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Original article

A dietary intervention with functional foods reduces metabolic endotoxaemia and attenuates biochemical abnormalities by modifying faecal microbiota in people with type 2 diabetes

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ABSTRACT

Aim. – To study the effects of a functional food-based dietary intervention on faecal microbiota and biochemical parameters in patients with type 2 diabetes (T2D).
Materials and methods. – This placebo-controlled, randomized, double-blind study included 81 patients with T2D divided into two 3-month treatment groups: one following a reduced-energy diet with a dietary portfolio (DP) comprising high-fibre, polyphenol-rich and vegetable-protein functional foods; the other taking a placebo (P). The primary outcome was the effect of the DP on faecal microbiota. Secondary endpoints were biochemical parameters, lipopolysaccharide, branched-chain amino acids, trimethylamine N-oxide, glycosylated haemoglobin (HbA_{1c}) and free fatty acids (FFAs).
Results. – Patients with T2D exhibited intestinal dysbiosis characterized by an increase in *Prevotella copri*. Dietary intervention with functional foods significantly modified faecal microbiota compared with P by increasing alpha diversity and modifying the abundance of specific bacteria, independently of antidiabetic drugs. There was a decrease in *P. copri* and increases in *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*, two bacterial species known to have anti-inflammatory effects. The DP group also exhibited significant reductions in areas under the curve for glucose, total and LDL cholesterol, FFAs, HbA_{1c} ($P < 0.05$), triglycerides and CRP, and an increase in antioxidant activity ($P < 0.01$) vs. the P group.
Conclusion. – Long-term adherence to a high-fibre, polyphenol-enriched and vegetable-protein-based diet provides benefits for the composition of faecal microbiota, and may offer potential therapies for improvement of glycaemic control, dyslipidaemia and inflammation.

Introduction

There has been a constant increase in the incidence and prevalence of type 2 diabetes (T2D) in both developed and underdeveloped countries [1]. Complex diseases such as diabetes are multifactorial, in which changes to the environment, diet and lifestyle are considered major causes. The usual Western diet, which is rich in saturated fats and simple sugars, may be associated with negative metabolic changes such as dysfunction of the insulin pathway leading to hyperinsulinaemia that, in turn, stimulates production of tumour necrosis factor (TNF)-α in adipose tissue, thereby increasing lipolysis, while also increasing free fatty acids (FFAs) and glycerol [2]. High levels of FFAs can induce deterioration of β-cell function, resulting in hyperglycaemia, oxidative stress, insulin resistance and β-cell dysfunction [3]. In addition to these risk factors for T2D, alterations of the microbial community living in our gut, called the 'microbiota', have emerged as a new candidate factor that may be linked to T2D.

The human gut microbiota comprises 2172 identified bacterial species so far, grouped mainly into four phyla: Firmicutes (Gram-positive); Protobacteria; Actinobacteria; and Bacteroidetes (Gram-

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negative) [4]. These microorganisms perform key functions that the human host is incapable of performing on its own, such as the breakdown of non-digestible carbohydrates contained in the diet [5]. Changes to gut microbiota have been linked to the increasing prevalence of T2D. Also, a body of evidence demonstrates that intestinal microbiota play a role in the onset of metabolic diseases in part due to the release of lipopolysaccharide (LPS), which produces chronic low-grade inflammation [6]. In addition, Gram-negative-bacteria-promoted metabolic endotoxaemia can activate an inflammatory response leading to insulin resistance [7].

There is also evidence of a pathophysiological contribution of gut microbiota to cardiovascular diseases linked to gut microbial choline metabolism. Modulation of gut microbiota may even modify turnover of choline and carnitine. These compounds are metabolized by gut microbiota to produce trimethylamine (TMA), which is rapidly oxidized by hepatic flavin monooxygenases to form trimethylamine N-oxide (TMAO), which is proatherogenic and associated with cardiovascular risk [8]. Furthermore, it has been reported that specific gut microbiota constitute another, independent contributing source of elevated branched-chain amino acids (BCAAs), which are associated with insulin resistance in T2D patients [9].

Therefore, the present clinical trial has investigated the effect of a dietary portfolio (DP) based on a combination of functional foods, including inulin, chia seeds, soy protein, dehydrated nopal (because of its prebiotic properties), omega-3 fatty acids, vegetable protein, polyphenols, and soluble and insoluble fibre, all known to reestablish the gut microbiota, and attenuate the biochemical abnormalities and metabolic endotoxaemia caused by dysbiosis of the microbiota in patients with T2D. Our hypothesis was that a dietary intervention with functional foods would reduce biochemical abnormalities and metabolic endotoxemia compared with P, and also induce changes to gut microbiota leading to a healthier metabolic profile.

Materials and methods

Participants and sample collection

Patients with T2D for 1–7 years and aged 30–60 years, with a body mass index (BMI) of 25–39.9 kg/m^2, and a healthy control group of subjects aged 20–40 years, with a BMI of 18.5–24.9 kg/m^2, were recruited through advertisements at the Department of Physiology of Nutrition at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City.

T2D participants were all receiving pharmacological treatment for control of glucose with metformin or a combination of glibenclamide and metformin, and followed from August 2014 to September 2016. Exclusion criteria for these T2D participants included $HbA_{1c} \geq 9.9\%$, fasting glucose $\geq$ 12.2 mM (220 mg/dL), total cholesterol $\geq$ 62.4 mM (240 mg/dL), triglycerides $\geq$ 3.99 mM (350 mg/dL), serum creatinine $>$ 106 μM (1.2 mg/dL) in women and $>$ 114.9 μM (1.3 mg/dL) in men, a history of cardiovascular events, weight loss of $\geq$ 3 kg within the last 3 months, cancer, acquired immunodeficiency syndrome (AIDS), kidney or liver disease, pregnancy, smoking, substance abuse and alcohol consumption. To ensure comparable data, patients were interviewed for their history of gastrointestinal diseases and use of probiotics. No participants in either the healthy control or T2D group had taken antibiotics during the previous 2 months.

The study was approved by the Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán (No. 1165), registered at www.clinicaltrials.gov (NCT03421301) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Study design

The study was divided into two stages:

- basal comparisons between healthy and T2D subjects to create a reference for faecal microbiota, LPS, BCAAs and TMAO;
- a single-centre randomized, controlled, double-blind parallel-group vs. P study, consisting of six clinical visits, to evaluate the effects of a dietary intervention on faecal microbiota in patients with T2D.

At the first visit, a screening evaluation was conducted to determine whether participants met the inclusion criteria (Fig. S1; see supplementary material associated with this article online). All eligible subjects were then invited to a second visit, which consisted of obtaining a medical history, undergoing a 2-h oral glucose tolerance test (OGTT), and collecting stool samples for DNA isolation and a 5-mL blood sample. All participants also received, as the first stage, a reduced-energy dietary plan adjusted to be 500 kcal less than their usual kcal consumption. At the third visit, all subjects were randomized to receive either the DP or P treatment in combination with the reduced energy diet for 1 month. At the fourth and fifth visits, separated by a 1-month interval, dietary assessment and compliance with the DP or P were evaluated. At each follow-up visit, a 24-h dietary recall was collected, a physical-activity questionnaire was filled out, and anthropometric and clinical parameters were assessed. At the sixth visit, a 2-h OGTT was performed, and a stool sample for DNA isolation and 5 mL of blood were again collected (for details, see the supplementary Appendix associated with this article online).

