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# Comparison of prebiotic candidates in ulcerative colitis using an *in vitro* fermentation model

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## Abstract

**Aims:** This study explored the effect of three different prebiotics, the human milk oligosaccharide 2′-fucosyllactose (2′-FL), an oligofructose-enriched inulin (fructo-oligosaccharide, or FOS), and a galacto-oligosaccaride (GOS) mixture, on the faecal microbiota from patients with ulcerative colitis (UC) using *in vitro* batch culture fermentation models. Changes in bacterial groups and short-chain fatty acid (SCFA) production were compared.

**Methods and results:** *In vitro* pH controlled batch culture fermentation was carried out over 48 h on samples from three healthy controls and three patients with active UC. Four vessels were run, one negative control and one for each of the prebiotic substrates. Bacterial enumeration was carried out using fluorescence *in situ* hybridization with flow cytometry. SCFA quantification was performed using gas chromatography mass spectrometry. All substrates had a positive effect on the gut microbiota and led to significant increases in total SCFA and propionate concentrations at 48 h. 2′-FL was the only substrate to significantly increase acetate and led to the greatest increase in total SCFA concentration at 48 h. 2′-FL best suppressed *Desulfovibrio* spp., a pathogen associated with UC.

**Conclusions:** 2′FL, FOS, and GOS all significantly improved the gut microbiota in this *in vitro* study and also led to increased SCFA.

## Impact Statement

Positively modulating the gut microbiota in ulcerative colitis (UC) could be a novel and powerful way to improve outcomes in this condition. Prebiotics offer a way to selectively alter the gut microbiota in UC. It is suggested that the human milk oligosaccharide 2′-fucosyllactose would be the preferred candidate to take forward to a clinical trial.

**Keywords:** prebiotic; ulcerative colitis; inflammatory bowel disease; gut microbiome; galacto-oligosaccharide; human milk oligosaccharide

## Introduction

Ulcerative colitis (UC) is a type of inflammatory bowel disease characterized by chronic inflammation of the mucosal layer of the colon, manifesting clinically as bloody diarrhoea. It has a rising prevalence worldwide, but is highest in western countries at 0.3% (Ng et al. 2017). Although mortality from the condition has declined significantly with improved medical therapy over the past 70 years, resultant morbidity and healthcare costs are high, and up to 20% of patients require surgery to manage the condition (Kühn et al. 2015).

Patients with UC have an altered gut microbiota, which is thought to play a key role in pathogenesis of the condition. Key differences include a reduction in abundance of the Firmicutes *Faecalibacterium prausnitzii* and *Roseburia hominis* in patients with UC, as well as reductions in *Lactobacillus* spp. and *Bifidobacterium* spp. (Bullock et al. 2004, Verma et al. 2010, Machiels et al. 2014). Another important difference is the increased presence of sulphate-reducing bacteria (SRB), such as *Desulfovibrio* spp. in patients with UC (Gibson et al. 1991, Rowan et al. 2010). These SRB produce hydrogen sulphide from glycans and dietary sulphates, which disrupts normal colonocyte metabolism and promotes inflammation. Whether these changes are cause or consequence of colonic inflammation in UC cannot be known for sure; the likelihood is a combination of the two.

A prebiotic is defined as ‘a substrate that is selectively utilised by host microorganisms conferring a health benefit’ (Gibson et al. 2017). Prebiotics acting on the human gut microbiota tend to be oligosaccharides that resist hydrolysis and pass through the upper gut unaltered, reaching the colon intact where they are preferentially fermented by bacteria which produce beneficial metabolites. The primary metabolites of interest are short-chain fatty acids (SCFAs), which not only act as fuel for colonocytes, but when absorbed systemically can reduce inflammation, regulate the immune system, suppress cancer growth, and positively influence metabolism (Xiong et al. 2022).

Examples of prebiotics include: inulin-type fructans, including short and long chain fructooligosaccharides (FOS); galacto-oligosaccharides (GOS); and human milk oligosaccharides (HMO). The latter are a family of carbohydrates produced by lactating mothers in breastmilk and are critical in nourishing the infant gut microbiota.

Modulating the ‘dysbiotic’ gut microbiota in UC using prebiotics may be a way to reduce inflammation and improve symptoms. A limited number of *in vitro* studies have been carried out on prebiotics in UC. Xylo-oligosaccharide has been shown to promote growth of *Lactobacillus* spp.,

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*Roseburia* spp., and *Bifidobacterium* spp. in a fermentation model of faeces from patients with UC in remission (Li et al. 2021). Arabinooligosaccharides derived from sugar beet pulp and FOS both selectively increased *Lactobacillus* spp. and *Bifidobacterium* spp. in the faecal microbiota of patients with UC (Vigsnæs et al. 2011). The HMO 2′-fucosyllactose (2′-FL) has been shown *in vitro* to increase *Bifidobacterium* spp., *Clostridium* cluster XIVa, and *Roseburia* spp. in the faecal microbiota from patients with UC, and to increase concentrations of the SCFAs acetate, butyrate, and propionate (Ryan et al. 2021). Several heterogeneous small clinical trials have also been carried out with prebiotics in patients with UC, using FOS, GOS, disaccharides, HMOs, hemicellulose, and non-saccharide prebiotics, with inconsistent results (Kanauchi et al. 2003, Casellas et al. 2007, Valcheva et al. 2019, Ryan et al. 2021, Wilson et al. 2021, Ikegami et al. 2023).

