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Randomized Control Trials

Effects of a 6-month multi-strain probiotics supplementation in endotoxemic, inflammatory and cardiometabolic status of T2DM patients: A randomized, double-blind, placebo-controlled trial

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SUMMARY

Objective: The aim of this trial was to characterize the beneficial effects of probiotics on decreasing endotoxin levels and other cardiometabolic parameters in Arab patients with type 2 diabetes mellitus (T2DM).

Methods: Saudi adults with naïve T2DM (n = 30; 12 males and 18 females) were randomly allocated to receive twice daily placebo or 2.5×10^9 cfu/g of Ecologic®Barrier (multi-strain probiotics; n = 31; 14 males and 17 females) in a double-blind manner over a 6 month period, respectively. Anthropometrics were measured and fasting blood samples were collected to analyze endotoxin, glycemic parameters [glucose, insulin, c-peptide and homeostasis model assessment for insulin resistance (HOMA-IR)], lipids [triglycerides, total cholesterol, low and high-density lipoprotein (LDL and HDL, respectively) cholesterol and total/HDL-cholesterol ratio], inflammatory markers [tumor-necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP)] and adipocytokines [leptin, adiponectin and resistin] at baseline and after 3 and 6 months of intervention.

Results: Multi-strain probiotics supplementation for 6 months caused a significant decrease in circulating levels of endotoxin by almost 70% over 6 months, as well as glucose (38%), insulin (38%), HOMA-IR (64%), triglycerides (48%), total cholesterol (19%), total/HDL-cholesterol ratio (19%), TNF-α (67%), IL-6 (77%), CRP (53%), resistin (53%), and a significant increase in adiponectin (72%) as compared with baseline. Only HOMA-IR had a clinically significant reduction (−3.4, 64.2%) in the probiotics group as compared to placebo group at all time points. No other clinically significant changes were observed between the probiotic or placebo group at 3 and 6 months in other markers.

Conclusion: Multi-strain probiotic supplementation over 6 months as a monotherapy significantly decreased HOMA-IR in T2DM patients, with the probiotic treatment group highlighting reduced inflammation and improved cardiometabolic profile. As such, multi-strain probiotics is a promising adjuvant anti-diabetes therapy.

Trial registration: ClinicalTrials.gov Identifier: NCT01765517.

Abbreviations: CRP, C-reactive protein; HDL, high density lipoprotein; HOMA IR, Homeostasis model assessment for insulin resistance; IL, interleukin; ITT, Intent to Treat; LOCF, Last observation carried forward; LDL, low density lipoprotein; LPS, lipopolysaccharides; MDC, minimum detectable concentration; PP, Per Protocol; T2DM, type 2 diabetes mellitus; TNF, tumor necrosis factor.

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1. Introduction

In recent years there has been intense commercial interest in understanding the role of human microbiome in diseases and factors that can relieve it, with the use of pre-biotic and pro-biotic often in inflammatory intestinal disorders making it an emerging biomedical industry projected to be worth $64.02 billion by 2022 [1]. Despite this interest there has been conflicting evidence into the effectiveness of probiotics in health and disease *per se* with limited insight into the use of prebiotics and probiotics for the management type 2 diabetes mellitus (T2DM) [2–5]; despite the knowledge that T2DM is also considered an inflammatory chronic condition. Prior studies in T2DM subjects has shown the importance of the gut derived gram negative bacterial fragment lipopolysaccharide (LPS, endotoxin) which can overgrow in the intestine, induce a leaky gut, and allow endotoxin to enter into the circulation and induce systemic inflammation [6]. Prior studies have also shown that the use of diet and/or surgery for weight reduction can lower endotoxin-induced inflammation [7–9], which, suggests that manipulation of the gut microbiota with an appropriate pro-biotic may also have significant health effects [10]. Since the gut microbiome is the main reservoir of endotoxin, probiotics supplementation may alter its levels by modifying its composition and strengthening the gut epithelial barrier [11,12].

Few studies to date have examined the effects of probiotics on systemic levels of endotoxin in chronic, non-communicable diseases. Those that have examined the specific impact of probiotics on endotoxin and associated metabolic diseases have shown conflicting outcomes. Probiotics use in cirrhotic patients has shown a positive 25% reduction in systemic endotoxin [13], while a more recent review indicated the effects on circulating endotoxin was minimal [14]. Although in animal studies, where diet is more easily controlled, more consistent evidence suggests that probiotics supplementation may be beneficial in the use of insulin-resistant diseases [15]. The few human intervention trials that have been conducted appear to support the animal studies with a recent meta-analysis of 12 studies implicating that probiotics give rise to significant improvements in HbA1c and fasting insulin amongst subjects with T2DM [16]. Nevertheless, the majority of the interventional studies conducted to date with probiotics use in subjects with T2DM have tended to be either short-term studies, no longer than 3 months and/or mono-strains were used as supplementation [17,18]. To the best of our knowledge, there is limited evidence on the effects of a long duration, multi-strain probiotics supplementation on systemic endotoxin levels amongst T2DM subjects. This study therefore sought to test the hypothesis that multi-strain probiotics supplementation reduces endotoxin levels and consequently improve cardiometabolic profile in an Arab T2DM population where metabolic risk is high.

