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# Cellobiose as a candidate prebiotic: enhanced butyrate production in an in vitro human gut fermentation model

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## Abstract

***Aims*** **While the prebiotic potential of cellobiose has been suggested previously, this study extends current knowledge by including more microbial responses in a controlled human gut model. The fermentation profile of cellobiose was compared with OF P95 and a negative control using faecal samples from healthy donors ($n = 3$).**

***Methods and results*** **Fluorescent *in situ* hybridization with flow cytometry was used to quantify key bacterial groups, and gas chromatography assessed organic acid production over 48 h. Both carbohydrates induced significant alterations in microbiota profiles and organic acid production compared to baseline and negative controls. Cellobiose fermentation significantly increased total organic acids, acetate, and butyrate from baseline, with significantly higher total organic acids and butyrate levels than the negative control at 48 h ($P = 0.002$ and $P = 0.016$, respectively). Distinct temporal shifts were observed for total bacteria and *Atopobium* with cellobiose, while *Bifidobacterium* was not significantly stimulated, contrasting with potent bifidogenic activity with OF P95 (e.g. T0–T48 increase, $P < 0.001$) and generally more pronounced total SCFA and acetate yields.**

***Conclusions*** **These findings validate prior indications but also extend current knowledge, showing that cellobiose has a distinct fermentation profile with potential for specific SCFA modulation, particularly butyrate.**

## Impact statement

**While certain fibres are known to modulate the gut microbiota, the search for novel and accessible prebiotics is a continuous process. This research provides key *in vitro* evidence that cellobiose, a simple disaccharide, possesses prebiotic potential by selectively promoting the growth of beneficial bacteria. This work identifies cellobiose as a promising candidate for future *in vivo* studies, providing the foundational knowledge required to establish its prebiotic status.**

**Keywords** cellobiose, prebiotic, gut microbiota, in vitro fermentation, short-chain fatty acid (SCFA)

## Introduction

The human gut microbiome is increasingly recognized as an important biomarker of host health, with many strategies being developed to beneficially modulate these complex microbial communities (Smith et al. 2019, Rebersek 2021, Wu et al. 2021, Pang et al. 2023). Prebiotics, defined as selectively fermented substrates that confer health benefits via modulation of the gut microbiota, have emerged as a key strategy for promoting beneficial microbial communities (Gibson et al. 2017).

Carbohydrate-based prebiotics are structurally diverse, varying significantly in their degree of polymerization (DP) and types of glycosidic linkages, which in turn dictates their fermentability and physiological effects (Guarino et al. 2020, You et al. 2022). Short-chain oligosaccharides (SCOs), characterized by a DP of 2–5, are often fermented more rapidly and potentially in more proximal regions of the colon compared to their long-chain structures (Stewart et al. 2008, van Trijp et al. 2024). These structural variations give opportunities to develop new prebiotics targeting specific microbial changes or metabolic outputs (Spacova et al. 2020). Emerging prebiotic candidates, including xylo-oligosaccharides (XOS), pectic-oligosaccharides (POS), manno-oligosaccharides (MOS), arabinoxylo-oligosaccharides (AXOS), and cello-oligosaccharides (COS), show considerable promise. For instance, XOS significantly increased faecal *Lactobacillus* spp. and *Bifidobacterium* spp. counts *in vivo* (Lin et al. 2016), while AXOS also elevated faecal *Bifidobacterium* concentrations *in vivo* (Maki et al. 2012); POS has demonstrated anti-inflammatory potential, linked to its promotion of *Eubacterium eligens*, a commensal bacterium with anti-inflammatory properties (Chung et al. 2017); MOS have shown positive effects on bacteria such as *Lactobacillus casei* and stimulated considerable short-chain fatty acids (SCFA) production (Jana

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et al. 2021). COS also present distinct prebiotic potential (Avila et al. 2021). However, current evidence for POS, COS, and MOS largely comes from traditional culture-based methods or animal models. Therefore, their comprehensive prebiotic assessment in human subjects requires further investigation. As emphasized by Hutkins et al. (2024), validating the prebiotic status of these candidates requires evidence of selective microbial fermentation, established health benefits, and strong statistical correlation between the two. Short-chain cello-oligosaccharides (SC-COSs; 2–5 units, $\beta$-1,4-linked) are proposed to be rapidly fermented by *Lactobacillus* species, enabling quick proximal colon fermentation into acetate and lactate, which lower pH, support colonocyte function, and strengthen the gut barrier (Rastall et al. 2022).

Cellobiose, a disaccharide, has been studied as a novel prebiotic candidate (Sanz et al. 2005). While *Lactobacillus* species typically lack native cellulase enzymes for cellulose degradation (Zúñiga et al. 2021), certain strains such as *Lactobacillus acidophilus* NCFM, could efficiently utilize cellobiose, presumably via expression of $\beta$-glucosidase enzymes. Other studies have explored the prebiotic potential of cellobiose using various methods, including *in vitro* fermentations with human faecal inocula (Sanz et al. 2005), pure bacterial cultures (Basholli-Salihu et al. 2013, Liu et al. 2024), animal models (Paßlack et al. 2020), and one human study where cellobiose was combined with probiotics (van Zanten et al. 2014). These studies suggest cellobiose can promote beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* species. However, some key parameters remain unexplored. For example, previous *in vitro* fermentations did not include dynamic pH control, nor have they used feasible doses for human subjects. Additionally, many existing studies focus primarily on short-chain fatty acids without examining the overall bacterial community in detail. Although animal experiments provide useful information, they do not fully reflect human microbial responses or health outcomes. Thus, the current study aims to evaluate the prebiotic potential of commercial cellobiose by performing pH-controlled anaerobic fermentations with human faecal samples.

