitoring lifestyle modification (Al-Daghri et al., 2014), and better insulin sensitivity including glycaemic profile among those receiving vitamin D supplements (Al-Daghri et al., 2013; Al-Shahwan et al., 2015; Al-Sofiani et al., 2015; Al-Jabri et al., 2010). From these limited interventional studies, it is clear that more prospective studies are required to provide further insights for prevention and control of DM. Furthermore and given the current evidence in the literature, it also appears that the Saudi T2DM population is more inclined to participate in trials involving nutritional supplements as adjuvant management for T2DM (Alfadda et al., 2011; Badar et al., 2017; Abd El-Kader et al., 2013; Al-Daghri et al., 2014; Al-Daghri et al., 2013; Al-Shahwan et al., 2015; Al-Sofiani et al., 2015; Al-Jabri et al., 2010).

1.3 Syndrome X

The concept of "Syndrome X" was first developed in 1988 by Professor Gerald Reaven which later evolved into what is commonly known now as the "Metabolic Syndrome" (MetS), a condition from a cluster of several independent cardiovascular risk factors that include obesity, hypertension, dyslipidaemia and hyperglycaemia, which, as a single entity linked centrally to insulin resistance. This cluster of factors is considered to compound the risk of the individual in progressing to full blown cardiovascular/atherosclerotic disease and or DM (Reaven, 1988). In 2006, the global prevalence of MetS according to IDF was estimated to be a quarter of the world's human adult population (Kaur 2014). Currently, several MetS definitions still exist and diagnosis is highly dependent on the definition used, creating considerable confusion among epidemiologists and clinicians, not to mention the lack of standard definition to other populations at risk such as children and adolescents (Kassi et al., 2011). As such, MetS management and prevention are focused more on reducing the

individual risk factors through lifestyle interventions targeting weight reduction (Case et al., 2002), increased physical activity (Zhang et al., 2017) and dietary modification (Steckhan et al., 2016).

In the Middle East and the Gulf countries in particular, the prevalence of MetS as of 2010 was relatively higher by 10-15% compared to other developed nations and higher amongst Arab women (Mabry et al., 2010). In Saudi Arabia, the single largest country-wide survey was undertaken on 17,293 subjects aged 30-70 years old from 1995-2000 and determined that the prevalence of MetS was 39.3% (Al-Nozha et al., 2005). More recent evidence indicates a steady and modestly decreasing prevalence in Saudi adults, with the highest prevalence reported among the age group 50-55 years (Figure 1.2.1), but an increasing incidence among Saudi children (Al-Daghri et al., 2011).

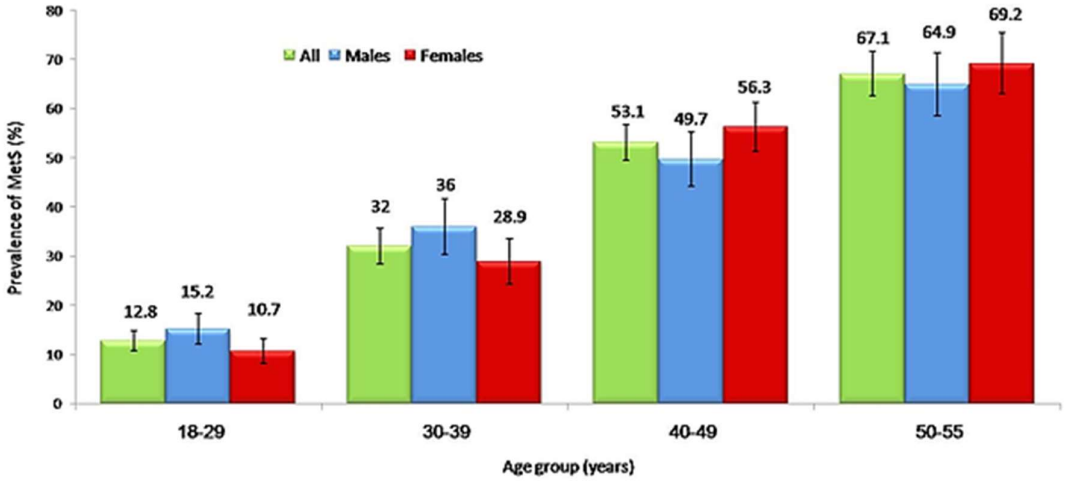


Figure 1.3.1. Increasing prevalence of MetS in Saudi adults according to age (Adopted from Al-Daghri et al., 2011).

The succeeding sub-section highlights the different MetS risk factors and their relevance in the Saudi Arabian context.

1.3.1 The Other Cardiometabolic Risk Factors of MetS

There is a worldwide consensus that despite varying definitions of MetS, they all agree that obesity (as discussed previously), dyslipidaemia, hypertension and elevated glucose are its core factors (Kassi et al., 2011).

1.3.1.1 Hypertension

Hypertension is defined as elevated systolic and/or diastolic blood pressure and is considered the leading preventable cause of premature death worldwide (Mills et al., 2016). The American Heart Association (AHA) defines hypertension as ≥140/90mmHg (Bertoia et al., 2012). As of 2010, the global prevalence of hypertension among adults was 31.1% (95% Confidence Interval 30.2-32.9%) (Mills et al., 2016). Uncontrolled hypertension greatly increases risk of target organ damage and as such, treatment has been focused on reducing cardiovascular and renal complications (Cushman, 2003). In Saudi Arabia, the most recent countrywide survey examining hypertension prevalence revealed that among 10,735 Saudis aged 15 and above, 15.2% and 40.6% of Saudis were hypertensive or borderline hypertensive, respectively with more than half of the hypertensive population unaware of their condition (El Bchearoui et al., 2014). It was also noted that being male, older, and diagnosed with diabetes were associated as increased risk factors elicited (El Bchearoui et al., 2014). The prevalence of hypertension is high even among Saudi women, with a recent meta-analysis of studies revealing a prevalence of 21.8% (Alshaikh et al., 2016). The prevalence is almost doubled in the presence of T2DM with almost half (45%) of Saudi patients co-currently presenting with hypertension as well (Al Slail et al., 2106).

1.3.1.2 Dyslipidaemia

Dyslipidaemia or abnormal lipid profile, is defined as elevated triglycerides and/or low levels of HDL-cholesterol (Musunuru, 2010). It has been associated with more than half of the global cases of ischemic heart disease (Smith 2007). In Saudi Arabia, low levels of HDL-cholesterol (<1.29mmol/l in females and <1.03mmol/l in males) is the most common cardiometabolic disorder amongst Saudis overtime, with a reported alarming prevalence of >85% in both children and adults (Al-Daghri et al., 2010; Al-Daghri et al., 2011) (Figure 1.2.1), affirming previous national survey on the prevalence of MetS in Saudi Arabia (Al-Nozha et al, 2005). Hypertriglyceridemia is the second most common MetS risk factor amongst Saudis with a prevalence of 33% in Saudi children and 34% in Saudi adults (Al-Daghri et al., 2010; Al-Daghri et al., 2011).

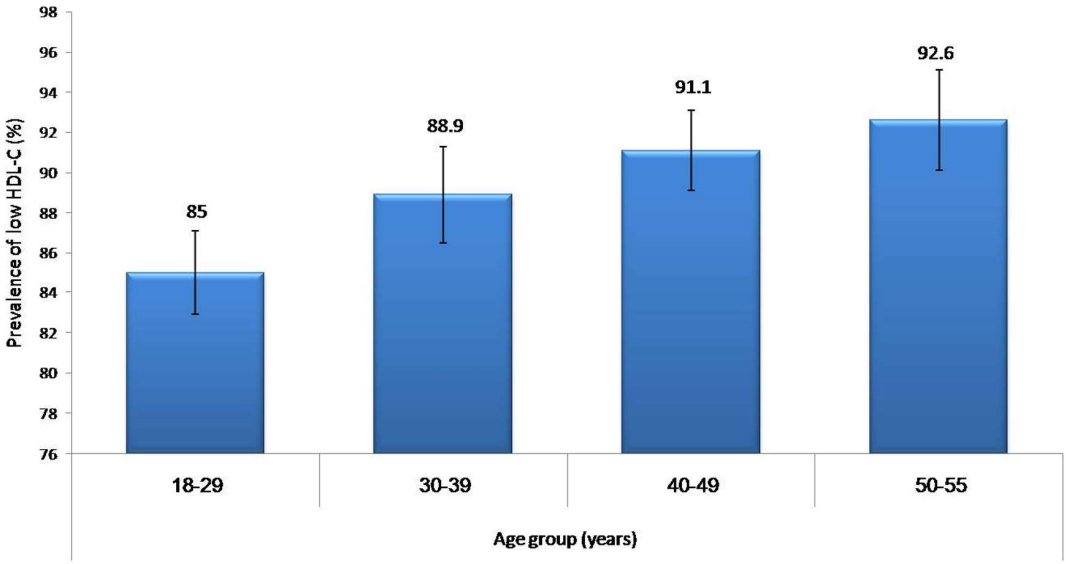


Figure 1.3.1.2 Prevalence of low-HDL cholesterol among Saudi adults according to age (Adopted from Al-Daghri et al., 2011)

1.4 Biomarkers of Metabolic Dysfunction

The adipose tissue was once known as a storage depot where accumulation of fat cells takes place. Physiologically, adipose tissue has been classified as white adipose tissue (WAT) and brown adipose tissue (BAT) (Saely et al., 2012; Reddy et al., 2014). The latter is highly vascularised with an abundance of mitochondria as opposed to the latter, hence its thermogenic function rather than storage. Metabolically active BAT has recently been shown in adults using magnetic resonance imaging-based method and was identified to be fairly static over long periods of time (Jones et al., 2017; Reddy et al., 2014). On the other hand, our understanding of the WAT has observed that fat cells (adipocytes) are not just for storage, but like BAT, also have both metabolic and endocrine functions, which, during weight gain, can alter their functionality and contribute to metabolic disorders (Jung and Choi, 2014; Baker et al., 2006). The adipose tissue as it is now known, produces a vast array of adipocyte-derived factors, known as adipocytokines (or adipokines) (Tilg and Moschen, 2006). Under normal physiological processes, adipocytokines play a significant role in energy homeostasis, triglyceride storage and the mobilization of fat (Leal and Mafra, 2013). However, when the volume of adipose tissue is enhanced, central abdominal fat in particular, it can initiate a cascade of other altered metabolic functions within fat leading to systemic metabolic consequences (McTernan et al., 2002; Harte et al., 2003; Valsamakis et al., 2004a; Lois et al., 2008; Freemantle et al., 2008; Genske et al., 2017). These major adipocytokines with adipose tissue include leptin, adiponectin, resistin complement components, plasminogen activator inhibitor-1, biomarkers of inflammation such as tumour necrosis factor (TNF-α), interleukin-6 (IL-6) and proteins of the rennin-angiotensin system (Kershaw and Flier, 2004; Harte et al., 2006). The endocrine functions of various adipocytokines to key metabolisms of the

human body has been hypothesised to connect obesity to most of the chronic non-communicable diseases since it mediates crosstalk between different cell groups not only within the adipose tissue but to other organs as well in maintaining energy homeostasis (Cao, 2014) (Figure 1.4.1.1). Hence, many studies have focused on the role of adipocytokines as major biomarkers of interest not only to monitor efficacy of nutritional interventions and obesity prevention/reduction programs but as therapeutic targets themselves in reversing obesity-induced, insulin resistance-related disorders (Valsamakis et al., 2004b; Borges et al., 2007; Quarta et al., 2016). Similar to insulin resistance and body fat distribution however, these biomarkers are affected by ethnicity and should be taken into consideration when conducting intervention studies (Mente et al., 2010; Sulistyoningrum et al, 2013).

Variations in adipocytokine expression have been demonstrated across ethnic groups (Parvaresh Rizi et al., 2015). In the Arab population, adipocytokines were demonstrated to be highly heritable, with parental adipocytokine patterns transmitted to offspring and manifesting as early as pre-teens (Al-Daghri et al., 2011b). Furthermore, adipocytokines exhibit differential expression according to sex (Al-Daghri et al., 2011c) and lifestyle modifications (Al-Daghri et al., 2015). This unusual combination of differing adipocytokine levels can be due to high degree of consanguineous marriages as well as the shared specific social and environmental exposures that led to aberrant heritability patterns that are yet to be demonstrated in other ethnic groups.

For the purpose of this thesis, the adipocytokines discussed in detail in the succeeding subsections were the parameters of interest measured in the trial studies.

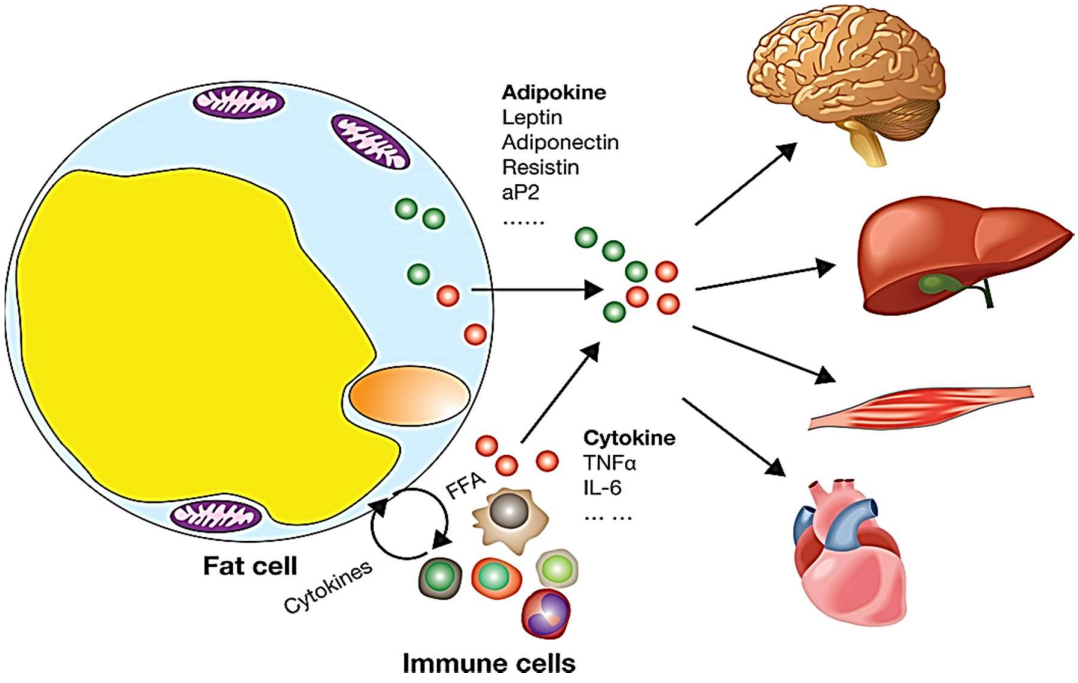


Figure 1.4.1.1. The metabolic adipose tissue and known adipocytokines (Adopted from Cao, 2014)

1.4.1 Leptin

Leptin was one of the first adipocytokines to be discovered in adipose tissue, it is a 167-amino acid protein with the first 21 amino acid residues cleaved as a peptide (John, 1998). It was first identified as the product of the *ob gene* in leptin-deficient obese (*ob/ob*) mice and was initially described as the adipocytokine associated with the regulation of appetite and energy homeostasis (John, 1998). The human leptin has 146 amino acid residues composed of four anti-parallel α-helices that are 5-6 turns long and is connected by cross-over links. Both crystal structure and nuclear magnetic resonance studies have revealed that leptin adopts a cytokine fold similar to that

exhibited by the short-helix subfamily of cytokine folds (Figure 1.3.1.2) (Zhang et al., 1997).

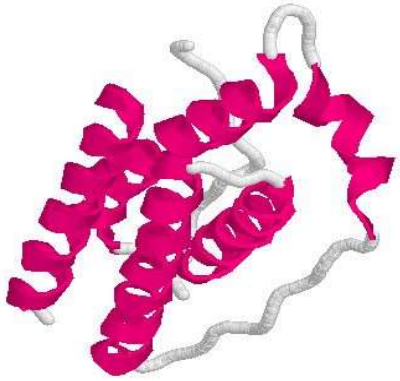


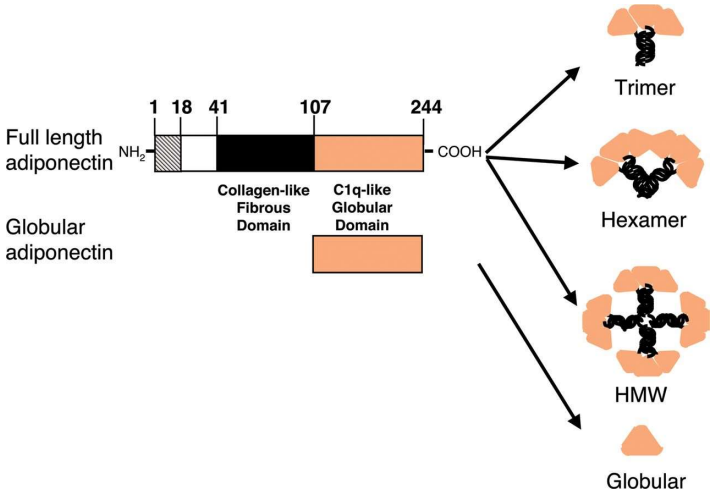
Figure 1.4.1.2 Crystal Structure of Leptin (Adopted from Zhang et al., 1997)

Elevated levels of circulating leptin is an integral feature of human obesity with total body fat mass being the best predictor of leptin levels, followed by % body fat and BMI as the least, among anthropometric measures (Sinha and Caro, 1998). Amongst humans, leptin has a highly conserved structure secretion within a 24-hour period. This circadian pattern is characterized by basal levels between 08:00 and 12:00 hours, ascending gradually to peak between 24:00 and 04:00 hours and constantly descending to its lowest point by 12:00 hours (Anubhuti and Arora, 2008).

Although the rate of leptin production is related to adiposity, a large portion of the inter-individual variability in plasma leptin concentration is independent of body fatness. It is leptin resistance and not leptin deficiency *per se* which is regarded as a pathogenic mechanism in human obesity (Al-Daghri et al., 2007). Among its essential functions, leptin acts via hypothalamic receptors that inhibit feeding and increase thermogenesis, resulting in weight loss (Jequier, 2002). Evidence also suggests that leptin has inhibitory role on insulin secretion, and levels above 20ng/ml help predict development of gestational diabetes mellitus (Maghbooli et al., 2007).

Evidence amongst the Saudi Arabian population have demonstrated the associations of leptin to MetS and coronary artery disease among Saudi patients (Al-Daghri et al., 2003), postmenopausal breast cancer among Saudi women (Assiri et al.,

2015) and obesity among non-diabetic Saudi men (Al-Sheikh, 2017) with higher leptin levels as compared to their T2DM counterparts (Anandaraj et al., 2017).



1.4.2 Adiponectin

Figure 1.4.2.1. Structure of Adiponectin (Adopted from Okamoto et al., 2006).

Adiponectin is a 30-kDa collagen-like protein, clinically noted to be anti-atherogenic and insulin sensitizing at higher levels (Al-Daghri et al., 2008). The protein forms the basic unit of a trimer, which self-associates to form hexamers then multimers of high molecular weight (HMW) (Figure 1.3.2.1) (Okamoto et al., 2006). HMW adiponectin seems to be the most active ones in relation to insulin sensitivity (Ferrarezi et al., 2007). AdipoR1 and AdipoR2 are the known receptors of adiponectin, with AdipoR1 being present in muscle tissues as high-affinity receptor for globular adiponectin and low affinity for full-length adiponectin, whereas AdipoR2 is abundantly noted in the liver and serves as intermediate-affinity receptors for both forms of adiponectin. The physiology of adiponectin in various glycaemic and lipid functions can be explained by the activation of AMP-activated protein kinase (AMPK) and stimulation of PPARα, which lead to elevated glucose uptake and oxidation of fatty acids in skeletal muscles and depressed hepatic glucose output (Adya et al., 2015). In skeletal muscle, adiponectin increases expression of molecules involved in fatty-acid transport such as CD36, in combustion of fatty acid such as acyl-coenzyme A oxidase, and in energy

dissipation such as uncoupling protein 2, leading to decreased triglyceride contents (Lai et al., 2015).

Adiponectin as an insulin-sensitizing hormone is reduced in the presence of insulin resistance and has thus been associated with diabetes and pre-diabetes risk (Mather et al., 2008; Jiang et al., 2016). As a biomarker, low-circulating levels of adiponectin has been a classic feature of endothelial dysfunction and insulin resistance (Al-Jiffri et al., 2016; Anandaraj et al., 2017). Owing to its inverse associations to various metabolic abnormalities including abdominal obesity, insulin resistance and dyslipidaemia, improvement in its levels owing to the simplest lifestyle and dietary modifications can therefore translate to reduction of risk.

In the Saudi population, adiponectin and other well-known biomarkers of obesity have been studied (Al-Daghri et al., 2013; Al-Daghri et al., 2015; Al-Attas et al., 2013; Alokail et al, 2013; Alokail et al., 2011; Al-Attas et al., 2010). Adiponectin, in particular, has been shown to be inversely associated with abdominal adiposity, insulin resistance and other anthropometric measures in adults (Al-Daghri et al., 2013); including vitamin D deficiency (Al-Daghri et al., 2015), cigarette smoking (Al-Attas et al., 2013), obesity-related malignancies such as breast cancer (Alokail et al, 2013), prostate cancer (Alokail et al., 2011) and premature biological aging (Al-Attas et al., 2010).

1.4.3 Resistin

Resistin is a cysteine-rich signalling molecule unique among the class of adipocytokines since it initially showed what appeared compelling evidence that directly linked obesity to diabetes, at least in animal models, hence the name resistin (resistance to insulin) (Steppan et al., 2001). Crystal structures of resistin and RELMbeta show an uncommon multimeric arrangement (Figure 1.3.3.1). with each protomer containing a carboxy-terminal disulfide-rich β-sandwich "head" domain and an amino-terminal α-helical "tail" sector (Patel et al, 2004).

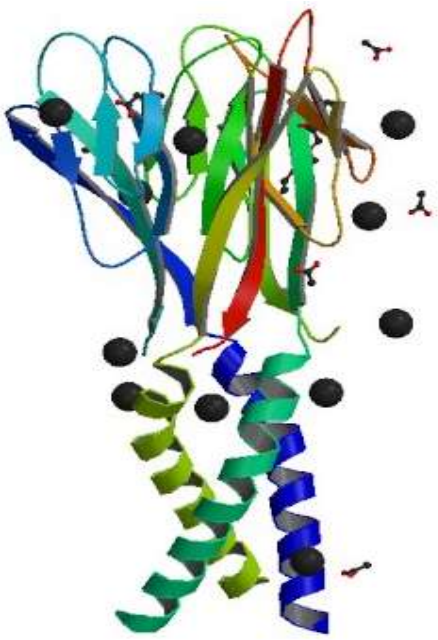


Figure 1.4.3.1. Crystal structure of resistin (Adopted from Patel et al., 2004)

Overtime, human studies highlighted that the function of resisitn appeared to have a more pro-inflammatory role via the integration of nuclear factor-kappaB (NFκB) and c-Jun NH2-terminal kinase (JNK) signaling pathways from human adipocytes (Kusminski et al., 2007). Resistin, together with the other pro-inflammatory adipocytokines, were shown to be modulated by nutrition as well as gut derived circulating gram negative bacterial fragments also known as endotoxin (Piya et al, 2013), with a modest effect in glycaemic metabolism but as dramatic as previously seen in animal studies (McTernan et al, 2003; McTernan et al., 2006; Kusminski et al., 2005).

Studies examining the adipocytokine resistin in the Saudi population has demonstrated higher levels of resistin among patients with T2DM (Habib 2005),

gestational diabetes mellitus (GDM) (Noureldeen et al., 2014), tuberculosis and *khat* addiction (a local shrub commonly chewed and acts as a stimulant) (Alvi et al., 2015) as well as obesity-related malignancies such as breast cancer (Assiri and Kamel 2016; Assiri et al., 2015). Furthermore, resistin gene (RETN) polymorphisms have shown differential expression among Saudi patients with colon cancer (Alharithy, 2014).

1.4.4. The Inflammatory Biomarkers: C - reactive protein, TNF-α and IL-6

Chronic, low-grade inflammation has been implicated in the pathogenesis of atherosclerosis and other chronic, non-communicable diseases (Geng et al., 2016). Tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) are classified as adipocytokines, and, together with the hepatocyte-derived acute phase reactant, C-reactive protein (CRP), are major players involved in local and systemic inflammation, respectively. The circulating biomarkers are consistently shown to be elevated in the presence of injury and infection, but systemic levels that are 2-3 times normal are classified as low-grade (Ross, 1999). Figure 1.3.3.4 shows a graphic representation of chronic, low-grade inflammation.

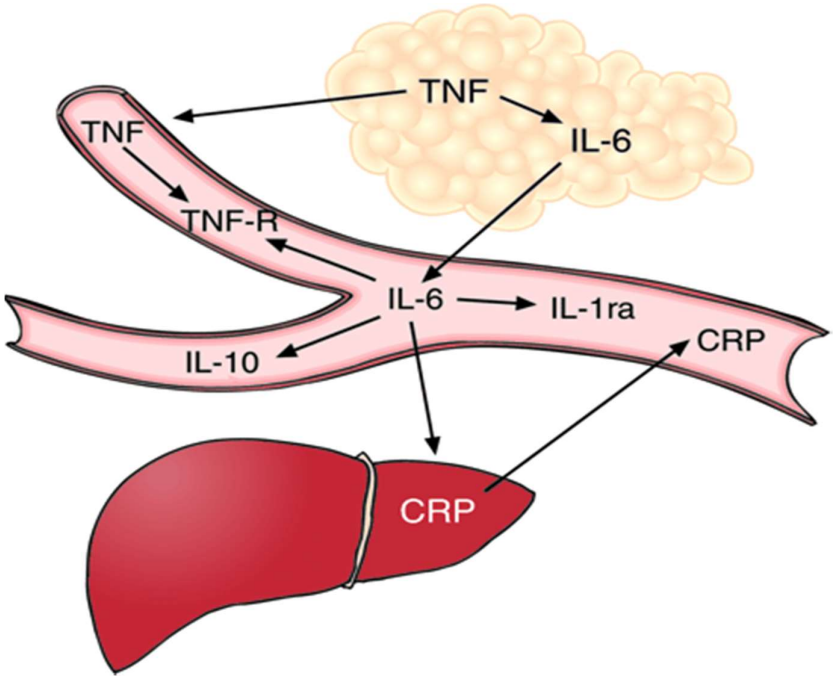


Figure 1.4.4.1. Chronic low-grade systemic inflammation. TNF-α is produced in the adipose tissue and stimulates the production of IL-6 in both adipose tissue and blood mononuclear cells. IL-6 enhances systemic levels of other inflammatory markers including CRP (Adopted from Petersen and Pedersen, 2005).

TNF-α has been shown to stimulate tumorigenesis by disruption of cell-epithelial adhesion and promotion of cell migration (Montesano et al, 2005; Alokail et al., 2014). IL-6, has also been shown to be elevated in most cancer types studied and are strongly associated with several phenotypic features of cancer (Culig, 2011; Alokail et al., 2014). CRP, as a first line of defence against pathogens, has been studied comprehensively since it is one of the biomarkers visible in atherosclerotic lesions and has consistently shown to be elevated in all other non-communicable diseases that involve chronic, low-grade inflammation (Danesh et al., 2004).

1.5 Gut Microbiota and Endotoxin

The study of the human gut microbiome has rapidly evolved through technological advancements and with it laid the foundations of the microbiome's influence in human health and disease not limited to the gastrointestinal system but to the an array of metabolic processes (Shreiner et al., 2015). As of 2016, it has been identified that the more realistic commensal bacteria to human host cell ratio is 1.3:1 (Sender et al., 2016), and while it did debunk the long standing accepted ratio of 10:1, it did not in any way minimize the significant importance of the gut microbiome in human physiology and metabolism. Among the multitude of bacteria residing in our bodies, majority belong only to two phyla: *Bacteroidetes* and *Firmicutes*, with low levels of the latter being more associated with a variety of metabolic disorders (Johnson et al., 2017). Furthermore, the largest and earliest source of microbial exposure in humans is the intestinal tract, which contains a large and diverse population of microbes. As a result, the intestinal tract is considered the most important postnatal source of microbial stimulation of the immune system (Rodriguez et al., 2015). The initial gut composition from exposure to maternal microbiome can significantly influence immune system development (Belkaid and Hand, 2014), including the transition from milk-based diet (whether from breast-feeding or infant formula) to solid foods (Tognini, 2017), as microbial colonization during infancy can set the stage for the microbiome in adulthood (Houghteling and Walker, 2015). Hence, disruption of this process in early life during a time of dynamic changes (Rodriguez et al., 2015; Belkaid and Hand, 2014) in the infant's gut might have long-term health effects. Some chronic metabolic disorders such as asthma and obesity often begin in early childhood, after the gut microbiota is established (Ly et al., 2011). Previous studies in both animal models and in humans have demonstrated relationships between

gut microbiota, atopic diseases (e.g., eczema, allergic rhinitis and asthma) and obesity (Sepp et al, 1997; Bjorksten et al, 1999; Bottcher et al, 2000; Murray et al, 2005; Penders et al, 2007; Adlerberth et al, 2007; Verhulst et al, 2008; Kalliomaki et al, 2001, 2008; Ley et al, 2005, 2006; Turnbaugh et al, 2009). Early-life factors (*e.g.*, diet, medications, hygiene, antioxidants and nutrients) associated with asthma, obesity, or both, might alter the gut milieu (Turnbaugh et al, 2009; Litonjua et al, 2008).

The metabolic state of obesity and weight gain has been observed to distort the microbial balance in the gut aside from modifying adipocyte functions (Figure 1.5.1) (John and Mullin, 2015). This alteration in the microbial balance undesirably impacts health by promoting low-grade chronic inflammatory states, the same feature found in T2DM and CVD (Chassaing and Gewirtz, 2014). The results of several recent studies suggest that low-grade systemic inflammation can result from the absorption of endotoxin across the intestinal tract (Creely et al, 2007; Brun et al, 2007; Al-Attas et al, 2009; Harte et al, 2010). The absorption of endotoxin is positively correlated with obesity (Ley et al, 2005; Triantafyllou et al, 2007; Cani et al, 2007b) and has been associated with a number of measurable clinical effects, which include T2DM (Creely et al, 2007; Cani et al, 2008), non-alcoholic steatohepatitis (Brun et al, 2007), cardiovascular disease (Miller et al, 2009) and multi-organ injury. Previous studies have shown that there is a 2-3 fold increase in circulating endotoxin in patients with insulin resistance and or T2DM compared with non-diabetic insulin sensitivity lean controls (Al-Attas, et al, 2009; Harte et al, 2010; Creely et al, 2007).

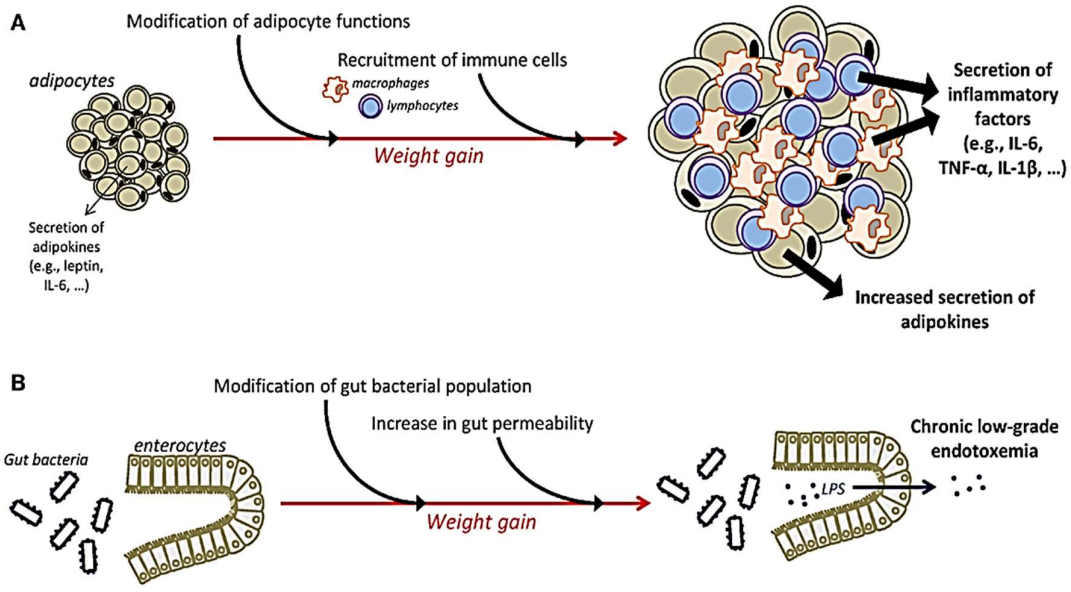


Figure 1.5.1: An overview of the potential impact of systemic endotoxin derived from the gut (Adopted from Castanon et al., 2014).

The intestinal mucosa provides a selectively permeable barrier between the circulation and intestinal lumen contents. Paracellular transport through the intact epithelial cell layer occurs through apical junctional complexes, which are composed of tight junctions (TJs) and adherens junctions. They regulate barrier permeability in response to a number of physiological and pathological stimuli, (Liu, et al., 2009) metabolic abnormalities and inflammation (Triantafyllou et al, 2007). TJs are also under cytokine control as well (Watson et al, 2005). The intestinal mucosa permits limited paracellular transport of bacterial lipopolysaccharide (LPS), another term for endotoxin (Watson et al, 2005), and TJ dysfunction increases intestinal permeability to these toxic luminal contents (Cani et al, 2008; Liu et al, 2009). Evidence from murine models suggests that obesity is associated with increased endotoxin absorption (Brun et al, 2007) and a number of mechanisms could explain the presence of endotoxemia in obese mice. First, diet may impair the intestine's barrier function through its effects on intestinal flora or motility; the intestines of mice fed a high-fat

diet are colonized by a greater proportion of LPS-containing bacteria [Cani et al, 2007]. The introduction of dietary fiber reduces the proportion of gram-negative bacteria in the gut lumen and hence reduces plasma endotoxemia (Cani et al, 2007). Second, the ecology of murine gut microbiota is altered by obesity (Ley et al, 2005), an effect potentially mediated by insulin resistance, because reduced intestinal motility and bacterial overgrowth are apparent in hyperglycemic and hyperinsulinemic states (Zietz et al, 2000; Cuoco et al, 2002; Triantafyllou et al, 2007). Hyperglycemia increases gut mucosal permeability in LPS-treated rats, independently of the plasma insulin concentration (Yajima et al, 2009). However, insulin can also act directly on the intestine to increase absorption (Westergaard, 1989). Finally, obesity is a disorder of chronic low-grade inflammation (Weisberg et al, 2003), and inflammation is implicated in impaired intestinal permeability. A previous study in patients with Crohn's disease showed increased absorption of polyethylene glycol 400 and lactulose (Katz et al, 1989) compared with healthy controls. Pro-inflammatory cytokines in obese patients may disrupt tight junctions, compromising the intestinal barrier to gut microbiota. TNF-α modifies permeability by targeting TJs and reduces the expression of p-glycoprotein MDR-1 (Belliard et al, 2004). TNF-α also alters the lipid composition and fatty acyl structure of phospholipids in microdomains at TJs (Li et al, 2008) and increases translocation of *Escherichia coli* through a monolayer of glutamine-starved epithelial cells *in vitro* (Clark et al, 2003). Treating Crohn's disease with the anti-TNF drug infliximab restores the intestinal barrier, although this may simply represent restoration of the normal mucosa (Suenaert et al, 2002).

Previous and recent evidence suggests a complex relationship between metabolic factors, inflammation, and intestinal permeability. However, the transport of luminal contents across the intestinal mucosa may initiate the innate pathway

through binding of toll-like receptors (TLRs) to bacterial antigens, such as LPS, a component of the gram-negative cell membrane (Nesto et al, 2004; Kaisho & Akira, 2002). Activation of TLRs results in transduction of nuclear factor κB (NFκB) to the nucleus and subsequent transcription of inflammatory mediators, such as interleukin (IL)-1, IL-6, and TNF-α (Muzio et al, 2004). LPS has been shown to upregulate TLR-2 expression and induce both IL-6 and TNF-α in human adipocytes (Lin et al, 2000; Creely et al, 2007; Song et al, 2006). Many studies have shown that impairments in gut barrier function result in plasma endotoxemia (Cani et al, 2007; Cani et al, 2008; Liu et al, 2009; Yajima et al, 2009). Circulating endotoxin may, in turn, aggravate intestinal barrier damage by promoting mucosal immunodeficiency (Liu et al, 2009). Murine studies have shown that continuous infusion of endotoxin increases gut permeability, as does high-fat dietary feeding (Cani et al, 2007; Brun et al, 2007). Although the underlying mechanism is poorly understood, LPS/endotoxin shows particular affinity to chylomicrons, the lipoproteins responsible for transporting fatty acids across the intestinal wall. This affinity has been implicated in the post-prandial inflammatory response (Ghoshal et al, 2009) and may account for translocation of LPS/endotoxin across the intestinal wall. Therefore, endotoxemia could at least partly explain the chronic low-grade inflammation associated with obesity. As such, there is intense interest in manipulation of the gut microbiota.

1.6 Probiotics

The definition of probiotics has been widely debated, but in 2001, the Food and Agriculture Organization (FAO) and the WHO defined them as "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host: (FAO/WHO, 2001). This universally accepted definition implies that a probiotic strain, unless protected by a capsule, should be intrinsically resistant to low pH, bile and pancreatic enzymes to ensure gastrointestinal transit in numbers adequate to elicit a defined benefit to the host. It was also recognized that the concept of probiotics can improve the host's health by modifying the composition of the intestinal microbiota. The recent advent of powerful molecular techniques has made it possible to monitor changes in the gut microbiota following probiotic administration, to better understand their functions. This has also helped researchers to identify that probiotics offer remarkable potential for the prevention and management of various infective and non-infectious disorders. Scientific evidence indicates that the ability of probiotic bacteria to confer their health effects largely depend on the strain being used (Tuohy et al, 2007). However, the host's own intestinal microbiota, which has a less diverse population of intestinal anaerobes in early life, appears to be associated with both atopic diseases and obesity (Forno et al, 2008; Turnbaugh et al, 2009).

Many different probiotics strains have been identified and the effects of these bacteria, either given in monoculture or as a cocktail of various strains, have been subject to increasing scientific evaluation in recent years. Probiotic bacteria, the most common of which are the lactose-fermenting *Lactobacilli*, inhibit the growth of pathogenic bacteria by acidifying the gut lumen, competing for nutrients, and producing antimicrobial substances (Gorbach et al, 2000; Liu et al, 2004).

Furthermore, they adhere to the gastrointestinal mucosa and are thought to prevent bacterial translocation from the gut (Chiva et al, 2000). The strongest evidence for the use of probiotics has been in the management of diarrheal diseases (Allen et al, 2010). Data extrapolated from a large number of studies, including systemic reviews (Allen et al, 2004; Checkley et al, 2008; Johnston et al, 2007) meta-analyses (Cremonini et al, 2002; Huang et al, 2002; Sazawal et al, 2006; Van Niel et al, 2002), open-label studies (Fang et al, 2009; Guandalini et al, 2000) and multicenter trials testing the efficacy of probiotics in preventing diarrhoea concluded that, in addition to having a good safety profile, probiotics significantly reduced the duration and frequency of acute diarrhea (Henker et al, 2007). In addition, trials have documented favourable effects of probiotics in other gastrointestinal diseases [*e.g.*, irritable bowel syndrome (IBS) and pouchitis]. A recent systematic review and meta-analysis identified 19 randomized clinical trials reporting the effect of probiotics on IBS symptoms (Moayyedi et al, 2010). From these 19 studies, 10 randomized clinical trials reported that the significant effects of probiotics were superior to placebo, and 15 out of 19 reported improved IBS scores in the probiotics group as an outcome. In the largest randomized clinical trial to date of probiotics in IBS using encapsulated doses of *Bifidobacterium infantis*, the authors reported a statistically significant benefit of *B. infantis* at a dose of 108 colony forming units (CFU) on abdominal pain, bloating, tenesmus and straining (Whorwell et al. 2006).

Studies of obesity have also shown altered gut microbial compositions in human subjects and in mice (Turnbaugh et al, 2009; Ley et al, 2005, 2006). The guts of obese human subjects had reduced numbers of *Bacteroidetes* and increased numbers of *Firmicutes* compared with lean people (Turnbaugh et al, 2009). In some obese humans, an increased proportion of fecal *Bacteroidetes* was found to parallel

weight loss on a hypocaloric diet during a 1-year intervention trial (Turnbaugh et al, 2009). Diet-induced obesity in animal models may also lead to increased *Mollicutes* (a class of *Firmicutes*), which appears to be reversible with dietary manipulation aimed at limiting weight gain (Ley et al, 2005). The finding that the microbial composition is reversible by dietary modification suggests that differences in the gut composition between obese and lean individuals are related to dietary factors independent of obesity (Hildebrandt et al, 2009; Cani et al, 2009). It should also be noted that not all of the studies have shown beneficial effects of probiotics, which means that caution should be taken in terms of the dosage and strains to be used, as these may have important ramifications on the effects observed.

Taken together, the current evidence supports a role for the gut microbiota in the pathogenesis of diet-induced obesity and related metabolic disorders, which might be reversible with dietary and/or gut microbiota manipulation (Ly et al, 2011). As the gut flora is the main source of endotoxin, treatment with probiotics may influence the circulating levels of endotoxin by altering the microbiota composition. To date, relatively few studies have examined the effects of endotoxin in metabolic diseases by using probiotics. To our knowledge, very few interventional studies have been performed other than in a small study of patients with cirrhosis in which a 25% reduction in endotoxin was reported (Backhed, et, al 2005). However, animal studies have revealed that treatment with probiotics may be beneficial in insulin-resistant states (Ley et al, 2005, 2006; Husebye et al, 2001). Studies by, Li and co-workers have reported that treatment with probiotics decreased liver inflammation in a mouse model of non-alcoholic fatty liver disease (Li, et al, 2003). More recently, it has been shown that probiotics can delay the onset of glucose intolerance in high-fructose-fed rats (O'Hara et al, 2006).

