

1 **Effects of a 6-month Multi-Strain Probiotics Supplementation in**
2 **Endotoxemic, Inflammatory and Cardiometabolic Status of T2DM Patients:**
3 **A Randomized, Double-Blind, Placebo-Controlled Trial**

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39 **Summary**

40 **Objective**

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

44 **Methods**

45 Saudi adults with naïve T2DM (n=61; 12 males and 18 females) were randomly allocated to
46 receive twice daily placebo or 2.5×10^9 cfu/gram of Ecologic®Barrier (multi-strain probiotics; 14
47 males and 17 females) in a double-blind manner over a 6 month period, respectively.
48 Anthropometrics were measured and fasting blood samples were collected to analyze endotoxin,
49 glycemic parameters [glucose, insulin, c-peptide and homeostasis model assessment for insulin
50 resistance (HOMA-IR)], lipids [triglycerides, total cholesterol, low and high-density lipoprotein
51 (LDL and HDL, respectively) cholesterol and total/HDL-cholesterol ratio], inflammatory markers
52 [tumor-necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and C-reactive protein (CRP)] and
53 adipocytokines [leptin, adiponectin and resistin] at baseline and after 3 and 6 months of
54 intervention.

55 **Results**

56 Multi-strain probiotics supplementation for 6 months caused a significant decrease in circulating
57 levels of endotoxin by almost 70% over 6 months, as well as glucose (38%), insulin (38%),
58 HOMA-IR (64%), triglycerides (48%), total cholesterol (19%), total/HDL-cholesterol ratio (19%),
59 TNF- α (67%), IL-6 (77%), CRP (53%), resistin (53%), and a significant increase in adiponectin
60 (72%) as compared with baseline. Only HOMA-IR had a clinically significant reduction (-3.4,
61 64.2%) in the probiotics group as compared to placebo group at all time points. No other clinically
62 significant changes were observed between the probiotic or placebo group at 3 and 6 months in
63 other markers.

64 **Conclusion**

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

69 **Trial Registration:** ClinicalTrials.gov Identifier: NCT01765517

70 **Key Words:** probiotics, endotoxin, type 2 diabetes mellitus

71 **1. Introduction**

72 In recent years there has been intense commercial interest in understanding the role of
73 human microbiome in diseases and factors that can relieve it, with the use of pre-biotic and pro-
74 biotic often in inflammatory intestinal disorders making it an emerging biomedical industry
75 projected to be worth \$46.56 billion by 2020 [1]. Despite this interest there has been conflicting
76 evidence into the effectiveness of probiotics in health and disease *per se* with limited insight into
77 the use of prebiotics and probiotics for the management type 2 diabetes mellitus (T2DM) [2-5];
78 despite the knowledge that T2DM is also considered an inflammatory chronic condition. Prior
79 studies in T2DM subjects has shown the importance of the gut derived gram negative bacterial
80 fragment lipopolysaccharide (LPS, endotoxin) which can overgrow in the intestine, induce a leaky
81 gut, and allow endotoxin to enter into the circulation and induce systemic inflammation [6]. Prior
82 studies have also shown that the use of diet and/or surgery for weight reduction can lower
83 endotoxin-induced inflammation [7-9], which, suggests that manipulation of the gut microbiota
84 with an appropriate pro-biotic may also have significant health effects [10]. Since the gut
85 microbiome is the main reservoir of endotoxin, probiotics supplementation may alter its levels by
86 modifying its composition and strengthening the gut epithelial barrier [11, 12].

87 Few studies to date have examined the effects of probiotics on systemic levels of endotoxin
88 in chronic, non-communicable diseases. Those that have examined the specific impact of
89 probiotics on endotoxin and associated metabolic diseases have shown conflicting outcomes.
90 Probiotics use in cirrhotic patients has shown a positive 25% reduction in systemic endotoxin [13],
91 while a more recent review indicated the effects on circulating endotoxin was minimal [14].
92 Although in animal studies, where diet is more easily controlled, more consistent evidence

93 suggests that probiotics supplementation may be beneficial in the use of insulin-resistant diseases
94 [15]. The few human intervention trials that have been conducted appear to support the animal
95 studies with a recent meta-analysis of 12 studies implicating that probiotics give rise to significant
96 improvements in HbA1c and fasting insulin amongst subjects with T2DM [16]. Nevertheless, the
97 majority of the interventional studies conducted to date with probiotics use in subjects with T2DM
98 have tended to be either short-term studies, no longer than 3 months and/or mono-strains were
99 used as supplementation [17, 18]. To the best of our knowledge, there is limited evidence on the
100 effects of a long duration, multi-strain probiotics supplementation on systemic endotoxin levels
101 amongst T2DM subjects. This study therefore sought to test the hypothesis that multi-strain
102 probiotics supplementation reduces endotoxin levels and consequently improve cardiometabolic
103 profile in an Arab T2DM population where metabolic risk is high.