# Materials and methods

## Materials

Unless otherwise stated, all reagents used in this experiment were sourced from Sigma–Aldrich (Merck, Gillingham, UK). The carbohydrates used in this study were as follows: The fructooligosaccharides (FOS) used were oligofructose P95 (OF P95) (Orafti® P95, DP 3–9, average DP 4; Purity: ∼95%; BENEO-Orafti, Tienen, Belgium), and the cellobiose used was a commercial novel sweetener (Purity: ∼99%; SAVANNA Ingredients GmbH, Germany).

## *In vitro* batch culture fermentation

### *Ethics*

All procedures performed in studies, involving faecal sample collection from healthy human participants were in accordance with the ethical standards and with the 1964 Helsinki declaration. The study was approved by the Ethics Committee of University of Reading Research Ethics Committee (UREC 15/20). Informed consent was obtained from all individual participants included in the study.

### *Faecal sample preparation*

Freshly voided faecal samples were obtained from three healthy adults aged between 18 and 35, who had not taken antibiotics for at least 6 months prior to the experiment and had no history of gastrointestinal disorders, were not regular consumers of prebiotics or probiotics.

Faecal samples were diluted 1 in 10 (w/v) using 0.1 mol $L^{-1}$ anaerobically prepared phosphate-buffered saline (PBS, Oxoid, Hampshire, UK), pH 7.4. Faecal samples were then homogenized in a stomacher (Seward, stomacher 80, Worthing, UK) for 120 s at 230 paddle beats per min. A volume of 15 mL of faecal slurry was immediately used to inoculate each batch culture vessel.

### *Basal batch culture nutrient medium*

Peptone water (2 g), yeast extract (2 g), NaCl (0.1 g), $K_2HPO$ (0.04 g), $KH_2PO$ (0.04 g), $MgSO{\cdot}7H_2O$ (0.01 g), $CaCl_2{\cdot}6H_2O$ (0.01 g), $NaHCO_3$ (2 g), L-cystine HCl (0.5 g), Tween 80 (2 mL), vitamin $K_1$ (10 $\mu$L), haemin (0.05 g), bile salts (0.05 g), and resazurin (4 mL) were added to 1 L of deionized water. The pH was adjusted to 7.0. A volume of 45 mL of the prepared medium was aliquoted into glass jars and autoclaved at 121°C for 15 min.

### *pH-controlled, stirred batch culture fermentation*

For each donor, three 100-mL vessels were prepared by aseptically adding 45 mL of basal nutrient medium into each. This setup was preconditioned overnight with continuous agitation and oxygen-free nitrogen flowing at 15 mL $min^{-1}$, a condition that was maintained throughout fermentation. Prior to inoculation with the faecal slurry, the medium was equilibrated in a water bath to 37°C, and its pH was kept between 6.7 and 6.9 by adjusting with either 0.5 mol $L^{-1}$ HCl or 0.5 mol $L^{-1}$ NaOH using an Electrolab pH controller (Tewksbury, UK). A magnetic stirrer ensured constant mixing of the faecal samples. For each donor, two substrate conditions were established: one vessel received a 1% (w/v) of oligofructose P95 and one vessel received a 1% (w/v) cellobiose, while the third vessel served as a negative control without added carbohydrates. All vessels were then inoculated with 5 mL of a 10% (w/v) faecal slurry (diluted in PBS). To facilitate subsequent bacterial and organic acid analyses via fluorescence *in situ* hybridization-flow cytometry (FISH-FLOW) and gas chromatography-flame ionization detection (GC-FID), respectively, 5-mL samples were withdrawn from each vessel at 0, 8, 24, and 48 h.

### *Enumeration of faecal microbial populations by flow cytometry fluorescence in situ hybridization (FISH-FLOW)*

A 750 $\mu$L sample of batch culture fermentation effluent was centrifuged at 11 337 $\times$ *g* for 5 min. The supernatant was then discarded, and the pellet suspended in 375 $\mu$L filtered 0.1 mol $L^{-1}$, pH 7.4 PBS solution. Filtered 4% paraformaldehyde (PFA) at 4°C (1125 $\mu$L) was added, and samples were stored at 4°C for 4 h. Samples were then washed thoroughly with PBS three times to remove PFA and resuspended in 150 $\mu$L PBS and 150 $\mu$L 99% ethanol. Samples were then stored at −20 °C, until FISH analysis by flow cytometry could be conducted. The probes used in this study are presented in Table 1.

A volume of 75 $\mu$L of fixed samples was mixed with 500 $\mu$L of filtered cold (4°C) 0.1 mol/L, pH 7.4 PBS and then centrifuged at 11 337 $\times$ *g* for 3 min. The resulting supernatant was then discarded,