The limited interventional studies performed on probiotics supplementation thus far have several limitations, including the study design, small sample size and short duration of intervention (Table 1.5.1). Furthermore and to the best of our knowledge, no randomised clinical trial has been conducted using a multi-strain probiotic (8 bacterial strains) in the T2DM population. For the purpose of this thesis, a summary of preliminary findings performed involving one or several of the probiotics strains used in the present interventional studies are presented in Table 1.5.1.

Table 1.6.1 Probiotic Strains Used in the Thesis

Probiotic Strain	**Effects**	**References**
Bifidobacterium bifidum	Decrease in FPG, CRP & increase in total antioxidant capacity (12 weeks)	Badehnoosh et al., 2017
	Decreased insulin and HOMA-IR (12 weeks)	Soleimani et al., 2017
	Increase in quantitative insulin sensitivity index; decreased triglycerides and VLDL concentrations (6 weeks)	Ahmadi et al., 2016
	Increased adiponectin mRNA and decreased monocyte chemoattractant protein 1 and IL-6 (obese mice 5 weeks)	Le et al., 2014
	Decrease in total cholesterol and triglycerides, increase in HDL cholesterol (elderly T2DM for 30 days)	Moroti et al., 2012
	Reduces abdominal adoposity and increases antioxidant enzyme in combination with other probiotic strains in overweight women (RCT, N=43, 8 weeks)	Gomes et al., 2017

Bifidobacterium lactis	Increases intestinal barrier integrity by increasing TEER in Caco-2 cells	Mokkaka et al., 2016.
	Reduces visceral fat accumulation and improves glucose tolerance by increasing gut acetate levels	Aoki et al., 2017
	Reduces glycemia in combination with metformin in animal models	Stenman et al., 2015
	Reduces abdominal adoposity and increases antioxidant enzyme in combination with other probiotic strains in overweight women (RCT, N=43, 8 weeks)	Gomes et al., 2017
Lactobacillus acidophilus	Decrease in FPG, CRP & increase in total antioxidant capacity	Badehnoosh et al., 2017
	Decreased insulin and HOMA-IR	Soleimani et al., 2017
	Increase in quantitative insulin sensitivity index; decreased triglycerides and VLDL concentrations (6 weeks)	Ahmadi et al., 2016
	Decrease in total cholesterol and triglycerides, increase in HDL cholesterol (elderly T2DM for 30 days)	Moroti et al., 2012
	Reduces abdominal adoposity and increases antioxidant enzyme in combination with other probiotic strains in overweight women (RCT, N=43, 8 weeks)	Gomes et al., 2017

	Modulates LPS-induced inflammatory activity by regulating TLR4 and NFκB expression in weaned pigs	Lee et al., 2016
Lactobacillus brevis	Attenuates hyperglycemia in diabetes-induced mice in STZ rat model	Marques et al., 2016
	Inhibits lipopolysaccharide production by E.coli, NFκB activation and p16 expression in aged mice	Jeong et al., 2016
Lactobacillus casei	Decrease in FPG, CRP & increase in total antioxidant capacity	Badehnoosh et al., 2017
	Decreased insulin and HOMA-IR	Soleimani et al., 2017
	Increase in quantitative insulin sensitivity index; decreased triglycerides and VLDL concentrations (6 weeks)	Ahmadi et al., 2016
	Reduces abdominal adiposity and increases antioxidant enzyme in combination with other probiotic strains in overweight women (RCT, N=43, 8 weeks)	Gomes et al., 2017b
	Improves glucose intolerance, dyslipidemia, immunoregulation and oxidative stress in high-fat diet and STC-induced T2DM in mice	Chen et al., 2014
	Anti-obesity effects observed in obese rats	Karmimi et al., 2015

Lactobacillus salivarius	Corrects diabetes-induced enteric dysbiosis by inhibition of gut iNOS protein expression (Lin et al, 2017) and through induction of non-defensin proteins (Chung et al, 2015). No effect on glycemic control among pregnant women with abnormal glucose tolerance (RCT, N =149).	Lin et al., 2017 Chung et al., 2016 Lindsay et al., 2015
Lactococcus lactis W19 *Lactococcus lactis* W58	Reverses diabetes in NOD mice in combination with low-dose Anti-CD3 Prevents hyperglycemia and improves glucose tolerance by inhibition of antigen-specific proliferation of T cells in NOD mice Reduces abdominal adiposity and increases antioxidant enzyme in combination with other probiotic strains in overweight women (RCT, N=43, 8 weeks) Inhibits increases in blood glucose levels after ingesting sucrose in silkworms	Takiishi et al., 2017 Liu et al., 2016 Gomes et al., 2017 Matsumoto et al., 2016

1.7 Study Hypothesis and Aims of the Study

The current research hypothesis is that, gut-derived components also known as endotoxin or lipopolysaccharides, act as a potent initiator of systemic low grade inflammation, that may be modulated by consumption of multi-strain probiotics, to reverse the damaging effects of endotoxaemia amongst people with T2DM.

In order to test this hypothesis, a randomised, double-blind, placebo-controlled trial has been conducted over a 3 and 6 month duration to determine the beneficial effects of a multi-strain probiotics supplementation among adult, medication naïve and newly diagnosed T2DM Saudi patients. Specifically, the main objectives of this thesis were:

1. To investigate the effects of probiotics supplementation on circulating endotoxin levels of Saudi patients with T2DM (primary outcome).
2. To define the effects of probiotics supplementation on different cardiometabolic indices including anthropometry, glycaemic, lipid, inflammatory and adipocytokine profiles of Saudi patients with T2DM.

Chapter 2

Materials and Methods

2.1 Study Design

The main study design is a randomised, double-blind, placebo-controlled trial, considered the gold standard of interventional studies due to its ability to demonstrate causality, elimination of unknown biases secondary to randomisation and reduction of confounding effects from interventions due to blinding (Misra, 2012). Parts of the study protocol has been previously published (Alokail et al., 2013) and has been registered at the US National Library of Medicine (USNLM) at the National Institute of Health (NIH): ClinicalTrials.gov Identifier: NCT01765517 (Appendix I). Records of the protocol have also been deposited and submitted to the Saudi Food and Drug Administration (SFDA) in Riyadh, Saudi Arabia, as a requirement to obtain approval for the conduct of clinical trial (Approval number 8/25/126307; date: April 7, 2013) (Appendix II) and the use of imported probiotic and placebo sachets (Code: 2-1434-1-8188-90-2; Approved date: June 26, 2013) (Appendix III).

2.2 Ethical approval

The study protocol has been approved by the Ethics Committee of the College of Science (Approval number 8/25/16519) (Appendix IV), King Saud University, Riyadh, Saudi Arabia and the Ministry of Health in Riyadh, Saudi Arabia, for the recruitment of participants from different primary care centres (Approval number 8/25/136164) (Appendix V). The study conforms to the revised ethical standards for the conduct of human research studies under the declaration of Helsinki (Carlson et al, 2004).

2.3 Sample Size Calculation

Prior to the conduct of the trial, the hypothesis was that treatment with probiotics should reduce mean endotoxin levels by 25–30%, while no change seen in the placebo group. To obtain 80% power and demonstrate a statistically significant difference (two-sided p-value = 0.05) between the two treatments, 100 participants must be treated (50 patients per arm). Since dropouts were anticipated, recruitment was made sure to exceed N=100. The sample size was calculated based on the estimated mean change during treatment (Δ-values) and corresponding standard deviation (SD) of the change. On the assumption that the correlation between 1 (placebo) and 2 (probiotics) had a measurement of 0.70, the SD for the Δ-value was 78% of the SD of separate measurements, as demonstrated in the following table.

Table 2.3.1 SD for single measurement, corresponding correlations and Δ-value

SD for each measurement	Correlation between measures	SD of the Δ-value
1	0.60	0.89
1	0.65	0.84
1	0.70	0.78
1	0.75	0.71
1	0.80	0.63

The table below shows the estimated sample sizes according to various assumptions of treatment effect and correlation between 1 and 2 measurements.

Table 2.3.2 Estimated Sample Sizes

Endotoxin baseline (U/L)	Difference in Δ-value (probiotics – placebo)	SD for change (common for both treatments)	Correlation for 1. and 2. measurement	Sample size (total) 2-tailed α: 0.05 Power: 80%	Sample size (total) 2-tailed α: 0.05 Power: 90%
10 (± 6)	–2	5.34	0.60	224	298
	–2	5.04	0.65	200	266
	–2	4.68	0.70	176	236
	–2	4.26	0.75	148	198
	–2	3.78	0.80	116	154
	–2.5	5.34	0.60	144	192
	–2.5	5.04	0.65	128	172
	–2.5	4.68	0.70	114	152
	–2.5	**4.26**	**0.75**	**96**	**128**
	–2.5	3.78	0.80	76	100

2.4 Participant Recruitment

A total of 150 adult Saudi male and female T2DM participants (medication naïve and without co-morbidities, aged 30-60 years) were initially recruited, 96 of whom were randomized, 78 completed 3 months and 61 completed the entire clinical trial (Figure 2.1.1). Interventions were performed at weeks 0, 12 and 26 in all participants who completed the trial. Participant enrolment was initially undertaken with collaborating primary care centres and the Out-Patient Department of King Salman Hospital in Riyadh, Saudi Arabia in Riyadh. Due to the low turnout of participants from primary care centres and their reluctance to participate compared with subjects attending King Salman Hospital, the study recruitment was performed

only on King Salman Hospital, a tertiary hospital, and as such the present trial was considered as a single-centre study.

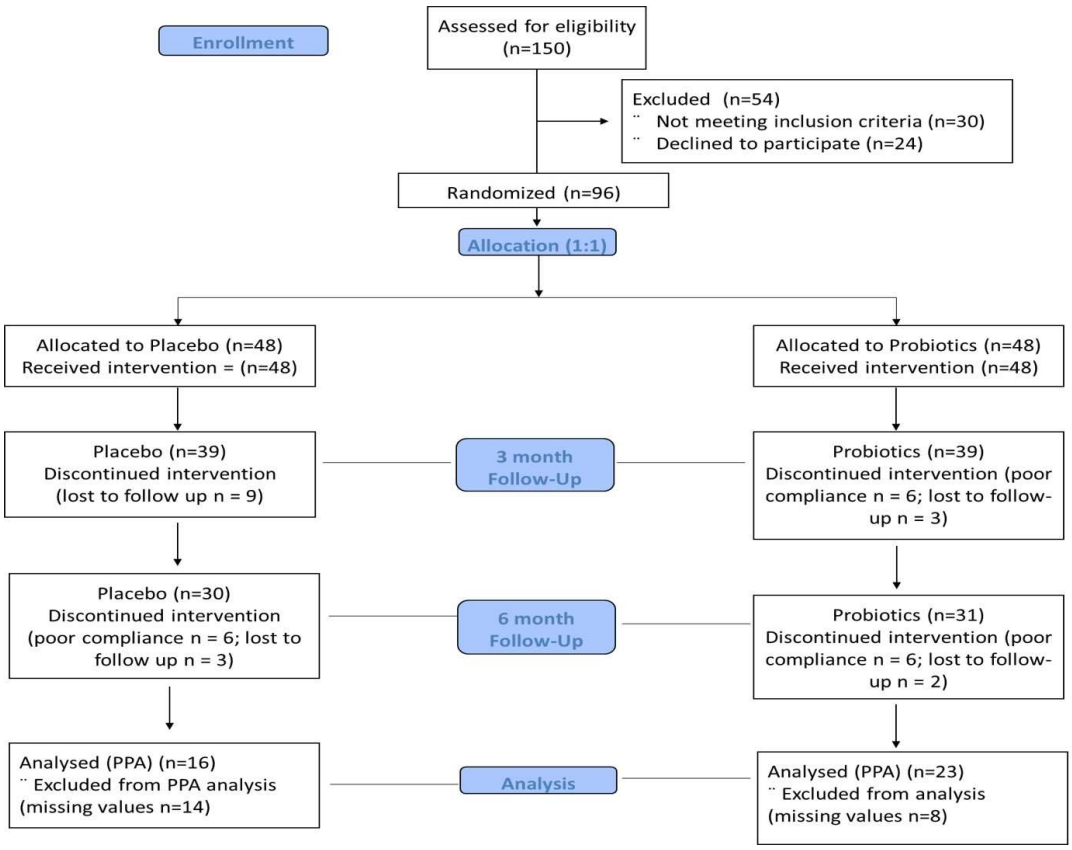


Figure 2.4.1. CONSORT Flowchart detailing number of participants at enrolment, allocation and treatment for the entire duration of the clinical trial.

2.4.1 Inclusion and Exclusion Criteria

Participants were initially recruited and screened at various primary care centres but since most of those attending these centres were determined to be long-term DM patients, the recruitment refocused to King Salman University Hospital in Riyadh, Saudi Arabia. Saudi adult males and females aged 30-60 years with stable and well controlled (HbA1c <7.5%), newly diagnosed T2DM (less than 6 months post-diagnosis), naïve to treatment (uninitiated in any oral hypoglycemic agents, including insulin), were invited to participate. They were oriented about the protocol and those who showed interest were asked for written informed consent prior to enrolment. Participants who were non-Saudis, with long standing gastrointestinal disease, intake of systemic antibiotics within six weeks before inclusion or use of probiotics supplements within three months before inclusion, regular intake of insulin or insulin analogues, antacids, H2-receptor blockers, proton pump inhibitors, loperamide, cholestryramine, ω3-unsaturated fatty acid supplements, fibrates, corticosteroids or sex steroids were excluded. In addition, those who were mentally incapable to give consent were excluded. Other criteria for exclusion included those who were actively participating in another clinical trial or participated in another intervention study within the last 6 months, lactating or pregnant and/or with known cardiovascular disease.

2.5 Allocation to Treatment

After confirmation of eligibility and obtaining written informed consent, participants were given a unique subject number by the research nurse. The randomization scheme was computer generated by Winclove using permuted blocks with block size equal to 4, whilst both the patients and clinicians at the primary care

centre and King Salman Hospital were blinded to the treatment received. Eligible participants were allocated (1:1) to treatment for 26 weeks with either the probiotics supplement or placebo. Participants allocated to the probiotics group received two sachets with two grams of freeze-dried powder of the probiotic mixture Ecologic®Barrier daily (Winclove, Amsterdam, Netherlands). Ecologic®Barrier ($2.5x10^9$ cfu/gram) contains the following bacterial strains: *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58. Participants in the placebo group received sachets consisting of the carrier of the probiotic product (maize starch and maltodextrins). Placebo content contains no probiotic bacteria, but is indistinguishable in colour, smell and taste from the probiotic sachets. A sample sachet used in the study and the actual sachets used commercially are shown in figure 2.5.1.

A B

Figure 2.5.1 Actual sachets used in the intervention study (A) versus commercial sachets (B)

Participants were instructed to dissolve all sachet contents in lukewarm water and consume it completely. This was performed twice: one sachet before breakfast and one sachet before bedtime. Store unused sachets at room temperature. Return unused sachets at designated appointment times to monitor compliance and to be given fresh refill. Unblinding was performed after the last participant submitted unused sachets and blood samples. The company responsible for the blinding and randomization (Winclove) was informed and the excel sheet containing allocation was then provided electronically.

Figure 2.5.2 shows the actual box with code and label provided for each participant containing sachets for the intervention study.

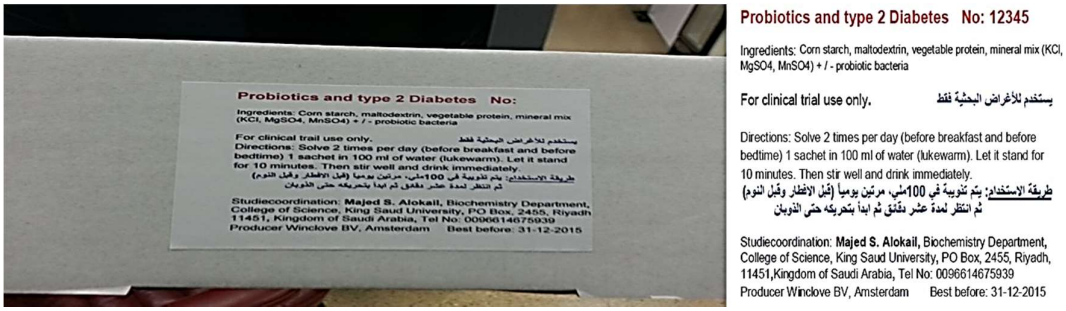


Figure 2.5.2 Refill box with code and instructions.

2.6 Data Handling and Record Keeping

Case report forms (CRF) were used to record data for all participants and completed by the research field nurse and data given to the research team to enter the data into an electronic database.

2.7 Study Schedule and Location

Following participants' induction into the study all further treatments were managed at the out-patient clinic in King Salman Hospital, Riyadh, Saudi Arabia

where all participants were initially screened and recruited (see Table 2.7.1 for scheduled visits). The assigned research nurse and research assistant were responsible for all contacts with patients and reported accordingly to the research team. The research team and the collaborating physician were available throughout the entire intervention period to ensure that participant concerns were addressed and medical queries were noted and attended to.

2.8 Acquisition of Clinical Data and Assessment of Compliance

Medical history including the presence of chronic diseases, medications regularly taken and other habits (*i.e.*, smoking) were recorded before inclusion. Changes in existing medications during the intervention stage of the study were also noted. All anthropometric and clinical measurements were performed at baseline and at weeks 12 and 26, with participants notified a week before the intended appointment. Compliance was monitored accordingly from unused sachets submitted by the participants during visits. Participants were free to refuse further participation during the intervention period.

Table 2.7.1 Participant Schedule of Visits

	Pre-Screening	**Screening**	**Inclusion**	**Weeks**		
				0	12	26
Participant visits out-patient clinic		●		●	●	●
Phone review	●					
Eligibility check	●	●				
Obtain informed consent			●			
Blood sampling				●	●	●
Provision of test product				●	●	
Return of unused product					●	●
Monitor compliance & adverse events					●	●

2.9 Acquisition of Anthropometric Measurements

Anthropometric measurements were performed using standardized methods. Height (cm) was measured using a stadiometre. Weight (kg) was taken with the participant in light clothing without shoes or items in pockets. Waist (cm) was measured as a horizontal plane at the level of the umbilicus and hip circumference (cm) was measured as a horizontal plane at the level of the trochanter major using a regular tape measure. Systolic and diastolic blood pressure (mmHg) were measured twice using an aneroid mercurial sphygmomanometer and the mean blood pressure calculated and noted accordingly. Body mass index (BMI) was calculated as the

quotient between weight (kg) and height in squared metres. Waist-to-hip (WHR) ratio was calculated as waist divided by hip circumference. Finally, mean arterial pressure (MAP) was calculated using the formula:

[(Diastolic Blood Pressure x 2 + Systolic Blood Pressure)]/3. All anthropometric and clinical measurements were repeated at weeks 0, 12, and 26.

2.10 Acquisition of Routine Biochemical Data and Biological Samples

2.10.1 Blood and Sample Collection

All eligible participants were requested to fast for 8-10 hours the night before scheduled appointment for collection of fasting blood samples and anthropometric measurements. Fasting blood samples were collected at baseline (week 0), week 12 and 26. Peripheral venous blood drawn were collected into pyrogen-free tubes without any anticoagulant. The tubes were allowed to coagulate, immediately positioned in ice and centrifuged (2500 ×*g* for 10 min at 4°C) within two hours of extraction for instant delivery to the Biomarkers Research Program, Biochemistry Department, College of Science, King Saud University, Riyadh, Saudi Arabia. Delivered serum samples were divided into 2 (1ml) aliquots and immediately stored at –20°C until use. At least 2ml serum samples were collected at each time-point.

2.10.2 Clinical and Biochemical Measurements

The materials used in the study is listed in Table 2.10.2.1 for the analysis of circulating endotoxin, glycaemic (glucose, insulin and C-peptide), lipid (triglycerides, HDL- and total cholesterol), adipocytokine (leptin, adiponectin and resistin) and inflammatory (TNF-α, C-reactive protein and IL-6) profiles of participants. Fasting serum glucose, triglycerides, total cholesterol and HDL-cholesterol were measured using a chemical analyser (Konelab, Espoo, Finland). Circulating insulin and c-peptide were measured using electrochemiluminiscence (ECL) assay by Roche ELECSYS® and Cobas e411 (Roche Diagnostics, Basel, Switzerland). Serum leptin, TNF-α and IL6 (human bone magnetic bead panel) as well as adiponectin and resistin (human adipokine magnetic bead panel) were measured using Milliplex Map® (Millipore, Billerica, MA, USA) multiple assays by Luminex® xMAP® (Luminex Corp, Austin, TX, USA). Finally, CRP was measured using enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, USA).

Table 2.10.2.1 Materials Used in the Study

MATERIALS	SUPPLIER
Chemicals and solutions	
70% ethanol	Fisher Chemical, VA, USA
Sheath fluid	Luminex Corp, TX, USA
SysWash	Roche, Basel, Switzerland
ISE cleaning solution	Roche, Basel, Switzerland
Kits	
Insulin	Roche, Basel, Switzerland
C-peptide	Roche, Basel, Switzerland
Human Adipokine Magnetic bead panel	EMD Millipore Corp, Germany
Human Bone Magnetic bead panel	EMD Millipore Corp, Germany
ELISA Human C-reactive protiein	R&D System, USA
Total Cholesterol	Thermo Scientific, VA, USA
Glucose	Thermo Scientific, VA, USA
Triglycerides	Thermo Scientific, VA, USA
HDL-Cholesterol	Thermo Scientific, VA, USA
Endotoxin	Lonza, MD, USA
Equipment	
Cobas e 411 analyzer	Roche, Basel, Switzerland
Microplate reader	Molecular devices, CA, USA
FlexMap 3D multiplex platform	Luminex Corp, TX, USA
Power Sonic405	Hwashin Tech, Korea
DCA Vantage	Siemens, USA
Vortex mixer	Velp Scientifica, Velate, Italy
Multichannel pipette	Eppendorf, Hamburg, Germany
Timer	Thermo Scientific, VA, USA

2.10.3 Sample Analysis Principles and Detailed Procedures

2.10.3.1 Insulin and C-peptide

The sandwich principle was applied to higher molecular weight analytes such as insulin and c-peptide. The total time period of the assay was 18 minutes. In the first incubation, insulin from 20μL sample, a biotinylated monoclonal insulin-specific antibody, and a monoclonal insulin-specific antibody labelled with a ruthenium complex formed a sandwich complex. In the second incubation, streptavidin-coated micro particles were added and the complex was bound to the solid phase via

interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the micro particles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell and provided tripropylamine (TPA), an essential compound for the ECL-reaction. Application of voltage to the electrode then induced chemiluminescent emission which was measured by a photomultiplier. Results were determined via a calibration curve which was provided via the reagent barcode. The lower detection limit for insulin was 0.2IU/ml with an intermediate precision of 2.5%-2.8% and a repeatability of 1.9%-2.0% specific for e cobas 411 analyzer. The lower detection limit for c-peptide was 0.01 ng/ml with an intermediate precision of 0.6%-1.5% and a repeatability of 1.6%-2.3%.

2.10.3.1.1 Reagents and Sample Preparation for Insulin and C-Peptide

Waste water was first replaced by a new one (1 litre deionized water with 10mL SysWash) followed by Cobas-e machine cleaning with the use of ISE cleaning solution placed in ProCell and finally replacement of all instrument's reagents (calibrators, and controls, ProCell and ControlCell). For the calibration step, calibrator powder bottles were left at room temperature for 15 minutes, dissolved (calibrators 1 and 2) in 1000 µl of deionized water and allowed to stand for 30 minutes to reconstitute. Mixing was performed carefully to avoid foam formation. Around 100 µl of the reconstituted calibrator was then transferred in the empty labelled cap. The system automatically regulated the temperature of the reagents and the opening/closing of the bottles. For the quality control step, control powder bottles were left at room temperature for 15 minutes, carefully dissolved (controls 1 and 2) in 3000 µl deionized water and allowed to stand for 30 minutes to reconstitute. Mixing was also performed carefully to avoid foam formation. Around 100 µl of the

reconstituted control was then transferred in the empty labelled cap and processed in the machine**.** Controls were run in parallel with participant samples, one per reagent kit, whenever a calibration was performed. The control interval and limits was adapted to the laboratory requirements. For the sample tests, serum samples were thawed to approximately 20°C and placed on the reagent disk (20°C) of the analyser. Samples were vortexed and 100 µl of each samples in the test cups was added, avoiding foam formation as much as possible. Cup positions and samples' serial number were entered manually in the system.

2.10.3.2 Glucose

Glucose oxidase catalysed the oxidation of glucose to gluconate. The formed hydrogen peroxide (H_2O_2) was detected by a chromogenic oxygen acceptor, phenol, 4-aminophenazone (4-AP) in the presence of peroxidase. The resulting colour is then automatically quantified spectrophotometrically at 505nm (Konelab, Espoo, Finland). The required reagents (Thermo Fisher Scientific Inc., Middletown, VA, USA) were ready for use. Presence of bubbles were avoided in the bottleneck or on the surface of the reagent whenever the reagent vials or vessels in the analyser were inserted. Reagents in unopened vials were stable at 2-8 °C until the expiration date printed on the label and were kept away from sunlight. The samples were processed using collection tubes, in accordance with the manufacturer's instructions to avoid erroneous results. Special attention was given to the pre-analytical variables such as mixing, standing time before centrifugation and centrifuge settings. Sample types such as unhemolysed serum, heparin or EDTA plasma can still be used in the Konelab analyser (Konelab, Espoo, Finland). All samples were taken from participants on a fasted state. All samples were separated from the cells as soon as possible after

collection in order to avoid glycolysis. If the sample was not separated or analysed without delay, a glycolytic inhibitor was used.

2.10.3.2.1 HOMA-IR

Homeostasis model assessment (HOMA-IR) was calculated as the product of insulin (IU/ml) and glucose (mmol/l) divided by 22.5 (Matthews et al., 1985).

2.10.3.3 Total Cholesterol

The approach used was the CHOD/POD method. Cholesterol esters were enzymatically hydrolysed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol was then oxidized to cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide combines with HBA and 4-aminoantipyrine to form a chromophore (quinonemine dye) which was quantified spectrophotometrically at 500-550nm. Results were automatically calculated by the instrument (Konelab, Espoo, Finland). The required reagents for total cholesterol were ready for use (Thermo Fisher Scientific Inc., Middletown, VA, USA) and the sample preparation was similar to glucose assessment (refer to section 2.8.2).

2.10.3.4 Triglycerides

Triglycerides were hydrolysed by lipase to glycerol and fatty acids. The glycerol was phosphorylated to glycerol-3-phosphate, which was then oxidised to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide reacted with 4-aminoantipyrine and 4-chlorophenol forming a quinoneimine dye. The absorbance of the formed colour was then automatically measured spectrophotometrically at 510nm (Konelab, Espoo, Finland). The required reagents

for triglycerides were ready for use (Thermo Fisher Scientific Inc., Middletown, VA, USA) and the sample preparation was similar to glucose assessment (refer to section 2.8.2).

2.10.3.5 High Density Lipoproteins (HDL)

Measurement of HDL-cholesterol was performed as a homogenous enzymatic colorimetric test which was in the presence of magnesium sulfate and dextran sulfate which selectively forms water-soluble complexes with low density lipoprotein (LDL), very low density lipoprotein (VLD) and chylomicrons, compounds resistant to polyethylene glycol-modified (PEG) enzymes. The cholesterol concentration of HDL was determined enzymatically by cholesterol oxidase coupled with PEG to the amino acid groups. The results were automatically calculated by the instrument (Konelab, Espoo, Finland). Reagents A and B were ready for use. The pink intrinsic colour of the reagent did not interfere with the test. Both serum and heparin plasma can be used. EDTA plasma can cause lower than actual values. Samples that contained precipitates were centrifuged before the assay was performed.

2.10.3.6 Low Density Lipoproteins (LDL)

LDL-cholesterol levels were not measured similar to the other lipids. In this study, the widely used Friedewald formula was chosen to calculate LDL-cholesterol levels (Whelton et al., 2017):

LDL = Total cholesterol – HDL-cholesterol – [Triglycerides/2.17mmol/l]

2.10.3.7 Human C-reactive protein (CRP)

This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for CRP has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any CRP present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for CRP was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour develops in proportion to the amount of CRP bound in the initial step. The colour development was halted and the intensity of the colour was measured. The intra-assay precision as mentioned in the procedure was 4.4%-8.3% whilst the inter-assay precision was 6.0%-7.0%. Materials were provided and storage conditions were kept at 2-8°C.

2.10.3.7.1 Preparation of CRP Reagents

A serum separator tube (SST), a polypropylene tube, was used to contain samples. The samples were allowed to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Serum was removed and assayed immediately or stored at ≤ -20 °C. Serum samples required a 100-fold dilution and as such 10 µL of sample + 990 µL of calibrator diluent RD5P (1X) was performed. The wash buffer bottle was warmed to room temperature and mixed gently until the crystals have been completely dissolved. Wash buffer concentrate (20 ml) was diluted into deionized or distilled water and 500 mL of wash buffer was prepared. The *calibrator diluent RD5P (1X)* **(**20 mL) was diluted into 80 mL of deionized or distilled water 100 mL of calibrator diluent RD5P (1X) was prepared. Colour reagents A and B for the substrate solution

were mixed together in equal volumes within 15 minutes of use and was protected from light. Around 200 µL of the resultant mixture was required per well. For the CRP standard, *polypropylene tubes were used*. Around 200 µL of the calibrator diluent RD5P (1X) was pipetted into each of the six tubes. Around 200 µL of the standard was added to the 25 ng/mL tube for the 2-fold dilution series. Each tube was mixed thoroughly before the next transfer. The 50 ng/mL standard served as the high standard. The calibrator diluent RD5P (1X) served as the zero standard (0 ng/mL). Stop solution (6ml of 2N H_2SO_4) was prepared with caution by wearing protective gloves, clothing and facial protection. The solution (0.337 ml of H_2SO_4) was slowly added to 105 ml deionized water and the final volume was adjusted to 6 ml with deionized water.

All reagents and samples were brought to room temperature before use. All standards, samples, and controls were assayed in duplicate. Detailed steps as provided by the manufacturer was followed.

2.10.3.8 Leptin, IL-6 and TNF-α

Leptin, interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) were measured simultaneously using the human bone magnetic bead panel assay [*bone metabolism multiplex assay analytes included*: ACTH, DKK-1, FGF-23, IL-1β, **IL-6**, insulin, **leptin**, osteocalcin, OPN - osteopontin, osteoprotegerin, PTH, SOST, **TNF-α**] (Milliplex Map ®) based on the Luminex ® xMAP ® technology. Serum samples were diluted 1:2 in the assay buffer provided in the kit.

2.10.3.8.1 Preparation of Leptin, IL-6 and TNF-α Reagents

For individual vials of beads [Anti-leptin beads (bead region 39); anti-IL6 beads (bead region 34 and anti-TNFα beads (bead region 55)], each antibody-bead

vial was sonicated for 30 seconds; then vortexed for 1 minute. 150µL from each antibody-bead vial added to the mixing bottle and final volume brought to 3.0mL with bead diluent. The mixed beads were vortexed well. Quality controls 1 and 2 were reconstituted with 250µL deionized water, vortexed and allowed to sit for 5-10 minutes. Wash buffer was prepared using 60 mL of 10X wash buffer (two bottles) diluted with 540mL deionized water. For serum matrix, 1.0 mL of deionized water and 1.0mL of assay buffer was added to the bottle containing lyophilized serum matrix, mixed well and allowed to rest for at least 10 minutes for complete reconstitution. For the standards, the human bone standard vial was reconstituted with 250 µL deionized water, vortexed for 10 seconds, allowed to sit for 5-10 minutes and labelled as standard 7. Six polypropylene microfuge tubes were then labelled as standards 1-6. Assay buffer (150 µL) was added to each of the six tubes and serial dilutions (1:4) were prepared by adding 50 µL of the reconstituted standard 7 to standard 6 tube onwards until standard 1.

2.10.3.8.2 Immunoassay Procedure for Leptin, IL-6 and TNF-α

All reagents were allowed to warm to room temperature (20-25ºC) before use in the assay. The placement of 8 standards [0 (Background), 1-7] controls 1 and 2, and samples were diagrammed on Well Map Worksheet in a vertical configuration. The assay was run in duplicate and the 96-well plate was prepared and arranged according to manufacturer's instructions. The plate was run on Luminex 200TM, HTS, FlexMAP 3DTM or MAGPIX® with xPONENT software. The intra-assay variation was 1.4%-7.9% and inter-assay variation of <21%. Minimum detectable concentrations (MDC) were as follows: leptin, 85.4 pg/ml, IL-6, 0.4 pg/ml and TNFα, 0.14 pg/ml.

2.10.3.9 Adiponectin and Resistin

Serum adiponectin and resistin were measured simultaneously using the human adipokine magnetic bead panel assay [*endocrine multiplex assay analytes included*: **adiponectin**, adipsin, lipocalin-2/NGAL, PAI-1 (total), **resistin**] (Milliplex Map®) based on the Luminex® xMAP® technology. Serum samples were diluted 1:400 in the assay buffer provided in the kit. The rest of the immunoassay procedure was similar to the human bone magnetic bead panel assay (refer to section 2.10.3.8.2**).** The intra-assay variation was 1.4%-7.9% and inter-assay variation of <21%. Minimum detectable concentrations (MDC) for adiponectin was adiponectin was 145.4 pg/ml and 6.7 pg/ml for resistin.

2.10.3.9.1 Preparation of Adiponectin and Resistin Reagents

For individual vials of beads, each antibody-bead vial [anti-human adiponectin (bead region 51) and anti-human resistin (bead region 64) was sonicated for 30 seconds then vortexed for 1 minute. The rest of the preparation reagents and immunoassay procedure were similar to the human bone magnetic bead panel (see sections 2.10.8.1 and 2.10.8.2).

2.10.3.10 Endotoxin

In this study, three different endotoxin quantifying kits were used to measure circulating endotoxin level in participants' sera. At the beginning, Limulus Amoebocyte Lysate (LAL) QCL-1000® kit was used, followed by PyroGene™ Recombinant Factor C Endotoxin Detection Assay, and finally the last kit, the Limulus Amoebocyte Lysate (LAL) Kinetic-QCL™. The last kit gave good results in terms of spike recovery.

2.10.3.10.1 Principle behind Endotoxin Quantification

Gram-negative bacterial endotoxin catalyses the activation of a proenzyme in the LAL. The initial rate of activation was determined by the concentration of endotoxin present. The activated enzyme catalyzes the release of pNA from the colourless substrate Ac-lle-Glu-Ala-Arg-pNA. The free pNA was measured photometrically at 405–410 nm after the reaction was stopped using the stop reagent. The correlation between the absorbance and the endotoxin concentration was linear in the 0.1–1.0 EU/ml range. The concentration of endotoxin in a sample was calculated from the absorbance values of solutions containing known amounts of endotoxin standard.

2.10.3.10.2 Reagents Supplied for Endotoxin

The kinetic-QCL reagent vial contained a lyophilized mixture of lysate prepared from the circulating amebocytes of the horseshoe crab, *Limulus polyphemus* and chromogenic substrate. This reagent was reconstituted immediately with 2.6 ml of LAL reagent water per vial and swirled gently to avoid foaming. It was used immediately. The LAL reagent water bottles contained 30 ml and were used in the reconstitution of all reagents and as a negative control (blank). Lastly, the E. coli endotoxin vial contained approximately 50 EU/ml. It was reconstituted with a specific volume of LAL reagent water and mixed vigorously for 15 minutes at high speed on a vortex mixer. The reconstituted stock endotoxin was stable for four weeks at 2-8°C.

2.10.3.10.3 Specimen Collection and Preparation for Endotoxin Assessment

Careful technique was used to avoid microbial or endotoxin contamination. All materials that came into contact with the specimen or test reagents were endotoxin free. Clean glassware and materials were rendered endotoxin free by heating at 250°C for 30 minutes. Appropriate precautions were taken to protect materials from subsequent environmental contamination. From experience, most sterile, individually wrapped, plastic pipettes and pipette tips were endotoxin free. However, these materials tested before regular use. Samples to be tested stored in such a way that all bacteriological activity is stopped or the endotoxin level may increase with time. For example, stored samples at 2-8°C for less than 24 hours; samples stored longer than 24 hours should be frozen. If the container of diluent used to rehydrate the reagents has been opened previously or was not supplied by Lonza, the diluent alone must be tested for endotoxin contamination.

2.10.3.10.4 Product Inhibition in Measuring Endotoxin

Product inhibition occurs when substances in the test sample interfere with the LAL reaction. In the Kinetic-QCL™ Assay, this inhibition results in a longer reaction time, indicating lower levels of endotoxin than may actually be present in the test sample. The lack of product inhibition should be determined for each specific sample, either undiluted or at an appropriate dilution. To verify the lack of product inhibition, an aliquot of test sample (or a dilution of test sample) was spiked with a known amount of endotoxin.

In an inhibition/enhancement assay, the spiked solution (PPC) was assayed along with the unspiked sample, their respective endotoxin concentrations, as well as the

endotoxin recovered in the spiked sample were automatically calculated. The endotoxin recovered should equal the known concentration of the spike within 50 – 200%. A spiked aliquot of the test sample (or dilution) was prepared: tube method (50 µl) of the 50.0 EU/ml solution was transferred into 4.95 ml of test sample (or dilution). This solution contained an endotoxin concentration of 0.5 EU/ml in test sample (or dilution). This sample was vigorously vortexed for one minute prior to use. Around 100 µl of this solution was transferred into the 96-well plate as directed by the assay template.

2.10.3.10.5 Reagents Preparation in Endotoxin Assessment

Reagents were allowed to equilibrate to room temperature prior to use. In order to calculate endotoxin concentrations in unknown samples, each Kinetic-QCL test was referenced to a valid standard curve. Due to the large concentration range over which endotoxin values can be determined, it was possible to adjust the quantitative range of any given test by adjusting the concentration of endotoxin standards used to generate the standard curve. A minimum of three standards were required. The Kinetic-QCL assay was optimized to be linear from 0.005 EU/ml to 50.0 EU/ml. Plastic tubes were never used for making endotoxin solutions.

A solution containing 5.0 EU/ml endotoxin was prepared by adding 0.1 ml of the 50.0 EU/ml endotoxin stock into 0.9 ml of LAL reagent water in a suitable container and labelled 5.0 EU/ml. This solution was vortexed vigorously for at least 1 minute before proceeding. Serial dilutions were done.

Due to the variability of this assay, all the samples were undertaken together on the same day. All lab areas, heat block, pipettes which used were cleaned with 70% ethanol. Cleaned and autoclaved (free endotoxin) reservoirs were used. Filtered tips

and unopened boxes of autoclaved multichannel tips were also used. The addition of all reagents in the LAL assay was made consistent. All tubes or microplate wells were treated in exactly the same manner in order to determine the proper endotoxin concentration. It was suggested that, in a series of tests, reagents should be pipetted in the same order from tube to tube or well to well, and at the same rate. A specific template for the test was created to be run. This template had the name of the analyst, type of assay, lot numbers of reagents, the number and concentration of endotoxin standards, number of replicates and how standards and samples were supposed to be organized on the microplate. The template was printed for use as a guide in placing standards and samples into the microplate. The template was used following the WinKQCL™ Software prompts. Around 100 µl LAL reagent water was dispensed at wells blank, endotoxin standards, product samples, positive product controls, etc. into the appropriate wells of the microplate. The filled plate was then placed in the microplate reader and the lid closed. The plate was pre-incubated for ≥10 minutes at 37°C ± 1°C. Near the end of the pre-incubation period, each of the appropriate number of Kinetic-QCL™ reagent vials were reconstituted with 2.6 ml LAL reagent water. It was then mixed gently but thoroughly. The lysate was not vortexed. The reagents were pooled into a reagent reservoir and mixed gently by shaking the reservoir from side to side. Using an eight channel pipette, a 100 µl of the Kinetic-QCL™ reagent was dispensed into all wells of the microplate beginning with the first column (A1-H1) and proceeding sequentially up to the last column used. Reagents were added as quickly as possible. Bubble formation was also avoided as much as possible. OK button was clicked immediately in the WinKQCL™ software to initiate the test and quantification of endotoxin.

2.11 Data Analysis

The actual sample size after the intervention period (N=39 per group) has an actual power of 83% based on within group analysis. All analyses were performed using SPSS (version 16.5 Chicago, IL, USA). Mean and standard deviations were used to represent the data for the continuous normal variables, while median and interquartile range were used to report continuous non-normal variables. Furthermore, frequencies and percentages (%) were reported for categorical data. Changes, which were differences between follow-up and baseline values, were also calculated as mean and as percentage (%). Bivariate correlations between endotoxin anthropometrics, glycaemic and lipid profile were measured using Spearman correlation coefficient. Independent sample Student T-test and Mann Whitney U test were used to determine metabolic and clinical differences between placebo and probiotic groups at baseline. Statistical analysis for within group comparisons were performed twice: using intention-to-treat (ITT) analysis, where missing data were dealt by using the last observation carried forward (LOCF) method. Per-protocol analysis (PPA) was also performed on participants who successfully completed 80% of the trial. Mixed method analysis of covariance (ANCOVA) was used to determine between group differences after adjusting for baseline observations. All non-normal variables were transformed prior to parametric testing. Intervention effects were presented at 95% confidence interval (CI). A p-value <0.05 was considered statistically significant. All analysis figures were plotted using MS Excel.

Chapter 3.

Effects of a 3-Month Daily Intake of a Multi-Strain Probiotic Supplement in Circulating Endotoxin Levels and Cardiometabolic Profiles of Naïve Saudi T2DM Patients

3.1. Introduction

In the last few years, the gut microbiome has gained considerable interest due to its ability to coexist with its human host and complement several key physiologic processes peacefully maintaining homeostasis and over-all human health (Backhed et al., 2005). One theory that may explain the contribution of gut microbes to metabolic disease progression is sub-chronic inflammation secondary to endotoxemia. This state occurs when fragments of gut-derived gram negative bacteria (lipopolysaccharides or endotoxin) traverse the intestinal mucosa to enter the circulation, and may represent an important mediator of low-grade systemic inflammation influenced by the host's own gut microbiota and metabolic state (Harte et al., 2010). Previous studies have also shown that endotoxin can stimulate an innate immune response from adipose, liver and skeletal muscle tissues, leading to increased production of pro-inflammatory cytokines (Creely et al., 2007; Miller et al., 2009; Piya et al., 2013; Al-Disi et al, 2015).