104 **2. Methods**

105 *2.1 Participants and study design*

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

111 For this study 150 adult Saudi participants [73 females (46 (63%) menopause), 77 males,
112 aged 30-60 years old) with newly diagnosed T2DM (<6 months) were initially recruited by the
113 research team for intervention from January 2014 to February 2016. All participants were patients
114 visiting the outpatient department of King Salman Hospital, Riyadh, Saudi Arabia. Patients with
115 diabetes complications (retinopathy, neuropathy, nephropathy, etc.) and poor glycemic control

116 (HbA1c > 7%) as noted in their medical records were excluded. Participants on prebiotics,
117 probiotics, or antibiotics treatment 6 weeks before inclusion, lactating or pregnant women, on
118 insulin or its analogues and those with gastrointestinal diseases were excluded. Sample size
119 calculation was previously done based on the primary outcome (endotoxin), considering 80%
120 power at $\alpha=0.05$ [19].

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

125 *2.2 Randomization and Blinding*

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

130 *2.3 Study Protocol*

131 The probiotics group was allocated with sachets [2g freeze-dried powder of the probiotic
132 mixture Ecologic®Barrier (Winlove probiotics, the Netherlands) (2.5×10^9 cfu/gram)] which
133 contains the following strains: *Bifidobacterium bifidum* W23, *Bifidobacterium*
134 *lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus*
135 *casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58.
136 This probiotic combination has been previously investigated for its ability to improve endothelial
137 barrier and its potency to inhibit mast cell activation, inhibit pro-inflammatory cytokines decrease
138 endotoxin load [20]. The placebo group was allocated the same sachets without the probiotic

139 strains (2 grams freeze-dried maize starch and maltodextrins). All participants were asked to
140 consume their assigned treatment twice daily (dissolving contents in glass of water) before
141 breakfast and before bed time. Anthropometrics were measured and included height (cm), weight
142 (kg), blood pressure (mmHg) waist and hip measurements (cm), body mass index (BMI kg/m²)
143 and waist-hip ratio (WHR) at baseline, 3 months and after 6 months of treatment. Fasting blood
144 samples were also collected during those time points. All blood samples were centrifuged, serum
145 samples separated, put on ice and immediately delivered to Prince Mutaib Chair for Biomarkers
146 of Osteoporosis (PMCO) in King Saud University (KSU) for storage at -20°C until further analysis.
147 To monitor compliance, subjects were asked to return once a month to be asked for side effects
148 and to return unused sachets for fresh refill.

149 *2.4 Biochemical Analyses*

150 Fasting serum samples were analyzed for glucose and lipid profile [total cholesterol, high
151 density lipoprotein (HDL) cholesterol and triglycerides] using Konelab routine analyzer (Konelab,
152 Espoo, Finland). LDL-cholesterol was calculated using the Friedewald equation [21]. Serum tumor
153 necrosis factor (TNF) α , interleukin (IL)-6, leptin, adiponectin and resistin were measured using
154 the Milliplex Map (Millipore, Billerica, MA, USA) in the FlexMAP 3D (Luminex Corp, Austin,
155 TX, USA) . Minimum detectable concentrations (MDC) were as follows: TNF α , 0.14pg/ml; IL-6,
156 0.4pg/ml; leptin, 85.4pg/ml; adiponectin, 145.4pg/ml and resistin, 6.7pg/ml. The intra-assay
157 variation was 1.4-7.9% and inter-assay variation of <21%. Serum insulin and C-peptide were
158 measured using electrochemiluminescence assay (Roche Diagnostics, Germany). C-reactive
159 protein (CRP) [intra-assay precision (4.4-8.3) and inter-assay precision (6.0-7.0)] (R&D Systems,
160 MN, USA). Homeostasis model assessment (HOMA IR) was calculated as the product of insulin
161 (uU/ml) and glucose (mmol/l) divided by 22.5 [22]. Endotoxin (primary endpoint) was measured

162 using a limulus ameocyte lysate (LAL) quantitative kinetic assay (Lonza, MD, USA). As serum
163 is very inhibitory to this assay a spike recovery was performed using a sample dilution of 1:40.
164 The recovery spike was 60% and was within the acceptable range of 50-200%. All serum samples
165 were analyzed at baseline, 3 months and after 6 months of treatment.