2. Methods

2.1. Participants and study design

The study was a 6-month, single-center, double-blind, randomized, placebo-controlled clinical trial. The trial protocol has been previously published and was also registered at the US National Institute of Health (NIH) (ClinicalTrials.gov Identifier: NCT01765517) [19]. Ethical approval was obtained from the Ethics Committee in the College of Science, King Saud University in Riyadh, Saudi Arabia.

For this study 150 adult Saudi participants [73 females (46 (63%) menopause), 77 males, aged 30–60 years old] with newly diagnosed T2DM (<6 months) were initially recruited by the research team for intervention from January 2014 to February 2016. All participants were patients visiting the outpatient department of King Salman Hospital, Riyadh, Saudi Arabia. Patients with diabetes complications (retinopathy, neuropathy, nephropathy, etc.) and poor glycemic control (HbA1c > 7%) as noted in their medical records were excluded. Participants on prebiotics, probiotics, or antibiotics treatment 6 weeks before inclusion, lactating or pregnant women, on insulin or its analogs and those with gastrointestinal diseases were excluded. Sample size calculation was previously done based on the primary outcome (endotoxin), considering 80% power at $\alpha = 0.05$ [19].

Circulating endotoxin level was measured as a primary outcome, whilst anthropometrics, glycemic parameters, lipid profile, inflammatory and adipocytokine markers were measured as secondary outcomes. Significant differences in the assessment between placebo and probiotics group after random allocation served as baseline covariate variables in this study.

2.2. Randomization and blinding

All participants were allocated (1:1) to receive either probiotics or placebo. The randomization scheme was computer generated by Winclove using permuted blocks with block size equal to 4. True allocation concealment was done since the research personnel involved cannot adjust randomization or discern the actual treatment the patient is given.

2.3. Study protocol

The probiotics group was allocated with sachets [2 g freeze-dried powder of the probiotic mixture Ecologic®Barrier (Winclove probiotics, the Netherlands) (2.5×10^9 cfu/g)] which contains the following strains: *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19 and *L. lactis* W58. This probiotic combination has been previously investigated for its ability to improve endothelial barrier and its potency to inhibit mast cell activation, inhibit pro-inflammatory cytokines decrease endotoxin load [20]. The placebo group was allocated the same sachets without the probiotic strains (2 g freeze-dried maize starch and maltodextrins). All participants were asked to consume their assigned treatment twice daily (dissolving contents in glass of water) before breakfast and before bed time. Anthropometrics were measured and included height (cm), weight (kg), blood pressure (mmHg) waist and hip measurements (cm), body mass index (BMI kg/m^2) and waist-hip ratio (WHR) at baseline, 3 months and after 6 months of treatment. Fasting blood samples were also collected during those time points. All blood samples were centrifuged, serum samples separated, put on ice and immediately delivered to Prince Mutaib Chair for Biomarkers of Osteoporosis (PMCO) in King Saud University (KSU) for storage at −20′C until further analysis. To monitor compliance, subjects were asked to return once a month to be asked for side effects and to return unused sachets for fresh refill.

2.4. Biochemical analyses

Fasting serum samples were analyzed for glucose and lipid profile [total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides] using Konelab routine analyzer (Konelab, Espoo, Finland). LDL-cholesterol was calculated using the Friedewald equation [21]. Serum tumor necrosis factor (TNF)α, interleukin (IL)-6, leptin, adiponectin and resistin were measured using the Milliplex Map (Millipore, Billerica, MA, USA) in the FlexMAP 3D (Luminex Corp, Austin, TX, USA). Minimum detectable concentrations (MDC) were as follows: TNFα, 0.14 pg/ml; IL-6, 0.4 pg/ml;

leptin, 85.4 pg/ml; adiponectin, 145.4 pg/ml and resistin, 6.7 pg/ml. The intra-assay variation was 1.4–7.9% and inter-assay variation of <21%. Serum insulin and C-peptide were measured using electrochemiluminescence assay (Roche Diagnostics, Germany). C-reactive protein (CRP) [intra-assay precision (4.4–8.3) and inter-assay precision (6.0–7.0)] (R&D Systems, MN, USA). Homeostasis model assessment (HOMA IR) was calculated as the product of insulin (uU/ml) and glucose (mmol/l) divided by 22.5 [22]. Endotoxin (primary endpoint) was measured using a limulus amebocyte lysate (LAL) quantitative kinetic assay (Lonza, MD, USA). As serum is very inhibitory to this assay a spike recovery was performed using a sample dilution of 1:40. The recovery spike was 60% and was within the acceptable range of 50–200%. All serum samples were analyzed at baseline, 3 months and after 6 months of treatment.

2.5. Data analyses

Data were analyzed using SPSS (version 16.5 Chicago, IL, USA). Statistical analysis was performed using Intention-to-treat (ITT) analysis, where missing data were dealt by using the last observation carried forward (LOCF) method. Per-protocol analyses was done only for primary endpoint (endotoxin). All normally distributed data were presented as mean and standard deviations, while non-normally distributed data was presented as median and interquartile range. Furthermore, categorical data was presented as frequencies and percentages (%). Independent sample Student T-test and Mann Whitney U test was used to determine significant differences between groups at baseline. Mixed method analysis of covariance (ANCOVA) was used to determine within and between group differences after adjusting for baseline covariates including WHR, leptin, TNF-α, IL-6, endotoxin, glucose and total cholesterol/HDL ratio. A further sub-analysis was done to determine the effect of sex in the intervention and repeated measures ANCOVA revealed no significant effect. All non-normal variables including glucose (mmol/l), insulin (IU/ml), c-peptide (ng/ml), HOMA-IR, TNF alpha (pg/ml), IL-6 (pg/ml), CRP (ug/ml), leptin (pg/ml), adiponectin (ug/ml), resistin (ng/ml) and endotoxin (IU/ml) (variables that did not follow a normal distribution curve) were transformed prior to parametric testing. Intervention effects were presented at 95% confidence interval (CI). A p-value < 0.05 was considered statistically significant.