There has been accumulating evidence pointing to the manipulation of the gut microbiome in the prevention and reversal of several chronic non-communicable diseases such as obesity, type 2 diabetes mellitus (T2DM) and the metabolic syndrome (MetS) (Brunkwall et al., 2017). It is now established that dietary intake and nutrition management are significant and clinically effective external factors in modifying the gut ecosystem (Singh et al., 2017). Specifically, probiotics, or live bacteria that naturally occur in the human body can confer health benefits, these have shown great potential as adjuvant therapies for a number of insulin-resistant diseases. Currently, randomized clinical trials in this area are limited and more research is required to strengthen the case.

In this chapter, studies were undertaken to establish whether a 12/13 week supplementation of a multi-strain probiotic could induce favourable changes in circulating endotoxin levels (primary outcome) and cardiometabolic profile (secondary outcome) of naïve T2DM subjects.

3.2. Materials and Methods

The present study was a 12-week single-centre, double-blind, randomised, placebo-controlled study. Ethics approval has been mentioned previously (Chapter 2.2.

3.2.1 Participants

A detailed description of participants has been described previsouly (Chapter 2.4). A flowchart is shown in figure 3.2.1.

3.2.2 Probiotic Supplements and Allocation Treatment

A detailed description of allocation to treatment has been described previously (Chapter 2.5).

3.2.3 Monitoring and Blood Sample Collection

A detailed description has been provided previously in Chapter 2.8-2.10

3.2.4 Biochemical Analyses

Fasting serum samples were analysed for glucose and lipid profile (Total cholesterol, HDL and triglycerides) using routine analyser (Konelab, Espoo, Finland). LDL-cholesterol was calculated using the Friedewald equation (Whelton et al., 2017). Serum insulin and c-peptide were measured using electrochemiluminescence assay (Roche Diagnostics, Germany). Coefficients of variation have been provided previously in Chapter 2.10.

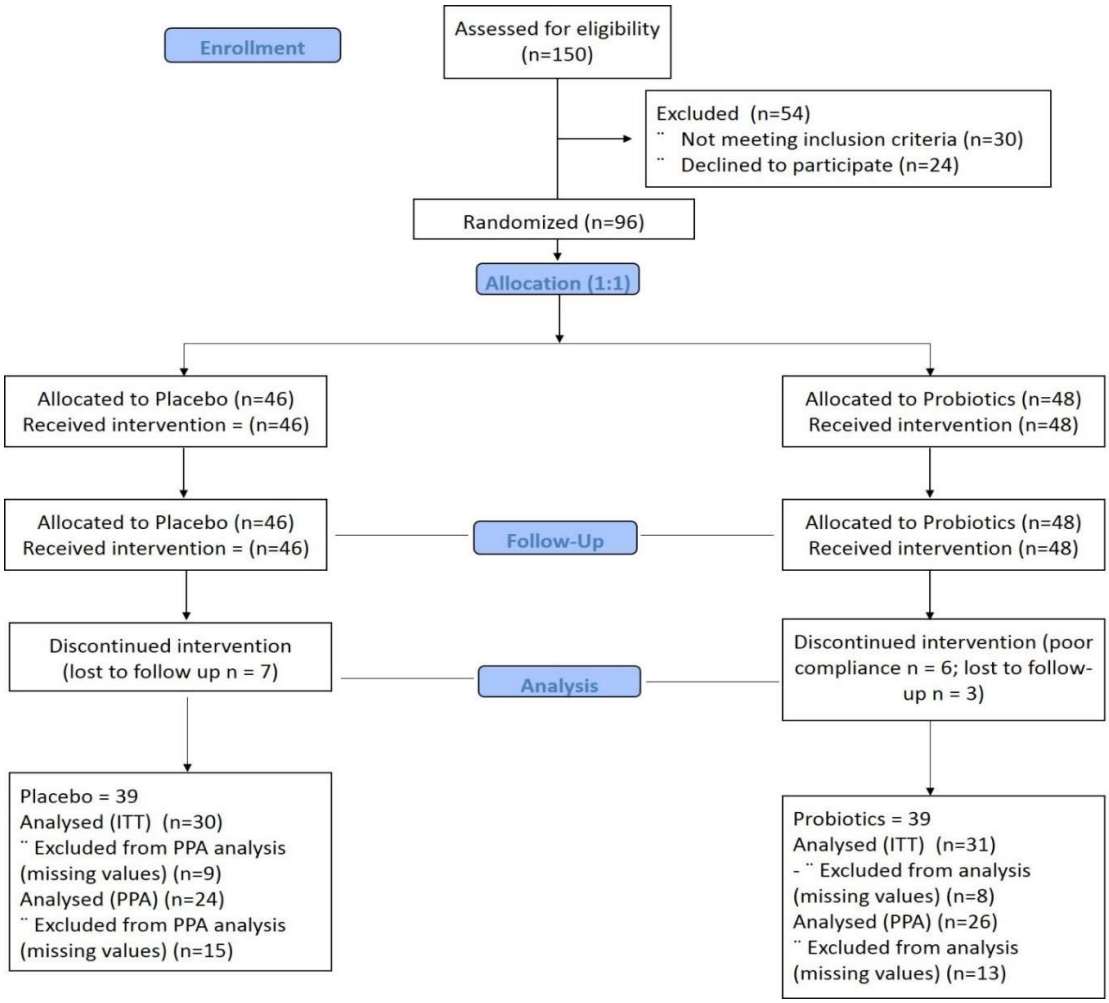


Figure 3.2.1 CONSORT Flowchart showing participants' screening, randomization and allocation throughout the 12/13 week intervention study. ITT - intention-to-treat; PPA - per protocol analysis

3.2.5 Data Analysis

In addition to the information previously provided in Chapter 2.11, mixed method analysis of covariance (ANCOVA) was used to determine within and between group differences after adjusting for baseline observations and covariates including WHR, MAP, Glucose (mmol/l), TC/HDL and endotoxin (IU/ml). Intervention effects were presented at 95% confidence interval (CI). P-value <0.05 was considered statistically significant.

3.3 Results

3.3.1 General Characteristics of Participants

The demographic characteristics of participants assigned to placebo [N=39, 21 males, 18 females] and probiotics [N=39, 20 males, 19 females] are shown in table 3.3.3.1. No statistically significant differences were noted in age (p=0.40), weight (p=0.22) and BMI (p=0.59). The placebo group had significantly higher mean waist-hip ratio than the probiotics group (placebo 1.0±0.1 vs probiotics 0.9±0.1; p=0.02). The placebo group also had a significantly lower diastolic (mmHg) (78.6±8.6 vs 83.6±11.8; p=0.04) and mean arterial blood pressure (95.5±7.7 vs 100.7±11.1; p=0.02) than the probiotics group. With regards to glycaemic and lipid parameters, the placebo group had significantly lower median glucose (mmol/l) levels [7.0 (5.7-11.2) vs 11.7 (8.4-16.4); p<0.001], as well as significantly lower mean circulating levels of total cholesterol (mmol/l) (5.2±1.0 vs 5.8±1.3; p=0.04), LDL-cholesterol (3.1±0.9 vs 3.7±1.3; p=0.05) and total/HDL-cholesterol ratio (5.0±1.3 vs 6.4±2.2; p=0.001) than the probiotics group. Lastly, median endotoxin (IU/ml) levels were significantly lower in the placebo than the probiotics group [2.1 (1.2 -4.4) vs 4.6 (2.4 -9.9); p=0.002]. The rest of the parameters were not significantly different from one another.

Table 3.3.1.1 Baseline Parameters According to Placebo and Probiotics.

Parameters	Placebo	Probiotics	P-value
N	39	39	
Males (%)	21 (56.8)	19 (51.4)	
Age (years)	46.6 ± 5.9	48.0 ± 8.3	0.40
Weight (kg)	79.5 ± 15.7	75.6 ± 11.0	0.22
BMI (kg/m^2)	30.1 ± 5.0	29.4 ± 5.2	0.59
Waist-Hip Ratio	1.0 ± 0.1	0.9 ± 0.1	0.02
Systolic BP (mmHg)	129.5 ± 10.3	134.8 ± 14.6	0.07
Diastolic BP (mmHg)	78.6 ± 8.6	83.6 ± 11.8	0.04
Mean Arterial Pressure (MAP)	95.5 ± 7.7	100.7 ± 11.1	0.02
Glucose (mmol/l)	7.0 (5.7 -11.2)	11.7 (8.4 -16.4)	<0.001
Insulin (uU/mL)	13.1 (7.7 -18.7)	9.9 (7.7 -16.4)	0.48
C-peptide (ng/ml)	0.1 (0.1 -0.5)	0.4 (0.0 -1.8)	0.22
HOMA-IR	4.1 (2.3 -7.3)	5.3 (3.5 - 10.2)	0.10
Triglycerides (mmol/l)	2.2 ± 1.4	2.5 ± 1.4	0.36
Total Cholesterol (mmol/l)	5.2 ± 1.0	5.8 ± 1.3	0.04
HDL-Cholesterol (mmol/l)	1.1 ± 0.3	1.0 ± 0.3	0.08
LDL-Cholesterol (mmol/l)	3.1 ± 0.9	3.7 ± 1.3	0.05
Total-Cholesterol/HDL Ratio	5.0 ± 1.3	6.4 ± 2.2	0.001
Endotoxin (IU/ml)	2.1 (1.2 -4.4)	4.6 (2.4 -9.9)	0.002

Note: Data presented as Mean ± SD for normal variables while non-normal variables are presented as Median (inter-quartile range).

3.3.2 Characteristics of Participants at Baseline and after Three Months

3.3.2.1 Endotoxin

Mean differences between placebo and probiotics group were presented in figure 3.3.2.1 and showed a significant difference in the probiotics group from baseline and after 3 months in both ITT ($p<0.001$) and PPA ($p<0.001$). This difference was not observed in the placebo group.

Within and between group effects of participants' characteristics using ITT and PPA as well as percentage changes were shown in tables 3.3.2.1.1 and 3.3.2.1.2. No difference was noted in endotoxin levels between groups [placebo mean change -0.20 (percentage change -9.5%) vs probiotics -2.40 (-52.2%); (95% Confidence Interval (CI): -0.05-0.36; p=0.15)]. Within group comparisons however showed a significant decrease in endotoxin levels in the probiotics group [baseline median = 4.6 (interquartile range 2.4-7.9) vs 3 months = 2.2 (1.2-3.6); $p<0.01$)]. This was not observed in the placebo group [2.2 (1.2-4.4) vs 1.9 (1.0-2.9); p=0.31)]. Within group comparisons using PPA showed the same significant difference in the probiotics group [5.1 (3.2-8.4) vs 2.3 (1.2-3.6); $p<0.01$)] and not in placebo [2.3 (1.2-4.6) 2.0 (1.1-4.7); p=0.14)] (Table 3.3.2.1.1).

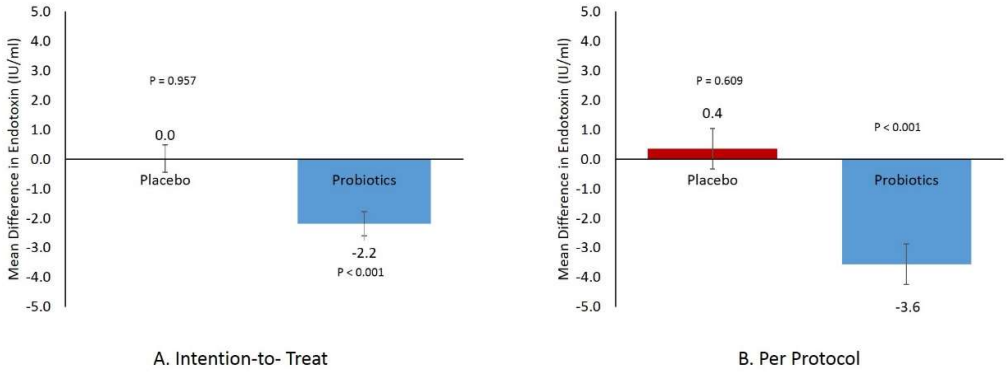


Figure 3.3.2.1 Mean differences in endotoxin levels in placebo versus probiotics using A) Intention-to-treat and B) Per Protocol Analysis

Table 3.3.2.1.1 Endotoxin Before and After Supplementation with Placebo or Probiotics Using ITT and PPA

	Placebo (N=39, 21 males, 18 females)				**Probiotics (N=39, 20 males, 19 females)**				**Intervention Effect**	
	Baseline	3-Months	Mean Change	P^a	Baseline	3-Months	Mean Change	P^a	Effect (95% CI)	P^b
ITT	2.1 (1.2 – 4.4)	1.9 (1.0 – 2.9)	-0.20	0.31	4.6 (2.4 – 7.9)	2.2 (1.2 – 3.6)	-2.40	<0.01	0.15 (-0.05 - 0.36)	0.15
	(N=24, 15 males, 9 females)				**(N=26, 14 males, 12 females)**					
PPA	2.3 (1.2 - 4.6)	2.0 (1.1 – 4.7)	-0.30	0.14	5.1 (3.2 - 8.4)	2.3 (1.2 - 3.6)	-2.80	<0.01	0.20 (-0.05 - 0.45)	0.12

Note: Endotoxin values presented as median (inter-quartile range); p^a and p^b denotes p-values for within group differences and between group differences respectively obtained from mixed model ANCOVA after adjusting for baseline covariates including WHR, MAP, Glu, (mmol/l), TC/HDL and Endo (IU/ml). Significant at $p<0.05$.

Table 3.3.2.1.2 Percentage Change (%) in Placebo and Probiotics

Parameters	**Intention-to-Treat**		**Per Protocol**	
	Placebo	**Probiotics**	**Placebo**	**Probiotics**
Endotoxin (IU/ml)	-9.52	-52.17	-13.04	-54.90

Note: Data presented as percentages (%).

3.3.2.2 Anthropometric and Clinical Measures

Using ITT and compared with the placebo group, participants in the probiotics groups had a significant improvement in WHR [placebo 0.0 (0.0%) vs probiotics -0.01 (-1.11%); (CI: -0.12- -0.01; p=0.02)] (Tables 3.3.2.2.1 and 3.3.2.2.3). No differences were noted in weight [0.42 (0.5%) vs -0.28 (-1.4%); (CI: -17.1-1.1; p=0.08)], BMI [0.15 (0.5%) vs -0.11 (-0.4%); (CI: -4.92-2.39; p=0.49)], systolic blood pressure [0.43 (0.3%) vs -5.84 (-0.4%0; (CI: -5.18-10.06; p=0.52)], diastolic blood pressure [1.22 (1.5%) vs -3.78 (-4.5%); (CI: -5.82-6.58; p=0.90)] and MAP [0.96 (1.0%) vs -4.47 (-4.4%); (CI: -4.58-6.72; p=0.71)].

Within group comparisons using ITT showed a significant decrease in systolic (baseline mean ± standard deviation = 135.0±15.0 vs 3 months 129.0±11.0; p<0.01), diastolic (84.0±12.0 vs 80.0±11.0; p=0.03) as well as mean arterial blood pressure (100.7±11.1 vs 96.2±9.7; p<0.01) in the probiotics group post intervention. These differences were not observed in the placebo group (p-values for SBP, DBP and MAP; 0.80, 0.44 and 0.60, respectively). Furthermore, p-values indicated no significant changes in either group with regards to weight (placebo = 0.71; probiotics = 0.08), BMI (placebo = 0.76; probiotics 0.86) and WHR (placebo = 0.32; probiotics = 0.75) after 3 months (Table 3.3.2.2.1).

Table 3.3.2.2.1 Anthropometric Characteristics Before and After Supplementation with Placebo or Probiotics Using Intention-to-Treat Analysis

Parameters	Placebo (N= 39, 21 males, 18 females)				Probiotics (N=39, 20 males, 19 females)				Intervention Effect	
	Baseline	3-Months	Mean Change	P[a]	Baseline	3-Months	Mean Change	P[a]	Effect (95% CI)	P[b]
Weight (kg)	79.5 ± 15.7	79.9 ± 15.9	0.42	0.71	75.6 ± 11.0	75.3 ± 11.3	-0.28	0.77	-8.00 (-17.1 – 1.1)	0.08
BMI (kg/m^2)	30.1 ± 5.0	30.2 ± 5.0	0.15	0.76	29.4 ± 5.2	29.3 ± 5.3	-0.11	0.86	-1.27 (-4.92 - 2.39)	0.49
WHR	1.0 ± 0.1	1.0 ± 0.1	0.00	0.32	0.9 ± 0.1	0.9 ± 0.1	-0.01	0.75	-0.07 (-0.12 - -0.01)	0.02
SBP (mmHg)	129.0 ± 10.0	130.0 ± 11.0	0.43	0.80	135.0 ± 15.0	129.0 ± 11.0	-5.84	<0.01	2.44 (-5.18 - 10.06)	0.52
DBP (mmHg)	79.0 ± 9.0	80.0 ± 8.0	1.22	0.44	84.0 ± 12.0	80.0 ± 11.0	-3.78	0.03	0.38 (-5.82 - 6.58)	0.90
MAP	95.5 ± 7.7	96.5 ± 7.8	0.96	0.60	100.7 ± 11.1	96.2 ± 9.7	-4.47	<0.01	1.07 (-4.58 - 6.72)	0.71

Note: Data presented as mean ± SD; p[a] and p[b] denotes p-values for within group differences and between group differences respectively obtained from mixed model ANCOVA after adjusting for baseline covariates including WHR, MAP, Glu, (mmol/l), TC/HDL and Endo (IU/ml). BMI, body mass index; WHR, waist-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; CI – confidence interval. Significant at $p<0.05$.

This significant reduction in WHR was also observed using PPA [0.0 (0.0%) vs -0.01 (-1.11%); (CI: -0.14- -0.03; p=0.01)] (Tables 3.3.2.2.2 and 3.3.2.2.3). Within group comparisons using the PPA showed no significant changes in all anthropometric measures post

intervention in both placebo (p-values for weight, BMI, WHR, SBP, DBP and MAP: 0.43, 0.96, 0.18, 0.65, 0.38, 0.59, respectively) and probiotics group (p-values for weight, BMI, WHR, SBP, DBP and MAP: 0.66, 0.98, 0.65, 0.25, 0.15, 0.11, respectively) (Table 3.3.2.2.2).

Table 3.3.2.2.2. Anthropometric Characteristics Before and After Supplementation with Placebo or Probiotics Using Per Protocol Analysis

Parameters	Placebo (N=24, 15 males, 9 females)				Probiotics (N=26, 14 males, 12 females)				Intervention Effect	
	Baseline	3-Months	Mean Change	P[a]	Baseline	3-Months	Mean Change	P[a]	Effect (95% CI)	P[b]
Weight (kg)	79.5 ± 15.7	79.9 ± 15.9	0.42	0.43	75.5 ± 10.9	75.4 ± 11.4	-0.07	0.66	-2.05 (-11.92 – 0)	0.72
BMI (kg/m^2)	30.1 ± 5.0	30.2 ± 5.0	0.15	0.96	29.4 ± 5.2	29.3 ± 5.4	-0.03	0.98	-0.77 (-4.67 - 3.14)	0.69
WHR	0.9 ± 0.1	0.9 ± 0.1	0.00	0.18	0.9 ± 0.1	0.9 ± 0.1	-0.01	0.65	-0.08 (-0.14 - -0.03)	0.01
SBP (mmHg)	129.5 ± 10.8	130.1 ± 11.7	0.57	0.65	132.7 ± 13.7	129.8 ± 12.7	-2.82	0.25	-2.44 (-11.93 - 7.05)	0.61
DBP (mmHg)	78.4 ± 9.1	80.0 ± 8.5	1.63	0.38	83.2 ± 12.4	80.0 ± 11.7	-3.24	0.15	2.51 (-5.97 - 10.98)	0.55
MAP	95.4 ± 8.5	96.7 ± 8.6	1.28	0.59	99.7 ± 11.2	96.6 ± 10.7	-3.10	0.11	0.86 (-6.77 - 8.49)	0.82

Note: Data presented as mean ± SD; p[a] and p[b] denotes p-values for within group differences and between group differences respectively obtained from mixed model ANCOVA after adjusting for baseline covariates including WHR, MAP, Glu, (mmol/l), TC/HDL and Endo (IU/ml). BMI, body mass index; WHR, waist-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; CI – confidence interval. Significant at $p<0.05$.

Table 3.3.2.2.3. Percentage Changes (%) in Anthropometric Characteristics in Treatment Groups by Analysis Type

Parameters	**Intention-to-Treat**		**Per Protocol**	
	Placebo	**Probiotics**	**Placebo**	**Probiotics**
BMI (kg/m^2)	0.50	-0.37	0.50	-0.10
Waist-Hip Ratio	0.00	1.11	0.00	1.11
Systolic BP (mmHg)	0.33	-4.33	0.44	-2.13
Diastolic BP (mmHg)	1.54	-4.50	2.08	-3.89
Mean Arterial Pressure (MAP)	1.01	-4.44	1.34	-3.11

Note: Data presented as percentages (%).

3.3.2.3 Glycaemic Profile

Between group comparisons using the ITT showed a clinically significant improvement in HOMA-IR observed in the probiotics group and not in placebo [placebo -0.50 (-12.2%) vs probiotics -3.20 (-60.4%); (CI: -0.34- -0.01; p=0.04)]. No differences were observed in glucose [placebo 1.0 (14.3%) vs probiotics -3.20 (-27.4%); (CI: -0.06-0.16; p=0.36)], insulin [placebo -2.40 (-18.3%) vs probiotics -3.0 (-30.3%); (CI: -0.24-0.07; p=0.29)] and C-peptide [placebo 0.0 (0.0%) vs probiotics -0.40 (80.0%); (CI: -0.38-0.53; p=0.74)] (Tables 3.3.2.3.1 and 3.3.2.3.3).

Within group comparisons using the ITT revealed significantly higher glucose levels in the placebo group after 3 months intervention [baseline median = 7.0 (interquartile range 5.7-11.2) vs 3 months = 8.0 (5.9-11.4); p=0.02)] (Table 3.3.2.3.1). Furthermore in the placebo group, no significant changes were observed post-intervention in circulating levels of insulin (p=0.72), C-peptide (p=0.12) and HOMA-IR (p=0.37). In contrast, post-intervention levels of glucose [11.7 (8.4-16.4) vs 8.5 (6.2-11.0); p<0.01)], insulin [9.9 (7.7-16.4) vs 6.9 (4.5-9.8); p<0.01)], C-peptide [0.5 (0.0-1.8) vs 0.1 (0.0-0.3); p=0.01)] and HOMA-IR [5.3 (3.5-10.2) vs 2.1 (1.5-5.2); p<0.01)] were significantly lower than baseline in the probiotics group using the ITT analysis (Table 3.3.2.3.1).

Table 3.3.2.3.1. Glycaemic Characteristics Before and After Supplementation with Placebo or Probiotics Using Intention-to-Treat Analysis

Parameters	Placebo (N= 39, 21 males, 18 females)				Probiotics (N=39, 20 males, 19 females)				Intervention Effect	
	Baseline	3-Months	Mean Change	P[a]	Baseline	3-Months	Mean Change	P[a]	Effect (95% CI)	P[b]
Glu (mmol/l) #	7.0 (5.7-11)	8.0 (5.9-11.4)	1.00	0.02	11.7 (8.4 - 16.4)	8.5 (6.2 – 11.0)	-3.20	<0.01	0.05 (-0.06 - 0.16)	0.36
Ins (IU/mL) #	13.1 (7.7-19)	10.7 (7.7–14.5)	-2.40	0.72	9.9 (7.7 - 16.4)	6.9 (4.5 - 9.8)	-3.00	<0.01	-0.08 (-0.2 - 0.07)	0.29
C-Pep(ng/ml) #	0.2 (0.1-0.5)	0.2 (0.1 - 0.9)	0.00	0.12	0.5 (0.0 - 1.8)	0.1 (0.0 - 0.3)	-0.40	0.01	0.08 (-0.38 - 0.53)	0.74
HOMA-IR #	4.1 (2.3–7)	3.6 (3.1 – 5.5)	-0.50	0.37	5.3 (3.5 - 10.2)	2.1 (1.5 - 5.2)	-3.20	<0.01	-0.17(-0.3- -0.01)	0.04

Note: Data presented as median (inter-quartile range); #median change presented instead of mean; all non-normal variables were transformed prior to parametric testing; p^a and p^b denotes p-values for within group differences and between group differences respectively obtained from mixed model ANCOVA after adjusting for baseline covariates including WHR, MAP, Glu, (mmol/l), TC/HDL and Endo (IU/ml). Glu, glucose; Ins, insulin; C-Pep, C-Peptide; HOMA-IR, homeostasis model for insulin resistance; CI – confidence interval. Significant at $p<0.05$.

A significant difference in glucose levels was observed using PPA in the placebo group [7.1 (5.7-11.2) vs 8.0 (5.9-11.4); p=0.01)] (Table 3.3.2.3.2). Between group comparisons using the PPA showed no significant differences in both groups (p-values for glucose, insulin, C-peptide and HOMA-IR: 0.30, 0.41, 0.79 and 0.11, respectively) (Tables 3.3.2.3.2 and 3.3.2.3.3). Furthermore in the placebo group, no significant changes were observed post-intervention in circulating levels of insulin (p=0.49), C-peptide (p=0.16) and HOMA-IR (p=0.29).

The same significant improvement in the glycaemic profile persisted even after using the PPA (p-values for glucose, insulin, C-peptide and HOMA-IR: <0.01, 0.04, 0.01 and <0.01, respectively) (Table 3.3.2.3.2).

Table 3.3.2.3.2. Glycaemic Characteristics Before and After Supplementation with Placebo or Probiotics Using Per Protocol Analysis

Parameters	Placebo (N=24, 15 males, 9 females)				Probiotics (N=26, 14 males, 12 females)				Intervention Effect	
	Baseline	3-Months	Mean Change	P[a]	Baseline	3-Months	Mean Change	P[a]	Effect (95% CI)	P[b]
Glu (mmol/l) #	7.1 (5.7 - 11.2)	8.0 (5.9 - 11.4)	0.90	0.01	11.7 (8.4 - 16.4)	8.5 (6.2-10.9)	-3.20	<0.01	0.06 (-0.06 - 0.19)	0.30
Ins (uU/mL) #	13 (7.5 - 18.7)	10.9 (7.7 - 15.5)	-2.10	0.49	9.8 (7.7 - 15.2)	6.8 (4.5 - 9.6)	-3.00	0.04	-0.08 (- 0.28 - 0.12)	0.41
C-Pep(ng/ml)#	0.2 (0.1 - 0.4)	0.3 (0.1 - 0.9)	0.10	0.16	0.7 (0.0 - 2.0)	0.1 (0.0 - 0.3)	-0.60	0.01	0.07 (-0.43 - 0.56)	0.79
HOMA-IR #	4.1 (2.3 - 7.5)	3.6 (3.1 - 6.0)	-0.50	0.29	5.2 (3.5 - 10.2)	2.1 (1.5 - 4.4)	-3.10	<0.01	-0.18 (-0.39 - 0.04)	0.11

Note: Data presented as median (inter-quartile range); #median change presented instead of mean; all non-normal variables were transformed prior to parametric testing; p^a and p^b denotes p-values for within group differences and between group differences respectively obtained from mixed model ANCOVA after adjusting for baseline covariates including WHR, MAP, Glu, (mmol/l), TC/HDL and Endo (IU/ml). Glu, glucose; Ins, insulin; C-Pep, C-Peptide; HOMA-IR, homeostasis model for insulin resistance; CI – confidence interval. Significant at $p<0.05$.

Table 3.3.2.3.3. Percentage Changes (%) in Glycaemic Characteristics in Treatment Groups by Analysis Type

Parameters	Intention-to-Treat		Per Protocol	
	Placebo	**Probiotics**	**Placebo**	**Probiotics**
Glucose (mmol/l)	14.29	-27.35	12.68	-27.35
Insulin (uU/ml)	-18.32	-30.30	-16.15	-30.61
C-peptide (ng/ml)	0.00	-80.00	50.00	-85.71
HOMA-IR	-12.20	-60.38	-12.20	-59.62

Note: Data presented as percentages (%).

3.3.2.4 Lipid Profile

Using the ITT analysis, between group comparisons showed no differences in all lipid indices: triglycerides [placebo -0.20 (-9.1%) vs probiotics -0.78 (-31.2%); (CI: -0.96-0.28; p=0.27)], total cholesterol [-0.53 (-10.2%) vs -0.63 (-10.9%); (CI: -0.52-0.75; p=0.72)], HDL-cholesterol [0.46 (-6.4%) vs 0.14 (-14.0%); (CI: -0.22-0.14; p=0.65)], LDL-cholesterol [-0.37 (-11.9%) vs -0.41 (-11.4%); (CI: -0.43-0.79; p=0.55)] and total/HDL cholesterol ratio [-0.11 (-2.2%) vs -1.07 (-16.7%); (CI: -0.72-2.38; p=0.29)] (Tables 3.3.2.4.1 and 3.3.2.4.3).

Within group comparisons using ITT showed significantly lower levels of total cholesterol after intervention in both placebo (baseline mean ± standard deviation 5.2±1.0 vs 3 months 4.7±0.9; $p<0.01$) and probiotics group (5.8±1.3 vs 5.1±0.9; $p<0.01$). Only the probiotics group, however, showed significantly lower circulating triglycerides (2.5±1.4 vs 1.7±0.7; p=0.04) and LDL-cholesterol (3.6±1.3 vs 3.2±0.9; p=0.02) after intervention. Both groups had no significant changes in HDL-cholesterol (placebo = 0.46; probiotics = 0.10) and total/HDL cholesterol (placebo = 0.67; probiotics = 0.35) ratios post-intervention (Table 3.3.2.4.1).

Table 3.3.2.4.1. Lipid Profile Characteristics Before and After Supplementation with Placebo or Probiotics Using Intention-to-Treat Analysis

Parameters	Placebo (N= 39, 21 males, 18 females)				Probiotics (N=39, 20 males, 19 females)				Intervention Effect	
	Baseline	3-Months	Mean Change	P[a]	Baseline	3-Months	Mean Change	P[a]	Effect (95% CI)	P[b]
TG (mmol/l)	2.2 ± 1.4	2.0 ± 0.8	-0.20	0.05	2.5 ± 1.4	1.7 ± 0.7	-0.78	0.04	-0.34 (- 0.96 - 0.28)	0.27
TC (mmol/l)	5.2 ± 1.0	4.7 ± 0.9	-0.53	<0.01	5.8 ± 1.3	5.1 ± 0.9	-0.63	<0.01	0.11 (-0.52 - 0.75)	0.72
HDL (mmol/l)	1.1 ± 0.3	1.0 ± 0.3	-0.07	0.46	1.0 ± 0.3	1.1 ± 0.3	0.14	0.10	-0.04 (-0.22 - 0.14)	0.65
LDL (mmol/l)	3.1 ± 0.9	2.8 ± 0.9	-0.37	0.12	3.6 ± 1.3	3.2 ± 0.9	-0.41	0.02	0.18 (- 0.43 - 0.79)	0.55
TC/HDL	5.0 ± 1.3	4.9 ± 1.4	-0.11	0.67	6.4 ± 2.2	5.3 ± 4.3	-1.07	0.35	0.83 (-0.72 - 2.38)	0.29

Note: Data presented as mean ± SD; p^a and p^b denotes p-values for within group differences and between group differences respectively obtained from mixed model ANCOVA after adjusting for baseline covariates including WHR, MAP, Glu, (mmol/l), TC/HDL and Endo (IU/ml). TG, triglycerides; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; Endo, endotoxin; CI – confidence interval. Significant at $p<0.05$.

The same non-significant changes in between group comparisons were observed using the PPA (p-values for triglycerides, total cholesterol, HDL- and LDL-cholesterol, total/HDL-cholesterol ratio: 0.26, 0.39, 0.31, 0.21, 0.13, respectively) (Table 3.3.2.4.2 and

3.3.2.4.3). Lastly, within group comparisons using PPA in the placebo group showed significantly lower levels of triglycerides (2.1±1.4 vs 2.0±0.8; p=0.05) and total cholesterol (5.2±1.0 vs 4.7±0.9; p<0.01) post-intervention. Significant improvement in total cholesterol levels was observed in the probiotics group (5.8±1.3 vs 5.1±0.9; p<0.01) as well as LDL-cholesterol (3.7±1.2 vs 3.3±0.9; p=0.03) after 3 months of intervention. The rest of the lipids not previously mentioned do not significantly differ from one another in both placebo and probiotics (Table 3.3.2.4.2).

Table 3.3.2.4.2. Lipid Profile Characteristics Before and After Supplementation with Placebo or Probiotics Using Per Protocol Analysis

Parameters	Placebo (N=24, 15 males, 9 females)				Probiotics (N=26, 14 males, 12 females)				Intervention Effect	
	Baseline	3-Months	Mean Change	P[a]	Baseline	3-Months	Mean Change	P[a]	Effect (95% CI)	P[b]
TG (mmol/l)	2.1 ± 1.4	2.0 ± 0.8	-0.20	0.05	2.5 ± 1.4	1.7 ± 0.7	-0.78	0.15	-0.41 (-1.13 - 0.31)	0.26
TC (mmol/l)	5.2 ± 1.0	4.7 ± 0.9	-0.53	<0.01	5.8 ± 1.3	5.1 ± 0.9	-0.63	<0.01	0.30 (-0.39 - 0.98)	0.39
HDL (mmol/l)	1.1 ± 0.3	1.0 ± 0.3	-0.07	0.15	1.0 ± 0.3	1.1 ± 0.3	0.15	0.20	-0.10 (-0.30 - 0.10)	0.31
LDL (mmol/l)	3.2 ± 0.9	2.8 ± 0.9	-0.41	0.11	3.7 ± 1.2	3.3 ± 0.9	-0.44	0.03	0.43 (-0.24 - 1.10)	0.21
TC/HDL	5.0 ± 1.3	4.9 ± 1.4	-0.13	0.88	6.5 ± 2.2	5.4 ± 4.4	-1.08	0.41	1.33 (-0.39 - 3.05)	0.13

Note: Data presented as mean ± SD; p^a and p^b denotes p-values for within group differences and between group differences respectively obtained from mixed model ANCOVA after adjusting for baseline covariates including WHR, MAP, Glu, (mmol/l), TC/HDL and Endo (IU/ml). TG, triglycerides; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; Endo, endotoxin; CI – confidence interval. Significant at p<0.05.

Table 3.3.2.4.3 Percentage Changes (%) in Lipid Characteristics in Treatment Groups by Analysis Type

Parameters	Intention-to-Treat		Per Protocol	
	Placebo	**Probiotics**	**Placebo**	**Probiotics**
Triglycerides (mmol/l)	-9.09	-31.20	-9.52	-31.20
Total Cholesterol (mmol/l)	-10.19	-10.86	-10.19	-10.86
HDL-Cholesterol (mmol/l)	-6.36	14.00	-6.36	15.00
LDL-cholesterol (mmol/l)	-11.94	-11.39	-12.81	-11.89
Total/HDL-Cholesterol Ratio	-2.20	-16.72	-2.60	-16.62

Note: Data presented as percentages (%).

3.3.5 Associations of Endotoxin to Anthropometrics, Glycaemic and Lipid Profiles Measured

Table 3.3.5.1 shows the bivariate associations between endotoxin and parameters measured. In all participants, endotoxin was significantly associated with diastolic BP (R=0.27; p=0.03) (Figure 3.3.5.1) and MAP (r=0.26; p=0.04) (Figure 3.3.5.2). HDL-cholesterol was inversely and significantly associated with endotoxin levels in all participants (R=-0.25; p=0.04) (Figure 3.3.5.3) and in the probiotics group (R=-0.35; p=0.05) (Figure 3.3.5.4). In the probiotics group, there were also significant associations between endotoxin and triglycerides (R=0.37; p=0.04) (Figure 3.3.5.5) and total/HDL cholesterol ratio (R=0.42; p=0.02) (Figure 3.3.5.6). The latter was also significant in all participants (R=0.32; p<0.01) (Figure 3.3.5.7). No significant associations were seen between endotoxin and any of the glycaemic parameters in all participants as well as after stratification to treatment groups.

Lastly, none of the participants complained of any serious side effects from the clinical trial. The most common complaint were minor gastrointestinal discomfort (feeling bloated and increased flatulence during the first week of treatment) (N=5, 1 in the placebo group and 4 in the probiotics group) which is common for first time probiotics users. This symptom gradually faded during the first weeks of treatment.

Table 3.3.5.1 Bivariate Correlations between Endotoxin, Anthropometrics, Glycaemic and Lipid Profiles of Participants at Baseline.

Parameters	ALL (N=78)		Placebo (N=39)		Probiotics (N=39)	
	R	P-value	R	P-value	R	P-value
Age (years)	-0.06	0.64	-0.13	0.48	-0.16	0.40
Weight (kg)	-0.11	0.38	0.00	0.99	-0.03	0.86
BMI (kg/m^2)	-0.09	0.48	-0.02	0.91	-0.06	0.78
Waist-Hip Ratio	-0.15	0.27	0.01	0.98	0.03	0.89
Systolic BP (mmHg)	0.25	0.05	0.16	0.40	0.21	0.28
Diastolic BP (mmHg)	**0.27**	**0.03**	0.25	0.19	0.10	0.59
Mean Arterial Pressure (MAP)	**0.26**	**0.04**	0.22	0.23	0.09	0.63
Glycaemic Profile						
Glucose (mmol/l)	0.22	0.08	0.15	0.44	-0.01	0.96
Insulin (uU/ml)	-0.12	0.35	-0.18	0.35	-0.10	0.59
C-peptide (ng/ml)	0.05	0.67	-0.21	0.27	-0.04	0.84
HOMA-IR	0.01	0.92	-0.11	0.56	-0.11	0.57
Lipid Profile						
Triglycerides (mmol/l)	0.21	0.09	-0.02	0.92	**0.37**	**0.04**
Total Cholesterol (mmol/l)	0.19	0.14	0.09	0.64	0.28	0.13
HDL-Cholesterol (mmol/l)	**-0.25**	**0.04**	0.09	0.63	**-0.35**	**0.05**
LDL-Cholesterol (mmol/l)	0.14	0.27	-0.03	0.88	0.23	0.22
Total/HDL Cholesterol Ratio	**0.32**	**<0.01**	0.07	0.73	**0.41**	**0.02**

Note: Data presented as Spearman Correlation coefficients; Numbers in bold indicate significance; significant at $p<0.05$.

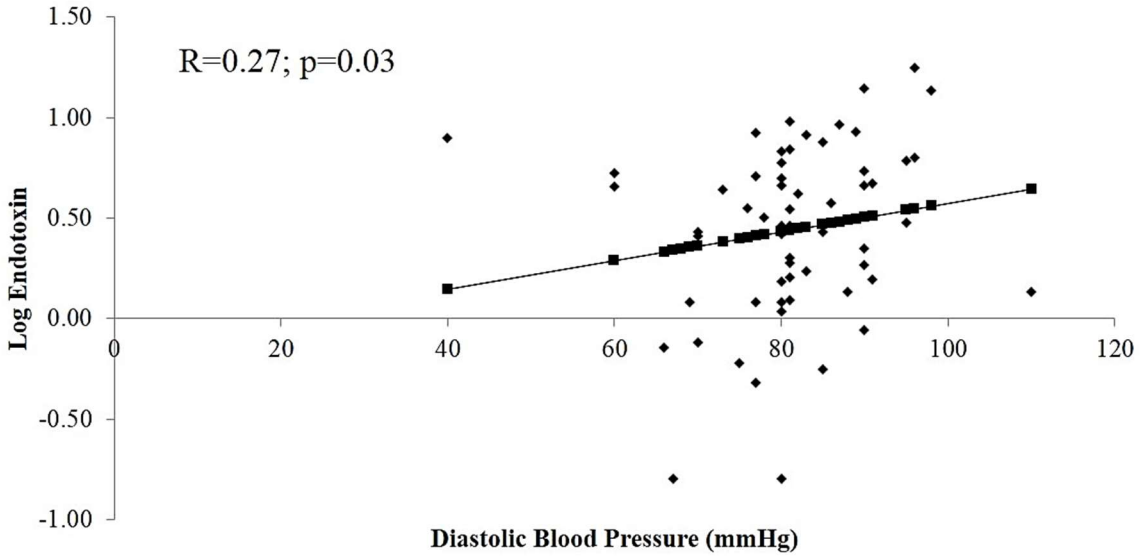


Figure 3.3.5.1. Significant positive association (R=0.27; p=0.03) between log endotoxin and diastolic blood pressure (mmHg) in all participants at baseline.

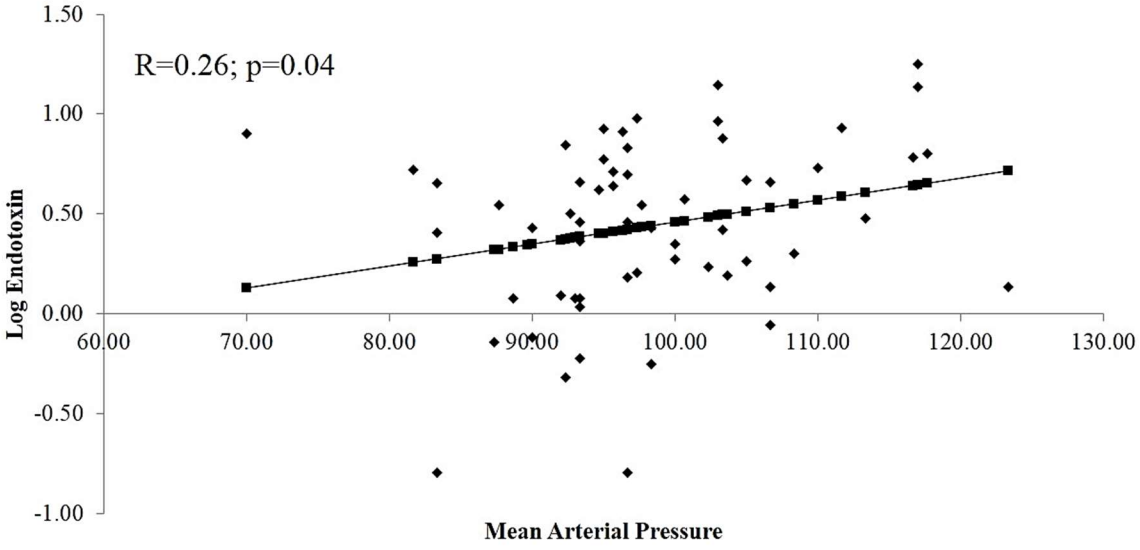


Figure 3.3.5.2. Significant positive association (R=0.26; p=0.04) between log endotoxin and mean arterial blood pressure in all participants at baseline.