No *in vitro* studies to date have directly compared prebiotic substrates head-to-head in a fermentation model of the faecal microbiota from patients with UC with a view to identifying the likely most effective prebiotic as a dietary therapy in UC. The aim of this work was to compare three major types of prebiotic thought to have positive health effects (an HMO, FOS, and GOS) in an *in vitro* batch culture fermentation model to assess their relative efficacy with respect to altering bacterial populations and metabolite production. This pre-competitive approach could then inform the choice of prebiotic to take forward to a human intervention trial.

## Materials and methods

### Substrates

Three prebiotic candidates were selected to be tested *in vitro*. The HMO used was 2′-FL, sourced from DSM, Copenhagen, Denmark. As an FOS, an oligofructose-enriched inulin, Orafti®Synergy1, was used, which was obtained from BENEO-Orafti, Obrigheim, Germany. The chosen GOS was B-GOS (Bimuno®), from Clasado Biosciences, Reading, UK.

### Upper gut simulation

HMOs, inulin, and oligofructose all pass through the upper gut intact and unaltered (Brand-Miller et al. 1998, Niness 1999). The patented B-GOS mixture used in this study contains 48% GOS, 22% lactose, 18% glucose, and 12% galactose. As the latter three components are altered or absorbed in the upper gastrointestinal tract prior to reaching the colon, to more faithfully replicate physiological conditions an upper gut simulation was carried out on this substrate as described by Mills et al. (2008) with slight modification as follows.

60 g of one batch of the B-GOS was dissolved in 150 ml distilled water and then stomached for 5 min. For the oral phase, 20 mg $\alpha$-amylase (Sigma, UK) in 6.25 ml calcium chloride (0.001 mol $l^{-1}$, pH 7.0) was added to the sample and incubated at 37°C on a shaker for 30 min. The pH was then lowered to 2.0, and for the gastric phase 2.7 g pepsin (Sigma, UK) in 25 ml hydrochloric acid (0.1 mol $l^{-1}$) were added and to the sample and the mixture was incubated at 37°C on a shaker for 2 h. For the small intestinal phase, 560 mg pancreatin (Sigma, UK), 3.5 g bile (Sigma, UK), and 125 ml sodium hydrogen carbonate (0.5 mol $l^{-1}$) was added and the pH adjusted to 7.0. The mixture was incubated at 37°C on a shaker for 3 h.

This sample solution was transferred to a Spectrum™ Spectra/Por™ Biotech Cellulose Ester Dialysis Membrane Tubing with a 0.5 kDa molecular weight cut-off to remove low molecular mass digestion products. Dialysis was then performed against 0.01 mol $l^{-1}$ sodium chloride for 15 h, then the dialysate was refreshed and further dialysis carried out for 2 h. The sample was then transferred to a freeze dryer for 96 h, with a pre- and post-drying weight measured in order to calculate the weight of end product equivalent to 1.5 g of the original B-GOS.

### Batch culture fermentation

Static *in vitro* anaerobic batch culture fermentation to simulate the distal colon was performed as described by Rycroft et al. (2001) with slight modifications. Batch culture vessels were assembled, consisting of a primary 300 ml volume inner chamber, an outer layer for a continuous water irrigation for temperature control, a nitrogen gas inlet and gas outlet, acid/base feeder, pH probe, access port, and magnetic stirrer. A volume of 135 ml of standard basal nutrient medium was sterilized by autoclaving at 121°C for 20 min and added aseptically to four sterile batch culture vessels, one negative control, and one for each substrate to be tested (Rycroft et al. 2001). Anoxic nitrogen gas was bubbled through the media for a minimum of 18 h prior to inoculation to ensure anaerobiosis. A circulating water bath was set at 37°C, and pH of each vessel was adjusted to 6.8, with a pH range maintained between 6.7 and 6.9 using pH controllers (Electrolab, Tewkesbury, UK). 1.5 g of each substrate (or equivalent post-upper gut digestion weight of B-GOS) was added to the relevant fermenters to give a final concentration of 1% w/v.