3. Results

Of the 150 participants that were recruited, 96 were randomized, 78 completed 3 months and 61 completed the entire trial (probiotics group, n = 31; placebo group, n = 30). The flowchart of the trial is presented in Fig. 1. Baseline comparison showed no significant differences in both groups except WHR, glucose, total cholesterol, total/HDL-cholesterol, TNF-α, IL-6, leptin and endotoxin (Table 1). The most common reasons for drop out included loss to follow-up and poor compliance. Flatulence was the most common complaint (N = 5, 1 in the placebo group and 4 in the probiotics group) during the first weeks of trial in both placebo and probiotics group (not included in tables).

3.1. Changes in anthropometrics and clinical measures

At baseline, the placebo group had a significantly higher WHR and a significantly lower mean arterial pressure than the probiotics group. Between-group comparisons showed no significant changes in all anthropometric and clinical measures post intervention (Table 2).

3.2. Changes in glycemic indices

Fasting glucose levels were significantly higher in the probiotics than the placebo group at baseline [11.7 mmol/l (8.4–16.4) versus

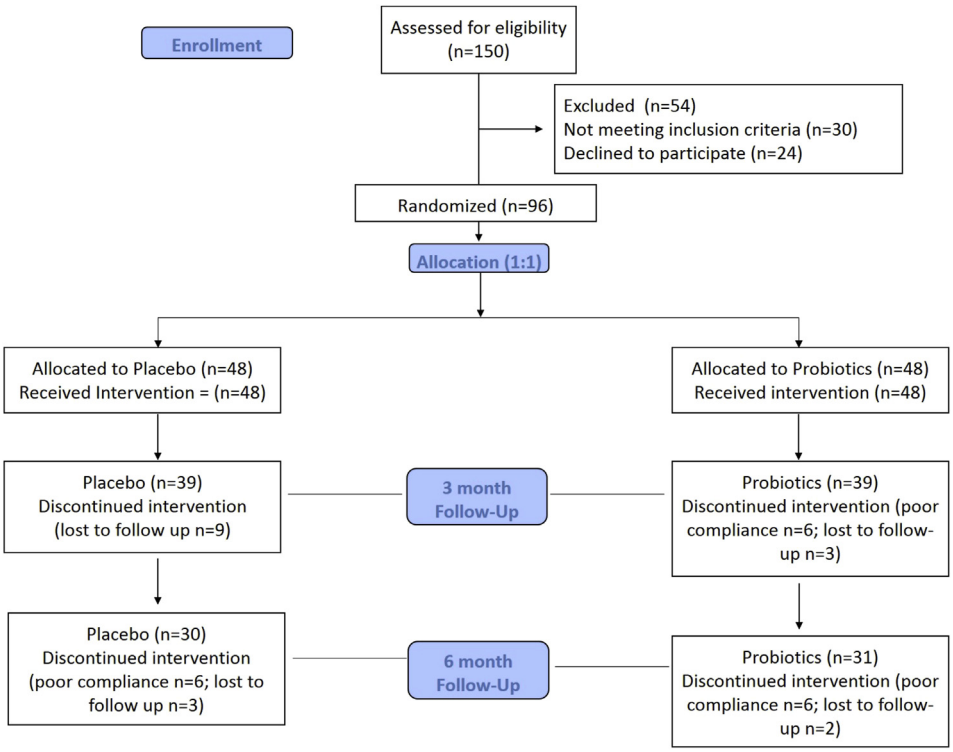

Fig. 1. CONSORT Flow Chart detailing participants' recruitment, randomization and allocation.

Table 1
Baseline characteristics according to intervention groups.

Parameters	Placebo	Probiotics	P-value
N	39	39	
M/F	21/18	19/20	
Age (Years)	46.6 ± 5.9	48.0 ± 8.3	0.40
BMI (kg/m^2)	30.1 ± 5.0	29.4 ± 5.2	0.56
Waist-Hip Ratio	1.0 ± 0.1	0.9 ± 0.1	0.02
Systolic BP (mmHg)	129.5 ± 10.3	133.4 ± 14.0	0.17
Diastolic BP (mmHg)	78.6 ± 8.6	83.2 ± 12.0	0.06
Mean Arterial Pressure (MAP)	95.5 ± 7.7	100.0 ± 10.9	0.05
Glycemic Profile			
Glucose (mmol/l)	7.1 (5.7–11.2)	11.7 (8.4–16.4)	0.001
Insulin (IU/ml)	13.0 (7.5–18.7)	9.9 (7.7–16.4)	0.62
C-peptide (ng/ml)	0.1 (0.1–0.4)	0.5 (0.0–1.9)	0.07
HOMA-IR	4.1 (2.3–7.5)	5.3 (3.5–10.2)	0.99
Lipid Profile			
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	0.09
LDL-cholesterol (mmol/l)	3.2 ± 0.9	3.7 ± 1.2	0.02
Total Cholesterol/HDL-Chol Ratio	5.0 ± 1.3	6.5 ± 2.2	0.001
Inflammatory Markers Profile			
TNF alpha (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 $p < 0.05$.