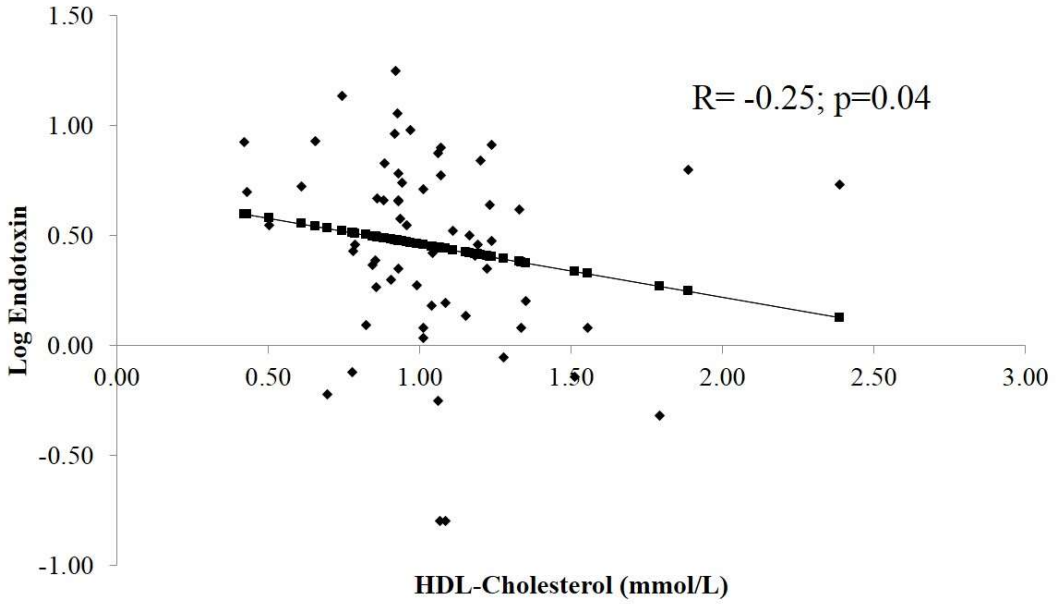


Figure 3.3.5.3. Significant inverse association (R=-0.25; p=0.04) between log endotoxin and HDL-cholesterol (mmol/L) in all participants at baseline.

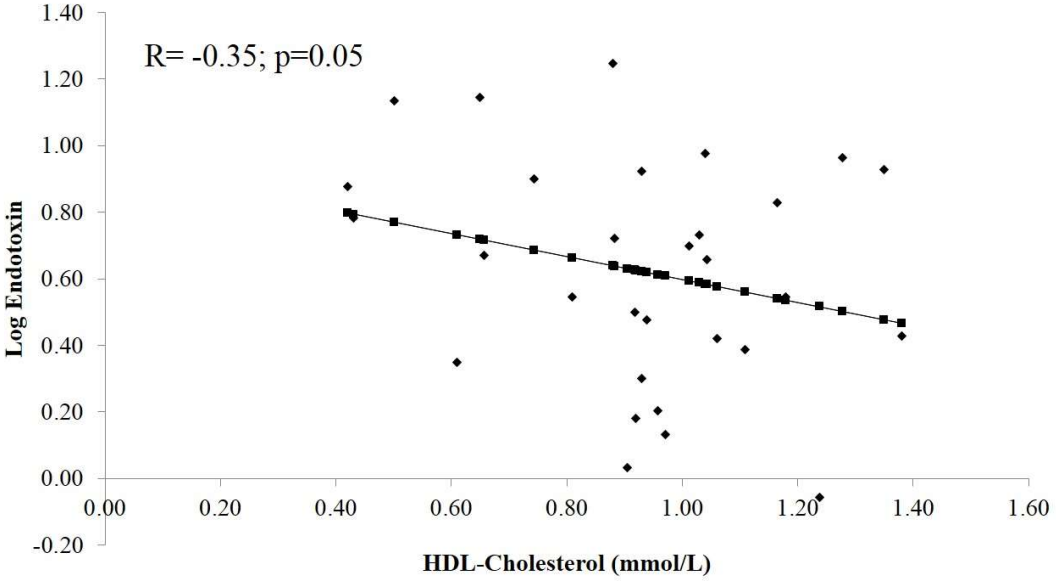


Figure 3.3.5.4. Significant inverse association (R= -35; p=0.05) between log endotoxin and HDL-cholesterol (mmol/L) in the probiotics group at baseline.

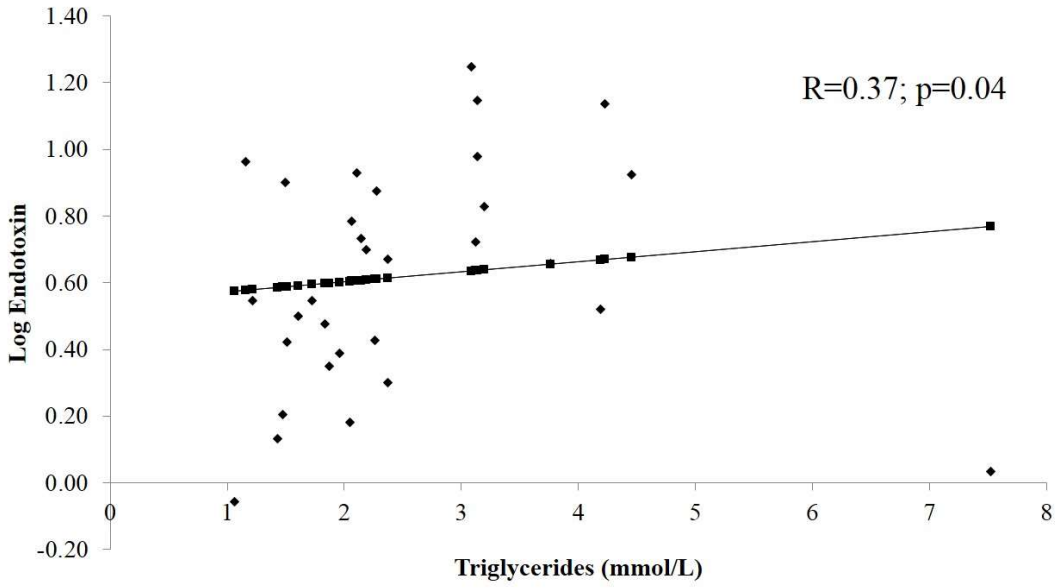


Figure 3.3.5.5. Significant positive association (R=0.37; p=0.04) between log endotoxin and triglycerides (mmol/L) in the probiotics group at baseline.

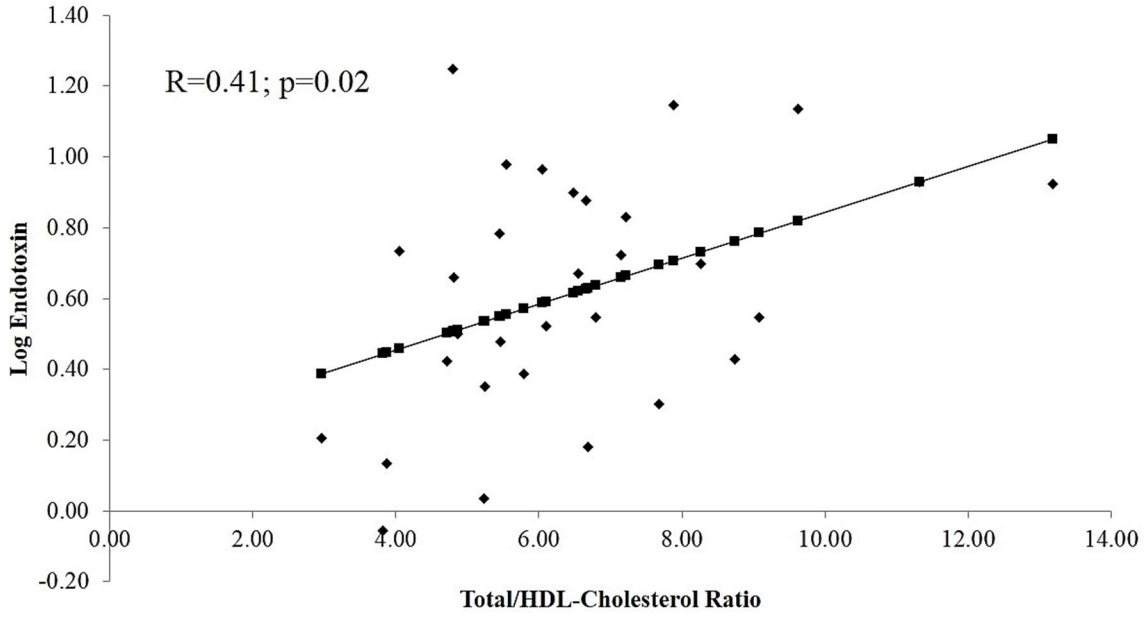


Figure 3.3.5.6. Significant positive association (R=0.42; p=0.02) between log endotoxin and total/HDL-cholesterol in all participants at baseline.

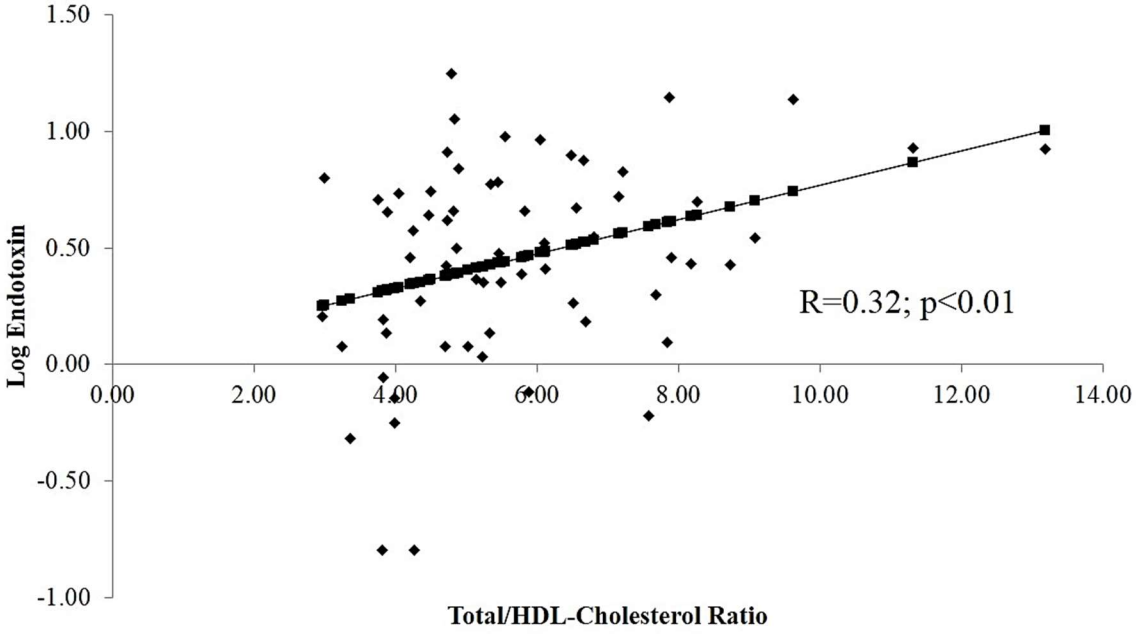


Figure 3.3.5.7. Significant positive association (R=0.32; p<0.01) between log endotoxin and total/HDL-cholesterol in the probiotics group at baseline.

3.4. Discussion

The present protocol is a 3-month randomized, double-blind, placebo-controlled clinical trial on the potential endotoxin-lowering effects of an 8-strain probiotics supplement among participants with T2DM. In this study, within-subject effects indicate significant and favourable changes in the probiotics group post-supplementation in terms of endotoxin, glycaemic and lipid reduction. However, it was observed that between-subjects effects, circulating endotoxin levels in the probiotics group were no different than placebo, yet clinically significant improvement in HOMA-IR and modest reduction in WHR (% change 1.1% versus 0 in placebo; $p<0.01$) in the probiotics group were noted.

The significant associations of endotoxin with lipid components as observed in the present study has been hypothesised to be due to endotoxin's high affinity with chylomicrons as it passes through the gastrointestinal mucosa (Ghoshal et al., 2009). Several other interventional studies demonstrated the immediate effect in lipid patterns as endotoxin levels are altered either through intravenous dose (Hudgins et al., 2003) or through high fat dietary intake (Harte et al., 2012). In the present study, the reduction of circulating endotoxin levels secondary to probiotics supplementation had a parallel improvement in the lipid profile of the probiotics group. Whilst this improvement between groups showed non-significance, it should be noted that even after randomisation, the probiotics group were metabolically worse at baseline, having higher endotoxin levels and worse lipid profile as opposed to the placebo group.

People with T2DM and those with persistent insulin-resistance commonly exhibit higher metabolic endotoxemia than their non-diabetic and non-insulin-resistant counterparts (Gomes et al., 2017). Animal studies demonstrated that increased levels of

circulating insulin may alter intestinal permeability, and this may partially explain higher circulating endotoxin levels among individuals with higher insulin levels. This increased permeability allows gut endotoxins to leak in the circulation, which, in turn, initiates a cascade of inflammatory reactions via the innate immune pathway, thus explaining the subclinical inflammation in obesity and insulin-resistant states (Brun et al., 2007). Some evidence also suggested that the use of probiotics as a supplement may strengthen a weakened intestinal barrier, preventing endotoxin influx in the circulation and ultimately reducing subclinical inflammation (Le Barz et al., 2015). As such by manipulating endotoxin levels through the introduction of probiotics in the digestive tract, it is believed that many endotoxin-induced metabolic disorders can be reversed, if not controlled.

A recent meta-analysis of RCTs among T2DM participants on probiotic supplementation revealed that multiple species of probiotics and interventions longer than 8 weeks had stronger metabolic benefits in terms of improved glucose control and lipid profiles (Hu et al., 2017). The use of the 8-strain probiotics supplement affirmed some of these beneficial effects in reducing abdominal adiposity (measured as WHR) and insulin resistance (HOMA-IR as noted in meta-analysis studies). The lack of significant changes in lipid profile and other indices assessed between groups do not supersede previous findings, as non-significant results may still be clinically meaningful but other factors in play such as the time effects and baseline differences between both groups may have affected the results. The current findings in this study were nevertheless in agreement with a recent double-blind, randomized trial, involving 43 participants (Placebo N=22 and Probiotic mix N=21) who were given 8 weeks supplementation of probiotic mix (*Lactobacillus acidophilus* and *casei*; *Lactococcus lactis*; *Bifidobacterium bifidum* and

lactis; 2×10^{10} colony-forming units/day) and noted a significant reduction in abdominal adiposity with no concomitant decrease in endotoxin levels (Gomes et al., 2017). The mentioned study in comparison to the present one had a shorter duration of intervention, lesser probiotic strains used and had a different primary endpoint as well as cohort used. Nevertheless, three probiotic species used in the former and the present study, namely, *Bifidobacterium bifidum, Lactobacillus acidophilus and Lactobacillus casei*, have been demonstrated to significantly improve glycaemic, inflammatory and lipid profiles of patients with gestational diabetes mellitus after 6 weeks of supplementation as well (Karamali et al., 2016; Badehnoosh et al., 2017). Whilst *Lactococcus lactis*, another potent probiotic species used in this study, has also recently reported to reverse type 2 diabetes in non-obese diabetic mice, in combination with low-dose anti-CD3, through a series of actions including decline in insulin autoantibody positivity and stable reversal of hyperglycemia (Takiishi et al., 2017). Addtionally another bacterial strain, *Lactobacillus salivarius* was also shown to reverse diabetes-induced intestinal defense impairment through reversal of enteric dysbiosis and decreased endotoxin levels in streptozotocin-induced diabetic mice (Chung et al., 2016). Studies using *Lactobacillus salivarius* as a stand-alone probiotic supplement for 4-6 weeks in women with gestational diabetes, however, was not associated with any improvement in metabolic health and pregnancy outcome (Lindsay et al., 2015). These highlight that the effects of probiotics are often species or strain/strains-specific. In this study, most likely, the cumulative potency of the 8 species employed may have contributed to the significant improvements in the HOMA-IR and WHR of the probiotics group. A recent randomized clinical trial involving 136 Malaysians with T2DM supplemented with either placebo or probiotics (*Bifidobacterium*

and *Lactobacillus*) for 12 weeks also showed improvement in terms of glycaemic control (Firouzi et al., 2017), similar to the findings of this study. Despite several trials conducted in the T2DM population, there is still lack in uniformity of findings and this in part may arise due to discrepancies in sample size, duration of treatment, different inclusion criteria and type of analyses performed during each research study.

The subjects in this current study given the probiotic did not elicit significant changes in BMI or body weight over time. This confirms several studies, including the recent meta-analysis by Park and Bae, who concluded limited efficacy of probiotics in weight management (Park and Bae, 2015). However, clinical trials overall are still very limited and therefore current evidence on probiotics, as weight loss agents are at most, suggestive. The significant reduction in abdominal adiposity in the probiotics group shown in the present study however is promising, but the actual changes may not be clinically meaningful, given the short duration of intervention and the small percent change. Furthermore, there was no significant improvements in blood pressure, although a recent study in animal models showed remarkable improvements in blood pressure after 8 week administration of *Lactobacillus casei* (Yap et al., 2014). A recent meta-analysis by Khalesi et al., from 9 clinical trials also concluded that probiotic administration may modestly improve blood pressure, and the potency maybe enhanced if multiple species and strains are taken for more than 8 weeks (Khalesi et al., 2014). Majority of the participants in the present study were normotensive and endothelial function may not have been that compromised. This may partially explain why no significant decrease in blood pressure was observed after probiotics supplementation. Nevertheless, the significant positive association between endotoxin and blood pressure confirms previous findings

(Andrade et al., 2017) and that the beneficial effects of probiotics in improving blood pressure maybe be tested using a duration of supplementation longer than the present study.

The present chapter has several limitations. Gut microbiome analysis was not measured, therefore, successful colonization of these strains in the intestinal tract cannot be confirmed. Dietary intake and physical activity of all participants were also not monitored and this could explain beneficial changes in the placebo group. Despite randomisation and blinding, there were still significant differences between placebo and probiotics group at baseline, with the probiotics group being metabolically worse than placebo, and these covariates were factored during data analyses. Corrections for p-value (Bonferoni) to reduce type 1 error were not done as this would be at the expense of increasing type 2 error, and the sample size is already at the minimal level where sound conclusions can still be derived, although positive results, whether elicited by chance, cannot be ruled out as well. The study's strengths include it's randomized, double-blind, placebo-controlled design and well defined cohort from a unique ethnic group. Despite the large dropout rate from participants, the study remained sufficiently powered and adequately blinded.

3.5. Conclusions

Despite the lack of difference in endotoxin levels between placebo and probiotics group, this study has demonstrated the beneficial effects of a 12-week, multi-strain probiotic supplementation in medication naïve T2DM individuals in terms of improved HOMA-IR and modest reduction in abdominal adiposity. A larger cohort and a longer duration of treatment may be necessary to confirm its effects in abdominal obesity as the present results, though significant, appears very small.

Chapter 4

Effects of a 6-Month Daily Intake of a Multi-Strain Probiotic Supplementation in Circulating Endotoxin Levels, Inflammation, Adipocytokines and Cardiometabolic Profiles of Naïve Saudi T2DM Patients

4.1 Introduction

Disequilibrium in the gut, also known as gut dysbiosis, significantly contributes in the pathogenesis of obesity-related diseases due to a weakened intestinal barrier which leads to chronic low-grade inflammation (Sato et al., 2017). One acceptable theory is that probiotics strengthen the intestinal barrier function, thus preventing leakage of pro-inflammatory stimulants in the circulation (Ling et al., 2016). This theory supports a role for the gut microbiota in the pathogenesis of diet-induced obesity and related metabolic disorders, which, theoretically, might be reversible with dietary and/or gut microbiota manipulation (Ly et al., 2011). In a recent review of 14 clinical trials ascertaining the effects of probiotics on weight loss and body fat, the beneficial effects of probiotics were noted to be strain-specific (Crovesy et al., 2017). In animal studies, treatment with probiotics may be beneficial in insulin-resistant states (Memmarast et al., 2017; Husebye et al., 2001). A few human intervention trials also support this concept, with a recent meta-analysis of 12 studies implicating a clinically improved HbA1c and circulating fasting insulin among patients with T2DM (Yao et al., 2017). Nevertheless, majority of the interventional studies in probiotics amongst patients with T2DM are either short term studies not longer than 3 months and/or that mono-strains were used as supplementation.

The previously known exclusive role of the human adipose tissue as a fat depot has been completely debunked with the discovery of adipocytokines, proteins which are known to mediate metabolism, inflammation and immunity (Tilg and Moschen, 2006). Expansion of adipose tissue during weight gain produces pro-inflammatory adipocytokines which can trigger systemic inflammation responsible for chronic low-grade inflammation observed in obesity-related diseases (*e.g.*, insulin resistance and the

metabolic syndrome) (Pereira and Alvarez-Leite, 2014). Furthermore, obesity-induced inflammation by itself is complex, as it also involves other factors such as the gut microbiota (Frazier et al., 2011). It is now well known that one of the hallmarks of insulin resistance and obesity-related complications is "metabolic endotoxemia", a by-product of a weak or "leaky" gut barrier (Shen et al, 2013). It makes sense that by improving gut health in general, or strengthening the permeability of intestinal barriers in particular, may hold the key in preventing and moderating some of the chronic metabolic disorders prevalent in developed and newly industrialized countries (Bischoff et al., 2014).

Gut microbiota manipulation in the reversal of diet-induced obesity and related metabolic disorders can be achieved most effectively through bariatric surgery as surgery-induced weight loss significantly reduces the amount of body fat, consequently decreasing food intake and altering gut microbiota composition, including levels of adipocytokines (Li and Richard, 2017; Adami et al., 2016). As with all invasive procedures however, bariatric surgery has its own list of risks and complications. Dietary interventions therefore, such as consumption of probiotics/prebiotics, may be the second best option, as these supplements can potentially strengthen the intestinal barrier leading to reduction of systemic endotoxin (lipopolysaccharides of commensal bacteria residing in the gastrointestinal tract). Endotoxins are known to promote sub-chronic inflammation (Noble et al., 2017). As the gut flora is the main source of endotoxin, treatment with probiotics may influence the circulating levels of endotoxin by altering the microbiota composition. To date, however, few studies have examined the effects of probiotics on the circulating levels of endotoxin in metabolic diseases over a 6 month period. Although a small study in patients with cirrhosis given probiotics did lead to a 25% reduction in

endotoxin (Backhed et al., 2005) whilst a further study showed no effects on endotoxin load (Horvath et al., 2016). In a short term study however by Simon and colleagues, they observed that a 4-week intake of *Lactobacillus reuteri* led to an improvement in insulin sensitivity among glucose tolerant individuals, yet no changes in endotoxin levels were observed with increased insulin levels were probably secondary to augmented incretin release (Simon et al., 2015). This lack of change in endotoxin maybe due to the short intervention period. Nevertheless, the ability of probiotics and *Lactobacilli* in particular, in strengthening intestinal integrity and potentially reducing endotoxin levels, have been demonstrated consistently, with the most recent study examining the permeability change in the gut through trans-epithelial resistance (TER) tests in rat models with necrotizing enterocolitis (NEC) injected with the probiotic strain (Blackwood et al., 2017). Other probiotic strains that were observed to reduce endotoxemia include the *Bacillus* species (McFarlin et al., 2017) and *Bifidobacteria* through enhancement of intestinal barrier function (Yang et al., 2017).

To the best of our knowledge, there is still limited evidence on the effects of a long duration, multi-strain probiotics supplementation on circulating endotoxin levels and their concomitant effects in adipocytokines and inflammatory markers among naïve T2DM patients. Furthermore, data is scarce with regards to the effects of these supplements in the Arabian population, an ethnic group highly predisposed to obesity and insulin-resistant-related diseases (Rahim et al., 2014). The present study therefore explored the potential beneficial effects of a 6-month multi-strain probiotics supplementation on these biomarkers and cardiometabolic parameters in adult Saudi participants with naïve T2DM.

4.2 Methods

This is a 6-month, single-centre, double-blind, randomised, placebo-controlled study. Ethical approval and trial registrations have been mentioned previously (Chapter 2.1, 2.2).

4.2.1 Participants

Detailed recruitment of participants has been mentioned in Chapter 2.5. A flowchart has been provided previously (Figure 2.4.1).

4.2.2 Probiotic Supplements and Allocation

Detailed trial protocol has been mentioned previously in Chapter 2.5

4.2.3 Monitoring and Blood Sample Collection

Measurements of all carediometabolic parameters, endotoxin, inflammation and adipocytokines have been described in detail in Chapter 2.9 and 2.10.

4.2.4 Data Analysis

Detailed data analysis has been previously mentioned in Chapter 2.11. In addition and specific to this chapter, mixed method analysis of covariance (ANCOVA) was used to determine within and between group changes adjusted for baseline covariates which included leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio. For the purpose of this chapter, the first 3 covariates were excluded from the presentation as they are discussed separately in the succeeding chapter. P-value <0.05 was considered statistically significant.

4.3 Results

4.3.1 Baseline Characteristics of Placebo and Probiotics Group

Baseline comparison of placebo and probiotics groups has been previously presented in table 3.3.1.1.

4.3.2 Changes in Anthropometrics and Clinical Measures in both Placebo and Probiotics Group Before and after 6-month Intervention

Table 4.3.2 shows within and between group comparisons in the anthropometric and clinical measures of both placebo and probiotics group using ITT analysis. In this approach, there was a significant over-all difference between placebo and probiotics in WHR (p=0.004) as well as both in 3 months (p=0.005) and 6 months of intervention (p=0.01) despite having no discernible difference in both groups in terms of percentage change (figure 4.3.2.1). There were no significant differences in BMI between placebo and probiotics over-all (p=0.35) as well as at 3 months [% change 0.10 vs -0.10; (CI: -6.35 – 2.14); p=0.32] and 6 months post-intervention [-0.40 vs 0.0; (CI: -6.09 - 2.34; p=0.38] (figure 4.3.2.2). Over-all at 6 months post-intervention, between group comparisons also showed no differences in systolic blood pressure (p=0.65) (Figure 4.3.2.3), diastolic blood pressure (p=0.83) (Figure 4.3.2.4) and mean arterial blood pressure (p=0.97) (Figure 4.3.2.5). Within group comparisons showed that both the placebo and probiotics groups also had no significant changes in all anthropometric and clinical measures after 3 and 6 months of intervention (Table 4.3.2).

Table 4.3.2. Anthropometric measures before and after intervention with placebo or probiotics among T2DM patients (ITT Analysis)

Parameters	Group		Intervention Effects (95% CI)		
	Placebo (N = 30)	Probiotics (N = 31)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
BMI (kg/m^2)					
Baseline	30.1 ± 5.0	29.4 ± 5.2	-2.10 (-6.35 - 2.14)	-1.88 (-6.09 - 2.34)	-1.96 (-6.20 - 2.24)
3 months	30.2 ± 5.0	29.3 ± 5.3			
6 months	29.7 ± 5.0	29.4 ± 5.2			
$\bar{X}$ (% Change) at 3 months	0.10 (0.33)	-0.10 (-0.34)	0.32	0.38	0.35
$\bar{X}$ (% Change) at 6 months	-0.40 (-1.33)	0.00 (0.00)			
Waist-Hip Ratio					
Baseline	1.0 ± 0.1	0.91 ± 0.1	-0.09 (-0.14 - -0.03)	-0.08 (-0.13 - -0.02)	-0.08 (-0.14 - -0.03)
3 months	1.0 ± 0.1	0.87 ± 0.1			
6 months	1.0 ± 0.1	0.86 ± 0.1			
$\bar{X}$ (% Change) at 3 months	0.0 (0.0)	0.03 (0.001)	0.005	0.01	0.004
$\bar{X}$ (% Change) at 6 months	0.0 (0.0)	0.5 (0.002)			
Systolic Blood Pressure (mmHg)					
Baseline	129.5 ± 10.3	134.8 ± 14.6	-2.33(-10.89 - 6.23)	-1.13 (-9.81 - 7.56)	-1.98 (-10.4 - 6.47)
3 months	129.9 ± 11.1	129.0 ± 11.4			
6 months	129.2 ± 11.3	130.6 ± 12.5			
$\bar{X}$ (% Change) at 3 months	0.40 (0.31)	-5.80 (-4.30)	0.59	0.80	0.64
$\bar{X}$ (% Change) at 6 months	-0.30 (-0.23)	-4.20 (-3.12)			
Diastolic Blood Pressure (mmHg)					
Baseline	78.6 ± 8.6	83.6 ± 11.8	0.45 (-6.99 - 7.88)	2.07 (-6.20 - 10.33)	0.81 (-6.74 - 8.37)
3 months	79.8 ± 8.1	79.8 ± 11.5			
6 months	77.3 ± 9.1	81.0 ± 11.7			
$\bar{X}$ (% Change) at 3 months	1.20 (1.53)	-3.80 (-4.55)	0.90	0.62	0.83
$\bar{X}$ (% Change) at 6 months	-1.30 (-1.65)	-2.60 (-3.11)			
Mean Arterial Pressure (mmHg)					
Baseline	95.5 ± 7.7	100.6 ± 11.1	-0.48 (-7.16 - 6.21)	1.00 (-6.16 - 8.17)	-0.12 (-6.81 - 6.57)
3 months	96.5 ± 7.8	96.2 ± 9.7			
6 months	100.7 ± 11.1	97.5 ± 9.9			
$\bar{X}$ (% Change) at 3 months	1.00 (1.05)	-4.40 (-4.37)	0.89	0.78	0.97
$\bar{X}$ (% Change) at 6 months	5.20 (5.45)	-3.10 (-3.08)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups overall; Results are obtained from mixed method ANCOVA after adjustment for baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio; CI – confidence interval; significance at $p<0.05$.

Table 4.3.3 shows within and between group comparisons in the anthropometric and clinical measures of both placebo and probiotics group using PPA. Between group comparison showed an over-all significant difference in BMI in both placebo and probiotics post-intervention [-0.50 vs 0.20; (CI: -10.7 - -0.14); p = 0.04] as well as WHR [0.0 vs 0.0; (CI: -0.16 - -0.01); p = 0.03]. Similar to the ITT analysis, there were no difference between groups over-all in systolic (p=0.51); diastolic (p=0.82) and mean arterial pressure (p=0.86). Within group comparisons showed no significant changes in both groups (Table 4.3.3). Mean values of anthropometric measures were plotted as bar charts in both placebo and probiotics group (Figures 4.3.1-4.3.5).

Table 4.3.3. Anthropometric measures before and after intervention with placebo or probiotics among T2DM patients (PP Analysis)

Parameter	Group		Intervention Effects (95% CI)		
	Placebo (N = 16)	Probiotics (N = 23)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
BMI (kg/m^2)					
Baseline	29.1 ± 4.9	27.3 ± 4.1	-1.59 (-6.65 - 3.46)	-5.43(-10.64- -0.23)	-5.44(-10.7 - -0.14)
3 months	29.4 ± 5.0	27.4 ± 4.2			
6 months	28.6 ± 4.8	27.5 ± 3.9			
$\bar{X}$ (% Change) at 3 months	0.30 (1.03)	0.10 (0.37)	0.53	0.04	0.04
$\bar{X}$ (% Change) at 6 months	-0.50 (-1.72)	0.20 (0.73)			
Waist-Hip Ratio					
Baseline	0.92 ± 0.08	0.89 ± 0.06	-0.09 (-0.16- -0.03)	-0.11 (-0.19 - -0.03)	-0.09 (-0.16 - -0.01)
3 months	0.91 ± 0.05	0.90 ± 0.08			
6 months	0.90 ± 0.06	0.90 ± 0.08			
$\bar{X}$ (% Change) at 3 months	0.0 (0.0)	0.0 (0.0)	0.005	0.01	0.03
$\bar{X}$ (% Change) at 6 months	0.0 (0.0)	0.0 (0.0)			
Systolic Blood Pressure (mmHg)					
Baseline	129.8 ± 8.1	133.4 ± 13.4	-4.74 (-15.75- 6.28)	-3.1 (-16.89- 10.7)	-4.4 (-18.23 - 9.39)
3 months	129.9 ± 8.7	128.3 ± 10.5			
6 months	128.6 ± 8.9	130.9 ± 11.1			
$\bar{X}$ (% Change) at 3 months	0.10 (0.08)	-5.10 (-3.82)	0.39	0.65	0.51
$\bar{X}$ (% Change) at 6 months	-1.20 (-0.92)	-2.50 (-1.87)			
Diastolic Blood Pressure (mmHg)					
Baseline	76.2 ± 9.0	83.9 ± 11.0	1.71 (-8.37 - 11.80)	3.77 (-7.74 - 15.28)	1.07 (-8.55 - 10.69)
3 months	81.1 ± 5.5	80.1 ± 7.5			
6 months	75.5 ± 9.3	81.8 ± 8.4			
$\bar{X}$ (% Change) at 3 months	4.90 (6.43)	-3.80 (-4.53)	0.73	0.50	0.82
$\bar{X}$ (% Change) at 6 months	-0.70 (-0.92)	-2.10 (-2.50)			
Mean Arterial Pressure (mmHg)					
Baseline	94.1 ± 7.1	100.4 ± 10.6	-0.44 (-9.46 - 8.58)	1.48 (-8.42 - 11.38)	-0.76 (-9.99 - 8.47)
3 months	97.4 ± 5.0	96.1 ± 7.6			
6 months	93.2 ± 7.5	98.1 ± 8.1			
$\bar{X}$ (% Change) at 3 months	3.30 (3.51)	-4.30 (-4.28)	0.92	0.76	0.86
$\bar{X}$ (% Change) at 6 months	-0.90 (-0.96)	-2.30 (-2.29)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups over-all; Results are obtained from mixed method ANCOVA after adjustment for baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio;; CI – confidence interval; significance at $p<0.05$.

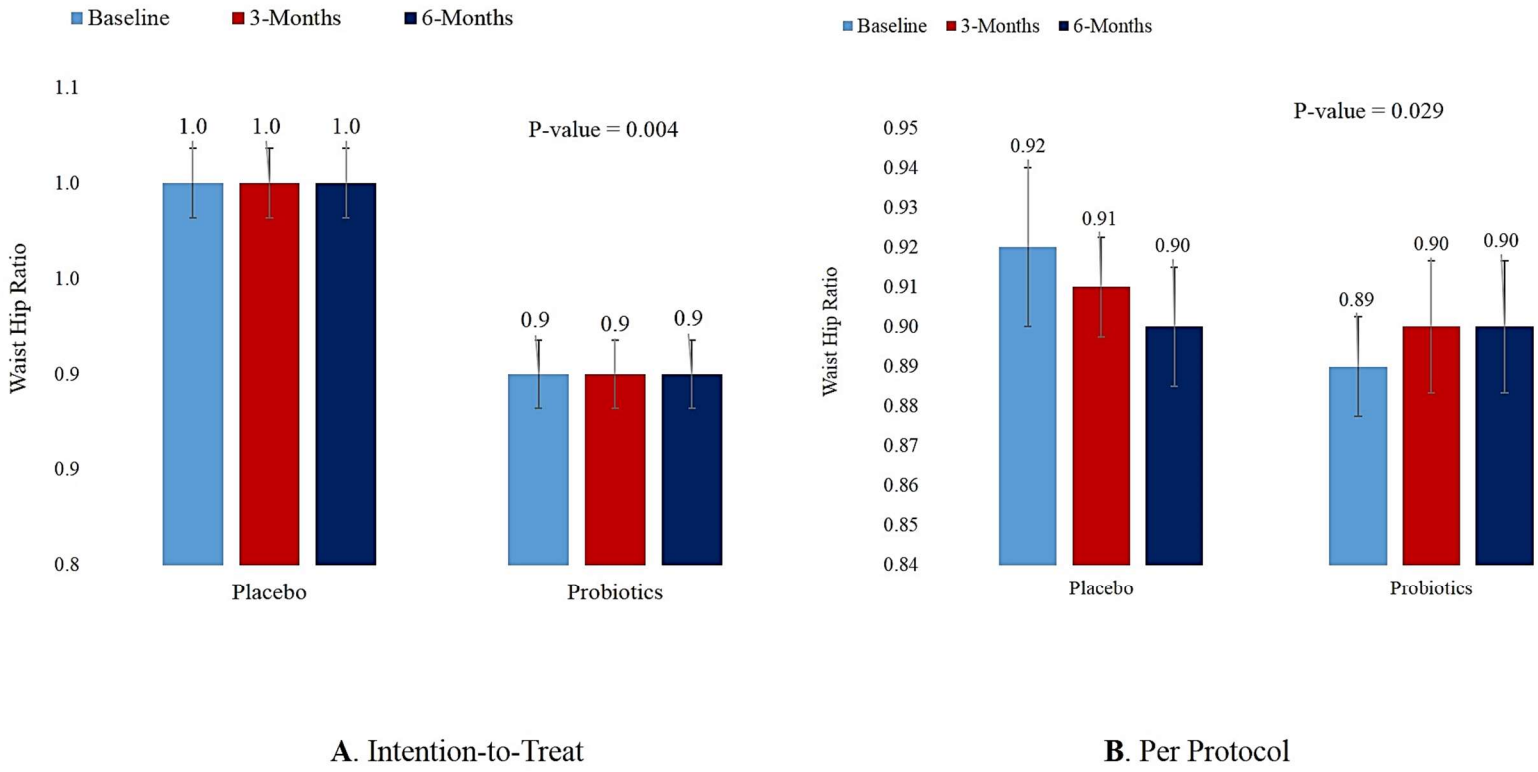


Figure 4.3.2.1 Mean waist-hip ratio before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; significant at $p<0.05$.

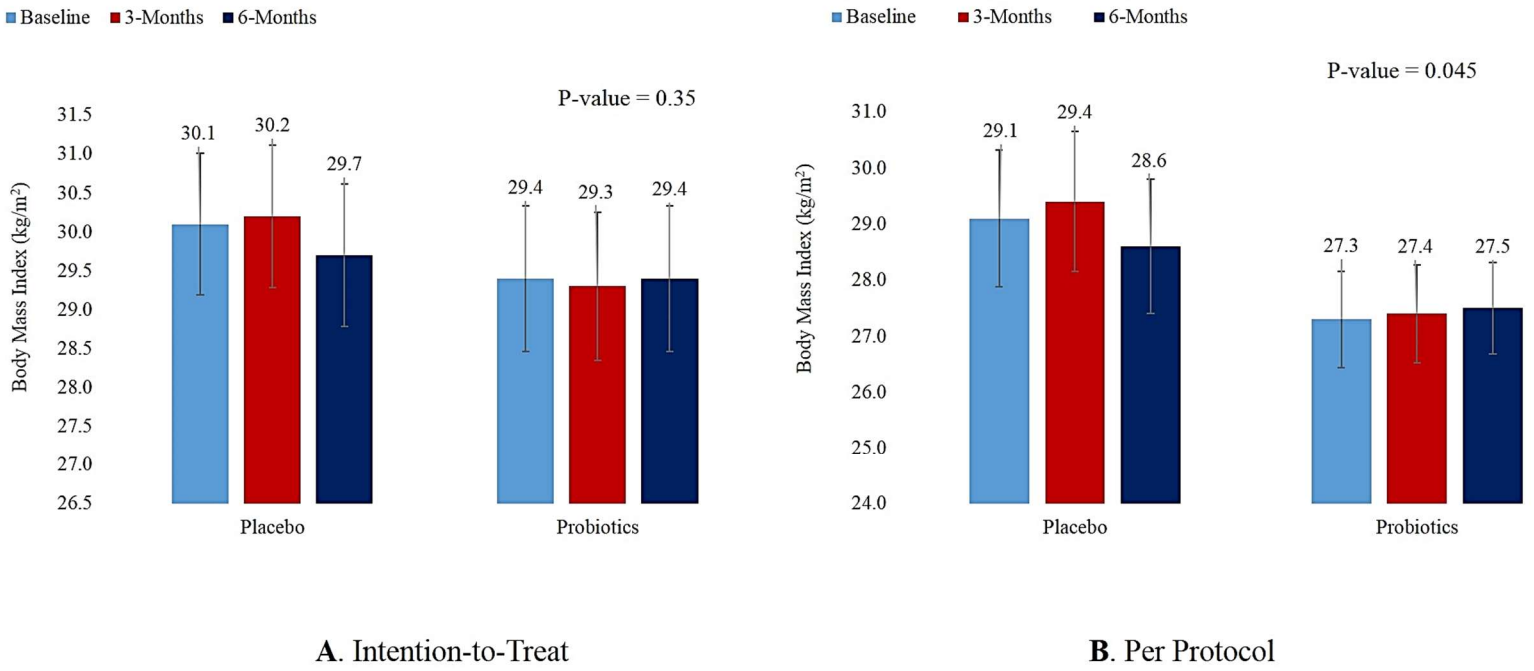


Figure 4.3.2.2 Mean BMI (kg/m^2) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; significant at $p<0.05$.