Fresh faecal samples were obtained from three healthy controls and three patients with UC. The study was carried out with ethical approval from the United Kingdom National Health Service Health Research Authority, Research Ethics Committee reference 23/NW/0080. Healthy controls (two males and one female) had no pre-existing diagnosis of any gastrointestinal disorders. Patients with UC (two males and one female) had at least moderate symptoms according to the Truelove and Witts criteria and Simple Clinical Colitis Activity Index, supported by a gastroenterologist opinion, faecal calprotectin $\geq$250 μg $g^{-1}$, recent endoscopic or histological evidence of inflammation, and/or elevated serum inflammatory indices (Truelove and Witts 1955, Walmsley et al. 1998). All patients were taking neither probiotic nor prebiotic products and had not received antibiotics within the past 3 months. Stool samples were collected into an anaerobic jar (gas phase $N_2$) using Thermo Scientific AnaeroGen 2.5 l anaerobic sachets (Oxoid, Basingstoke, UK), and inoculated into the fermenters within 2 h of defecation.

A 10% weight by volume faecal slurry was formed by stomaching 20 g of the faecal sample with 180 ml phosphate buffered saline (10 mM phosphate, pH 7.4). 15 ml of this faecal slurry was then inoculated into the basal medium in each vessel to establish a final faecal concentration of 1% (w/v), and samples were taken immediately for baseline values. Samples were then taken at 8, 24, and 48 h post-inoculation.

### Organic acid quantification using gas chromatography-mass spectrometry

In preparation for gas chromatography (GC), after being removed from the fermenter, 1.5 ml of sample was centrifuged at

**Table 1.** Probe name, DNA sequence, and bacterial group targeted by probes used in FISH-FC in this study.

| Probe name and reference | DNA sequence (5′–3′) | Targeted bacteria |
|---|---|---|
| Non Eub (Wallner et al. 1993) | ACTCCTACGGGAGGCAGC | Complementary probe to Eub338 (negative control) |
| Eub338 I (Amann et al. 1990) | GCTGCCTCCCGTAGGAGT | Most bacteria |
| Eub338 II (Daims et al. 1999) | GCAGCCACCCGTAGGTGT | Planctomycetales |
| Eub338 III (Daims et al. 1999) | GCTGCCACCCGTAGGTGT | Verrucomicrobiales |
| Bif164 (Langendijk et al. 1995) | CATCCGGCATTACCACCC | *Bifidobacterium* spp. |
| Lab158 (Harmsen et al. 1999) | GGTATTAGCAYCTGTTTCCA | *Lactobacillus* spp. and *Enterococcus* |
| Bac303 (Manz et al. 1996) | CCAATGTGGGGGACCTT | Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae |
| Erec482 (Franks et al. 1998) | GCTTCTTAGTCARGTACCG | Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb) |
| Rrec584 (Walker et al. 2005) | TCAGACTTGCCGYACCGC | *Roseburia* spp. |
| Ato291 (Harmsen et al. 2000) | GGTCGGTCTCTCAACCC | *Atopobium* cluster |
| Prop853 (Walker et al. 2005) | ATTGCGTTAACTCCGGCAC | Clostridial cluster IX |
| Fprau655 (Hold et al. 2003) | CGCCTACCTCTGCACTAC | *Faecalibacterium prausnitzii* |
| DSV687 (Devereux et al. 1992) | TACGGATTTCACTCCT | *Desulfovibrio* genus |
| Chis150 (Franks et al. 1998) | TTATGCGGTATTAATCTYCC TTT | Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II) |

11 600 × *g* for 10 min, and the supernatant was transferred and stored at −20°C. Samples were processed as described by Richardson et al. (1989). Samples were defrosted and vortexed, and 1 ml of sample was transferred to a flat-bottomed 100 mm × 16 mm glass tube. 50 µl of internal standard (0.1 M 2-ethylbutyric acid) was added to each tube, along with 0.5 ml concentrated hydrochloric acid and 3 ml diethyl ether. Samples were then vortexed for 1 min at 1500 rpm, and then centrifuged for 10 min at 2000 × *g*. The upper layer of diethyl ether with dissolved volatiles was transferred into a new flat-bottomed 100 mm × 16 mm glass tube. 400 µl of this solution was then added to 50 µl of *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (Merck, UK) in a screw-cap GC vial, and left to derivatize for 72 h.

SCFA analysis was performed using an Agilent 7890B Gas Chromatograph (Agilent, UK) with an HP-5MS column (length 30 m, diameter 0.25 mm, 0.25 µm coating, Agilent, UK). The temperatures of injector and detector were set at 275°C, with the column temperature programmed from 63°C to 190°C at 10°C $min^{-1}$ with a hold time of 2 min and a total run time of 17.7 min. Helium was the carrier gas at a flow rate of 6.5 ml $min^{-1}$ and pressure of 24.11 psi, and sample injection volume was 1 µl. An external standard containing all SCFAs was run after every batch of samples as a quality control. This external standard contained 30 mM acetic acid, 20 mM propionic acid, 5 mM iso-butyric acid, 20 mM *n*-butyric acid, 5 mM iso-valeric acid, 5 mM *n*-valeric acid, and 10 mM lactic acid (all Sigma, UK). Individual standards of each organic acid were run at specific concentrations for calibration purposes. Agilent OpenLab ChemStation software (Agilent Technologies, Cheadle, UK) was used for peak integration, and concentrations were calculated using the Internal Response Factor method described in Liu et al. (2016).

