Host-microbiome interactionsin human type 2 diabetes following prebiotic fibre (galactooligosaccharide) intake

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Abstract

Aberrant microbiota composition and function have been linked to several pathologies, including type 2 diabetes. In animal models, prebiotics induce favourable changes in the intestinal microbiota, intestinal permeability (IP) and endotoxaemia which are linked to concurrent improvement in glucose tolerance. This is the first study to investigate the link between intestinal permeability, glucose tolerance, and intestinal bacteria in human type 2 diabetes. Twenty-nine males with well-controlled type 2 diabetes were randomised to a prebiotic (galactooligosaccharide mixture) or placebo (maltodextrin) supplement (5.5g/day for 12 weeks). Intestinal microbial community structure, IP, endotoxaemia, inflammatory markers and glucose tolerance were assessed at baseline and post-intervention. IP was estimated by the urinary recovery of oral ^{51}Cr-EDTA and glucose tolerance by insulin modified IVGTT. Intestinal microbial community analysis was performed by high-throughput Next-Generation Sequencing of 16S rRNA amplicons and quantitative PCR. Prebiotic fibre supplementation had no significant effects on clinical outcomes or bacterial abundances compared with placebo; however, changes in the bacterial family *Veillonellaceae* correlated inversely with changes in glucose response and IL-6 levels (r = -0.90, P = 0.042 for both) following prebiotic intake. The absence of significant changes to the microbial community structure at a prebiotic dosage/length of supplementation shown to be effective in healthy individuals is an important finding, We propose that concurrent metformin treatment and the high heterogeneity of human type 2 diabetes may have played a significant role. It is also plausible that prebiotics may play a more important role in prevention rather than in the treatment of human type 2 diabetes.

INTRODUCTION

Evidence from animal studies supports a causal link between low grade inflammation, insulin resistance and impaired intestinal barrier function[1,2]; however, we recently demonstrated for the first time that intestinal permeability (IP) is compromised in type 2 diabetes (T2D) patients compared with healthy age and BMI matched volunteers[3]. Increased small IP as measured by urinary excretion of orally administered ^{51}Cr EDTA was significantly and positively correlated with the inflammatory marker tumour necrosis factor alpha (TNF-α). This may indicate that the chronic systemic low-grade inflammation characterising metabolic diseases such as T2D is associated with a leaky gut in humans.

It is hypothesised that the impaired intestinal barrier leads to an increased translocation of the gram-negative bacteria cell membrane component lipopolysaccharide (LPS) (as well as whole bacteria and other luminal antigens) into the circulation which results in metabolic endotoxaemia. LPS is a ligand of the toll-like receptor 4 (TLR-4). Activation of TLR-4 signalling by LPS results in a low-grade inflammation which affects insulin signalling and thus induces insulin resistance[1]. Interestingly circulating LPS is indeed elevated in T2D compared to healthy controls[4,5]. However, whether this is due to increased paracellular movement or due to fat-induced LPS absorption through increased chylomicron formation is unclear[6].

Intestinal dysbiosis in T2D has been observed in a number of cross-sectional studies[7–12]. Larsen *et al.*[7] found that *Betaproteobacteria* and the *Bacteroidetes* to *Firmicutes* ratio correlated positively with plasma glucose concentrations. Thus, as a potential therapeutic target, altering intestinal bacterial community structure and thereby reducing LPS load and uptake may be beneficial in T2D. An approach to changing the intestinal bacterial composition by diet is with the use of prebiotics and probiotics. Studies in rodents suggest that prebiotics, probiotics and synbiotics may improve intestinal barrier function and glucose control[2,13–15]. However, few studies have investigated the use of prebiotic supplementation in human T2D[16–22] and none in the terms of the potential mechanistic effects on the intestinal barrier. This is the first study to investigate the effects of prebiotic supplementation on intestinal bacteria, IP, endotoxaemia, and glucose tolerance concurrently in T2D patients.

MATERIALS AND METHODS

This was a randomised double-blind, placebo controlled parallel study comparing effects of prebiotic supplementation to placebo treatment for 12 weeks on glucose control, IP, intestinal bacterial composition, endotoxaemia and inflammatory markers in patients with T2D. The protocol was approved by the Central London NRES Committee (REC reference no. 11/LO/1141) and the University of Surrey Ethics Committee and was conducted according to the declaration of Helsinki. The trial was registered at the UKCRN portfolio database under trial identifier ISRCTN07813749.

Subjects

Males with well-controlled T2D aged 42-65 years were recruited through local GP practices and advertisement in a local newspaper. Due to repeated administration of the radioactive compound ^{51}Cr-EDTA and the potential influence of the menstrual cycle on outcomes, women were excluded from the study. All patients provided written informed consent. Exclusion criteria included use of antibiotics in the previous three months, use of anti-inflammatory medications (except a low dose (75mg/day) aspirin), diuretics, proton-pump inhibitors, inflammatory bowel disease, Crohn's disease, coeliac disease and irritable bowel syndrome. Patients were asked to exclude probiotic products and prebiotic supplements (other than the study supplement) from their diet for two weeks prior to the first study visit and throughout the study. Furthermore, they were asked not to change their lifestyle during the study. The sample size for this study was based on the primary outcome measure of changes to IP and based on our own published pilot data using this method in patients with well controlled T2D[3]. 30 Patients in this parallel design study provided 80% power to detect a treatment difference between groups of 1.6% in total permeability, using the calculated SD in this cohort of 1.57 (alpha 0.05).

Study protocol

Following the screening procedure patients were randomised to either prebiotic fibre (galactooligosaccharide mixture, GOS mixture [Bi^{2}muno]) or placebo (maltodextrin) supplementation for 12 weeks according to a randomisation scheme generated at randomization.com. Both supplements were supplied by Clasado Ltd (Milton Keynes, UK) as dry white powders in sachets each containing 5.5g and were readily mixed into beverages or food. The GOS mixture has been used in previous trials and is described by Vulevic *et al.*[23]. A dose of 5.5g GOS mixture has previously been demonstrated to have an bifidogenic effect in healthy individuals of this age and BMI, and be well tolerated in terms of gastrointestinal effects[23,24]. Patients were contacted twice during the 12 weeks supplementation to monitor side-effects and compliance. Patients returned unused sachets following the supplementation to verify compliance. Dietary intake data (7-day diet diary), clinical data and faecal samples were collected at baseline and at the end of

the intervention. The diet diaries were analysed in DietPlan6 (Forestfield Software Ltd, Horsham, UK). Faecal samples were collected into sterile universal polystyrene containers and were kept refrigerated. Faecal samples were stored at -20°C initially and at -80°C freezer for long term storage.

The coprimary outcomes of the study were changes in intestinal permeability, endotoxaemia and glucose tolerance. Secondary outcomes were changes in intestinal bacterial composition, inflammatory markers, lipids, blood pressure and anthropometric measurements. Use of metformin was considered a confounding factor. However, as 13 out of 14 patients in the prebiotic group were metformin treated, it was not possible to perform a subgroup analysis to explore a potential interaction between metformin and prebiotic treatment.

Intestinal permeability

IP was measured by 24h urinary excretion of orally administered ^{51}Cr-EDTA as previously described[3]. We utilized ^{51}Cr-EDTA as a probe as it is stable in the colonic luminal environment allowing assessment of colonic permeability and it is easily detected in the urine [25].

Anthropometric and blood pressure measurements

Having fasted overnight, patients attended the CEDAR centre of the Royal Surrey County Hospital. Body weight and body composition was measured by bioimpedance (Tanita, Arlington Heights, IL, USA). Waist circumference was measured at the level of the navel with a tape measure. Blood pressure was measured on the non-dominant arm after 5 minutes rest in a semi-upright position and the mean of three readings was calculated (Omron MX3 Plus, Omron Healthcare Europe, Milton Keynes, UK).

Glucose tolerance, inflammatory markers and lipids

Glucose tolerance was assessed using a frequently sampled insulin modified IV glucose tolerance test (IVGTT) as previously described[26]. Blood was collected into EDTA tubes for glucose, insulin and C-peptide and HbA1c measurements and into serum tubes containing clotting activator or pyrogen free tubes for measurements of inflammatory markers, lipids and LPS in serum. Aprotinin was added to blood samples (200 kallikrein inhibiting units/ml blood) collected for C-peptide measurement. Blood samples were centrifuged at 3000 x g at 4°C for 10 minutes and serum and plasma were stored at -20°C or -80°C.

