Gut Microbiome (2026), **7**, e13, 1–19
doi:10.1017/gmb.2026.10032

GUTMICROBIOME

RESEARCH ARTICLE

Exploring the prebiotic potential of commercial cellobiose: a randomized, controlled trial

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(Received 16 December 2025 UTC; revised 19 May 2026 UTC; accepted 02 June 2026 UTC)

Abstract

Cellobiose, a β-(1 → 4)-linked disaccharide indigestible by humans, is a novel candidate prebiotic. Evidence from controlled human trials on its effects on the gut microbiota and metabolites remains limited. We conducted an exploratory randomised, double-blind, placebo-controlled trial in 37 healthy adults. Participants were allocated to three arms for 4 weeks, received 5 g/day and 10 g/day for cellobiose, oligofructose P95, or maltodextrin placebo. The primary endpoint was change in absolute abundance of *Bifidobacterium* and *Lactobacillus* (measured by 16S rRNA gene sequencing). Secondary endpoints included other taxa, diversity, faecal short-chain fatty acids (SCFAs) measured by gas chromatography–mass spectrometry and gastrointestinal (GI) tolerability. Cellobiose did not significantly change *Bifidobacterium* or *Lactobacillus* versus baseline or placebo ($P > 0.05$). Oligofructose P95 induced a borderline increase in *Bifidobacterium* ($P = 0.049$). Overall community composition remained unchanged. However, network analyses under cellobiose revealed tighter positive correlations among key genera. Faecal SCFA levels were not altered. GI symptom rates were low and similar across all arms. Cellobiose, at doses up to 10 g/day, is safe and well tolerated but did not enrich classic beneficial taxa. The findings suggest cellobiose drives subtle community shifts towards butyrate-producing bacteria.

Keywords: cellobiose; prebiotic; gut microbiome; quantitative microbiome profiling; dietary intervention trial

Introduction

Modulation of the gut microbiota is an approach to support human health (Schmidt et al., 2018; Valdes et al., 2018; Hou et al., 2022; Ugwu et al., 2024). Some bacterial species are significantly associated with various health effects (Zafar and Saier, 2021; Negishi et al., 2025). *Bifidobacterium* and *Lactobacillus* have served as key genera due to their barrier-protection effects against inflammatory diseases and association with enhanced host immune system (O'Callaghan and van Sinderen, 2016; Dempsey and Corr, 2022). Prebiotics are substrates, selectively utilized by host microorganisms, conferring health benefits (Gibson et al., 2017). Most established prebiotics (e.g., inulin-type fructans, galacto-oligosaccharides) only target a narrow group of taxa (primarily bifidobacteria). Novel prebiotic candidates are needed to expand the range of selectively stimulated microbial communities beyond *Bifidobacterium*.

Cellobiose, a β-(1 → 4) linked glucose disaccharide, is potentially a novel prebiotic. It can be either derived enzymatically from cellulose in plant-based biomass or synthesized enzymatically from glucose and sucrose. Recent EU regulatory approval for its use in foods (Regulation 2023/943), based on a safety assessment submitted by Turck et al. (2022), allows human consumption of cellobiose, enabling dietary intervention studies. Although safety is established, the specific impact on the human gut microbiota at a

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community-wide level remains unknown. This study addresses this gap. To our knowledge, this is the first randomised control human trial to apply high throughput 16S rRNA gene sequencing to investigate how cellobiose modulates gut microbiota structure and metabolic outputs. The aim of the study was to move beyond the analysis of targeted bacterial groups by profiling the full bacterial community, allowing detection of broad shifts in diversity and specific compositional changes.

Methods

Ethics

The study was given ethical approval by the University of Reading Research Ethics Committee [UREC (24/20)] and was conducted in accordance with the Declaration of Helsinki. The study was registered at clinicaltrials.gov (NCT07097389). All participants gave written consent prior to study entry.

Volunteer recruitment and screening

Healthy adults (18 years old and above) were recruited in Reading, UK. Inclusion required no history of gastrointestinal (GI) disorders or food allergies, and regular bowel habits (≥3 movements/week). Exclusion criteria were antibiotic use within last 6 months; anaemia or any chronic/acute illness (e.g., pre-diabetes); cancer diagnosis or history of bowel resection; active smoking, alcohol/drug misuse; pregnancy or lactation.

Study design and interventions

This randomized, double-blind, parallel-group, placebo-controlled trial involved a 4-week intervention: 5 g/day (Week 0–1; adaptation) increasing to 10 g/day (Weeks 2–4). We chose a parallel design instead of a cross-over design to avoid unpredictable carry-over effects, ensuring that each group's baseline remained completely independent.

Prior to enrolment, eligible participants received both verbal and written information about the study and provided informed consent. Participants maintained their habitual diet but avoided other prebiotics/probiotics. The study interventions included: commercial cellobiose (Savanna GmbH, Germany), oligofructose (Orafti® P95, DP 3–9, average DP 4; Beneo-Orafti, Tienen, Belgium), and a maltodextrin placebo (Special Ingredients Ltd, UK). Participants were randomized (1:1:1) using a computer-generated sequence (https://www.randomizer.org/), and allocated via an online tool (https://ctrandomization.cancer.gov/). Blinding was ensured by identical packaging labelled with random letter codes; the decoding key was withheld from all personnel until analysis. All intervention bottles looked identical and bore only the letter code.

Stool samples were collected at baseline (V1) and post-intervention (V5). Compliance (≥95%) was verified via returned bottles and daily diaries. Each entry recorded the date and time of product consumption and daily tick-boxes for GI events (bloating; nausea; gas/flatulence; vomiting; abdominal pain or cramps; changes in stool consistency; diarrhoea; constipation; and frequency of bowel movements). All data were captured and managed using the secure Research Electronic Data Capture system hosted at the University of Reading, which facilitates validated data entry, audit trails, automated exports, and the integration of external data.

Outcome measures

Primary outcomes: Change in absolute abundance of faecal *Bifidobacterium* spp. and *Lactobacillus* spp. (16S rRNA sequencing and FISH-FLOW), changes in total faecal short-chain fatty acid (SCFA) concentration from V1 to V5.

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Secondary outcomes: Magnitude and pattern of overall gut-microbiota shift from V1 to V5 and GI tolerance and safety (daily symptom checkboxes; adverse events) during Days 1–28. Exploratory outcomes: SCFA-microbe correlations for each subject, $\log_2$-fold-changes (V5 vs. V1) in stool acetate, propionate, and butyrate. Spearman correlation coefficients (ρ) with 95% confidence intervals (CIs) were calculated to assess the relationship between the change in each SCFA and the $\log_2$-fold-changes in *Bifidobacterium*. For other key genera (e.g., *Faecalibacterium*, *Blautia*, *Roseburia*, and *Bacteroides*), compositional changes were reported as mean $\log_2$-fold-changes with 95% CIs. As an exploratory analysis, no formal hypothesis tests were performed; all results are presented as effect-size estimates only.

Faecal sample collection and processing

Participants received sterile stool collection containers for sample collection on V1 and V5. Immediately after collection, samples were placed in insulated bags with ice packs and retrieved from the participants' residences within 2 hours of voiding. Additionally, a 5 g portion of each sample was diluted at a ratio of 1:10 (w/w) in anaerobic phosphate-buffered saline (PBS; 0.1 M, pH 7.4) and homogenized using a stomacher at 230 paddle beats per minute for 2 minutes at room temperature. Following homogenization, 750 µL aliquots of the faecal slurry were centrifuged at 13,000 g for 5 minutes. Supernatants were removed, and pellets resuspended in 375 µL of PBS (0.1 M, pH 7.4) before fixing in 1125 µL of 4% (w/v) paraformaldehyde for 4 hours at 4 °C. After fixing, the samples were centrifuged again at 13,000 g for 5 minutes at room temperature, washed with 1 mL of PBS, and washing was repeated twice. The final pellet was resuspended in 300 µL of PBS and stored in a 1:1 (v/v) PBS–ethanol solution at −20 °C until further analysis by FISH. In parallel, 1.5-mL aliquots of the faecal slurry were centrifuged at 13,000 g for 10 minutes, and the supernatants were transferred to 1.5-mL tubes and stored at −80 °C for subsequent analyses. The pellets were saved for bacterial DNA extraction at −80 °C.

