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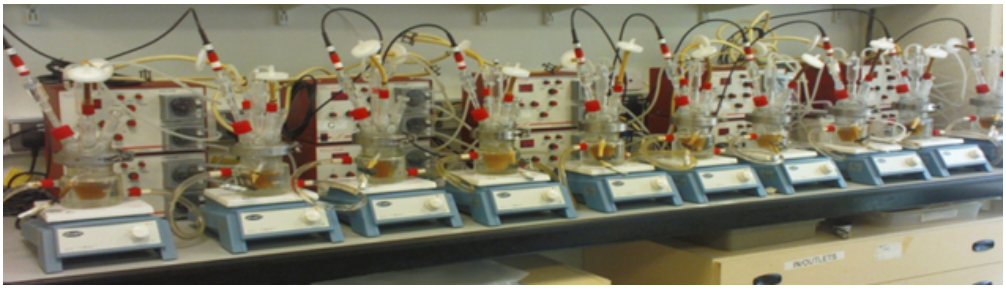

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Development of a prebiotic blend to influence in vitro fermentation effects, with a focus on propionate, in the gut.

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Key words

Propionate; Colonic model; Prebiotic; Arabinoxylan; Resistant starch; Inulin; Polydextrose, β-1,4 glucan; α-gluco-oligosaccharide; xylo-oligosaccharide.

Abstract

Short chain fatty acids (SCFAs) derived from the human gut microbiota, and in particular propionate, may beneficially influence metabolic processes such as appetite regulation. Development of prebiotics that induce high propionate levels during fermentation is desirable. Eleven candidate prebiotics were screened to investigate their fermentation characteristics, with a focus on propionate production in mixed anaerobic batch culture of faecal bacteria. Further to this, a continuous 3-stage colonic fermentation model (simulating the human colon) was used to evaluate changes in microbial ecology, lactate and SCFA production of three 50:50 blends, comprising both slow and rapidly fermented prebiotics. In mixed batch culture: xylo-oligosaccharide, polydextrose and α-gluco-oligosaccharide were associated with the greatest increase in propionate. Polydextrose, α-gluco-oligosaccharide, β-1,4 glucan and oat fibre induced the greatest reductions in the acetate to propionate ratio. The most bifidogenic prebiotics were the oligosaccharides. Fermentation of a 50:50 blend of inulin and arabinoxylan, through the continuous 3-stage colonic fermentation model, induced a substantial and sustained release of propionate. The sustained release of propionate through the colon, if replicable *in vivo*, could potentially influence blood glucose, blood lipids and appetite regulation, however, dietary intervention studies are needed. Bifidogenic effects were also observed for the inulin and arabinoxylan blend and an increase synthesis of butyrate and lactate, thus indicating wider prebiotic potential.

Introduction

Short chain fatty acids (SCFA), produced via fermentation in the gut, exert diverse physiological and metabolic effects, thus potentially influencing the health of the host (Chambers 2018). Colonic derived propionate for example, is mainly metabolised in the liver, where it can act as a precursor for gluconeogenesis, thus influencing metabolic homeostasis (De Vadder 2014). Propionate also interacts with host cell free-fatty-acid receptors stimulating the release of satiety signals (Nøhr 2013), and may therefore decrease food-seeking behaviours (Canfora & Blaak 2015) and reduce body weight (Chambers 2015). Propionate has also been shown to decrease systemic inflammation (Chambers 2019). Therefore, targeted manipulation of the activity of the gut microbiota to increase propionate production is likely to be of benefit to human health. Bacteria from within the gut microbiota utilise the acrylate, succinate and propanediol pathways for propionate production, although the pathway of choice is

dependent on the fermentation substrate, the microbial species involved, and the physicochemical environment within the colon. Primary utilisers of the acrylate pathway include *Megasphaera* spp. and *Coprococcus catus*; these may utilise lactate, produced by lactobacilli or *Bifidobacterium* spp. for example, as a substrate (Flint 2015). Primary polysaccharide degraders from the *Bacteroidia* genus may utilise dietary residues to feed the succinate pathway (Reichardt 2014), but specialist secondary degraders, such as *Veillonella parvula* and *Phascolarctobacterium succinatutens*, are capable of utilising derived succinate as a substrate for propionate synthesis (Watanabe 2012). Thus, cross-feeding, competitive, and symbiotic microbial interactions, add complexity to predicting propionate production in response to diet.

A prebiotic is defined as 'a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson 2017). Several oligosaccharides, hemicellulose, resistant starches, and to a lesser extent, plant polyphenols have been investigated for their prebiotic properties (Sanders 2019). Many of these have been shown to increase SCFA synthesis by gut bacteria (Roberfroid 2010), however investigations to date have not focused on using individual or combined prebiotics to increase propionate concentrations in the gut. We hypothesise that blending prebiotic substrates may increase propionate synthesis along diverse pathways and thus lead to an elevated total propionate yield. Further, we hypothesise that competition for diverse substrates will lead to sustained propionate synthesis throughout the colon, with potential benefits for metabolic health.

Here, we explored these hypotheses, firstly by screening 11 candidate prebiotics for propionate synthesis, using 48-hour anaerobic mixed-batch-culture fermentations with human faecal inoculum. These experiments were controlled for temperature and pH to approximate conditions in the distal region of the human colon. This initial screen was used to investigate the prebiotic characteristics of individual blends such as bifidogenic effects, SCFA production and lactic acid production. High propionate producing substrates were then selected to be developed into 3 blends for further analysis, in a 16 day 3-stage continuous mixed colonic culture system. This model is designed to simulate three anatomically distinct (proximal (V1), transverse (V2), and distal (V3)) regions of the colon. The model has been microbiologically validated against necropsy specimens by Gibson *et al*. (Gibson 1993) and has been widely used

to study prebiotic activity in the human colon (Gibson 1993; Grimaldi 2017; Liu 2016; Sarbini 2013).

The purpose of this work is to develop novel blends of prebiotics that, when consumed, may lead to a targeted beneficial increase in propionate production in the human colon.

Methods

Mixed batch culture fermentation

Faecal Donors

Faecal samples were collected on the day of inoculation and kept in an anaerobic container (10% H_2, CO_2 and 80% N_2) for no more than 1.5hrs prior to inoculation. A different faecal donor was used for each of the three repetitions. Donors were 2 females, 1 male, all with a BMI of between 19 and 28kg/m^2, they were non-smokers aged 25-40, and had no history of gastrointestinal disorders, no antibiotic use in the previous 6 months and had not consumed prebiotic/probiotic enriched functional foods within 3 weeks prior to participating. All volunteers gave verbal consent to use of faecal samples in these experiments, in accordance with University of Reading procedures and the Human Tissue Act 2004. Samples were diluted 1:10 in anaerobically stored phosphate buffered saline (PBS; 0.1 M, pH 7.4), then homogenised in a stomacher (Steward 400) for 2 min at 240 paddle beats per min.