Biochemical analyses

Whole blood glucose concentrations were measured on an YSI 2300 STAT Plus™ (YSI Life Sciences, Fleet, UK) with an average intra-assay CV of 4.8% and inter-assay CV of 5.8%. Plasma

insulin and C-peptide were analysed in duplicate using radioimmunoassays (Millipore, Billerica, MA) with average intra-assay CVs of 7.7% and 4.2% and inter-assay CVs of 12.6% and 6.4%, respectively. HbA1c and serum hsCRP were measured by the Surrey Pathology Partnership, an accredited laboratory, and serum IL-6 and TNF-α were measured using a Luminex platform and Biorad bio-plex kits and software. Serum triglycerides (TAGs), total cholesterol, HDL cholesterol, and non-esterified fatty acids (NEFA) were measured on an ILab650 using commercially available kits (Randox Laboratories, UK, and Instrumentation Laboratory, UK). All intra-assay CVs were <2% and inter-assay CVs ≤ 3% for lipids measurements. LDL cholesterol concentration was calculated using the Friedewald formula(27). LPS was measured in duplicate using Endosafe-MCS (Charles River Laboratories, Lyon, France) as previously described[15]. Serum LPS binding protein (LBP) and sCD14 concentrations were measured using commercially available kits according to the manufacturer's instructions (Hycult Biotechnology, Uden, the Netherlands). The average intra-assay CVs were 3.9% and 8.5% for LBP and sCD14, respectively.

Amplification and High-Throughput Sequencing

Amplification and sequencing were performed as previously described by Ellis et al.[28]. Further details are provided in the supplementary information.

Bioinformatics

The sequences were processed in Qiime[29] using the AmpliconNoise[30] pipeline that utilises flowgram information of the sequences to correct for errors. The samples were demultiplexed by exact matching of both barcode and primer and the sequences were filtered and trimmed based on the identification of low quality signals[31]. The filtered flowgrams were clustered to remove platform-specific errors and converted into sequences using the PyroNoise algorithm. The sequences had barcodes and degenerate primers removed prior to trimming at 400 base pairs (bp). They were then further clustered by SeqNoise to remove PCR single base errors. In the final step, the Perseus algorithm was used to identify chimeras.
The denoised sequences were classified using the standalone RDP classifier[32]. From this, taxa frequencies at five different levels: Phylum, Class, Order, Family and Genus; were calculated. Additionally, a non-supervised approach was used, operational taxonomic units (OTUs) were generated at 3% divergence following pair-wise global sequence alignment and hierarchical clustering with an average linkage algorithm. After generating the abundance tables, multivariate statistical analyses in the context of metadata were done in R utilising Vegan package (http://cran.r-project.org/web/packages/vegan/) for obtaining alpha- and beta-diversity estimates as well as permutation ANOVA using distance measures (adonis function). For calculating alpha-diversity measures, the samples were rarefied to the minimum sample size, where as for other statistics, we

log-normalised the abundance tables. Where appropriate, P-Values were adjusted using the Benjamini-Hochberg method to control the false discovery rate (FDR).

Quantification of bacterial groups by quantitative PCR

Total bacteria, *Bifidobacterium*, *Roseburia*, *Lactobacillus*, Enterobacteriaceae, *Clostridium leptum*, and *Clostridium coccoides* groups were quantified using quantitative real-time PCR (qPCR). The qPCR methods are described in the Supplementary Information.

Statistical analysis

Clinical outcomes and diet data are presented as mean ± SEM or median (interquartile range) as appropriate. Baseline values between groups was compared using an unpaired t-test or Mann-Whitney test and within group changes with a paired t-test or Wilcoxon matched pairs signed rank test as appropriate. Treatment effects were assessed by comparing differences in changes from baseline between groups using ANCOVA with baseline values as covariates or the Mann-Whitney test if log transformation did not normalise data distribution. Area under the curve (AUC) for glucose, insulin and C-peptide was calculated using the trapezoid rule. Glucose and insulin data were modelled using Bergman's minimal model (MINMOD Millennium version) as previously described(26). HOMA %S (insulin sensitivity), %B (β-cell function) and IR (insulin resistance) were calculated using the HOMA2 Calculator (http://www.dtu.ox.ac.uk/). Associations between changes in gut bacteria abundance, diet and clinical outcomes were assessed by Kendall's rank correlations. Analysis of qPCR data were performed on $\log_{10}$ transformed values. The level of significance was set at $P < 0.05$. Data were analysed in GraphPad Prism 6, SPSS versions 21 and 22 and R.

RESULTS

Figure 1 shows the flowchart for the study. Of the thirty-two patients recruited two patients withdrew from the study due to gastrointestinal upset (n = 1) and antibiotic treatment (n = 1). Another participant in the prebiotic group was excluded from the data analysis due to antibiotic treatment. Characteristics of the 29 patients who were included in the final data analyses are shown in Table 1. All patients had been on a stable treatment for at least three months prior to taking part in the study and had no changes to their medications during the study. Two patients in the placebo group did not undergo a full post-supplementation IVGTT due to venous access problems; however, a fasting blood sample was obtained from one of the patients and data from the initial 20 min of the IVGTT for the second patient were included in the data analysis.

Compliance, assessed by the number of unused sachets of supplement, was 96% (range: 84-100%) for both treatments. No adverse side effects were reported by the participants. There were no

significant differences between groups in clinical outcomes at baseline; however, Enterobacteriaceae were higher (P = 0.0379) (Supplementary Figure S2e) and Peptostreptococcaceae levels lower (P = 0.0019) in the prebiotic group at baseline.

Anthropometrics and blood pressure

Supplementation with the prebiotic fibre had no significant effects on body weight, BMI, body fat percentage, waist circumference or blood pressure when compared with placebo (Table 1).

Intestinal permeability

Prebiotic supplementation had no significant effect on IP as measured by urinary recovery of ^{51}Cr EDTA when compared with placebo (Figure 2).

Glucose tolerance

Prebiotic treatment had no significant effect on glucose, insulin and C-peptide fasting concentrations or responses during IVGTT compared with placebo (Table 2). The change in glucose effectiveness at zero insulin (GEZI) in the placebo group was significantly different from the prebiotic group.

Inflammatory markers and lipids

There were no significant effects of prebiotic treatment on inflammatory markers, LPS, or lipids, although the prebiotic tended to reduce total and LDL cholesterol (Supplementary Table S1).

Dietary assessment

At baseline the energy intake in the prebiotic group was 8929 ± 538 kJ/day with percentage of energy obtained from carbohydrate, sugar, fat, saturated fat and protein 42.1 ± 2.5%, 14.5 ± 1.7%, 36.6 ± 1.5%, 12.5 ± 0.8%, and 15.7 ± 0.9%, respectively. In the placebo group the mean daily energy intake was 8683 ± 581 kJ and carbohydrate, sugar, fat, saturated fat and protein provided 40.0 ± 1.5%, 14.3 ± 1.0%, 37.7 ± 1.5%, 12.1 ± 0.4% and 16.8 ± 0.8% of total energy, respectively. The percentage dietary energy from protein increased by 1.1% in the placebo group and this was significantly different from that observed in the prebiotic group (Supplementary Table S2). No other significant differences in dietary intakes were observed between groups.

Gut microbiota composition

Prebiotic fibre treatment did not induce significant changes in diversity, evenness (the relative abundance of species) and richness (the number of species per sample) indices when compared with placebo. However, bacterial diversity as assessed by the Shannon and inverse Simpson indices and richness increased significantly within the prebiotic group (Supplementary Table S3).

Faecal bacterial DNA extraction was unsuccessful (DNA concentration <50 ng/μL) for some samples resulting in $n = 11$ in the prebiotic group and $n = 12$ in the placebo group for the qPCR data set. After removing samples with <400bp the metagenomics data set consisted of $n = 7$ in the prebiotic group and $n = 9$ in the placebo group.

Consistent with previous reports on composition of the gut microbiota in humans, *Bacteroidetes* and *Firmicutes* were the two dominant phyla followed by *Proteobacteria*, unclassified bacteria and *Actinobacteria* (data not shown). Bacterial community structure in the treatment groups changed only slightly during the study, but the change was greater in the prebiotic group as can be observed in the NMDS plot (Supplementary Figure S1A). The change in the placebo group was mainly due to changes in metformin-treated patients (Supplementary Figure S1B). However, comparison of bacteria abundances at all taxonomic levels did not reveal any significant effect of treatment when adjusted for multiple testing (data not shown). Nonetheless, permutation ANOVA showed a trend towards an effect of treatment ($P = 0.099$) at the OTU level. When metformin was included as a cofactor, metformin had a significant effect on bacterial community structure at the genus level ($R^2 = 0.084$, $P = 0.009$) whereas only a trend was detected when the analysis were performed on OTUs ($R^2 = 0.039$, $P = 0.078$).