Dietary intervention

T2D patients followed a 15-day reduced-energy diet tailored to provide a 500 kcal/day deficit compared with their usual diet, as recommended by the US National Institutes of Health (NIH) [10]. The diet plan was 45–55% carbohydrate, 15–20% protein, 25–35% fat ($<$ 7% saturated fat), 200 mg/day of cholesterol, 20–35 g of fibre and 2000–3000 mg/day of sodium, based on total energy consumed. After this 15-day period, participants continued to follow their reduced-energy diets, but with the addition of either the DP (combination of functional foods) or P. The DP provided 200 kcal, subtracted from the diet, comprising 14 g of dehydrated nopal, 4 g of chia seeds, 30 g of soy protein and 4 g of inulin, while the P consisted of 28 g of calcium caseinate and 15 g of maltodextrin. The kcal number, appearance and flavour were similar between the DP and P, and both were given in packets in dehydrated form ready to be dissolved in water. The DP was divided into two packets: the first contained 17.3 g of DP or P to be consumed at breakfast, dissolved in 250 mL of water; the second packet was consumed at dinnertime (1500–1600 h) and contained 34.7 g of P or DP to be dissolved in 300 mL of water.

Statistical analyses

Continuous variables were expressed as means ± standard deviation (SD), while dichotomous variables were expressed as frequencies and percentages. Variables were assessed using the Kolmogorov–Smirnov Z test to examine distribution type. To evaluate the basal characteristics between groups (DP vs. P), an independent-samples *t*-test was used and, to compare proportions, a Chi^2 test was used. Differences in the percentage changes between the DP and P groups were expressed as medians and 95% confidence intervals (CIs), and analyzed using the Mann–Whitney U test. Comparisons between healthy controls and T2D patients were performed using the independent-samples *t*-test. Correlations between two variables

were evaluated by Pearson's test if the distribution was normal, or by Spearman's test if it was not. A $P < 0.05$ was considered significant. Data were analyzed using SPSS version 25 software for Macintosh (IBM Corp., Armonk, NY, USA).

Results

Anthropometric and biochemical variables in healthy and T2D subjects

As expected, all anthropometric parameters were significantly higher in patients with T2D: body weight, BMI, waist circumference, body fat and visceral area were 35%, 40%, 27%, 51% and 98% higher, respectively, than in the healthy control subjects. Furthermore, those with T2D had significantly higher systolic and diastolic blood pressure compared with healthy subjects, but were not classified as elevated according to the American Heart Association. Also, as expected, the biochemical variables of glucose and lipid metabolism during fasting were significantly higher with T2D than in the healthy controls. In addition, glucose tests and homoeostasis model assessment for insulin resistance (HOMA-IR) revealed that the T2D group had hyperglycaemia associated with insulin resistance, leading to significantly higher HbA_{1c}. These abnormalities were accompanied by alterations in lipid metabolism, with T2D patients having 104% and 32% higher serum triglyceride and low-density lipoprotein cholesterol (LDL-C), respectively, than healthy subjects. In addition, the LDL-C/high-density lipoprotein (HDL)-C ratio was 3.2 for T2D patients whereas, in healthy subjects, it was 1.5. Interestingly, there was a significant increase in alanine aminotransferase (ALT) (Table S1; see supplementary material associated with this article online).

Glucose metabolism

To determine to what extent biochemical differences were contributing to the presence of T2D, different biomarkers were measured in the healthy controls and T2D patients. The results showed that those with T2D had significantly higher areas under the curve (AUCs) for glucose after OGTT than healthy subjects and that this was dependent on the number of years of living with diabetes: the more years since the onset of T2D, the greater the AUC ($P < 0.0001$). In contrast, circulating levels of insulin decreased with evolution of the disease, as observed for the insulin AUC ($P = 0.001$; Fig. 1A). As expected, T2D patients had significantly higher levels of the incretin glucose-dependent insulinotropic polypeptide (GIP) and plasma FFAs compared with healthy subjects (Fig. 1B and C). In fact, there was a significant correlation between plasma FFAs and serum glucose, indicating increased lipolysis from adipose tissue in those with T2D, with a similar pattern observed between plasma FFAs and HbA_{1c} (Fig. 1D). Experimental evidence suggests that low levels of HDL-C may also be contributing to the pathophysiology of T2D: in the present study, patients with T2D showed 33% lower HDL-C than healthy subjects, whereas their HOMA-IR was 2.8-fold higher than in healthy subjects (Fig. 1E). Interestingly, T2D subjects had approximately 54.2-fold higher circulating levels of LPS compared with healthy subjects (Fig. 1F), thereby indicating a chronic low-grade inflammation termed 'metabolic endotoxaemia' and caused by possible dysbiosis in faecal microbiota.

Faecal microbiota

Clustering bacterial communities using principal coordinates analysis (PCoA) revealed that the faecal microbiota of T2D patients was unlike those in healthy subjects [analysis of similarities (ANOSIM): $r = 0.6065$, $P = 0.001$; Fig. 2A]. According to the Shannon diversity index (see Fig. 6B), there was no difference in alpha diversity between healthy and T2D subjects. The relative abundance of the three main phyla (Bacteroidetes, Firmicutes and Proteobacteria) represented approximately 99.32% of all sequences at the phylum level. However, patients with T2D showed an increase of 10.4% in relative abundance of Firmicutes compared with healthy subjects (Fig. 2B). At genus level, our analysis revealed a marked difference in faecal microbiota between T2D and healthy subjects (Fig. 2C). Linear discriminant analysis effect size (LEfSe) indicated a clear difference in faecal microbiota between T2D and healthy subjects, with increased levels of *Prevotella copri*, *Bacteroides plebeius* and *Bacteroides eggerthii* with T2D, and of *Bacteroides fragilis*, *Akkermansia muciniphila*, *Bacteroides uniformis* and *Bacteroides ovatus* in healthy controls (Fig. 2D).

There is also evidence that gut microbiota are modified by oral hypoglycaemic agents (OHAs) [11] as, in the present study, patients with T2D were taking different types of OHAs, metformin or metformin plus glibenclamide. Interestingly, the faecal microbiota showed several differences between groups at the species level in response to medication type (Fig. 2E), particularly on a heat map, which revealed that T2D patients treated with metformin had the greatest abundance of *P. copri*, followed by the metformin plus glibenclamide group (Fig. 2F). Interestingly, this latter group also showed the greatest abundance of *Escherichia coli*. In contrast, healthy subjects showed the greatest abundance of *Faecalibacterium prausnitzii* and *B. fragilis*.

TMAO, choline and betaine concentrations

Given the close relationship between diabetes and cardiovascular disease, and the link between gut microbiota and TMAO formation, TMAO concentrations and its related metabolites were measured in healthy and T2D subjects: those with T2D had a 48.2% higher TMAO concentration than healthy subjects (Fig. 3A), while men with T2D had a 47% higher TMAO concentration than women (Fig. 3B). In contrast, betaine in T2D was significantly lower (20.4%) than in healthy subjects, whereas there was no difference in choline concentrations between healthy and T2D subjects (Fig. 3C and D). TMAO concentrations and AUCs for glucose were significantly higher in T2D than in healthy subjects (Fig. 3E). On investigating the association between TMAO and vascular risk factors, T2D patients showed an inverse correlation between TMAO and HDL-C concentrations ($r = -0.265$, $P = 0.019$; Fig. 3F).