Basal medium g/L

Basal medium was prepared by heating and stirring the following substrates: peptone water (2 g), yeast extract (2g/L), NaCl (0.1 g/L) (Fisher Scientific, Loughborough, UK), K_2HPO_4 (0.04 g/L), KH_2PO_4 (0.04 g/L), $MgSO_4.7H_2O$ (0.01 g/L) (Fisher Scientific, Loughborough, UK), $CaCl_{2.6}H_2O$ (0.01 g/L), $NaHCO_3$ (2 g/L), Tween 80 (2ml/L), Hemin (0.05 g/L), Vitamin K (10 ml/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L) and resazurin (0.25 g/L). All substrates were sourced from Sigma Aldrich (Poole, UK), unless otherwise stated. The medium was then autoclaved at 121˚C for 15 min and aseptically added to individual sterile batch culture vessels, totalling 135mL, then adjusted to pH 6.8. The fermenters were purged with O_2 free N_2 (15 ml/min), they were magnetically stirred and were pH and temperature controlled (37^{o}C).

Substrates

Eleven commercially available fibres were assessed using batch culture; their physicochemical properties are described in **Table 1;** They were long-chain fructo-oligosaccharide (LC-FOS), (Fine Foods & Pharmaceuticals, Italy), oligofructose (OFS), (Orafti P95, Beneo, Germany), resistant starch (RS), (Himaize 260 – Sigma potato starch, Ingredion, Germany), resistant maltodextrin (R-MLX), (Promitor T&L, UK), α-gluco-oligosaccharide, (GLOS), (Solabia, Biocolians, France), β-1,4 glucan (β-GLU), (Glucagel), oat fibre (OAT), (Fine Foods & Pharmaceuticals, Italy), low viscosity arabinoxylan (LV-AX), (Megazyme, Ireland), medium viscosity arabinoxylan (MV-AX), (Megazyme, Ireland), xylo-oligosacchairde, (XOS), (Santori, Japan), Polydextrose (PDX), (Danisco, USA).

The batch culture method has been previously described by Vulevic *et al.* (Vulevic 2004) briefly 3 separate fermentation experiments were carried out using faecal samples from 3 healthy donors and assessed in triplicate. In each experiment, twelve sterile batch culture fermenters (300mL volume) were aseptically filled with 135mL autoclaved medium. Each vessel was inoculated with 15 ml faecal slurry (1:10 w/w), then fermentation was initiated with the addition of 1% w/v individual substrate into the fermenters. Inulin was chosen as a positive prebiotic control (1% w/v) and there was an additional 12th vessel containing no substrate included as a negative control vessel. Culture pH was maintained between 6.7–6.9 using pH controllers (Fermac 260; Electrolab, Tewksbury UK) and automatically adjusted by adding 0.5 M NaOH or HCl to the vessels as required. The pH of 6.8 and temperature of 37˚C was controlled to simulate conditions of the distal region of the human large intestine. Batch culture fermentations ran for 48 hours, and samples were collected at 0, 24 and 48 hours (5ml from each vessel) for analyses of bacterial populations and metabolite production. Fermentation of each substrate was performed in triplicate for each volunteer.

3-Stage continuous colonic model

Gut model medium g/L

Gut medium was prepared by heating and stirring the following substrates: starch (5g), peptone water (5g), tryptone (5g), yeast extract (4.5g), NaCl (4.5g) (Fisher Scientific, Loughborough, UK), KCl (4.5g), mucin (porcine) (4g), casein (3g), pectin (2g),

beechwood derived xylan (2g), arabinogalactan (2g), $NaHCO_3$ (1.5g), $MgSO_4$ (1.25g) (Fisher Scientific, Loughborough, UK), guar gum (1g), inulin (1g) (BENEO-Orafti, Tienen, Belgium), L-cysteine (0.8g), HCl (0.8g), KH_2PO_4 (0.5g), bile salts (0.4g), $CaCl_2 \cdot 6H_2O$ (0.15g), $FeSO_4.7H_2$ (0.005g), (0.05g) hemin (0.05g), Tween 80 (1mL), vitamin K (10µL), resazurin (4 ml). All substrates were sourced from Sigma Aldrich (Poole, UK), unless otherwise stated.

50:50 Blended prebiotic substrates

Inulin (LC-FOS) was chosen as a base oligosaccharide for each of the three 50:50 prebiotic blends; it is an established prebiotic with well documented bifidogenic effects, both *in vitro* and *in vivo*. We hypothesised that the fermentation of this LC-FOS would lead to an increase in acetate and lactate in the system and that these metabolites could be utilised in cross-feeding by propionate producing bacterial groups.

The secondary substrates for three prebiotic blends were selected based on the total propionate yield and based on the relative speed of propionate production in the batch culture screen. GLOS induced the highest total production of propionate in the single substrate batch culture; it was also observed that it yielded propionate primarily in the first 24 hours of batch culture. RS and ABX also produced an abundance of propionate, these two substrates demonstrated significant continued propionate production in the second 24 hour period of fermentation, and were thus selected for the other two blends. The physicochemical properties of the substrates used in the three 50/50 (w/w) prebiotic blends are given in **Table 1.** These blends were inulin (Fruitafit, Chimab, Italy) and resistant starch (I+RS) (Ingredion, USA); inulin and α-gluco-oligosaccharide (I+GLOS) (Bioecolia, Italy); and inulin and arabinoxylan (I+AX), (Megzyme, Ireland). A daily 2.66g dose of each blend was administered as two 1.33g doses (10.30am & 3pm). This daily dose is reflective of the scaled down model used here, previously described by Macfarlane *et al*. (Macfarlane 1998) and is approximately 33% of the prebiotic dose administered to human adults.

Faecal samples were collected from donors as described in the 48-hour batch culture experiments, with the exception that all three donors were female and aged between 25-38 yrs. A three-stage continuous gut model system that simulates the physio-chemical conditions of anatomically distinct areas in the large intestine was used to investigate the fermentation characteristics of fibre blends. This relies upon gradients

of pH, substrate concentration and microbial growth rate (vessel transit time) to simulate the proximal, transverse and distal colon (Gibson 1993; Macfarlane 1998). A trio of connected glass fermentation vessels cascaded in descending order of smallest to largest volume corresponding with the caecum (V1, 80mL at pH=5.5), transverse (V2, 100mL at pH=6.2), and distal colon (V3, 120mL at pH=6.8). The models were inoculated with a 20% (wt:v) faecal homogenate using freshly voided faeces from one of the 3 healthy weight donors aged 25-38 yrs. Following inoculation, the gut model was left for 24h to equilibrate, at which time the medium feed pump was switched on and fed into V1 and via gravity, through to the connected vessels V2 and V3. The system was run for 8 full turnovers to allow steady state (SS1) to be achieved, which took 15 days. Each turnover represented the rate at which the full volume (300mL) was replenished by medium. SS1 was determined by HPLC analysis of SCFA over 3 consecutive days (±6%) at which time the intervention (prebiotic addition) commenced. The system was operated at a retention time of 48hr, with a flow rate of 6.25mL until day 31 when a second steady state (SS2) was determined, again by HPLC analysis of SCFAs (±6%) over 3 consecutive days. Blends were tested in triplicate, for each donor, in the continuous culture colonic model.

Biochemical analysis

A 1mL sample collected from each of the fermentation models, was centrifuged at 13,000 x g for 10min and the resultant supernatant filtered using 0.22µm membrane (Millipore, Burlington USA). Total and individual SCFAs were then measured as previously described by Salazar *et al*. (Salazar 2009). In a cuvette, filtered supernatant (20µL) plus internal standard (I/S) of 10mM 2-ethylbutiric acid was injected into the Agilent II HPLC system (Agilent, Manchester UK) with an eluent of 0.0025M H_2SO_4 passing through an organic acid column REZEX-ROA (Phenomenex, Torrence USA) at a flow rate of 0.5ml/min, heated to 84°C, with a 45 min run time to allow all peaks required to be measured. Agilent Chemstation (Hewlett Packard, California USA) was used to integrate peaks and quantification obtained using calibration curves of individual SCFAs and lactate (Sigma Aldrich, Gillingham UK): acetic, propionic and butyric acid of increasing concentration (12.5, 25, 50, 75, 100mM).