Quantification of bacterial groups by qPCR

Prebiotic treatment had no significant effect on *Bifidobacterium* or any of the other bacteria measured (Supplementary Figure S2). *Bifidobacterium* levels increased in both groups; however, the change within the prebiotic group was greater and close to significance ($P = 0.0582$).

Correlations between changes in bacteria, clinical outcomes and dietary intakes

As an *a priori* aim was to investigate the role of prebiotic fibre intake specifically for hypothesis generation, correlations were calculated for each treatment group separately. The correlations differed between the two groups as can be observed from the different patterns in the heat maps (Supplementary Figures S3A-E). Changes in large bowel permeability (^{51}Cr EDTA 6-24h excretion) were positively correlated with bacterial changes at all taxonomic levels in the prebiotic group. The strongest correlations were for *Verrucomicrobia* and *Euryarchaeota* and *Methanobacteria* (Figure S3A,B), *Rikenellaceae* and unclassified *Clostridiales* (Figure S3D) and six genera, including *Alistipes*, *Shigella* and *Flavonifractor* (Figure S3E). Furthermore, changes in small intestinal and total intestinal (^{51}Cr EDTA 0-6h and 0-24h excretion, respectively) permeability correlated positively with changes in Enterobacteriaceae measured by qPCR ($r = 0.527$, $P = 0.024$, adj. $P = 0.51$ for both small intestinal and total tract permeability) in the prebiotic group. In contrast, only few bacteria correlated with changes in glucose tolerance outcomes;

279 *Actinobacteria* and *Bifidobacterium* correlated positively and *Veillonellaceae* and *Clostridium*
280 cluster XVIII inversely with glucose tAUC (Figures S3A-D). Unclassified Enterobacteriaceae
281 correlated positively with fasting glucose, insulin sensitivity (SI), hsCRP and waist circumference
282 (Figure S3D).

283 In the prebiotic group the strongest correlations between bacteria and inflammatory markers were
284 observed for sCD14 which correlated inversely with *Verrucomicrobia* and unclassified bacteria
285 ,*Erysipelotrichales* and *Verrucomicrobiales*, *Verrucomicrobiacea*, *Lactobacillaceae* and
286 *Erysipelotrichaceae* (Figures S3A,C,D). *Actinobacteria* and *Firmicutes* correlated positively with
287 IL-6 and TNF-α, respectively (Figure S3A). Furthermore, IL-6 correlated positively with
288 *Bifidobacterium* and negatively with *Veillonellaceae* and *Dialister* (Figures S3C,D,E). Changes in
289 small IP correlated with glucose response (iAUC) and carbohydrate energy percentage (r = -0.429,
290 P = 0.033 for both) and colon IP correlated with protein intake (r = 0.464, P = 0.021) in the
291 prebiotic group. However, due to the small sample size, apart from the association between
292 *Veillonellaceae* and IL-6 and glucose tAUC (r = -0.90, adj. P = 0.042 for both) none of these
293 correlations in the prebiotic group were statistically significant after adjustment for multiple testing.

294

DISCUSSION

In this study 12 weeks of prebiotic fibre supplementation did not have a significant beneficial effect on glucose tolerance outcomes in individuals with well controlled T2D. Although there was a decrease in the IP in the prebiotic group this was not statistically significant. Due to the number of patients presenting with permeability values within the normal range being higher than expected based on our previous work (50% versus 28%)[3], in future, it would be deemed necessary to test the role of prebiotics in those with a demonstrated impairment in barrier function to assess the true functionality of this dietary fibre.

Bifidobacterium levels increased in both treatment groups, although there was a trend towards post-intervention levels being higher in the prebiotic group. GOS has previously been shown to increase bifidobacteria levels, although it was noted that some volunteers were non-responders[23,24,33,34] and one study did not find a significant bifidogenic effect of GOS compared with placebo treatment[35]. Interestingly, others have reported a poorer bifidogenic effect of GOS in males and overweight individuals[34]. However, other factors may play a role in these negative findings including the type and dosage of GOS administered, background diet, as well as and the methods of analysis of *Bifidobacterium*[33]. As for the background diet, particularly the relatively high dietary fibre intake (>20g/day) in this cohort may have diminished the effect of the prebiotic supplement.

We used a dose of 5.5g prebiotic per day which may be considered to be low compared to other studies in which doses of 10g or more prebiotic were consumed[16,18,19]. Twelve weeks may not have been sufficient to elicit a significant effect on clinical outcomes although would have been ample time for changes in the microbiota to become apparent. Resistant starch (which is also a prebiotic) improves first-phase insulin secretion and insulin sensitivity in individuals at risk of T2D within this timescale[26,36], however, shows less efficacy in those already with T2D[37]. An unexpected finding was a decrease in first-phase insulin secretion and an increase in HbA1c in both groups in addition to an increase in fasting glucose within the prebiotic group. This suggests that short-term treatment with a low dose prebiotic fibre does not prevent further deterioration of key clinical parameters in T2D. The metabolic derangements in established T2D may be difficult to reverse as shown by the fact that prebiotic supplementation[18,19,37] does not improve glucose control in T2D, whereas a high-efficacy is shown in metabolic syndrome.

Metformin had a significant effect on the intestinal bacterial composition at the genus level, although it only explained a small part (<10%) of the variation in bacterial composition. Others have recently demonstrated a profound effect of metformin on intestinal bacterial community, bile acids, gut architecture, intestinal glucose utilization as well as circulating glucagon-like peptide 1,

LBP and LPS[9,38–43]. The effect of metformin on glucose control may partly be mediated by these intestinal effects; the increase in the mucin-degrading bacteria *Akkermansia muciniphila* following metformin treatment is thought to be beneficial[15,40]. Prebiotics have been shown to increase *A. muciniphila* in mice[15]; however, we did not observe significant changes in *A. muciniphila* levels following prebiotic treatment. However, it is a limitation of this study that all 13 for whom bacterial data was available in the prebiotic group were on metformin whilst only seven participants in the placebo group were on metformin. It seems plausible that metformin may have masked the effects of the prebiotic in the present study, and is a possible explanation underlying the discrepancy with both animal work and metabolic syndrome, as metformin treatment would not be administered in animal models of T2D.

The fact that the cohort in this study consisted of patients with well-controlled T2D may also play a role. Inflammatory markers were generally low in this group and this may have been due to a favourable combination of lifestyle factors and medication. However, inflammatory markers are often low in patients with T2D. This may be due some of the antihypertensive and lipid-lowering medications taken by the patients in this study have anti-inflammatory properties and these types of medications may also influence gut bacterial composition[44]. No clear links between IP and intestinal bacteria were found in this study. The positive correlation between Enterobacteriaceae and ^{51}Cr-EDTA recovery was not significant after adjustment for multiple testing although has been useful in hypothesis generating for future work. Others have suggested that a potential link exists between gut health and Enterobacteriaceae due to endotoxin-producing opportunistic pathogens in this bacterial family[45]. Nevertheless we found a significant inverse association between changes in *Veillonellaceae* and IL-6 and glucose tAUC suggesting a link between this bacterial family, inflammation and glucose response. *Veillonellaceae* comprises several acetate and propionate producers[46] and it has been suggested that short-chain fatty acids may mediate some of the beneficial effects of prebiotics on host metabolism[47]. The limitations in this study are primarily related to the small sample size which makes it difficult to detect subtle effects of a low dose of prebiotic in a heterogeneous study cohort and the potential confounding effects of various medications. In this study a decision was made at the outset to include numerous clinical and bacterial outcomes, in order to be hypothesis generating for future more focussed clinical studies.

In conclusion, supplementation with a low dose prebiotic for 12 weeks in metformin treated T2D patients did not improve glucose control, this is now in line with other work showing lack of efficacy of dietary fibres in the treatment of T2D in contrast to their beneficial role in T2D prevention[37]. However, our study was limited by the small sample size. Prior to adjustment for multiple testing, many significant associations between changes in intestinal bacteria and clinical

outcomes were observed during this study, providing focus and avenues for further work. The commonly used drug metformin is now known to be a significant confounder in the study of bacterial populations in T2D and must be accounted for in future work in this cohort.

