

# *Host-microbiome interactions in human type 2 diabetes following prebiotic fibre (galactooligosaccharide) intake*

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Accepted Version

Pedersen, C., Gallgher, E., Horton, F., Ellis, R. J., Ijaz, U. Z., Wu, H., Jaiyeola, E., Diribe, O., Duparc, T., Cani, P. D., Gibson, G. R. ORCID: <https://orcid.org/0000-0002-0566-0476>, Hinton, P., Wright, J., La Ragione, R. and Robertson, M. D. (2016) Host-microbiome interactions in human type 2 diabetes following prebiotic fibre (galactooligosaccharide) intake. *British Journal of Nutrition*, 116 (11). pp. 1869-1877. ISSN 0007-1145 doi: 10.1017/S0007114516004086 Available at <https://centaur.reading.ac.uk/68889/>

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To link to this article DOI: <http://dx.doi.org/10.1017/S0007114516004086>

Publisher: Cambridge University Press

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**Host-microbiome interactions in human type 2 diabetes following prebiotic fibre  
(galactooligosaccharide) intake.**

Camilla Pedersen<sup>1</sup>, Edith Gallagher<sup>2</sup>, Felicity Horton<sup>2</sup>, Richard J. Ellis<sup>3</sup>, Umer Z. Ijaz<sup>4</sup>, Huihai Wu<sup>1</sup>,  
Etana Jaiyeola<sup>1</sup>, Onyinye Diribe<sup>1</sup>, Thibaut Duparc<sup>5</sup>, Patrice D. Cani<sup>5</sup>, Glenn R. Gibson<sup>6</sup>, Paul  
Hinton<sup>2</sup>, John Wright<sup>1,7</sup>, Roberto La Ragione<sup>1</sup>, and M. Denise Robertson<sup>1</sup>

**Affiliations**

1. Faculty of Health and Medical Sciences, University of Surrey, UK
2. Medical Physics - Nuclear Medicine, Royal Surrey County Hospital, UK
3. Animal and Plant Health Agency, Addlestone, UK
4. School of Engineering, University of Glasgow, UK
5. Louvain Drug Research Institute, Catholic University of Louvain, Belgium
6. Department of Food and Nutritional Sciences, University of Reading, UK
7. CEDAR Centre, Royal Surrey County Hospital, UK

**Corresponding author**

Dr M Denise Robertson  
Leggett Building  
Faculty of Health and Medical Sciences  
University of Surrey  
Guildford  
GU2 7WG  
E: m.robertson@surrey.ac.uk  
Tel: +44 (0)1483 68 6407  
Fax: +44 (0) 1483 688 501

**Short title:** Prebiotic supplementation in type 2 diabetes

**Keywords:** prebiotic, diabetes, gut microbiota, intestinal permeability, endotoxaemia

**Word count:** main text: 3902, abstract: 251

**Figures and Tables:** 4

Supplementary Figures and Tables: 6

References: 47

## 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  $^{51}\text{Cr}$ -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.

## 53 INTRODUCTION

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

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

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

81

## 82 MATERIALS AND METHODS

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

### 89 Subjects

90 Males with well-controlled T2D aged 42-65 years were recruited through local GP practices and  
91 advertisement in a local newspaper. Due to repeated administration of the radioactive compound  
92 <sup>51</sup>Cr-EDTA and the potential influence of the menstrual cycle on outcomes, women were excluded  
93 from the study. All patients provided written informed consent. Exclusion criteria included use of  
94 antibiotics in the previous three months, use of anti-inflammatory medications (except a low dose  
95 (75mg/day) aspirin), diuretics, proton-pump inhibitors, inflammatory bowel disease, Crohn's  
96 disease, coeliac disease and irritable bowel syndrome. Patients were asked to exclude probiotic  
97 products and prebiotic supplements (other than the study supplement) from their diet for two weeks  
98 prior to the first study visit and throughout the study. Furthermore, they were asked not to change  
99 their lifestyle during the study. The sample size for this study was based on the primary outcome  
100 measure of changes to IP and based on our own published pilot data using this method in patients  
101 with well controlled T2D<sup>(3)</sup>. 30 Patients in this parallel design study provided 80% power to detect a  
102 treatment difference between groups of 1.6% in total permeability, using the calculated SD in this  
103 cohort of 1.57 (alpha 0.05).