## Bacterial enumeration

After sampling directly from the fermenter, 750 µl sample was taken in anticipation of bacterial enumeration using fluorescence *in situ* hybridization (FISH) combined with flow cytometry (FC). The samples were prepared as described by Grimaldi *et al.* (2017). The 750 µl of sample was centrifuged at 11 600 × *g* (consistent for all centrifuging throughout this process), and the supernatant was discarded. The pellet was resuspended in 375 µl filter-sterilized PBS, pre-filtered using a sterile 0.22 µm polyvinylidene fluoride syringe filter, and fixed in 1125 µl 4% paraformaldehyde. Samples were then incubated at 4°C for 4 h to achieve permeabilization, washed twice with filter-sterilized PBS, and resuspended in a mixture of 300 µl filter-sterilized PBS and 300 µl filtered ethanol.

75 µl of the suspension was then taken and suspended further in 500 µl filter-sterilized PBS. Samples were centrifuged at 11 600 × *g* for 3 min, and the pellet was suspended in 100 µl of solution containing 0.1 M Tris/HCl (pH 8.0), 0.05 M EDTA (pH 8.0), and 1 mg $ml^{-1}$ lysozyme. This was incubated in the dark at room temperature for 10 min then re-centrifuged and washed with filter-sterilized PBS. The pellet was then suspended in hybridization buffer (containing 5 M sodium chloride, 1 M Tris/HCl (pH 8.0), 10% sodium dodecyl sulphate, and 30% formamide) and 50 µl aliquoted into Eppendorf tubes, to which 4 µl of each oligonucleotide probe (see Table 1) and 4 µl of the Eub338 I-II-III total bacteria probe (an equimolar combination of Eub338 I, Eub338 II, and Eub338 III) were added (all probes Eurofins Genomics, UK). The working solution was incubated at 35°C in a heating block overnight.

Following incubation, the samples were centrifuged and washed using washing buffer [containing 5 M sodium chloride, 1 M Tris/HCl (pH 8.0), 0.5 M EDTA (pH 8.0), and 10% sodium dodecyl sulphate], then centrifuged again at 11 600 × *g* for 3 min and suspended in 300 µl filter-sterilized PBS. FC was performed using an Accuri C6 flow cytometer and analysed using the Accuri CFlow Sampler software (both BD Biosciences, UK). Bacterial counts were calculated using cytometry counts and appropriate dilution factor.

## Statistical analysis

Statistical analysis and graphing were performed using GraphPad Prism 10 for macOS, Version 10.0.1. Baseline bacterial populations between healthy controls and patients with UC were compared using an unpaired *t* test with Welch's correction. Multiple comparisons were corrected for using the Holm–Sìdàk method. Changes in log transformed bacterial populations and SCFA concentra-

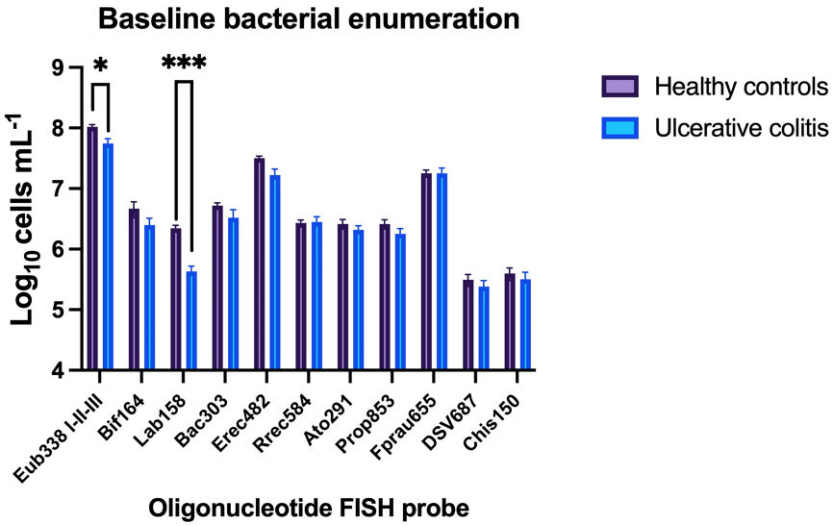


**Figure 1.** Baseline bacterial counts at timepoint zero for both healthy control and UC samples expressed as $\log_{10}$ cells $ml^{-1}$ of sample for each oligonucleotide probe. Error bars represent standard error of the mean, pairwise comparisons annotated where statistical significance reached: $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$.

tions over time were compared using repeated measures two-way ANOVA with the Geisser–Greenhouse correction, correcting for multiple comparisons using Dunnett's test. Where missing data were present a mixed-effects model was used.