Enumeration of faecal microbial populations by *FISH-FLOW*

FISH-FLOW analysis was performed following Jackson et al. (2023). The probes used are detailed in Table 1. Fluorescence measurements were taken at 488 and 640 nm using a BD Accuri C6 Plus flow cytometer. To minimize background noise, thresholds were set at 9000 for the forward scatter and 3000 for the side scatter, with a gate established to capture 90% of the primary event population. The flow rate was maintained at 10 µL/min, and data were acquired until 100,000 events were collected, as managed by the Accuri CFlow Sampler software. Bacterial counts were calculated by adjusting flow cytometry data according to PBS dilution, with results expressed as the logarithm ($\log_{10}$) of cells per gram of fresh, wet faeces.

Bacterial DNA extraction

Fresh stool (1.5 g) was stored at −80 C for metabolic profiling analysis conducted after the intervention study was completed. Bacterial DNA was extracted from faecal samples using QIAamp PowerFaecal

Table 1. Name, sequence, and target group of oligonucleotide probes used in bacterial enumeration.

Probes	Sequence (5′ to 3′)	Targeted groups	Reference
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338; non bacteria	Wallner et al. (1993)
Eub 338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	Amann et al. (1990)
Eub 338 I-II	GCAGCCACCCGTAGGTGT	Most bacteria	Daims et al. (1999)
Eub 338 I-II-III	GCTGCCACCCGTAGGTGT	Most bacteria	Daims et al. (1999)

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(QIAGEN) according to the manufacturer's instructions. In summary, faecal samples were homogenized and aliquoted into 2 mL screwcap tubes containing 0.6 g 0.1 mm glass beads. Bead beating was performed using a Fastprep24 (MP Biomedicals) for 4 cycles of 45 s at speed 5. Then, 500 µL of raw extract were then used for DNA isolation.

DNA isolation, library preparation, and 16S rRNA gene sequencing

Extracted bacterial DNA was amplified by polymerase chain reaction (PCR) of the V4 region of the 16S rRNA gene with two-stage Nextera PCR libraries using the primer pairs 515F (50-GTG YCA GCM GCC GCG GTA A-30) and 806R (50-GGA CTA CNV GGG TWT CTA AT-30).

Raw sample extracts were diluted to 2.5 ng/µL using Tris buffer, and 5 µL were used in first step PCR, together with 5x HOT FIREPol MultiPlex Mix (Solis BioDyne) and 4 µM primer mix (forward and reverse) 515F/806R (Microsynth). First step PCR samples were purified with NGS Clean Beads (Labgene). Bead ratio was 1:1:2, and beads were washed with 75% ethanol, air-dried, and resuspended in the Tris buffer. In second-step PCR, each sample was individually barcoded, using Nextera XT Index Kit v2 (Illumina, San Diego, California) and 5x HOT FIREPol MultiPlex Mix (Solis BioDyne). Second-step PCR samples were purified with NGS Clean Beads (Labgene). The final second-step PCR products were quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). Amplicons were pooled in equimolar amounts prior to sequencing. The final pool was quantified using a Quant-iT Pico-Green dsDNA Assay Kit (Thermo Fisher Scientific) and Fragment analyser (Agilent).

Subsequent PCR libraries were sequenced on an Illumina MiSeq platform using a v2 500 (2250 bp read length). Pools were diluted to 9.2 pM and loaded together with 15% PhiX (Illumina, FC-110-3001) to increase diversity resulting in a raw cluster density of 631 and a cluster passed filter rate of 98%. Paired-end reads that passed Illumina's chastity filter were subjected to demultiplexing and trimming of Illumina adaptor residuals using Illumina's bcl2fastq software version v2.20.0.422. The quality of the reads was checked with the software FastQC version 0.11.8, and sequencing reads that fell below an average Q-score of 20 or had any uncalled bases (N) were removed from further analysis. The locus-specific V4 primers were trimmed from the sequencing reads with the software cutadapt v3.2. Paired-end reads were discarded if the primer could not be trimmed. Trimmed forward and reverse reads of each paired-end read were merged to reform *in silico* the sequenced molecule considering a minimum overlap of 15 bases using the software USEARCH version 11.0.667. Merged sequences were again quality filtered, allowing a maximum of one expected erroneous base per merged read. Reads that contain ambiguous bases or were outliers regarding the amplicon size distribution were also discarded. Samples that resulted in less than 5000 merged reads were discarded so as not to distort the statistical analysis. Remaining reads were denoised using the UNOISE algorithm implemented in USEARCH to form amplicon sequencing variants (ASVs), discarding singletons and chimeras in the process. The resulting ASV abundance table was then filtered for possible barcode bleed-in contaminations using the UNCROSS algorithm. ASV sequences were compared to the reference sequences of the RDP 16S database provided by https://www.drive5.com/usearch/manual/sintax_downloads.html, and taxonomies were predicted considering a minimum confidence threshold of 0.5 using the SINTAX algorithm implemented in USEARCH. The resulting library was then corrected by taking into consideration numbers of 16S copies and rarefying to an even sampling intensity to reduce bias in diversity metric calculations and quantified as described by Vandeputte et al. (2017).

Organic acids by gas chromatography-flame ionization detection

Faecal organic acids were extracted and quantified by gas chromatography-flame ionization detection (GC-FID) according to a modified Richardson et al. (1989) protocol. Briefly, the previously collected faecal fluids (1.5 mL) were thawed, and centrifuged at 11,337 × g for 10 min. One millilitre of sample was transferred into a labelled 100 mm × 16 mm glass tube (International Scientific Supplies Ltd, Bradford, UK) and 50 µL of 2-ethylbutyric acid (0.1 mol/L, internal standard), 500 µL concentrated HCl and 3 ml

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diethyl ether were added to each glass tube before vortexing for 1 min. Samples were centrifuged at 2,000 × g for 10 min. The resulting diethyl ether (upper) layer of each sample was transferred to clean 100 mL screw top glass tubes. Ether extract (400 µL) and 50 µL *N*-tert-butyldimethylsilyl)-*N*-methyl trifluoroacetamide were added into a GC screw-cap vial. Samples were left at room temperature for 72 h to allow samples to completely derivatise. An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30 m × 0.25 mm column with a 0.25 µm coating (cross-linked [5%-phenyl]-methylpolysiloxane, Hewlett Packard) was used for analysis of organic acids. Temperatures of injector and detector were 275 °C, with the column temperature programmed from 63 °C to 190 °C at 15 °C/min followed by 190 °C for 3 minutes. Helium was the carrier gas (flow rate 1.7 ml/min; head pressure 133 KPa). A split ratio of 100:1 was used. Calibration curves were generated using acetic, propionic, butyric, lactate, iso-butyric, iso-valeric, valeric, and succinic acids (12.5–100 mmol/L). Raw concentrations (mmol/L) were converted to total SCFA per gram of starting material by multiplying by the extract volume and dividing by the wet weight of faeces. Final SCFA levels are reported as µmol SCFA per g wet faeces (mean ± standard error [SE]).