### 104 Study protocol

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

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

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

### 126 **Intestinal permeability**

127 IP was measured by 24h urinary excretion of orally administered <sup>51</sup>Cr-EDTA as previously  
128 described<sup>(3)</sup>. We utilized <sup>51</sup>Cr-EDTA as a probe as it is stable in the colonic luminal environment  
129 allowing assessment of colonic permeability and it is easily detected in the urine <sup>(25)</sup>.

### 130 **Anthropometric and blood pressure measurements**

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

### 137 **Glucose tolerance, inflammatory markers and lipids**

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

### 145 **Biochemical analyses**

146 Whole blood glucose concentrations were measured on an YSI 2300 STAT Plus™ (YSI Life  
147 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- $\alpha$  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  $\leq$  3% for lipids measurements. LDL cholesterol concentration was calculated using the Friedewald formula<sup>(27)</sup>. LPS was measured in duplicate using Endosafe-MCS (Charles River Laboratories, Lyon, France) as previously described<sup>(15)</sup>. 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.<sup>(28)</sup>. Further details are provided in the supplementary information.

## **Bioinformatics**

The sequences were processed in Qiime<sup>(29)</sup> using the AmpliconNoise<sup>(30)</sup> 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<sup>(31)</sup>. 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<sup>(32)</sup>. 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



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

#### 184 **Quantification of bacterial groups by quantitative PCR**

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

#### 188 **Statistical analysis**

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

#### 203 **RESULTS**

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

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

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

### 218 **Anthropometrics and blood pressure**

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

### 221 **Intestinal permeability**

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

### 224 **Glucose tolerance**

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

### 229 **Inflammatory markers and lipids**

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

### 232 **Dietary assessment**

233 At baseline the energy intake in the prebiotic group was  $8929 \pm 538$  kJ/day with percentage of  
234 energy obtained from carbohydrate, sugar, fat, saturated fat and protein  $42.1 \pm 2.5\%$ ,  $14.5 \pm 1.7\%$ ,  
235  $36.6 \pm 1.5\%$ ,  $12.5 \pm 0.8\%$ , and  $15.7 \pm 0.9\%$ , respectively. In the placebo group the mean daily energy intake  
236 was  $8683 \pm 581$  kJ and carbohydrate, sugar, fat, saturated fat and protein provided  $40.0 \pm 1.5\%$ ,  $14.3 \pm$   
237  $1.0\%$ ,  $37.7 \pm 1.5\%$ ,  $12.1 \pm 0.4\%$  and  $16.8 \pm 0.8\%$  of total energy, respectively. The percentage dietary  
238 energy from protein increased by 1.1% in the placebo group and this was significantly different  
239 from that observed in the prebiotic group (Supplementary Table S2). No other significant  
240 differences in dietary intakes were observed between groups.

### 241 **Gut microbiota composition**

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

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

250 Consistent with previous reports on composition of the gut microbiota in humans, *Bacteroidetes*  
251 and *Firmicutes* were the two dominant phyla followed by *Proteobacteria*, unclassified bacteria and  
252 *Actinobacteria* (data not shown). Bacterial community structure in the treatment groups changed  
253 only slightly during the study, but the change was greater in the prebiotic group as can be observed  
254 in the NMDS plot (Supplementary Figure S1A). The change in the placebo group was mainly due  
255 to changes in metformin-treated patients (Supplementary Figure S1B). However, comparison of  
256 bacteria abundances at all taxonomic levels did not reveal any significant effect of treatment when  
257 adjusted for multiple testing (data not shown). Nonetheless, permutation ANOVA showed a trend  
258 towards an effect of treatment ( $P = 0.099$ ) at the OTU level. When metformin was included as a  
259 cofactor, metformin had a significant effect on bacterial community structure at the genus level ( $R^2$   
260  $= 0.084$ ,  $P = 0.009$ ) whereas only a trend was detected when the analysis were performed on OTUs  
261 ( $R^2 = 0.039$ ,  $P = 0.078$ ).