**Table 1** Name, sequence, and target group of oligonucleotide probe used in this study for FISH of 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) |
| Bif164 | CATCCGGCATTACCACCC | *Bifidobacterium* | Langendijk et al. (1995) |
| Lab158 | GGTATTAGCAYCTGTTTCCA | *Lactobacillus, Leuconostoc Weissella*, *Lactococcus lactis;* all *Enterococcus, Vagococcus, Melisococcus, Catellicoccus, Tetragenococcus, Pediococcus, Paralactobacillus* spp | Franks et al. (1998) |
| Bac303 | CCAATGTGGGGGACCTT | Most *Bacteroidaceae* and *Prevotellaceae* | Manz et al. (1996) |
| Erec482 | GCTTCTTAGTCARGTACCG | Most of the bacteria in the *Clostridium coccoides—Eubacterium rectale* group (*Clostridium* clusters XIVa and XIVb) | Franks et al. (1998) |
| Rrec584 | TCAGACTTGCCGYACCGC | *Roseburia* genus (*E. rectale*, *R. intestinalis*) | Walker et al. (2005) |
| Ato291 | GGTCGGTCTCTCAACCC | *Atopobium* cluster | Harmsen et al. (2000) |
| Prop853 | ATTGCGTTAACTCCGGCAC | *Clostridium* cluster IX | Walker et al. (2005) |
| Fprau655 | CGCCTACCTCTGCACTAC | *Faecalibacterium prausnitzii* and relatives | Devereux et al. (1992) |
| DSV687 | TACGGATTTCACTCCT | Most Desulfovibrionales (excluding *Lawsonia*) and many Desulforomonales | Hold et al. (2003) |
| Chis150 | TTATGCGGTATTAATCTYCCTTT | Most of the bacteria in the *Clostridium histolyticum* group (*Clostridium* clusters I and II) | Franks et al. (1998) |

and pellets resuspended in 100 $\mu$L of TE-FISH (Tris/HCl mol $L^{-1}$ pH 8, EDTA 0.5 M pH 8, and filtered distilled water with the ratio of 1:1:8) containing lysozyme solution (1 mg $mL^{-1}$ of 50 000 U $mg^{-1}$ protein). Samples were then incubated for 10 min in the dark at room temperature and centrifuged at 11 337 $\times$ g for 3 min. Supernatants were discarded, and pellets washed with 500 $\mu$L filtered cold PBS by aspiration to disperse the pellet. Samples were then centrifuged at 11 337 $\times$ g for 3 min and supernatants discarded.

Pellets were resuspended in 150 $\mu$L of hybridization buffer, aspirated using a pipette and gently vortexed. Samples were centrifuged at 11 337 $\times$ *g* for 3 min and supernatants discarded. Pellets were resuspended in 1 mL of hybridization buffer. Aliquots (50 $\mu$L) of samples were placed in labelled 1.5 mL Eppendorf tubes and 4 $\mu$L of specific probes (50 ng $\mu L^{-1}$) were added. Samples were incubated at 35°C for at least 10 h in the dark.

Following incubation, 125 $\mu$L of hybridization buffer were added to each tube and vortexed gently. Samples were then centrifuged at 11 337 $\times$ g for 3 min and supernatants were discarded. Pellets were then washed with 175 $\mu$L of washing buffer solution and gently vortexed. Samples were incubated at 37°C for 20 min and centrifuged at 11 337 $\times$ *g* for 3 min. Supernatants were discarded and different volumes of filtered cold PBS (300, 600, and 1200 $\mu$L) were added based on flow cytometry load. Samples were kept at 4°C in the dark until flow cytometry measurements could be conducted. Fluorescence measures were performed on an BD AccuriTM C6 Plus (BD, Erembodegem, Brussels) measuring at 488 and 640 nm. A threshold of 9000 in forward scatter (FSC-A) and 3000 inside scatter (SSC-A) was placed to discard background noise, a gated area was applied in the main density dot to include 90% of the events. Flow rate was 35 $\mu$L $min^{-1}$, limit of collection was set for 100 000 events and analysed with Accuri Cflow Sampler software. Bacterial counts were then calculated through consideration of flow cytometry reading and PBS dilution.

### *Organic acids by GC-FID*

Samples (1.5 mL) of batch culture fluid were collected and centrifuged at 11 337 $\times$ *g* for 10 min. Supernatants were transferred to 1.5 mL Eppendorf tubes and stored at −80°C until analysis could be conducted. Sample extractions were performed according to Richardson et al. (1989) with modifications. Briefly, 1 mL of sample was transferred into a labelled 100 mm $\times$ 16 mm glass tube (International Scientific Supplies Ltd., Bradford, UK) and 50 $\mu$L of 2-ethylbutyric acid (0.1 mol $L^{-1}$, internal standard), 500 $\mu$L concentrated HCl, and 3 mL diethyl ether were added to each glass tube before vortexing for 1 min. Samples were centrifuged at 2000 $\times$ *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 $\mu$L) and 50 $\mu$L N-tert-butyldimethylsilyl)-N-methyl trifluoroacetamide (MTBSTFA) were added into a GC screw-cap vial. Samples were left at room temperature for 72 h to allow samples to completely derivatize.

An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30 m $\times$ 0.25 mm column with a 0.25 $\mu$m 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 to 190°C at 15°C $min^{-1}$ followed by 190°C for 3 min. Helium was the carrier gas (flow rate 1.7 mL $min^{-1}$; head pressure 133 kPa). A split ratio of 100:1 was used. Quantification of organic acids was achieved by calibration with acetic, propionic, butyric, lactate, iso-butyric, iso-valeric, valeric, and succinate acids in concentrations between 12.5 and 100 mmol $L^{-1}$. Mean metabolite concentrations were expressed as mmol $L^{-1}$.

## Statistical analysis

Statistical Package for Social Science version 27 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Changes in bacteriology and organic acid production were analysed using a general linear model (GLM) to assess repeat measures. Post hoc comparisons were also performed to determine any significant differences between interventions at 8, 24, and 48 h in bacteriology and organic acid. All tests were two-tailed and *P*-values were considered significant at $P \leq 0.05$ and are displayed by specified *P*-values. All post hoc pairwise comparisons were corrected for type 1 errors using Bonferroni adjustment within each GLM. Additional pairwise comparison was applied to assess the significance of single pairs of data. Graphs were generated using GraphPad Prism version 10.0.0 for iOS/Mac, GraphPad Software, San Diego, California, www.graphpad.com

# Results

## Bacterial population changes during fermentation

To understand bacterial changes induced by cellobiose and oligofructose (OF P95), bacterial populations were measured at 0, 8, 24, and 48 h using fluorescence *in situ* hybridization (FISH) probes targeting total bacteria and specific bacterial groups. OF P95 served as a positive control, and basal medium was the negative control (Table 2).