7.1 mmol/l (5.7–11.2)]. After adjusting for baseline covariates, between group-comparisons showed no significant difference in glucose levels between placebo and probiotics groups at 3 months [1.0 mmol/l (14.3%) vs −3.2 mmol/l (−27.4%)] and after 6 months [1.1 mmol/l (15.7%) vs −4.5 mmol/l (−38.5%)]. No difference was also observed in C-peptide levels [0.80 ng/ml (800%) vs −0.30 ng/ml (−75%)] at 6 months. A borderline significant difference was observed in insulin levels [-0.30 IU/ml (−2.4%) vs −3.80 IU/ml (−38.4%)] at 6-month comparison and clinically significant differences were noted in HOMA-IR at 3 months [0.0 (0%) vs −3.2 (−60.4%)] and after 6 months [0.80 (20.5%) vs −3.40 (−64.2%)] in favor of the probiotics group. 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. The rest of the glycemic parameters in the placebo group did not significantly change over time. In the probiotics group, a significant decrease was observed in glucose, insulin and HOMA-IR values overtime. Median levels of C-peptide significantly decreased only after 6 months (Table 3).

3.3. Changes in lipid profile

LDL- and total cholesterol as well as total/HDL-cholesterol ratio were significantly higher in the probiotics group than placebo at baseline. Between group comparisons showed no differences in placebo and probiotics groups over-all in levels of triglycerides [−0.10 mmol/l (−4.6%) vs −1.20 mmol/l (−48%)], total cholesterol [−0.30 mmol/l (−5.8%) vs −1.10 mmol/l (−19%)], HDL-cholesterol [−0.10 mmol/l (−9.1%) vs −0.30 mmol/l (30%)], LDL-cholesterol [−0.10 mmol/l (9.7%) vs − 0.80 mmol/l (−22.2%)] and total/HDL-cholesterol ratio [−0.30 (−5.8%) vs −1.10 (−19%)]. Within group analysis showed no changes in the placebo group over time. In the probiotics group, significant improvements were observed in

Table 2
Anthropometric measures before and after intervention with placebo or probiotics in T2DM patients.

Parameter	Placebo (N = 30)			Probiotics (N = 31)			Intervention Effects (CI 95%)		
	Baseline	3 months	6 months	Baseline	3 months	6 months	0–3 months	0–6 months	Over-all
BMI (kg/m^2)	30.1 ± 5.0	30.2 ± 5.0	29.7 ± 5.0	29.4 ± 5.2	29.3 ± 5.3	29.4 ± 5.2	−2.10 (−6.4–2.1)	−1.88 (−6.1–2.3)	−1.96 (−6.2–2.2)
Change (%) at 3 m		0.1 (0.3)			−0.10 (−0.3)				
Change (%) at 6 m		−0.4 (−1.3)			0.0 (0.0)				
WHR	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	−0.09 (−0.1–−0.03)	−0.08 (−0.1–−0.02)	−0.08 (−0.1–−0.03)
Change (%) at 3 m		0.0 (0.0)			0.0 (0.0)				
Change (%) at 6 m		0.0 (0.0)			0.0 (0.0)				
SBP (mmHg)	129.5 ± 10.3	129.9 ± 11.1	129.2 ± 11.3	134.8 ± 14.6	129.0 ± 11.4	130.6 ± 12.5	−2.33 (−10.9–6.2)	−1.13 (−9.8–7.6)	−1.98 (−10.4–6.5)
Change (%) at 3 m		0.4 (0.3)			−5.8 (−4.3)				
Change (%) at 6 m		−0.3 (−0.2)			−4.2 (−3.1)				
DBP (mmHg)	78.6 ± 8.6	79.8 ± 8.1	77.3 ± 9.1	83.6 ± 11.8	79.8 ± 11.5	81.0 ± 11.7	0.45 (−7.0–7.9)	2.07 (−6.2–10.3)	0.81 (−6.7–8.4)
Change (%) at 3 m		1.2 (1.5)			−3.8 (−4.6)				
Change (%) at 6 m		−1.3 (−1.6)			−2.6 (−3.1)				
MAP (mmHg)	95.7 ± 7.7	96.5 ± 7.8	100.7 ± 11.1	100.6 ± 11.1	96.2 ± 9.7	97.5 ± 9.9	−0.48 (−7.2–6.2)	1.00 (−6.2–8.2)	−0.12 (−6.8–6.6)
Change (%) at 3 m		1.0 (1.0)			−4.4 (−4.4)				
Change (%) at 6 m		5.2 (5.4)			−3.1 (−3.1)				

Note: Data was presented as mean ± SD. Results were obtained from mixed method ANCOVA adjusted for baseline covariates; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; 3 m, 3 months; 6 m, 6 months.

Table 3
Glycemic parameters before and after intervention with placebo or probiotics in T2DM patients.