Total and branched-chain amino acids

Serum levels of amino acids, mainly BCAAs, have previously been associated with insulin resistance and, recently, it was also suggested that specific bacteria might be involved in the synthesis of these amino acids [9]. Total serum amino acids (except for glutamine) in T2D patients were 1.3-fold higher than in healthy subjects (Fig. 4A), and serum BCAAs leucine, valine and isoleucine were the most increased (by 2.46-fold) compared with healthy subjects (Fig. 4B). In addition, experimental studies have brought evidence that the structure and function of the gut is preserved by glutamine [12], which is also able to regulate intestinal permeability [13]. In the present study, there was wide variability in serum glutamine concentrations in those with T2D, with 32% showing very low concentrations of glutamine at 24–69 μM, values well below normal (205.44 μM), indicating possible deterioration of gut permeability. Interestingly, T2D patients with low glutamine concentrations (< 100 μM) had significantly ($P = 0.001$) higher serum LPS levels than those with glutamine concentrations > 100 μM, indicative of altered gut permeability (Fig. 4C).

On the other hand, our data also showed that T2D patients with an average HbA_{1c} of 7.2 ± 1.2% had significantly higher BCAA serum

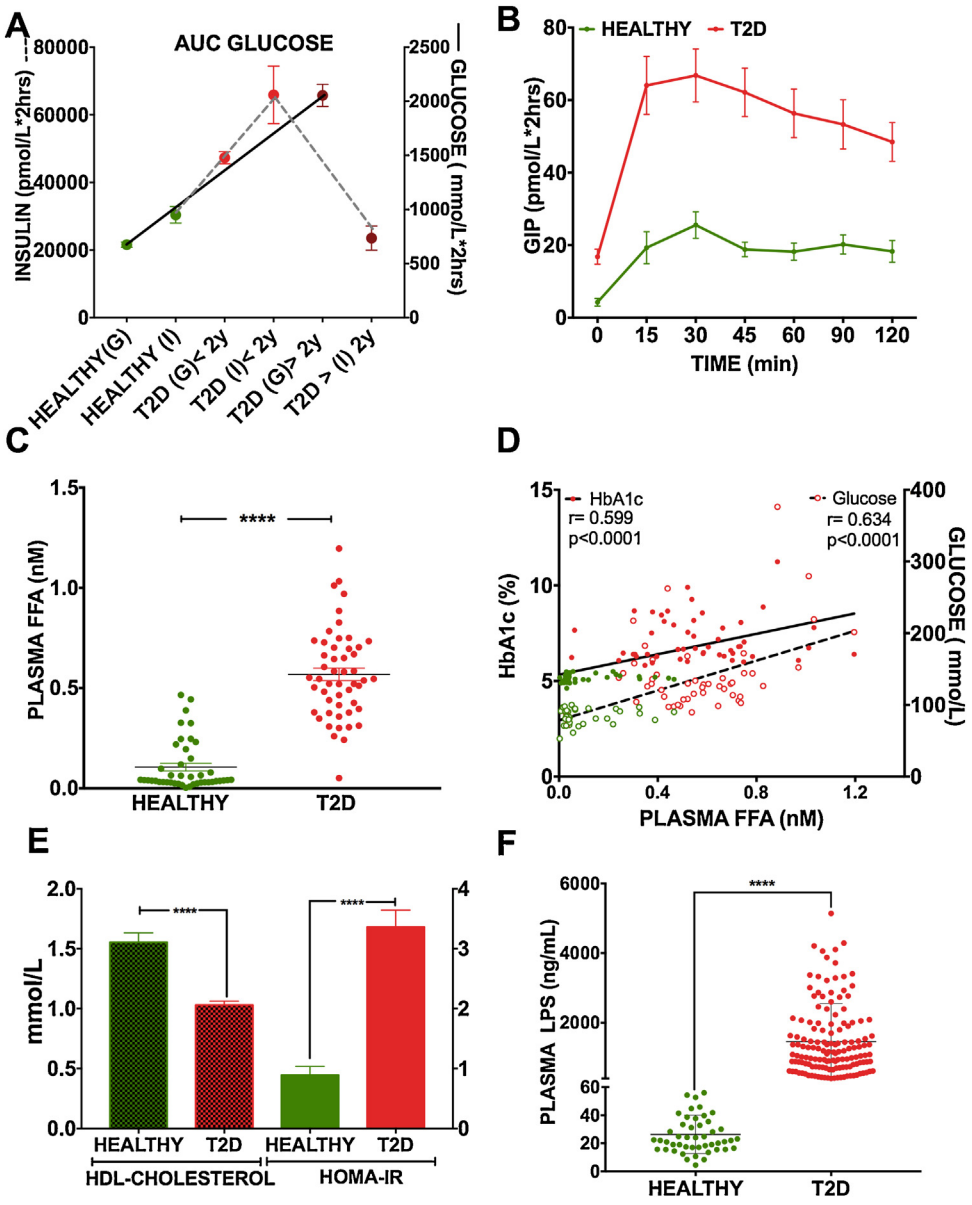

Fig. 1. Biochemical and hormonal parameters in healthy controls and type 2 diabetes (T2D) patients: (A) area under the curve (AUC) for glucose (G) and insulin (I) concentrations (n = 25 subjects in each group); (B) concentrations of serum glucose-dependent insulinotropic polypeptide (GIP) after oral glucose tolerance tests in healthy controls and patients with T2D (n = 34 in each group); (C) plasma free fatty acids (FFA) in healthy and T2D subjects (n = 50 in each group); (D) correlations between serum glucose and plasma FFA, and between HbA_{1c} and plasma FFA in healthy and T2D subjects; (E) high-density lipoprotein (HDL) cholesterol and homoeostasis model assessment for insulin resistance (HOMA-IR) in healthy and T2D subjects; and (F) plasma lipopolysaccharide (LPS) in healthy (n = 50) and T2D subjects (n = 151) assessed for eligibility. Data are means ± SEM. $^{****}P < 0.0001$. 2y: 2 years (since diabetes diagnosis).

concentrations than healthy subjects with an average HbA_{1c} of 5.1 ± 0.23% (Fig. 4D). Indeed, recent studies in rodents have demonstrated that, in particular, *P. copri is* associated with insulin resistance and glucose intolerance, along with augmented circulating levels of BCAAs. In our present T2D patients, it was observed that increasing levels of BCAAs were associated with an increase in *P. copri* abundance (Fig. 4E). Moreover, our results have also shown that the higher the BCAA serum concentration, the higher the serum LPS concentration (Fig. 4F).

Dietary intervention with functional foods

The American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) have devised recommendations for glycaemic control in people with T2D through dietary interventions with healthy foods in accordance to their individual preferences and cultures [14]. The DP intervention was developed using foods rich in soluble fibre, omega-3 fatty acids, prebiotics, and vegetable proteins of good quality and low glycaemic index (GI). This dietary intervention with functional foods has led to better compliance with the dietary changes.