Bacterial enumeration

Faecal bacterial groups from both sets of *in vitro* experiments were enumerated using fluorescent *in-situ* hybridisation (FISH) in conjunction with flow cytometry, using the exact same method, the only difference was the way samples were collected. For batch culture, samples were collected at 0, 24 and 48h whereas for the 3-stage continuous model**,** samples were taken from all vessels (V1-V3) over 3 consecutive days at SS1 & SS2.

For both batch and continuous culture: a 750 µL sample was taken and centrifuged for 5 min at 11,330 x *g* for 5 min. The supernatant was then removed, and the remaining pellet re-suspended in 375 µl filtered PBS and 1125 µL of 4% (v/v) paraformaldehyde (PFA). Following incubation at 4˚C for 4 hours, samples were centrifuged at 11,300 x g for 5 min and then washed in 1mL of PBS to remove the PFA. The washing step was repeated 3 times, and the resultant pellet re-suspended in 150 µl filtered PBS and 150 µl ethanol (99%) then stored at -20˚C as previously described by Hobden *et al*. (Hobden 2013).

For permeabilisation: 75 µl of the fixed sample was mixed with 500 µl of 4°C PBS and centrifuged at 11330 × *g* for 3 min, the supernatant was then removed, and the remaining pellet re-suspended with 100 µL of TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, distilled H_2O) containing 1mg/mL lysozyme. Following incubation in the dark for 10 min, hybridisation steps were carried out, whereby samples were centrifuged at 11,300 x g for 3 min, the remaining pellet washed with 500 µL of cold PBS, then washed again with 150 µL of hybridisation buffer (5 M NaCl, 1 M Tris/HCl at pH 8, 30% formamide, double distilled dH_2O, 10% SDS), centrifuged at 11,300 × *g* for 3 min. The pellet was re-suspended into 1 mL hybridisation buffer and aliquoted (50 µL) into tubes for both total and individual bacterial groups to be enumerated using specific nucleotide probes that target specific areas of RNA and are detailed in: **Table 2:** (Daims 1999; Devereux 1992; Franks 1998; Harmsen 2002; Hold 2003; Langendijk 1995; Manz 1996; Reichardt 2014; Walker 2005; Wallner 1993). For hybridisation**:** EUB338-I-II-III (4 µL) probe, which is attached with fluorescent Alexa 488 at the 5' end, was added to each tube to target total bacterial species. EUB338 I alone is considered insufficient for the detection of all bacterial groups so EUB I II & III were mixed together (in equal measure) to allow for more accurate quantification (Daims 1999). A specific probe (4 µL) was then added, which is distinguished from EUB388-I-II-III as it attaches

with fluorescent Alexa 647. Non-EUB was used as a negative control on its own, attaching with fluorescent Alexa 647 at the 5' end. All tubes were then incubated in a heating block in the dark at 35°C for a minimum of 10 hours to enable hybridisation. Following hybridisation, samples were centrifuged for 3 min at 13000 x *g* and the supernatant removed and the pellet washed with 200 µL of washing buffer. The washing buffer consisted of: 12.8 µl of 5 M NaCl, 20 µl of 1 M Tris-HCl (pH 8), 10 µl of 0.5 M EDTA (pH 8), and 1 µl of 10% SDS in 956.2 µl of filtered cold distilled water, incubated for 20 min at 37°C for 20 min, centrifuges at 11,330 x *g* for 3 min, and re-suspended in in 4°C PBS. The amount of PBS used was dependant on the preparation of samples and calculated using a dilution factor. Events/µl were then determined from NON EUB338 and EUB I-II-III probes using a flow cytometer (BD Accuri™ C6) according to manufacturer instructions to determine background noise, which was then subtracted from the analysis.

Statistical analysis

Statistical analysis was performed using SPSS for windows version 20.0. Tests for normality were carried out using Shapiro-Wilk. Most data were normally distributed and analysed by two-way ANOVA and Tukeys post-hoc testing. Additional paired t-tests were applied to assess the significance of single pairs of data. Statistical significance was accepted at $P < 0.05$ for all analyses.

Results

Phase 1. 48-hour Mixed batch culture fermentation

Short chain fatty acid concentrations

Propionate

Propionate production differed significantly across all 3 timepoints (0, 24, 48 hours) ($P<0.001$) and among fermentations ($P<0.001$). Between 0-24 hours, GLOS induced the greatest propionate increase, 33 mM above levels measured in the control vessel at this time. Other high propionate producers between 0-24 hours, with an average increase of 17 ± 2 mM above control vessel, were OFS and PDX. Between 24-48 hours, the greatest changes ([42-24]) in propionate concentrations were observed with

XOS: 19 mM, β-GLU: 12 mM, RS: 10 mM, PDX: 11 mM and MV-AX: 10 mM, above control **(Figure 1A)**.

Acetate

There were significant differences (P<0.001) in concentrations of acetate across all time points (0, 24, 48 hours) and these differed among fermentations relative to control (P<0.001). Acetate production was highest in the first 24 hours with the prebiotic substrates, with the exception of OAT and β-GLU, neither of these two substrates induced significant increases in acetate, above control. The magnitude of increase in acetate ([24-0]), was greatest with OFS, XOS, LV-AX, R-MLX, RS and LC-FOS, with an average 52 ± 5 mM increase above levels measured in the control vessel. LC-FOS and RS induced the greatest increase in acetate concentrations between 24-48 hours, with an average 37 ± 5 mM increase above control. Other notable acetate producers at this time were: R-MLX, MV-AX and XOS with an average 17 ± 2 mM increase above control. **(Figure 1B).**

Butyrate

Butyrate production differed significantly across all timepoints 0, 24, 48 hours (P< 0.05) and among fermentations (P<0.05). Between 0-24 hours, the greatest increase in butyrate concentrations ([24]-[0]) were recorded for β-GLU and XOS, averaging 14 ± 3 mM above control. The greatest increase in butyrate concentrations between 24 and 48 hours ([48]-[24]) were observed with LC-FOS, PDX and RS, these substrates induced an average 5 ± 2 mM increase above control **(Figure 1C)**.

Changes in microbial community structure

Changes that occurred to microbial ecology in the mixed faecal 48 hour batch culture at 0, 24 and 48 hours are presented in **Figure 2**.

Eub I-III – Total bacteria

Significant increases in Eub383 I-II-III labelled cells (total bacterial cell counts) were observed across the 3 time points (0. 24, 48 hours) (P<0.001). The magnitude of these changes differed significantly among prebiotic substrates and relative to the control (P<0.001). There was an increase in Eub383 I-II-III labelled cells during all fermentations between 0-24 hours and the greatest increases ([24]-[0]) were observed

with LC-FOS, R-MLX, OFS, GLOS and MV-AX, with an average 1.02 ± 0.12 log_{10} (cells/mL) increase above control.