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Declaration of interest: The authors declare that there is no duality of interest associated with this manuscript.

AUTHOR CONTRIBUTION STATEMENT

MDR: Obtained the funding, designed and supervised the research. CP, EG, FH, PH, MDR: Conducted the clinical experiments. RJE: performed the Next-Generation sequencing. EJ: performed the DNA extraction. OD: performed qPCR. TD and PDC: performed inflammatory marker and LPS measurements. CP, UZI and HW: analysed the data. JW and DR-J: provided medical supervision. CP, UZI, RJE, OD and MDR: wrote the manuscript. RLR, GRG, OD and PDC: edited the manuscript.

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Figure legends

Figure 1. Flow chart showing the recruitment and retention of patients in the study.

Figure 2. Intestinal permeability estimated by ^{51}Cr EDTA (mean and SEM) excreted in urine following 12 weeks of prebiotic (black bars, n = 14) or placebo (grey bars, n = 15) supplementation. A. % ^{51}Cr EDTA excreted before (pre) and after supplementation (post) and B. change in ^{51}Cr EDTA excreted. There were no significant differences between treatment groups (P = 0.322, P = 0.235 and P = 0.176 (ANCOVA) for small intestinal (0-6h), colon (6-24h) and total tract (0-24h) permeability, respectively).

Figure S1A. Ordination plots using Bray-Curtis distances. No clustering of samples was observed and the beta-diversity changed slightly in both treatment groups.

Figure S1B. Ordination plots using Bray-Curtis distances. The placebo group was split into metformin treated (Yes) and non-metformin treated (No) patients. The ordination plot shows that the change in the placebo group was mainly due to changes in metformin treated patients. All patients in the prebiotic group were metformin treated.

Figure S2: Quantification of bacteria using quantitative real time PCR (n = 11 in prebiotic groups and n = 12 in placebo group). Boxes show 25 and 75% percentiles, the line is the median and whiskers show maximum and minimum $\log_{10}$ rDNA copies per g faeces (wet weight). e: Enterobacteriaceae levels were significantly higher in the prebiotic group at baseline (unpaired t-test, $P = 0.0379$). *Bifidobacterium* levels increased in 8 patients in both treatment groups; however, the increase within the prebiotic group was on the cusp of significance ($P = 0.058$, paired t-test). Prebiotic treatment had no effect on total bacteria, *Lactobacillus*, *Roseburia*, Enterobacteriaceae, *Clostridium leptum* or *Clostridium coccoides* groups.

Figure S3A. Correlation heat maps showing associations (Kendall's rank correlations) between changes in clinical outcomes and bacteria abundances at the phylum level (not adjusted for multiple testing).

Figure S3B. Correlation heat maps showing associations (Kendall's rank correlations) between changes in clinical outcomes and bacteria abundances at the class level (not adjusted for multiple testing).

Figure S3C. Correlation heat maps showing associations (Kendall's rank correlations) between changes in clinical outcomes and bacteria abundances at the order level (not adjusted for multiple testing).

Figure S3D. Correlation heat maps showing associations (Kendall's rank correlations) between changes in clinical outcomes and bacteria abundances at family level (not adjusted for multiple testing). Among the biochemical outcomes only correlations between IL-6 and glucose tAUC and *Veillonellaceae* (r = -0.90, adj. P = 0.042 for both) were significant after correction of P-values for multiple testing.

Figure S3E. Correlation heat maps showing associations between changes in clinical outcomes and bacteria abundances at genus level (not adjusted for multiple testing).

Table 1: Characteristics of the treatment groups at baseline (Pre) and post-supplementation (Post) and diabetes medications[a].

	Prebiotic		Placebo		
	Pre	**Post**	**Pre**	**Post**	**P-value[f]**
Age (years)	56.7 ± 1.6	-	58.1 ± 1.7	-	-
Time since diagnosis (years)	4.6 ± 0.6	-	4.0 ± 0.8	-	-
Ethnicity (*n*)		-		-	
Caucasian	11		14		
Asian	2		0		
Black	1		1		
Body weight (kg)	87.0 ± 3.5	87.6 ± 3.6	86.7 ± 3.2	86.8 ± 3.2	0.335
BMI (kg/m^2)	28.0 ± 1.1	28.2 ± 1.1	28.4 ± 0.9	28.5 ±0.9	0.333
Body fat (%)[b]	26.5 ± 1.3	27.3 ± 1.3[e]	26.0 ± 1.5	26.5 ± 1.4	0.514
Waist circumference (cm)[c]	101.3 ± 3.1	101.7 ± 3.6	101.5 ± 2.7	101.2 ± 2.6	0.451
Blood pressure (sys) (mmHg)[c]	136 ± 2	133 ± 3	136 ± 3	132 ± 4[e]	0.942
Blood pressure (dia) (mmHg)[c]	86 ± 2	83 ± 2	84.0 ± 1.7	81.1 ± 1.6	0.909
Diabetes medications (n)[d]					
Metformin	7		3		
Metformin and gliclazide	3		2		
Metformin and sitagliptin	1		2		
Metformin, gliclazide, and sitagliptin	1		0		
Metformin, sitagliptin, and thiazolidinedione	1		0		
Sitagliptin and gliclazide	1		1		
Gliclazide	0		1		

[a]Means and SEM presented. $n = 14$ in the prebiotic group and $n = 15$ in the placebo group unless otherwise stated. There were no differences in baseline (Pre) values between groups ($P > 0.05$, unpaired t-test). [b]$n = 13$ in Placebo group. [c]$n = 13$ in prebiotic group. [d]The remaining 6 patients in the placebo group were diet/exercise controlled. [e]Significant within group change ($P < 0.05$, paired t-test). [f]The P-value is for the comparison of the change between groups with Pre value as covariate (ANCOVA). Other medications (n) used by patients in the prebiotic group were statins (11), blood pressure medication (8), Fenofibrate (2), Omeprazole (2), low-dose aspirin (1), Levothyroxine sodium (1) and citalopram (1). Other medications used in the placebo group were statins (8), blood pressure medication (8), low-dose aspirin (5), Omeprazole (2), benign prostate hyperplasia medications (2), hay fever medication (2), Betahistine hydrochloride (1), asthma medication (1), medications for incontinence (2), sleep medication (1) and anti-fungal medication (1).

570 **Table 2: Glucose tolerance outcomes at baseline and after 12 weeks supplementation[a].**

	Prebiotic		Placebo		
	Pre	**Post**	**Pre**	**Post**	**P-value[f]**
Glucose, fasting (mmol/L)[b]	6.1 ± 0.4	6.8 ± 0.4[c]	6.2 ± 0.3	6.5 ± 0.3	0.227
Glucose $tAUC_{180\ min}$ (mM*min)	1319 ± 74	1414 ± 84[c]	1234 ± 89	1289 ± 98	0.485
Glucose $iAUC_{180min}$ (mM * min)	222 ± 33	197 ± 32	153 ± 32	170 ± 35	0.221
Insulin, fasting (pmol/L)[b,d]	83.5 ± 14.7	94.0 ± 18.7	94.6 ± 15.3	83.0 ± 13.0	0.543
Insulin $tAUC_{180\ min}$ (pM*min)[d]	6026 ± 774	7121 ±948	6867 ± 1091	6274 ± 821	0.112
Insulin $iAUC_{180min}$ (pM min)	3522 ± 355	4301 ± 449[c]	3892 ± 626	3784 ±568	0.171
Insulin $tAUC_{10min}$ (pM*min)[e]	176 ± 28	175 ± 33	182 ± 33	151 ± 24	0.355
Insulin $iAUC_{10min}$ (pM min)[e]	37 ± 14	18 ± 6	23 ± 19	16 ± 12	0.946
C-peptide $tAUC_{180\ min}$ (pM min)	339 ± 30	403 ± 41	342 ± 41	333 ± 44	0.166
C-peptide $iAUC_{180\ min}$ (pM min)	71 ± 9	94 ± 11	73 ± 14	59 ± 20	0.111
HbA1c (mmol/mol)[b,d]	51.2 ± 3.1	53.1 ± 3.2	46.3 ± 1.8	48.4 ± 2.4	0.946
HbA1c (%)[b]	6.8 ± 0.3	7.0 ± 0.3	6.4 ± 0.2	6.6 ± 0.2	-
AIRg ($mU\ L^{-1}\ min^{-1}$)	39.1 ± 13.4	21.2 ± 5.2	38.3 ± 15.6	23.1 ± 10.5	0.856
DI[l]	38 (5.5 – 119.1)	49.8 (2.7 – 111.3)	53.6 (0 – 172.4)	20.6 (0.1 – 36.8)	0.4507
SI[l] ($(mU/L)^{-1}\ min^{-1}$)	1.95 (0.95 – 3.98)	2.18 (0.16 -4.32)	4.48 (1.31 – 172.5)	1.91 (0.22 – 4.84)	0.2358
GEZI (min^{-1})	0.022 (0.011-0.025)	0.0175 (0.0045-0.026)	0.015 (-0.2165-0.021)	0.02 (0.0155-0.0225)	**0.0212**
Beta-cell function (mU/mM)	173.1 ± 30.4	139.5 ± 24.8	165.9 ± 26.7	113.2 ± 14.6[c]	0.350
IR ($mmol\ mU\ L^{-2}$)	3.6 ± 0.8	4.3 ± 0.9	4.3 ± 0.8	3.9 ± 0.9	0.337
HOMA2%B[b]	100.4 ± 10.9	90.2 ± 11.6	100.2 ± 10.9	81.3 ± 7.0[c]	0.362
HOMA2%S[b]	62.6 (46.0-97.2)	59.0 (37.6-92.3)	54.1 (36.4-87.2)	65.5 (39.1-82.4)	0.2147
HOMA2IR[b]	1.60 (1.03-2.18)	1.7 (1.08-2.68)	1.88 (1.15-2.77)	1.58 (1.27-2.56)	0.1994