For the dietary intervention stage of our study, 81 T2D patients were randomly assigned to either the DP or P group, although nine subjects from each group discontinued the study, mostly due to the patients' own decision. At the end of the study, 28 and 25 subjects from the DP and P groups, respectively, were analyzed (Fig. S1; see supplementary material associated with this article online); their anthropometric and biochemical variables are presented in Table S2 (see supplementary material associated with this article online). There were no significant differences for such variables of glucose and lipid metabolism or in blood pressure between groups. However, T2D patients following the DP showed significant reductions in specific biochemical parameters

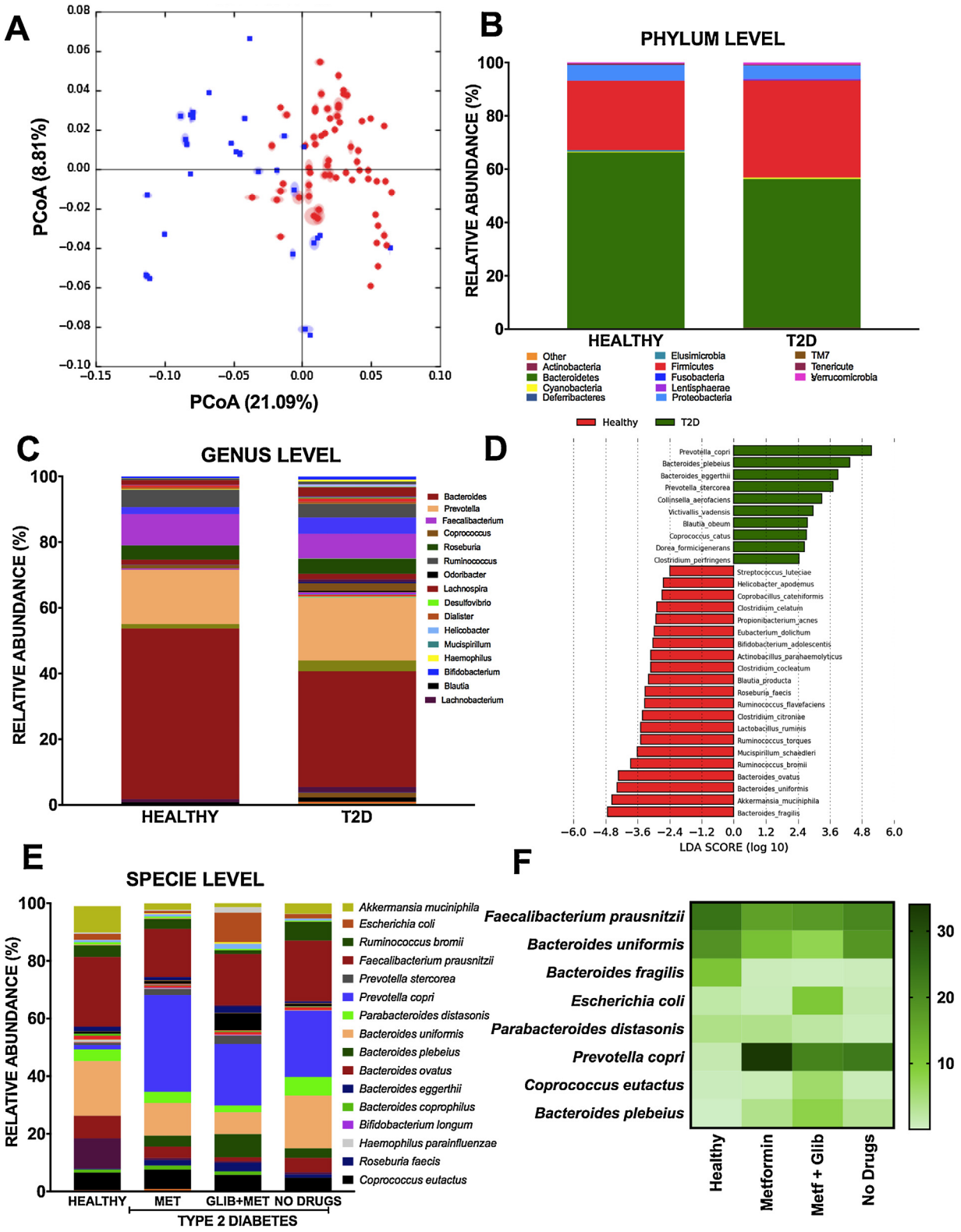

Fig. 2. Type 2 diabetes (T2D) modifies faecal microbiota in Mexican adults: (A) principal coordinates analysis (PCoA) of healthy (n = 22) and T2D (n = 58) subjects based on unweighted UniFrac distance measures (red circles: T2D patients; blue squares: healthy subjects); (B) taxonomic summary of faecal microbiota at phylum level and (C) at genus level; (D) discriminative taxa at species level as determined by linear discriminant analysis (LDA) effect size; (E) taxonomic summary of faecal microbiota at species level by type of medication used to control blood glucose; and (F) heat map of eight bacterial species with the biggest differences in healthy and T2D subjects according to type of antidiabetic medication. MET, Metf: metformin; GLIB: glibenclamide.

compared with the P group: AUCs for glucose, triglycerides, total cholesterol and LDL-C showed reductions of −8.7%, −23%, −7.8% and −9.9%, respectively, compared with basal values (Figs. 5 and 6A). In addition, T2D patients in the DP group had significantly reduced levels of FFAs, HbA_{1c} and C-reactive protein (CRP) by −15.6%, −7.2% and −13%, respectively, vs basal values. In addition, LPS concentrations decreased in both groups (−65% in the DP group, −52% in the P group), indicating that modifications in diet can help to reduce metabolic endotoxaemia