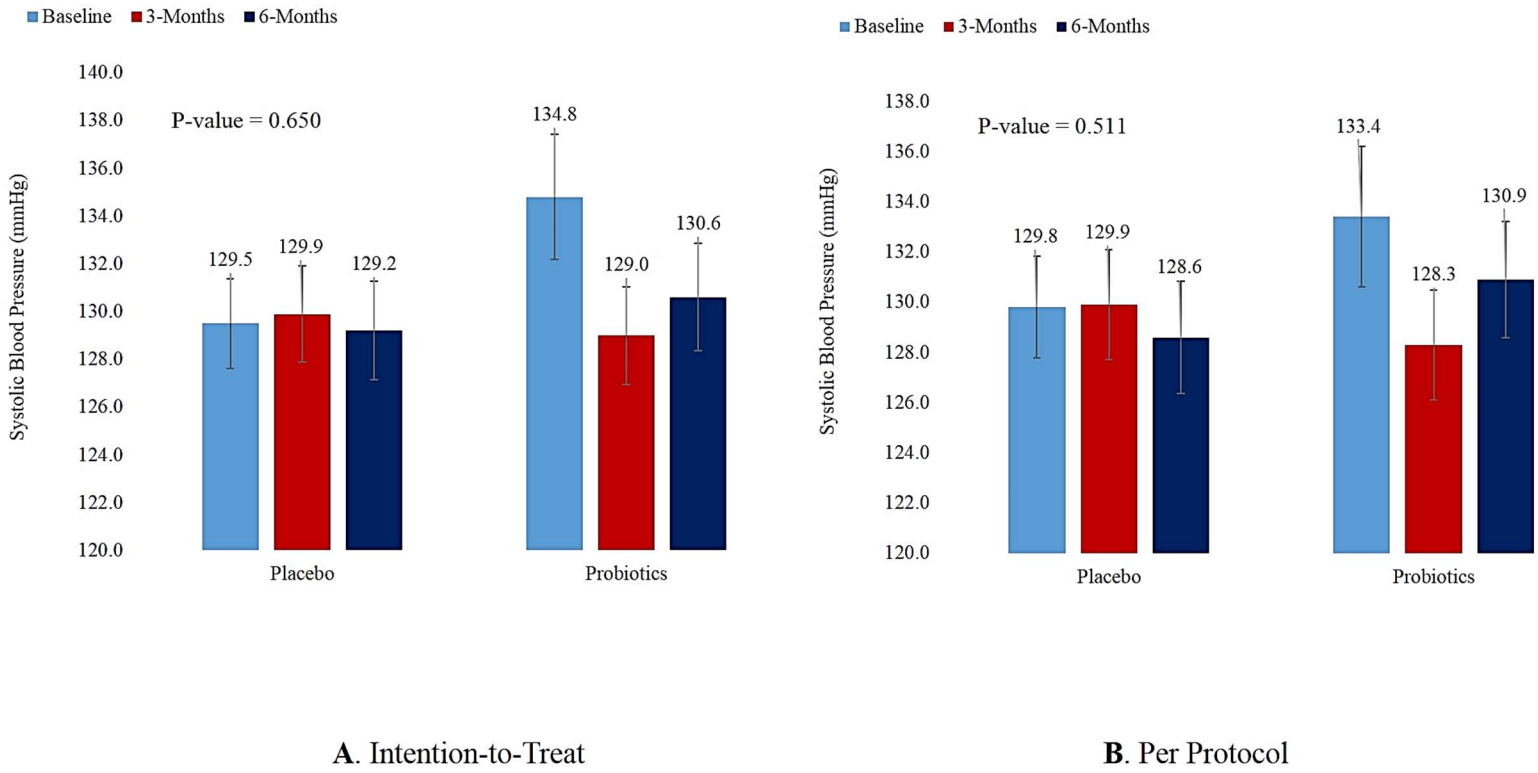


Figure 4.3.2.3 Mean systolic blood pressure (mmHg) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; significant at $p<0.05$.

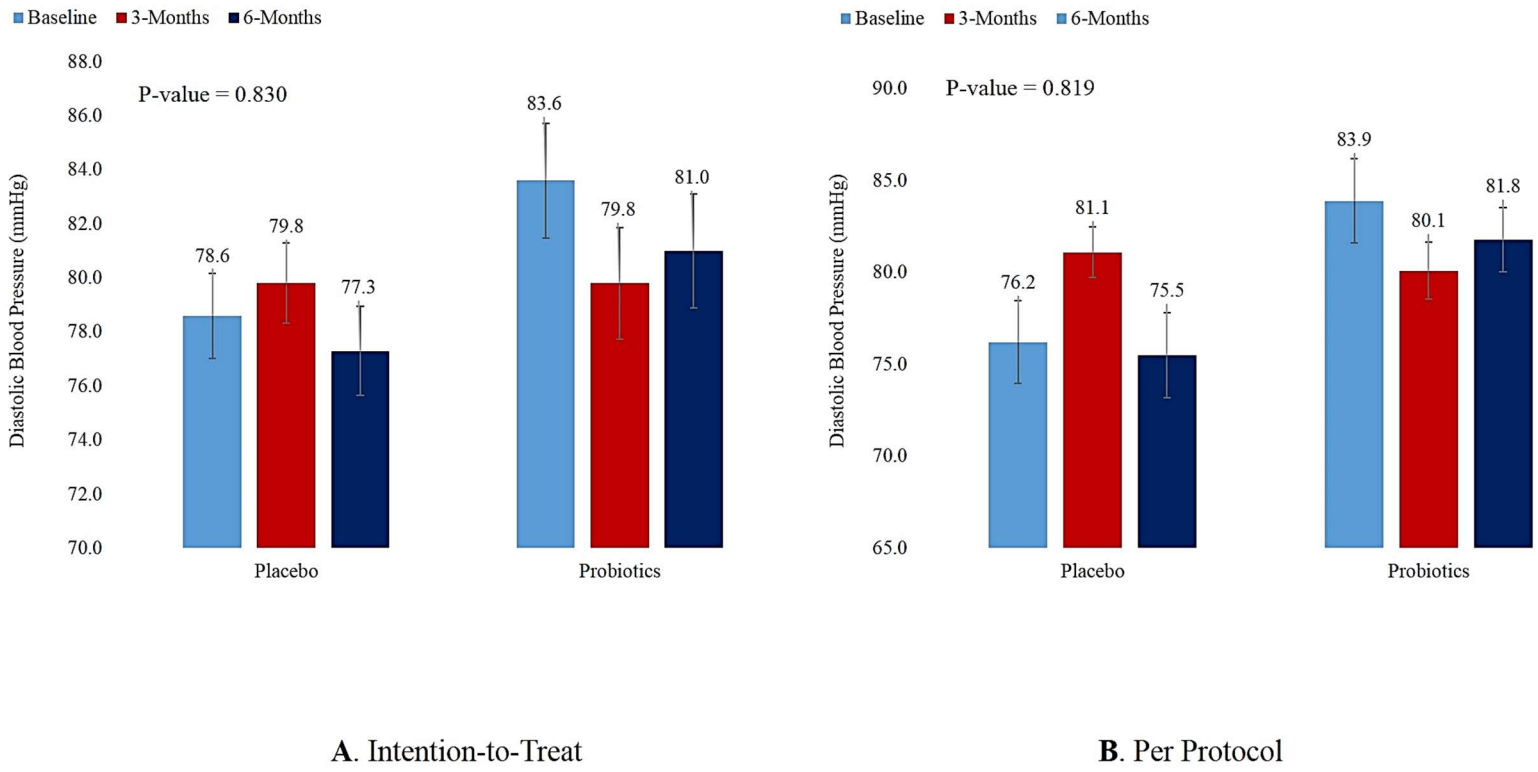


Figure 4.3.2.4 Mean diastolic blood pressure (mmHg) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; significant at $p<0.05$.

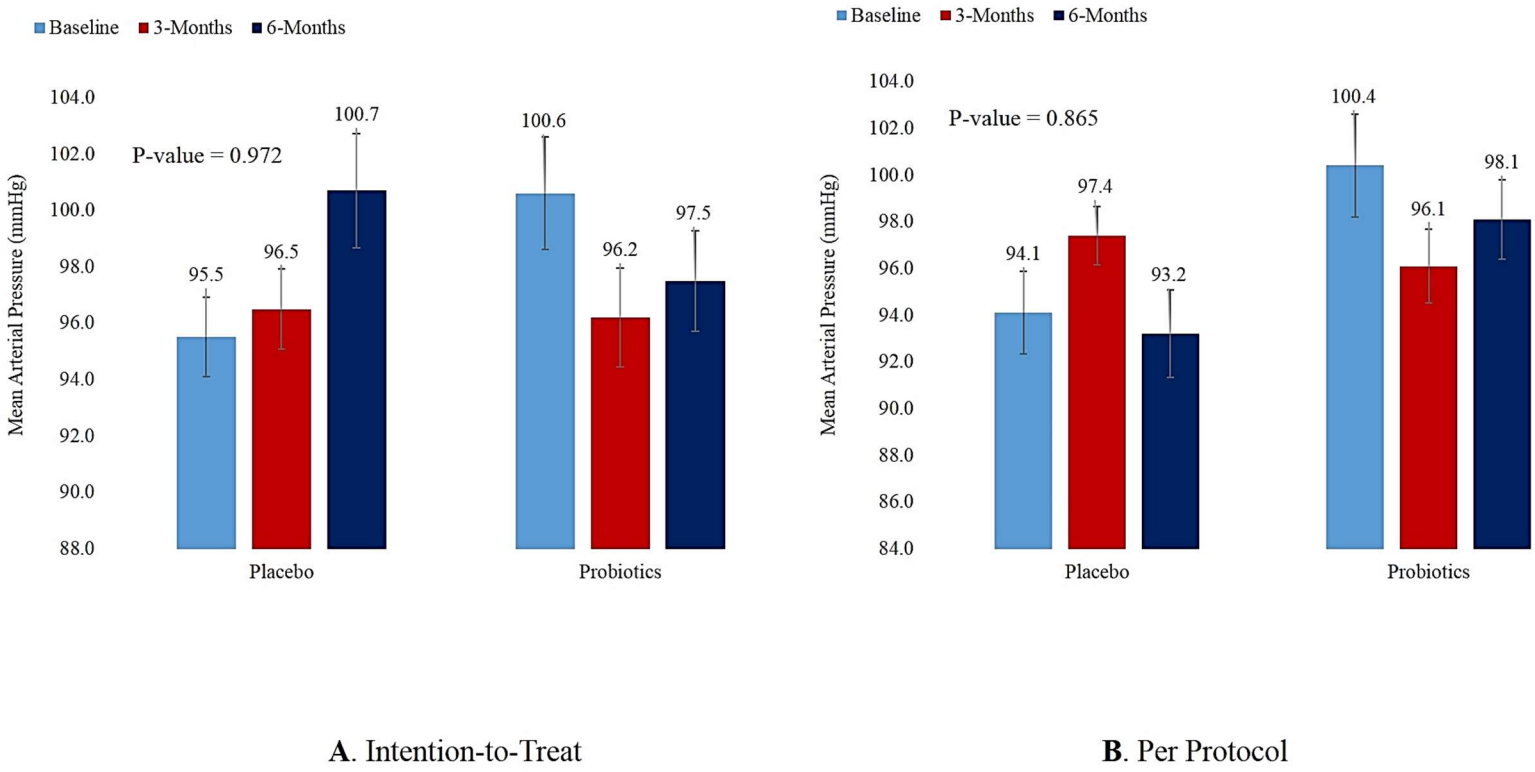


Figure 4.3.2.5 Mean arterial pressure (mmHg) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; significant at $p<0.05$.

4.3.3 Changes in Glycaemic Indices in both Placebo and Probiotics Group Before and after 6-month Intervention

Table 4.3.4 shows the between and within group comparisons between placebo and probiotics groups over time in terms of glycaemic indices. No difference was observed over-all in glucose levels between placebo and probiotics groups [1.1 vs -4.5 (CI: -0.07 – 0.14); p = 0.54] (figure 4.3.3.1). A borderline significance was observed in insulin levels [-0.30 vs – 3.80 (CI: -0.40 – 0.01); p = 0.07] (figure 4.3.3.2). No difference was observed in C-peptide levels [0.80 vs -0.30 (CI:-0.22 – 0.61); p = 0.34] (Figure 4.3.3.3) and an over-all significant difference was noted in HOMA-IR [0.80 vs -3.40 (CI: -0.59 - -0.17); p = 0.001] (Figure 4.3.3.4). Within group comparisons showed that in the placebo group, there was a significant increase in c-peptide levels at 6 months as compared to both baseline and 3 months ($p<0.05$). The rest of the glycaemic parameters in the placebo group did not significantly change over time. In the probiotics group, a significant decrease was observed in median levels of glucose and insulin after 3 months and a further significant decrease after 6 months. Median levels of c-peptide significantly decreased after 6 months in the probiotics group. A significant decrease was also noted in the HOMA-IR over time in both 3 months ($p<0.05$) and 6 months ($p<0.05$) post-intervention (Table 4.3.4).

Table 4.3.4. Glycaemic parameters before and after intervention with placebo or probiotics among T2DM patients (ITT Analysis)

Parameters	Group		Intervention Effects (95% CI)		
	Placebo (N = 30)	Probiotics (N = 31)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
Glucose (mmol/L)					
Baseline	7.0 (5.7 - 11.2)	11.7 (8.4 - 16.4)	0.10 (-0.01 - 0.21)	0.07 (-0.04 - 0.18)	0.03 (-0.07 - 0.14)
3 months	8.0 (5.9 - 11.4)	8.5 (6.2 - 10.9)^A			
6 months	8.1 (6.9 - 11.4)	7.2 (5.3 - 9.1)^{AB}			
$\bar{X}$ (% Change) at 3 months	1.00 (14.29)	-3.20 (-27.35)	0.08	0.19	0.54
$\bar{X}$ (% Change) at 6 months	1.10 (15.71)	-4.50 (-38.46)			
Insulin (IU/ml)					
Baseline	12.4 (8.0 - 18.7)	9.9 (7.7 - 16.4)	-0.12(-0.31 - 0.07)	-0.19(-0.41 - 0.03)	-0.20(-0.40 - 0.01)
3 months	10.8 (8.3 - 15.5)	6.9 (4.5 - 9.8)^A			
6 months	12.1 (8.0 - 17.4)	6.1 (3.6 - 9.6)^A			
$\bar{X}$ (% Change) at 3 months	-1.60 (-12.90)	-3.00 (-30.30)	0.20	0.09	0.07
$\bar{X}$ (% Change) at 6 months	-0.30 (-2.42)	-3.80 (-38.38)			
C-peptide (ng/ml)					
Baseline	0.1 (0.1 - 0.5)	0.4 (0.0 - 1.8)	0.44 (-0.02 - 0.90)	0.24 (-0.16 - 0.65)	0.20 (-0.22 - 0.61)
3 months	0.2 (0.1 - 0.9)	0.1 (0.0 - 0.3)^A			
6 months	0.9 (0.1 - 1.9)^A	0.1 (0.0 - 0.4)			
$\bar{X}$ (% Change) at 3 months	0.10 (100.00)	-0.30 (-75.00)	0.06	0.23	0.34
$\bar{X}$ (% Change) at 6 months	0.80 (800.00)	-0.30 (-75.00)			
HOMA-IR					
Baseline	3.9 (2.3 - 6.5)	5.3 (3.5 - 10.2)	-0.21(-0.41- -0.02)	-0.34(-0.55- -0.12)	-0.38(-0.59- -0.17)
3 months	3.9 (3.3 - 6.0)	2.1 (1.5 - 5.2)^A			
6 months	4.7 (3.6 - 6.7)	1.9 (1.2 - 3.1)^A			
$\bar{X}$ (% Change) at 3 months	0.00 (0.00)	-3.20 (-60.38)	0.03	0.004	0.001
$\bar{X}$ (% Change) at 6 months	0.80 (20.51)	-3.40 (-64.15)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups over-all; ^{A} denotes significance within groups compared to baseline; ^{B} denotes significance within groups compared to 3 monthsResults are obtained from mixed method ANCOVA after adjustment for baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio; CI – confidence interval; significance at $p<0.05$.

Table 4.3.5 shows the same glycaemic comparisons between groups using the PPA. No difference was noted in levels of glucose [0.6 vs -5.30; (CI: -0.05 – 0.230; p = 0.19], insulin [2.6 vs -6.7; (CI:-0.56 – 0.11); p = 0.18] and C-peptide [1.2 vs -1.0; (CI: -0.52 – 0.53); p = 0.99]. A borderline significant difference was observed in HOMA-IR [1.44 vs -5.30; (CI: -0.76 – 0.01); p = 0.05]. Within group comparisons showed a significant decrease in glucose levels after 3 months ($p<0.05$) and 6 months ($p<0.05$) in the probiotics group as well as insulin (both p-values <0.05 at 3 and 6 months, respectively), c-peptide (both p-values <0.05 at 3 and 6 months, respectively) and HOMA-IR (both p-values <0.05 at 3 and 6 months, respectively). In the placebo group, c-peptide levels were significantly higher after 6 months of intervention compared to baseline ($p<0.05$) (see Table 4.3.5). Changes in all glycaemic indices over time are also presented in Figures 4.3.3.1-4.3.3.4.

Table 4.3.5. Glycaemic measures before and after intervention with placebo or probiotics among T2DM patients (PP Analysis)

Parameter	Group		Intervention Effects (95% CI)		
	Placebo (N = 16)	Probiotics (N = 23)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
Glucose (mmol/L)					
Baseline	6.9 (5.3 - 8.0)	12.3 (8.7 - 16.9)	0.09 (-0.04 - 0.23)	0.12 (-0.02 - 0.27)	0.09 (-0.05 - 0.23)
3 months	7.2 (5.9 - 13.1)	8.5 (6.5 - 10.2)^A			
6 months	7.5 (6.7 - 11.4)	7.0 (5.3 - 8.4)^A			
$\bar{X}$ (% Change) at 3 months	0.30 (4.35)	-3.80 (-30.89)	0.18	0.09	0.19
$\bar{X}$ (% Change) at 6 months	0.60 (8.70)	-5.30 (-43.09)			
Insulin (IU/ml)					
Baseline	14.6 (8.8 - 24.9)	12.1 (8.8 - 14.7)	-0.14(-0.37 - 0.08)	-0.18(-0.56 - 0.20)	-0.22(-0.56 - 0.11)
3 months	13.6 (9.6 - 19.3)	6.9 (4.5 - 9.5)^A			
6 months	17.2(12.1 - 21.3)	5.4 (3.6 - 9.1)^A			
$\bar{X}$ (% Change) at 3 months	-1.00 (-6.85)	-5.20 (-42.98)	0.21	0.34	0.18
$\bar{X}$ (% Change) at 6 months	2.60 (17.81)	-6.70 (-55.37)			
C-peptide (ng/ml)					
Baseline	0.2 (0.1 - 0.5)	1.1 (0.2 - 2.0)	0.55 (0.03 - 1.07)	-0.14(-0.63 - 0.34)	0.00 (-0.52 - 0.53)
3 months	0.1 (0.1 - 0.6)	0.2 (0.1 - 0.3)^A			
6 months	1.4 (0.5 - 2.0)^{AB}	0.1 (0.1 - 0.2)^A			
$\bar{X}$ (% Change) at 3 months	-0.10 (-50.00)	-0.90 (-81.82)	0.04	0.54	0.99
$\bar{X}$ (% Change) at 6 months	1.20 (600.00)	-1.00 (-90.91)			
HOMA-IR					
Baseline	4.06 (2.5-12.3)	7.2 (4.7-11.0)	-0.24(-0.49 - 0.01)	-0.24(-0.66 - 0.18)	-0.38(-0.76 - 0.01)
3 months	4.5 (3.1-6.5)	2.6 (1.7-4.5)^A			
6 months	5.5 (4.1-6.7)	1.9 (1.2-2.5)^A			
$\bar{X}$ (% Change) at 3 months	0.44 (10.84)	-4.60 (-63.89)	0.06	0.24	0.05
$\bar{X}$ (% Change) at 6 months	1.44 (35.47)	-5.30 (-73.61)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups over-all; ^{A} denotes significance within groups compared to baseline; ^{B} denotes significance within groups compared to 3 months; Results are obtained from mixed method ANCOVA after adjustment for baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio; CI – confidence interval; significance at $p<0.05$.

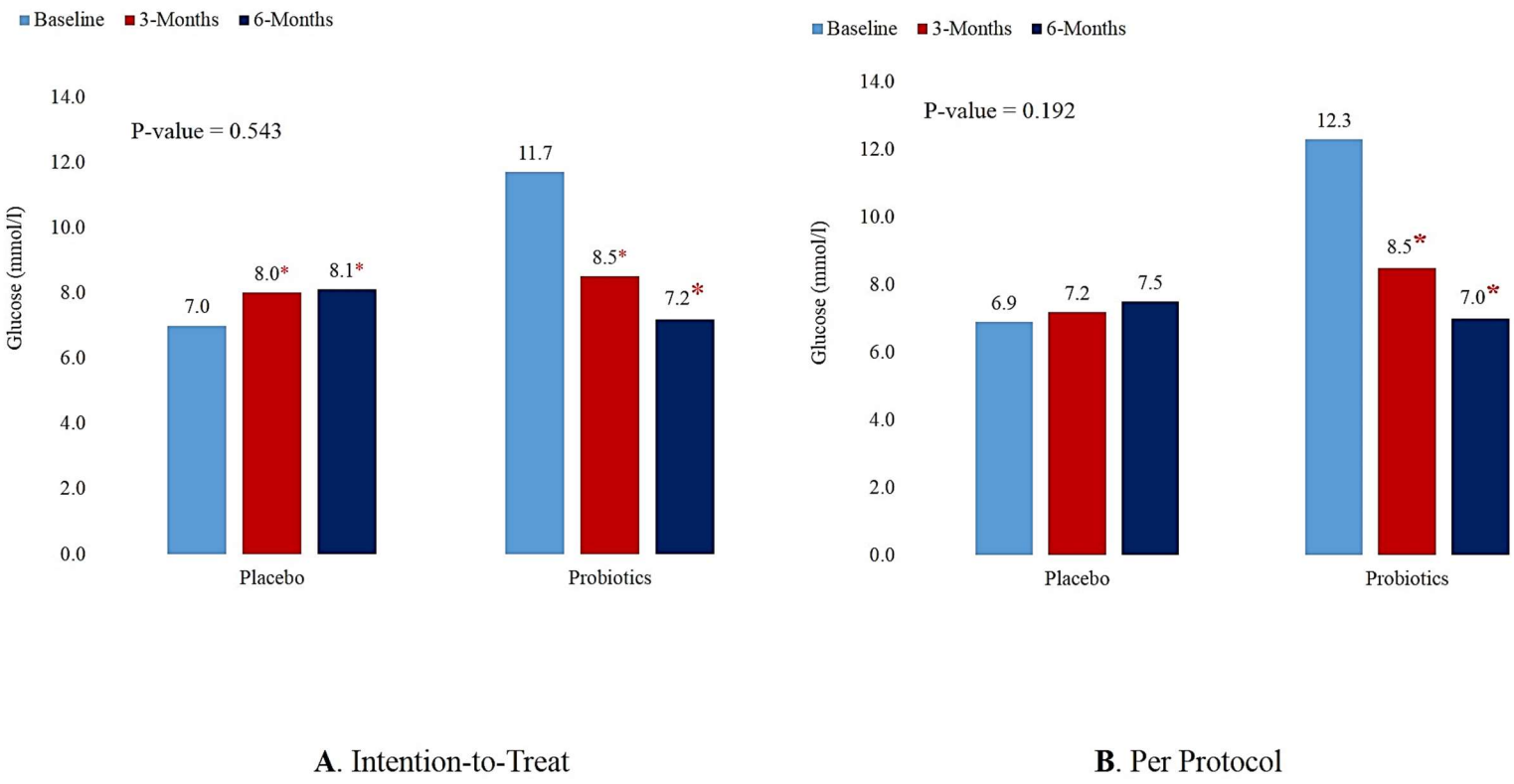


Figure 4.3.3.1 Median glucose (mmol/L) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

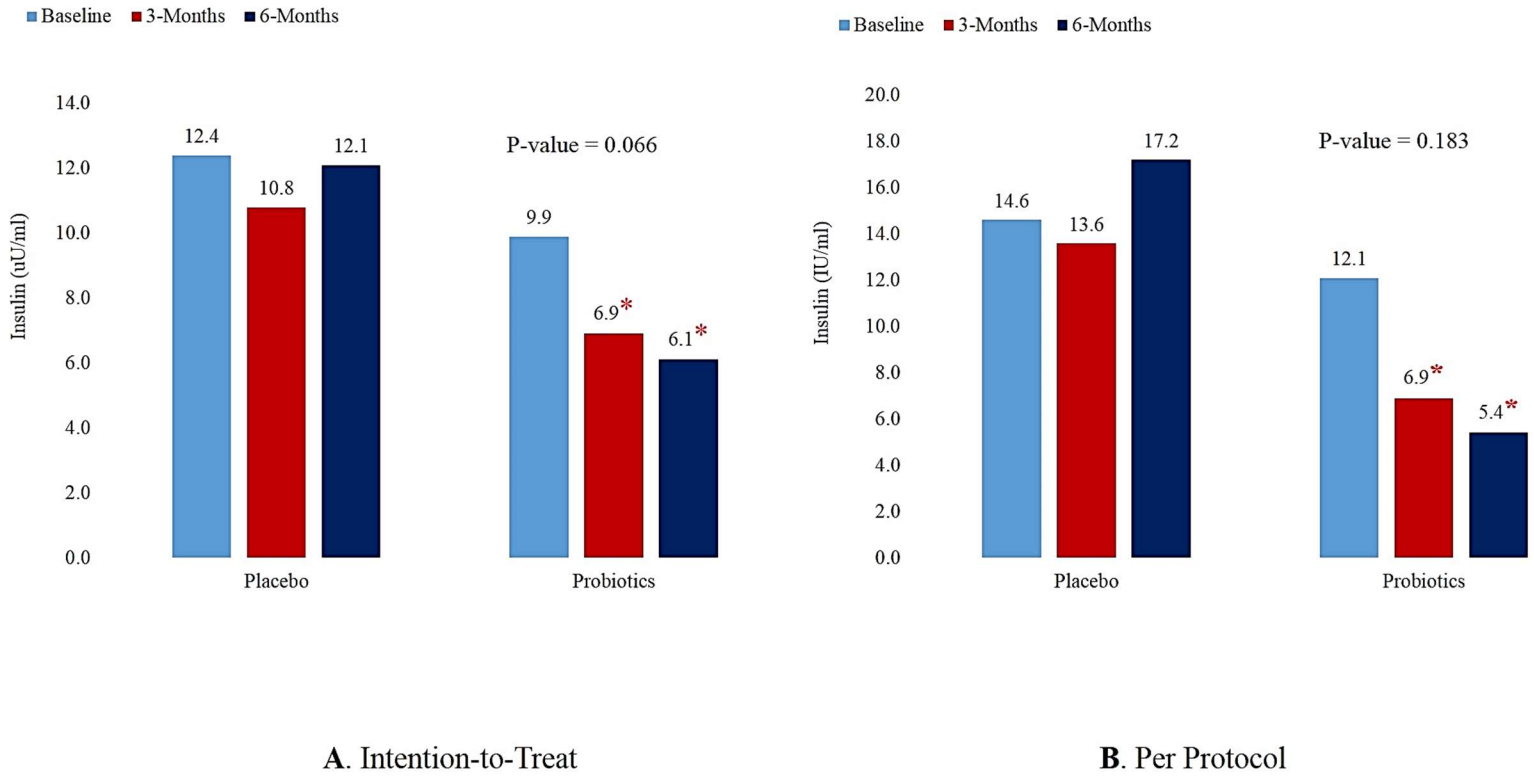


Figure 4.3.3.2 Median insulin (IU/ml) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

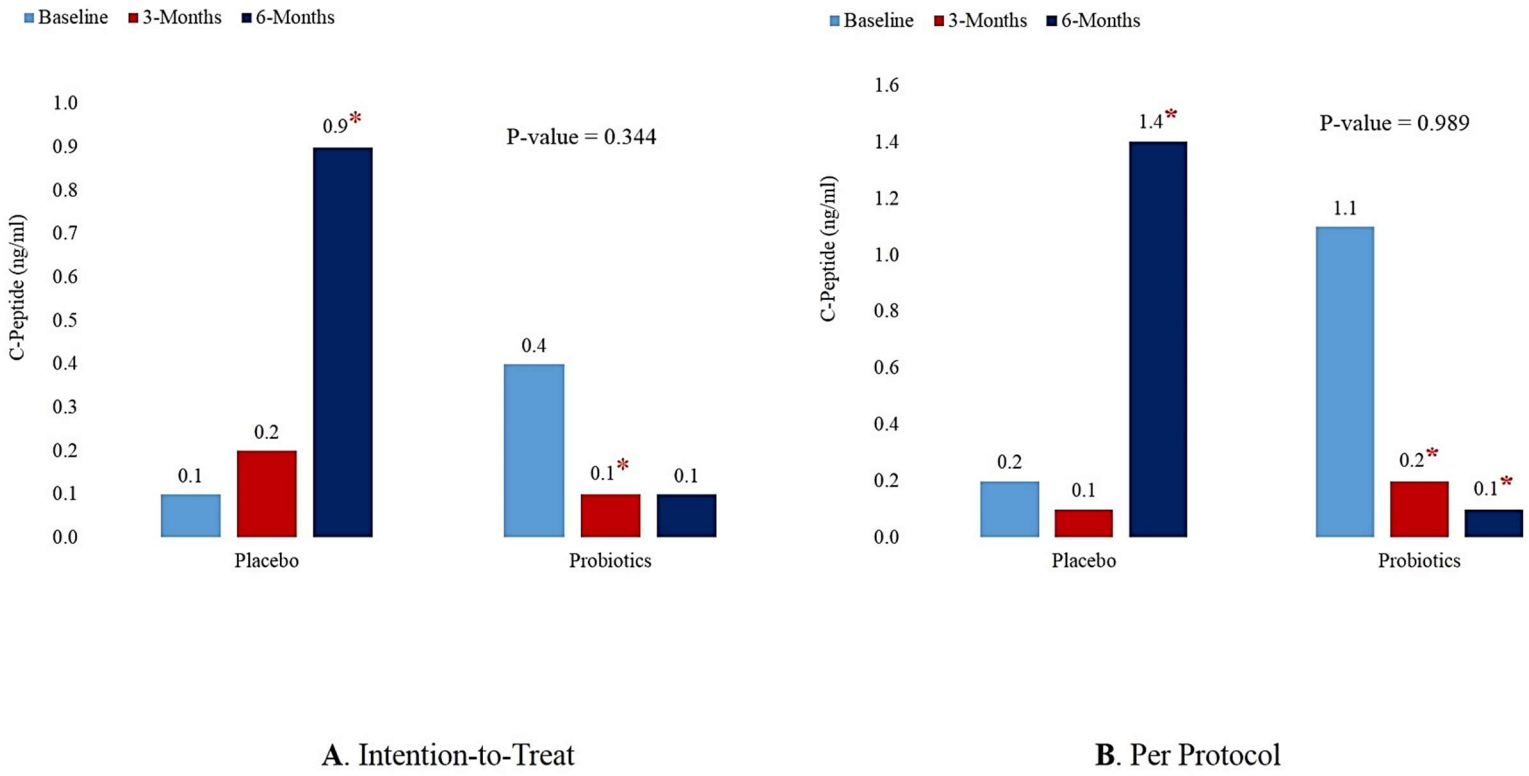


Figure 4.3.3.3 Median C-peptide (ng/ml) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

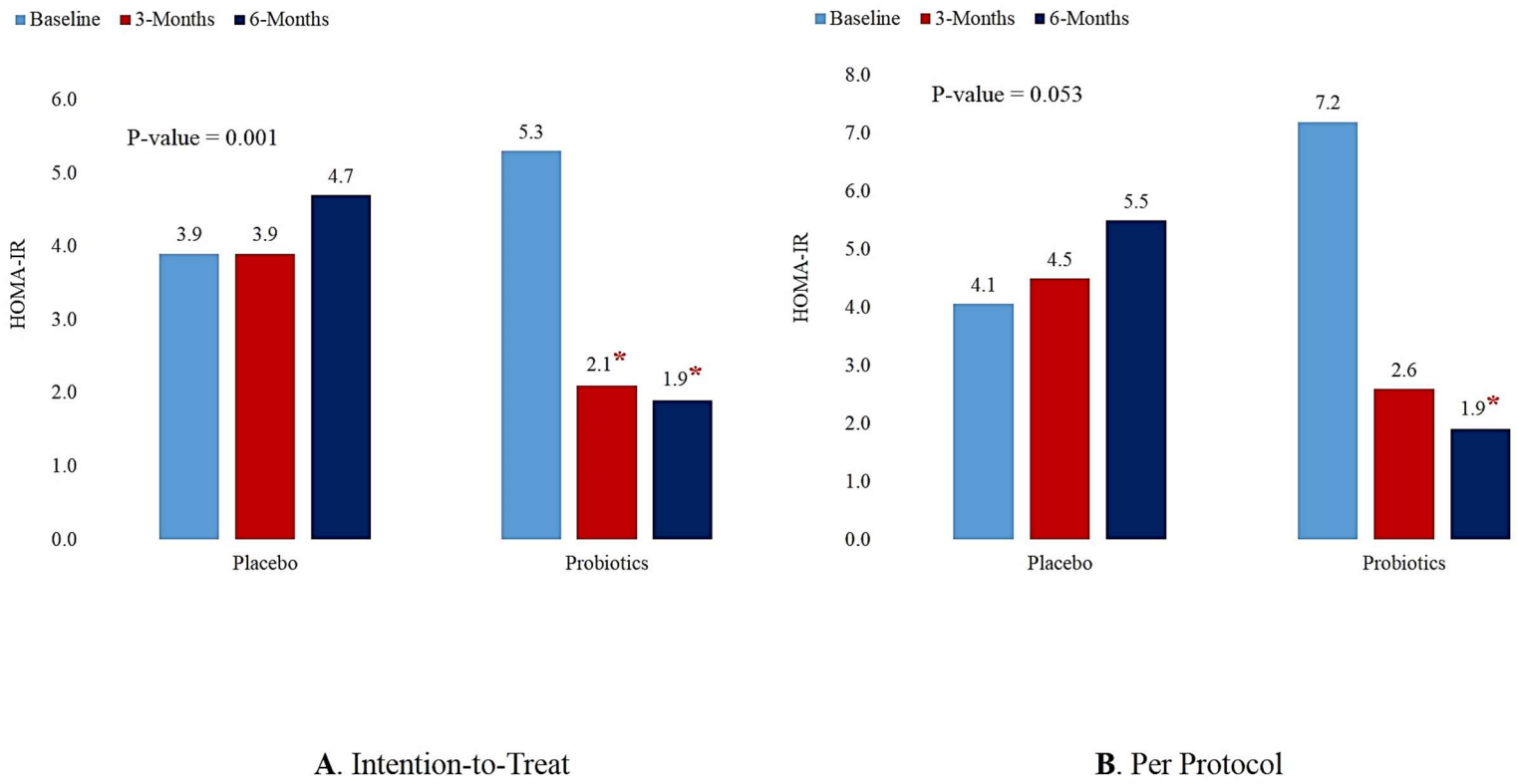


Figure 4.3.3.4 Median HOMA-IR before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

4.3.4 Changes in Lipid Profile in both Placebo and Probiotics Group Before and after 6-month Intervention

Changes in lipid profile in both groups using the ITT analysis were shown in Table 4.3.6. Between group comparisons showed no differences in placebo and probiotics groups over-all in levels of triglycerides [-0.10 vs -1.20; (CI: -1.19-0.17; p = 0.14] (Figure 4.3.4.1), total cholesterol [-0.30 vs -1.10; (CI: -1.17 – 0.220; p = 0.18] (Figure 4.3.4.2), HDL-cholesterol [-0.10 vs -0.30; (CI: -0.82 – 0.39); p = 0.66] (Figure 4.3.4.3), LDL-cholesterol [-0.10 vs – 0.80; (CI: -0.82 – 0.39); p = 0.48] (Figure 4.3.4.4) and total/HDL-cholesterol ratio [-0.30 vs -1.10; (CI: -0.81 – 1.80); p = 0.45) (Figure 4.3.4.5). Within group analysis showed no significant changes in the placebo group over time. In the probiotics group however and as compared to baseline, significant improvements were observed in terms of decreased triglycerides after 3 months ($p<0.05$) and 6 months ($p<0.05$), total cholesterol (p-values <0.05) and total/HDL cholesterol ratio after 6 months of intervention ($p<0.05$).

Table 4.3.6 Lipid profile before and after intervention with placebo or probiotics among T2DM patients (ITT Analysis)

Parameters	Group		Intervention Effects (95% CI)		
	Placebo (N = 30)	Probiotics (N = 31)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
Triglycerides (mmol/L)					
Baseline	2.2 ± 1.4	2.5 ± 1.4	-0.04 (-0.71 - 0.63)	-0.65 (-1.48 - 0.19)	-0.51 (-1.19 - 0.17)
3 months	2.0 ± 0.8	1.7 ± 0.7[A]			
6 months	2.1 ± 1.6	1.3 ± 0.6[A]			
$\bar{X}$ (% Change) at 3 months	-0.20 (-9.09)	-0.80 (-32.00)	0.92	0.13	0.14
$\bar{X}$ (% Change) at 6 months	-0.10 (-4.55)	-1.20 (-48.00)			
Total Cholesterol (mmol/L)					
Baseline	5.2 ± 1.0	5.8 ± 1.3	-0.35 (-1.07 - 0.36)	-0.63 (-1.41 - 0.14)	-0.47 (-1.17 - 0.22)
3 months	4.7 ± 0.9	5.1 ± 0.9			
6 months	4.9 ± 1.0	4.7 ± 1.1[A]			
$\bar{X}$ (% Change) at 3 months	-0.50 (-9.62)	-0.70 (-12.07)	0.32	0.10	0.18
$\bar{X}$ (% Change) at 6 months	-0.30 (-5.77)	-1.10 (-18.97)			
HDL-Cholesterol (mmol/L)					
Baseline	1.1 ± 0.3	1.0 ± 0.3	-0.05 (-0.21 - 0.12)	-0.06 (-0.25 - 0.13)	-0.04 (-0.21 - 0.14)
3 months	1.0 ± 0.3	1.1 ± 0.3			
6 months	1.0 ± 0.4	1.3 ± 0.4			
$\bar{X}$ (% Change) at 3 months	-0.10 (-9.09)	0.10 (10.00)	0.56	0.54	0.66
$\bar{X}$ (% Change) at 6 months	-0.10 (-9.09)	0.30 (30.00)			
LDL-Cholesterol (mmol/L)					
Baseline	3.1 ± 0.9	3.6 ± 1.3	-0.30 (-0.94 - 0.34)	-0.28 (-0.95 - 0.39)	-0.22 (-0.82 - 0.39)
3 months	2.8 ± 0.9	3.2 ± 0.9			
6 months	2.8 ± 1.0[A]	2.7 ± 1.0			
$\bar{X}$ (% Change) at 3 months	-0.30 (-9.68)	-0.40 (-11.11)	0.35	0.40	0.48
$\bar{X}$ (% Change) at 6 months	-0.10 (-9.68)	-0.80 (-22.22)			
Total Cholesterol/HDL-Cholesterol Ratio					
Baseline	5.2 ± 1.0	5.8 ± 1.3	1.12 (-0.65 - 2.89)	0.19 (-0.72 - 1.10)	0.49 (-0.81 - 1.80)
3 months	4.7 ± 0.9	5.1 ± 0.9			
6 months	4.9 ± 1.0	4.7 ± 1.1[A]			
$\bar{X}$ (% Change) at 3 months	-0.50 (-9.62)	-0.70 (-12.07)	0.21	0.67	0.45
$\bar{X}$ (% Change) at 6 months	-0.30 (-5.77)	-1.10 (-18.97)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups over-all; [A] denotes significance within groups compared to baseline; Results are obtained from mixed method ANCOVA after adjustment for baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio; CI – confidence interval; significance at $p<0.05$.

Table 4.3.7 shows the changes in lipid profile in both groups using the PP analysis. Between group comparisons showed borderline significant differences in placebo and probiotics groups over-all in levels of triglycerides [-0.30 vs -1.30; (CI: -2.12 -0.12; p = 0.08] and total cholesterol [-0.10 vs -1.20; (CI: -2.03 – 0.06; p = 0.06]. No differences were observed in HDL-cholesterol [-0.10 vs -0.40; (CI: -0.24 – 0.29); p = 0.84], LDL-cholesterol [0.10 vs – 1.0; (CI: -1.25 – 0.59); p = 0.46] and total/HDL-cholesterol ratio [1.0 vs -3.4; (CI: -2.15 – 2.41); p = 0.91). Similar to the ITT comparisons, within group analysis in PP showed no significant changes in the placebo group over time. In the probiotics group, significant improvements were observed in terms of decreased triglycerides after 3 months ($p<0.05$) and 6 months ($p<0.05$) and total cholesterol (p-values <0.05) after 6 months of intervention. The rest of the lipid profile in the probiotics group had no significant change over time. Changes in lipid profile in both groups are also presented in figures 4.3.4.1-4.3.4.5.