[a]Means ± SEM or median (interquartile ranges) presented. n = 13 for placebo group and n=14 for prebiotic group unless otherwise stated. There were no differences in baseline (PRE) values between groups (P > 0.05, unpaired t-test or Mann-Whitney test). [b]n=15 for placebo group. [c]Significant within group change (P < 0.05, paired t-test or Wilcoxon matched pairs signed rank test). [d]ANCOVA performed on log-transformed values. [e]n=14 for placebo group. [f]The P-value is for the comparison of the change between groups with Pre value as covariate (ANCOVA). tAUC: total area under the curve. iAUC: incremental area under the curve. AIRg: Acute insulin response to glucose. DI: Disposition index. SI: insulin sensitivity. GEZI: Glucose effectiveness at zero insulin. IR: insulin resistance. %B: % beta-cells. %S: % sensitivity.

580 **Figure 1**. Flow chart showing the recruitment and retention of patients in the study.

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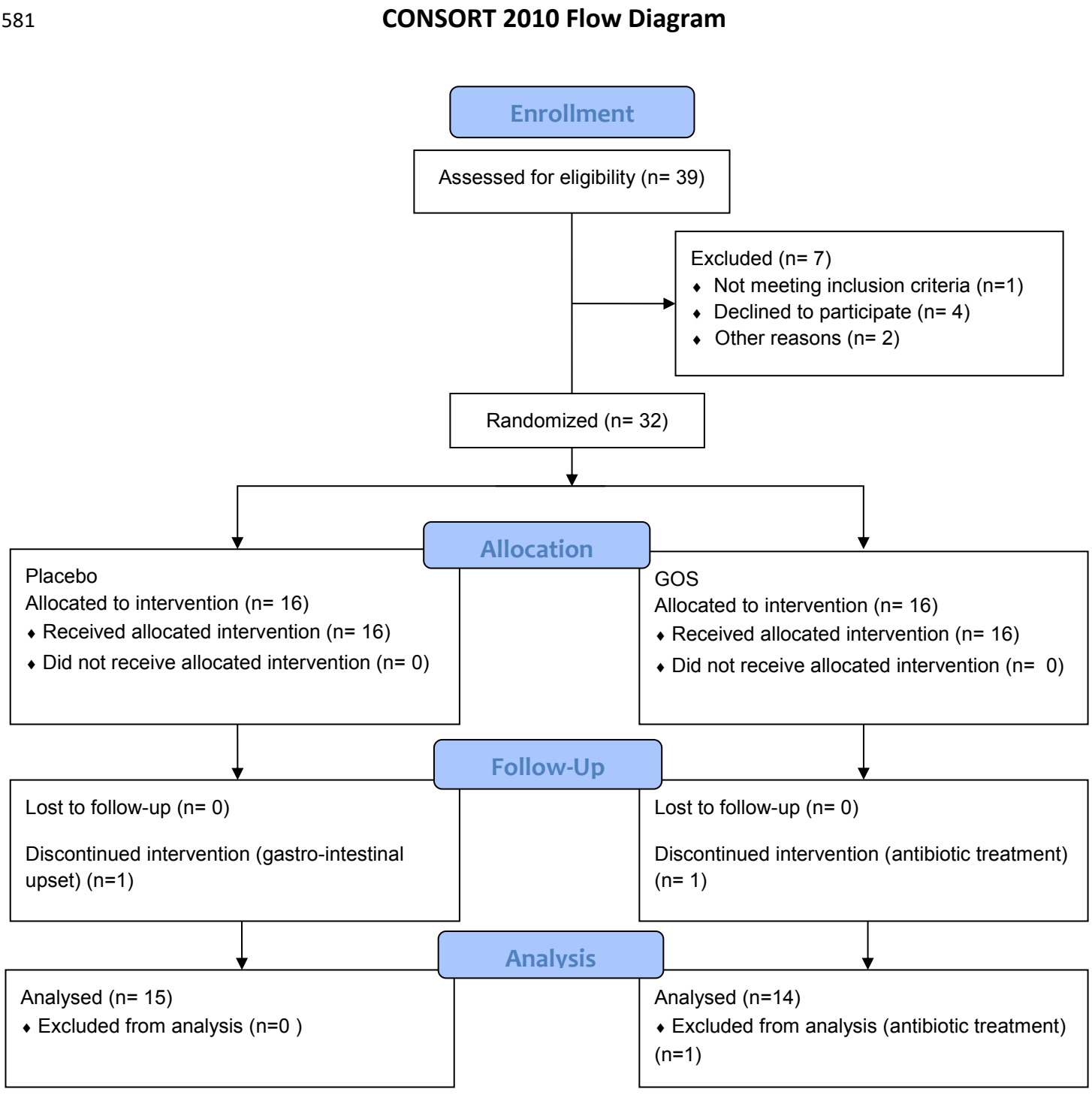

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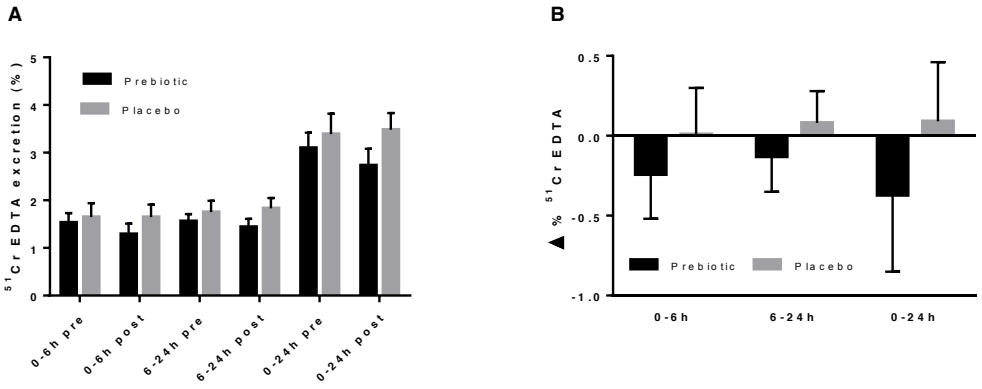

583

584 **Figure 2**. Intestinal permeability estimated by ^{51}Cr EDTA (mean and SEM) excreted in urine following 12
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587 significant differences between treatment groups (P = 0.322, P = 0.235 and P = 0.176 (ANCOVA) for small
588 intestinal (0-6h), colon (6-24h) and total tract (0-24h) permeability, respectively).

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SUPPLEMENTARY MATERIALS AND METHODS

DNA extraction

DNA was extracted from faecal samples using the PowerFecal™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA concentration and quality were measured by NanoDrop 2000 (Thermo Scientific) and Qubit 2.0 fluorometer (Invitrogen).