Sample size and statistical analysis

Data were processed and analysed in RStudio 4.2 and Statistical Package for Social Science version 29.0.2.0 (SPSS Inc.). All graphs were generated in GraphPad Prism Version 10.00 for MacOS (GraphPad Software). To investigate the primary, secondary and exploratory outcomes, different statistical analysis were applied. Power analysis (80%, α =0.05) indicated 11 subjects per arm to detect a 20% change in *Lactobacillus* abundance. Twelve subjects per arm (n = 36) were planned to be enrolled to account for 10% dropout. In practice, 37 participants were randomised. All randomised participants were included in the intention-to-treat analyses; models are robust to unequal group sizes.

Assumption checks were first applied to check the normality of each outcome (FISH-FLOW, quantitative microbiome profiling [QMP] and metabolic outputs) and of model residuals was assessed by Kolmogorov–Smirnov and Shapiro–Wilk tests, together with Q-Q plots, homogeneity of variances was evaluated by Levene's test (based on means, medians, and trimmed means). Linearity, homoscedasticity, and homogeneity of regression slopes were examined in the context of the general linear model (GLM; scatterplots of covariate vs. dependent variable, residuals vs. predicted values, and tests of covariate × factor interactions).

For primary and secondary outcomes that met normality and variance-equality assumptions, repeated-measures GLMs (time as within-participant factor; treatment as between-participant factor) were applied to test change over time and treatment × time interactions in bacteriology outcomes. To adjust for baseline differences and participant sex, factorial analysis of covariance (ANCOVA) was performed on change-from-baseline values with baseline and sex as covariates. Pair-wise comparisons within each ANOCVA, repeated-measures general linear analysis of variance were Bonferroni-adjusted for type I errors. Outcomes failing normality of residuals (even after log- or other transformations) were analysed non-parametrically. Wilcoxon signed-rank tests were applied for within-intervention changes, and the Kruskal–Wallis H tests were also used for between-intervention comparisons, followed by Dunn's post-hoc tests with Benjamini–Hochberg false discovery rate (BH-FDR) adjustment. For exploratory outcomes, correlations between bacterial taxa and SCFAs were analysed using log [fold change (post-pre)] using non-parametric Spearman's rank correlation corrected for using FDR. Tests were two-tailed and P value <= 0.05 were considered statistically significant.

Results

Participant flow and baseline characteristic

In total, there were 44 volunteers who expressed interest and were screened for eligibility, of whom 39 were randomised (n = 13 in each group). Two volunteers withdrew from the trial, 37 volunteers

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completed it (14 males and 23 females) (n = 12, 12, 13 for each group) and were assessed in analysis for all primary, secondary, and exploratory outcomes. The Consolidated Standards of Reporting Trials (CONSORT) flow diagram is presented in Figure 1 in accordance with the CONSORT 2025 statement (Hopewell et al., 2025). Participants were recruited for the study between August 2024 and March 2025. The follow-up period for all participants concluded in April 2025, and the trial was completed as planned.

Based on returned bottle counts and consumption diaries, the mean adherence rate was 98% in the cellobiose group, 97% in the maltodextrin group and 99% in the OF P95 group. All participants included in the final analysis met the pre-defined compliance threshold of ≥95%. Table 2 summarizes baseline subject characteristics.

GI tolerability and bowel habit

For each GI symptom (bloating, nausea, gas/flatulence, vomiting, abdominal pain or cramps, diarrhoea, constipation), stool-frequency change, and stool-consistency change, we calculated the proportion of days (0–1) that was reported per participant (weekly counts ÷ 7). Median weekly proportions did not differ significantly across cellobiose, OF P95, and maltodextrin arms for any GI symptom (all Kruskal–Wallis qFDR >0.05). Gas/flatulence was the most reported GI symptom, occurring on a mean of 16.6% of study days across all participants (vs. 13.1% for stool-frequency changes and 10.4% for bloating). One participant in the maltodextrin arm withdrew early due to excessively frequent bowel movements that conflicted with their work. No other adverse events were reported. In short, these results indicate comparable GI tolerability among cellobiose, oligofructose, and maltodextrin at the tested doses, with low and stable rates of symptoms and stool changes throughout the 4-week intervention.

Bacterial enumeration by FISH-FLOW

All 37 participants provided paired stool samples at baseline (Visit 1) and after 4 weeks (Visit 5). Total bacterial counts, as measured by FISH-FLOW, did not change significantly from baseline to the end of the intervention in any of the three groups (Figure 2). A repeated-measures ANOVA confirmed that there was no significant effect of the intervention on total bacterial counts (see Supplementary Table 1 for full statistical results).

Microbiome profiling analysis–QMP

The core microbiome was defined as genera present in at least 50% of the 37 participants at either baseline (Visit 1) or the end of the intervention (Visit 5). The mean absolute abundances of the 25 most abundant genera at baseline are presented for each intervention at both time points (Figure 3). Overall, there were no significant changes in the composition of the core microbiome from baseline to the end of the intervention in any of the three groups.

The effects of the interventions on major phyla were assessed. Repeated-measures ANOVA revealed no significant changes from baseline to the end of the intervention in either the cellobiose or maltodextrin groups. However, the OF P95 group showed a significant decrease in two phyla: Euryarchaeota (P = 0.05) and Pseudomonadota (P = 0.021). A full statistical analysis is available in Supplementary Table 2. At the genus level, responses to the interventions varied. In line with the primary outcome, Figure 4 showed that the abundance of *Bifidobacterium* was significantly increased from baseline to the end of the intervention in the OF P95 group (P = 0.049). No significant changes in *Bifidobacterium* or *Lactobacillus* were observed in the cellobiose or maltodextrin groups. Full statistical results are available in Supplementary Table 3.

As shown in Figure 3, on an aggregate level, genus-level QMP abundance remained relatively stable across timepoints; however, underlying individual responses to the interventions were highly

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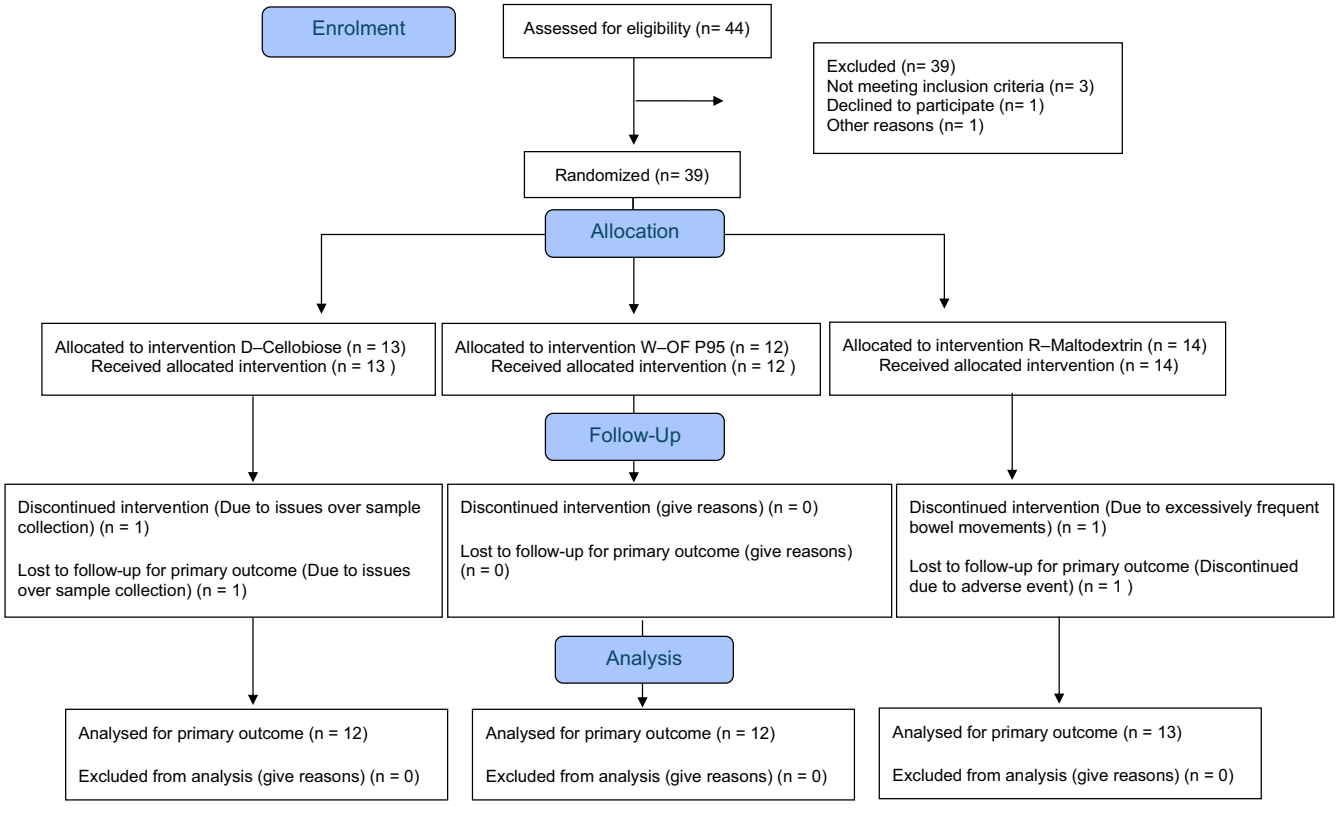