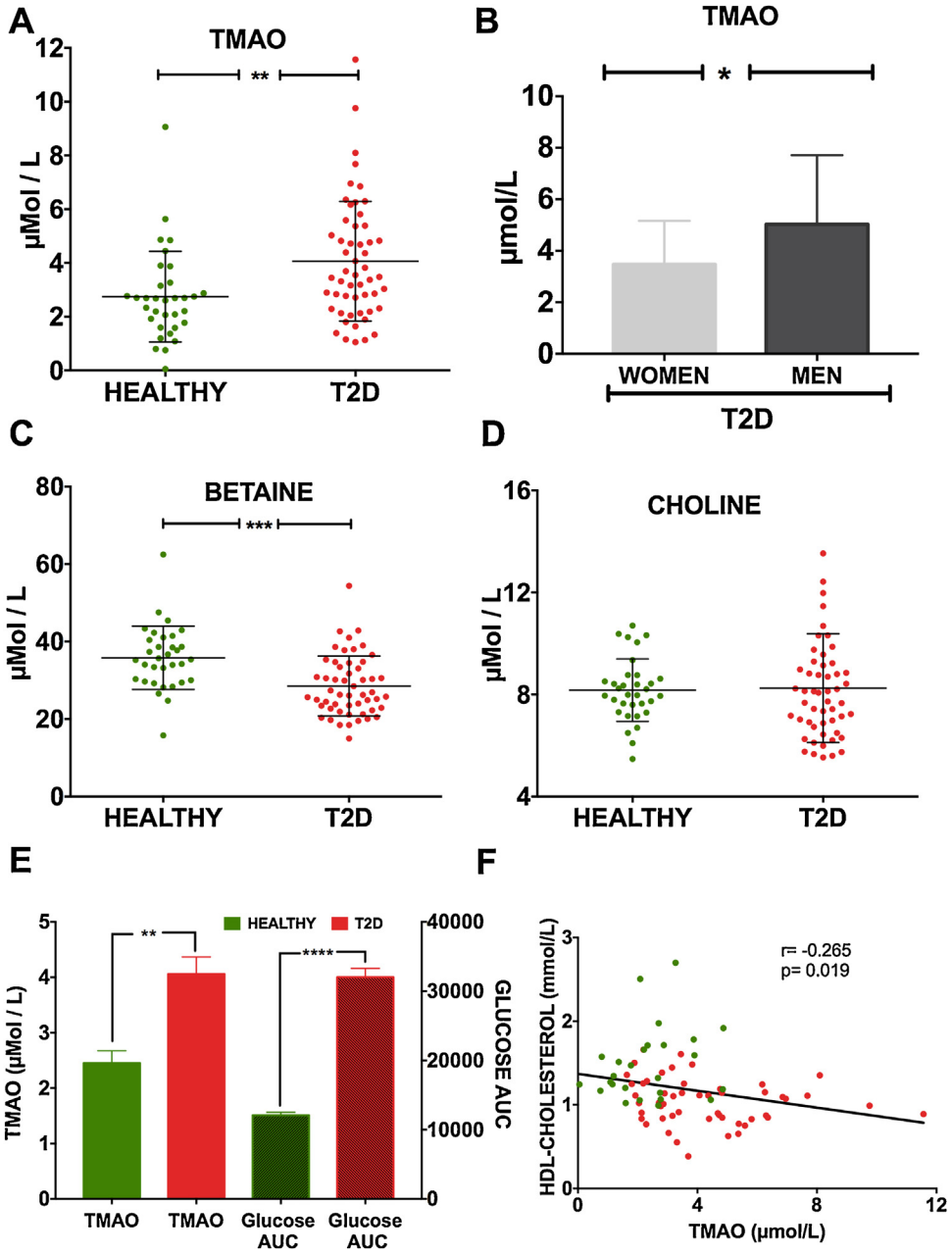

Fig. 3. Plasma trimethylamine N-oxide (TMAO), betaine and choline in 33 healthy and 53 type 2 diabetes (T2D) subjects: (A) TMAO; (B) plasma TMAO concentrations by gender (33 women, 20 men); (C) plasma betaine and (D) choline concentrations; (E) plasma TMAO and glucose area under the curve (AUC); and (F) correlation between high-density lipoprotein (HDL) cholesterol and plasma TMAO. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

mediated by LPS. On the other hand, plasma antioxidant activity was significantly increased only in subjects following the DP (5.6%) compared with their basal levels (Fig. 5B).

Faecal microbiota after dietary intervention

As previously demonstrated, the faecal microbiota in healthy subjects differ significantly from those of patients with T2D. Thus, targeting the intestinal microbiota could offer new possibilities for diabetes treatment. Consumption of the DP modified faecal microbiota in those with T2D at the species level. In fact, DP consumption for 12 weeks increased levels *of F. prausnitzii* and *A. muciniphila* by approximately 34% and 125%, respectively, while reducing levels of *P. copri* by 13%. Remarkably, DP consumption also stimulated the abundance of *Bifidobacterium longum* (Fig. 6A), shown to improve insulin signalling [15]. In addition, there was an increase of *B. fragilis*, which has a strong capacity to use a wide range of dietary polysaccharides [16]. Interestingly, only after the DP dietary intervention was there a significant difference in alpha diversity (Fig. 6B). The species associated with biochemical parameters were *B. plebeius* with TMAO, and *Lactobacillus reuteri* and *Mucispirillum schaedleri* with CRP; however, there were also associations between LPS and HbA_{1c}, HOMA and BCAAs, and tyrosine and phenylalanine (Fig. 6C). Furthermore, there was a cross-sectional association of amino acids with plasma LPS in T2D participants (Fig. 6D).

Discussion

The total number of people living with diabetes worldwide is projected to rise from 171 million in 2000 to 366 million in 2030 [1]. It is estimated that, in Mexico, there are approximately 16.4 million people with T2D, placing Mexico in the top

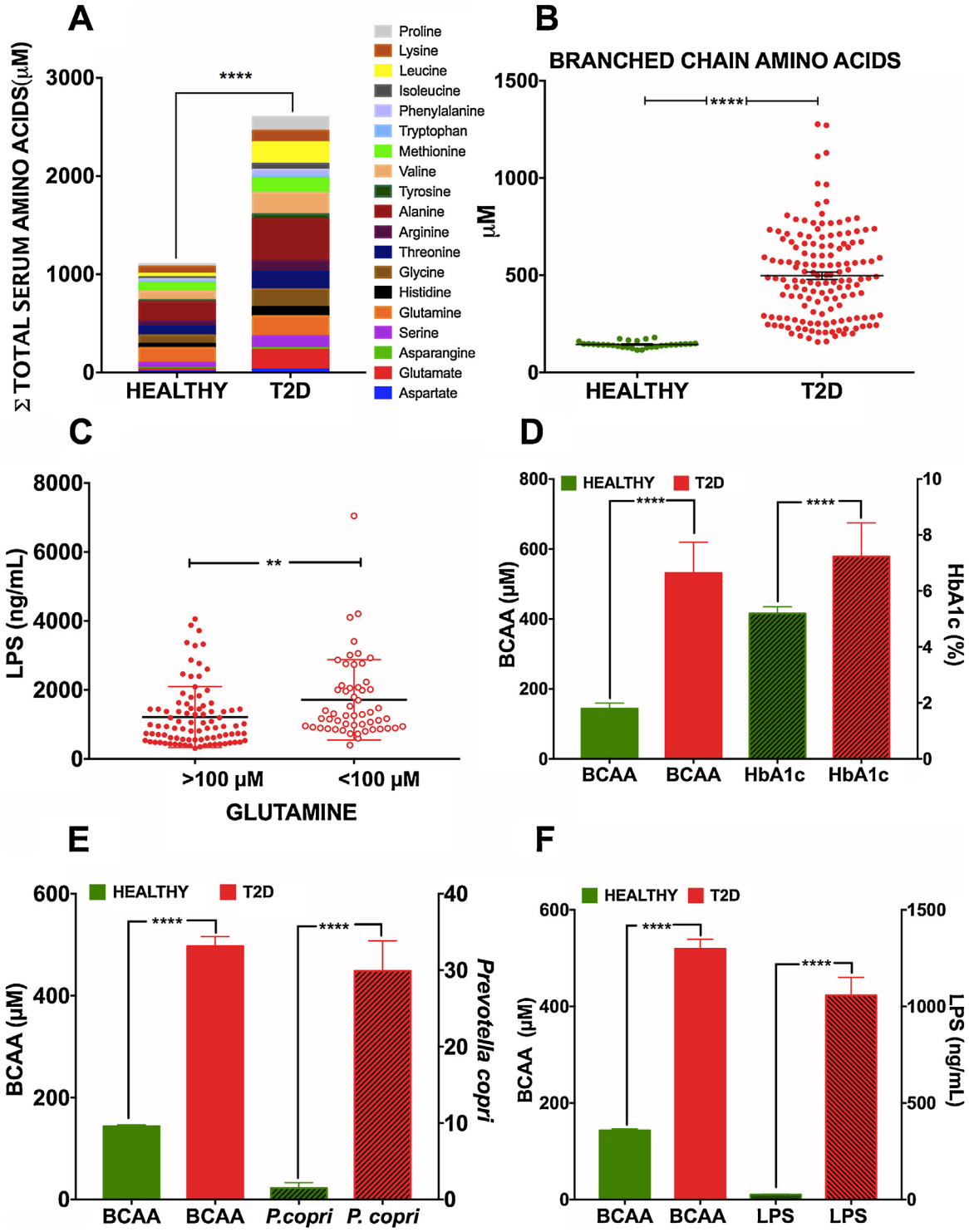

Fig. 4. Plasma amino acids in 51 healthy and 157 type 2 diabetes (T2D) subjects: (A) Σ serum total amino acids; (B) branched-chain amino acids (BCAA); (C) serum lipopolysaccharide (LPS) in T2D patients by glutamine concentration; (D) BCAA concentration and HbA_{1c}; (E) plasma BCAA concentrations and *Prevotella copri* abundance; and (F) serum LPS and plasma BCAA concentration. $^{**}P < 0.01$, $^{****}P < 0.0001$.