Amplification and High-Throughput Sequencing

Briefly, the V4 and V5 region of the bacterial 16S rRNA gene was amplified from extracted DNA with universal primers (U515F: 5'-GTGYCAGCMGCCGCGGTA and U927R: 5'-CCCGYCAATTCMTTTRAGT). Forward fusion primers consisted of the GS FLX Titanium primer A and the library key (5' -CATCTCATCCCTGCGTGTCTCCGACTCAG) together with one of a suite of sixteen 10-base multiplex identifiers (MIDs 1–16) (Roche Diagnostics Ltd, UK). Reverse fusion primers included the GS FLX Titanium primer B and the library key (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG). Amplification was performed with FastStart HiFi Polymerase (Roche Diagnostics Ltd, UK) using the following cycling conditions: 94°C for 3 min; 30 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min; followed by 72°C for 8 min. Ampure XP magnetic beads (Beckman Coulter) were used for purification of amplicons. Amplicon concentration was assessed using the fluorescence-based Picogreen assay (Invitrogen) and concentrations normalized before pooling. Amplicon pools were immobilized and amplified on beads by emulsion PCR using Lib-L emPCR kits (Roche Diagnostics Ltd, UK). Unidirectional sequencing from the forward primer was performed on the 454 GS FLX Titanium platform according to the manufacturer's instructions (Roche Diagnostics Ltd, UK).

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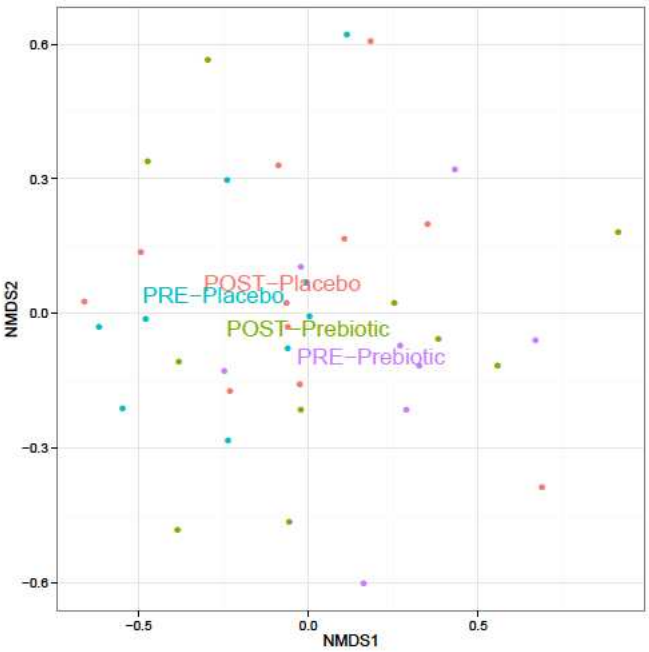
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PRE−Prebiotic
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NMDS2