Bifidobacterium: Significant changes in numbers of Bif164-labelled cells (*Bifidobacterium*) were observed across all 3 timepoints (0, 24, 48 hours) (P<0.001). The bifidogenic effects also differed across prebiotic fermentations (P<0.001). Between 0 and 24 hours the magnitude of increase in Bif164-labelled cells was greatest with OFS, XOS, GLOS, R-MLX and LV-AX, with an average 0.90 ± 0.14 log_{10} increase in Bif164-labelled cells/mL above the control. Between 24 and 48 hours, the magnitude of change ([42-24]) in abundance of Bif164-labelled cells was greatest with LC-FOS and PDX, which induced an average 0.74 ± 0.02 log_{10} increase in cells/mL above control. Whereas GLOS, MV-AX, RS and R-MLX induced an average 0.39 ± 0.08 log_{10} increase in cells/mL above control.

Clostridium Cluster XIVa (Clostridium coccoides):

There were changes in the abundance of Erec482-labelled cells (*Clostridium coccoides*), measured across all time-points (0, 24, 48 hours) (P<0.001) and these changes differed significantly among prebiotic fermentations (P<0.05) compared to control. LC-FOS and R-MLX induced the greatest increase in Erec482-labelled cells with an average 0.70 ± 0.02 log_{10} cells/mL increase, above control. Fermentation of OAT, PDX, LV-AX and XOS however, led to an average 0.45 ± 0.09 log_{10} cells/mL decrease in Erec482-labelled cells, relative to the control. For many of the substrates there was a slight decrease in the numbers of Erec482-labelled cells in the 24-48 hours period, however RS induced a 0.67 log_{10} increase in cells/mL and XOS and MV-AX induced an average 0.31 ± 0.1 log_{10} increase in cells/mL above control.

Across all 3 timepoints (0, 24, 48 hours), there were significant differences in cell numbers of: Prop183-labelled cells (*Propionobacteria)*, Lab158-labelled cells (*Lactobacillus)*, Rrec584-labelled cells (*Roseburia/Eubacterium rectale)* and DSV687-labelled cells (*Desulfovibrio)* (P<0.05). However, changes in cell numbers did not significantly differ among prebiotic fermentations, relative to the control vessel.

Phase II: 16-day continuous culture 3-stage gut model

Short chain fatty acid and organic acid concentrations

Conditions in each vessel were simulated to approximate conditions in specific regions of the adult human colon, V1: proximal region, V2: transverse region and V3: distal region. SCFA concentrations through time in each of the fermentation vessels are presented in **Figure 3**.

Following 16 days of supplementation, propionate levels differed significantly among prebiotic blends in V1 and V3 ($P<0.05$). The greatest increase in propionate concentrations in V1 was observed during the I+RS fermentation (5.7 mM). I+AX induced the greatest increase in propionate levels in V3 with a rise of 4.5 mM.

Acetate production differed across all stages of the colonic model following supplementation, which significantly differed among prebiotic blends in V1 and V2 ($P<0.05$). In V1, fermentation of I+AX induced the greatest increase in acetate, with a rise of 47 mM. Whereas in V2, I+RS induced the greatest rise in acetate levels (46 mM). Significant differences were observed, among prebiotics, in the production of butyrate in V1 ($P<0.05$) with I+AX inducing the greatest increase (10.06 mM). Differences in the levels of lactate between fermentations, were close to significance in V2 ($P=0.055$), with I+AX fermentation inducing the largest rise in lactate (2.0mM) (**Figure 4**).

Changes in microbial ecology

Changes in microbial ecology in the continuous culture models are presented in **Figure 5**. The abundance of Lab158-labelled cells (*Lactobacillus*) significantly increased in both V1 and V2 ($P<0.05$). The magnitude of this increase was differed among blends in V2 and was close to significance ($P=0.055$). In V1, the greatest increase in Lab158-labelled cells occurred following fermentation with I+RS with a 0.72 $\log_{10}$ rise in cells/mL. In V2, the greatest increase was observed with I+GLOS fermentation with a 0.51 $\log_{10}$ rise in Lab158-labelled cells/mL. Numbers of Bac303-labelled cells (*Bacteroidia*) differed among prebiotic treatments in V1 ($P<0.05$), with I+RS inducing the greatest 0.14 $\log_{10}$ increase in Bac303-labelled cells/mL. Conversely, with I+GLOS fermentation there was a reduction of 0.14 $\log_{10}$ in Bac303-labelled cells/mL in V1. Changes in the abundance of Erec482-labelled cell numbers (*Clostridium coccoides*) significantly differed among the 3 blends ($P<0.05$) with the

highest 0.14 log_{10} increase in cells/mL observed during I+GLOS fermentation. Rrec584-labelled cell numbers (*Roseburia/Eubacterium rectale)* significantly differed among all 3 prebiotic blends (P<0.05), with fermentation of I+GLOS inducing the greatest 0.45 log_{10} increase in Erec482-labelled cells/mL.There was a significant increase in the abundance of Fprau655-labelled cells (*Faecalbacterium prausnitzii*) in V1 (P<0.05). The greatest increase was observed following fermentation of I+RS with a 0.63 log_{10} (cells/mL) increase in Fprau655-labelled cells **(Figure 5).** Following 16 days of fermentation**,** changes in cell-labelled Prop853 (*Propionobacterium*) were nearing significance (P=0.077) in V2 and these changes differed among the 3 prebiotic blends (P=0.078), with I+AX inducing the greatest 0.56 log_{10} increase in cells/mL. In V3 there was a significant effect of time on changes to cell-labelled Prop853 numbers with fermentation of I+AX inducing the greatest 1.09 log_{10} (cells/mL) increase. Notably, I+GLOS also induced a large 0.73 log_{10} (cells/mL) increase cell-labelled Prop853 in V3.

Discussion

The human gut microbial community is home to an array of specialist carbohydrate active enzymes which may precipitate diverse metabolic, and ecological, responses to different glycans according to their chemical structure (Cartmell 2018). This may be exploited through dietary intervention to elicit specific functional changes in the composition, and/or metabolic activity, of the intestinal microbial community (Scott 2020). We set out to develop a prebiotic blend specifically capable of maximising, and importantly sustaining, propionate production through the human colon. Initially, we screened eleven candidate substrates for prebiotic potential using a 48-hour anaerobic batch culture fermentation model with faecal inoculate.

Of all the substrates assessed in the batch culture screen, propionate production was greatest with α-gluco-oligosaccharide; polydextrose and oligofructose.

This increase in propionate was accompanied by an increase in total SCFA, however, the ratio of acetate to propionate was only lowered in the fermentations with α-gluco-oligosaccharide, β-1,4 glucan, oat fibre and polydextrose. The ratio of acetate to propionate may be of interest metabolically, with acetate being implicated in cholesterol metabolism, whilst propionate may help regulate blood glucose and

suppress appetite. Our observations are consistent with the findings of Hu *et al*. (Hu 2020) who demonstrate higher synthesis of propionate with gluco-oligosaccharide relative to inulin, and with the findings of Carlson *et al*. (Carlson 2017), Fehlbaum *et al*. (Fehlbaum 2018) and Hughes *et al*. (Hughes 2008) who demonstrated a reduction in the acetate:propionate ratio with fermentations enriched in oat fibre, or β-1,4 glucan, versus relative increases in acetate observed in fermentations with inulin and xylo-oligosaccharide. From a metabolic perspective, slow and sustained propionate release might be as desirable as producing efficacious quantities, it was notable however, that propionate production was relatively speedy for several of the most efficacious prebiotics; for example, by 24 hours the fermentation supernatant of α-gluco-oligosaccharide was already at 94% of the final concentration observed at 48 hours. β-1,4 glucan, long-chain fructo-oligosaccharide (inulin), resistant starch, low and medium viscosity arabinoxylan, and xylo-oligosaccharide, all showed evidence of slower, propionate synthesis, with substantial increases in propionate concentrations being observed between 24 and 48 hours. Low viscosity arabinoxylan, characterised by less branching units than medium viscosity arabinoxylan, induced greater acetate and butyrate in batch culture. This is in line with research indicating structural differences can strongly impact SCFA output and as branching units increase, so does the propiogenic effect of the arabinoxylan(Tuncil 2020).