Figure 1. CONSORT flow diagram of participant progress through the phases of a three group, parallel randomised trial (enrolment, intervention allocation, follow-up, and data analysis). Abbreviations: OF P95, oligofructose; CONSORT, Consolidated Standards of Reporting Trials.

Table 2. Baseline participant characteristics

Characteristic	Cellobiose (*n* = 12)	OF P95 (*n* = 12)	Maltodextrin (*n* = 13)	Total (*n* = 37)
Age (y)	33.8 ± 17.2 (22–72)	37.1 ± 15.2 (20–82)	34.1 ± 12.0 (20–59)	35.0 ± 14.6 (20–82)
Male, n (%)	3 (25%)	6 (50%)	5 (38.5%)	14 (37.8%)
Female, n (%)	9 (75%)	6 (50%)	8 (61.5%)	23 (62.2%)

Note: Data are presented as standard error (SE) (range) for age, and n (%) for sex distribution. Abbreviation: OF P95, oligofructose P95.

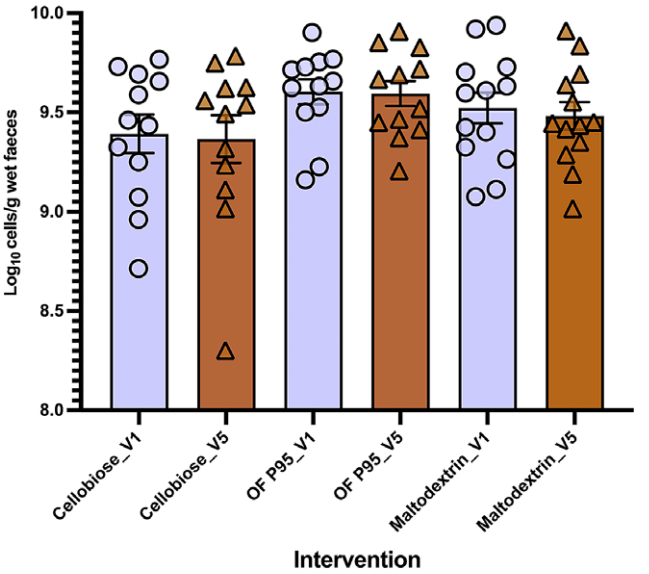


Figure 2. Differences in total bacteria measured by FISH-FLOW (log10 cells/g wet faeces) at visit 5 of the intervention using the Eub I-II-III probes. Values are mean and standard error (SE) (all points) expressed as log10 cells/g wet faeces. Results that are statistically significant between interventions are displayed by specified P values.

heterogeneous, with some participants exhibiting large increases in specific genera while others remained unchanged or declined. To account for this variability and control for baseline differences and sex, a two-way ANCOVA was performed (factors: intervention, gender; covariate: V1; dependent variable: V5). Baseline abundance was a strong predictor of follow-up levels ($F(1,30) = 21.44$, $P < 0.001$, partial $\eta^2 = 0.42$), and sex had a modest but significant effect ($F(1,30) = 5.01$, $P = 0.033$, $\eta^2 = 0.14$). The main effect of intervention was borderline ($F(2,30) = 3.31$, $P = 0.050$, $\eta^2 = 0.18$), and Bonferroni-adjusted pairwise comparisons between cellobiose, OF P95, and maltodextrin were all non-significant ($P > 0.05$), although OF P95 tended to exceed maltodextrin (mean difference = 2.54×10^8, $P = 0.053$).

The ANCOVA residuals for *Lactobacillus* abundance remained non-normal even after log-transformation (Shapiro–Wilk $P = 0.006$), necessitating the use of non-parametric analysis. At the end of the intervention (Visit 5), the median *Lactobacillus* abundance for all participants was 1.18×10^7 cells/g (interquartile range: 6.33×10^6–3.01×10^7). A Kruskal–Wallis H test found no significant difference in the distribution of *Lactobacillus* abundance across the three intervention groups ($H(2) = 1.19$, $P = 0.551$). A median test likewise showed no difference in the proportion of participants above vs. below the overall median ($\chi^2(2) = 0.999$, $P = 0.607$). These results indicate that post-treatment *Lactobacillus* abundance did not differ among the cellobiose, OF P95, and maltodextrin groups.

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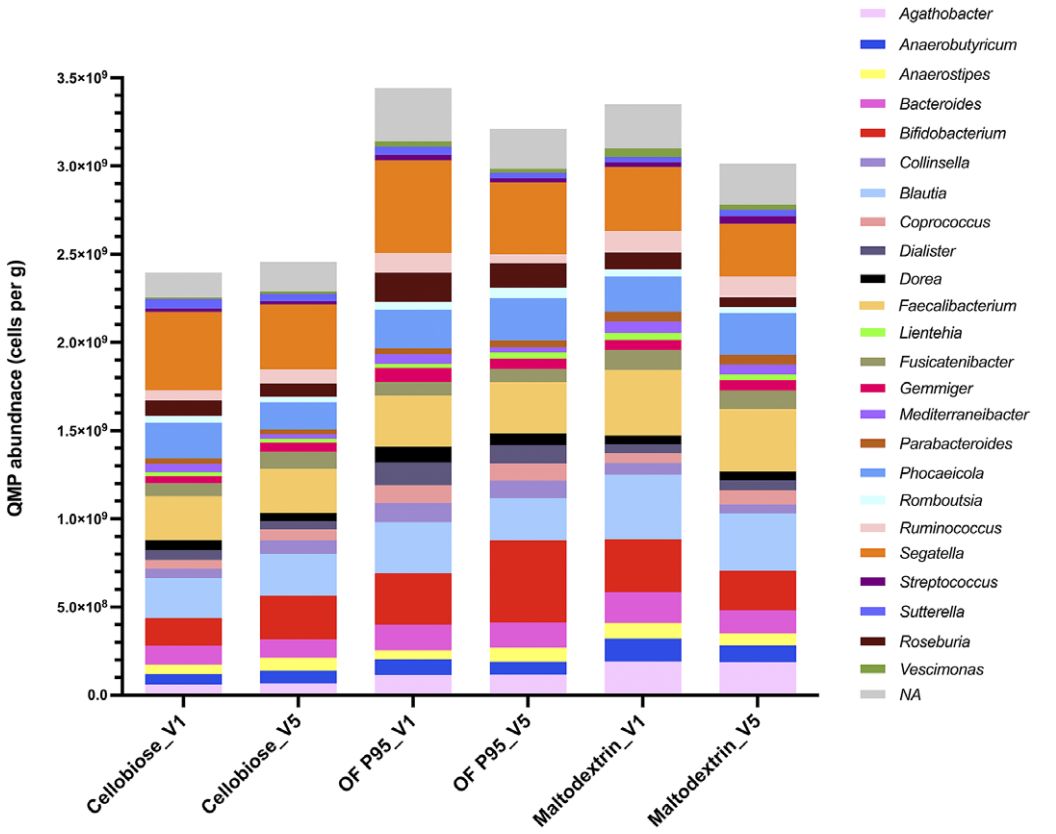


Figure 3. Quantitative microbiome profile (QMP) based on 16S rRNA sequencing across all interventions at V1 and V5. Numbers are expressed as cells/g faeces.