Parameter	Placebo (N = 30)			Probiotics (N = 31)			Intervention Effects (CI 95%)		
	Baseline	3 months	6 months	Baseline	3 months	6 months	0–3 months	0–6 months	Over-all
Glucose (mmol/l)	7.0 (5.7–11.2)	8.0 (5.9–11.4)	8.1 (6.9–11.4)	11.7 (8.4–16.4)	8.5[a] (6.2–10.9)	7.2[ab] (5.3–9.1)	0.10 (−0.01–0.2)	0.07 (−0.04–0.2)	0.03 (−0.07–0.1)
Change (%) at 3 m		1.0 (14.3)			−3.2 (−27.4)				
Change (%) at 6 m		1.1 (15.7)			−4.5 (−38.5)				
Insulin (IU/ml)	12.4 (8.0–18.7)	10.8 (8.3–15.5)	12.1 (8.0–17.4)	9.9 (7.7–16.4)	6.9[a] (4.5–9.8)	6.1[a] (3.6–9.6)	−0.12 (−0.3–0.1)	−0.19 (−0.4–0.03)	−0.20 (−0.4–0.01)
Change (%) at 3 m		−1.6 (−12.9)			−3.0 (−30.3)				
Change (%) at 6 m		−0.3 (−2.4)			−3.8 (−38.4)				
C-peptide (ng/ml)	0.1 (0.1–0.5)	0.2 (0.1–0.9)	0.9[a] (0.1–1.9)	0.4 (0.0–1.8)	0.1[a] (0.0–0.3)	0.1 (0.0–0.4)	0.44 (−0.02–0.9)	0.24 (−0.2–0.6)	0.20 (−0.2–0.6)
Change (%) at 3 m		0.1 (100.0)			−0.3 (−75.0)				
Change (%) at 6 m		0.8 (800.0)			−0.3 (−75.0)				
HOMA-IR	3.9 (2.3–6.5)	3.9 (3.3–6.0)	4.7 (3.6–6.7)	5.3 (3.5–10.2)	2.1[a] (1.5–5.2)	1.9[a] (1.2–3.1)	−0.21[*] (−0.4 to −0.02)	−0.34[**] (−0.6 to −0.12)	−0.38[**] (−0.6 to −0.17)
Change (%) at 3 m		0.0 (0.00)			−3.2 (−60.4)				
Change (%) at 6 m		0.80 (20.5)			−3.4 (−64.2)				

Note: Data was presented as median (interquartile range). Results were obtained from mixed method ANCOVA adjusted for baseline covariates; superscript "a" denotes significance compared to baseline; superscript "b" denotes significance compared to 3 months; * denotes significance at $p < 0.05$; ** denotes significance at $p < 0.01$; 3 m, 3 months; 6 m, 6 months. Significant at $p < 0.05$.

terms of decreased triglycerides, total cholesterol and total/HDL cholesterol ratio (Table 4).

3.4. Changes in inflammatory markers

At baseline, the probiotics group had a significantly higher median levels of TNFα and IL6 than placebo group. Between-group comparisons post-intervention showed no significant differences in placebo and probiotic groups in levels of TNFα [−0.20 pg/ml (−40%) vs −0.60 pg/ml (−66.7%)], IL-6 [−2.8 pg/ml (−77.8%) vs −3.9 pg/ml (−76.5%)] and C-reactive protein [0.40 ug/ml (13.3%) vs −2.9 ug/ml (−52.7%)]. Within group comparisons however showed that all inflammatory markers significantly improved over time in the probiotics group and these changes were not observed in the placebo group (Table 5).

3.5. Changes in endotoxin levels and adipocytokine profile

Endotoxin was significantly higher in the probiotics group than placebo at baseline. Furthermore, no differences in baseline adipocytokines were observed except for levels of leptin being significantly higher in the probiotics than the placebo group. Between group comparisons after 6 months showed no differences in both groups in levels of endotoxin [0.80 IU/ml (38.1%) vs. −3.20 IU/ml (−69.6%)], leptin [−1.1 pg/ml (−28.2%) vs. −2.7 pg/ml (−46.6%)], adiponectin [0.0 μg/ml (0%) vs. 6.1 μg/ml (71.8%)], and resistin [5.0 ng/ml (79.4%) vs. −6.8 ng/ml (−58.1%)]. Within group comparisons showed a significant increase in resistin levels after 6 months compared to baseline ($p < 0.05$) as well as a significant increase in endotoxin levels after 6 months as compared to 3 months in the placebo group. In the probiotics group post-intervention, there was a significant improvement in endotoxin (Fig. 2) and adiponectin levels, and a significant decrease in resistin. No significant changes in either group were noted in leptin levels (Table 6).

4. Discussion

The ambition of this randomized controlled study was to determine primarily the systemic endotoxin-lowering capability of a multi-strain probiotic supplementation and whether such treatment would result in improved cardiometabolic profile in patients with T2DM. From this study, it was observed that circulating endotoxin levels were significantly reduced post-intervention in the probiotics group, whilst the placebo group remained unchanged by time. In addition, comparison between groups also showed a clinically significant difference in HOMA-IR with improvement in insulin sensitivity in the probiotic group. The noted associated improvement in endotoxin levels and HOMA-IR has been observed in other diet or medicinal intervention studies using T2DM subjects [7,8]. In conjunction with reduction in endotoxin levels in the probiotic group at six months there were also associated improvements in cholesterol, Total cholesterol/HDL ratio, and glycemic control from baseline in group analysis supporting the concept that probiotics can provide cardiometabolic protective effects. Noting that the placebo group did not appear comparable to the probiotic group from baseline biochemical data gathered.

