1. ABOUT THE DATASET
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Title: Dataset supporting the article ‘Fermentation of human milk oligosaccharides using faecal microbiota from healthy and Irritable Bowel Syndrome donors using in vitro batch cultures’.

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Rights-holder: University of Reading.

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Description: This dataset presents the statistical analysis results from a study exploring the effects of different human milk oligosaccharides (HMOs), solely and in combination, on the gut microbiome and short-chain fatty acid (SCFA) production using 280ml anaerobic in vitro batch culture fermenters. 7 IBS and 3 healthy volunteers were included and samples from each fermentation were taken at timepoints 0, 4, 8, 24 and 48 hours. The aim was to assess prebiotic effects of HMOs in faecal samples from Irritable Bowel Syndrome (IBS) and healthy controls, and to determine a best-performing HMO in IBS. Fluorescence in situ hybridisation coupled with flow cytometry was utilised to study the microbiota at 0 and 24 hours. SCFA production was assessed by gas chromatography at 0, 8, 24 and 48 hours. The data outputs were kept separately for both laboratory techniques. Baseline microbial profiles were analysed to determine whether there were significant differences between healthy and IBS volunteers and whether there was an IBS signature. IBS donors had different starting microbial profiles compared to healthy controls, and lower levels of SCFA. In response to HMOs, there was a shift in both the healthy and IBS faecal microbiomes. In IBS donors, *Bifidobacterium*, *Faecalibacterium* and total bacterial numbers significantly increased post-HMO intervention. There was a shift in both the healthy and IBS faecal microbiomes and an increase in SCFA production at 24 hours.

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2. TERMS OF USE
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3. PROJECT AND FUNDING INFORMATION
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Title: A human intervention study using human milk oligosaccharides to improve symptoms in irritable bowel syndrome through targeting of the gut microbiota.

Dates: September 2021-September 2025

Funding organisation: FoodBioSystems DTP, BBSRC and Nestlé.

Grant no.: Ref: BB/T008776/1

4. CONTENTS
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File listing

 - Excel Spreadsheets Dataset:

File name: in\_vitro\_data.xlsx

Spreadsheets in file:

1. Supplemental data table 1 (Bacterial Enumeration - IBS vs Healthy and paired tests).
2. Supplemental data table 2 (Bacterial enumeration - T0 vs T24 all vessels combined).
3. Supplemental data table 3 (IBS and Healthy all - Organic acids - T0, T8, T24 and T48).
4. Supplemental data table 4 (IBS vs Healthy - Organic acids: T0 vs T24).
5. Supplemental data table 4 (2) (IBS vs Healthy - Organic acids: T0 vs T48).
6. Supplemental data table 5 (IBS vs Healthy - Organic acids: all vessels combined T0 vs T24).
7. Supplemental data table 5 (2) (IBS vs Healthy - Organic acids: all vessels combined T0 vs T48).

5. METHODS
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The dataset was generated in the Food and Nutritional Sciences Unit at The University of Reading from 1 March 2022 to 24 September 2023.
Methods explained in the relevant paper used to obtain these results:

*Human milk oligosaccharides*

HMOs (2’FL, 3-fucosyllactose (3’FL), 3-sialyllactose (3’SL), 6-sialyllactose (6’SL), lacto-neotetraose (LNT) and LNnT) were provided by Glycom (Denmark) (batch numbers CPN6817 1000217 FD, CPN291300215, CPN5115 1000916 FD, CPN4215 00115). 1.5g of each HMO were used individually and in combination, using in vitro batch culture vessels (1) to give a final concentration of 1% per vessel.

*Faecal sample preparation*

Ethical approval for collecting faecal samples from volunteers was obtained from the University of Reading Research Ethics Committee (UREC 18/02). Fresh faecal samples were provided by 3 healthy donors free from GI disorders (age 24-27) and 7 IBS donors (4 IBS-D, 2 IBS-C and 1 IBS-M, age 22-64), all females. Donors were not regular users of pre/probiotic supplements or consumers of live yoghurt and had not consumed antibiotics in the 3 months prior to donating. Samples were collected and placed in an anaerobic jar (O2 ≤0.1%; CO2: 7-15%) using Thermo Scientific AnaeroGen™️ 2.5 L anaerobic sachets (Oxoid, Basingstoke, UK). Samples were used for inoculation within 2 hours of production. To form a 10% (w/v) faecal slurry, 20 g of weighed faecal sample was homogenised with 180 mL of anaerobically prepared phosphate buffered saline at 0.1 mol l−1 (PBS, Oxoid, Hampshire, UK), pH 7.4 for 2 minutes using a stomacher (Seward, stomacher 80, Worthing, UK) at 240 paddle beats/min.