#### 262 **Quantification of bacterial groups by qPCR**

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

#### 266 **Correlations between changes in bacteria, clinical outcomes and dietary intakes**

267 As an *a priori* aim was to investigate the role of prebiotic fibre intake specifically for hypothesis  
268 generation, correlations were calculated for each treatment group separately. The correlations  
269 differed between the two groups as can be observed from the different patterns in the heat maps  
270 (Supplementary Figures S3A-E). Changes in large bowel permeability ( $^{51}\text{Cr}$  EDTA 6-24h  
271 excretion) were positively correlated with bacterial changes at all taxonomic levels in the prebiotic  
272 group. The strongest correlations were for *Verrucomicrobia* and *Euryarchaeota* and  
273 *Methanobacteria* (Figure S3A,B), *Rikenellaceae* and unclassified *Clostridiales* (Figure S3D) and  
274 six genera, including *Alistipes*, *Shigella* and *Flavonifractor* (Figure S3E). Furthermore, changes in  
275 small intestinal and total intestinal ( $^{51}\text{Cr}$  EDTA 0-6h and 0-24h excretion, respectively)  
276 permeability correlated positively with changes in Enterobacteriaceae measured by qPCR ( $r =$   
277  $0.527$ ,  $P = 0.024$ , adj.  $P = 0.51$  for both small intestinal and total tract permeability) in the prebiotic  
278 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*, *Verrucomicrobiaceae*, *Lactobacillaceae* and  
286 *Erysipelotrichaceae* (Figures S3A,C,D). *Actinobacteria* and *Firmicutes* correlated positively with  
287 IL-6 and TNF- $\alpha$ , 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

## 295 DISCUSSION

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

303 *Bifidobacterium* levels increased in both treatment groups, although there was a trend towards post-  
304 intervention levels being higher in the prebiotic group. GOS has previously been shown to increase  
305 bifidobacteria levels, although it was noted that some volunteers were non-responders<sup>(23,24,33,34)</sup> and  
306 one study did not find a significant bifidogenic effect of GOS compared with placebo treatment<sup>(35)</sup>.  
307 Interestingly, others have reported a poorer bifidogenic effect of GOS in males and overweight  
308 individuals<sup>(34)</sup>. However, other factors may play a role in these negative findings including the type  
309 and dosage of GOS administered, background diet, as well as and the methods of analysis of  
310 *Bifidobacterium*<sup>(33)</sup>. As for the background diet, particularly the relatively high dietary fibre intake  
311 (>20g/day) in this cohort may have diminished the effect of the prebiotic supplement.

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

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

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

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

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

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

365

## 366 **ACKNOWLEDGEMENTS**

367 The research was supported by the National Institute for Health Research (NIHR) Clinical Research  
368 Network (CRN): Kent, Surrey and Sussex. Thank you to the staff at the CEDAR centre, Royal  
369 Surrey County Hospital, and Dr Caroline Bodinham and Dr Martin Whyte, University of Surrey, for  
370 their assistance in the clinical experiments, Amandine Bever, Catholic University of Louvain, for  
371 assistance in ELISAs and to the study participants for their time and support. Thank you to Clasado  
372 Ltd. for providing the supplements. MDR is the guarantor of this work and, as such, had full access  
373 to all the data in the study and takes responsibility for the integrity of the data and the accuracy of  
374 the data analysis.