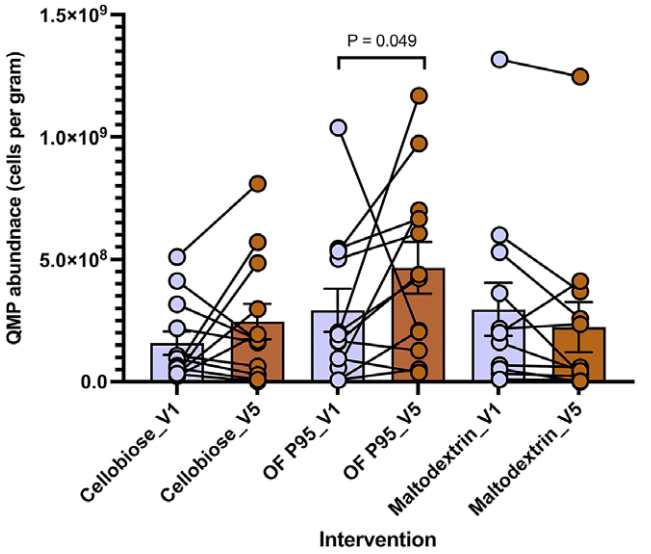


Figure 4. Quantitative microbiome profile differences in *Bifidobacterium* based on 16S rRNA sequencing at baseline (V1) and completion (V5) of the intervention in 37 adults. Individual, mean, and standard error (SE) were presented and expressed as numbers of cells/g faeces. Repeated-measure ANOVA was used to calculate intervention effect. Results that are statistically significant between interventions are displayed by specified *P* values. Abbreviation: OF P95, Oligofructose P95.

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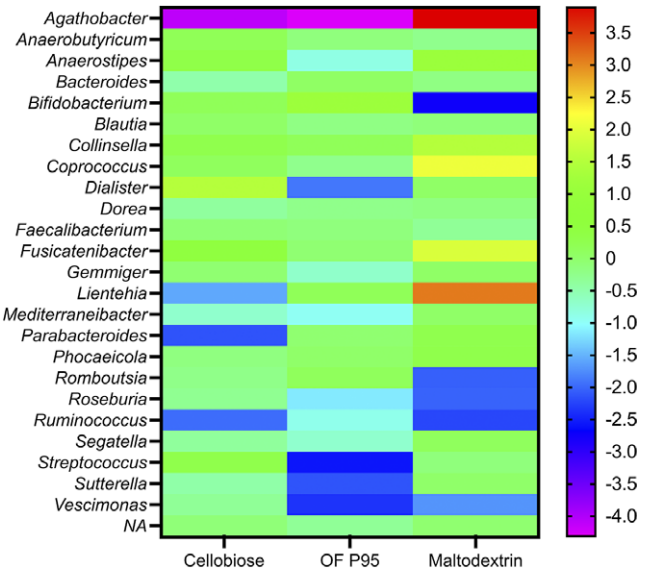


Figure 5. Heatmap of mean $\log_2$ fold-change in top 25 selected genus level for each arm across the full cohort (n = 37). Log_2 fold-change values range from −4 (strong negative) to +4 (strong positive); colour intensity reflects the magnitude and direction (purple = negative, red = positive). Abbreviations: OF P95, oligofructose P95; NA, not assigned at the sequencing level.

Besides major bacterial taxa, the magnitude and pattern of overall gut-microbiota shift from V1 to V5 was also investigated. Log_2-fold-changes (V5/V1) were calculated and plotted in a heatmap (Figure 5) for each genus within each arm, they were compared across arms using ANOVA with FDR correction. Overall stability was observed across the core community: 17 of the 25 genera exhibited mean $\log_2$-fold-changes between −2 and +2 in all three arms, corresponding to less than a twofold shift in abundance. None of the 25 genera show a statistically significant difference in mean Log_2-fold-changes across arms (adjusted p-values [q-FDR] > 0.05). To further understand the correlations between these changes, a correlation heatmap was generated among the top 25 genera, grouping by each intervention, as an exploratory, hypothesis-generating analysis. Figure 6 and Supplementary Table 4 show the genus-genus correlation matrix. In the cellobiose group, strong positive correlations ($\rho > 0.6$) were widespread. *Anaerostipes* formed the most links, correlating positively with 12 of the top 25 genera, nearly half of the network. Its strongest associations were with *Blautia* (Spearman's $\rho = 0.98$, $P = 9.3 \times 10^{-6}$), *Coprococcus* (Spearman's $\rho = 0.92$, $P = 7.1 \times 10^{-4}$), *Faecalibacterium* (Spearman's $\rho = 0.92$, $P = 5.6 \times 10^{-4}$), *Fusicatenibacter* (Spearman's $\rho = 0.92$, $P = 5.6 \times 10^{-4}$), and *Gemmiger* (Spearman's $\rho = 0.93$, $P = 3.0 \times 10^{-4}$). *Bifidobacterium* was also found to be positively correlated with *Blautia* (Spearman's $\rho = 0.83$, $P = 0.006$), *Coprococcus* (Spearman's $\rho = 0.84$, $P = 0.005$), *Dorea* (Spearman's $\rho = 0.87$, $P = 0.002$), *Faecalibacterium* (Spearman's $\rho = 0.85$, $P = 0.003$), *Fusicatenibacter* (Spearman's $\rho = 0.86$, $P = 0.003$), *Gemmiger* (Spearman's $\rho = 0.90$, $P = 0.001$), *Segatella* (Spearman's $\rho = 0.70$, $P = 0.031$), and *Streptococcus* (Spearman's $\rho = 0.67$, $P = 0.050$). Conversely, OF P95 produced a fragmented network, *Anaerobutyricum* was found to be the most connected, correlating positively with *Bifidobacterium* (Spearman's $\rho = 0.80$, $P = 0.027$), *Blautia* (Spearman's $\rho = 0.91$, $P = 0.006$), *Collinsella* (Spearman's $\rho = 0.88$, $P = 0.009$), *Dialister* (Spearman's $\rho = 0.79$, $P = 0.033$), *Fusicatenibacter* (Spearman's $\rho = 0.74$, $P = 0.050$), and *Gemmiger* (Spearman's $\rho = 0.86$, $P = 0.013$). While the maltodextrin group maintained only a few significantly positive correlations, reflecting a baseline community structure.