10 countries with the highest incidences of T2D [17]. In our study population, those with T2D of 2 years' duration also showed, in addition to hyperglycaemia and low insulin concentrations, elevated concentrations of the incretin GIP, indicating that its insulinotropic effect is almost totally lost in T2D, which is in agreement with a previous report [18]. Elevated GIP levels induce cytokine expression [19], lipolysis [20] and insulin resistance in human adipocytes [21], whereas a significant increase in plasma FFA concentrations was observed as a result of enhanced lipolysis that, in turn, inhibited insulin-stimulated glucose uptake in muscle, thereby leading to insulin resistance [22]. Thus, the higher the levels of circulating FFAs, the higher the serum glucose and HbA_{1c} concentrations.

The susceptibility of the Hispanic population to having low HDL concentrations has already been demonstrated [23]. Our present subjects with T2D showed significantly lower HDL-C concen-

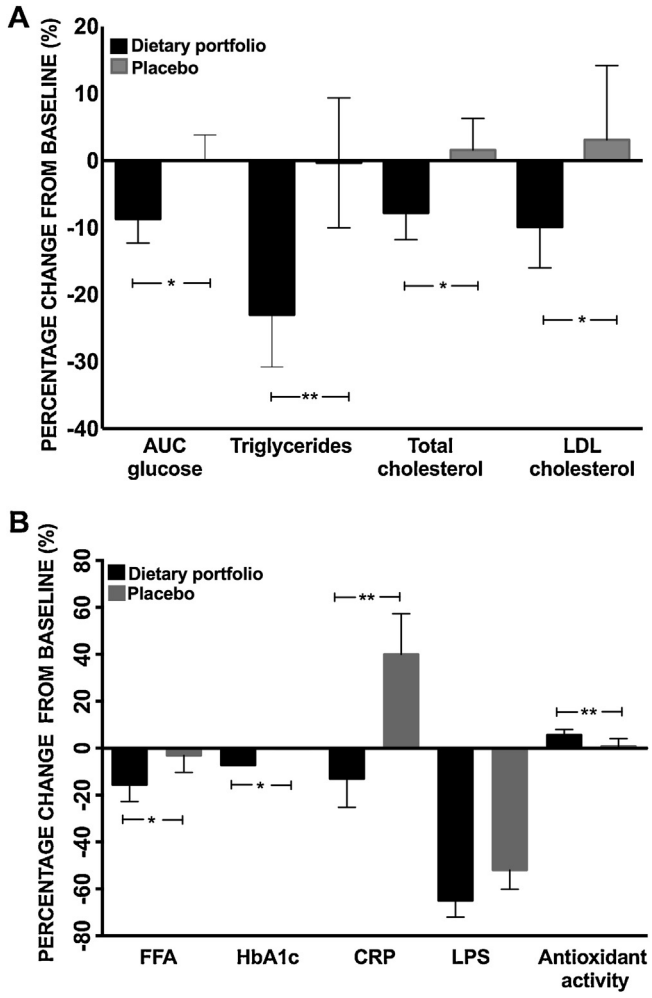

Fig. 5. Change (%) in biochemical parameters in type 2 diabetes patients after a dietary intervention comprising functional foods (n = 28, black) or placebo (n = 25, grey): (A) area under the curve (AUC) for glucose, serum triglycerides, total cholesterol and low-density lipoprotein (LDL) cholesterol; and (B) plasma free fatty acids (FFA), glycosylated haemoglobin (HbA_{1c}), C-reactive protein (CRP), lipopolysaccharide (LPS) and antioxidant activity. Bars are means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. placebo).

trations and higher HOMA-IR index scores than healthy subjects, which is in agreement with evidence supporting an association between low HDL-C levels and T2D [24]. Moreover, it has been determined that insulin resistance is associated with the low-grade chronic systemic inflammation known as metabolic endotoxaemia, mediated by LPS [6]; the latter is a major component of the Gram-negative bacteria that play important roles in the activation of proinflammatory pathways when bound to their toll-like receptor (TLR)-4, leading to an increase in inflammatory cytokines [25]. Indeed, our T2D patients showed a 55-fold increase in serum LPS compared with healthy subjects, which is indicative of marked chronic metabolic endotoxaemia. LPS infiltration also increases the intestinal epithelial tight-junction permeability [26] associated with dysbiosis in gut microbiota.

Several clinical trials have demonstrated changes in both the composition and function of gut microbiota in association with hyperglycaemia and T2D [27]. Our present results have revealed that the gut microbiota in T2D subjects differed from phyla to species levels compared with healthy subjects. The main species increased in T2D were *P. copri*, *B. plebeius* and *B. eggerthii* whereas, in healthy subjects, these were *B. fragilis*, *A. muciniphila* and *B. uniformis*. In fact, *P. copri*, an LPS-producing bacteria, has been linked to a low-grade inflammation that impairs barrier function, and triggers systematic inflammation and synthesis of BCAAs, LPS and insulin resistance in mice [9].

Our present study has demonstrated that patients with T2D have significant increases in plasma BCAAs, HbA_{1c}, *P. copri* and insulin resistance, with a significant association between LPS and plasma BCAAs. It has also been suggested that increased concentrations of BCAAs are associated with the development of insulin resistance due to a reduced breakdown of BCAAs in adipose tissue [28], and that the gut microbiota might be another independent contributing source of elevated serum BCAA levels in T2D. In addition, the amino-acid glutamine, involved in the regulation of intestinal permeability, also showed wide variability in plasma in those with T2D, with around 30% having glutamine concentrations < 100 μM, which were associated with a 41.4% higher LPS concentration than in those with glutamine concentrations > 100 μM, thereby indicating diminished intestinal integrity and expression of multiple tight-junction proteins [29]. Thus, glutamine deprivation exacerbates proinflammatory cytokine production, whereas glutamine supplementation limits the inflammatory response in vitro and protects the intestinal mucosa in different models of intestinal injury [13].

It is important to point out that the use of medications to treat T2D significantly modified gut microbiota in such patients compared with healthy subjects before the dietary intervention. Indeed, the use of metformin alone or in combination with glibenclamide reduced *F. prausnitzii*, a Gram-positive species associated with anti-inflammatory properties [31] and thought to be a sensor of intestinal health. While use of metformin plus glibenclamide increased *E. coli, Coprococcus eutactus* and *B. plebeius*, the combination also reduced *B. uniformis* and *A. muciniphila*. There is even evidence that metformin increases the relative abundance of *Escherichia* species [11], thereby promoting tolerance to diverse diseases [30].