## 375 **FINANCIAL SUPPORT**

376 This study was funded by an EFSD clinical research grant.

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

## 379 **AUTHOR CONTRIBUTION STATEMENT**

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

386

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## 516 Figure legends

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

518 **Figure 2.** Intestinal permeability estimated by  $^{51}\text{Cr}$  EDTA (mean and SEM) excreted in urine  
519 following 12 weeks of prebiotic (black bars,  $n = 14$ ) or placebo (grey bars,  $n = 15$ ) supplementation.  
520 A. %  $^{51}\text{Cr}$  EDTA excreted before (pre) and after supplementation (post) and B. change in  $^{51}\text{Cr}$   
521 EDTA excreted. There were no significant differences between treatment groups ( $P = 0.322$ ,  $P =$   
522  $0.235$  and  $P = 0.176$  (ANCOVA) for small intestinal (0-6h), colon (6-24h) and total tract (0-24h)  
523 permeability, respectively).

524

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

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

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

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

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

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

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

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

555

556 **Table 1: Characteristics of the treatment groups at baseline (Pre) and post-supplementation**  
557 **(Post) and diabetes medications<sup>a</sup>.**

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

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

568

569

**Table 2: Glucose tolerance outcomes at baseline and after 12 weeks supplementation<sup>a</sup>.**

	Prebiotic		Placebo		P-value <sup>f</sup>
	Pre	Post	Pre	Post	
Glucose, fasting (mmol/L) <sup>b</sup>	6.1 ± 0.4	6.8 ± 0.4 <sup>c</sup>	6.2 ± 0.3	6.5 ± 0.3	0.227
Glucose tAUC <sub>180 min</sub> (mM * min)	1319 ± 74	1414 ± 84 <sup>c</sup>	1234 ± 89	1289 ± 98	0.485
Glucose iAUC <sub>180min</sub> (mM * min)	222 ± 33	197 ± 32	153 ± 32	170 ± 35	0.221
Insulin, fasting (pmol/L) <sup>b,d</sup>	83.5 ± 14.7	94.0 ± 18.7	94.6 ± 15.3	83.0 ± 13.0	0.543
Insulin tAUC <sub>180 min</sub> (pM * min) <sup>d</sup>	6026 ± 774	7121 ± 948	6867 ± 1091	6274 ± 821	0.112
Insulin iAUC <sub>180min</sub> (pM min)	3522 ± 355	4301 ± 449 <sup>c</sup>	3892 ± 626	3784 ± 568	0.171
Insulin tAUC <sub>10min</sub> (pM * min) <sup>e</sup>	176 ± 28	175 ± 33	182 ± 33	151 ± 24	0.355
Insulin iAUC <sub>10min</sub> (pM min) <sup>e</sup>	37 ± 14	18 ± 6	23 ± 19	16 ± 12	0.946
C-peptide tAUC <sub>180 min</sub> (pM min)	339 ± 30	403 ± 41	342 ± 41	333 ± 44	0.166
C-peptide iAUC <sub>180 min</sub> (pM min)	71 ± 9	94 ± 11	73 ± 14	59 ± 20	0.111
HbA1c (mmol/mol) <sup>b,d</sup>	51.2 ± 3.1	53.1 ± 3.2	46.3 ± 1.8	48.4 ± 2.4	0.946
HbA1c (%) <sup>b</sup>	6.8 ± 0.3	7.0 ± 0.3	6.4 ± 0.2	6.6 ± 0.2	-
AI Rg (mU L <sup>-1</sup> min <sup>-1</sup> )	39.1 ± 13.4	21.2 ± 5.2	38.3 ± 15.6	23.1 ± 10.5	0.856
DI <sup>f</sup>	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 <sup>f</sup> ((mU/L) <sup>-1</sup> min <sup>-1</sup> )	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 <sup>-1</sup> )	0.022 (0.011-0.025)	0.0175 (0.0045-0.026)	0.015 (-0.2165-0.021)	0.02 (0.0155-0.0225)	<b>0.0212</b>
Beta-cell function (mU/mM)	173.1 ± 30.4	139.5 ± 24.8	165.9 ± 26.7	113.2 ± 14.6 <sup>c</sup>	0.350
IR (mmol mU L <sup>-2</sup> )	3.6 ± 0.8	4.3 ± 0.9	4.3 ± 0.8	3.9 ± 0.9	0.337
HOMA2 % B <sup>b</sup>	100.4 ± 10.9	90.2 ± 11.6	100.2 ± 10.9	81.3 ± 7.0 <sup>c</sup>	0.362
HOMA2 % S <sup>b</sup>	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
HOMA2 IR <sup>b</sup>	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