Polydextrose; α-gluco-oligosaccharide and oligofructose all elicited increased growth of *Lactobacillus*, *Bifidobacterium,* and *Propionibacterium*, although increases in the abundance of these genera was not markedly different from those observed with the other less efficient substrates. In fact, none of the microbial groups that we quantified could be exclusively linked to high propionate production. It is feasible that rapidly synthesised lactate/acetate, produced though preferential and speedy bifidobacterial metabolism of α-gluco-oligosaccharide, may have functioned as intermediate metabolites for *Propionibacterium*, or other unidentified bacteria such as the *Lachnospiraceae* to use in the production of propionate.

In phase 2 of the study, we elected to explore the effects of blending fast and slow fermenting prebiotic substrates to optimise and sustain propionate production in a continuous 3-stage batch culture model. Given the commercial availability, and the level of characterisation of the fermentative capacity of long chain fructo-oligosaccharide as a prebiotic (Liu 2016), we selected inulin for use in three 50:50 prebiotic blends because we know inulin is strongly bifidogenic and the aim one of the

aims was to sustain metabolite production. Inulin was combined with either α-gluco-oligosaccharide as a rapid propionate producer, or with arabinoxylan, or resistant starch as slower propionate producers. Fermentation characteristics of these three blends were then evaluated in a validated three-stage model of the colon described by Macfarlane et al (Macfarlane 1998). This culture model was continuously fed with fresh prebiotic substrate over 16 days between steady states 1 and 2, thus allowing for a full ecological adaptation to the substrate and estimates upon site of fermentation in the colon.

Based on the findings of phase 1, we had hypothesised that an α-gluco-oligosaccharide/inulin (fast/steady) blend would yield the greatest concentration of propionate in each of the three model vessels corresponding to the proximal, transverse and distal colon. The assumption here was that α-gluco-oligosaccharide would serve as the predominant substrate and fermented early on (e.g. simulating the proximal colon), sparing inulin for fermentation in vessels 2 and 3. However, whilst fermentation of the inulin/α-gluco-oligosaccharide resulted in propionate production, of the three blends, it was the combination of two slower acting substrates, inulin with arabinoxylan, that yielded highest propionate, and total SCFA. Fermentation of both the inulin/arabinoxylan, and the inulin/α-gluco-oligosaccharide blends induced comparable propionate in vessel 1 (proximal colon). The arabinoxylan blend, however led to a greater propionate concentration in vessel 2 (transverse colon) and sustained higher concentrations in vessel 3 (descending colon), thus demonstrating an effective and sustained synthesis of propionate. The blend of inulin and resistant starch did not prove effective at producing propionate beyond vessel 1, perhaps due to its different effect on microbial ecology or through differing effects on redox balance or hydrogen availability in the system (Navone 2018). The inulin/arabinoxylan mixture was the most bifidogenic of the three substrate blends, with significant and progressive increases in bifidobacterial cell counts through each of the three continuous fermentation vessels; *Bifidobacteria* are known to metabolise carbohydrate to acetate and lactate through the bifid shunt (Devika & Raman 2019), any lactate produced from this bifidogenic metabolism may be leveraged by *Propionibacterium,* for example, conversion to propionate via the acrylate pathway, or by other members of the gut microbiota which can convert lactate to propionate via the succinate pathway (Reichardt 2014).

Increased lactate production during inulin/arabinoxylan fermentation may have also induced an increase in butyrate in vessel 1. *Clostridium coccoides*/*Eubacterium*

rectale, was the only butyrate producing group to notably grow in all vessels during inulin/arabinoxylan fermentation. Clostridia are well documented to utilise lactate to produce butyrate (Chai 2019). In contrast, the inulin/resistant starch blend, whilst also being bifidogenic, did not significantly increase the abundance of *Propionibacterium*, favouring instead the growth of *Lactobacillus*, *Bacteroidia* and *Feacalibacterium prausnitzi* in vessel 1 and of *Roseburia* in vessel 3.

Whilst it did increase the abundance of *Bifidobacterium*, the inulin/α-gluco-oligosaccharide blend was found to be the least bifidogenic of the three substrates, it also moderately increased abundance of *Propionibacterium*. Of the three blends it was most associated with increases in the abundance of *Bacteroidia, Roseburia* and *Lactobacillus* in vessels 2 and 3. The observed increase in abundance of *Roseburia* might best explain the significant increase in butyrate concentrations. *Roseburia* are well characterised butyrate producers, that can utilise acetate, lactate, and oligosaccharide degradation products made available by keystone degraders such as *Bacteroidia* (Louis & Flint 2009).

Whilst all three blends precipitated a rise in total SCFA, and elucidated a plethora of changes in microbial ecology that would be considered of prebiotic potential, disparities between the distinct effects of the substrates when cultured alone in batch culture versus synergistic effects when combined in continuous culture emphasises the importance of understanding the influence of these products when consumed either as functional food blends, or on their own but as part of habitual dietary patterns, this is of particular importance when seeking targeted changes in the gut microbiota. Colonic diseases such as inflammatory bowel disease and colon cancer develop mainly in the distal region of the colon, in part due to increased production of health deleterious metabolites from proteolytic metabolism such as phenols and hydrogen sulphide (Chambers 2018). Slowing fermentation through blending potential prebiotics may enhance microbial communities distally, thereby increasing production of more health beneficial metabolites from this saccharolytic metabolism such as SCFAs and reduce exposure to more harmful metabolites in the distal region.

The data generated across phase 1 of the study increases understanding about the potential gut microbial responses to a broad panel of putative prebiotic substrates, this comparative characterisation may be of considerable use when considering other targeted interventions for a specific metabolite, or microbial response. We noted for, example, that substrates elucidating greatest increases in acetate were, oligofructose,

xylo-oligosaccharide, low viscosity arabinoxylan, resistant dextrin and oat fibre, whilst those that elucidated the greatest increases in butyrate were oligofructose, xylo-oligosaccharide and resistant maltodextrin. These findings need to be interpreted in the context of the biological capacity of our faecal donors and does not confirm these prebiotic effects, but may inform future functional prebiotic blends particularly if taken forward to be tested *in vivo*.

The limitations of this work are that it uses *in vitro* models of human gut microbial fermentation with likely loss of microbial diversity from the in vivo situation. Further, these models do not capture microbial activity at the intestinal epithelial interface, and they do not account for changes in metabolite concentrations through intestinal absorption and host cell metabolism. As substrates were added directly to the culture, and with no enzymatic pre-treatment simulating the small intestine, monosaccharide content of some of the less purified substrates, may have led to changes to bacterial ecology that may have impacted subsequent metabolic output that differs from that with pre-treatment. However, the strength of these models is that they remove biological heterogeneity that confounds microbiota research in human volunteers, and importantly they allow real time sampling through the fermentation stream.