Previous studies have tried to evaluate the beneficial effects of probiotics in T2DM with the ultimate cardiometabolic benefits requiring more than 3 months, with our study suggesting 6 month follow up may highlight promising findings [16,23–26]. Our study is, to our knowledge, the first to demonstrate the effects of a multi-strain probiotic supplement given over 6-months in the Arab T2DM population, using endotoxin as the primary endpoint. It is also

Table 4
Lipid profile before and after intervention with placebo or probiotics among T2DM patients.

Parameter	Placebo (N = 30)			Probiotics (N = 31)			Intervention Effects (CI 95%)		
	Baseline	3 months	6 months	Baseline	3 months	6 months	0–3 months	0–6 months	Over-all
TG (mmol/l)	2.2 ± 1.4	2.0 ± 0.8	2.1 ± 1.6	2.5 ± 1.4	1.7 ± 0.7[a]	1.3 ± 0.6[a]	−0.04 (−0.7–0.6)	−0.65 (−1.5–0.2)	−0.51 (−1.2–0.2)
Change (%) at 3 m		−0.2 (−9.1)			−0.8 (−32.0)				
Change (%) at 6 m		−0.1 (−4.6)			−1.2 (−48.0)				
T.Chol (mmol/l)	5.2 ± 1.0	4.7 ± 0.9	4.9 ± 1.0	5.8 ± 1.3	5.1 ± 0.9	4.7 ± 1.1[a]	−0.35 (−1.1–0.4)	−0.63 (−1.4–0.1)	−0.47 (−1.2–0.2)
Change (%) at 3 m		−0.5 (−9.6)			−0.7 (−12.1)				
Change (%) at 6 m		−0.3 (−5.8)			−1.1 (−19.0)				
HDL-C (mmol/l)	1.1 ± 0.3	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.3	1.1 ± 0.3	1.3 ± 0.4	−0.05 (−0.2–0.1)	−0.06 (−0.2–0.1)	−0.04 (−0.2–0.1)
Change (%) at 3 m		−0.1 (−9.1)			0.1 (10.0)				
Change (%) at 6 m		−0.1 (−9.1)			0.3 (30.0)				
LDL-C (mmol/l)	3.1 ± 0.9	2.8 ± 0.9[a]	2.8 ± 1.0	3.6 ± 1.3	3.2 ± 0.9	2.7 ± 1.0	−0.30 (−0.9–0.3)	−0.28 (−0.9–0.4)	−0.22 (−0.8–0.4)
Change (%) at 3 m		−0.3 (−9.7)			−0.4 (−11.1)				
Change (%) at 6 m		−0.1 (−9.7)			−0.8 (−22.2)				
T.Chol/HDL ratio	5.2 ± 1.0	4.7 ± 0.9	4.9 ± 1.0	5.8 ± 1.3	5.1 ± 0.9	4.7 ± 1.1[a]	1.12 (−0.6–2.9)	0.19 (−0.7–1.1)	0.49 (−0.8–1.8)
Change (%) at 3 m		−0.5 (−9.6)			−0.7 (−12.1)				
Change (%) at 6 m		−0.3 (−5.8)			−1.1 (−19.0)				

Note: Data was presented as mean ± SD. Results were obtained from mixed method ANCOVA adjusted for baseline covariates; superscript "a" denotes significance compared to baseline; TG, triglycerides, T.Chol, total cholesterol; 3 m, 3 months; 6 m, 6 months. Significant at $p < 0.05$.

Table 5
Inflammatory markers before and after intervention with placebo or probiotics among T2DM patients.

Parameter	Placebo (N = 30)			Probiotics (N = 31)			Intervention Effects (CI 95%)		
	Baseline	3 months	6 months	Baseline	3 months	6 months	0–3 months	0–6 months	Over-all
TNF-α (pg/ml)	0.5 (0.2–0.8)	0.5 (0.2–0.8)	0.3 (0.2–0.8)	0.9 (0.4–1.2)	0.6 (0.3–0.9)	0.3[ab] (0.2–0.7)	0.16 (−0.03–0.3)	0.07 (−0.1–0.3)	0.05 (−0.1–0.2)
Change (%) at 3 m		0 (0)			−0.3 (−33.3)				
Change (%) at 6 m		−0.2 (−40.0)			−0.6 (−66.7)				
IL-6 (pg/ml)	3.6 (1.4–11.4)	0.8 (0.6–4.4)	0.8 (0.7–3.8)	5.1 (2.7–18.8)	1.4[a] (0.7–18.0)	1.2[a] (0.8–3.6)	−0.20 (−0.6–0.2)	−0.14 (−0.5–0.2)	−0.21 (−0.6–0.2)
Change (%) at 3 m		−2.8 (−77.8)			−3.7 (−72.6)				
Change (%) at 6 m		−2.8 (−77.8)			−3.9 (−76.5)				
CRP (μg/ml)	3.0 (1.9–6.2)	2.9 (1.5–4.7)	3.4 (2.6–5.6)	5.5 (2.7–6.1)	3.1[a] (1.4–5.7)	2.6[a] (1.2–4.9)	−0.11 (−0.4–0.2)	−0.20 (−0.5–0.1)	−0.23 (−0.5–0.1)
Change (%) at 3 m		−0.1 (−3.3)			−2.4 (−43.6)				
Change (%) at 6 m		0.4 (13.3)			−2.9 (−52.7)				

Note: Data was presented as median (interquartile range). Results were obtained from mixed method ANCOVA adjusted for baseline covariates; superscript "a" denotes significance compared to baseline; 3 m, 3 months; 6 m, 6 months. Significant at $p < 0.05$.