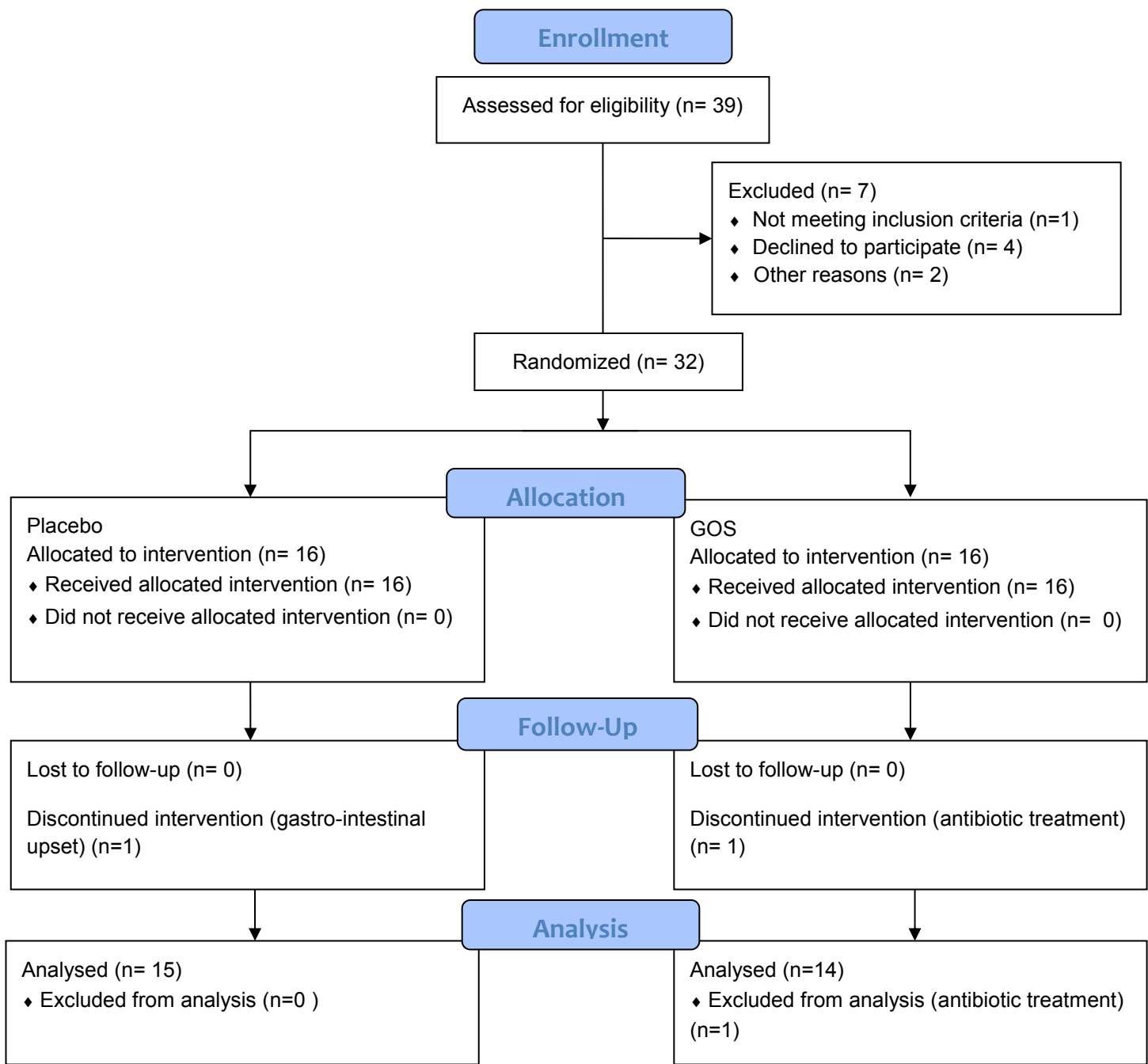


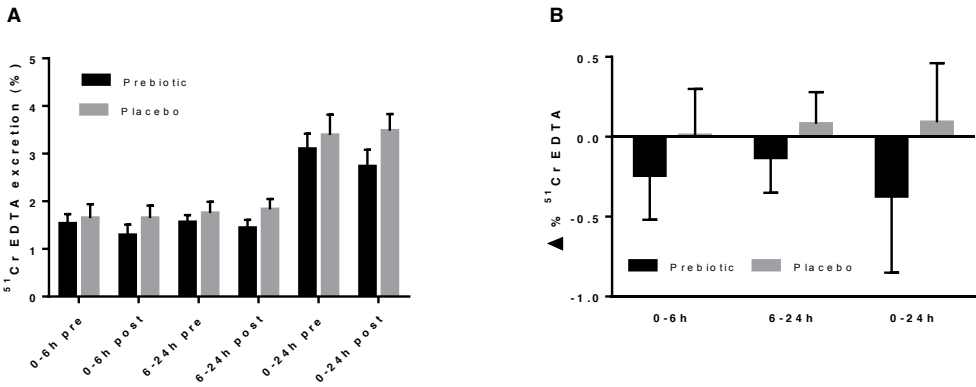
571 <sup>a</sup>Means  $\pm$  SEM or median (interquartile ranges) presented.  $n = 13$  for placebo group and  $n = 14$  for prebiotic  
572 group unless otherwise stated. There were no differences in baseline (PRE) values between groups ( $P > 0.05$ ,  
573 unpaired t-test or Mann-Whitney test). <sup>b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

579

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

581 **CONSORT 2010 Flow Diagram**





583