SCFA quantification

Metabolic profiles of faecal samples across three interventions at baseline and completion were analysed. Figure 7 and Supplementary Table 5 summarize the concentrations of total organic acids, acetate,

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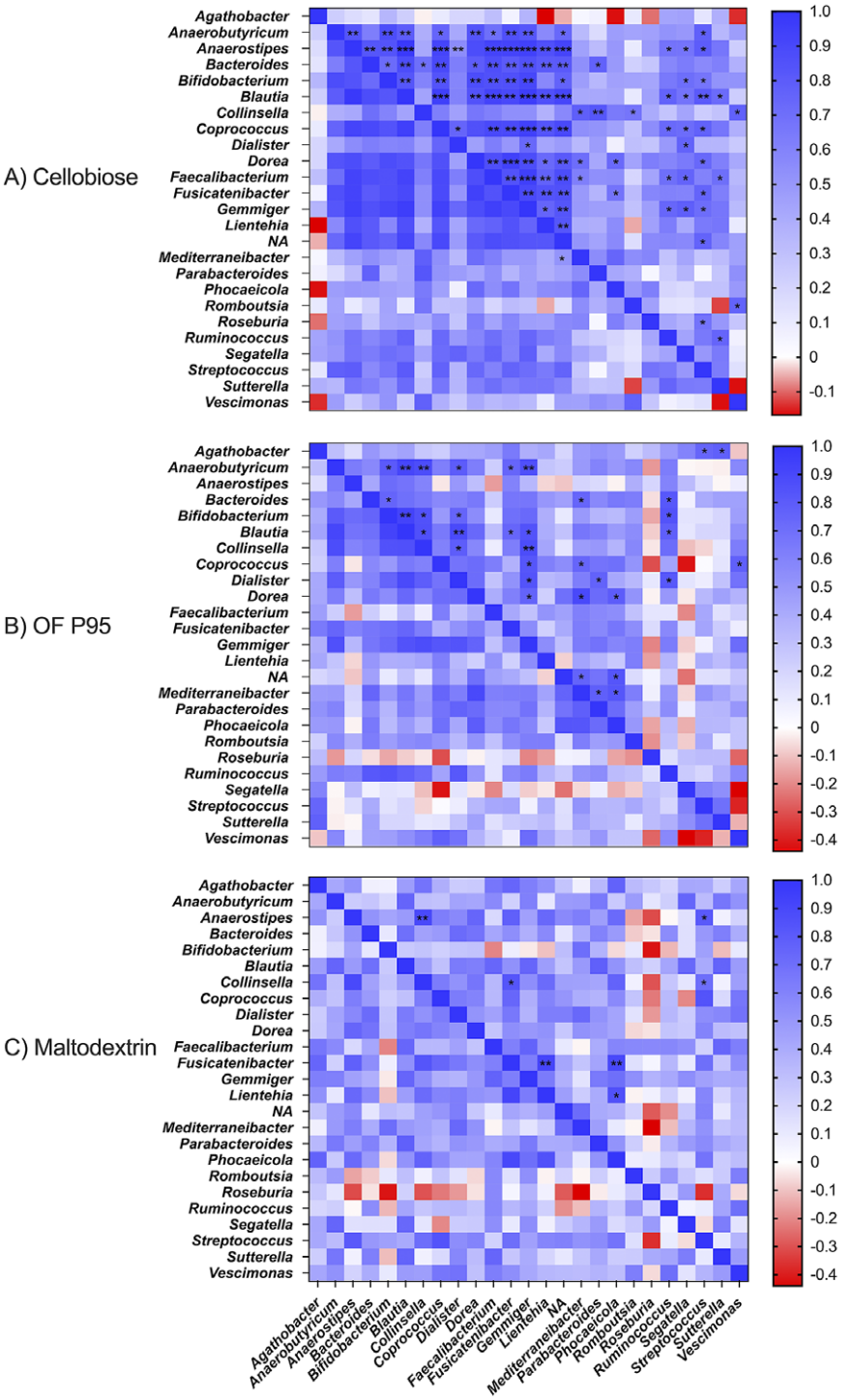


Figure 6. Heatmap of Spearman genus–genus correlations within each intervention. Cell colour reflects correlation coefficient (blue = positive, red = negative). Asterisks (*$P \leq 0.5$; **$P \leq 0.01$; ***$P \leq 0.001$) denote correlations significant after BH-FDR correction; only the upper triangle of each symmetric matrix is annotated to avoid duplication. Abbreviations: OF P95, oligofructose P95; NA, not assigned at the sequencing level.

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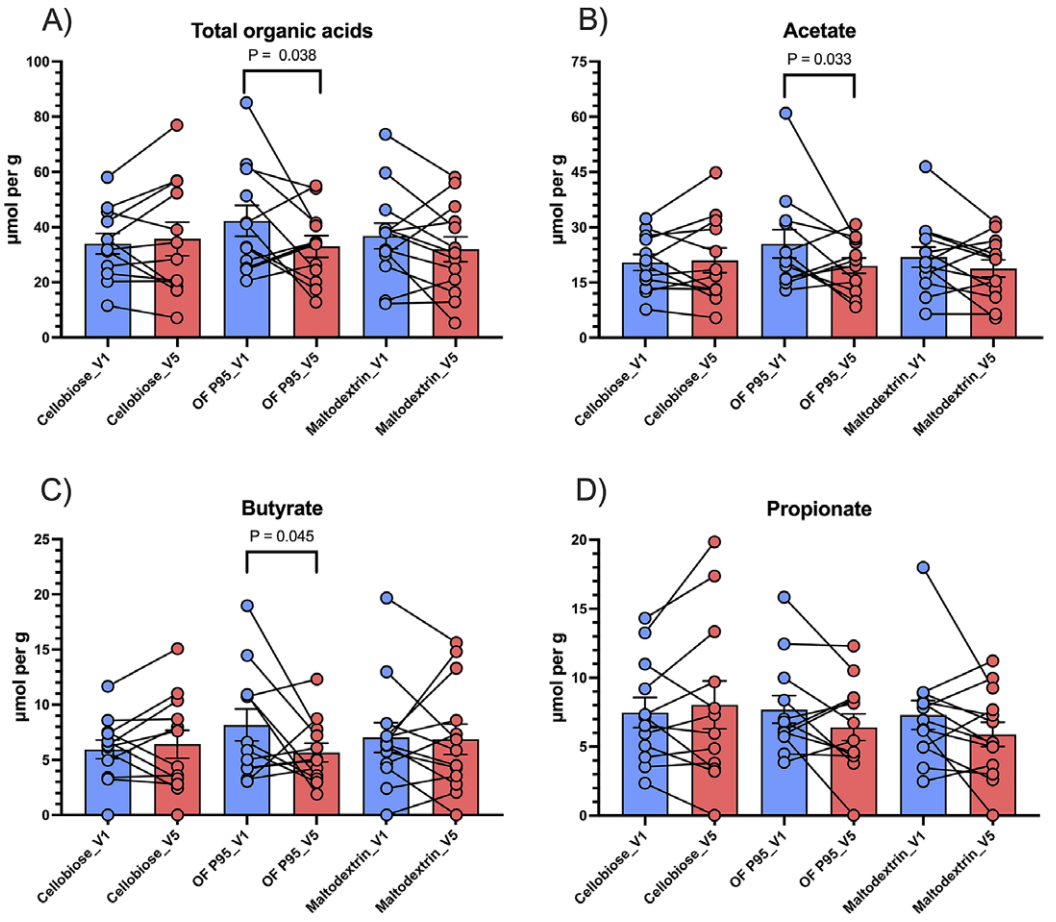


Figure 7. Amount of total organic acids (A), acetate (B), butyrate (C) and propionate (D) from fresh wet faeces measured by gas chromatography at V1 and V5. Individual, mean and SE were presented and expressed as numbers of µmol/g faeces. Results that are statistically significant within respective treatments are displayed by specified P-values. Error bars represent SE. Abbreviations: OF P95, Oligofructose P95.

butyrate, and propionate. Organic acids were unchanged from V1 to V5 in cellobiose and maltodextrin ($P > 0.05$), while OF P95 induced a significant decrease ($P = 0.038$). Acetate and butyrate showed no significant differences over time in cellobiose and maltodextrin ($P > 0.05$). Propionate levels remained stable with no significant changes over time across all intervention groups. Crucially, when comparing the net changes from baseline between the different groups, no statistically significant differences were observed for any of the analysed metabolites.

OF P95 again showed a significance decrease over time in acetate ($P = 0.038$) and butyrate ($P = 0.045$). Propionate showed no significant differences over time in both interventions. However, between-group comparisons of change from baseline were not significant.