Table 4.3.7. Lipid profile before and after intervention with placebo or probiotics among T2DM patients (PPA)

Parameter	Group		Intervention Effects (95% CI)		
	Placebo (N = 16)	Probiotics (N = 23)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
Triglycerides (mmol/L)					
Baseline	2.0 ± 1.0	2.5 ± 1.4	0.14 (-0.66 - 0.93)	-1.20 (-2.58 - 0.17)	-1.00 (-2.12 - 0.12)
3 months	2.2 ± 0.8	1.7 ± 0.7[A]			
6 months	2.3 ± 2.0	1.2 ± 0.5[A]			
$\bar{X}$ (% Change) at 3 months	0.20 (10.00)	-0.80 (-32.00)	0.73	0.08	0.08
$\bar{X}$ (% Change) at 6 months	0.30 (15.00)	-1.30 (-52.00)			
Total Cholesterol (mmol/L)					
Baseline	5.2 ± 1.0	5.8 ± 1.3	-0.18 (-1.05 - 0.68)	-1.18 (-2.38 - 0.02)	-0.99 (-2.03 - 0.06)
3 months	4.8 ± 0.9	5.1 ± 0.9			
6 months	5.1 ± 1.0	4.6 ± 1.0[AB]			
$\bar{X}$ (% Change) at 3 months	-0.40 (-7.69)	-0.70 (-12.07)	0.67	0.05	0.06
$\bar{X}$ (% Change) at 6 months	-0.10 (-1.92)	-1.20 (-20.69)			
HDL-Cholesterol (mmol/L)					
Baseline	1.1 ± 0.3	0.9 ± 0.3	-0.07 (-0.27 - 0.12)	0.01 (-0.29 - 0.31)	0.03 (-0.24 - 0.29)
3 months	1.0 ± 0.3	1.1 ± 0.3			
6 months	1.0 ± 0.5	1.3 ± 0.4			
$\bar{X}$ (% Change) at 3 months	-0.10 (-9.09)	0.20 (22.22)	0.46	0.96	0.84
$\bar{X}$ (% Change) at 6 months	-0.10 (-9.09)	0.40 (44.44)			
LDL-Cholesterol (mmol/L)					
Baseline	3.1 ± 0.8	3.6 ± 1.2	-0.18 (-0.99 - 0.63)	-0.34 (-1.36 - 0.68)	-0.33 (-1.25 - 0.59)
3 months	2.8 ± 1.0	3.2 ± 0.8			
6 months	3.2 ± 0.8	2.6 ± 0.8			
$\bar{X}$ (% Change) at 3 months	-0.30 (-9.68)	-0.40 (-11.11)	0.66	0.50	0.46
$\bar{X}$ (% Change) at 6 months	0.10 (3.23)	-1.00 (-27.78)			
Total Cholesterol/HDL-Cholesterol Ratio					
Baseline	4.9 ± 1.3	6.9 ± 2.3	1.76 (-0.30 - 3.82)	-0.24 (-1.55 - 1.07)	0.13 (-2.15 - 2.41)
3 months	5.0 ± 1.2	5.8 ± 5.0			
6 months	5.9 ± 2.7	3.5 ± 0.8			
$\bar{X}$ (% Change) at 3 months	0.10 (2.04)	-1.10 (-15.94)	0.09	0.71	0.91
$\bar{X}$ (% Change) at 6 months	1.00 (20.41)	-3.40 (-49.28)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups over-all; [A] denotes significance within groups compared to baseline; [B] denotes significance within groups compared to 3 months; Results are obtained from mixed method ANCOVA after adjustment for baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio; CI – confidence interval; significance at $p<0.05$.

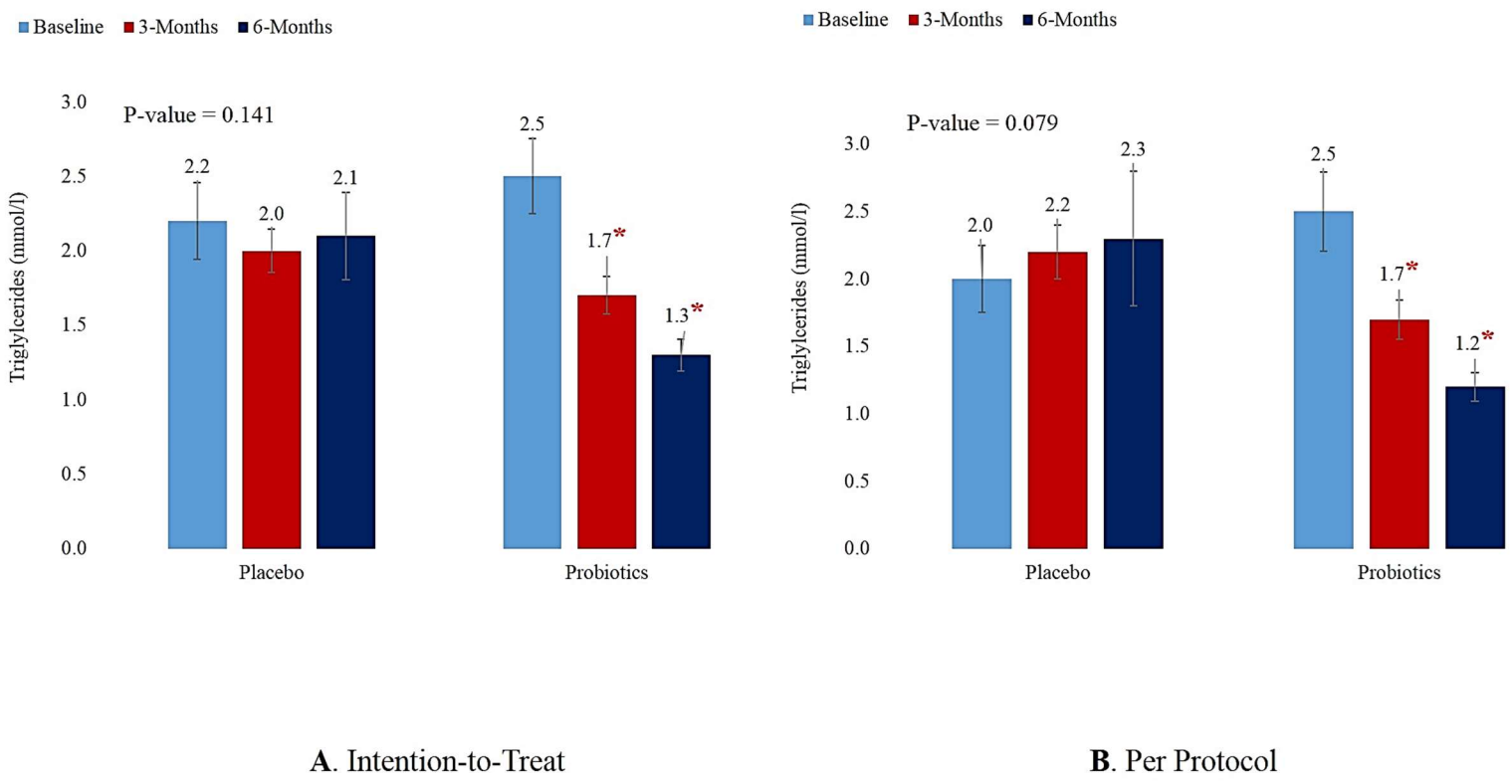


Figure 4.3.4.1 Mean triglycerides (mmol/L) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

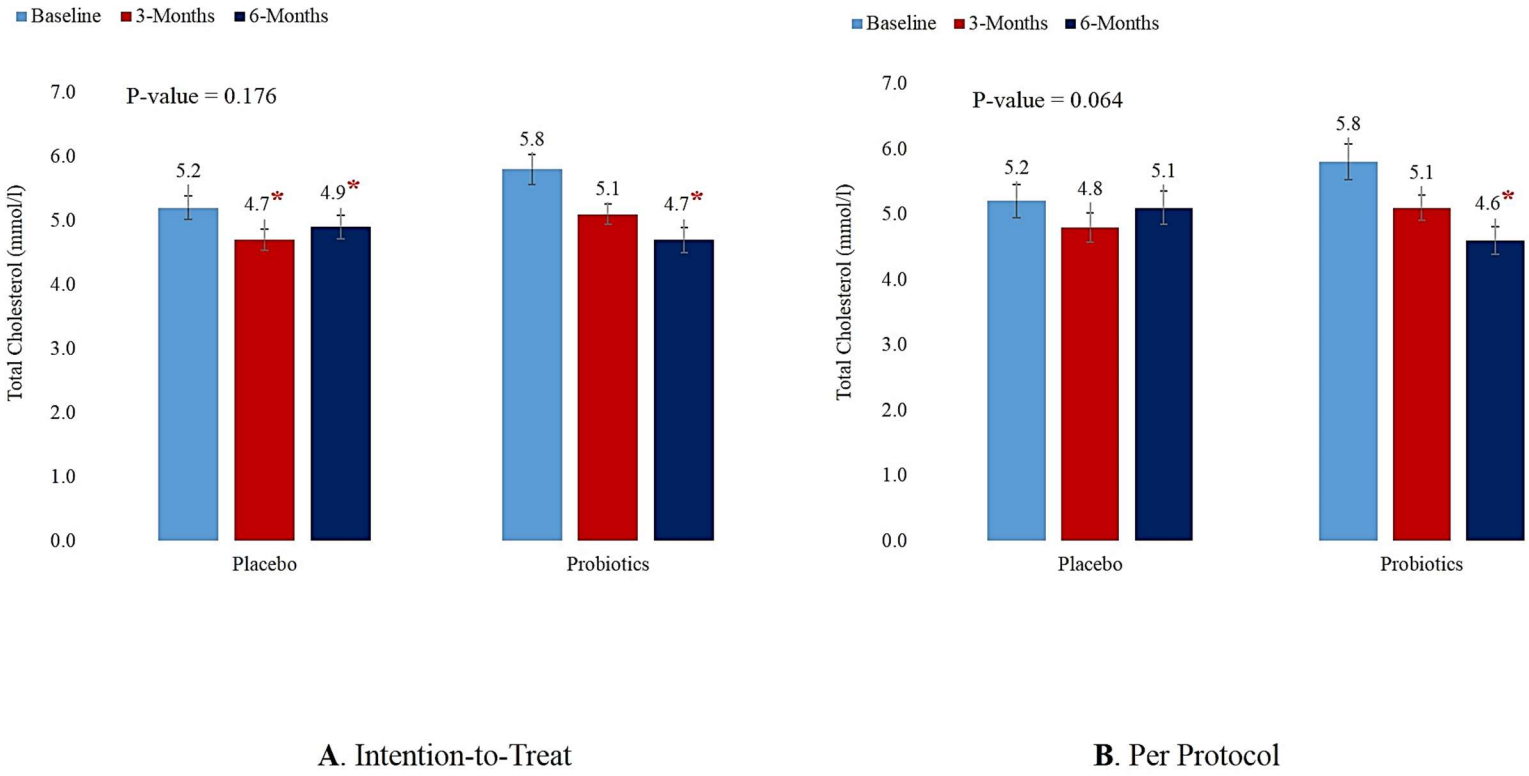


Figure 4.3.4.2 Mean total cholesterol (mmol/L) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

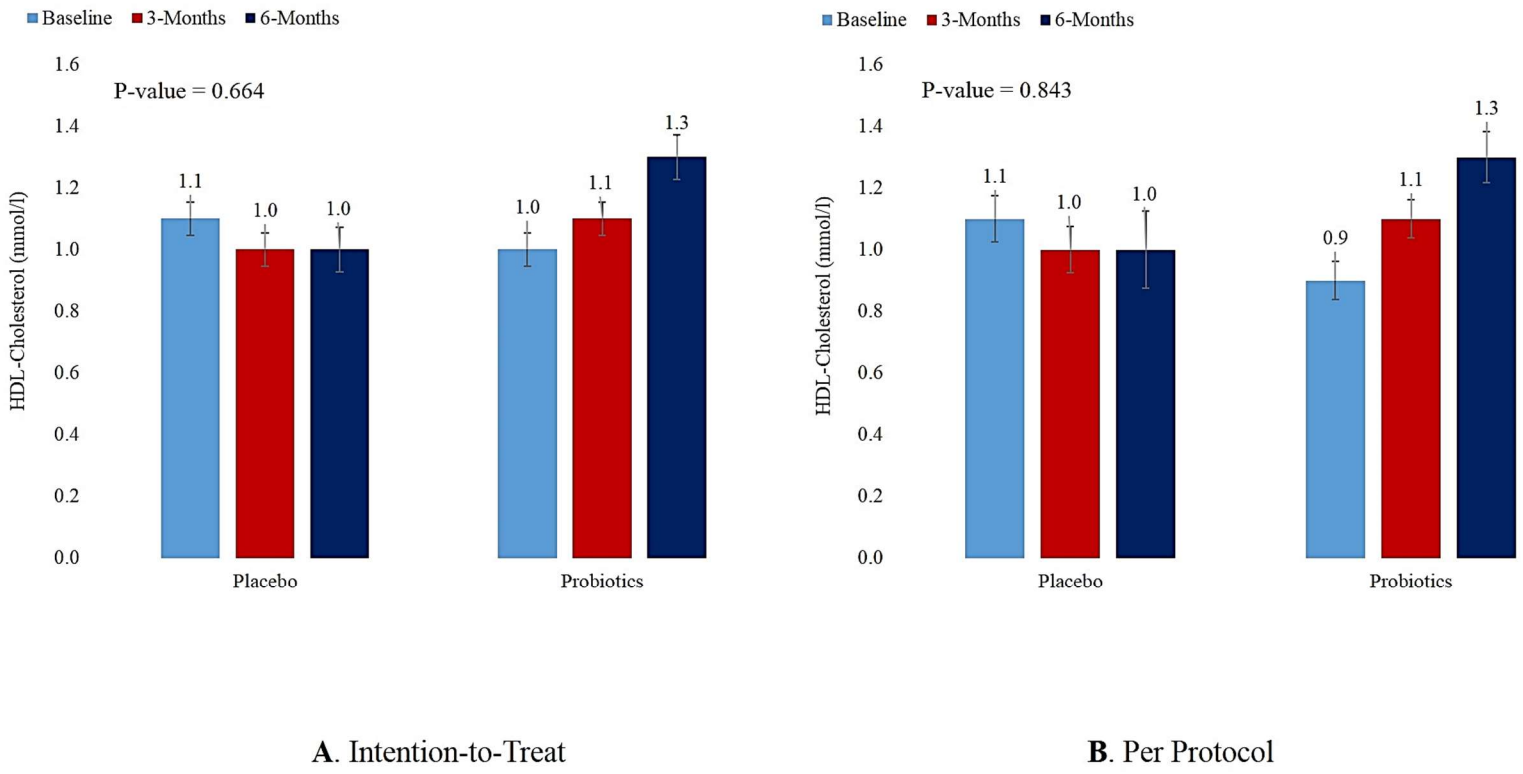


Figure 4.3.4.3 Mean HDL-cholesterol (mmol/L) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; significant at $p<0.05$.

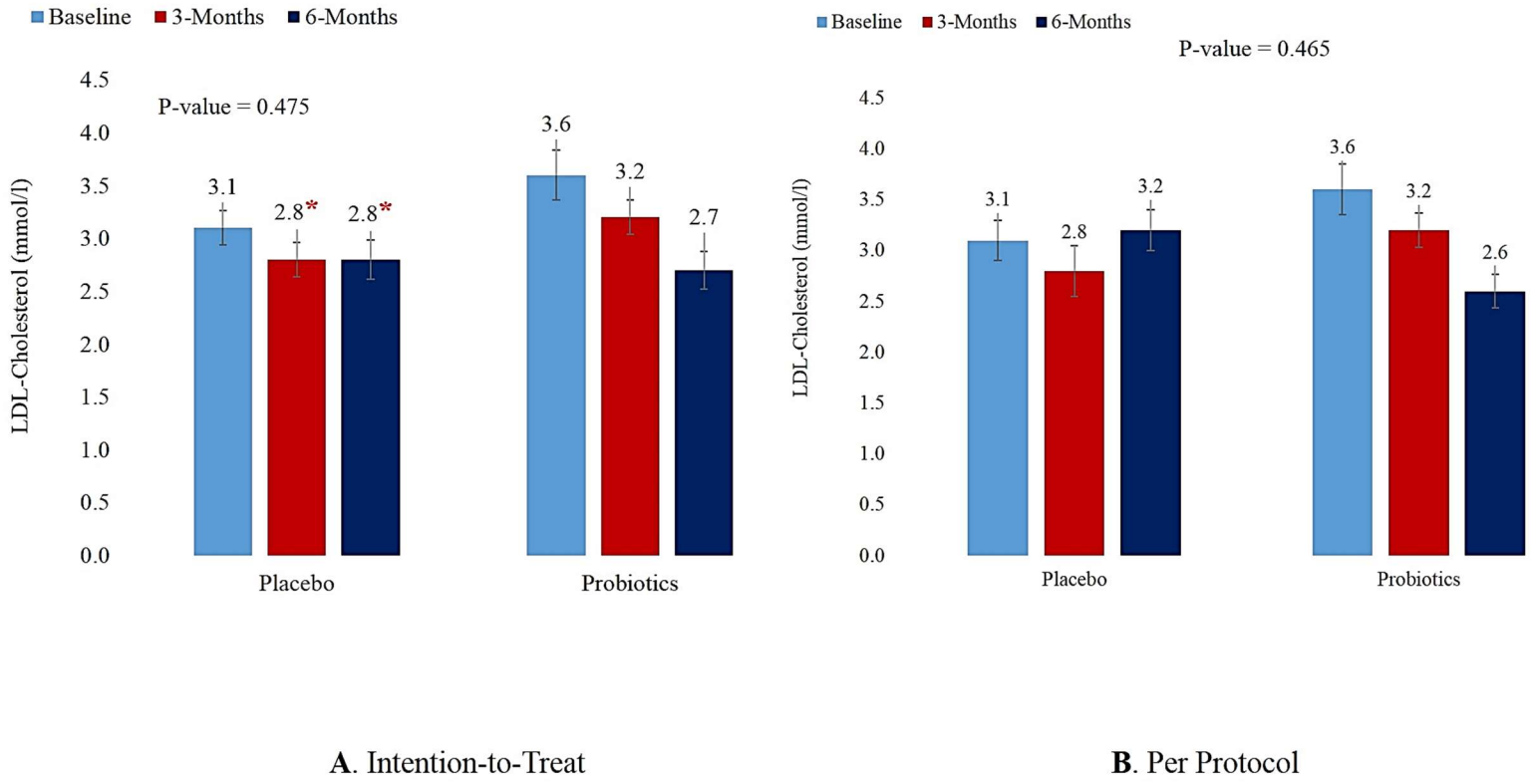


Figure 4.3.4.4 Mean LDL-cholesterol (mmol/L) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

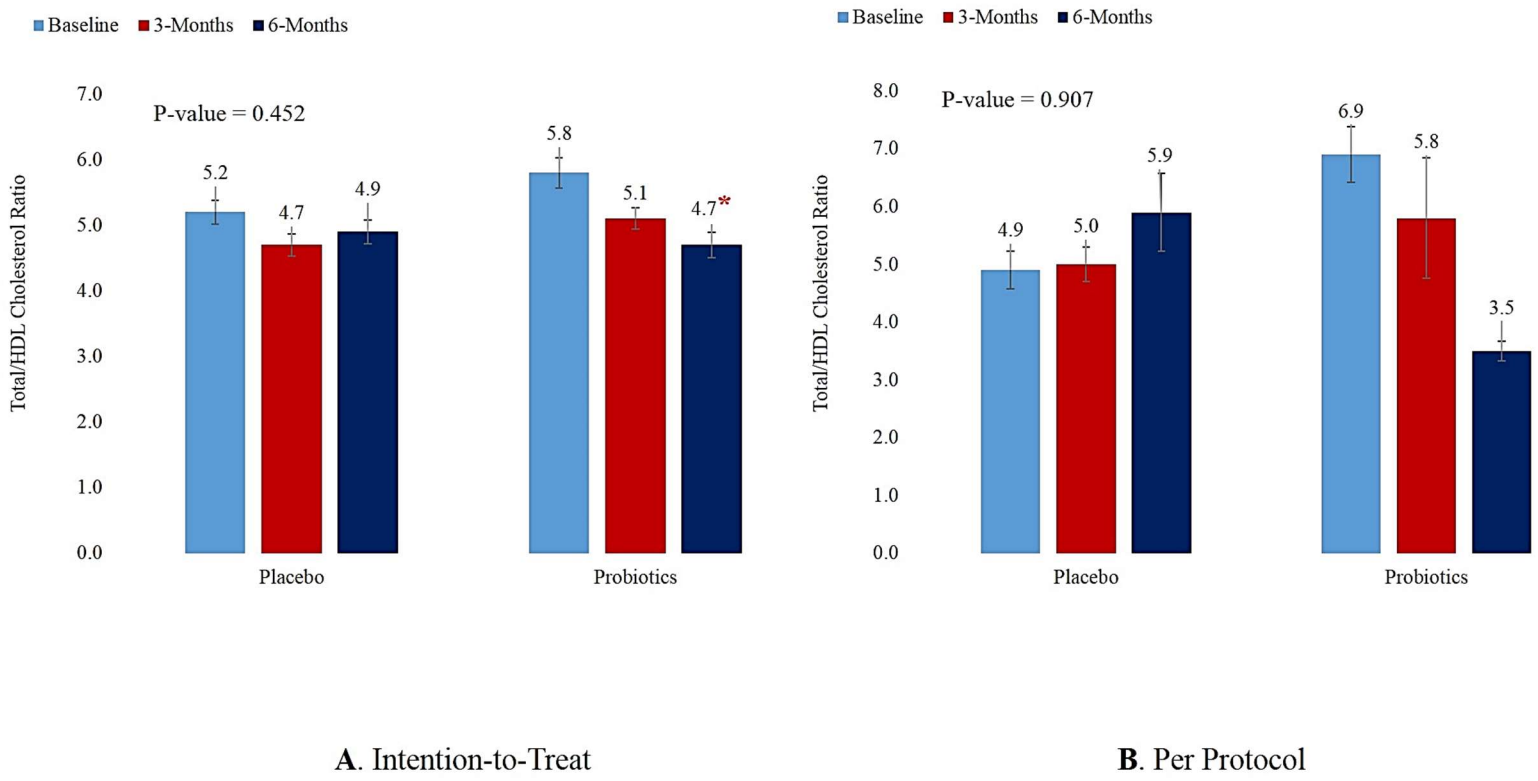


Figure 4.3.4.5 Mean total/HDL-cholesterol (mmol/L) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

4.3.8 Baseline Characteristics of Placebo and Probiotics Group in Endotoxin, Inflammation and Adipocytokine Profiles

Baseline comparison of placebo and probiotics groups are presented in Table 4.3.8. Both groups are comparable in terms of age and BMI (not shown in table). At baseline, the probiotics group had a significantly higher median levels of TNFα and IL-6 (p-values 0.01 and 0.04, respectively). With regards to adipocytokine profile, the probiotics group had a significantly higher median levels of leptin than the placebo group (p=0.04). Endotoxin, which is the primary endpoint of the study, was significantly higher in the probiotics group than placebo at baseline (p=0.002) (table 4.3.8).

Table 4.3.8. Baseline Characteristics according to Intervention Groups

Parameters	Placebo	Probiotics	P-value
N	39	39	
M/F	21/18	19/20	
Inflammatory Markers Profile			
TNF α (pg/ml)	0.5 (0.2 - 0.9)	0.9 (0.3 - 1.3)	0.01
IL-6 (pg/ml)	3.7 (1.9 - 11.4)	5.6 (3.0 - 19.1)	0.04
CRP (ug/ml)	2.7 (1.9 - 6.2)	5.6 (2.8 - 6.4)	0.29
Adipocytokine Profile			
Leptin (pg/ml)	3.6 (1.4 - 7.6)	5.8 (2.5 - 17.2)	0.04
Adiponectin (ug/ml)	11.4 (8.7 - 16.4)	8.3 (6.5 - 18.0)	0.09
Resistin (ng/ml)	6.3 (4.2 - 11.4)	10.8 (5.3 - 16.9)	0.12
Endotoxin (IU/ml)	2.2 (1.2 – 4.5)	4.8 (2.6 - 8.4)	0.002

Note: Data presented as Mean ± SD for normally distributed data while non-normally normally distributed data are presented as Median (inter-quartile range). P-value significant at $p<0.05$.

4.3.9 Changes in Inflammatory Markers in both Placebo and Probiotics Group Before and after 6-month Intervention

Changes in inflammatory markers in both the placebo and probiotic group is shown in Table 4.3.9 using the ITT analysis. After 6 month intervention, no significant difference in placebo and probiotics were observed in TNFα [-0.40 vs -0.60; (CI:-0.12 – 0.21); p = 0.57] (Figure 4.3.9.1), IL-6 [-2.8 vs -3.9; (CI:-0.61 – 0.18); p = 0.28] (Figure 4.3.9.2) and C-reactive protein [0.40 vs -2.9; (CI:-0.54 – 0.07); p = 0.13] (Figure 4.3.9.3). Within group comparisons however showed that all these inflammatory markers improved over time in the probiotics group, with levels of TNFα decreasing significantly after 6 months ($p<0.05$), as well as IL-6 in both 3 months ($p<0.05$) and 6 months ($p<0.05$) and CRP ($p<0.05$). These within group changes were not observed in the placebo group (Table 4.3.9).

Table 4.3.9 Inflammatory Markers before and after intervention with placebo or probiotics among T2DM patients (ITT Analysis)

Parameters	Group		Intervention Effects (95% CI)		
	Placebo (N = 30)	Probiotics (N = 31)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
TNFα (pg/ml)					
Baseline	0.5 (0.2 - 0.8)	0.9 (0.4 - 1.2)	0.16 (-0.03 - 0.34)	0.07 (-0.12 - 0.26)	0.05 (-0.12 - 0.21)
3 months	0.5 (0.2 - 0.8)	0.6 (0.3 - 0.9)			
6 months	0.3 (0.2 - 0.8)	0.3 (0.2 - 0.7)^{AB}			
$\bar{X}$ (% Change) at 3 months	0.00 (0.00)	-0.30 (-33.33)	0.10	0.46	0.57
$\bar{X}$ (% Change) at 6 months	-0.20 (-40.00)	-0.60 (-66.67)			
IL-6 (pg/ml)					
Baseline	3.6 (1.4- 11.4)	5.1 (2.7 - 18.8)	-0.20 (-0.59 - 0.19)	-0.14 (-0.51 - 0.22)	-0.21 (-0.61 - 0.18)
3 months	0.8 (0.6 - 4.4)	1.4 (0.7 - 18.0)^{A}			
6 months	0.8 (0.7 - 3.8)	1.2 (0.8 - 3.6)^{A}			
$\bar{X}$ (% Change) at 3 months	-2.80 (-77.78)	-3.70 (-72.55)	0.31	0.43	0.28
$\bar{X}$ (% Change) at 6 months	-2.80 (-77.78)	-3.90 (-76.47)			
C-Reactive Protein (ug/ml)					
Baseline	3.0 (1.9 - 6.2)	5.5 (2.7 - 6.1)	-0.11 (-0.40 - 0.18)	-0.20 (-0.47 - 0.07)	-0.23 (-0.54 - 0.07)
3 months	2.9 (1.5 - 4.7)	3.1 (1.4 - 5.7)^{A}			
6 months	3.4 (2.6 - 5.6)	2.6 (1.2 - 4.9)^{A}			
$\bar{X}$ (% Change) at 3 months	-0.10 (-3.33)	-2.40 (-43.64)	0.44	0.14	0.13
$\bar{X}$ (% Change) at 6 months	0.40 (13.33)	-2.90 (-52.73)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups over-all; ^{A} denotes significance within groups compared to baseline; ^{B} denotes significance within groups compared to 3 months; Results were obtained from mixed method ANCOVA with baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio; CI- confidence interval; significance at $p<0.05$.

Changes in the circulating inflammatory markers in both groups using PPA is shown in table 4.3.10. Similar to the ITT, no significant difference in placebo and probiotics were observed in levels of TNFα [-0.30 vs -0.90; (CI:-0.30 – 0.37); p = 0.81], IL-6 [-5.0 vs -16.0; (CI:-0.99 – 0.63); p = 0.63] and C-reactive protein [1.8 vs -2.3; (CI:-0.46 – 0.58); p = 0.78]. Within group comparisons however showed significant improvements over time in the probiotics group in levels of TNFα ($p<0.05$) and IL-6 in both 3 months ($p<0.05$) and 6 months ($p<0.05$). No significant improvement in CRP levels were seen in the probiotics group. No improvement were seen in all inflammatory markers in the placebo group (Table 4.3.10). Changes in inflammatory profile in both groups using PPA are also presented in figures 4.3.9.1-4.9.3.3.

Table 4.3.10. Inflammatory markers before and after intervention with placebo or probiotics among T2DM patients (PP Analysis)

Parameter	Group		Intervention Effects (95% CI)		
	Placebo (N = 16)	Probiotics (N = 23)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
TNFα (pg/ml)					
Baseline	0.5 (0.3 - 0.6)	1.1 (0.7 - 1.4)	0.27 (0.04 - 0.51)	0.00 (-0.41 - 0.40)	0.04 (-0.30 - 0.37)
3 months	0.6 (0.3 - 0.9)	0.8 (0.6 - 0.9)			
6 months	0.2 (0.1 - 0.2)	0.2 (0.1 - 0.3)^{AB}			
$\bar{X}$ (% Change) at 3 months	0.10 (20.0)	-0.30 (-27.3)	0.03	0.99	0.81
$\bar{X}$ (% Change) at 6 months	-0.30 (-60.0)	-0.90 (-81.8)			
IL-6 (pg/ml)					
Baseline	5.8 (4.3 - 9.2)	18.0 (5.1 - 20.8)	-0.23 (-0.78 - 0.32)	-0.07 (-0.47 - 0.33)	-0.18 (-0.99 - 0.63)
3 months	0.5 (0.2 - 1.5)	10.7 (0.3 - 18.9)^{A}			
6 months	0.8 (0.7 - 1.6)	2.0 (0.8 - 2.8)^{A}			
$\bar{X}$ (% Change) at 3 months	-5.30 (-91.4)	-7.30 (-40.6)	0.40	0.70	0.63
$\bar{X}$ (% Change) at 6 months	-5.00 (-86.2)	-16.00 (-88.9)			
C-Reactive Protein (ug/ml)					
Baseline	3.8 (2.2 - 6.6)	6.4 (5.8 - 6.6)	-0.16 (-0.58 - 0.26)	-0.09 (-0.51 - 0.32)	0.06 (-0.46 - 0.58)
3 months	2.1 (1.2 - 6.2)	4.9 (3.8 - 6.0)			
6 months	5.6 (3.8 - 5.7)	4.1 (2.6 - 4.9)			
$\bar{X}$ (% Change) at 3 months	-1.70 (-44.74)	-1.50 (-23.44)	0.43	0.61	0.78
$\bar{X}$ (% Change) at 6 months	1.80 (47.37)	-2.30 (-35.94)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups over-all; ^{A} denotes significance within groups compared to baseline; ^{B} denotes significance within groups compared to 3 months; Results were obtained from mixed method ANCOVA with baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio; CI - confidence interval; significance at $p<0.05$.

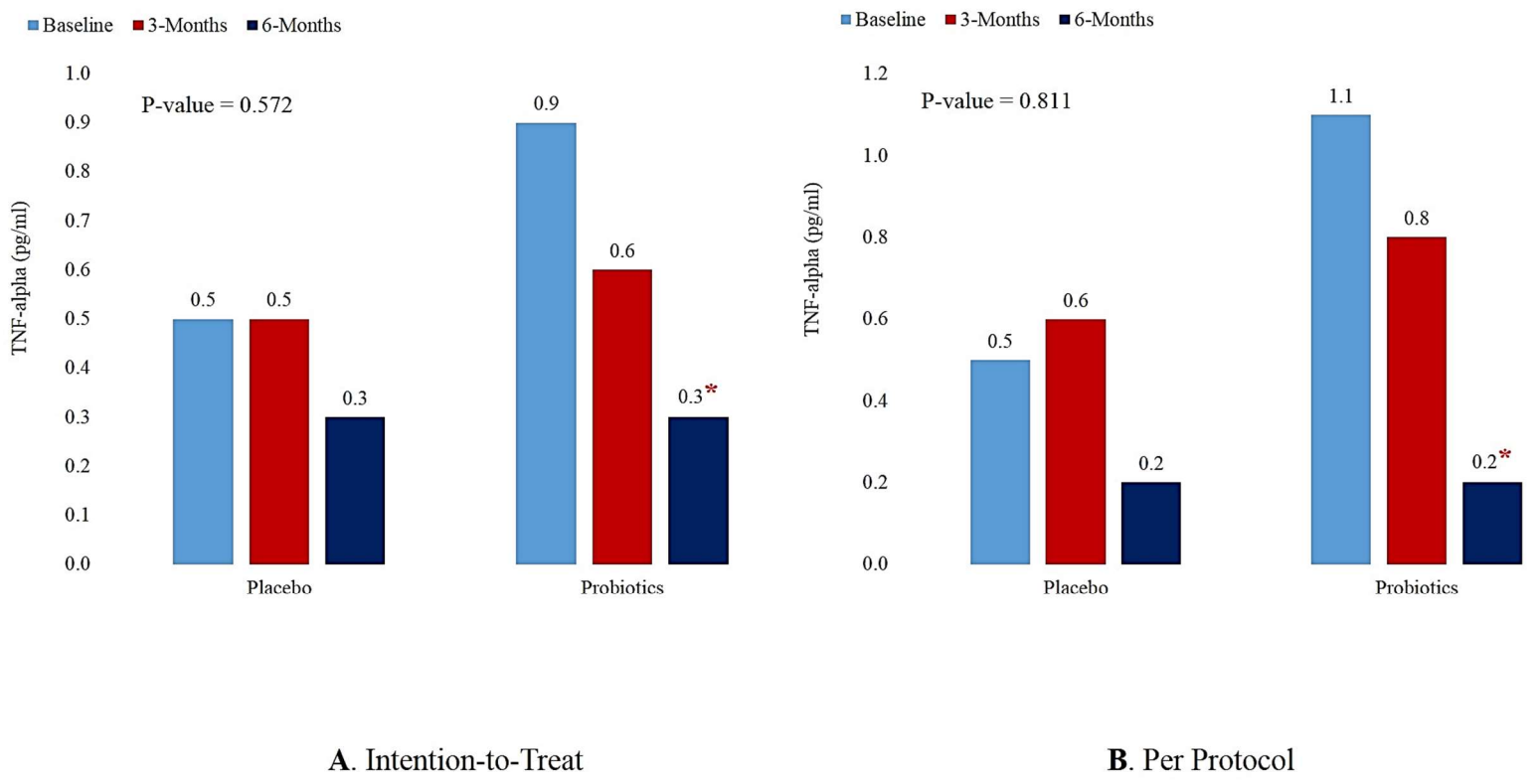


Figure 4.3.9.1 Median TNF-α (pg/ml) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

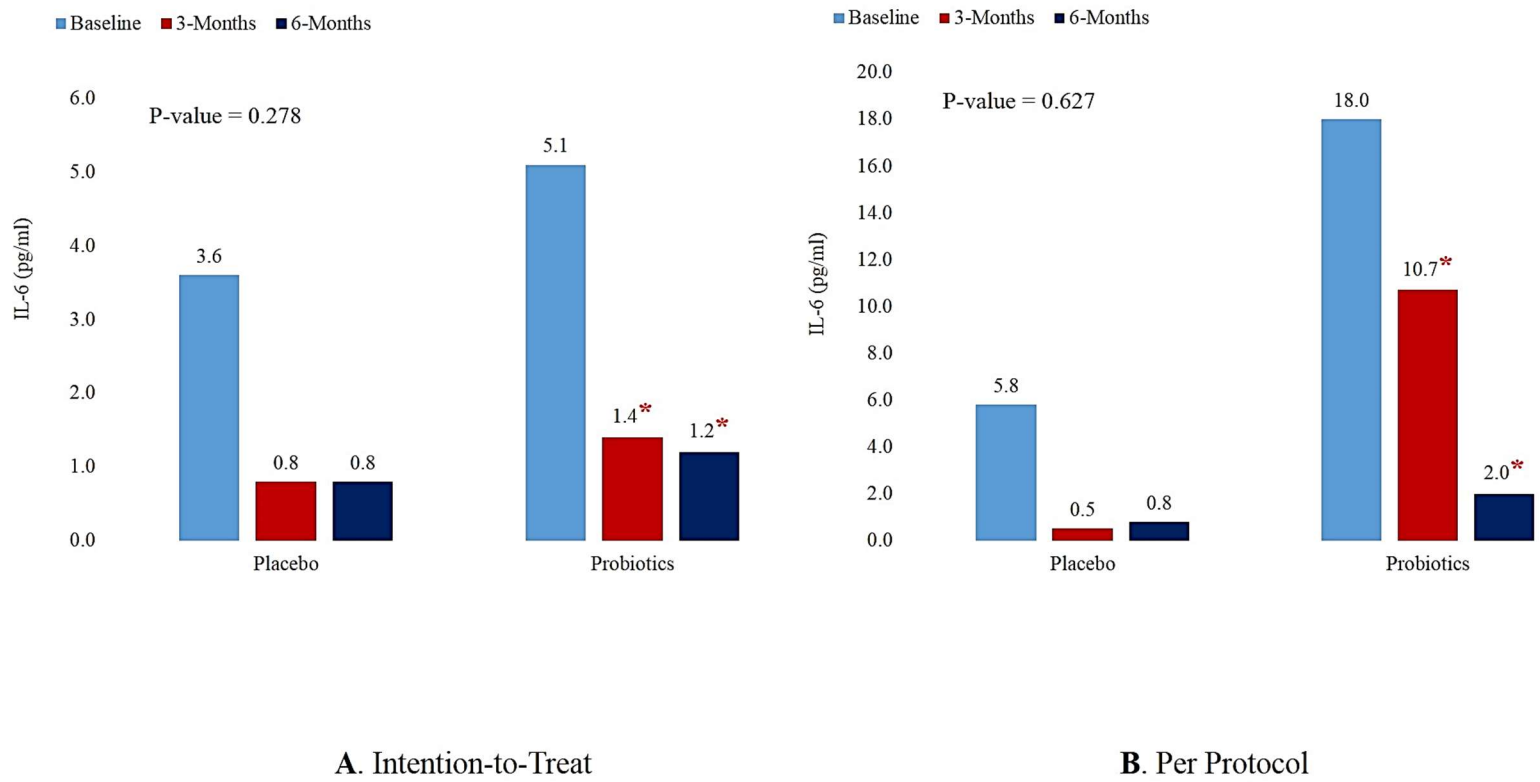


Figure 4.3.9.2 Median IL-6 (pg/ml) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

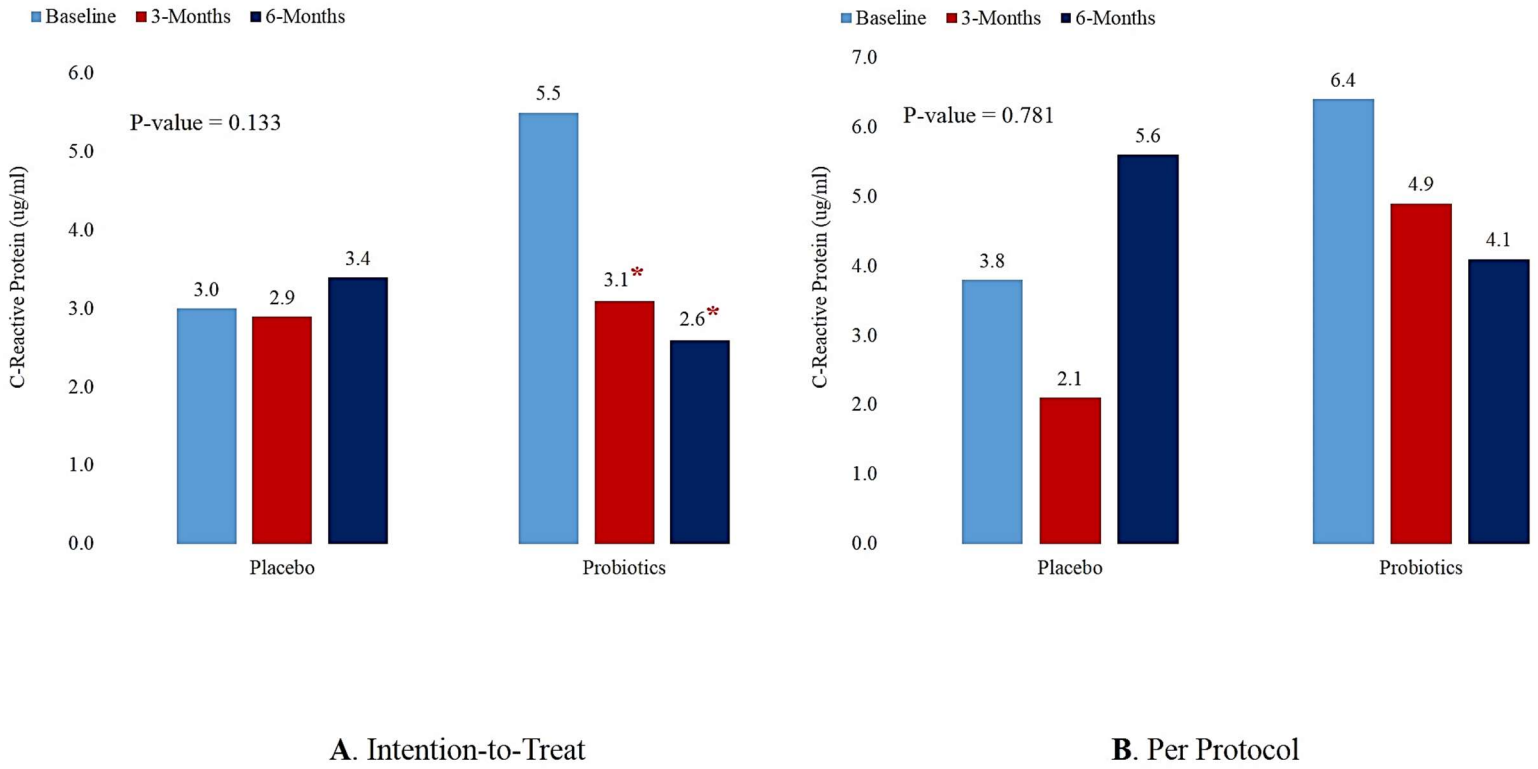


Figure 4.3.9.3 Median C-Reactive protein (µg/ml) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

4.3.10. Changes in Adipocytokine Profile and Endotoxin levels in both Placebo and Probiotics Group Before and after 6-month Intervention

Changes in circulating adipocytokines and endotoxin in both groups using the ITT analysis are shown in Table 4.3.11. No differences were observed in placebo and probiotics with regards to levels of leptin [-1.1 vs -2.7; (CI:-0.18 – 0.61); p=0.27] (Figure 4.3.10.1), adiponectin [0.0 vs 6.1; (CI:-0.22-0.18); p = 0.84] (Figure 4.3.10.2), resistin [5.0 vs -6.8; (CI: -0.30-0.13); p = 0.44] (Figure 4.3.10.3) and endotoxin [0.80 vs -3.20; (CI:-0.33-0.13); p = 0.38] (Figure 4.3.10.4). Within group comparisons showed that in the placebo group, there was a significant increase in resistin levels after 6 months compared to baseline ($p<0.05$) as well as a significant increase in the endotoxin group after 6 months as compared to 3 months ($p<0.05$). Within group comparison in the probiotics group showed a significant increase in circulating adiponectin levels after 6 months ($p<0.05$), a significant decrease in resistin levels after 6 months ($p<0.05$) and a significant improvement in endotoxin levels after 6 months of intervention ($p<0.05$). No significant changes in both groups were noted in leptin.