166 *2.5 Data Analyses*

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

184

185 **3. Results**

186 Of the 150 participants that were recruited, 96 were randomized, 78 completed 3 months and
187 61 completed the entire trial (probiotics group, n=31; placebo group, n=30). The flowchart of the
188 trial is presented in figure 1. Baseline comparison showed no significant differences in both groups
189 except WHR, glucose, total cholesterol, total/HDL-cholesterol, TNF- α , IL-6, leptin and endotoxin
190 (Table 1). The most common reasons for drop out included loss to follow-up and poor compliance.
191 Flatulence was the most common complaint (N=5, 1 in the placebo group and 4 in the probiotics
192 group) during the first weeks of trial in both placebo and probiotics group (not included in tables).

193 *3.1 Changes in Anthropometrics and Clinical Measures*

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

197 *3.2 Changes in Glycemic Indices*

198 Fasting glucose levels were significantly higher in the probiotics than the placebo group at
199 baseline [11.7mmol/l (8.4-16.4) versus 7.1mmol/l (5.7-11.2)]. After adjusting for baseline
200 covariates, between group-comparisons showed no significant difference in glucose levels
201 between placebo and probiotics groups at 3 months [1.0mmol/l (14.3%) vs -3.2mmol/l (-27.4%)]
202 and after 6 months [1.1mmol/l (15.7%) vs -4.5mmol/l (-38.5%)]. No difference was also observed
203 in C-peptide levels [0.80ng/ml (800%) vs -0.30ng/ml (-75%)] at 6 months. A borderline significant
204 difference was observed in insulin levels [-0.30IU/ml (-2.4%) vs -3.80IU/ml (-38.4%)] at 6-month
205 comparison and clinically significant differences were noted in HOMA-IR at 3 months [0.0 (0%)
206 vs -3.2 (-60.4%)] and after 6 months [0.80 (20.5%) vs -3.40 (-64.2%)] in favor of the probiotics
207 group. Within group comparisons showed that in the placebo group, there was a significant

208 increase in C-peptide levels at 6 months as compared to both baseline and 3 months. The rest of
209 the glycemic parameters in the placebo group did not significantly change over time. In the
210 probiotics group, a significant decrease was observed in glucose, insulin and HOMA-IR values
211 overtime. Median levels of C-peptide significantly decreased only after 6 months. (Table 3).

212 *3.3 Changes in Lipid Profile*

213 LDL- and total cholesterol as well as total/HDL-cholesterol ratio were significantly higher
214 in the probiotics group than placebo at baseline. Between group comparisons showed no
215 differences in placebo and probiotics groups over-all in levels of triglycerides [-0.10mmol/l (-
216 4.6%) vs -1.20mmol/l (-48%)], total cholesterol [-0.30mmol/l (-5.8%) vs -1.10mmol/l (-19%)],
217 HDL-cholesterol [-0.10mmol/l (-9.1%) vs -0.30mmol/l (30%)], LDL-cholesterol [-0.10mmol/l
218 (9.7%) vs -0.80mmol/l (-22.2%)] and total/HDL-cholesterol ratio [-0.30 (-5.8%) vs -1.10 (-19%)].
219 Within group analysis showed no changes in the placebo group over time. In the probiotics group,
220 significant improvements were observed in terms of decreased triglycerides, total cholesterol and
221 total/HDL cholesterol ratio (Table 4).

222 *3.4 Changes in Inflammatory Markers*

223 At baseline, the probiotics group had a significantly higher median levels of TNF α and IL6
224 than placebo group. Between-group comparisons post-intervention showed no significant
225 differences in placebo and probiotic groups in levels of TNF α [-0.20pg/ml (-40%) vs -0.60pg/ml
226 (-66.7%)], IL-6 [-2.8pg/ml (-77.8%) vs -3.9pg/ml (-76.5%)] and C-reactive protein [0.40ug/ml
227 (13.3%) vs -2.9ug/ml (-52.7%)]. Within group comparisons however showed that all inflammatory
228 markers significantly improved over time in the probiotics group and these changes were not
229 observed in the placebo group (Table 5).