Overall, total bacterial counts increased significantly with both OF P95 and cellobiose ($P \leq 0.05$), particularly in early fermentation stages (Fig. 1). However, significant decreases were observed in cellobiose from T8 to T48 ($P = 0.001$) and from T24 to T48 ($P = 0.007$). OF P95 strongly stimulated growth of *Bifidobacterium* at all time points ($P \leq 0.05$), the highest increase averaging at 2.117 $\pm$ 0.29 (SE) log10 cells $mL^{-1}$ at 24 h (T24) ($P = 0.002$), whereas cellobiose did not significantly increase *Bifidobacterium* compared to controls. *Lactobacillus* populations remained stable across all conditions, indicating neither substrate significantly stimulated their growth. Both OF P95 and cellobiose initially increased Bac303-targeted bacterial populations, although this effect diminished by 48 h. Notably, cellobiose significantly increased *Atopobium* (Ato291) at 8 h, averaging a 1.567 $\pm$ 0.33 (SE) log10 cells $mL^{-1}$ ($P = 0.019$), whereas OF P95 had a delayed but significant effect at 24 h ($P = 0.047$). *Faecalibacterium* (Fprau655) populations generally decreased over 48 h in all groups, without significant differences between cellobiose and negative controls. In summary, while OF P95 consistently showed clear bifidogenic effects, cellobiose demonstrated more varied and transient changes on bacterial groups in this experiment.

## Organic acid production

At baseline (T0), total organic acid concentrations did not differ significantly between groups (Fig. 2). Both OF P95 and cel-

**Table 2** Average bacterial concentration (log10 cells/mL batch culture) at 0, 8, 24, and 48 h of fermentation during *in vitro* pH-controlled batch culture fermentations using faecal inocula (mean values with their standard errors; $n = 3$).

| | | Negative control | Oligofructose P95 | Cellobiose |
|---|---|---|---|---|
| **Total bacteria (Eub mix)** | **0** | 7.873 (0.264) | 7.850 (0.252)$^{a}$ | 7.863 (0.305)$^{ab}$ |
| | **8** | 7.793 (0.270) | 8.690 (0.155)$^{b}$ | 8.390 (0.304)$^{a}$ |
| | **24** | 7.880 (0.181) | 8.663 (0.123)$^{b}$ | 8.307 (0.298)$^{ab}$ |
| | **48** | 7.410 (0.329) | 8.377 (0.114)$^{ab}$ | 7.630 (0.324)$^{b}$ |
| **Ato291** | **0** | 5.873 (0.358) | 5.780 (0.276)$^{a}$ | 5.790 (0.285)$^{a}$ |
| | **8** | 5.870 (0.240) | 7.020 (0.505)$^{a}$ | 7.357 (0.634)$^{b}$ |
| | **24** | 6.100 (0.100) | 7.283 (0.143)$^{b}$ | 6.837 (0.745)$^{a}$ |
| | **48** | 5.820 (0.284) | 6.857 (0.378)$^{a}$ | 6.400 (0.743)$^{a}$ |
| **Bac303** | **0** | 6.670 (0.350) | 6.580 (0.371)$^{ab}$ | 6.593 (0.401)$^{ab}$ |
| | **8** | 6.677 (0.217) | 7.423 (0.355)$^{a}$ | 7.210 (0.247)$^{ab}$ |
| | **24** | 6.333 (0.222) | 6.827 (0.367)$^{ab}$ | 6.737 (0.339)$^{a}$ |
| | **48** | 5.860 (0.367) | 6.223 (0.148)$^{b}$ | 5.733 (0.351)$^{b}$ |
| **Bif164** | **0** | 6.480 (0.250) | 6.313 (0.240)$^{a}$ | 6.433 (0.300) |
| | **8** | 6.340 (0.225) | 7.860 (0.369)$^{*,b}$ | 6.440 (0.285) |
| | **24** | 6.756 (0.164) | 8.430 (0.290)$^{*,b}$ | 6.783 (0.207) |
| | **48** | 6.267 (0.302) | 8.117 (0.144)$^{*,b}$ | 6.230 (0.505) |
| **Lab158** | **0** | 5.537 (0.368) | 5.630 (0.282) | 5.593 (0.343) |
| | **8** | 5.357 (0.188) | 6.290 (0.092) | 5.693 (0.288) |
| | **24** | 4.447 (0.881) | 6.403 (0.187) | 5.953 (0.183) |
| | **48** | 4.957 (0.497) | 5.897 (0.093) | 5.393 (0.439) |
| **Chis150** | **0** | 5.290 (0.358) | 5.183 (0.206) | 5.173 (0.290) |
| | **8** | 5.503 (0.082) | 5.757 (0.382) | 6.973 (0.479) |
| | **24** | 4.123 (0.748) | 4.833 (0.917) | 4.880 (0.940) |
| | **48** | 4.470 (0.595) | 5.520 (0.158) | 4.673 (0.818) |
| **DSV687** | **0** | 4.913 (0.213) | 4.997 (0.234) | 5.130 (0.206) |
| | **8** | 5.237 (0.107) | 5.347 (0.333) | 5.750 (0.059) |
| | **24** | 4.390 (0.699) | 4.753 (0.877) | 4.720 (0.860) |
| | **48** | 4.703 (0.864) | 5.557 (0.160) | 4.763 (0.882) |
| **Erec482** | **0** | 7.463 (0.243) | 7.437 (0.243) | 7.473 (0.283) |
| | **8** | 7.213 (0.419) | 7.323 (0.531) | 7.203 (0.496) |
| | **24** | 7.233 (0.331) | 7.867 (0.217) | 7.423 (0.356) |
| | **48** | 6.620 (0.289) | 7.227 (0.358) | 6.517 (0.424) |
| **Fprau655** | **0** | 7.180 (0.289) | 7.157 (0.259)$^{ab}$ | 7.120 (0.339) |
| | **8** | 6.930 (0.420) | 7.263 (0.266)$^{ab}$ | 6.690 (0.711) |
| | **24** | 6.930 (0.229) | 7.317 (0.147)$^{a}$ | 6.543 (0.502) |
| | **48** | 6.237 (0.478) | 6.223 (0.441)$^{b}$ | 5.927 (0.419) |
| **Prop853** | **0** | 6.297 (0.344) | 6.293 (0.377) | 6.383 (0.324) |
| | **8** | 6.287 (0.428) | 6.547 (0.467) | 6.477 (0.448) |
| | **24** | 6.553 (0.208) | 6.523 (0.443) | 6.307 (0.361) |
| | **48** | 5.840 (0.316) | 6.590 (0.447) | 5.860(0.356) |
| **Rrec584** | **0** | 6.227 (0.251) | 6.240 (0.263) | 6.203 (0.335) |
| | **8** | 5.980(0.312) | 5.867 (0.416) | 5.860 (0.388) |
| | **24** | 4.563 (1.102) | 5.880 (0.030) | 4.663 (0.841) |
| | **48** | 3.787 (0.787) | 4.713 (0.897) | 4.487 (0.833) |