584 **Figure 2.** Intestinal permeability estimated by <sup>51</sup>Cr EDTA (mean and SEM) excreted in urine following 12  
585 weeks of prebiotic (black bars, n = 14) or placebo (grey bars, n = 15) supplementation. A. % <sup>51</sup>Cr EDTA  
586 excreted before (pre) and after supplementation (post) and B. change in <sup>51</sup>Cr EDTA excreted. There were no  
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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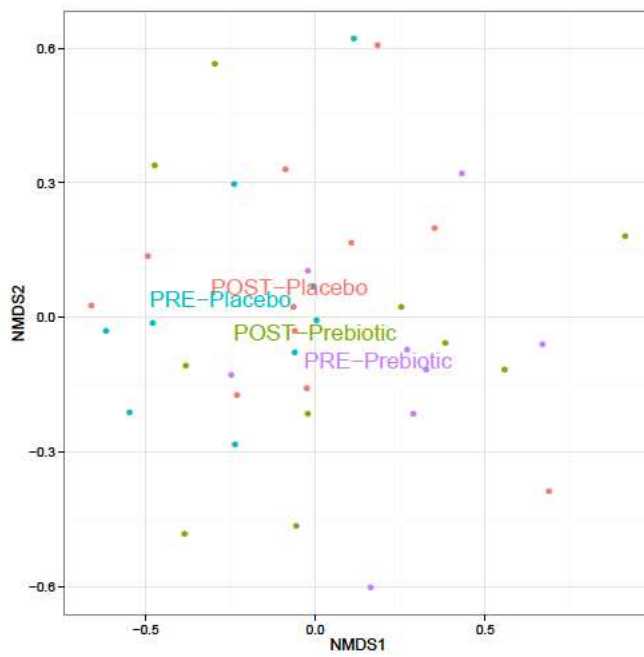
## **SUPPLEMENTARY MATERIALS AND METHODS**