230 *3.5 Changes in Endotoxin levels and Adipocytokine Profile*

231 Endotoxin was significantly higher in the probiotics group than placebo at baseline.
232 Furthermore, no differences in baseline adipocytokines were observed except for levels of leptin
233 being significantly higher in the probiotics than the placebo group. Between group comparisons
234 after 6 months showed no differences in both groups in levels of endotoxin [0.80IU/ml (38.1%)
235 vs. -3.20IU/ml (-69.6%)], leptin [-1.1pg/ml (-28.2%) vs. -2.7pg/ml (-46.6%)], adiponectin
236 [0.0µg/ml (0%) vs. 6.1µg/ml (71.8%)], and resistin [5.0ng/ml (79.4%) vs. -6.8ng/ml (-58.1%)].
237 Within group comparisons showed a significant increase in resistin levels after 6 months compared
238 to baseline ($p<0.05$) as well as a significant increase in endotoxin levels after 6 months as
239 compared to 3 months in the placebo group. In the probiotics group post-intervention, there was a
240 significant improvement in endotoxin (Figure 2) and adiponectin levels, and a significant decrease
241 in resistin. No significant changes in either group were noted in leptin levels (Table 6).

242 **4. Discussion**

243 The ambition of this randomized controlled study was to determine primarily the systemic
244 endotoxin-lowering capability of a multi-strain probiotic supplementation and whether such
245 treatment would result in improved cardiometabolic profile in patients with T2DM. From this
246 study, it was observed that circulating endotoxin levels were significantly reduced post-
247 intervention in the probiotics group, whilst the placebo group remained unchanged by time. In
248 addition, comparison between groups also showed a clinically significant difference in HOMA-IR
249 with improvement in insulin sensitivity in the probiotic group. The noted associated improvement
250 in endotoxin levels and HOMA-IR has been observed in other diet or medicinal intervention
251 studies using T2DM subjects [7, 8]. In conjunction with reduction in endotoxin levels in the
252 probiotic group at six months there were also associated improvements in cholesterol, Total
253 cholesterol/HDL ratio, and glycemic control from baseline in group analysis supporting the

254 concept that probiotics can provide cardiometabolic protective effects. Noting that the placebo
255 group did not appear comparable to the probiotic group from baseline biochemical data gathered.

256 Previous studies have tried to evaluate the beneficial effects of probiotics in T2DM with the
257 ultimate cardiometabolic benefits requiring more than 3 months, with our study suggesting 6
258 month follow up may highlight promising findings [23-27]. Our study is, to our knowledge, the
259 first to demonstrate the effects of a multi-strain probiotic supplement given over 6-months in the
260 Arab T2DM population, using endotoxin as the primary endpoint. It is also important to stress that
261 the probiotic supplementation in this present study was used as a standalone treatment given in the
262 absence of exercise and diet-related modifications in the intervention or lifestyle control in a
263 culture with easy access to excess food. While this is not the first interventional study undertaken
264 on the effects of probiotics in patients with T2DM, our protocol addressed previous trials concerns
265 for a longer duration and use of multiple strains, which highlighted cardiometabolic benefits in the
266 probiotic group from baseline to six months. Clearly the 6 month time point was important to
267 observe changes as the most significant changes were noted which affirms a recent meta-analysis
268 of Hu and colleagues observed, where trials with longer durations of intervention using multiple
269 probiotic strains had more beneficial cardiometabolic effects in patients with T2DM [28].

270 This present study showed significant improvements in the endotoxin levels of the probiotic
271 group overtime, although not clinically significant as compared to placebo group at 6 months.
272 However, the T2DM patients in the probiotic group began the study at a significantly higher
273 baseline endotoxin level, despite noted comparability for BMI, age and gender. Furthermore,
274 biochemically the probiotic intervention group began the study with significantly raised glucose
275 levels, diastolic blood pressure and inflammatory status as well. This therefore would have affected
276 the 6-month comparison as the baseline groups were not comparable which may have been a

277 challenge with using newly diagnosed T2DM patients; despite best efforts to limit confounders in
278 the study. Such discrepancies between the two groups could also have been due to sample
279 size difference, duration of intervention and patient selection [29]. However, there was a noted
280 70% drop in endotoxin level in those subjects on the probiotic over six months compared with a
281 net effect of zero change in the control placebo group over the same period.