Different letters indicate significant differences ($P < 0.05$) in bacterial populations over time for a single treatment group on Bonferroni-adjusted pairwise comparisons.
Different symbols (∗,†) indicate significant differences ($P < 0.05$) in bacterial populations between treatment groups at that time point based on Bonferroni-adjusted pairwise comparisons.

lobiose significantly increased total organic acids by 48 h compared to baseline ($P < 0.001$), with OF P95 showing the largest increase. Although organic acids also increased slightly in the negative control, the increases in both OF P95 and cellobiose groups were significantly higher, demonstrating their capacity to enhance fermentation beyond background levels. As is depicted in Fig. 3, butyrate concentrations significantly increased over time with both substrates. At 48 h, cellobiose showed the greatest increase in butyrate, averaging 17.780 ± 2.39 (SE) mmol $L^{-1}$, which significantly higher than the negative control ($P = 0.002$),

**Table 3** Average SCFA concentrations and total organic acids (mM) at 0, 8, 24, and 48 h of fermentation during *in vitro* pH-controlled batch fermentations using faecal inocula (mean values with their standard errors; $n = 3$).

| Organic acid | Time | Negative control | OF P95 | Cellobiose |
|---|---|---|---|---|
| **Acetate** | **0** | 0.68 (0.04) | 0.79 (0.15)$^{a}$ | 0.72 (0.15)$^{a}$ |
| | **8** | 10.00 (5.97) | 39.89 (11.52)$^{b}$ | 9.53 (1.78)$^{a}$ |
| | **24** | 11.40 (0.49) | 38.55 (2.23)$^{c}$ | 29.02 (4.40)$^{b}$ |
| | **48** | 12.19 (0.77) | 47.33 (5.96)$^{c}$ | 29.33 (5.08)$^{c}$ |
| **Propionate** | **0** | 0.08 (0.08) | 0.08 (0.08)$^{a}$ | 0.07 (0.07)$^{a}$ |
| | **8** | 2.43 (1.29) | 6.21 (4.59)$^{a}$ | 2.45 (0.98)$^{a}$ |
| | **24** | 2.94 (0.16) | 12.85 (5.36)$^{ab}$ | 8.85 (2.46)$^{ab}$ |
| | **48** | 3.29 (0.12) | 13.84 (4.58)$^{b}$ | 10.01 (0.52)$^{b}$ |
| **Butyrate** | **0** | 0.08 (0.04) | 0.09 (0.04)$^{a}$ | 0.08 (0.04)$^{a}$ |
| | **8** | 2.20 (1.97) | 2.02 (1.68)$^{a}$ | 0.79 (0.47)$^{a}$ |
| | **24** | 2.72 (0.30) | 11.88 (2.91)$^{b}$ | 17.96 (1.72)$^{b}$ |
| | **48** | 3.46 (0.59) | 14.54 (3.66)$^{c}$ | 17.86 (1.87)$^{c}$ |
| **Iso-valerate** | **0** | 0.00 (0.00)$^{a}$ | 0.02 (0.02) | 0.00 (0.00) |
| | **8** | 0.05 (0.05)$^{a}$ | 0.00 (0.00) | 0.05 (0.03) |
| | **24** | 0.32 (0.04)$^{b}$ | 0.19 (0.05) | 0.23 (0.09) |
| | **48** | 0.75 (0.23)$^{b}$ | 0.32 (0.09) | 0.29 (0.08) |
| **Iso-butyrate** | **0** | 0.00 (0.00) $^{a}$ | 0.00 (0.00) | 0.00 (0.00)$^{ab}$ |
| | **8** | 0.05 (0.03)$^{*,a}$ | 0.03 (0.03)$^{\dagger}$ | 0.00 (0.00)$^{\ddagger,a}$ |
| | **24** | 0.69 (0.20)$^{ab}$ | 0.46 (0.20) | 0.55 (0.32)$^{ab}$ |
| | **48** | 0.99 (0.20)$^{b}$ | 0.70 (0.20) | 0.71 (0.20)$^{b}$ |
| **Lactate** | **0** | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| | **8** | 9.93 (6.56) | 17.98 (6.30) | 1.11 (0.68) |
| | **24** | 0.00 (0.00) | 0.00 (0.00) | 1.19 (1.19) |
| | **48** | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| **Valerate** | **0** | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| | **8** | 0.21 (0.21) | 0.19 (0.19) | 0.11 (0.11) |
| | **24** | 0.55 (0.27) | 0.45 (0.36) | 0.57 (0.47) |
| | **48** | 0.79 (0.32) | 1.61 (0.79) | 0.81 (0.39) |
| **Total Organic acids** | **0** | 0.85 (0.14)$^{a}$ | 0.97 (0.25)$^{a}$ | 0.87 (0.24)$^{a}$ |
| | **8** | 24.86 (18.82)$^{ab}$ | 66.32 (18.16)$^{bc}$ | 14.03 (3.27)$^{ab}$ |
| | **24** | 18.62 (0.34)$^{b}$ | 64.38 (5.83)$^{bc}$ | 58.36 (0.97)$^{b}$ |
| | **48** | 21.12 (0.62)$^{b}$ | 77.90 (5.67)$^{bc}$ | 59.02 (4.65)$^{b}$ |