## Results

### Total bacteria and *Lactobacillus* counts were reduced at baseline in patients with UC

Figure 1 demonstrates baseline counts of bacterial groups identified using FISH-FC in both the healthy controls and patients with UC. Counts of total bacteria measured with the Eub338 I-II-III probe were decreased in patients with UC compared to healthy controls (7.74 vs 8.02 $\log_{10}$ cells $ml^{-1}$, $P = .02$), as were counts of *Lactobacillus* spp. (5.63 vs 6.34 $\log_{10}$ cells $ml^{-1}$, $P = .0006$). No significant differences at baseline were found for other bacterial groups after correcting for multiple comparisons.

### All substrates had a positive effect on the UC microbiota *in vitro*

Figure 2 shows bacterial enumeration data at baseline, 8 and 24 h post-inoculation in the negative control vessel and three vessels containing prebiotic substrates. Statistically significant increases from baseline to 8 h were seen in total bacteria with 2′-FL (from 7.51 to 8.05 $\log_{10}$ cells $ml^{-1}$, $P = .0235$) and GOS (from 7.59 to 8.09 $\log_{10}$ cells $ml^{-1}$, $P = .0362$), and in *Lactobacillus* spp. with FOS (from 5.49 to 6.33 $\log_{10}$ cells $ml^{-1}$, $P = .0296$).

Although not reaching statistical significance, a bifidogenic effect was seen with all substrates, most pronounced with 2′-FL. All substrates increased *Lactobacillus* spp. at 24 h, and 2′-FL was the only substrate to reduce *Desulfovibrio* spp. abundance. No real changes were seen in *F. prausnitzii* and *Roseburia* spp. with any substrate. Using linear regression to model rate of change from baseline to 8–24 h, a significant deviation in slope gradient from zero was seen in *Bifidobacterium* spp. ($P = .0414$) with GOS and with the *Clostridium coccoides-Eubacterium rectale* group ($P = .0297$) and *Atopobium* cluster ($P = .0072$) with 2′-FL.

### 2′-FL reduced the proportion of *Desulfovibrio* spp. in the UC microbiota

Figure 3 shows the relative abundance of *Desulfovibrio* spp. over time with each of the tested substrates. A clear decline was seen with 2′-FL but not with FOS or GOS. This pattern was maintained when data were combined with that of healthy controls (not shown).

### Prebiotics effect a greater change in bacterial counts in patients with UC compared to healthy controls

Figure 4 gives the ratio of change in individual bacterial groups from baseline to 24 h into the fermentations. Although no changes reached statistical significance, in general it was apparent that there were greater changes across the bacterial populations in UC samples compared to controls. In particular, 2′-FL resulted in the greatest ratio change in *Bifidobacterium* spp. and *Lactobacillus* spp., and both 2′-FL and FOS saw a noteworthy rise in the *Clostridium coccoides—Eubacterium rectale* group. 2′-FL saw the smallest increase in *Desulfovibrio* spp. compared to the other substrates.

### All substrates lead to increased organic acid production, with 2′-FL increasing acetate and total SCFA levels the most

Figure 5 shows how the concentration of organic acids in each vessel changed from baseline to 8, 24, and 48 h post-inoculation. Graphs are shown for the three most abundant and physiologically important SCFAs (acetate, butyrate, and propionate), as well as total SCFA concentration (sum of acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, and lactate). 2′-FL was the only substrate to significantly increase acetate concentrations from baseline to 48 h (from 0.952 to 49.9 mmol $l^{-1}$, $P = .0474$). Acetate levels following 48 h of fermentation with 2′-FL were significantly greater than