282 The reduction in systemic endotoxin level in probiotic group may have arisen as probiotics are
283 known to alter the gut microbiome, act as competitive inhibition with other bacterial components
284 via adherence to the mucosa and epithelium, strengthen the intestinal epithelial barrier function
285 translating to reduced circulating endotoxin, and modification of the immune response in favor of
286 the host [30, 31]. The use of 8 strains in our study most likely provided a cumulative effect on
287 changes to the gut, strengthened by the longer duration of intervention.

288 The effects of the probiotic supplementation on weight loss was not observed. Other studies
289 have noted changes in weight but these have tended to be when the probiotic is taken as part of a
290 either a hypocaloric diet and/or use of bioactive compounds, factors that were not included in our
291 study [32]. Furthermore, no substantial effect was observed in blood pressure despite the longer
292 duration of treatment in this study. Prior studies have noted changes in animal studies but these
293 again have tended to be when taken with other agents such as prebiotics and symbiotics [33] or in
294 human studies when part of a prescribed dietary regimen [34].

295 It was also observed in this present study the use of the probiotics led to improvement in
296 adipocytokines with a reduction in TNF α , IL-6, CRP, resistin and a rise in adiponectin at six
297 months, which was not observed in the placebo group, even though interaction effects at set
298 intervals noted no significant difference. This lack of effect between groups largely appeared to

299 arise due to the raised baseline endotoxin and adipokine levels in the probiotic group compared
300 with the placebo group.

301 Previous observations have suggested that endotoxins from non-commensal bacteria may
302 affect adipocytokine levels secondary to translocation induction of several intestinal microbial
303 antigens into the circulation, creating an altered adipokine profile and intestinal dysbiosis [35].
304 Certain probiotics, specifically lactic acid bacteria strains, have demonstrated *in vitro* that they can
305 differentially modulate adipokine expression and the inflammatory response [36]. It is noteworthy
306 that 6 of the 8 probiotic strains used in this study belong to the lactic acid bacteria class. However,
307 how probiotics directly or indirectly influence adipocytokine levels requires further evaluation, as
308 the effects may be secondary to improved insulin sensitivity and stronger intestinal barrier
309 function.

310 The authors acknowledge several limitations. Successful colonization of probiotics in the
311 intestinal tract were not obtained, although absence of gut microbiome data does not necessarily
312 mean absence of efficacy [37]. The study also had a low response rate, partly because majority of
313 the patients who initially showed interest to participate declined to continue after a few days,
314 probably because the concept of ingesting live bacteria to improve metabolic status is relatively
315 unheard of in this part of the world. The actual sample size was below the proposed sample size,
316 therefore, the actual power was compromised producing impacting the final clinical findings. The
317 use of prebiotics instead of probiotics might prove to be more beneficial in the region, given the
318 reluctance to use probiotics. Another limitation is the persistent discrepancy between baseline
319 values of the probiotics and the placebo group despite randomization, as is the nature of clinical
320 trials. Baseline characteristics show that while BMI, age and gender were matched for both placebo
321 and probiotics group, the probiotics group were actually cardiometabolically less metabolically

322 healthy than the placebo group. While this was addressed by adjusting analyses for baseline
323 differences, the additional adjustments of covariates made it more difficult to elicit the desired
324 treatment effect because of the added statistical stringency due to the small cohort. Finally, analysis
325 was not controlled for diet or exercise, which were not assessed, factors that may considerably
326 affect the gut microbiota.

327 Despite the limitations and the rigorous analyses undertaken, a significant improvement
328 was observed in terms of decreased HOMA-IR over time. As HOMA-IR is intricately linked to
329 most of the cardiometabolic indices measured, the clinically significant improvement suggests that
330 probiotics supplementation do confer beneficial effects when consumed by the T2DM population.
331 The present clinical trial is the first in the Arab T2DM population; hence, the present findings may
332 prove clinically beneficial for this region. The present study is also one of the longest randomized
333 controlled trials to demonstrate the beneficial effects of a multi-strain probiotic supplementation
334 in improving the HOMA-IR of T2DM patients. Clinical trials on probiotic supplementation in the
335 Arabic T2DM population has never been performed previously. This is important since the gut
336 microbiome is highly affected not only by the health status of the individual, but more so by
337 geography and ethnicity [38]. Findings of the present study therefore add value to the current
338 literature in terms of ethnic-specific effects of probiotics supplementation among patients with
339 T2DM.