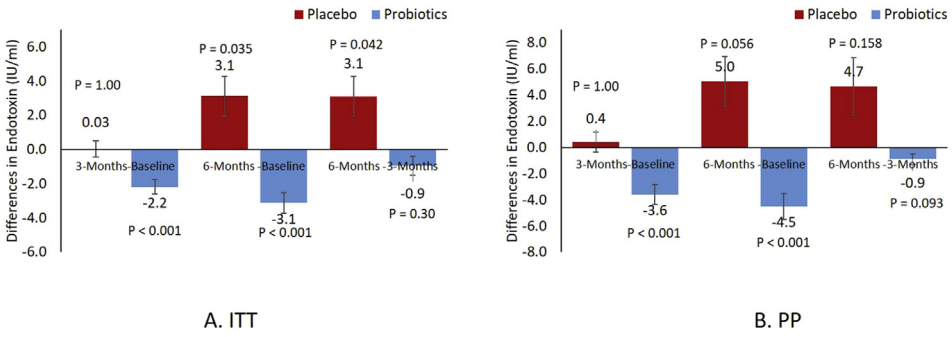

Fig. 2. Changes in endotoxin levels in probiotics and placebo group using A) Intent-to-Treat (ITT) and B) Per-Protocol Analyses.

important to stress that the probiotic supplementation in this present study was used as a standalone treatment given in the absence of exercise and diet-related modifications in the intervention or lifestyle control in a culture with easy access to excess food. While this is not the first interventional study undertaken on the effects of probiotics in patients with T2DM, our protocol addressed previous trials concerns for a longer duration and use of multiple strains, which highlighted cardiometabolic benefits in the probiotic group from baseline to six months. Clearly the 6 month time point was important to observe changes as the most significant changes were noted which affirms a recent meta-analysis of Hu and colleagues observed, where trials with longer durations of intervention using multiple probiotic strains had more beneficial cardiometabolic effects in patients with T2DM [27].

This present study showed significant improvements in the endotoxin levels of the probiotic group overtime, although not clinically significant as compared to placebo group at 6 months. However, the T2DM patients in the probiotic group began the study at a significantly higher baseline endotoxin level, despite noted comparability for BMI, age and gender. Furthermore, biochemically the probiotic intervention group began the study with significantly raised glucose levels, diastolic blood pressure and inflammatory status as well. This therefore would have affected the 6-month comparison as the baseline groups were not comparable which may have been a challenge with using newly diagnosed T2DM patients; despite best efforts to limit confounders in the study. Such discrepancies between the two groups could also have been due be due to sample size difference, duration of intervention and patient selection [28]. However, there was a noted 70% drop in endotoxin level in those subjects on the probiotic over six months compared with a net effect of zero change in the control placebo group over the same period.

The reduction in systemic endotoxin level in probiotic group may have arisen as probiotics are known to alter the gut microbiome, act as competitive inhibition with other bacterial components via adherence to the mucosa and epithelium, strengthen the intestinal epithelial barrier function translating to reduced circulating endotoxin, and modification of the immune response in favor of the host [29,30]. The use of 8 strains in our study most likely provided a cumulative effect on changes to the gut, strengthen by the longer duration of intervention.

The effects of the probiotic supplementation on weight loss was not observed. Other studies have noted changes in weight but these have tended to be when the probiotic is taken as part of a either a hypocaloric diet and/or use of bioactive compounds, factors that were not included in our study [31]. Furthermore, no substantial effect was observed in blood pressure despite the longer duration of treatment in this study. Prior studies have noted changed in animal studies but these again have tended to be when taken with other agents such as prebiotics and symbiotics [32] or in human studies when part of a prescribed dietary regimen [33].

It was also observed in this present study the use of the probiotics led to improvement in adipocytokines with a reduction in TNFα, IL-6, CRP, resistin and a rise in adiponectin at six months, which was not observed in the placebo group, even though interaction effects at set intervals noted no significant difference. This lack of effect between groups largely appeared to arise due to the raised baseline endotoxin and adipokine levels in the probiotic group compared with the placebo group.

Previous observations have suggested that endotoxins from non-commensal bacteria may affect adipocytokine levels secondary to translocation induction of several intestinal microbial antigens into the circulation, creating an altered adipokine profile and intestinal dysbiosis [34]. Certain probiotics, specifically lactic acid bacteria strains, have demonstrated *in vitro* that they can differentially modulate adipokine expression and the inflammatory response [35]. It is noteworthy that 6 of the 8 probiotic strains used in this study belong to the lactic acid bacteria class. However, how probiotics directly or indirectly influence adipocytokine levels requires further evaluation, as the effects may be secondary to improved insulin sensitivity and stronger intestinal barrier function.