### **DNA extraction**

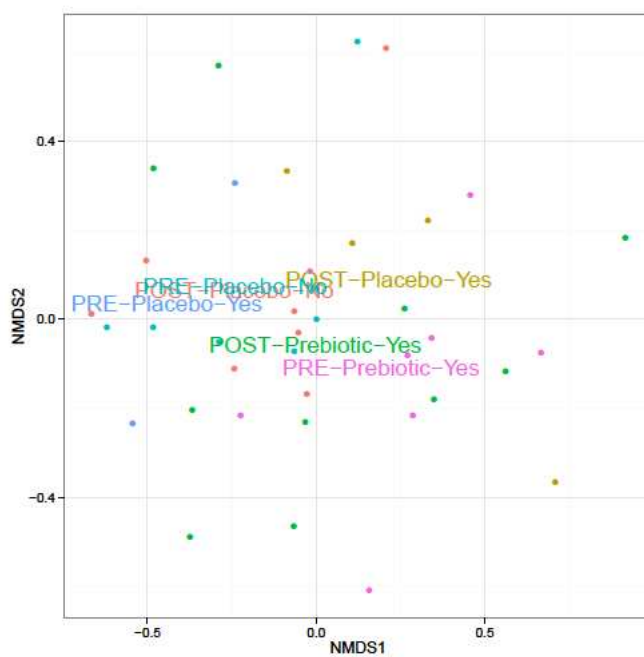
DNA was extracted from faecal samples using the PowerFecal™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA concentration and quality were measured by NanoDrop 2000 (Thermo Scientific) and Qubit 2.0 fluorometer (Invitrogen).