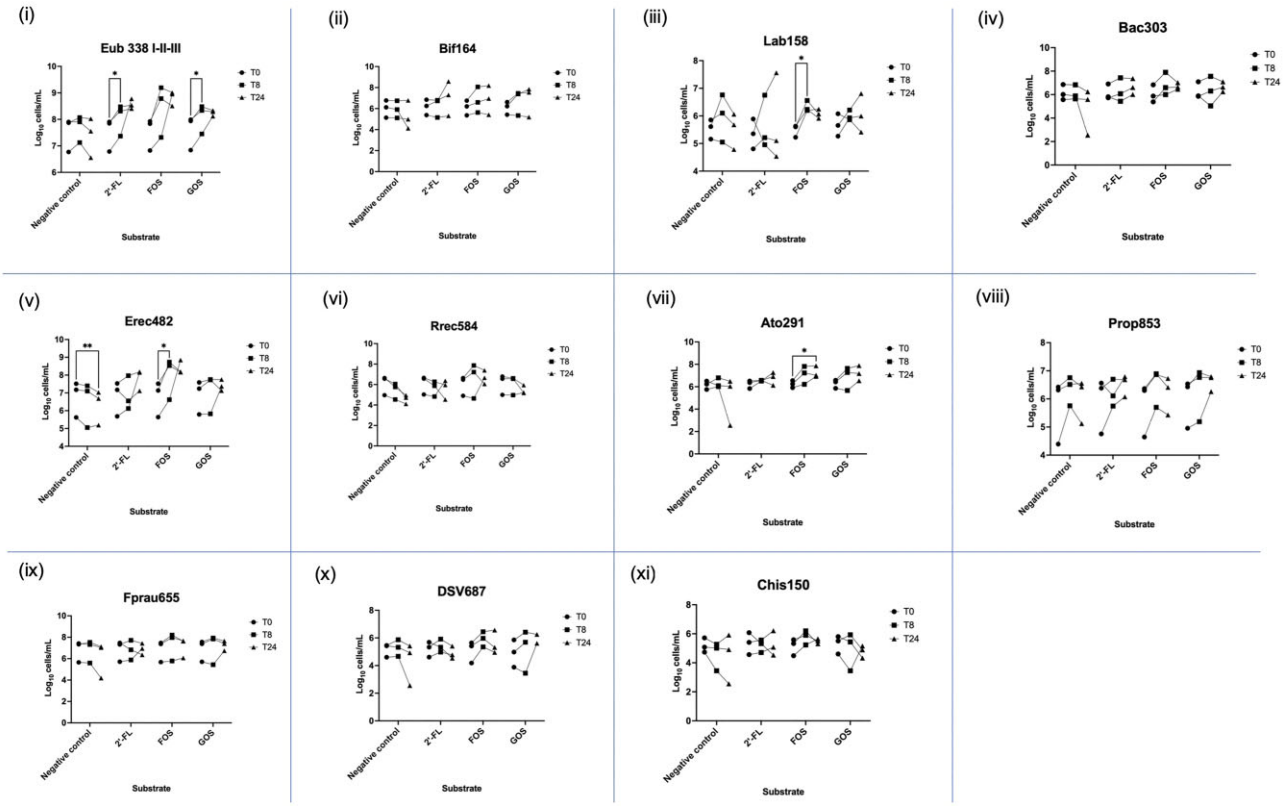


**Figure 2.** Bacterial counts from baseline microbiota to 8 and 24 h with each oligonucleotide probe in patients with UC in negative control vessel and with each substrate. Individual panels shown for Eub 338 I-II-III (i), Bif164 (ii), Lab158 (iii), Bac303 (iv), Erec482 (v), Rrec584 (vi), Ato291 (vii), Prop853 (viii), Fprau655 (ix), DSV687 (x), and Chis150 (xi). Pairwise comparisons annotated where statistical significance reached between mean values: $^{*}P \leq .05$, $^{**}P \leq .01$.

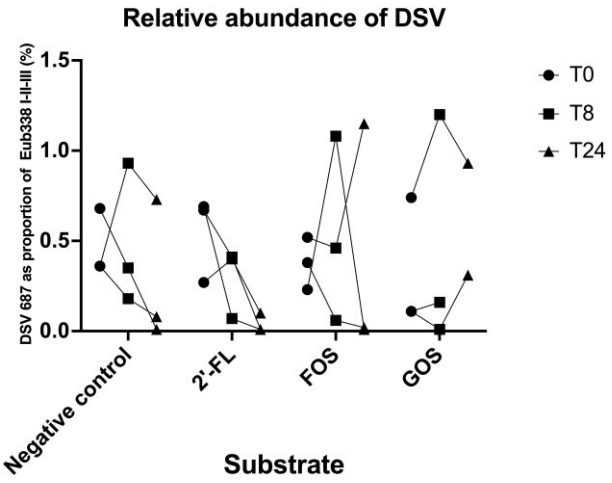


**Figure 3.** Relative abundance of *Desulfovibrio* spp. expressed as percentage of total bacteria identified, and error bars denote standard error of the mean.

those with GOS (49.9 mmol $l^{-1}$ vs 20.07 mmol $l^{-1}$, $P = .958$), but not FOS (49.9 mmol $l^{-1}$ vs 34.46 mmol $l^{-1}$, $P = .53$).

GOS was the only substrate to significantly increase butyrate concentration from baseline to 48 h (from 0.216 to 6.635 mmol $l^{-1}$, $P = .042$). No significant differences between butyrate values at 48 h were observed between 2′-FL, FOS, and GOS.

All three substrates significantly increased propionate and total SCFA concentrations from baseline to 48 h. Directly comparing values at 48 h for total SCFA for the different substrates, there was no significant difference between 2′-FL and FOS (79.60 mmol $l^{-1}$ vs 75.18 mmol $l^{-1}$, $P = .958$), but 2′-FL did have a significantly increased peak total SCFA concentration compared to GOS (79.60 mmol $l^{-1}$ vs 41.52 mmol $l^{-1}$, $P = .0424$).