Different superscript letters ($^{a, b, c}$) within a row (changes over time for a single treatment group) indicate significant differences ($P < 0.05$) over time for that treatment group based on Bonferroni-adjusted pairwise comparisons, and different symbols ($*$, †, ‡) within a column indicate significant differences ($P < 0.05$) between treatment groups at that time point based on Bonferroni-adjusted pairwise comparisons.

while OF P95 showed significant increases earlier (at 24 h) but did not significantly differ from the control at 48 h. Acetate was the most abundant organic acid produced. OF P95 and cellobiose both significantly increased acetate production by 48 h. Although OF P95 showed numerically higher acetate production, this difference was not statistically significant compared to cellobiose. Propionate, valerate, iso-valerate, and iso-butyrate concentrations increased significantly over time across all treatments, but there were no significant differences between substrates and controls by 48 h. Lactate peaked at 8 h, especially with OF P95, before declining, but neither substrate led to significantly higher lactate concentrations compared to the negative control at 48 h. In summary, OF P95 and cellobiose significantly enhanced total organic acids, cellobiose particularly increased butyrate. However, effects on other organic acids such as propionate, valerate, iso-valerate, iso-butyrate, and lactate were minimal or transient (Table 3).

# Discussion

We hypothesized that cellobiose would modulate the gut microbiota, similar to established prebiotics. To test this, we compared cellobiose with OF P95 and a no-substrate control, observing *Bifidobacterium* and *Lactobacillus* as primary indicators. As expected, OF P95 produced a robust bifidogenic response. Cellobiose also elevated *Bifidobacterium* levels but did not reach statistical significance, which could be a possible consequence of the higher substrate dose, longer incubation, and strict pH control compared with Sanz et al. (2005).

In contrast to the sustained effect OF P95, cellobiose induced a rapid, transient increase in total bacterial counts and most targeted groups, peaking at 8 h before declining by 48 h. This pattern reflects the swift hydrolysis of its single $\beta$-1,4-glucosyl linkage, followed by substrate depletion or metabolite-driven community shifts, indicating that some unmeasured taxa contribute