Table 4.3.11 Adipocytokines and Endotoxin Before and After Intervention with Placebo or Probiotics among T2DM Participants (ITT Analysis)

Parameters	Group		Intervention Effects (95% CI)		
	Placebo (N = 30)	Probiotics (N = 31)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
Leptin (pg/ml)					
Baseline	3.9 (1.6 - 7.6)	5.8 (2.5 - 17.2)	0.24 (-0.13 - 0.62)	0.20 (-0.22 - 0.62)	0.22 (-0.18 - 0.61)
3 months	4.0 (1.6 - 7.0)	3.5 (2.2 - 10.0)			
6 months	2.8 (0.9 - 6.9)	3.1 (2.1 - 9.7)			
$\bar{X}$ (% Change) at 3 months	0.10 (2.56)	-2.30 (-39.66)	0.20	0.35	0.27
$\bar{X}$ (% Change) at 6 months	-1.10 (-28.21)	-2.70 (-46.55)			
Adiponectin (pg/ml)					
Baseline	11.1 (8.7 - 16.6)	8.5 (6.4 - 14.6)	-0.08 (-0.29- 0.13)	-0.04 (-0.23- 0.15)	-0.02(-0.22 - 0.18)
3 months	9.7 (5.1 - 16.8)	10.4 (7.2 - 18.7)			
6 months	11.1 (5.7 - 16.0)	14.6 (7.8 - 24.4)[A]			
$\bar{X}$ (% Change) at 3 months	-1.40 (-12.61)	1.90 (22.35)	0.44	0.64	0.84
$\bar{X}$ (% Change) at 6 months	0.00 (0.00)	6.10 (71.76)			
Resistin (ng/ml)					
Baseline	6.3 (4.2 - 11.4)	11.7 (6.4 - 18.8)	0.05 (-0.18 - 0.27)	-0.02(-0.25 - 0.21)	-0.08(-0.30 - 0.13)
3 months	11.8 (6.2 - 19.1)	6.2 (3.7 - 14.5)			
6 months	11.3 (5.3 - 15.2)[A]	4.9 (3.1 - 8.3)[A]			
$\bar{X}$ (% Change) at 3 months	5.50 (87.30)	-5.50 (-47.01)	0.67	0.86	0.44
$\bar{X}$ (% Change) at 6 months	5.00 (79.37)	-6.80 (-58.12)			
Endotoxin (IU/ml)					
Baseline	2.1 (1.2 – 4.4)	4.6 (2.4 – 7.9)	0.13 (-0.12 - 0.38)	-0.10(-0.35 - 0.14)	-0.10(-0.33 - 0.13)
3 months	1.9 (1.0 – 2.9)	2.2 (1.2 – 3.6)[A]			
6 months	2.9 (1.9 - 7.0)[B]	1.4 (1.0 - 2.1)[A]			
$\bar{X}$ (% Change) at 3 months	-0.20 (-9.52)	-2.40 (-52.17)	0.30	0.40	0.38
$\bar{X}$ (% Change) at 6 months	0.80 (38.10)	-3.20 (-69.57)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups over-all; [A] denotes significance within groups compared to baseline; Results were obtained from mixed method ANCOVA with baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio; CI - confidence interval; significance at $p<0.05$.

Changes in circulating adipocytokines and endotoxin in both groups using the PP analysis are shown in Table 4.3.12. No differences over-all were observed in placebo and probiotics with regards to levels of leptin [-1.6 vs -2.8; (CI:-1.15 – 0.73); p=0.62], adiponectin [-1.8 vs 1.3; (CI:-0.23-0.25); p = 0.96], resistin [5.0 vs -7.2; (CI: -0.45-0.23); p = 0.44] and endotoxin [0.90 vs -3.60; (CI:-0.21-0.42); p = 0.50]. Within group comparisons showed no changes in all adipocytokine markers in the placebo group. Endotoxin levels in the placebo group however showed a significant increase over time as compared to baseline ($p<0.05$). In the probiotics group, a significant decrease in leptin levels were observed after intervention ($p<0.05$) as well as a significant decrease in resistin levels ($p<0.05$). Endotoxin levels also significantly decreased overtime and this was apparent in both 3 months ($p<0.05$) and 6 months intervention ($p<0.05$). Changes in adipocytokine profile and endotoxin levels in both groups using PPA are also presented in figures 4.3.10.1-4.3.10.4.

Table 4.3.12 Adipocytokines and Endotoxin Before & After Intervention with Placebo or Probiotics among T2DM Participants (PP Analysis)

Parameter	Group		Intervention Effects (95% CI)		
	Placebo (N = 16)	Probiotics (N = 23)	P^a (0 vs 3 M)	P^b (0 vs 6 M)	P^c
Leptin (pg/ml)					
Baseline	2.1 (1.4 - 9.3)	5.8 (3.3 - 20.0)	0.43 (-0.07 - 0.93)	-0.22 (-1.33- 0.90)	-0.21 (-1.15- 0.73)
3 months	4.4 (3.2 - 15.2)	5.7 (3.1 - 9.8)			
6 months	0.5 (0.3 - 3.5)	3.0 (0.8 - 9.6)^{AB}			
$\bar{X}$ (% Change) at 3 months	2.30 (109.52)	-0.10 (-1.72)	0.09	0.67	0.62
$\bar{X}$ (% Change) at 6 months	-1.60 (-76.19)	-2.80 (-48.28)			
Adiponectin (ug/ml)					
Baseline	10.8 (8.7 - 14.3)	9.4 (5.6 - 18.0)	0.16 (-0.10 - 0.42)	-0.03(-0.30 - 0.23)	0.01 (-0.23 - 0.25)
3 months	9.5 (5.0 - 17.2)	9.4 (6.8 - 14.8)			
6 months	9.0 (5.4 - 12.9)	10.7 (7.2 - 19.8)			
$\bar{X}$ (% Change) at 3 months	-1.30 (-12.04)	0.00 (0.00)	0.22	0.80	0.96
$\bar{X}$ (% Change) at 6 months	-1.80 (-16.67)	1.30 (13.83)			
Resistin (ng/ml)					
Baseline	6.3 (4.2 - 11.4)	11.7 (4.6 - 19.6)	0.07 (-0.24 - 0.38)	-0.11 (-0.53- 0.30)	-0.11 (-0.45- 0.23)
3 months	15.4 (6.2 - 22.5)	7.7 (4.6 - 14.1)			
6 months	11.3 (4.8 - 15.8)	4.5 (3.1 - 7.6)^{A}			
$\bar{X}$ (% Change) at 3 months	9.10 (144.44)	-4.00 (-34.19)	0.64	0.58	0.50
$\bar{X}$ (% Change) at 6 months	5.00 (79.37)	-7.20 (-61.54)			
Endotoxin (IU/ml)					
Baseline	1.7 (0.9 – 2.6)	5.0 (3.2 - 8.5)	0.29 (-0.01 - 0.58)	0.13 (-0.24 - 0.50)	0.11 (-0.21 - 0.42)
3 months	1.7 (0.9 – 2.7)	2.4 (1.3 - 4.3)^{A}			
6 months	2.6 (1.6 - 12.7)^{A}	1.4 (0.9 - 3.4)^{A}			
$\bar{X}$ (% Change) at 3 months	0.00 (0.00)	-2.60 (-52.00)	0.06	0.47	0.50
$\bar{X}$ (% Change) at 6 months	0.90 (52.94)	-3.60 (-72.00)			

Note: P^a denotes p-value between groups at baseline and 3 months; P^b denotes p-value between groups at baseline and 6 months; P^c denotes p-value between groups over-all; ^{A} denotes significance within groups compared to baseline; ^{B} denotes significance within groups compared to 3 months; Results were obtained from mixed method ANCOVA with baseline covariates including leptin, TNF-α, endotoxin, WHR, glucose and total cholesterol/HDL ratio; significance at $p<0.05$.

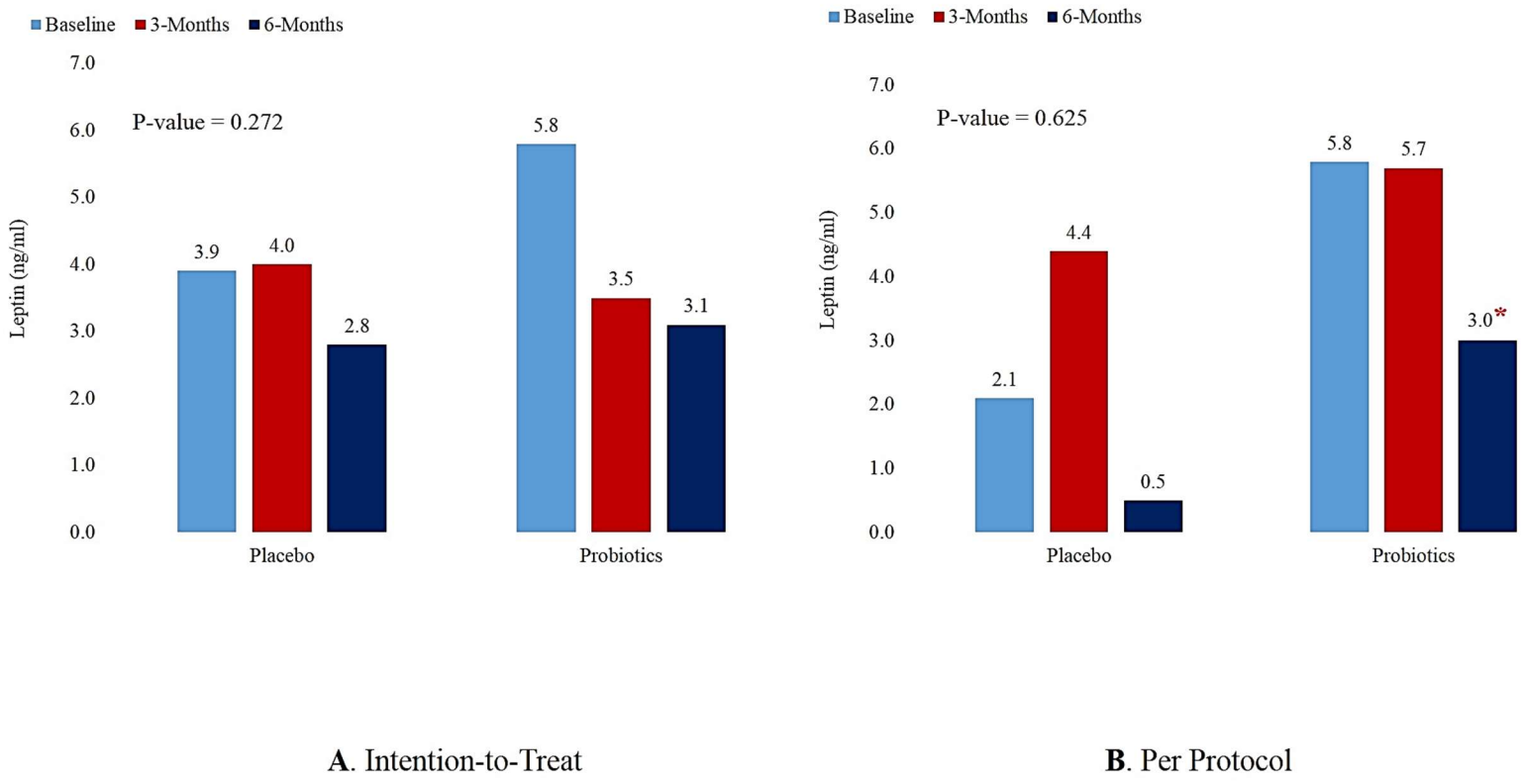


Figure 4.3.10.1 Median leptin (ng/ml) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

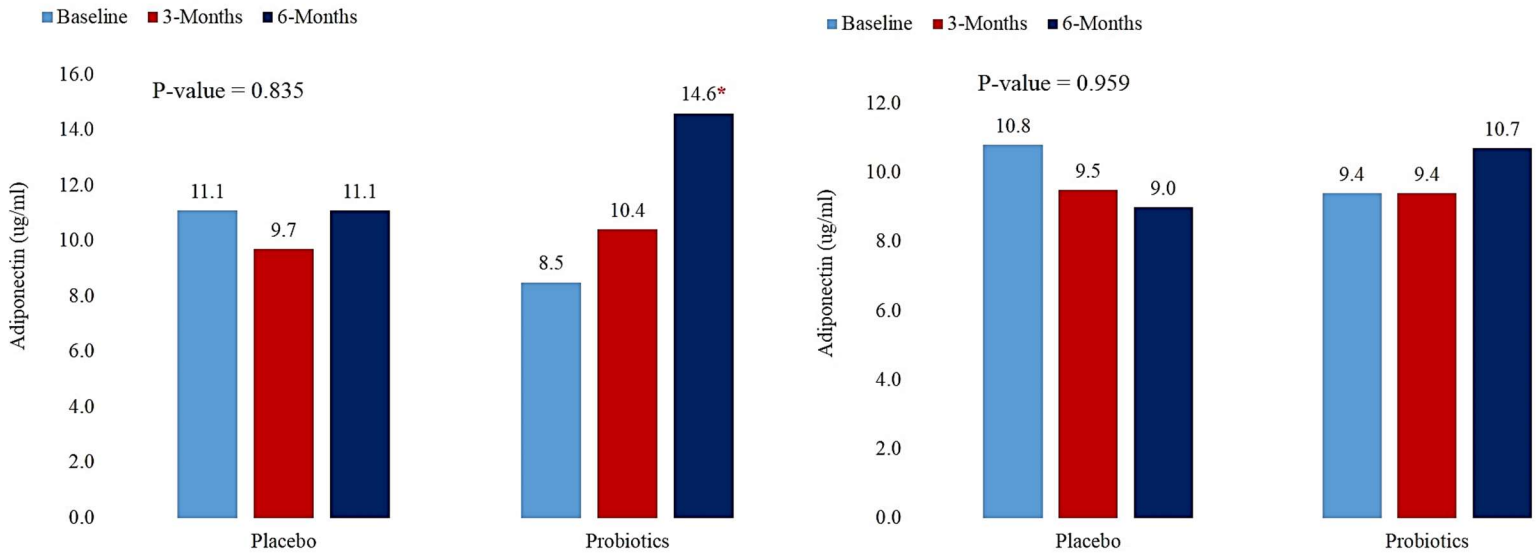


Figure 4.3.10.2 Median adiponectin (µg/ml) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

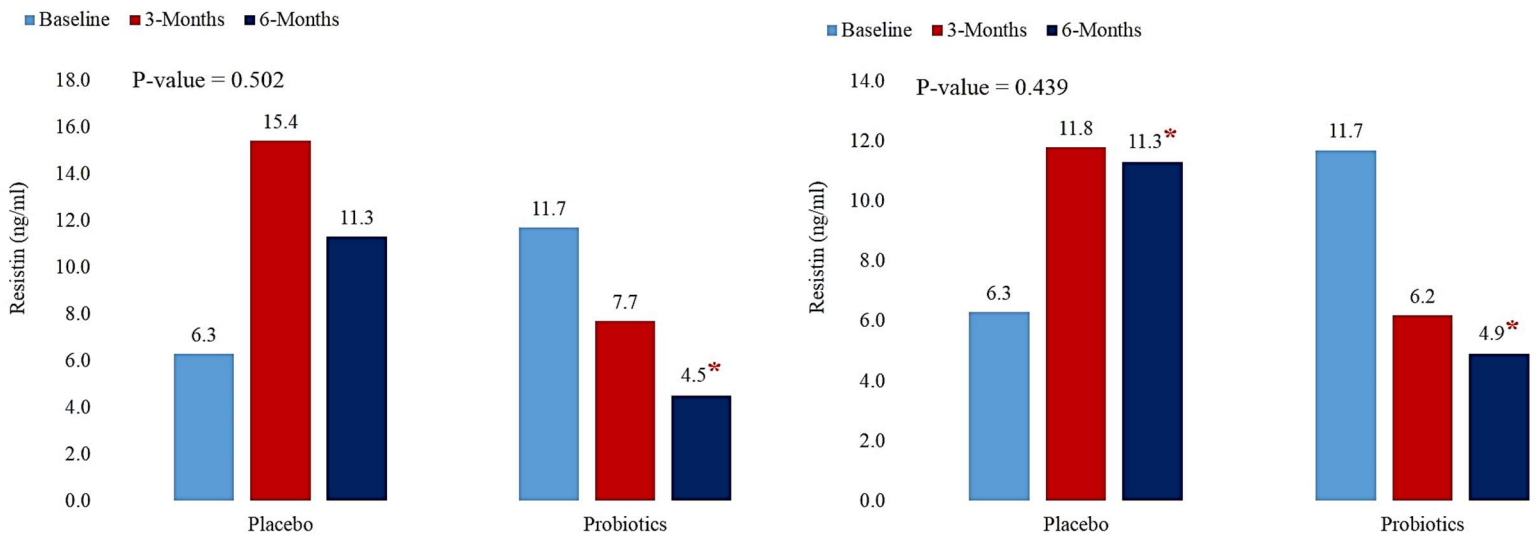


Figure 4.3.10.3 Median resistin (ng/ml) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

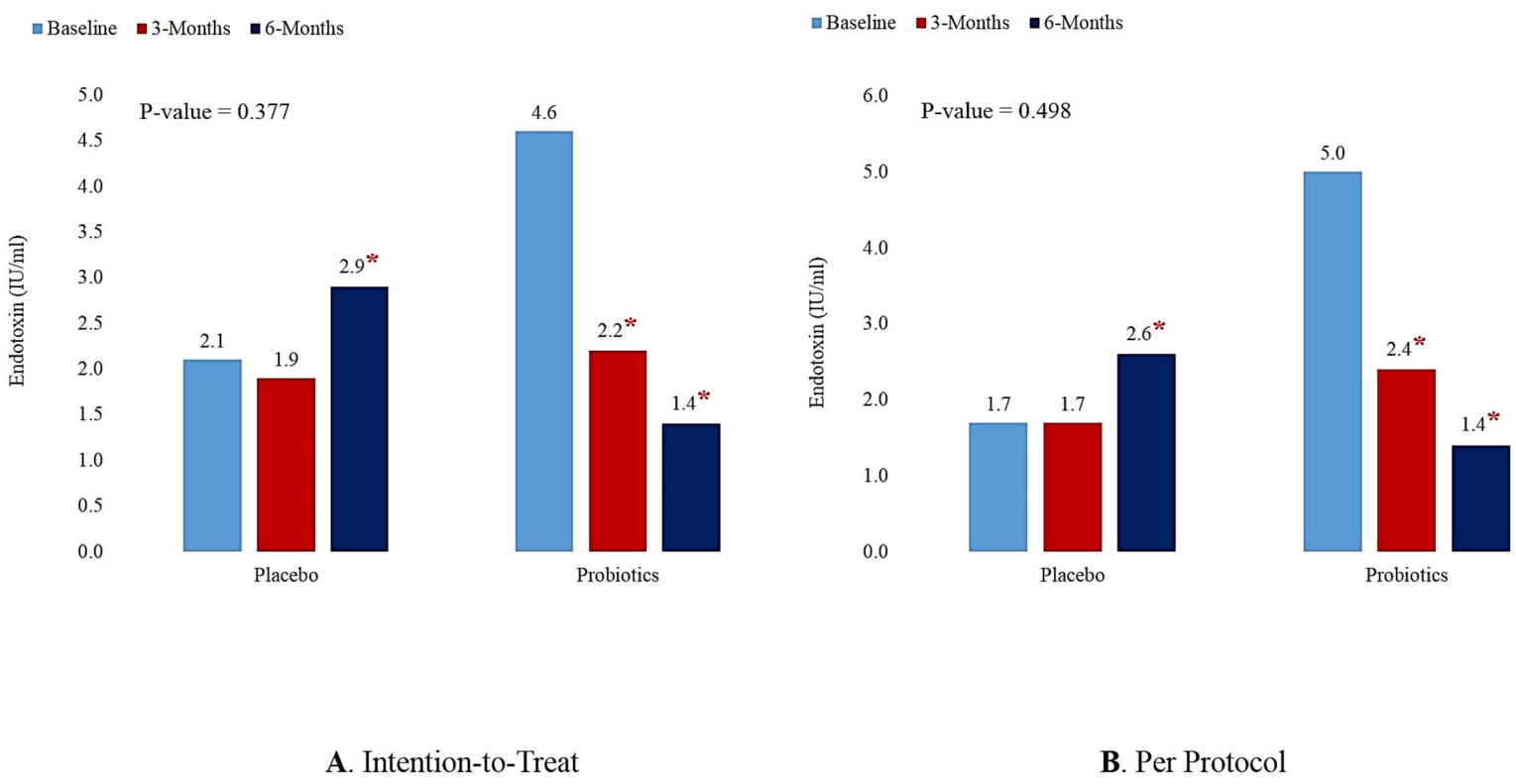


Figure 4.3.10.4 Median endotoxin (IU/ml) before and after intervention in placebo and probiotics group using A) Intention-to-Treat and B) Per Protocol Analysis; P-value denotes between group difference over-all; * denotes significance compared to baseline in within group comparison; significant at $p<0.05$.

4.4 Discussion

In this randomised controlled study, the aim was to determine the beneficial effects of 6-month probiotic supplementation in endotoxemia, inflammation, and cardiometabolic parameters among naïve T2DM patients. Comparison between groups noted significant clinical difference in HOMA-IR, in favour of the probiotics group. No significant between group differences were observed in terms of endotoxin (primary endpoint), lipid profile, other glycaemic indices and anthropometrics, with the exception of WHR, in favour of placebo.

In this study, whilst it was identified that circulating endotoxin levels were significantly reduced post-intervention within the probiotics group, this wasn't significant over-all compared to placebo. Depending on the type of analysis used, significant improvements were also noted in the inflammatory and anti-inflammatory adipocytokines markers in the probiotics group over time. However when compared to placebo, no significant differences were observed in all these markers. Several intervention studies confirm the endotoxin-lowering effects of certain probiotic strains including *Bifidobacteria* and *Lactobacillus* on peritoneal dialysis patients (Wang et al., 2015), among novice long distance triathletes in combination with antioxidants and prebiotics (Roberts et al., 2016) and in patients with cirrhosis (Bajaj et al., 2014); although findings from cirrhotic patients are inconsistent as some studies show no effect in endotoxin levels (Horvath et al., 2016). It is worthy to note that variations in the endotoxin lowering effects of probiotics are highly related to probiotic strain used, duration of intervention and baseline metabolic status of patients. The study of Horvath for instance (Horvath et al., 2016), used the same probiotic formulation used in this study and while they also noted

no significant difference between endotoxin levels after intervention in both arms, it is difficult to compare patient populations as liver functions significantly influences markers of intestinal permeability and possibly endotoxin levels (Arab et al., 2017).

Our study is to our knowledge, one of the very few to demonstrate the effects of a medium term multi-strain probiotics supplementation in several adipocytokines, such as leptin, adiponectin and resistin amongst T2DM patients. Certain probiotics, specifically, lactic acid bacteria strains have demonstrated *in vitro* that they can differentially modulate adipokine expression and the inflammatory response (Fabersani et al., 2017). Worthy to note is that 6 of the 8 probiotics strains used in the present study belong to the lactic acid bacteria class. In alignment with previous findings therefore, this study demonstrated improved levels of an anti-inflammatory adipocytokine, adiponectin as well as decreased levels of inflammatory markers in the probiotics group although no dramatic change was not in the placebo, which appears masked when group interaction effects over-all point to no clinically significant difference.

Previous observations have shown that endotoxins from non-commensal bacteria may affect adipocytokine levels secondary to translocation induction of several intestinal microbial antigens into the circulation, creating altered adipokine profile and intestinal dysbiosis (Cani et al., 2009). The endotoxin-reducing ability of probiotics by creating a stronger intestinal barrier function may partially explain improved adipocytokine levels among those taking probiotic supplements. In animal studies, induced conditions such as non-alcoholic fatty liver disease (NAFLD) where intestinal barrier was compromised, inclusion of probiotic mixture in the diet demonstrated improved lipids, better

adipocytokines (leptin and resistin) and healthier levels of inflammatory markers (TNF-α and IL-6) than those fed without the probiotics mixture (Al-Muzafar and Amin, 2017).

While this is not the first interventional study to examine the effects of probiotics on patients with T2DM, the present study addressed a previous meta-analysis of randomised trials which suggested that probiotics consumption for a longer duration and use of multiple strains may potentially increase the modest benefits of probiotics supplementation in glucose metabolism (Zhang et al., 2016). Furthermore, the recent meta-analysis of Hu and colleagues observed that trials with longer durations of intervention using multiple probiotic strains had more beneficial cardiometabolic effects on patients with T2DM (Hu et al., 2017). The use of 8 strains in the present study most likely provided a cumulative potency in the probiotics intervention aside from the longer duration of 6 months. A separate clinical trial that used a different set of probiotics strains also showed modest changes, with an improved metabolic status in T2DM patients, this discrepancy could be due to sample size difference, duration of intervention and patient selection, amongst others (Firouzi et al., 2017). Over-all, most meta-analyses of interventional studies reaffirm that probiotics intake among patients with T2DM can modestly decrease insulin resistance and improve glycaemic indices when taken as a standalone supplement (Li et al, 2016; Yao et al., 2017; Sun and Buys 2016; Zhang et al., 2016). Effects on blood pressure were also not observed despite the longer duration of treatment even though it was observed in animal studies in combination with other agents such as prebiotics and synbiotics (Tunanpong et al., 2017).

The authors acknowledge several limitations. Successful colonization of probiotics in the intestinal tract cannot be confirmed since stool samples were not

obtained. The study also has a lower uptake and completion rate than desired and therefore potentially affecting the study outcomes. Worthy to note however (as also mentioned in the previous chapters) is that the probiotics group was metabolically worse than placebo; had a poorer glucose control, higher lipids, endotoxin and adiponectin at baseline and so whilst no changes between placebo and probiotics group were observed, it should be taken into consideration that they didn't start at the same level even after randomisation. Despite limitations, this is the first and longest randomised controlled trial to ascertain the effects of a multi-strain probiotic supplementation in reducing endotoxin, inflammatory and adipocytokine profiles in Saudis with T2DM.

4.5 Conclusions

In summary, the present study is the first and the longest clinical trial done to ascertain the effects of probiotics in endotoxemia, inflammation and cardiometabolic parameters in naive Saudi T2DM patients. This randomised clinical trial demonstrated that a daily multi-strain probiotic supplementation for 6 months significantly reduced endotoxin levels and improved inflammatory and anti-inflammatory adipocytokine profiles among Saudi T2DM participants in the probiotics group, but comparison with the placebo group revealed no apparent significant changes. Furthermore, probiotic supplementation for 6 months can significantly reduce HOMA-IR and modestly improve lipids in this population. The present findings also suggest that probiotics supplementation as a monotherapy may not be clinically effective for weight loss. As participants in the present study were treatment naïve, further studies on the effects of probiotics compared with standard therapies for T2DM are needed.

Chapter 5

Final Discussion

5.1 Discussion

The concept of gut microbiota manipulation to reverse several known diseases, T2DM included, through probiotic supplementation, has only recently gained considerable interest among nutritionists and biomedical scientists. The abundance of successful preliminary animal model studies where metabolomics and metagenomics approaches have been performed has reignited interest in probiotic intervention studies to shift to human subjects (Panwar et al., 2013). Furthermore, currently probiotics as a functional food (Stanton et al., 2001) is a multi-billion dollar industry, gaining momentum only in recent years despite largely unverified claims. As such interest has developed as researchers seek to evaluate such functional food and assess probiotic supplements leading to increased publications in the field (Zheng et al., 2017).

Within this context studies were undertaken in this thesis to initiate a randomised, double-blind, placebo-controlled clinical trial approach, to determine the different beneficial effects of an 8-strain probiotic supplementation amongst Saudi adults with T2DM over a 6 month duration. These effects were observed at different points over time in several indices including circulating endotoxin, anthropometrics, glycaemic, lipid, inflammatory and adipocytokine profiles. The studies revealed that while substantial improvements in the indices of interest were more apparent in the probiotics group over time, these effects and with the exception of HOMA-IR, were not clinically significant when compared with placebo. Furthermore after randomisation, it was clear that the probiotics group were more insulin-resistant and metabolically worse compared to the placebo group and this has somehow compromised effects that can be otherwise deemed clinically significant. While the present finding is not new since many recent meta-

analyses conducted on the effects of probiotics in patients with T2DM universally conclude the clinical benefits of probiotic supplementation in improving glycaemic parameters (Wang et al., 2017; Sun and Buys, 2016; Hu et al., 2017), the present studies conducted still contributed new insights. First is the probiotic supplements themselves and the study design. The use of an 8-strain probiotic supplement to be given over a 6-month in the present study has never been tested in the T2DM population. The same probiotics supplement however has been tested in other populations over shorter duration with mostly beneficial outcomes. One such study by Steenbergen and colleagues provided first evidence that intake of probiotics reduce negative thoughts associated with sad mood (Steenbergen et al., 2015). Other benefits of the probiotics supplements used in the previous study included the reduction of migraines (de Roos et al., 2015) and improved immune function via increased neopterin levels and reactive oxygen species production by neutrophils amongst cirrhotic patients (Horvath et al., 2016). The last study also showed minimal influence of probiotics in gut endothelial function, as observed by no discernible changes in endotoxin levels, similar to the present study. The formulation and choice of the 8-strain probiotics supplement is also worthy of mention. This probiotic combination has been investigated for its ability to not only improve endothelial barrier but also for its potency to inhibit mast cell activation, inhibit pro-inflammatory cytokines and more importantly, to decrease endotoxin load (van Hemert S and Ormel G, 2014), which is the main endpoint of the present studies conducted.

The second novelty in the present studies conducted is the choice of cohort. Clinical trials on probiotic supplementation in the Arabic T2DM population, has also never been performed previously. This is important since the gut microbiome, although

mostly populated by *Firmicutes* and *Bacteroidetes* is highly affected not only by the health status of the individual, but more so by geography and ethnicity (Gupta et al, 2017). These diversity in gut microbiome has been observed as early as the first year of life (Stearns et al., 2017). The effects therefore of probiotics are not only strain-specific but also highly varied depending on the individual's gut microbiome make up and health status. Findings of the present study therefore adds value to the current literature in terms of ethnic-specific effects of probiotics supplementation among patients with T2DM.

Exactly how probiotic supplementation reverses abnormal metabolism has been studied extensively. Some of the well-known mechanisms of actions of probiotics include beneficial alteration of the gut microbiome, competitive inhibition with other bacterial components via adherence to the mucosa and epithelium, strengthening of the intestinal epithelial barrier function and modification of the immune response in favour of the host (Bermudez-Brito et al., 2012; Thomas and Versalovic, 2010). It is worth mentioning that significant improvements in the probiotics group were demonstrated over time in terms of reduction of endotoxin, glycaemic, lipid, adipocytokine and inflammatory profiles. Whilst these effects were not demonstrated in the placebo group, both arms (placebo and probiotics) were not equal in insulin resistance, inflammatory and CVD risk status at baseline. Therefore these positive effects observed in the probiotics group may largely be due to an over-all improved epithelial barrier secondary to probiotics supplementation. The significant reduction of circulating endotoxin levels, in the probiotics group in particular, may have directly caused these effects, since previous studies from the same population have consistently demonstrated the significant associations of endotoxin with several cardiometabolic factors in the same ethnic group and having the same disease

(T2DM), including the metabolic syndrome (Al-Disi et al., 2015; Harte et al., 2012; Al-Attas et al., 2009). Since endotoxin is largely stored within the gut, it makes sense that prevention of endotoxin from leaking out of the gut through a strengthened intestinal barrier would translate to a better and healthier cardiometabolic profile.

Lastly, the clinically significant difference in WHR in favour of the probiotics group at 3 months intervention and in placebo at 6 months is in contradiction to one another yet also confirms the conflicting results from various meta-analyses on the anti-obesity effects of probiotics consumption in humans (Nova et al., 2016; Crovesy et al., 2017; Sayon-Orea et al., 2017). Currently, the beneficial effects of probiotics appear to be more successful in animal models (Karimi et al., 2017; Kobyliak et al., 2017). Worthy to mention is that the probiotics supplementation in the present studies of the thesis was used as a standalone treatment given in the absence of exercise and diet-related modifications in the intervention as well as a lesser controlled environment. A recent randomised clinical trial by Gomes and colleagues (2017) however parallels the present thesis' finding on abdominal obesity reduction, but this was in combination with a prescribed dietary regimen, hence the higher percentage change (>5%) reduction in waist circumference as compared to the WHR assessed in the present thesis (<0.01%). Whether anti-obesity efficacy of probiotics will be enhanced in combination with the mentioned strategies remain to be proven. Nevertheless, the over-all evidence for weight loss secondary to probiotics is still scarce. Furthermore, the efficacy of probiotics are strain specific and highly dependent on various intrinsic components within the individual and this could probably explain the inconsistencies of findings in the literature.

5.2 Limitations of the Present Studies

Two major limitations in the present studies were noted. First is the sample size. A priori sample size determination is mandatory for all successful clinical trials. In this case, it was calculated that at N=60 per arm group (total of 120 participants), the effect size will have 80% power to detect a statistically significant difference. The actual number of participants who completed the 6-month trial was 39 (N=16 for placebo and N=23 for probiotics). This explains why strong and significant changes in the probiotics group over time did not translate into clinically meaningful changes when compared with placebo.

The second limitation is the persistent discrepancy between baseline values of the probiotics and the placebo group despite randomisation, as is the nature of clinical trials. Baseline characteristics show that whilst age and BMI were matched for both placebo and probiotics group, the probiotics group were actually cardiometabolically worse than placebo. Whilst this was addressed by adjusting analyses for baseline differences, the additional adjustments of covariates made it more difficult to elicit the desired treatment effect because of the added statistical stringency to the small cohort. This is worth highlighting because the probiotics group made a more substantial improvement and hence the disparity with the control. Nevertheless despite the limitations and the rigorous analyses done, a significant improvement was observed in terms of decreased insulin resistance over time, in favour of the probiotics group. As insulin resistance is intricately linked to most of the cardiometabolic indices measured, the clinically significant improvement suggests that probiotics supplementation do confer beneficial effects when consumed by the T2DM population.

Other limitations as mentioned previously, albeit minor, include the lack of evidence to prove successful gut colonization of probiotic bacteria since RT-PCR was not performed at this stage. Indeed, whilst the need for the probiotic bacteria to be alive after ingestion is mandatory, the practical aspect of determining whether successful colonization occurred would support the concept, although absence of gut microbiome data does not necessarily mean absence of efficacy (Rowland et al., 2010).

5.3 Future Directions

In light of the present findings, additional clinical trials are clearly warranted, especially in the Middle Eastern region. Before addressing this however, the general population should be given to determine the importance of public health awareness of the benefits of consuming probiotics supplements. The concept of probiotics is largely unheard of and people generally were unaware that probiotics have been a steady part of the Arabian diet in the form of fermented products such as yoghurt and laban (fermented milk). As awareness is heightened it is expected that this may reduce dropout rates as subsequent probiotics trials are conducted. Clinical trials in Saudi Arabia in particular is still at its infancy. A recent observation from Jamjoom and colleagues (2015) revealed that there were only 39 clinical trials conducted in Saudi Arabia and where a Saudi Arabian institution was principally responsible over a span of 13 years and this was severely dwarfed in comparison to 807 clinical trials registered over a span of three years in one German university alone. Other recommendations include conducting several probiotics clinical trials to other populations such as pregnant women and children using

different doses, probiotics strains, intervention duration and in combination with other agents such as diet, exercise, prebiotics and other supplements, to name a few.

5.4 Conclusions

The present thesis performed a randomised, double-blind, placebo-controlled clinical trial of 6-month duration to determine the effects of a multi-strain probiotic supplementation in reducing endotoxin levels and altering the statuses of anthropometry, glycaemia, lipids, inflammatory and adipocytokines in the Saudi adult population with newly diagnosed T2DM. Findings from the thesis as conducted in the several studies presented offer important information that will expand our current understanding on how multi-strain probiotic supplements work in the diabetic population coming from a relatively homogenous ethnic background. The findings also shed light on the challenges of conducting randomised clinical trials in this area of the world where such studies that offer high level of evidence are still evolving and would require greater input and participation from the general population. It is clear that whilst further interventional trials that meet the required statistical power are necessary to reaffirm the present findings, the significant improvement in insulin resistance in favour of the probiotics group despite the low sample size turn post intervention and the rigorous analysis performed merit clinical attention. Whether the same effects will be elicited in the presence of other medications and diabetes-related complications remains to be investigated. Nevertheless and in light of this positive result in the present thesis, probiotics supplementation appears useful as an adjuvant therapy in medication naïve patients with known insulin resistance and early phase T2DM.

List of Publications, Records and Papers Extracted from Thesis

ClinicalTrials.Gov. Bethesda (MD): National Library of Medicine (US) 2013 January 10. Identifier: NCT01765517, Study to explore the effects of probiotics on endotoxin levels in type 2 diabetes mellitus patients. Available online: https://clinicaltrials.gov/ct2/show/NCT01765517

Sabico S, Alokail M, Al-Daghri N. McTernan Philip. Effects of probiotics in patients with diabetes mellitus type 2: a study protocol for a randomized, double-blind, placebo-controlled trial. J Clin Gastroenterol 2016; 50: S230.

Sabico SLB, Al-Daghri NM, McTernan P. Use of probiotics in subclinical inflammatory conditions: review of evidence. J Food Process Technol 2012; 3:10. (Conference Proceeding)

Alokail MS, **Sabico S**, Al-Saleh Y, Al-Daghri NM, Alkharfy KM, Vanhoutte PM, McTernan PG. Effects of probiotics in patients with diabetes mellitus type 2: study protocol for a randomized, double-blind, placebo controlled trial. Trials 2013; 14: 195.

Sabico S, Al-Mashharawi A, Al-Daghri NM, Yakout S. Alnaami AM, Alokail MS. McTernan PG. Daily intake of a multi-strain probiotic supplement for 12 weeks improves cardiometabolic profiles of native T2DM patients. J Transl Med 2017; 15(1): 249.

Sabico S, Al-Mashharawi A, Al-Daghri NM, Amer OE, Hussain DS, Wani K, Masoud MS, Alokail MS, McTernan PG. Effects of a 6-month multi-strain probiotics supplementation in endotoxemia, inflammation and cardiometabolic status of T2DM patients: a randomized, double-blind, placebo-controlled trial. **Submitted to Clinical Nutrition (For resubmission pending revision, January 22, 2018)**

List of Conferences/Symposiums Attended Relevant to Thesis

1. International Conference and Exhibition on Probiotics. November 19-21, 2012, San Antonio, TX, USA (Oral Presenter)
2. Probiota 2014 Conference. February 4-5 2014, Amsterdam, Netherlands (Poster Presenter)
3. Warwick Medical School Post Graduate Research Symposium, May 21, 2015, Warwick University, Coventry, UK (Attendee)
4. 8th Probiotics, Prebiotics & New Foods - for microbiota and human health. September 13-15, Rome, Italy. (Poster Presenter)
5. Warwick Medical School Post Graduate Research Symposium, June 6-7, 2017, Warwick University, Coventry, UK (Oral Presenter)
6. ENDO 2018 – March 17-20, 2018, McCormick Place West, Chicago, Illinois, USA (Poster Presenter)


Name
Shaun Louie B. Sabico
Organization
King Saud University, Saudi Arabia
OMICS Group
Conferences
Accelerating Scientific Discovery
Speaker
International Conference and Exhibition on
Probiotics
November 19-21, 2012 Hilton San Antonio Airport, USA

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Confirmation of Poster Presenter Invitation for Shaun Sabico

Dear Shaun,

Further to our conversations, I am delighted to formally confirm our invite to you to attend the forthcoming *Probiota 2014* Conference, taking place on 4th & 5th February 2014 in Amsterdam, Netherlands.

We are pleased that you can attend the event as a poster presenter and we look forward to welcoming you to the conference. Please be aware the delegates to the conference are responsible for payment of all registration fees and any travel and accommodation costs in connection with their attendance.

If I can be of any further assistance, please let me know.

With best regards,

JONATHAN WORSFOLD

On behalf of Probiota 2014 Conference Team

Poster presented in Amsterdam and Italy

EFFECTS OF PROBIOTICS IN PATIENTS WITH DIABETES MELLITUS TYPE 2: A STUDY PROTOCOL FOR A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL

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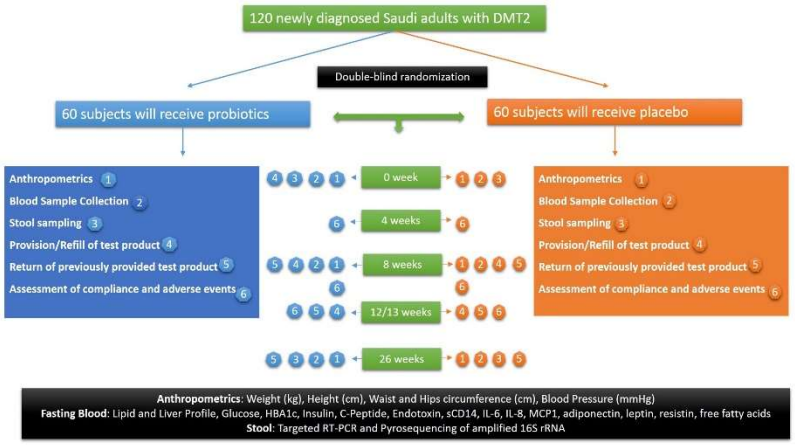
Background

Low grade chronic inflammation and elevated serum endotoxin levels are observed in patients with DMT2. Probiotics may influence circulating endotoxin levels by altering gut microbiota and gut barrier function in a beneficial manner to reduce inflammation. No information is available whether or not they do so in patients with DMT2. We hypothesize that treatment with probiotics will reduce mean endotoxin levels [1]. Therefore, the aim of this study is to characterize the beneficial effects of probiotics on circulating endotoxin levels and other biomarkers related to systemic low-grade inflammation in patients with DMT2.