## Discussion

In this study, we observed the impact of three different substrates, a human milk oligosaccharide, an oligofructose-enriched inulin, and a galactooligosaccharide mixture, on the faecal microbiota from patients with UC in a batch culture fermentation model. All substrates positively altered the gut microbiota with respect to the relative proportions of beneficial microbes and elevated organic acid profiles following fermentation. In this model, 2′-FL appeared to outperform both FOS and GOS in terms of modification of the microbial community of UC microbiota *in vitro*. This is based on suppression of *Desulfovibrio* spp. and improvement of more beneficial bacterial classes, including *Bifidobacterium* spp. and the Firmicutes *Clostridium* cluster XIVa and XIVb, along with the improvement in individual and total SCFA profile.

The paucity of *Lactobacillus* spp. found at baseline in patients with active UC compared to healthy controls is consistent with data from previous studies (Bullock et al. 2004, Vigsnæs et al. 2011). Interestingly, no baseline difference in *F.*

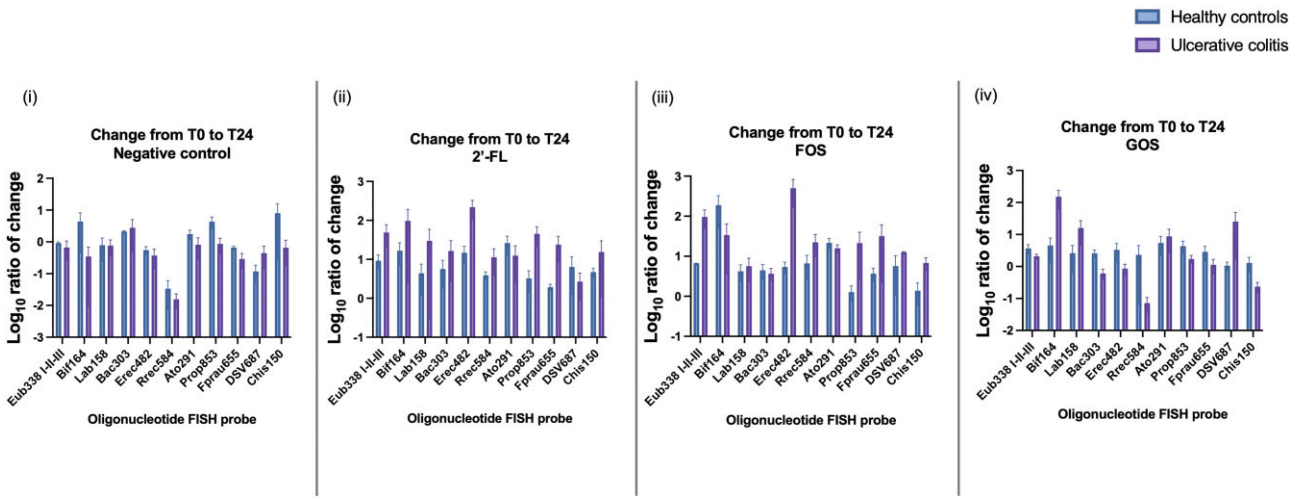


**Figure 4.** Ratio of change from baseline microbiota to 24 h with each oligonucleotide probe in both healthy controls and patients with UC for negative control and each substrate. Separate graphs shown for negative control (i), 2′-FL (ii), FOS (iii), and GOS (iv). Error bars represent standard error of the mean.

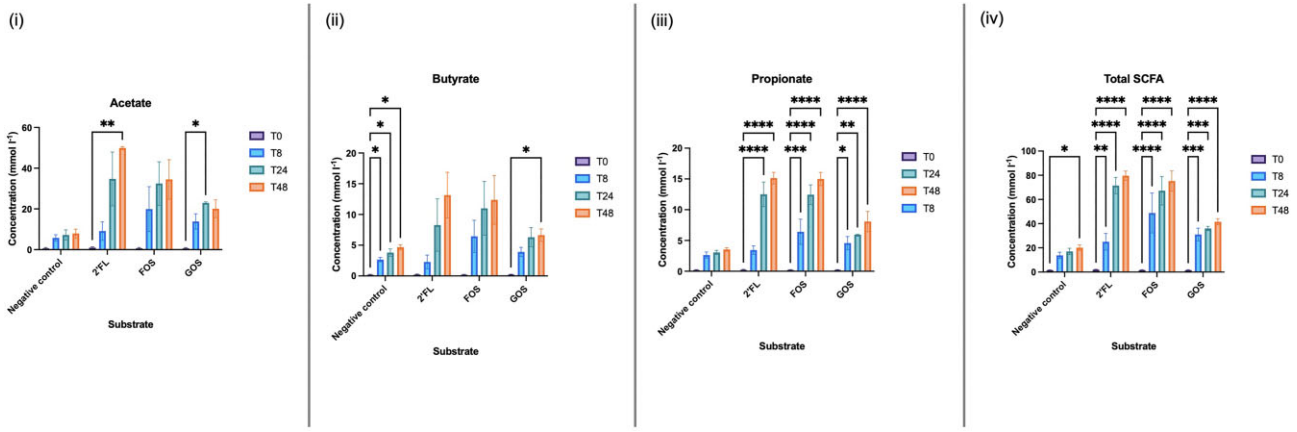


**Figure 5.** Concentrations of organic acids at baseline, and then at 8, 24, and 48 h post-inoculation for the negative control vessel and for each substrate in samples taken from patients with UC. Separate graphs shown for acetate (i), butyrate (ii), propionate (iii), and total SCFA (iv). Error bars denote standard error of the mean, pairwise comparisons annotated where statistical significance reached: $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, $^{****}P \leq .0001$.