Multiple studies have established that gut microbiota significantly contribute to a variety of cardiometabolic diseases [32], and that T2D is associated with altered gut microbial choline and carnitine metabolism [33]. Choline is metabolized by gut microbiota to produce TMA, which is oxidized by hepatic flavin monooxygenases to form TMAO, which is proatherogenic and associated with increased cardiovascular risk [34]. Our present study revealed a 48.2% increase in TMAO in T2D patients, associated with an increased AUC for glucose and low levels of plasma HDL-C. It has also been demonstrated that omnivorous subjects produce significantly more TMAO than vegetarians through a microbiota-dependent mechanism [34], while the polyphenols derived from dietary plant intakes have protective effects on vascular endothelial cells possibly via antioxidants that prevent LDL oxidation [35]. In fact, a significant decrease in LDL-C concentration was observed only in T2D patients consuming our combination of plant-based functional foods.

Modulation of the host microbiota through long-term adherence to a high-fibre plant-based diet has been suggested as a potential treatment for T2D, glycaemic control and restoration of microbiota function [36]. Dietary supplementation with complex carbohydrates modifies the composition of gut microbiota and may have a major potential to prevent and treat cardiometabolic disease [37]. Inulin, chia seeds and nopal contain complex carbohydrates that are non-digestible by the host, but able to modulate gut microbiota [38,39]. In addition, nopal has been reported to protect against metabolic endotoxaemia [40], and to reduce postprandial peaks of glucose in patients with T2D [41]. After dietary treatment with a combination of functional foods, significant decreases were observed in the AUCs for glucose, triglycerides, total cholesterol, LDL-C, FFAs, HbA_{1c} and CRP, with an

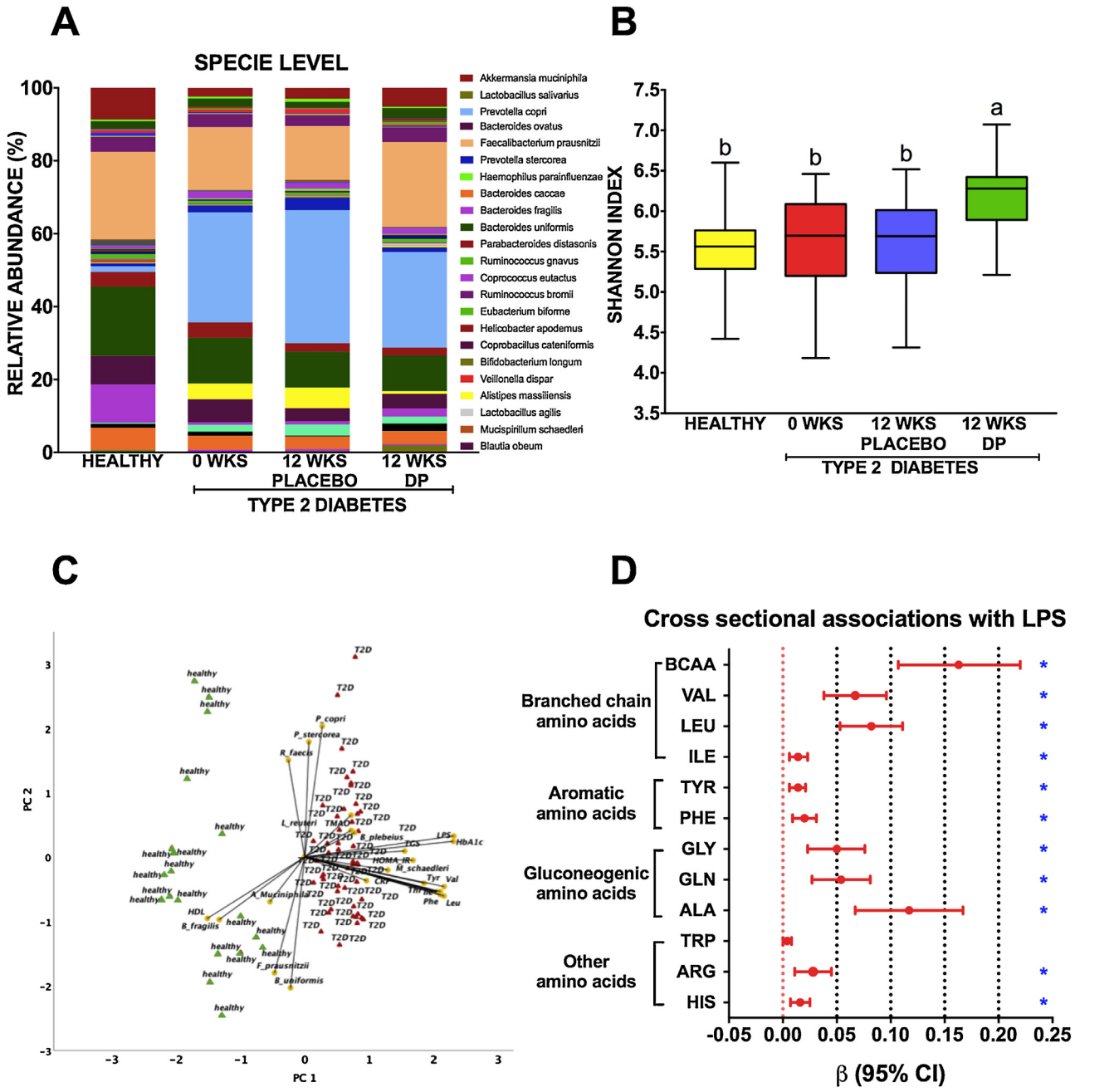

Fig. 6. (A) Faecal microbiota in type 2 diabetes (T2D) before and after 12 weeks (WKS) of placebo or a dietary portfolio (DP) comprising functional foods; (B) Shannon index of alpha diversity with placebo or DP in T2D; (C) overall covariation of taxonomic profiles and biochemical parameters in T2D and healthy subjects (PC: principal components); and (D) cross-sectional associations of amino acids with lipopolysaccharide (LPS) from linear regression models. Error bars are 95% confidence intervals; $^{*}P < 0.05$. BCAA: branched-chain amino acids; VAL: valine; LEU: leucine; ILE: isoleucine; TYR: tyrosine; PHE: phenylalanine; GLY: glycine; GLN: glutamine; ALA: alanine; TRP: tryptophan; ARG: arginine; HIS: histidine.

increase in serum antioxidant activity, compared with the P group. Notably, these changes were accompanied by an increase in alpha diversity and, in particular, a decrease in levels of *P. copri*, with increases in *F. prausnitzii* and *A. muciniphila* independently of the antidiabetic medications used by T2D patients.

Thus, the results of our present work demonstrate that a combination of different functional foods can modify the faecal microbiota that, in turn, improve carbohydrate and lipid metabolism while reducing metabolic endotoxaemia. Nevertheless, it is still necessary to study longer-term dietary interventions that reverse dysbiosis in faecal microbiota while improving other metabolites related to insulin resistance and cardiovascular risk.

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Authors' contributions

N.T. was responsible for the conception and design of the study. I.M.-V., M.G. and A.F. conducted the study and participated in data collection. I.M.-V. was responsible for statistical considerations in the analysis. M.S. performed the microbiota and bioinformatics analysis. A.A. and O.G. performed the amino-acid analysis. N.T., I.M.-V., A.T.,

L.N. and M.L.F. participated in critically reviewing and interpreting the data for the manuscript. All authors had full access to all of the study data and share the final responsibility for the decision to submit this report for publication. N.T. is the guarantor of this work and, as such, had full access to all of the study data, and takes responsibility for the integrity of the data and accuracy of the data analysis.

Disclosure of interest

The authors declare that they have no competing interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found, in the online version, at https://doi.org/10.1016/j.diabet.2018.09.004.