As a result of this work, we have developed a blend of prebiotics that induces a sustained metabolites in colonic fermentation, subsequently we hope to investigate (in human intervention trials) whether or not we can use this blend to sustain propionate release in the human gut.

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Figure Legends

Figure 1. HPLC analysis of SCFA concentrations measured in the supernatant of broth collected from vessels 1 to 12 at baseline, 24 and 48 hours: long chain fructo-oligosaccharide (LC-FOS), oligofructose (OFS), xylo-oligosaccharide (XOS), α-gluco-oligosaccharide (GLOS), polydextrose (PDX), resistant malto-dextrin (R-MLX), medium viscosity arabinoxylan (MV-AX), low viscosity arabinoxylan (LV-AX), oat fibre (OAT), βeta-glucan (β-GLU), control and representing the mean (n=3) of the data (mM). **A)** acetate, **B)** buryate, **C)** propionate.

Figure 2. Bacterial groups measured by FISH-FLOW (Log_{10} cells/mL) using probes: total bacteria (Eub338 I-II-III), *Bifidobacterium* spp. (Bif164), *Lactobacillus* spp. (Lab158), most Bacteroidacae and Prevotellaceae (Bac303), *Clostridium coccoides-Eubacterium rectale* group (Erec482), *Roseburia* (Rrec584), *Faecalibacterium prausnitzii* (Fprau655), *Desulfovibrio* spp. (DSV867), *Clostridium histolyticum* (Chis150). Collected from the culture supernatant at time points 0, 24 and 48 hours from each vessel 1 to 12: long chain fructo-oligosaccharide (LC-FOS), oligofructose (OFS), xylo-oligosaccharide (XOS), α-gluco-oligosaccharide (GLOS), polydextrose (PDX), resistant malto-dextrin (R-MLX), medium viscosity arabinoxylan (MV-AX), low viscosity arabinoxylan (LV-AX), oat fibre (OAT), βeta-glucan (β-GLU), control. Values represent the mean of the data (n=3) ± SEM.

Figure 3. HPLC analysis of SCFA concentrations measured in the supernatant of broth collected from all vessels: V1, V2, V3 of a continuous *in vitro* colonic model both before (SS1) and after (SS2) the administration of 1.33g/d prebiotic blends (n=3): inulin and resistant starch (I+RS), inulin and α-gluco-oligosaccharide (I+GLOS) and inulin and arabinoxylan (I+AX). Values represent the mean (n=3) of data ± SEM (mM). **A)** acetate, **B)** butyrate, **C)** propionate.

Figure 4. HPLC analysis of lactic acid concentrations measured in the supernatant of broth collected from all vessels: V1, V2, V3 of a continuous *in vitro* colonic model both before (SS1) and after (SS2) the administration of 1.33g/d prebiotic blends (n=3): inulin and resistant starch (I+RS), inulin and α-gluco-oligosaccharide (I+GLOS) and inulin and arabinoxylan (I+AX). Values represent the mean (n=3) of data ± SEM (mM).

Figure 5. Bacterial groups measured by FISH-FLOW (Log cells/mL) sampled from the supernatant of broth collected from all vessels: V1, V2, V3, of a 3-stage continuous colonic model, both before (SS1) and after (SS2) the administration of 1.33g/d prebiotic blends (n=3): inulin and resistant starch (I+RS), inulin and α-gluco-oligosaccharide (I+GLOS) and inulin and arabinoxylan (I+AX). Probes used: *Bifidobacterium* spp. (Bif164), *Lactobacillus* spp. (Lab158), most Bacteroidacae and Prevotellaceae (Bac303), *Roseburia* (Rrec584) and *Faecalibacterium prausnitzii* (Fprau655). Values represent the mean (n=3) of data ± SEM.

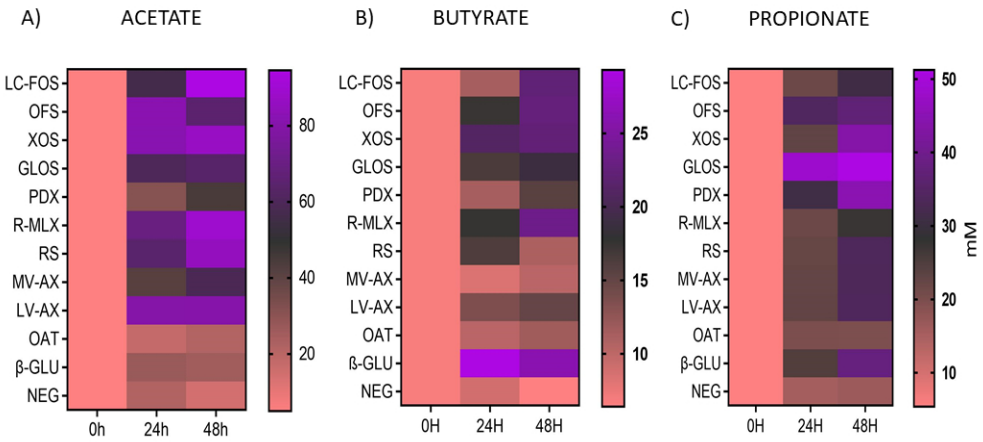

Figure 1

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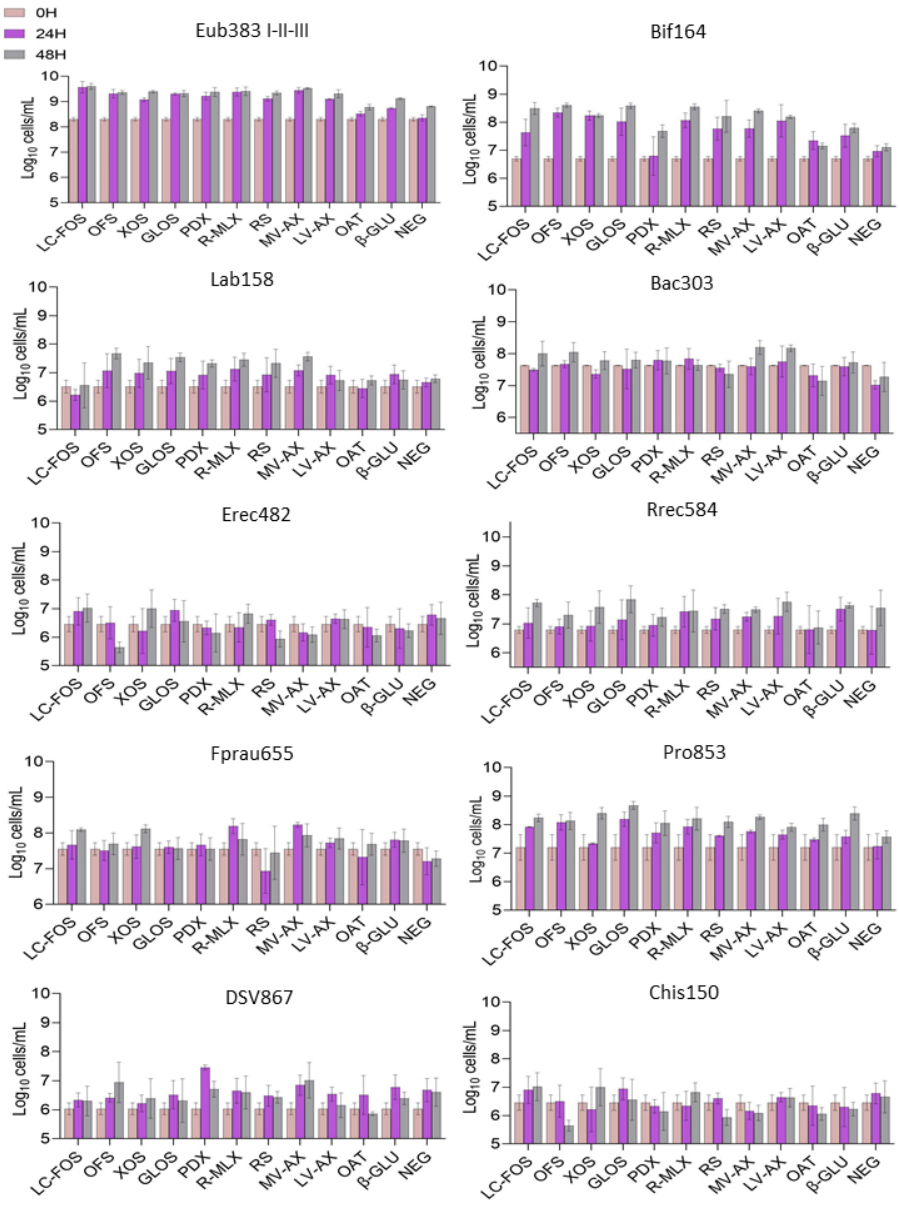