### **Amplification and High-Throughput Sequencing**

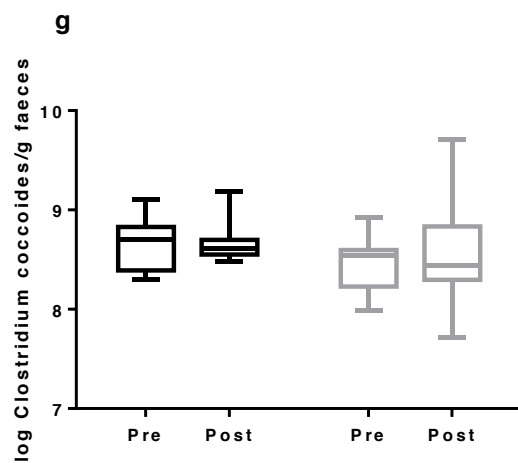
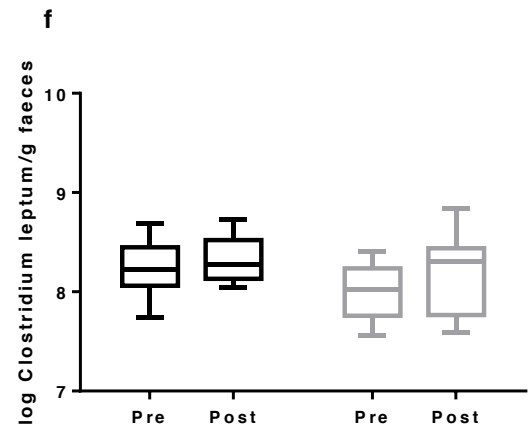
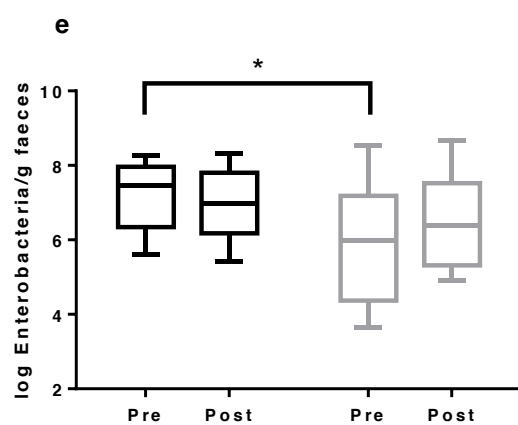
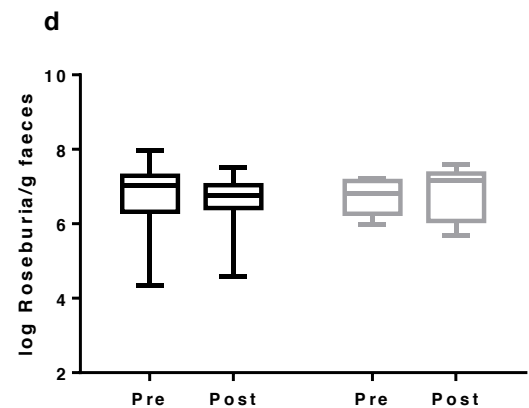
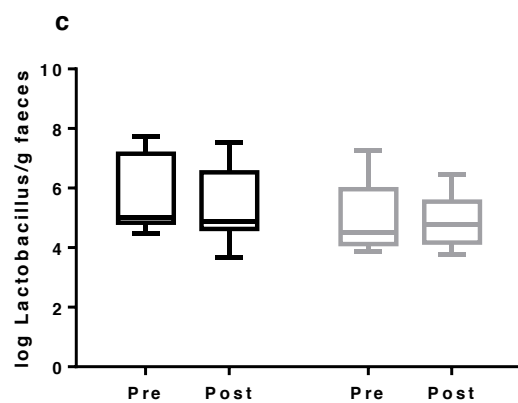
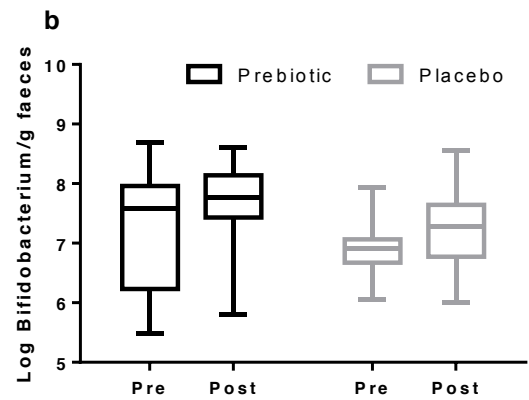
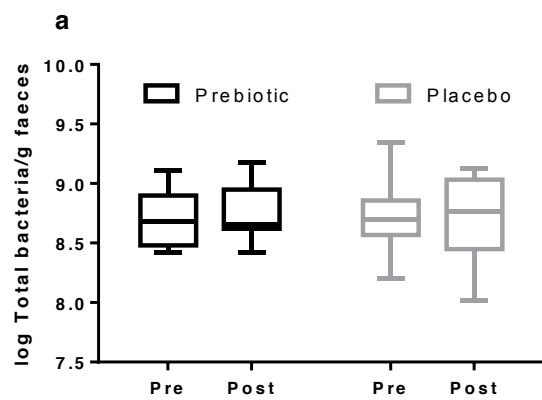
Briefly, the V4 and V5 region of the bacterial 16S rRNA gene was amplified from extracted DNA with universal primers (U515F: 5'-GTGYCAGCMGCCGCGGTA and U927R: 5'-CCCGYCAATTCMTTTRAGT). Forward fusion primers consisted of the GS FLX Titanium primer A and the library key (5' -CATCTCATCCCTGCGTGTCTCCGACTCAG) 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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