In short, neither total nor individual SCFAs were significantly affected by cellobiose and maltodextrin, while OF P95 exhibited a significant decrease in total SCFAs and key organic acids. The individual subject trajectories revealed a split: most subjects show an increase in total SCFA (and in each of acetate, propionate, and butyrate) in the cellobiose group, while most subjects induced a decrease in those same SCFA in OF P95 and maltodextrin.

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Correlations between bacteriology and SCFA quantification

To investigate the relationships between changes in taxa and SCFA quantification, a correlation matrix using the fold change [Log2 (post/pre)] for of the QMP genera and SCFA was constructed (Figure 9). The data were analysed using a non-parametric Spearman's rank correlation analysis, further adjusted using BH-FDR. Figure 8 and Supplementary Table 6 summarize the overall correlation heatmap between SCFA and top 25 bacteria taxa. No correlation reached statistical significance after BH-FDR correction (all $q > 0.05$). But 22 out of 25 genera had positive Spearman's ρ with acetate fold-change (mean ρ = 0.11, range −0.20 to 0.34), yet 19 of 25 genera had negative Spearman's ρ with propionate fold-change (mean ρ = −0.1, range −0.30 to 0.14).

No genus–SCFA pair reached significance ($q < 0.05$) in any intervention. However, there are some clear trends (Figure 9); in the cellobiose group, most genera correlated positively with acetate (median ρ = +0.12). *Mediterraneibacter* had the strongest correlation with acetate (ρ = 0.85; raw $P = 0.0005$; $q = 0.052$). Correlations with butyrate and propionate were weak and variable (median $|\rho| < 0.05$). In OF P95, genera tended to correlate negatively with butyrate (median ρ = −0.18) and propionate (median ρ = −0.15), mirroring the overall decline in these SCFAs despite increased bacterial counts. Acetate correlations were small (median ρ = −0.02). Last but not the least, in the maltodextrin group, correlations clustered around zero for all SCFAs (median ρ ≈ 0.04), in line with minimal changes in both bacterial abundance and SCFA levels.

Discussion

This randomised, controlled trial used QMP and a pre-specified analysis plan to test three carbohydrate interventions, cellobiose, oligofructose P95 (OF P95), and maltodextrin. The primary endpoints were *Bifidobacterium* and *Lactobacillus* absolute abundances. Neither genus changed significantly from baseline after cellobiose consumption. In contrast, OF P95 produced a borderline increase in

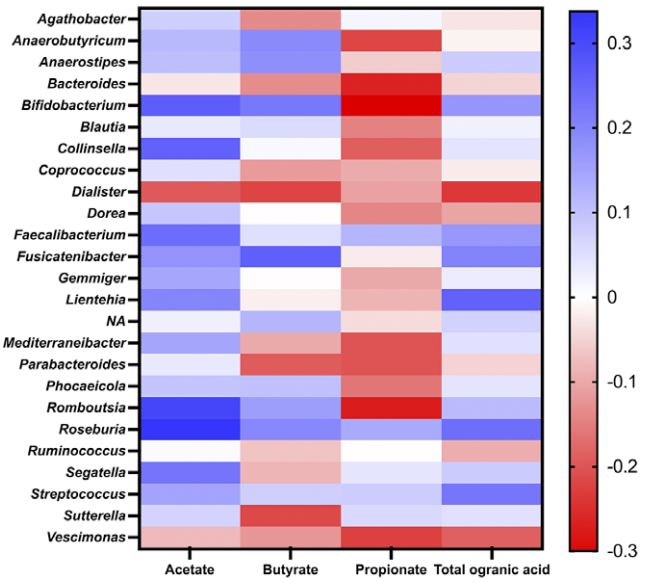


Figure 8. Heatmap of Spearman's rank correlations between bacterial genera and SCFA $\log_2$–fold-changes across the full cohort (n = 37). Spearman's ρ (two-sided) was calculated for each genus–SCFA pair and adjusted for multiple testing with the Benjamini–Hochberg FDR. ρ values range from −0.3 (strong negative) to +0.3 (strong positive); colour intensity reflects the magnitude and direction of ρ (blue = negative, red = positive). Asterisks denote correlations surviving FDR (q < 0.05). Abbreviations: NA, not assigned at the sequencing level.

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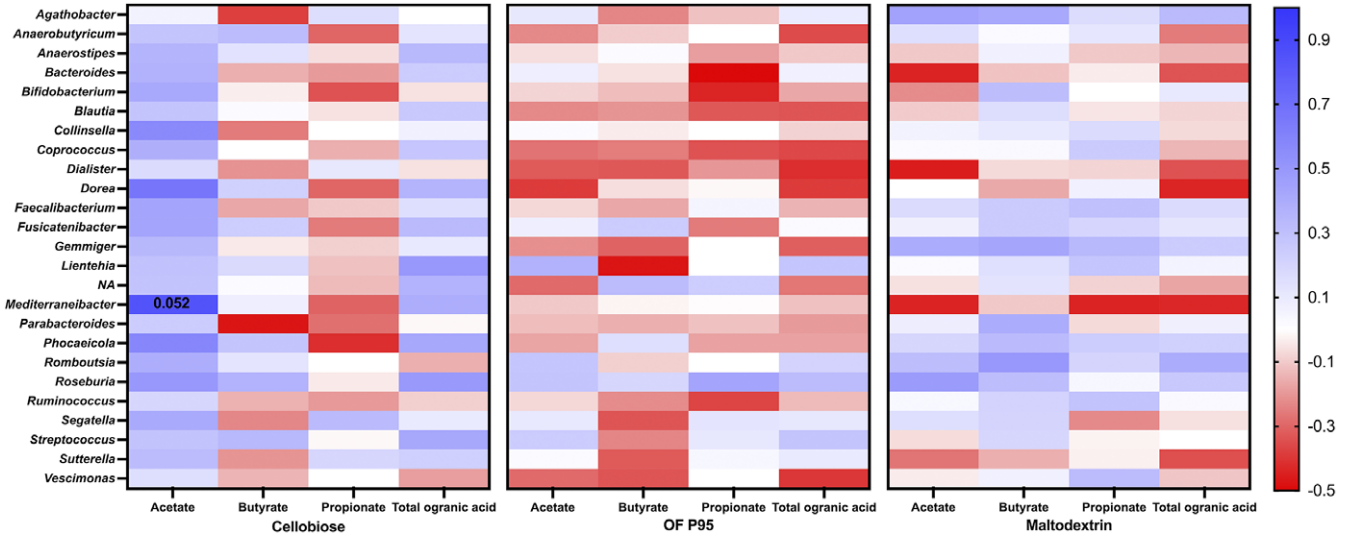


Figure 9. Heatmap of Spearman's rank correlations between bacterial genera and SCFA $\log_2$-fold-changes across the full cohort (n = 37), grouped by intervention. Spearman's ρ (two-sided) was calculated for each genus–SCFA pair and adjusted for multiple testing with the BH-FDR. ρ values range from −0.5 (strong negative) to +1 (strong positive); colour intensity reflects the magnitude and direction of ρ (blue = positive, red = negative). Specific value denotes correlations surviving FDR ($q < 0.05$). Abbreviations: OF P95, oligofructose P95; NA, not assigned at the sequencing level.