Key Words: Type 2 Diabetes Mellitus, Endotoxin, Microbiota, Probiotics

Methods

A randomized placebo controlled trial with 120 consenting adult Saudi DMT2 patients with placebo or probiotics (Ecologic®Barrier, Winclove, Netherlands) The probiotics ($2.5x10^9$ cfu/gram) contain the following bacterial strains: *B. bifidum* W23, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, *Lc. lactis* W19 and *L. lactis* W58). Blood and stool samples will be analysed (see figure).



Discussion

The bacterial strains for this study are selected based on different *in vitro* screening criteria. Among the criteria were the inhibition of cytokine-induced barrier dysfunction of the epithelial cell line Caco-2, the capacity to induce expression of interleukin-10 [2], as this anti-inflammatory cytokine has a protective function on the epithelial barrier [3], the ability to break-down lipopolysaccharides and the inhibition of mast cell activation [4]. It is expected that the probiotic product employed will induce beneficial changes in gut microbiota, reduce systemic endotoxin
levels and, as such, decrease the systemic inflammatory response observed in DMT2 subjects.

Trial Status: On-going recruitment
Trial Registration: ClinicalTrials.gov Identifier: NCT01765517
Grant: National Plan for Science and Technology (NPST 11-MED2114-02)

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8TH PROBIOTICS, PREBIOTICS & NEW FOODS

for microbiota and human health

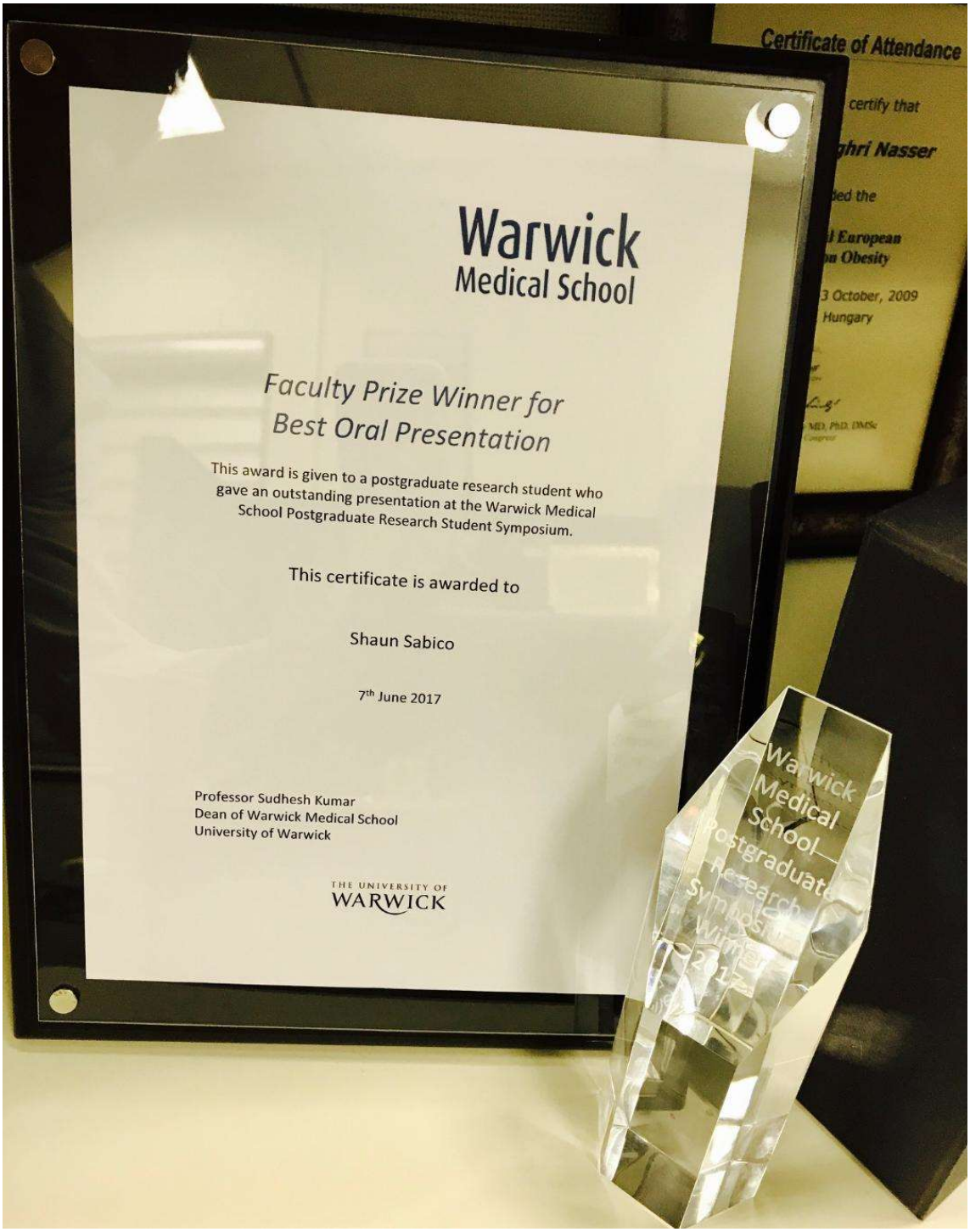
Certificate of Attendance

This is to certify that

SHAUN LOUIE SABICO

has attended the Meeting

Rome - September 13/15, 2015 - Università Urbaniana


Warwick
Medical School
Faculty Prize Winner for
Best Oral Presentation
This award is given to a postgraduate research student who gave an outstanding presentation at the Warwick Medical School Postgraduate Research Student Symposium.
This certificate is awarded to
Shaun Sabico
7th June 2017
Professor Sudhesh Kumar
Dean of Warwick Medical School
University of Warwick
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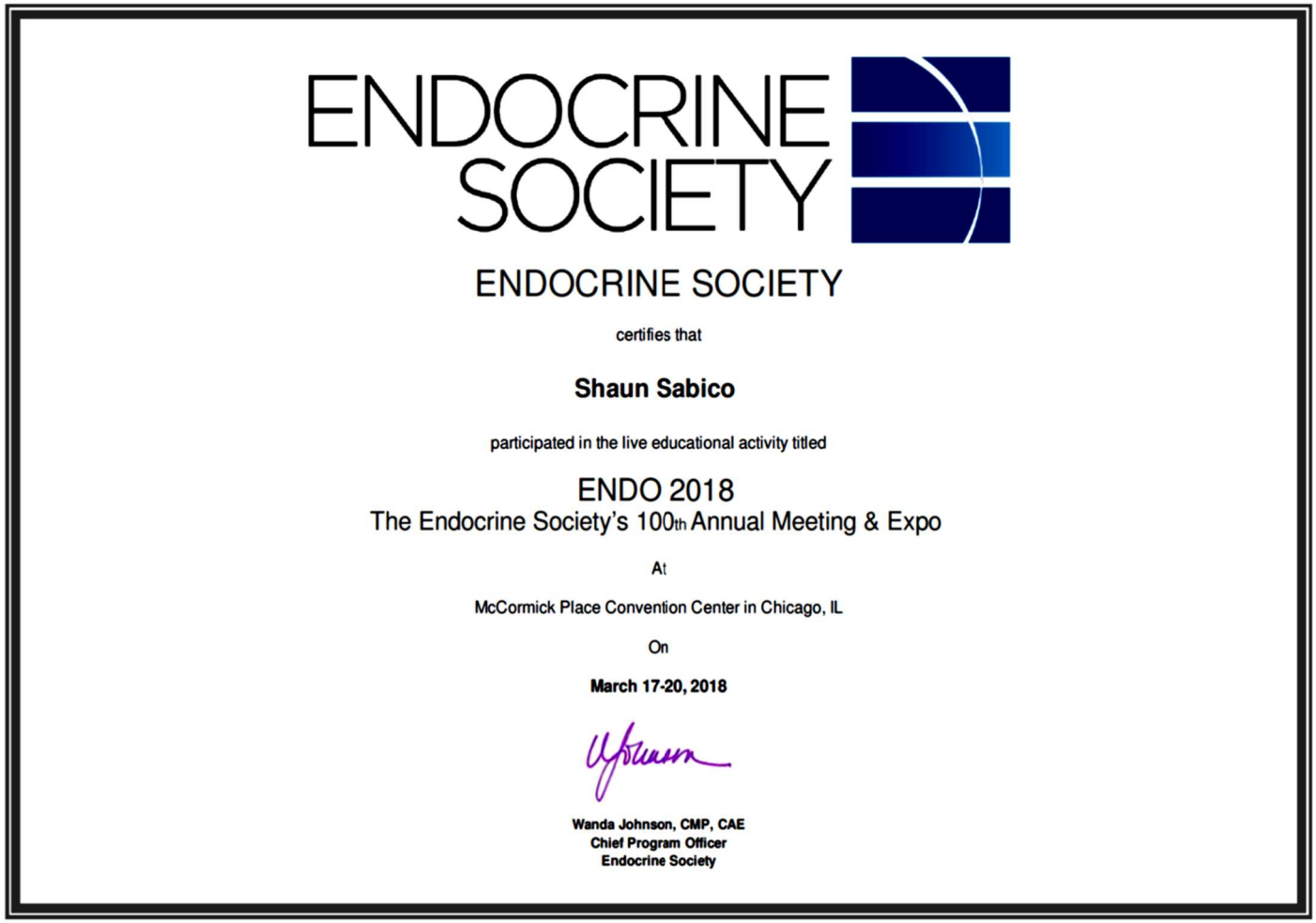

ENDOCRINE SOCIETY

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certifies that

Shaun Sabico

participated in the live educational activity titled

ENDO 2018
The Endocrine Society's 100th Annual Meeting & Expo

At

McCormick Place Convention Center in Chicago, IL

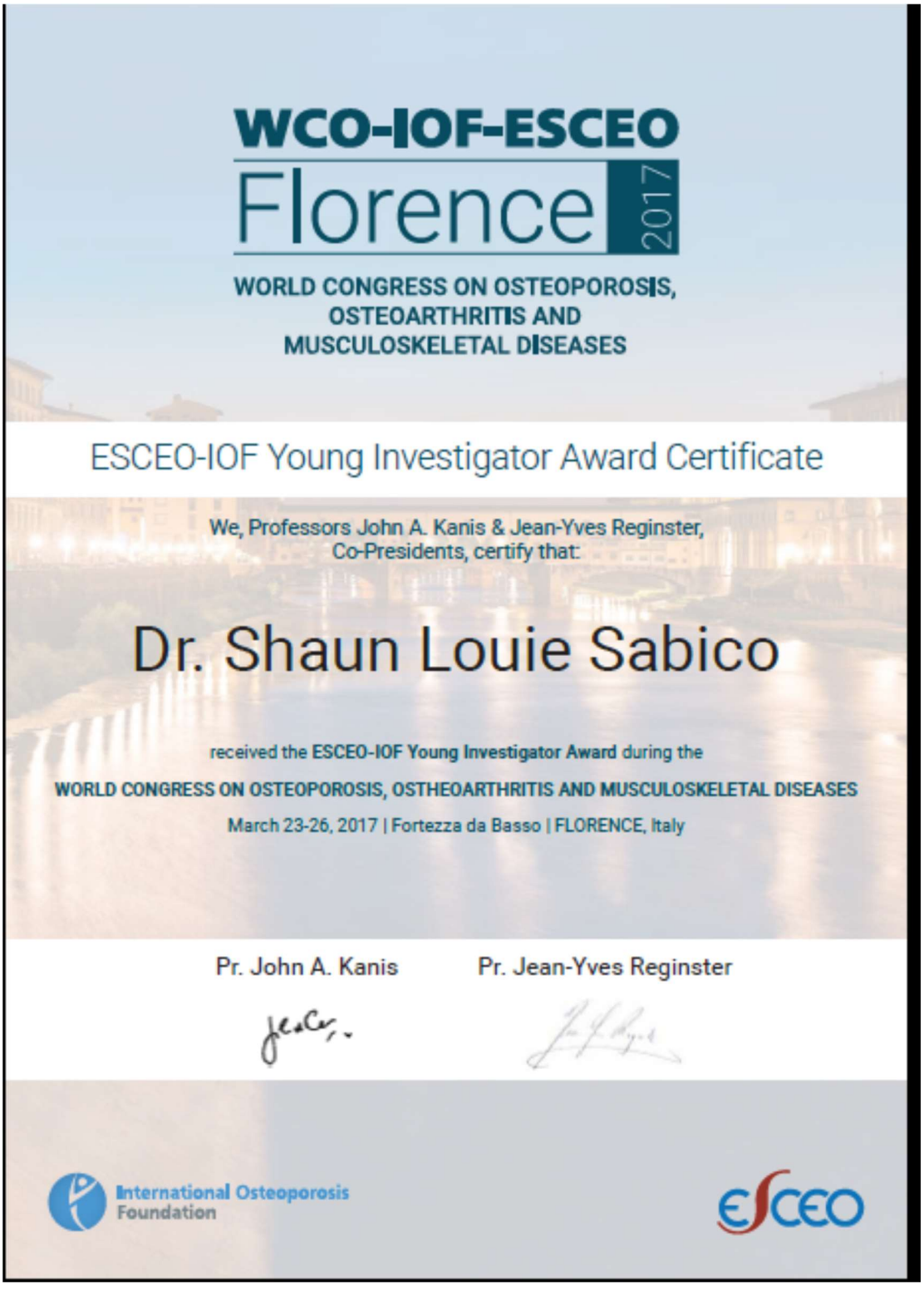
On

March 17-20, 2018

Wanda Johnson, CMP, CAE
Chief Program Officer
Endocrine Society

List of Awards, Scholarships, Grants and Community Service during PhD Program

1. **Research Grant as Co-Investigator** (£298,000.00): A 26-week, Randomized, Double-blind, Placebo-controlled Study to Explore the Effects of Probiotics on Endotoxin Levels in Patients with Type 2 Diabetes Mellitus. National Plan for Science and Technology (NPST) (Grant Number: 11-MED2114-02), Awarded in 2012.
2. **Guest Lead Editor,** Journal of Diabetes Research (February 27, 2015- July 17, 2015)
3. **Eli Lilly Scholarship Award** ($1500.00). WCO-IOF-ESCEO April 14-17, 2016, Malaga, Spain.
4. **IOF Young Investigator Award** (€1000.00). WCO-IOF-ESCEO March 23-26, 2017, Florence, Italy
5. **Best Oral Presentation** (£250.00). WMS Post-Graduate Research Student Symposium. Warwick Medical School, June 7, 2017, Coventry, UK
6. **First Prize Oral Presentation** (£600.00). Fifth Annual Clinical Congress of the Gulf Chapter of the American Association of Clinical Endocrinologists. October 5-7, 2017, Dubai, United Arab Emirates
7. **IOF Young Investigator Award** (€1000.00). WCO-IOF-ESCEO April 19-22, 2018, Krakow, Poland


WCO-IOF-ESCEO
Florence 2017
WORLD CONGRESS ON OSTEOPOROSIS, OSTEOARTHRITIS AND MUSCULOSKELETAL DISEASES
ESCEO-IOF Young Investigator Award Certificate
We, Professors John A. Kanis & Jean-Yves Reginster, Co-Presidents, certify that:
Dr. Shaun Louie Sabico
received the ESCEO-IOF Young Investigator Award during the
WORLD CONGRESS ON OSTEOPOROSIS, OSTHEOARTHRITIS AND MUSCULOSKELETAL DISEASES
March 23-26, 2017 | Fortezza da Basso | FLORENCE, Italy
Pr. John A. Kanis
Pr. Jean-Yves Reginster
International Osteoporosis Foundation
ESCEO


GULF CHAPTER
Free Communication Prize Certificate
The Board of Directors
would like to express its congratulations
Shaun Sabico
for winning the first prize in the category of oral presentations at the
The Fifth Annual Clinical Congress
of the Gulf Chapter of the American Association of Clinical Endocrinologists
held on Thursday 5th to Saturday 7th of October, 2017 at the Grand Hyatt Hotel, Dubai, United Arab Emirates
Salem Beshyah
Chapter's President
Ebaa Al-Ozairi
Chapter's Treasurer
Nasser Aljuhani
Chapter's Secretary

Hindawi

Journal of Diabetes Research

Special Issue on
Gut Microbiota and Diabetes

Call for Papers

There is an expanding research on the remarkable influence of the human gut microbiota in health and disease. We already know that interactions between the gut flora and human physiology occur at several levels, and bacterial fragments such as endotoxin can enter the circulation which, in turn, may influence our metabolic state. Recent advances have also uncovered the promising effects of both pre- and probiotics in human health. In this special issue for the Journal of Diabetes Research, we aim to highlight the expanding field of gut microbiota research and its potential role in the pathophysiology of diabetes mellitus.

We invite our colleagues and investigators to contribute original research and review articles that will broaden our still limited understanding of the human gut microbiota and how manipulation of the gut flora may hold clinical implications in the management of insulin resistance-related diseases such as diabetes mellitus. Potential topics include, but are not limited to:

- Recent developments in the gut microflora and diabetes research
- Role of probiotics in the management of diabetes mellitus
- Influence of nutrition in the gut microflora of patients with diabetes
- Advances in probiotic strain selection techniques specific for diabetes management
- Advances in endotoxin and diabetes research
- Advances in the bioavailability and efficacy of probiotic supplements for insulin resistance-related diseases such as obesity, metabolic syndrome and diabetes
- Emerging biomarkers in diabetes and gut microflora

Before submission authors should carefully read over the journal's Author Guidelines, which are located at http://www.hindawi.com/journals/jdr/guidelines. Prospective authors should submit an electronic copy of their complete manuscript through the journal Manuscript Tracking System at http://mts.hindawi.com/submit/journals/jdr/gmd according to the following timetable:

Manuscript Due	February 27, 2015
First Round of Reviews	May 22, 2015
Publication Date	July 17, 2015

Lead Guest Editor

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Subject: ESCEO-IOF Young Investigator Awards - Notification - WCO-IOF-ESCEO 2018 Krakow

From: leisten@humacom.com
To: eaglescout01@yahoo.com
Date: Tuesday, February 27, 2018, 10:53:26 AM GMT+3

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Dear Doctor Sabico,

We would like to thank you very much for having submitted an application for the

2018 ESCEO-IOF Young Investigator Award

It is our pleasure to inform you that due to the outstanding quality of your work, you are selected as being one of the recipients of this prestigious prize.

The prize will be presented to you during the Cocktail Ceremony on Saturday April 21, 2018, from 18h40 to 19h40, at the ICE Krakow Congress Center. You are requested to attend this private cocktail offered by ESCEO and IOF to all recipients of prizes.

Failure to attend will result in the cancellation of the award.

Please note that prizes are contingent on being personally present in Krakow.

We sincerely congratulate you and looking forward to meeting you in Krakow, we remain sincerely yours,

Congress Chairpersons
Jean-Yves Reginster (President ESCEO) and **John A. Kanis** (Honorary President IOF)

Scientific Committee Chairpersons
Cyrus Cooper (President IOF) and **René Rizzoli** (Chairman ESCEO SAB)

List of Publications Relevant to PhD Thesis

1. Al-Daghri NM, Ansari MGA, **Sabico S**, Aljohani NJ, Al-Saleh Y, Alfawaz H, Alharbi M, Alokail MS, Wimalawansa SJ. Efficacy of different modes of vitamin D supplementation strategies in Saudi adolescents. J Steroid Biochem Mol Biol 2018; pii: S0960-0760(18)30063-3.

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8. Al-Daghri NM, Rahman S, **Sabico S**, Yakout S, Wani K, Al-Attas OS, Saravanan P, Tripathi G, McTernan PG, Alokail MS. Association of Vitamin B12 with Pro-Inflammatory Cytokines and Biochemical Markers Related to Cardiometabolic Risk in Saudi Subjects. Nutrients. 2016 Sep 6;8(9).

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11. Al-Daghri NM, Aljohani NJ, Al-Attas OS, Al-Saleh Y, Alnaami AM, **Sabico S**, Amer OE, Alharbi M, Kumar S, Alokail MS. Comparisons in childhood obesity and cardiometabolic risk factors among urban Saudi Arab adolescents in 2008 and 2013. Child Care Health Dev. 2016 Sep;42(5):652-7.

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accentuated in patients with Type 2 diabetes mellitus: a body mass index-independent role of adiponectin? J Endocrinol Invest. 2013 Jan;36(1):1-6.

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47. Al-Daghri NM, Alkharfy KM, Al-Saleh Y, Al-Attas OS, Alokail MS, Al-Othman A, Moharram O, El-Kholie E, **Sabico S**, Kumar S, Chrousos GP. Modest reversal of metabolic syndrome manifestations with vitamin D status correction: a 12-month prospective study. Metabolism. 2012 May;61(5):661-6.

48. Al-Daghri NM, Al-Attas OS, Alokail MS, Alkharfy KM, Hussain T, **Sabico S**. Gender differences exist in the association of leptin and adiponectin levels with insulin resistance parameters in prepubertal Arab children. J Pediatr Endocrinol Metab. 2011;24(7-8):427-32.

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circulating adipocytokines and ghrelin levels in teen-age girls. Endocr J. 2010;57(10):915-23.

55. Al-Daghri NM, Al-Attas OS, Alokail MS, Alkharfy KM, **Sabico SL**, Chrousos GP. Decreasing prevalence of the full metabolic syndrome but a persistently high prevalence of dyslipidemia among adult Arabs. PLoS One. 2010 Aug 13;5(8):e12159.

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58. Al-Daghri N, Alokail M, Al-Attas O, **Sabico S**, Kumar S. Establishing abdominal height cut-offs and their association with conventional indices of obesity among Arab children and adolescents. Ann Saudi Med. 2010 May-Jun;30(3):209-14.

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62. Al-Daghri NM, Al-Attas OS, Hussain T, **Sabico S**, Bamakhramah A. Altered levels of adipocytokines in type 2 diabetic cigarette smokers. Diabetes Res Clin Pract. 2009 Feb;83(2):e37-9.

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List of Published Abstracts Relevant to PhD Thesis

1. Al-Daghri N, Khan N, Al-Attas S, **Sabico S**, Alokail M, Kumar S. Association of serum sex hormone-binding globulin with features of metabolic syndrome in children. FASEB J 2016; 4(1): 628.1

2. Al-Disi D, Al-Daghri N, Khan N, Alsaif A, Alfadda A, **Sabico S**, Tripathi G, Mcternan PG. A 3-Month Balanced Diet with Complex Carbohydrate Improves Cardiometabolic Profile, Metabolic Endotoxaemia, and the Capacity to Manage a Damaging High-Fat Meal Challenge More Appropriately in Obese T2DM Subjects. Diabetes 2014; 6(1): A192.

3. Al-Daghri N, Aljohani N, Al-Fawaz H, Al-Saleh Y, Al-Yousef M, **Sabico S**, Kumar S. A 6-Month Modest Lifestyle Modification with Increased Sunlight Exposure Improves Vitamin D Status, Lipid Profile, and Glycemic Status in Overweight and Obese Saudi Adults with Vitamin D Deficiency and Varying Glycemic Levels. Diabetes 2014; 6(1): A590.

4. Alkharfy K, Al-Daghri N, **Sabico S**, Al-Othman A, Moharram O, Alokail M, Al-Saleh Y, Kumar S, Chrousos G. Vitamin D supplementation in patients with diabetes mellitus type 2 on different therapeutic regimens: a one-year prospective study. FASEB J 2014; 4(1): 575.4

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7. Alkharfy K, Al-Daghri N, Al-Othman A, Moharram O, Alokail M, Al-Saleh Y, **Sabico S**. Vitamin D supplementation as influenced bu diabetic therapies. FASEB J 2013; 27(1): 791.5

8. Kumsaiyai, Harte A, Al-Naji F, Al-Daghri N, Kyrou I, Barber T, **Sabico S**, Tripathi G, McTernan P. Human abdominal subcutaneous adipocytes as an active source of LpPLA2, influenced by fat depot and metabolic state, with LpPLA2

converting LDL into more potent atherogenic Ox-LDL, in vitro. Bioscientifica 2013; 31.

9. Harte A, Varma M, Tripathi G, McGee K, Al-Daghri N, Al-Attas O, **Sabico S**, O'Hare J, Ceriello A, Saravanan P, Kumar S, McTernan P. Post-prandial high fat intake leads to acute exposure to circulating endotoxin in type 2 diabetes mellitus subjects. Bioscientifica 2012; 28.

Appendices

Appendix 1. Ethical Approval from KSU

بسم الله الرحمن الرحيم

المملكة العربية السعودية
وزارة التعليم العالي
جامعة الملك سعود
كلية العلوم

الرقم: ٨/ ٢٦٥١٩/ ٢٥
التاريخ: ١ / ٢ / ١٤٣٢هـ
المرفقات: .....
الموضوع: .....

سعادة الدكتور / ماجد بن صالح العقيل **حفظه الله**

السلام عليكم ورحمة الله وبركاته

نفيد سعادتكم بأن لجنة أخلاقيات البحوث الحيوية العلمية بكلية العلوم ناقشت في الاجتماع الأول يوم الأحد الموافق١٦ / ١ / ١٤٣٣ هـ الموافق ١١ / ١٢ / ٢٠١١ م البحث المرسل من سعادتكم تحت عنوان " A 26-week, Randomized, Double-blind, Placebo-controlled Study to Explore the Effects of Probiotics on Endotoxin Levels in Patients with Type 2 Diabetes Mellitus ".

وقد وافقت اللجنة على المشروع.

وتقبلوا خالص تحياتي وتقديري ،،،

ودمتم بخير ،،،،

رئيس لجنة أخلاقيات البحوث الحيوية العلمية

د. ناصر بن محمد الداغري

Appendix II Approval from Ministry of Health to Recruit in Primary Care Centres

Kingdom Of Saudi Arabia

Ministry Of Health

General Directorate Of Health Affairs
In Riyadh

المملكة العربية السعودية
وزارة الصحة
مديرية الشؤون الصحية في منطقة الرياض
إدارة الصحة العامة

سعادة / مشرف القطاع الصحي (جميع القطاعات داخل مدينة الرياض) المحترم

السلام عليكم ورحمة الله وبركاتة

اشارة الى كتاب المشرف على كرسي الامير متعب بن عبدالله لابحاث المؤشرات الحيوية لهشاشة العظام رقم ١٣٦١٦٤ / ٢٥ / ٨ بتاريخ ٦ / ٤ / ١٤٣٤ هـ بخصوص دراسة بعنوان " دراسة عشوائية لمدة ٢٦ أسبوع حول تأثير البروبيوتيك على مستويات الأندوتكسين لدى المرضى المصابين بداء السكري من النوع الثاني " والتي تتطلب جمع عينات من المراكز الصحية والمستشفيات التابعة لمنطقة الرياض وبناء على موافقة لجنة أخلاقيات البحوث العلمية الحيوية بكلية العلوم .

علية نأمل الاطلاع والعمل على تسهيل المهمة .

وتقبلو أطيب تحياتي ،،،

م / المدير العام للصحة العامة

د / منصور بن علي اليوسف

المديرية العامة للشؤون الصحية بمنطقة الرياض
الادارة العامة المساعدة للصحة العامة
رقم الصادر: ٠٧٤١٩١
التاريخ : ١٤٣٤-٠٥-٢٥
المشفوعات : الصحة المهنية

ريخ : / / ١٤٣٤هـ المشفوعات :

Appendix III SFDA Approval for Probiotics Dispatch

(2 Separate Batches)

الهيئة العامة للغذاء والدواء
Saudi Food & Drug Authority
رقم الصادر : 8188/ع
وحدة التسجيل : إدارة الإتصالات الإدارية
تاريخ الصادر : 17 / 08 / 1434 هـ
جهة الإحالة : أفراد

المملكة العربية السعودية
الهيئة العامة للغذاء والدواء
(٢٥٥)
قطاع الدواء

إذن استيراد مستحضرات لدراسة سريرية			
تاريخ الإصدار:	١٤٣٤/٠٨/١٧هـ	تاريخ الإنتهاء:	١٤٣٥/٠٢/١٧هـ

المكرم ناصر الداغري المحترم

السلام عليكم ورحمة الله وبركاته ،،،

إشارة إلى خطابكم المقيد لدينا برقم ٥٧٥٢/ع وتاريخ ١٤٣٤/٠٨/١٧هـ المتضمن طلب إذن استيراد للمستحضر الموضح أدناه لعمل الدراسة السريرية الموضحة أدناه:

"a 26-week, Randomized, double blined, placebo-controlled study to explore the effects of probiotics on endotoxin levels in patient with type 2 Diabetes Mellitus"

اسم العينة	الكمية
Winclove 849 (Probiotics) 2gm	240 Boxes / 96 Sachets

عليه نفيدكم بالموافقة على استيراد المستحضر بالكمية المذكورة أعلاه لصالح الدراسة السريرية الموضحة أعلاه ، وفي حال وصول الشحنة بإمكانكم إنهاء إجراءات الفسح عن طريق مكتب قطاع الدواء بمنفذ الوصول مع تقديم أصل الخطاب.

- يجب نقل المستحضرات وفق ظروف التخزين الموصى بها من الشركة الصانعة مع إرفاق مؤشر لدرجات الحرارة .

مع خالص تحياتي وتقديري،،،

نائب الرئيس التنفيذي لقطاع الدواء

أ.د. صالح بن عبدالله باوزير

عن/ص/حمد بن تركي العنزي

الفسح المركزي

٣٢٩٢ الطريق الدائري الشمالي - حي الربيع - الرياض ١٣٣٨٢ - ٦٢٨٨ - المملكة العربية السعودية - هاتف: ٢٧٥٩٢٢٢ ١ ٩٦٦+ فاكس: ٢٠٥٧٦٣٥ ١ ٩٦٦+
3292 Northern Ring Rd. - Al Rabie District - Riyadh 13312-6288 - Kingdom of Saudi Arabia - Tel.: +966 1 2759222 - Fax: +966 1 2057635
www.sfda.gov.sa

بسم الله الرحمن الرحيم

المملكة العربية السعودية
الهيئة العامة للغذاء والدواء
(٢٥٥)
قطاع الدواء

الهيئة العامة للغذاء والدواء
Saudi Food & Drug Authority
رقم الصادر : 10347/ع
وحدة التسجيل : إدارة الإتصالات الإدارية
تاريخ الصادر : 08 / 03 / 1435 هـ
جهة الإحالة : أفراد
2-1435-1-10347-69-2

المحترم
المكرم ناصر الداغري

السلام عليكم ورحمة الله وبركاته ،،،

إشارة إلى خطابكم المقيد لدينا برقم ٦٩٥١/ع وتاريخ ١٤٣٥/٠٣/٠٨هـ المتضمن طلب إذن استيراد للمستحضر الموضح أدناه لعمل الدراسة السريرية الموضحة أدناه:

"A 26-week, Randomized, double blind, placebo-controlled study to explore the effects of probiotics on endotoxin levels in patient with type 2 Diabetes Mellitus"

اسم العينة	الكمية
Winclove 849 (Probiotics) 2gm	304 Boxes / 96 Sachets

عليه نفيدكم بالموافقة على استيراد المستحضر بالكمية المذكورة أعلاه لصالح الدراسة السريرية الموضحة أعلاه ، وفي حال وصول الشحنة بإمكانكم إنهاء إجراءات الفسح عن طريق مكتب قطاع الدواء بمنفذ الوصول مع تقديم أصل الخطاب.

- يجب نقل المستحضرات وفق ظروف التخزين الموصى بها من الشركة الصانعة مع إرفاق مؤشر لدرجات الحرارة .

مع خالص تحياتي وتقديري،،،

نائب الرئيس التنفيذي لقطاع الدواء

أ.د. صالح بن عبدالله باوزير

عنه/ حمد بن تركي العنزي

الهيئة العامة للغذاء والدواء
الفسح المركزي لقطاع الدواء
Drug Sector

Appendix IV SFDA Clearance

Kingdom of Saudi Arabia
Saudi Food & Drug Authority
(255)
Drug Sector

المملكة العربية السعودية
الهيئة العامة للغذاء والدواء
(٢٥٥)
قطاع الدواء

مع ضرورة تزويد الهيئة بتقرير سنوي عن الدراسة يشتمل على معلومات المأمونية و الفعالية.

كما نود الإشارة إلى ضرورة الالتزام بمتطلبات الهيئة أثناء إجراء الدراسة وبعد الانتهاء منها كما هو موضح في المرفق. مع العلم بأن مفتشي الممارسة السريرية الجيدة (GCP) ومفتشي الممارسة الجيدة للمختبرات (GLP) سوف يقومون بزيارة مكان إجراء الدراسة للتأكد من إتباع الأنظمة واللوائح المعتمدة من قبل الهيئة.

و يمكن الحصول على إذن استيراد للمنتج عن طريق وحدة الفسح المركزي بقطاع الدواء بالهيئة مع ضرورة إرفاق نسخة من خطاب الموافقة لوحدة الفسح المركزي.

وفي حال وجود أي استفسارات فيمكنكم التواصل معنا عن طريق البريد الالكتروني: CT.drug@sfda.gov.sa أو الاتصال على هاتف ٢٠٣٨٢٢٢- ٠١ تحويله ٥٧٧٤ أو ٢٣٤٢ أو ٥٧٩٢ إدارة الدراسات السريرية.

وتقبلوا خالص التحية والتقدير..

نائب الرئيس التنفيذي لقطاع الدواء

أ.د. صالح بن عبدالله باوزير

عنه / د. هاجد بن محمد بن هاجد

المملكة العربية السعودية - الرياض ١٣٣١٢ - ٦٢٨٨ - حي النفل - ٣٢٩٢ الطريق الدائري الشمالي - هاتف ٢٧٥٩٢٢٢ ١ ٩٦٦+ فاكس ٢٠٥٧٦٣٣ ١ ٩٦٦+
Kingdom of Saudi Arabia - Riyadh 13312 - 6288 - Al Nafal District - 3292 Northern Ring Rd. - Tel.: +966 1 2759222 - Fax: +966 1 2057633
www.sfda.gov.sa

Kingdom of Saudi Arabia
الهيئة العامة للغذاء والدواء
Saudi Food & Drug Authority
رقم الصادر : 17505/ع
وحدة التسجيل : قطاع الدواء
تاريخ الصادر : 29 / 05 / 1434 هـ
جهة الواجهة : جامعة الملك سعود بالرياض
12-1434-1-17505-06-2

المملكة العربية السعودية
الهيئة العامة للغذاء والدواء
(٢٥٥)
قطاع الدواء

(خطاب موافقة)

المحترم

سعادة الدكتور ماجد بن صالح العقيل

أستاذ الكيمياء الحيوية بكلية العلوم - جامعة الملك سعود

السلام عليكم ورحمة الله وبركاته...

إشارة إلى خطابكم رقم (٨/٢٥/١٢٦٣٠٧) وتاريخ ١٤٣٤/٣/٢٨هـ الوارد للهيئة العامة للغذاء والدواء بتاريخ ١٤٣٤/٣/٢٩هـ بخصوص طلب الموافقة على إجراء دراسة سريرية بعنوان:

"A 26-week, Randomized, Double-blined, Placebo Controlled Study to Explore the Effects of Probiotics on Endotoxin Levels in Patients with Type 2 Diabetes Mellitus"

نفيدكم بأنه لا مانع لدينا من إجراء الدراسة السريرية المشار إليها أعلاه في المراكز التالية (فقط):

١. **مستشفى الملك خالد الجامعي بجامعة الملك سعود بالرياض.**
٢. **مستشفى الملك عبدالعزيز الجامعي بجامعة الملك سعود بالرياض.**
٣. **مستشفيات ومراكز الرعاية الأولية التابعة لوزارة الصحة.**

كما نود التأكيد بأنه يتوجب على الشركة ضرورة إبلاغ الهيئة عن جميع الأعراض الجانبية الخطرة والغير متوقعة للمستحضرات التي تخضع للدراسة بصورة مستعجلة خلال ١٥ يوم، أما في حال حدوث أي عرض جانبي خطير (Serious Adverse Event) يؤدي إلى أحد النتائج التالية: (الوفاة، تهديد الحياة، دخول المستشفى للمعالجة، حدوث العجز أو الإعاقة، أو ظهور عيب خلقي) فيلزم إبلاغ الهيئة فوراً وخلال مدة أقصاها ٧ أيام

Appendix V Study Questionnaire

Kingdom Of Saudi Arabia

Ministry Of Higher Education

King Saud University

Biomarkers Research Program

Serial No. : _____

المملكة العربية السعودية

وزارة التعليم العالي

جامعة الملك سعود

مركز أبحاث المؤشرات الحيوية

Date: / / 2013

National ID: _-____-____- **رقم الهوية**

Name: _____ **الاسم**

Sex الجنس : **Male ☐ ذكر** **Female ☐ أنثى**

Age: _____ **العمر**

Birth Date / / تاريخ الولادة Place _____ المكان Phone _____ التلفون

الحالة الاجتماعية	أعزب	متزوج	مطلق	أرمل	طفل
Marital status	☐	☐	☐	☐	☐
	Single	Married	Divorced	Widowed	Child

Family history التاريخ العائلي

سكري أقارب من الدرجة الأولى	سكري أقارب من الدرجة الثانية	ارتفاع ضغط الدم
☐	☐	☐
Diabetes 1st degree	Diabetes 2nd degree	Hypertension

ارتفاع الدهون		سمنة	سرطان	حدد
☐	☐	☐	☐	_____
Hyperlipidemia	Chronic Gastrointestinal Disease	Obesity	Cancer	

☐ Others أخرى : _____-

_____

Smoking التدخين

☐ Smoker مدخن ☐ Sheshah شيشه

☐ Ex-Smoker مدخن سابق

☐ Never smoked لم يدخن أطلاقاً

- # of packs/day _____ عدد علب السجائر في اليوم
- Duration (years) _____ المدة بالسنوات
- Years quitted _____ سنوات الإقلاع

For Female Subjects Only أسئلـــــة للسيـــــدات فقـــط

Are you pregnant [ ] Yes نعم [ ] No لا هل أنت حامل ؟

Medications: Please list all medications you are currently taking in the space provided

Please Answer the Following Questions: Check if appropriate:

	YES	NO
1. Do you have any gastrointestinal disorder?	___	___
2. Have you used antibiotics for the past 6 months?	___	___
3. Have you used probiotics regularly for the past 3 months?	___	___

- Almarai Vetal Laban
- Protexin Capsule
- Other Dairy products (yoghurt, low-fat, high-fat, skimmed) With probiotics label

4. Clinical trial participation in the past 6 months?	___	___
5. Are you taking any of the following <u>regularly</u>?		

- Insulin/insulin analogs ___ ___
- Corticosteroids ___ ___
- Antacids (For hyperacidity) ___ ___
- H2-receptor blockers (For hyperacidity, ulcer) ___ ___
- Proton Pump Inhibitors (For hyperacidity, ulcer) ___ ___
- Loperamide (For diarrhea) ___ ___
- Cholestryramine (For high cholesterol) ___ ___
- Omega-3 supplements (cod liver oil) ___ ___
- Sex steroids ___ ___

For DMT2 Patients

1. Did your anti-DM medication change in the past 6 mos? ___ ___
2. Will your medications change within 1 year? ___ ___

Anthropometrics

	Values		
Date Taken	**Baseline**	**Week 8**	**Week 26**
Height (cm)			
Weight (kg)			
Waist (cm)			
Hip (cm)			
Systolic BP (mmHg)			
Diastolic BP (mmHg)			

Blood Tests

	Values		
Date Taken	**Baseline**	**Week 8**	**Week 26**
Fasting glucose			
HBA1c			
Insulin			
C-Peptide			
Triglycerides			
Total Cholesterol			
LDL-Cholesterol			
HDL-Cholesterol			
Endotoxin			
IL-6			
CRP			
TNF-α			
Leptin			
Adiponectin			
Resistin			

CONSENT

I fully agree to participate in this study as a subject [A 26-week, Randomized, Double-blind, Placebo-controlled Study to Explore the Effects of Probiotics on Endotoxin Levels in Patients with Type 2 Diabetes Mellitus].

I am aware that I will be receiving intervention and that blood will be collected from me at different time points.

The doctors and investigators in-charge have oriented me about the study in a language that I can understand, as well as the risk factors and problems that I may encounter. They were able to answer all my questions and doubts about the study and my level of participation.

The doctors and investigators in-charge can go through my medical records in relation to the study providing full confidentiality of my information.

I am aware that there will be no problem if in case I decide to stop the intervention.

I have the right to withdraw from this study at any time without mentioning reasons.

Patient's Full Name, Signature and Date

Appendix VI Letter of Probiotics Provider

WINCLOVE PROBIOTICS

WINCLOVE B.V.
HULSTWEG 11
1032 LB AMSTERDAM
T + 31 (0)20 435 02 35
F + 31 (0)20 435 02 36
E WINCLOVE@WINCLOVE.NL

September 4, 2013

PROFESSOR MAJED S. ALOKAIL
College of Science, King Saud University
PO Box 2455, Riyadh, 11451
Kingdom of Saudi Arabia

Dear Prof. Alokail,

We thank you for consulting our company for the probiotic products that you will use in your clinical trial entitled "**A 26-week, Randomized, Double-blind, Placebo-controlled Study to Explore the Effects of Probiotics on Endotoxin Levels in Patients with Type 2 Diabetes Mellitus**". We take this opportunity to highlight why our probiotic products will best produce the results you desire for your study.

The rationale of Winclove 849 uniqueness and its superiority over other probiotic products is that first, **it is specifically formulated for patients with Diabetes Mellitus Type 2**. It is by far **the only probiotic product in the world formulated for the type of population in your study**. Since you will be conducting a clinical trial I also would like to emphasize that we are also one of the few companies, if not **the only company who manufactures placebo for use in probiotic clinical trials**.

I also would like to call your attention that as of now, **we have no agents or distributors in Saudi Arabia or any other country within the GCC region**, and therefore distribution of this product is exclusive to your study for clinical trial use.

Together with this letter you will find our quotation providing in detail the expenses needed to obtain both the placebo and supplement that you will use in your study.

We are looking for your response the soonest.

Best regards,

Dr. Saskia van Hemert
Senior Scientist
Winclove Probiotics
Hulstweg 11, 1032 LB Amsterdam
T: +31 20 435 02 35
E: saskiavanhemert@winclove.nl, W: www.winclove.com

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