*prausnitzii* or *Roseburia* spp. was seen in this study in patients with UC compared to healthy controls, as has been suggested previously (Machiels et al. 2014).

It is interesting to note that although final counts at 24 h were similar, patients with UC appeared to have a greater capacity for change in microbial counts with the same substrates compared to healthy controls. This was particularly true in important groups such as *Bifidobacterium*, *F. prausnitzii*, *Lactobacillus* spp., and the butyrate producing *Clostridium coccoides—Eubacterium rectale* group. This suggests that given the initial dysbiosis in patients with UC, there is greater 'room to grow' with the introduction of prebiotics in these patients compared to healthy controls.

The important role of the SRB *Desulfovibrio* spp. in the pathogenesis of UC would suggest that any potential intervention to reduce inflammation in the condition should ideally reduce SRB abundance, or at the least not increase it. Of the prebiotics tested here, only 2′-FL had a suppressive effect on *Desulfovibrio* spp.

The significant improvements in organic acids demonstrated with the prebiotic candidates are likely be clinically relevant. Patients with UC have reduced levels of total SCFAs, acetate, and propionate compared to healthy controls, and patients with active UC have reduced levels of acetate, butyrate, and propionate compared to those in remission (Hold et al. 2003). Butyrate acts as the main source of energy for colonocytes, and SCFAs act via G protein coupled receptors to modulate the immune system (Parada Venegas et al. 2019, Xu et al. 2022). Increasing luminal SCFA concentration therefore is likely to have a beneficial effect in reducing inflammation in UC.

Several potential limitations exist for this study. Active UC is characterized by loose stool and increased frequency, and so it is worth considering whether the timepoints described here are appropriate given the potential decrease in colonic transit time in UC. Previous studies have shown, however, that it is the rectosigmoid irritability due to colonic inflammation, rather than reduced transit time that leads to these symptoms (Rao et al. 1987).

The reason 2′-FL may have outperformed FOS and GOS in this *in vitro* model could be due to its bifidogenic effect. This has been demonstrated *in vivo* in dietary intervention studies

in healthy adults (Elison et al. 2016). HMOs such as 2′-FL in the infant gut are predominantly metabolized by *Bifidobacterium* spp., which are in turn the most dominant genus in the infant colon (Hill et al. 2017, Sakanaka et al. 2020). Given reduction in abundance of *Bifidobacterium* spp. is implicated in active UC, targeting this deficiency with HMOs to shift the gut microbiota to a more 'healthy' profile is a logical approach.

The ultimate assessment of efficacy of prebiotics in disease states is through human intervention studies, the gold standard being the double-blind, placebo controlled randomized controlled trial (RCT). Two human studies to date have used this design to look at prebiotics in UC, both looking at inulin-type fructans (Casellas et al. 2007, Ikegami et al. 2023). The most recent, a 2023 study compared the effect of the trisaccharide FOS 1-kestose vs placebo in 40 patients (Ikegami et al. 2023). The trial met its primary endpoint, with a significant improvement in clinical activity index in the prebiotic group compared to placebo. Significant decreases in a number of genera were observed following kestose treatment, but no change in *Bifidobacterium* spp. or *Faecalibacterium* spp. was reported, and there were no differences in SCFA production between intervention and placebo groups. Crucially, FOS was well tolerated and low-risk, which is mirrored in all prebiotic trials to date in healthy controls, UC, and other conditions.

No RCT studies on the role of HMOs in UC have so far been done. A single arm, open label trial using a proprietary nutritional formula containing 2′-FL (along with a host of micro- and macronutrients, amino acids, and *iso*-maltooligosaccharide) in 12 patients, including four patients with UC found improvement in Gastrointestinal Quality of Life Index following 6 weeks' intervention (Ryan et al. 2021). In addition, increases in stool total SCFA, butyrate, and acetate were observed, as were increases in *Bifidobacterium* spp. and *F. prausnitzii*.

Overall, this study is the first to directly compare 2′-FL, FOS, and GOS in terms of relative efficacy in improving the gut microbiota and metabolic profile in UC. This *in vitro* approach would support the progression to a well-designed human dietary intervention trial of the HMO 2′-FL in patients with UC, to determine whether improvements in clinical, microbiological, and metabolic parameters can be observed with consumption of this prebiotic.

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## Author contributions

James M. Kennedy (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing), Aminda De Silva (Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing), Gemma E. Walton (Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing), Carlos Poveda (Data curation, Investigation, Project administration, Resources, Writing – review & editing), and Glenn R. Gibson (Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing)

## Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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