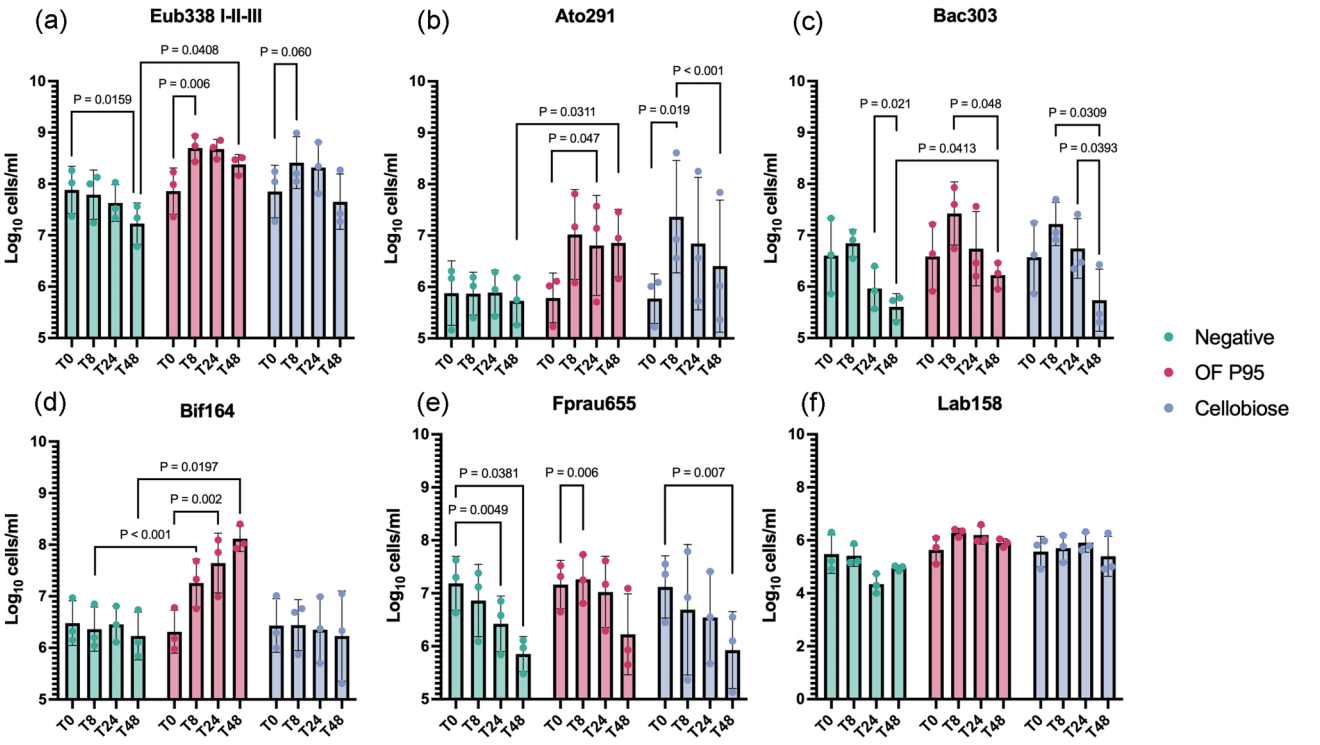


**Figure 1** Bacterial groups measured by FISH-FLOW (log10 cells/mL) using probes: (a) total bacteria (Eub338 I–II–III), (b) *Atopobium* cluster (Ato291), (c) most *Bacteroidaceae* and *Prevotellaceae* (Bac303), (d) *Bifidobacterium* spp. (Bif164), and (e) *Faecalibacterium prausnitzii* (Fprau655) and (f) *Lactobacillus* (Lab158) at 0, 8, 24, and 48 h. Mean and SE (all data points; $n = 3$). Green represents negative control. Results that are statistically significant within respective treatments are displayed by specified *P*-values. Abbreviations: OF P95 = oligofructose P95.

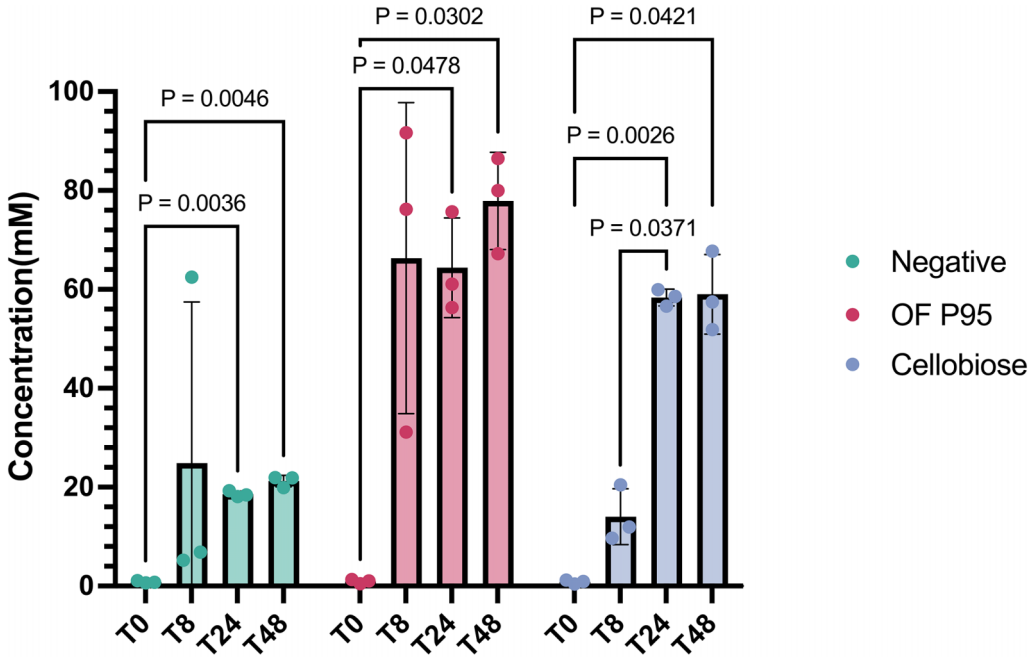


**Figure 2** Concentration of total organic acids from anaerobic batch culture fermenters with faecal inocula measured by gas chromatography (in mmol/L) at 0, 8, 24, and 48 h total organic acids (expressed as the sum of acetate, propionate, butyrate, iso-butyrate, iso-valerate, valerate, and lactate) for three healthy donors are presented in with negative control, OF P95, and cellobiose. Results that are statistically significant within respective treatments are displayed by specified *P*-values. Error bars represent SD.