The authors acknowledge several limitations. Successful colonization of probiotics in the intestinal tract were not obtained, although absence of gut microbiome data does not necessarily mean absence of efficacy [36]. The study also had a low response rate, partly because majority of the patients who initially showed interest to participate declined to continue after a few days, probably because the concept of ingesting live bacteria to improve metabolic status is relatively unheard of in this part of the world. The actual sample size was below the proposed sample size, therefore, the actual power was compromised producing impacting the final clinical findings. The use of prebiotics instead of probiotics might prove to be more beneficial in the region, given the reluctance to use probiotics. Another limitation is the persistent discrepancy between baseline values of the probiotics and the placebo group despite randomization, as is the nature of clinical trials. Baseline characteristics show that while BMI, age and gender were matched for both placebo and probiotics group, the probiotics group were actually cardiometabolically less metabolically healthy than the placebo group. While 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 due to the small cohort. Finally, analysis was not controlled for diet or exercise, which were not assessed, factors that may considerably affect the gut microbiota.

Table 6
Adipocytokines and endotoxin before and after intervention with placebo or probiotics among T2DM patients.

Parameter	Placebo (N = 30)			Probiotics (N = 31)			Intervention Effects (CI 95%)		
	Baseline	3 months	6 months	Baseline	3 months	6 months	0–3 months	0–6 months	Over-all
Leptin (pg/ml)	3.9 (1.6–7.6)	4.0 (1.6–7.0)	2.8 (0.9–6.9)	5.8 (2.5–17.2)	3.5 (2.2–10.0)	3.1 (2.1–9.7)	0.24 (−0.1–0.6)	0.20 (−0.2–0.6)	0.22 (−0.2–0.6)
Change (%) at 3 m		0.1 (2.6)			−2.3 (−39.7)				
Change (%) at 6 m		−1.1 (−28.2)			−2.7 (−46.6)				
Adipo (μg/ml)	11.1 (8.7–16.6)	9.7 (5.1–16.8)	11.1 (5.7–16.0)	8.5 (6.4–14.6)	10.4 (7.2–18.7)	14.6[a] (7.8–24.4)	−0.08 (−0.3–0.1)	−0.04 (−0.2–0.2)	−0.02 (−0.2–0.2)
Change (%) at 3 m		−1.4 (−12.6)			1.9 (22.4)				
Change (%) at 6 m		0 (0)			6.1 (71.8)				
Resistin (ng/ml)	6.3 (4.2–11.4)	11.8 (6.2–19.1)	11.3 (5.3–15.2)	11.7 (6.4–18.8)	6.2 (3.7–14.5)	4.9[a] (3.1–8.3)	0.05 (−0.2–0.3)	−0.02 (−0.2–0.2)	−0.08 (−0.3–0.1)
Change (%) at 3 m		5.5 (87.3)			−5.5 (−47.0)				
Change (%) at 6 m		5.0 (79.4)			−6.8 (−58.1)				
Endo (IU/ml)	2.1 (1.2–4.4)	1.9 (1.0–2.9)	2.9[b] (1.9–7.0)	4.6 (2.4–7.9)	2.2[a] (1.2–3.6)	1.4[a] (1.0–2.1)	0.13 (−0.1–0.4)	−0.10 (−0.4–0.1)	−0.10 (−0.3–0.1)
Change (%) at 3 m		−0.2 (−9.5)			−2.4 (−52.2)				
Change (%) at 6 m		0.8 (38.1)			−3.2 (−69.6)				

Note: Data was presented as median (interquartile range). Results were obtained from mixed method ANCOVA adjusted for baseline covariates; superscript "a" denotes significance compared to baseline; superscript "b" denotes significance compared to 3 months; Adipo, adiponectin; Endo, endotoxin; 3 m, 3 months; 6 m, 6 months. Significant at $p < 0.05$.

Despite the limitations and the rigorous analyses undertaken, a significant improvement was observed in terms of decreased HOMA-IR over time. As HOMA-IR 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. The present clinical trial is the first in the Arab T2DM population; hence, the present findings may prove clinically beneficial for this region. The present study is also one of the longest randomized controlled trials to demonstrate the beneficial effects of a multi-strain probiotic supplementation in improving the HOMA-IR of T2DM patients. Clinical trials on probiotic supplementation in the Arabic T2DM population has never been performed previously. This is important since the gut microbiome is highly affected not only by the health status of the individual, but more so by geography and ethnicity [37]. Findings of the present study therefore add value to the current literature in terms of ethnic-specific effects of probiotics supplementation among patients with T2DM.

In summary, a daily multi-strain probiotic supplementation for 6 months can significantly improve HOMA-IR, reduce endotoxin and inflammatory adipokine levels amongst Arab T2DM subjects. The significant improvement in insulin resistance in favor of the probiotics group despite the low sample size and the rigorous analysis performed merit clinical attention. Findings from the study offer important information that will expand our current understanding on how multi-strain probiotic supplements work in the diabetic population arising from a relatively homogenous and understudied ethnic population. The findings also shed light on the challenges of conducting randomized 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. This study nonetheless recommends the use of multiple-strain probiotics as a supplemental therapy in subjects with T2DM.

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Authors' contributions

S.S., N.M.A, M.S.A and P.G.M conceived and designed the experiments; S.S., A.A., K.W., O.E.A, M.G.A. and M.S.M. performed the experiments; S.S. and S.D.H analyzed the data; K.W., O.E.A., M.G.A and M.S.M. contributed reagents/materials/analysis tools; S.S. wrote the paper. All authors have seen and approved the final version of the manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.08.009.

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