340 In summary, a daily multi-strain probiotic supplementation for 6 months can significantly
341 improve HOMA-IR, reduce endotoxin and inflammatory adipokine levels amongst Arab T2DM
342 subjects. The significant improvement in insulin resistance in favor of the probiotics group despite
343 the low sample size and the rigorous analysis performed merit clinical attention. Findings from the
344 study offer important information that will expand our current understanding on how multi-strain

345 probiotic supplements work in the diabetic population arising from a relatively homogenous and
346 understudied ethnic population. The findings also shed light on the challenges of conducting
347 randomized clinical trials in this area of the world where such studies that offer high level of
348 evidence are still evolving and would require greater input and participation from the general
349 population. This study nonetheless recommends the use of multiple-strain probiotics as a
350 supplemental therapy in subjects with T2DM.

351

352 **Abbreviations**

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

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363 **Conflict of interests**

364 The authors declare that they have no competing interests.

365 **Authors' contributions**

366 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.,
367 M.G.A. and M.S.M. performed the experiments; S.S. and S.D.H analyzed the data; K.W., O.E.A.,

368 M.G.A and M.S.M. contributed reagents/materials/analysis tools; S.S. wrote the paper. All authors
369 have seen and approved the final version of the manuscript.

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507 **Legends to Figures**

508 **Figure 1.** CONSORT Flow Chart detailing participants' recruitment, randomization and allocation.

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

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517 **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

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

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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/m²)	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 3m	0.1 (0.3)			-0.10 (-0.3)					
Change (%) at 6m	-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 3m	0.0 (0.0)			0.0 (0.0)					
Change (%) at 6m	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 3m	0.4 (0.3)			-5.8 (-4.3)					
Change (%) at 6m	-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 3m	1.2 (1.5)			-3.8 (-4.6)					
Change (%) at 6m	-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 3m	1.0 (1.0)			-4.4 (-4.4)					
Change (%) at 6m	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; 3m, 3 months; 6m, 6 months.

Table 3. Glycaemic 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 3m	1.0 (14.3)			-3.2 (-27.4)					
Change (%) at 6m	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 3m	-1.6 (-12.9)			-3.0 (-30.3)					
Change (%) at 6m	-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 3m	0.1 (100.0)			-0.3 (-75.0)					
Change (%) at 6m	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- -0.02)	-0.34** (-0.6- -0.12)	-0.38** (-0.6- -0.17)
Change (%) at 3m	0.0 (0.00)			-3.2 (-60.4)					
Change (%) at 6m	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; 3m, 3 months; 6m, 6 months. Significant at p<0.05.

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 3m	-0.2 (-9.1)			-0.8 (-32.0)					
Change (%) at 6m	-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 3m	-0.5 (-9.6)			-0.7 (-12.1)					
Change (%) at 6m	-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 3m	-0.1 (-9.1)			0.1 (10.0)					
Change (%) at 6m	-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 3m	-0.3 (-9.7)			-0.4 (-11.1)					
Change (%) at 6m	-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 3m	-0.5 (-9.6)			-0.7 (-12.1)					
Change (%) at 6m	-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; 3m, 3 months; 6m, 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 3m	0 (0)			-0.3 (-33.3)					
Change (%) at 6m	-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 3m	-2.8 (-77.8)			-3.7 (-72.6)					
Change (%) at 6m	-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 3m	-0.1 (-3.3)			-2.4 (-43.6)					
Change (%) at 6m	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; 3m, 3 months; 6m, 6 months. Significant at $p < 0.05$.

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 3m	0.1 (2.6)			-2.3 (-39.7)					
Change (%) at 6m	-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 3m	-1.4 (-12.6)			1.9 (22.4)					
Change (%) at 6m	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 3m	5.5 (87.3)			-5.5 (-47.0)					
Change (%) at 6m	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 3m	-0.2 (-9.5)			-2.4 (-52.2)					
Change (%) at 6m	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; 3m, 3 months; 6m, 6 months. Significant at p<0.05.