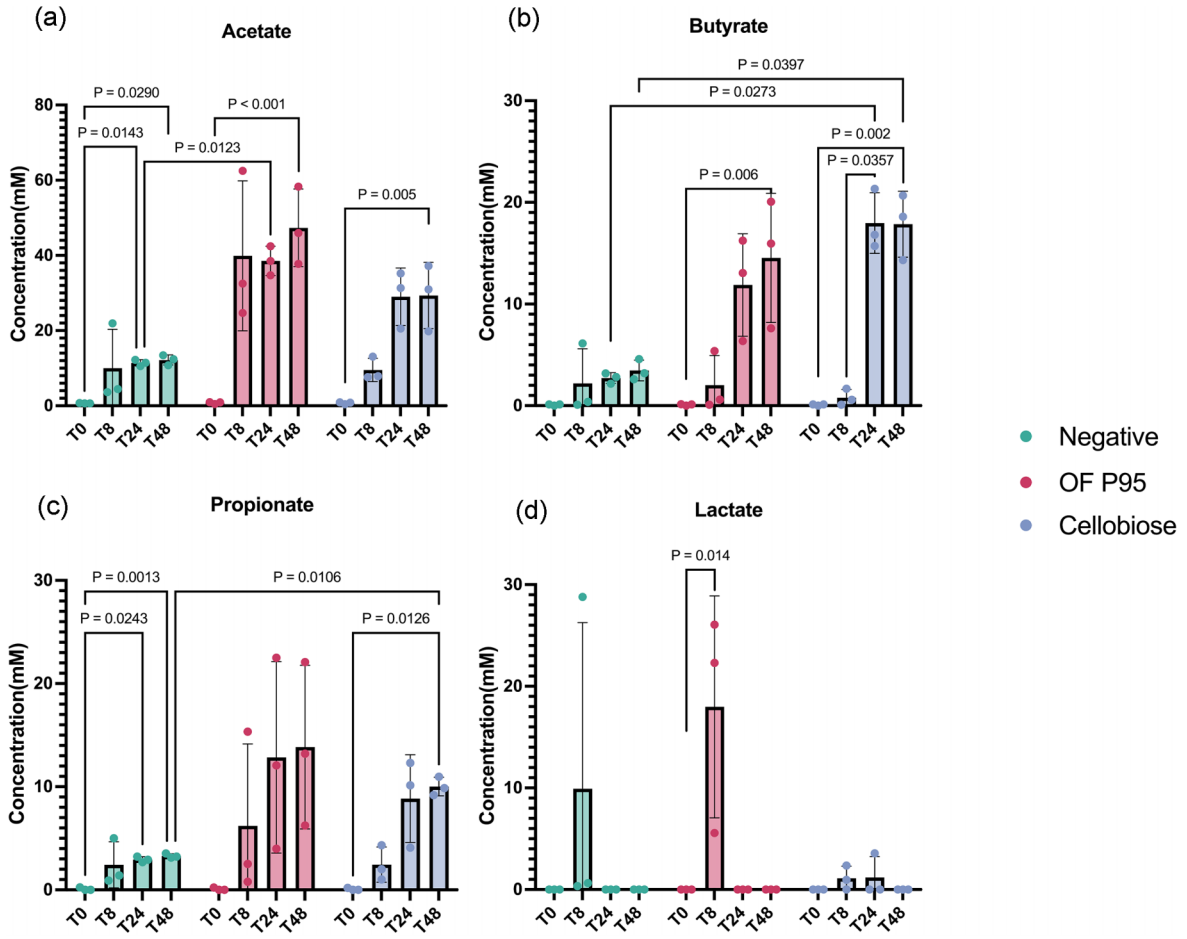


**Figure 3** Concentration of acetate (a), butyrate (b), propionate (c), and lactate (d) from anaerobic batch culture fermenters with faecal inocula measured by gas chromatography (in mmol/L) at 0, 8, 24, and 48 h, for three healthy donors are presented in with negative control, OF P95, and cellobiose. Results that are statistically significant within respective treatments are displayed by specified *P*-values. Error bars represent SD.

to early fermentation. A key finding was the significant increase in *Atopobium* spp. following cellobiose supplementation. To our knowledge, this is the first report of cellobiose selectively stimulating *Atopobium* (Fig. 1(b)), as this genus is not typically promoted by traditional prebiotics. Pectic oligosaccharides with high arabinose content have, however, been shown to enhance *Atopobium* levels in human faecal fermentations (Di et al. 2017). Our findings suggest that cellobiose may target different microbial groups.

A key metabolic finding was that cellobiose drove a significant increase in butyrate by 48 h, exceeding both the negative control and OF P95. Interestingly, this butyrogenic effect occurred despite a decline in *Faecalibacterium prausnitzii* and stable counts for the Erec482 group. Although *Eubacterium rectale* and *Roseburia* spp. are traditionally recognized as major butyrate producers (Louis and Flint 2009), our FISH data suggests they were not the primary drivers. Given the limited FISH probes utilized, it is probable that other butyrate-producing genera acted as the functional drivers. Butyrate is known as the primary energy source for colonocytes, for its impact on intestinal barrier function, and its anti-inflammatory actions (Singh et al. 2022). These findings suggest that cellobiose fits the functional profile of a candidate prebiotic, as it is selectively utilized to produce metabolites with potential health-conferring properties. Furthermore, this distinct metabolic activity differentiates cellobiose from traditional prebiotics like FOS.

As preliminary work, our *in vitro* fermentations revealed clear trends but require confirmation with larger cohorts. While targeted FISH probes allowed precise quantification of selected taxa, untargeted sequencing approaches (e.g. 16S rRNA gene or shotgun metagenomics) will be essential to identify the full spectrum of cellobiose-responsive microbes. Future studies should use *in vivo* trials to validate these initial findings and explore the health implications of cellobiose supplementation. Moreover, comparing cellobiose with other disaccharides like kojibiose, isomaltose, mannobiose, and inulinobiose would be insightful to understand the structure-function relationships of DP2 oligosaccharides in the human gut.

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## Author contributions

Manxi Huang (Conceptualization, Investigation, Writing – original draft), Peter Jackson (Methodology, Supervision, Writing – review & editing), Afroditi Chatzifragkou (Project administration, Supervision, Writing – review & editing), Robert A. Rastall (Conceptualization, Project administration, Supervision, Writing – review & editing)

## Conflicts of interest

None declared.

## Funding

None declared.

## Data availability

The datasets generated and analysed during the current study, including raw bacterial enumeration and metabolite concentration data, are openly available in the University of Reading Research Data Archive at https://doi.org/10.17864/1947.001505

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