References

[1] Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004;27:1047–53.
[2] Mahendran Y, Cederberg H, Vangipurapu J, Kangas AJ, Soininen P, Kuusisto J, et al. Glycerol and fatty acids in serum predict the development of hyperglycemia and type 2 diabetes in Finnish men. Diabetes Care 2013;36:3732–8.
[3] Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? Diabetes 2003;52:1–8.
[4] Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J 2017;474:1823–36.
[5] Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut Microbes 2012;3:289–306.
[6] Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761–72.
[7] Allin KH, Nielsen T, Pedersen O. Mechanisms in endocrinology: Gut microbiota in patients with type 2 diabetes mellitus. Eur J Endocrinol 2015;172:R167–77.
[8] Tang WH, Wang Z, Li XS, Fan Y, Li DS, Wu Y, et al. Increased trimethylamine n-oxide portends high mortality risk independent of glycemic control in patients with type 2 diabetes mellitus. Clin Chem 2017;63:297–306.
[9] Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BA, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. Nature 2016;535(7612):376–81.
[10] Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults – The evidence report. National Institutes of Health. Obes Res 1998;6(Suppl. 2):51S–209S.
[11] Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature 2015;528:262–6.
[12] van der Hulst RR, van Kreel BK, von Meyenfeldt MF, Brummer RJ, Arends JW, Deutz NE, et al. Glutamine and the preservation of gut integrity. Lancet 1993;341:1363–5.
[13] Achamrah N, Dechelotte P, Coeffier M. Glutamine and the regulation of intestinal permeability: from bench to bedside. Curr Opin Clin Nutr Metab Care 2017;20:86–91.
[14] Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M, et al. Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). Diabetes Care 2012;35:1364–79.
[15] Gomes AC, Bueno AA, de Souza RG, Mota JF. Gut microbiota, probiotics and diabetes. Nutr J 2014;13:60.
[16] Wexler HM. Bacteroides: the good, the bad, and the nitty-gritty. Clin Microbiol Rev 2007;20:593–621.
[17] Whiting DR, Guariguata L, Weil C, Shaw J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract 2011;(94):311–21.
[18] Vilsboll T, Krarup T, Madsbad S, Holst JJ. Defective amplification of the late phase insulin response to glucose by GIP in obese type II diabetic patients. Diabetologia 2002;45:1111–9.
[19] Goralska J, Razny U, Polus A, Stancel-Mozwillo J, Chojnacka M, Gruca A, et al. Pro-inflammatory gene expression profile in obese adults with high plasma GIP levels. Int J Obes (Lond) 2018;42:826–34.
[20] Getty-Kaushik L, Song DH, Boylan MO, Corkey BE, Wolfe MM. Glucose-dependent insulinotropic polypeptide modulates adipocyte lipolysis and reesterification. Obesity (Silver Spring) 2006;14:1124–31.
[21] Timper K, Grisouard J, Sauter NS, Herzog-Radimerski T, Dembinski K, Peterli R, et al. Glucose-dependent insulinotropic polypeptide induces cytokine expression, lipolysis, and insulin resistance in human adipocytes. Am J Physiol Endocrinol Metab 2013;304:E1–13.
[22] Boden G, Shulman GI. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. Eur J Clin Invest 2002;323:14–23.
[23] Guevara-Cruz M, Tovar AR, Larrieta E, Canizales-Quinteros S, Torres N. Increase in HDL-C concentration by a dietary portfolio with soy protein and soluble fiber is associated with the presence of the ABCA1R230C variant in hyperlipidemic Mexican subjects. Mol Genet Metab 2010;101:268–72.
[24] Wilson PW, Meigs JB, Sullivan L, Fox CS, Nathan DM, D'Agostino Sr . Prediction of incident diabetes mellitus in middle-aged adults: the Framingham Offspring Study. Arch Intern Med 2007;167:1068–74.
[25] Boutagy NE, McMillan RP, Frisard MI, Hulver MW. Metabolic endotoxemia with obesity: Is it real and is it relevant? Biochimie 2016;124:11–20.
[26] Nighot M, Al-Sadi R, Guo S, Rawat M, Nighot P, Watterson MD, et al. Lipopolysaccharide-induced increase in intestinal epithelial tight permeability is mediated by toll-like receptor 4/myeloid differentiation primary response 88 (MyD88) activation of myosin light chain kinase expression. Am J Pathol 2017;187:2698–710.
[27] Delzenne NM, Cani PD, Everard A, Neyrinck AM, Bindels LB. Gut microorganisms as promising targets for the management of type 2 diabetes. Diabetologia 2015;58:2206–17.
[28] Serralde-Zuniga AE, Guevara-Cruz M, Tovar AR, Herrera-Hernandez MF, Noriega LG, Granados O, et al. Omental adipose tissue gene expression, gene variants, branched-chain amino acids, and their relationship with metabolic syndrome and insulin resistance in humans. Genes Nutr 2014;9:431.
[29] Li N, Neu J. Glutamine deprivation alters intestinal tight junctions via a PI3-K/Akt mediated pathway in Caco-2 cells. J Nutr 2009;139:710–4.
[30] Cabreiro F. Metformin joins forces with microbes. Cell Host Microbe 2016;19:1–3.
[31] Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 2012;490:55–60.
[32] Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57–63.
[33] Shan Z, Sun T, Huang H, Chen S, Chen L, Luo C, et al. Association between microbiota-dependent metabolite trimethylamine-N-oxide and type 2 diabetes. Am J Clin Nutr 2017;106:888–94.
[34] Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19:576–85.
[35] Tuso P, Stoll SR, Li WW. A plant-based diet, atherogenesis, and coronary artery disease prevention. Perm J 2015;19:62–7.
[36] Jardine M. Nutrition considerations for microbiota health in diabetes.. Diabetes Spectr 2016;29:238–44.
[37] Vinke PC, El Aidy S, van Dijk G. The role of supplemental complex dietary carbohydrates and gut microbiota in promoting cardiometabolic and immunological health in obesity: lessons from healthy non-obese individuals. Front Nutr 2017;4:34.
[38] Ramnani P, Costabile A, Bustillo AG, Gibson GR. A randomised, double-blind, cross-over study investigating the prebiotic effect of agave fructans in healthy human subjects. J Nutr Sci 2015;4:e10.
[39] Lopez-Palacios C, Pena-Valdivia CB, Rodriguez-Hernandez AI, Reyes-Aguero JA. Rheological flow behavior of structural polysaccharides from edible tender cladodes of wild, semidomesticated and cultivated 'Nopal' (Opuntia) of Mexican highlands. Plant Foods Hum Nutr 2016;71:388–95.
[40] Sanchez-Tapia M, Aguilar-Lopez M, Perez-Cruz C, Pichardo-Ontiveros E, Wang M, Donovan SM, et al. Nopal (Opuntia ficus indica) protects from metabolic endotoxemia by modifying gut microbiota in obese rats fed high fat/sucrose diet. Sci Rep 2017;7:4716.
[41] Lopez-Romero P, Pichardo-Ontiveros E, Avila-Nava A, Vazquez-Manjarrez N, Tovar AR, Pedraza-Chaverri J, et al. The effect of nopal (Opuntia ficus indica) on postprandial blood glucose, incretins, and antioxidant activity in Mexican patients with type 2 diabetes after consumption of two different composition breakfasts. J Acad Nutr Diet 2014;114:1811–8.