*Batch culture fermentation*

Batch culture fermenters were inoculated with 15mL of fresh faecal slurry from each donor. First, 135 mL of standard basal nutrient medium (2) was autoclaved at 121°C for 15 min. Media was then steamed for 20 minutes and aseptically added to autoclaved 280 mL vessels. The basal medium (per litre) consisted of: 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 0.04 g K2HPO4, 0.04 g KH2PO4, 0.01 g MgSO4.7H2O, 0.01 g CaCl.6H2O, 2 g NaHCO3, 2 mL Tween 80, 0.05 g hemin, 0.01 mL vitamin K1, 0.5 g L-cysteine-HCl, 0.5 g bile salt and 4 mL resazurin solution (0.25 g/L). Vessels were then left to gas overnight using oxygen free N2 at a rate of 15 mL/min to achieve anaerobic conditions. Vessels were maintained at 37◦C using a circulating water bath. The media were adjusted to pH 6.8 and subsequently maintained between 6.7 and 6.9 using pH controllers (Fermac 260, Electrolab, Tewkesbury, UK) connected to 0.5 M solutions of HCL and NaOH. This pH was selected to mimic conditions of the distal colon (3, 4). Immediately prior to faecal inoculation, 1.5g of each HMO intervention was added to each vessel (1% w/v final concentration). In addition, each fermentation run included a negative control vessel, to which only the faecal slurry was added, and a positive prebiotic control vessel, to which fructooligosaccharide (FOS) derived from inulin (Orafti® P95, Beneo, Belgium) (1.5 g) was added as an additional substrate. All vessels were inoculated with 15 mL of faecal slurry (10% w/v) to give a final concentration of 1% faeces (w/v). Baseline samples (T0) were taken immediately post-inoculation, and further samples were collected at 4, 8, 24, 48 and 72 hours. Samples T0, T8, T24 and T48 were analysed by GC and T0 and T24 by flow-FISH. Stable pH and anaerobic conditions were maintained throughout.

*Preparation of samples*

Samples were aliquoted into 1.5 mL Eppendorfs™ (Fisher Scientific) for Gas Chromatography (GC) analysis (short-chain fatty acids), and Fluorescence in situ Hybridisation (FISH) (enumeration of bacteria), 1.5 mL and 0.75 mL, respectively. For GC, samples were centrifuged at 11,600 g for 10 minutes before transferring the supernatant into a fresh vial, and storing the pellet at −20◦C. For FISH, samples were centrifuged at 11,600 g for 5 min. After removing supernatant, the pellet was resuspended in 375μL of PBS before adding 1125μL of 4% paraformaldehyde. These samples were then stored at 4◦C for 4–8 hours before being washed twice with 1 mL of PBS and resuspending the pellet in 150μL of PBS. Finally, 150μL of ethanol was added, the samples were vortexed to homogenise and then stored at −20◦C pending analysis.

Fluorescence in situ hybridisation with flow cytometry (Flow-FISH)

Preparation of samples followed the protocol of Grimaldi et al. (5). Briefly, samples were removed from storage at −20◦C and vortexed to redisperse. Then, 75μL of sample were suspended in 500μL of PBS before vortexing and centrifuging for 3 minutes at 15,115 g (consistent for all centrifuging during this process). For permeabilisation of the bacterial cell wall, supernatant was discarded, and the pellets resuspended in TE-FISH (tris-HCl 1M pH 8.0, EDTA 0.5M pH 8.0, water at 10:10:80) containing lysozyme (1 mg/mL) and incubated in the dark for 10 min at room temperature. Samples were then re-centrifuged and washed using 500μL PBS. For in situ hybridisation, pellets were resuspended in 150μL of hybridisation buffer (5 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.01% (w/v) sodium dodecyl sulphate, 30% formamide), centrifuged, and resuspended again in 1 mL of hybridisation buffer. Then, 50μL of this solution was added to each Eppendorf containing 4μL of the oligonucleotide probe solutions, which were vortexed and incubated overnight at 35◦C using heating blocks. Following incubation, 125μL of hybridisation buffer was added, and Eppendorfs were vortexed and centrifuged. After discarding the supernatant, pellets were resuspended in 175μL of washing buffer (5 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate), vortexed to homogenise and then incubated at 37◦C for 20 minutes in the heating block. The washed pellets were then centrifuged again, resuspended in 300μL of PBS, vortexed, and then stored in the dark at 4◦C ready for flow cytometry. Enumeration of bacteria was conducted using an Accuri C6 flow cytometer and analysed using the Accuri C Flow Sampler software (6). Ten oligonucleotide probes (Table 1) were selected for inclusion, targeting a range of functionally relevant bacterial populations. Additionally, a mixed 338EUB probe was used to enumerate total bacteria.