Bifidobacterium, consistent with its well-documented bifidogenic activity (Kolida et al., 2002; Meyer and Stasse-Wolthuis, 2009; Jackson et al., 2023; Van Harsselaar et al., 2024), while maltodextrin had no clear effect. Secondary analyses of overall composition showed only small, non-significant differences between interventions. Exploratory co-abundance networks revealed intervention-specific patterns. Cellobiose was associated with dense, positive correlations among butyrate-linked genera, *Anaerobutyricum*, *Anaerostipes*, and *Coprococcus*, with *Blautia* positioned as a major acetate producer (Shetty et al., 2020; Hosomi et al., 2022; Sheridan et al., 2022; Notting et al., 2023). *Bifidobacterium* clustered alongside *Gemmiger*, *Faecalibacterium*, and *Dorea* (strong positive ρ). This pattern fits known cross-feeding relationships between bifidobacteria (lactate producers) and butyrate producers (lactate consumers) (Duncan et al., 2004; Belenguer et al., 2006; Vital et al., 2014). In contrast, OF P95 networks were more fragmented, and maltodextrin largely preserved baseline structure. The correlation between *Mediterraneibacter* $\log_2$-fold-change and acetate under cellobiose was strong but borderline. Furthermore, it is worth noting that maltodextrin, while utilized as a placebo control in this trial, may not be entirely inert. Emerging evidence indicates that maltodextrin can subtly modulate gut microbial composition and metabolic pathways (Almutairi et al., 2022), a factor that should be kept in mind when evaluating comparative baseline-to-endpoint changes between the groups.

As for the tolerability and safety, GI symptoms and stool measures were similar across interventions, indicating good tolerability of the tested cellobiose at 5–10 g/day. This result is supported by a previous human trial where cellobiose was shown to be highly tolerable, with single doses of 20 g causing no issues and repeated 15 g doses causing only minor, temporary symptoms (Moré et al., 2020). Compared with the broader prebiotic literature, inulin-type fructans/oligofructose show a dose-dependent GI profile, gas/bloating rise above ~10 g/day, although typical prebiotic doses (≈5–10 g/day) are generally acceptable, placing our findings within expected tolerability bands for fermentable carbohydrates (Kolida and Gibson, 2007; Bonnema et al., 2010; Le Bourgot et al., 2022).

Given that fructan-type substrates are preferentially fermented by bifidobacteria, the observed bifidogenic effect of OF P95 is expected. The lack of a *Lactobacillus* response is also expected, as *Lactobacillus* is typically a minor member of adult faecal communities and is more characteristic of the upper gut (Heeney et al., 2018; Jensen et al., 2023); hence, it is often hard to detect stable increases in stool collection studies. These taxa are also strain-specific and often respond to different carbohydrate niches than fructans (Kaplan and Hutkins, 2000; Walton et al., 2012; Zúñiga et al., 2018; Zheng et al., 2020; Huang et al., 2022). In short, the data suggest that while OF P95 was specifically bifidogenic, the effect of cellobiose on *Bifidobacterium* was not statistically significant. Instead, cellobiose appears to alter community interactions among various butyrate-producing bacteria rather than driving a large shift in any single genus.

A strength of this study is the use of QMP to report absolute counts (cells/g faeces) rather than relative abundances. Percentages can be misleading when increases in one taxon mathematically lower others' percentages (Gloor et al., 2017). This method has been used for linking community variation to microbial load (Vandeputte et al., 2017). To improve the interpretability of our data, we focused our analysis on a predefined group of core genera that were prevalent across most participants. This approach reduces the statistical noise from rare taxa, which can show misleadingly large fold-changes even if they are only present in one or two individuals (Weiss et al., 2017).

This study's ability to detect genus-level microbial shifts was likely limited by its brief duration and the use of modest doses (5–10 g/day). These factors, combined with a relatively small sample size ($n = 37$), mean that more subtle or strain-specific changes may have been missed. Furthermore, while participants remained on their normal diets to improve the external validity of the findings, this introduced dietary variability as a confounding factor that could have masked smaller prebiotic effects.

The exploratory multi-taxon analyses involved many statistical comparisons, which increases the likelihood of finding false positives by chance, even after applying a FDR correction. To address this, our interpretation strategy prioritized biologically meaningful signals over simple statistical significance. We therefore focused on the magnitude of change (effect sizes) and consistent trends across related bacterial groups, treating these exploratory results as hypothesis-generating findings for future research.

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Finally, QMP provides stool-based absolute counts and therefore characterises luminal communities; it does not capture the mucosa-associated microbiota and cannot localise changes to specific colonic regions; conclusions are therefore restricted to stool. Likewise, faecal SCFA concentrations are conservative endpoints: because most SCFA are absorbed and metabolised in the large intestine, stool levels reflect the residual rather than the production rate and are sensitive to transit and dilution effects.

In summary, this *in vivo* intervention trial confirmed the selective fermentation of OF P95 by *Bifidobacterium* and suggested that cellobiose may reshape co-abundance patterns among butyrate-linked genera. The core community remained stable in absolute terms. While no statistically significant differences were observed between the intervention groups, the data suggest a trend towards an increase in beneficial bacteria following cellobiose consumption.

We did not find major shifts in total microbial numbers or endpoint metabolite levels. However, this does not mean cellobiose has no biological activity. These results show the limits of testing a healthy, resilient gut using only static "before and after" measurements, which can easily miss fluid ecosystem changes happening during the intervention. Because a single human study does not give a definitive result, continuous *in vivo* testing is necessary to consistently validate these ingredients. Future human trials must inevitably continue to rely on robust pre- and post-intervention designs to provide essential clinical data.

To complement these essential human trials and better validate underlying mechanisms, future work can pair *in vivo* studies with dynamic *in vitro* gut models, such as continuous fermentation systems. These laboratory systems offer a controlled platform to track rapid community interactions and metabolic fluxes in real time. Using *in vitro* tools to resolve these transient pathways will provide key mechanistic insights that can then be used to better design and validate subsequent, fully powered human interventions.

Nevertheless, these proof-of-concept data from our human trial indicate that cellobiose is well-tolerated and shows a promising trend towards modulating the gut microbiota.

Establishing the definitive prebiotic status of cellobiose will require a broader body of evidence, as outlined by consensus panels (Gibson et al., 2017; Hutkins et al., 2024). Future work should also build on the findings of this study by employing larger, longer-duration clinical trials with predefined health endpoints. Furthermore, applying methods such as shotgun metagenomics and metabolomics in future trials would allow for a mechanism analysis, directly linking specific microbial genes to the metabolic products of cellobiose fermentation in the human gut.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/gmb.2026.10032.

Data availability statement. Processed data supporting the findings of this article will be publicly deposited in the University of Reading Research Data Archive upon acceptance for publication. The data are fully de-identified to comply with ethical and privacy guidelines and the Helsinki Declaration. The data are currently available from the corresponding author, R.A.R., upon reasonable request and for peer review purposes. The study protocol was pre-registered at ClinicalTrials.gov (Identifier: NCT07097389) and the full protocol document is provided within the Supplementary Material accompanying this article.

Acknowledgements. The authors would like to express their sincere gratitude to Dr. Abigail Basson and Dr Patricia Sanz Morales for their invaluable guidance with 16S rRNA sequencing and GC-FID. We also thank Christy Chung for her technical assistance. Finally, the authors acknowledge Savanna Ingredient GmbH for providing the cellobiose used in this study.

Author contribution. Conceptualization, M.H. and R.A.R.; Methodology, P.P.J.J.; Investigation, M.H.; Project administration, M.H., A.C., and R.A.R.; Supervision, P.P.J.J., A.C., and R.A.R.; Writing—original draft, M.H.; Writing—review and editing, P.P.J.J., A.C., and R.A.R.

Funding. This research received no specific grant from any funding agency, commercial, or not-for-profit sectors.

Disclosure statement. No conflict of interest declared from author(s).

https://doi.org/10.1017/gmb.2026.10032 Published online by Cambridge University Press

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Cite this article: Huang M, Jackson PPJ, Chatzifragkou A and Rastall RA 2026. Exploring the prebiotic potential of commercial cellobiose: a randomized, controlled trial. *Gut Microbiome,* **7**, e13, 1–19. https://doi.org/10.1017/gmb.2026.10032

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