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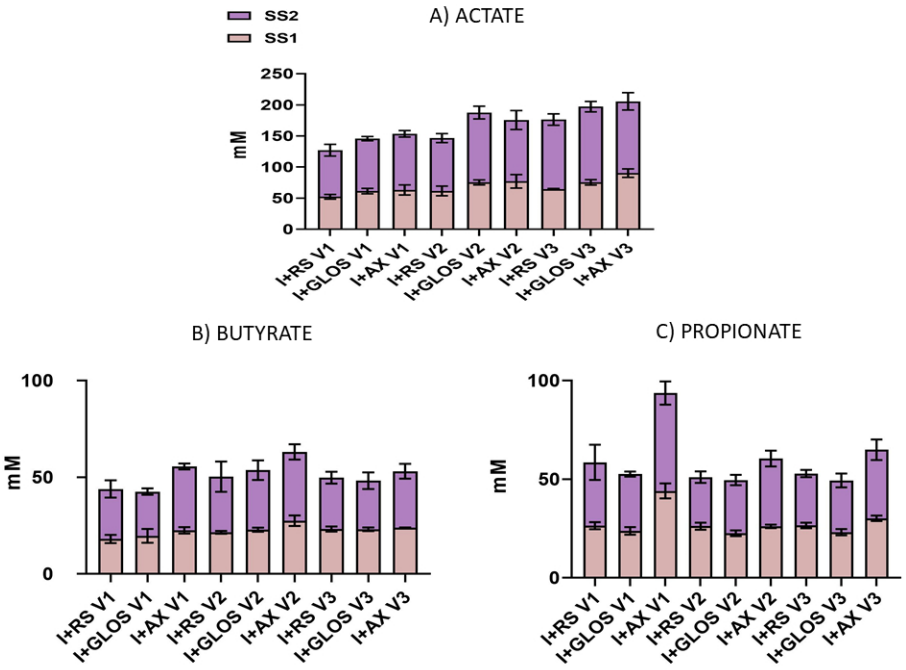

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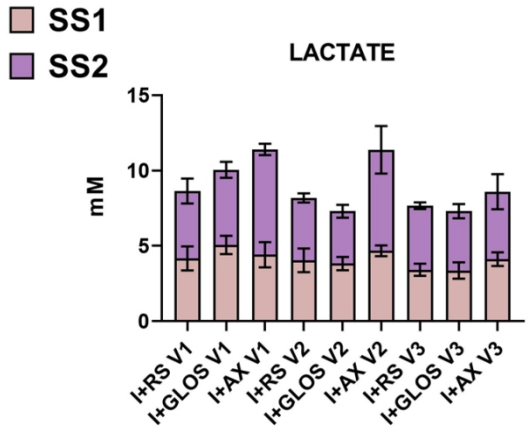

Figure 4. HPLC analysis of lactic acid concentrations measured in the supernatant of broth collected from all vessels: V1, V2, V3 of a continuous in vitro colonic model both before (SS1) and after (SS2) the administration of 1.33g/d prebiotic blends (n=3): inulin and resistant starch (I+RS), inulin and α-gluco-oligosaccharide (I+GLOS) and inulin and arabinoxylan (I+AX). Values represent the mean (n=3) of data ± SEM (mM).

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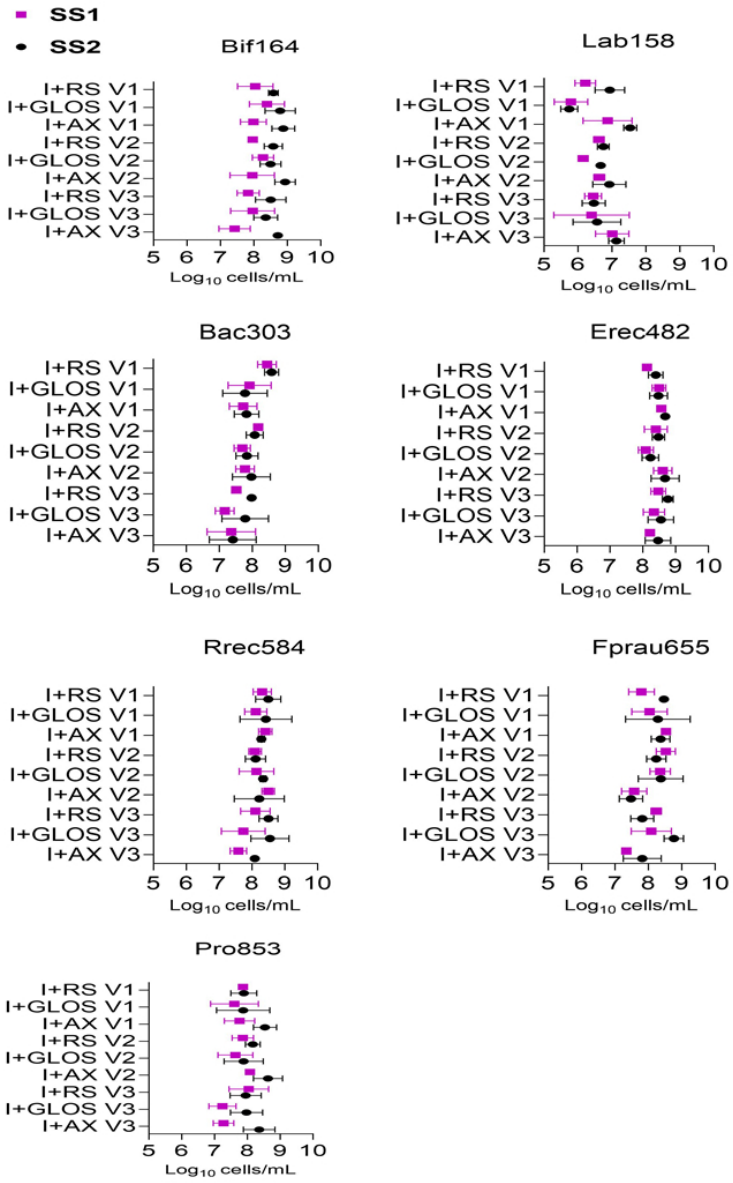

Figure 5. Bacterial groups measured by FISH-FLOW (Log cells/mL) sampled from the supernatant of broth collected from all vessels: V1, V2, V3, of a 3-stage continuous colonic model, both before (SS1) and after (SS2) the administration of 1.33g/d prebiotic blends (n=3): inulin and resistant starch (I+RS), inulin and α-gluco-oligosaccharide (I+GLOS) and inulin and arabinoxylan (I+AX). Probes used: Bifidobacterium spp. (Bif164), Lactobacillus spp. (Lab158), most Bacteroidacae and Prevotellaceae (Bac303), Roseburia (Rrec584) and Faecalibacterium prausnitzii (Fprau655). Values represent the mean (n=3) of data ± SEM.