Table 1. Oligonucleotide probe sequences and corresponding target species.

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| --- | --- | --- | --- |
| Probe name  |  Sequence (5’ to 3’)  |  Targeted groups  |  Reference  |
| Non Eub  | ACTCCTACGGGAGGCAGC  | Control probe complementary to EUB338 | (7) |
| Eub338‡  | GCTGCCTCCCGTAGGAGT  | Most bacteria  | (8) |
| Eub338II‡  | GCAGCCACCCGTAGGTGT  | Planctomycetales  | (8) |
| Eub338III‡  | GCTGCCACCCGTAGGTGT  | Verrucomicrobiales  | (8) |
| Bif164  | CATCCGGCATTACCACCC  | Bifidobacterium spp.  | (9) |
| Lab158  | GGTATTAGCAYCTGTTTCCA  | Lactobacillus and Enterococcus  | (10) |
| Bac303  | CCAATGTGGGGGACCTT  | Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae  | (11) |
| Erec482  | GCTTCTTAGTCARGTACCG  | Most of the Clostridium coccoides-Eubacterium rectale group (Clostridium cluster XIVa and XIVb)  | (12) |
| Rrec584  | TCAGACTTGCCGYACCGC  | Roseburia genus  | (13) |
| Ato291  | GGTCGGTCTCTCAACCC  | Atopobium cluster  | (14) |
| Prop853  | ATTGCGTTAACTCCGGCAC  | Clostridial cluster IX  | (13) |
| Fprau655  | CGCCTACCTCTGCACTAC  | Faecalibacterium prausnitzii and relatives  | (15) |
| Dsv687  | TACGGATTTCACTCCT  | Desulfovibrio genus  | (16) |
| Chis150  | TTATGCGGTATTAATCTYCCTTT  | Most of the Clostridium histolyticum group (Clostridium cluster I and II)  | (12) |

*Gas chromatography*

Preparation of samples for GC was carried out in line with the method previously described by Richardson and colleagues (17). Samples were defrosted, centrifuged for 30 seconds at 15,115 g, and 1mL transferred to 100 mm × 16 mm glass vials, together with 50μL internal standard (0.1 M 2-ethylbutyric acid) 0.5 mL concentrated HCl and 3 mL diethyl ether. Vials were vortexed for 1 min and centrifuged for 10 min at 2000 g (Eppendorf 5804 R, Stevenage UK). The upper diethyl ether layer was extracted and transferred to new vials, from which 400μL was taken and added with 50μL of N-Methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) to screwcap HPLC vials. The vials were protected from light and stored at room temperature for 72 hours prior to analysis to allow for all SCFAs, including lactate, to derivatise.

Samples were analysed using a 5690 series Gas Chromatograph (Hewlett Packard, London, UK) with HP-5 ms column (L×I.D. 30 m×0.25 mm, 0.25μm film thickness) coating of crosslinked (5%-phenyl)- ethylpolysiloxane (Agilent, Santa Clara, CA, USA). Then, 1μL of each sample was injected with a run time of 17.7 min. Injector and detector temperatures were 275◦C and the column temperature was programmed from 63◦C to 190◦C at 5◦C per min and held at 190◦C for 3 minutes. Helium was used as the carrier gas at a flow rate of 1.7 mL/min (head pressure, 133 KPa). A split ratio of 100:1 was used. The external standard solution included acetic acid (30 mM); propionic acid (20 mM); n-butyric acid (20 mM); n-valeric acid (5 mM); iso-butyric acid (5 mM); iso-valeric acid (5 mM) (all Sigma-Aldrich). Quality control samples of external standard solution were included for every 20 samples to maintain accurate calibration. Peak integration was performed using Agilent Chem station software (Agilent Technologies, Cheadle, UK), and quantification of each SCFA (mM) was calculated using internal response factors as described previously (1).

*Statistical analysis*

Statistical Package for Social Science version 27 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. To assess changes in bacteriology and organic acid production a general linear model was constructed to assess repeat measures (Treatment\*Time). Comparisons were also performed to assess any potential significant differences between health status within each treatment (Health status\*Treatment\*Time) at T0 and T24 in both bacteriology and total organic acid production. Post hoc comparisons were also performed to identify any significant differences between organic acid production at 48 h within IBS and Healthy treatments. All post hoc pairwise comparisons were corrected for type 1 errors using Bonferroni adjustment within each general linear model. All tests were two tailed and P values were considered significant at P ≤ 0.05 and are displayed by \* = P ≤ 0.05, \*\* = P ≤ 0.01 and \*\*\* = P ≤ 0.001. Graphs were generated in GraphPad Prism version 10.0.2 for Windows, GraphPad Software, San Diego, California, www.graphpad.com.

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