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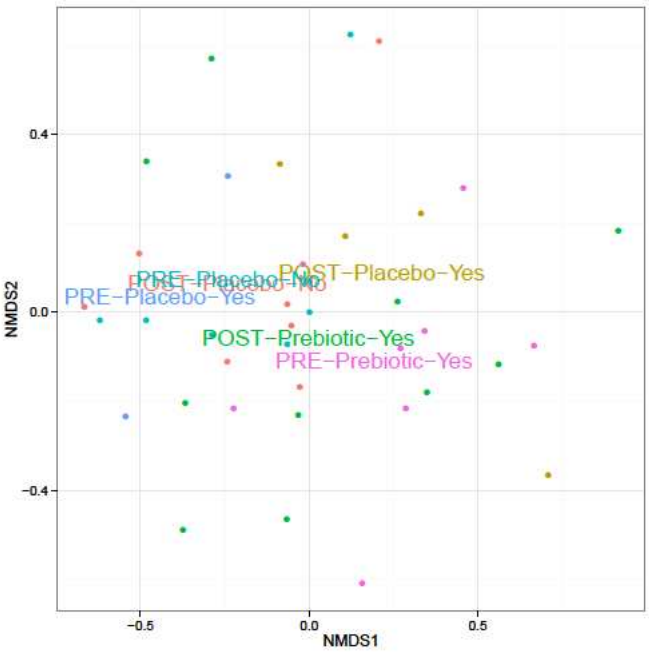
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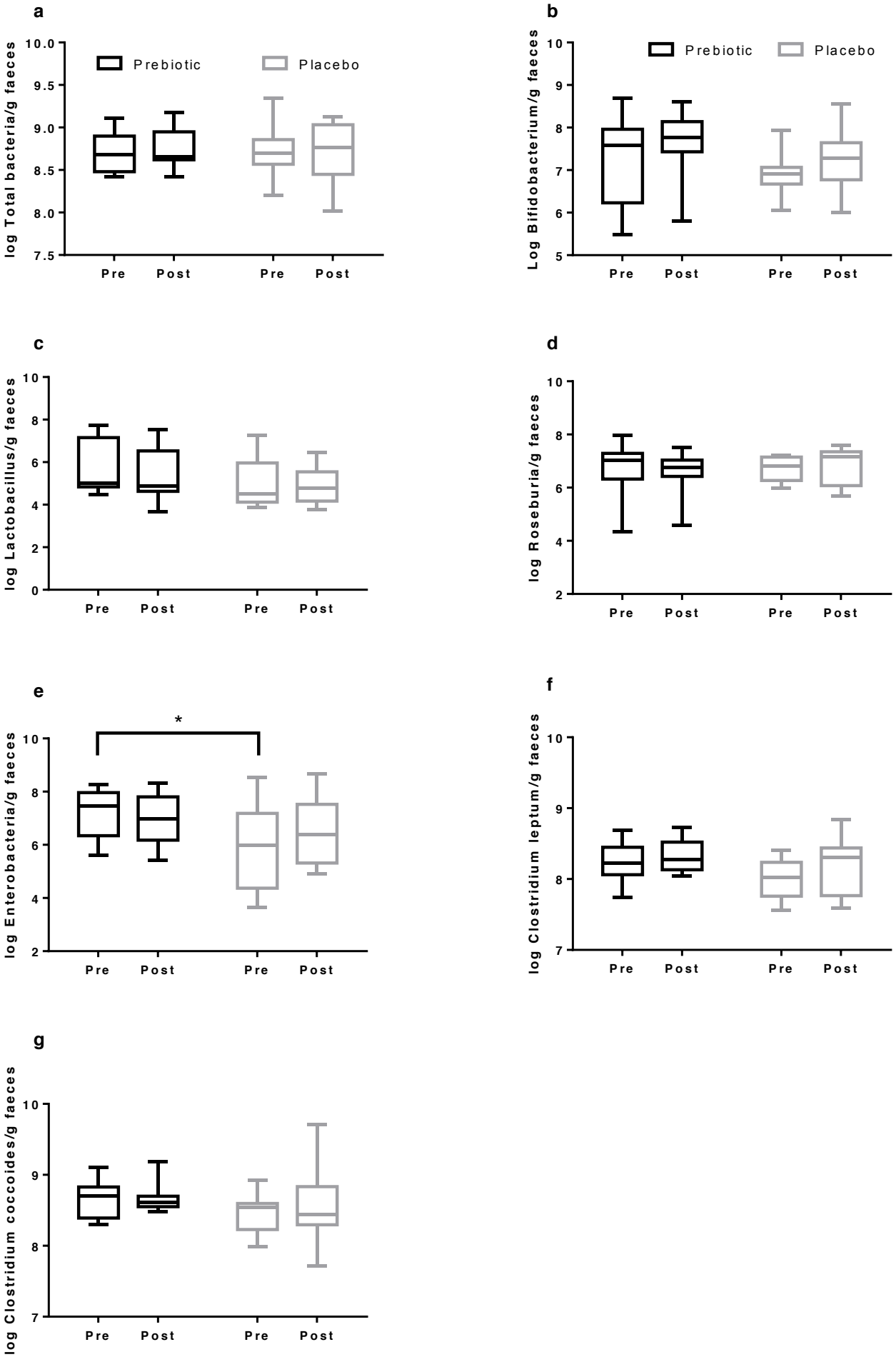
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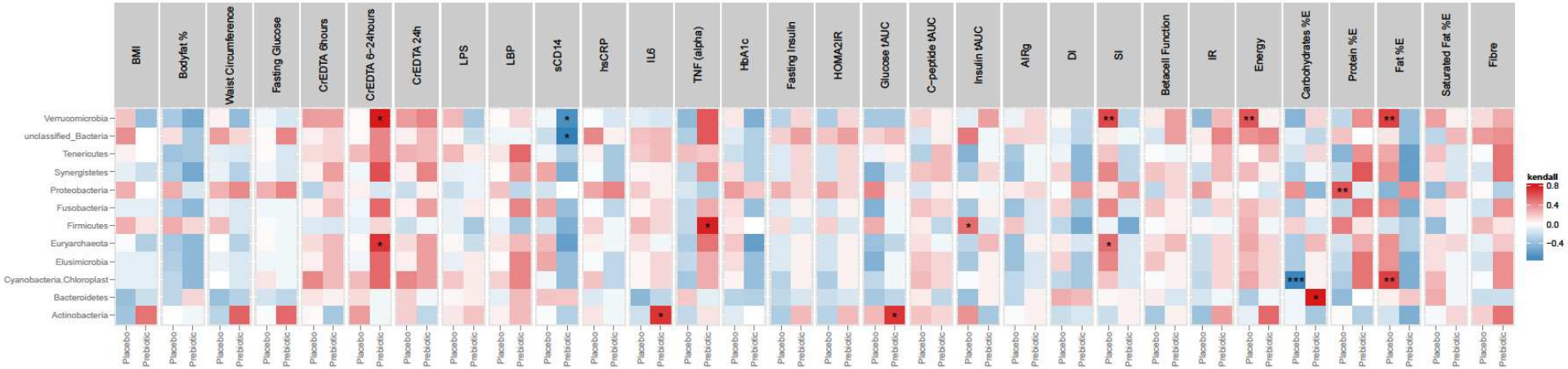
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TNF (alpha)
HbA1c
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HOMA2IR
Glucose tAUC
C−peptide tAUC
Insulin tAUC
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SI
Betacell Function
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Protein %E
Fat %E
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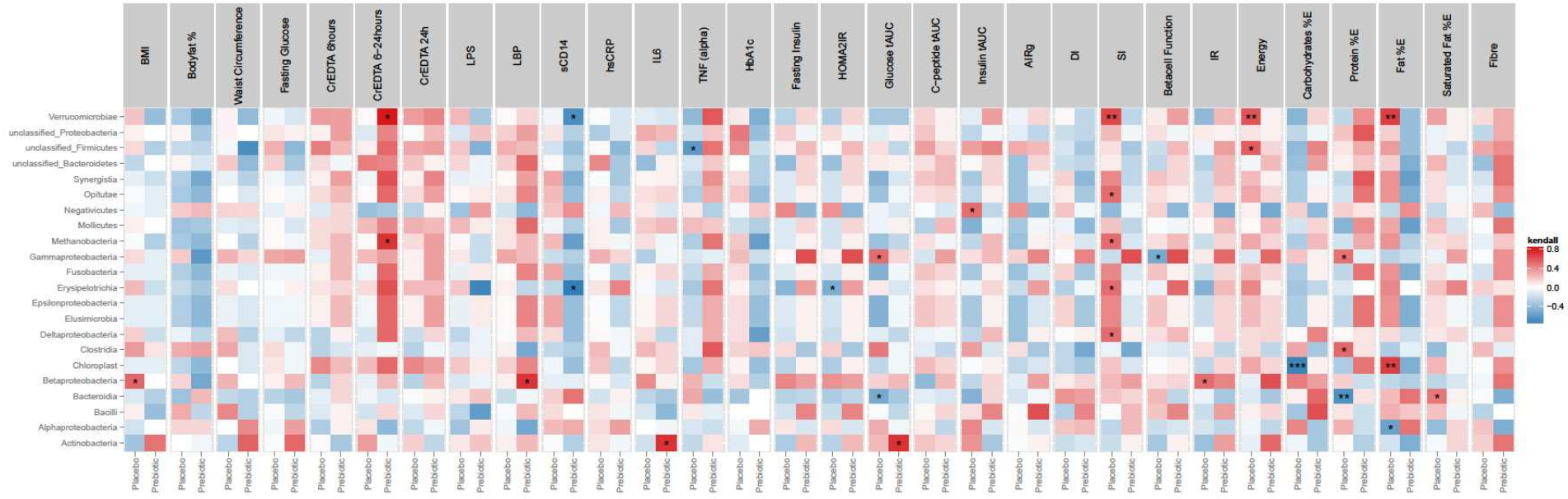
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CrEDTA 6-24hours
CrEDTA 24h
LPS
LBP
sCD14
hsCRP
IL6
TNF (alpha)
HbA1c
Fasting Insulin
HOMA2IR
Glucose tAUC
C-peptide tAUC
Insulin tAUC
AIRg
DI
SI
Betacell Function
IR
Energy
Carbohydrates %E
Protein %E
Fat %E
Saturated Fat %E
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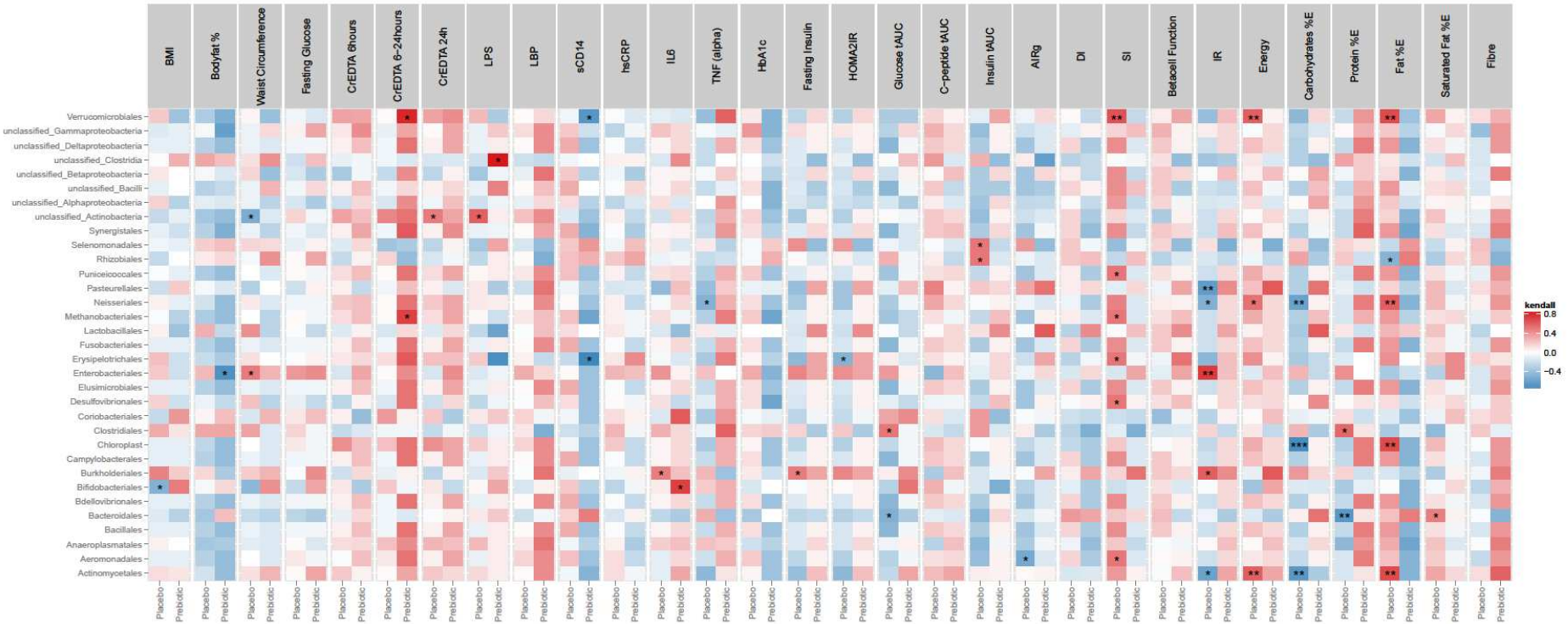
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CrEDTA 6-24hours
CrEDTA 24h
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TNF (alpha)
HbA1c
Fasting Insulin
HOMA2IR
Glucose tAUC
C-peptide tAUC
Insulin tAUC
AIRg
DI
SI
Betacell Function
IR
Energy
Carbohydrates %E
Protein %E
Fat %E
Saturated Fat %E
Fibre
Verrucomicrobiales
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Erysipelotrichales
Enterobacteriales
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Coriobacteriales
Clostridiales
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Campylobacterales
Burkholderiales
Bifidobacteriales
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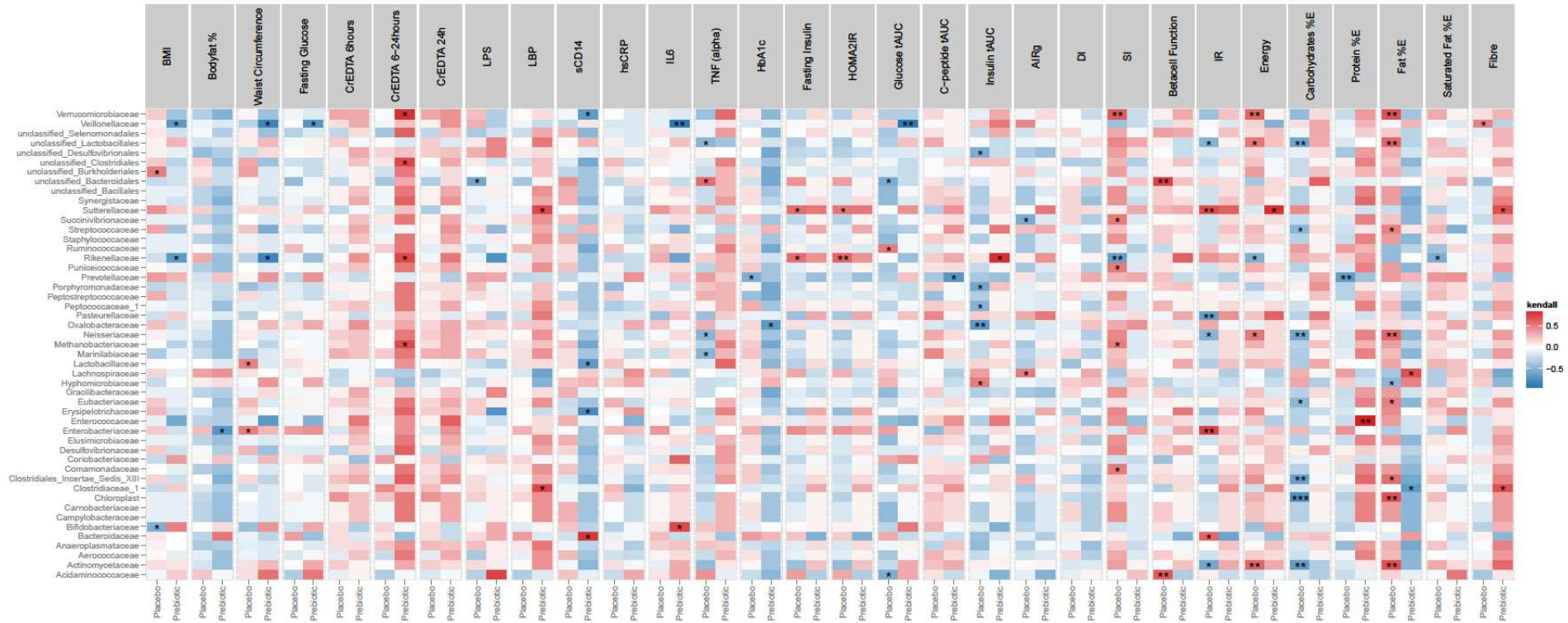
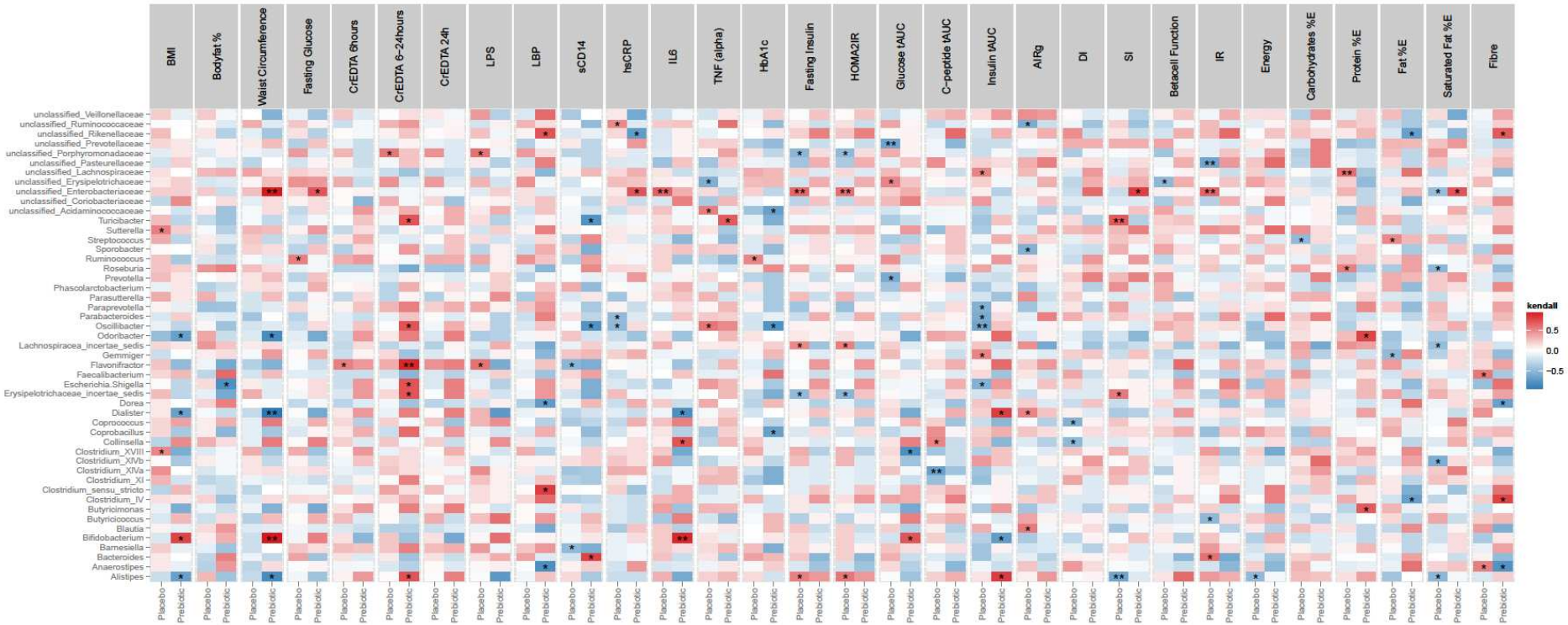
Bacteroidales
Bacillales
Anaeroplasmatales
Aeromonadales
Actinomycetales
Placebo
Prebiotic
kendall
0.8
0.4
0.0
−0.4