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Table 1. The physico-chemical characterisation of substrates used in batch culture fermentation. Information provided by technical specification, supplier information and previous research.

Substrates	Physio-chemical properties	Purity
For phase I batch culture - long chain fructo-oligosaccharides (LC-FOS) (Fine Foods & pharmaceuticals, Italy)	Polysaccharide of fructose monomers with β (2,1) linkages. DP: ~8, Mw: 522.5 g/mol	~90% purity (~10% monosaccharide: fructose, glucose)
For phase II 3-stage gut model - long chain fructo-oligosaccharides (LC-FOS) (Fruitafit, Chimab, Italy)	Polysaccharide of fructose monomers with β (2,1) linkages. ~DP: 8-13, Mw: 522.5 g/mol	~90% purity (~10% monosaccharide: fructose, glucose & dissaccharide: sucrose)
Oligofructose (OFS) (Raftilose P95 from Orafti, Belgium)	Oligosaccharide of fructose monomers with β (2,1) linkages. DP: 2-9, Mw: 504 g/mol	~93-97% purity (2.5 - 6.8% monosaccahride: fructose, glucose & sucrose)
Hi-Maize 260 resistant starch (RS) (Ingredion, United States)	Polysaccharide of amylose (helical polymer of glucose with α(1, 4) linkages) DP: 6000 and Mw 105 g/mol and soluble polysaccharide amylopectin (branched polymers of glucose with α(1,4) and α(1,6) linkages) DP ~500, Mw: 108 g/mol	~48 % amylose & ~41% amylopectin, 0% monosaccharide
Resistant maltodextrin (R-MLX) (Promitor, Tate & Lyle, England)	Polysaccharide of glucose with α-1,4 linkages. DP ~12, Mw: 504 g/mol	~70% purity (~10% monosaccharide: fructose, glucose)
Gluco-oligosaccharides (GLOS) (Bioecolians, Italy)	Oligosaccharide of α-(1,3)/α-(1,6)-linked glucose residues. DP: 6-17, Mw: 8-65 kDa	~87% purity (~13% monosaccharide: maltose & disaccharide: sucrose)
β-glucan (β-GLU) (Dansico, Denmark)	Polysaccahride of glucose residues linked by β-(1,4) and β-(1,3) DP: 5-28, Mw: 504.4 g/mol	~75% purity (~18% monosaccharide: glucose)

Oat Fibre (OAT) (Fine Foods & Pharmaceuticals, Italy)	Polysaccharide of glucose residues β-(1, 4) and β-(1, 3). Linear d-glucopyranosyl residues β-(1, 4), β-(1, 3). DP: 5-28, Mw: 504.4 g/mol	~75% purity (~18% monosaccharide: glucose)
Low viscosity arabinoxylan (LV-AX) (Megazyme, Ireland)	Polysaccharide of linear xylopyranosyl residues ß-D (1, 4) α-L-Arabinofuranosyl (1, 3). DP ~36, Mw: 56.7 kDa. Viscosity ~10 cSt.	~95% purity (monosaccharide % at 38:62 arabinose:xylose
Medium viscosity arabinoxylan, (MV-AX) (Megazyme, Ireland)	Polysaccharide of linear xylopyranosyl residues ß-D (1, 4) α-L-Arabinofuranosyl (1, 3) linkages. DP ~36, Mw: 323 kDa. Viscosity ~20-30 cSt	~95% purity (monosaccharide % at 38:62 arabinose: xylose)
Xylo-oligosaccharides (XOS) (Suntory, Japan)	Oligosaccharide of xylose β (1, 4) linkages. DP: 2-4, Mw: 414.36 g/mol	70% purity (~15-30% monosaccharide: glucose, xylose, arabinose)
Polydextrose (PDX) (Danisco, Denmark)	Polysaccharide of glucose α- and β-(1, 2) through to (1,6) linkages. DP: 3-60, Mw: 342.3 g/mol	~99.5% purity (~1% monosaccharide: glucose)

Table 2. Oligonucleotide probes used in both batch culture and continuous colonic model fermentations for fluorescent *in situ* hybridisation and enumeration of bacterial groups with flow-cytometry.

Probe Name	Target species	Sequence 5' to 3'	Reference
Non Eub	Non bacteria	ACTCCTACGGGAGGCAGC	Wallner *et al.,* 1993
Eub338‡	Most bacteria	GCTGCCTCCCGTAGGAGT	Daims *et al*., 1995
Eub338II‡	Most bacteria	GCAGCCACCCGTAGGTGT	Daims *et al*., 1995
Eub338III‡	Most bacteria	GCTGCCACCCGTAGGTGT	Daims *et al*., 1995
Bif164	*Bifidobacterium*	CATCCGGCATTACCACCC	Langendijk *et al*., 1995
Lab158	*Lactobacillus, Leuconosto Weissella Lactococcus lactis;* all*Enterococcus, Vagococcus, Melisococcus, Catellicoccus, Tetragenococcus, Pediococcus*, *Paralactobacillus* spp.	GGTATTAGCAYCTGTTTCCA	Harmsen *et al*., 2002
Bac303	Most *Bacteroidaceae* and P*revotellacea*	CCAATGTGGGGGACCTT	Manz *et al.,* 1996
Erec482	*Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa and XIVb)	GCTTCTTAGTCARGTACCG	Franks *et al*., 1998
Rrec584	*Roseburia - Eubacterium rectale*	TCAGACTTGCCGYACCGC	Walker *et al.*, 2005

Ato291	*Atopobium, Colinsella, Olsenella Eggerthella Cryptobacterium curtum; Mycoplasma equigenitalium Mycoplasma elephantis*	GGTCGGTCTCTCAACCC	Harmsen *et al*., 2002
Prop853	*Propionibacterium* (Clostridial Cluster IX)	ATTGCGTTAACTCCGGCAC	Walker *et al.*, 2005
Fprau655	*Faecalibacterium prausnitzii*	CGCCTACCTCTGCACTAC	Devereux *et al.*, 1992
DSV687	Most *Desulfovibrionales* and *Desulfuromonales*	TACGGATTTCACTCCT	Hold *et al*. 2003
Chis150	*Clostridium histolyticum* (*Clostridium* cluster I and II)	TTATGCGGTATTAATCTYCCTTT	Franks *et al*., 1998