600


BMI
Bodyfat %
Waist Circumference
Fasting Glucose
CrEDTA 6hours
CrEDTA 6-24hours
CrEDTA 24h
LPS
LBP
sCD14
hsCRP
IL6
TNF (alpha)
HbA1c
Fasting Insulin
HOMA2IR
Glucose tAUC
C-peptide tAUC
Insulin tAUC
AIRg
DI
SI
Betacell Function
IR
Energy
Carbohydrates %E
Protein %E
Fat %E
Saturated Fat %E
Fibre
Verrucomicrobiaceae
Veillonellaceae
unclassified_Selenomonadales
unclassified_Lactobacillales
unclassified_Desulfovibrionales
unclassified_Clostridiales
unclassified_Burkholderiales
unclassified_Bacteroidales
unclassified_Bacillales
Synergistaceae
Sutterellaceae
Succinivibrionaceae
Streptococcaceae
Staphylococcaceae
Ruminococcaceae
Rikenellaceae
Punicicoccaceae
Prevotellaceae
Porphyromonadaceae
Peptostreptococcaceae
Peptococcaceae_1
Pasteurellaceae
Oxalobacteraceae
Neisseriaceae
Methanobacteriaceae
Marinilabiaceae
Lactobacillaceae
Lachnospiraceae
Hyphomicrobiaceae
Gracilibacteraceae
Eubacteriaceae
Erysipelotrichaceae
Enterococcaceae
Enterobacteriaceae
Elusimicrobiaceae
Desulfovibrionaceae
Coriobacteriaceae
Comamonadaceae
Clostridiales_Incertae_Sedis_XIII
Clostridiaceae_1
Chloroplast
Carnobacteriaceae
Campylobacteraceae
Bifidobacteriaceae
Bacteroidaceae
Anaeroplasmataceae
Aerococcaceae
Actinomycetaceae
Acidaminococcaceae
Placebo
Prebiotic
kendall
0.5
0.0
−0.5

601



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